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Lysine Analogues

Asymmetric Synthesis of Lysine Analogues with Reduced Basicity, and their Incorporation into Proteasome Inhibitors

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Abstract: Most known β2-selective proteasome inhibitors suffer from relatively poor cell permeability as the result of a net positive charge caused by the basic moiety at P1. In this paper, 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-yne). These amino acids, as well as histidine and diaminopropionic-acid-glycine, were incorporated at the P1 and/or P3 positions of oligopeptide vinyl sulfones. All inhibitors were found to inhibit β2, but with a loss of potency compared to our most potent and selective β2 inhibitor, LU-102. These results notwithstanding, our results provide important insights for the future design of β2-selective proteasome inhibitors.

Introduction

Proteasomes are large proteolytic machineries that are 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, and various proteasome inhibitors are currently being evaluated in clinical trials against 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 an 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 for targetting in drug development, and indeed, bortezomib, carfilzomib, and various clinical candidates were developed to target the β5 subunits.[4] However, bortezomib efficiently inhibits its β1c and β1i with potency similar to that against the β5 active sites;[5] 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 coinhibition 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). NC-022 is the most potent proteasome inhibitor of these two, but its Arg residues render the molecule impermeable to cells. Using NC-002, we showed that selective β2 inhibition sensitises MM cells to bortezomib and carfilzomib.[9] With the aim of overcoming the lability of the arginine epoxyketone (intramolecular attack of the guanidine group onto 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 synthesised on large scale. The 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 came at the cost of cell permeability (Figure 1).

With LU-102, we found that selective β2 inhibition not only sensitises MM cells to bortezomib and carfilzomib,[10] but also overcomes resistance to bortezomib and carfilzomib, a major problem that arises when patients are treated with proteasome inhibitors.[6] LU-102 shows nanomolar potency in cell lysates, but 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. 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 values closer to physio-

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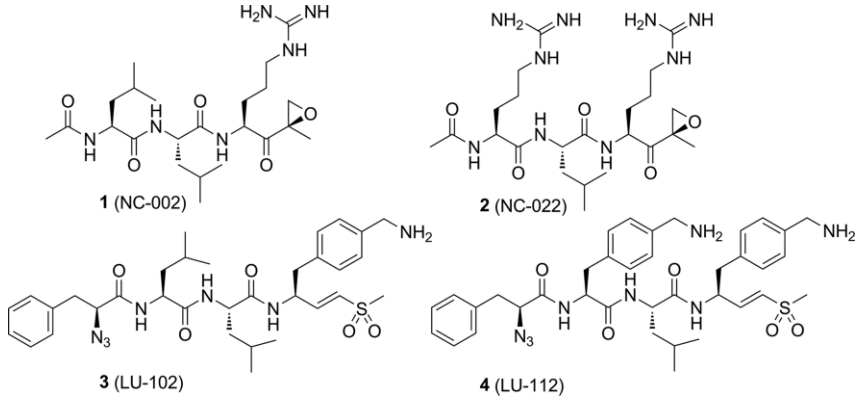
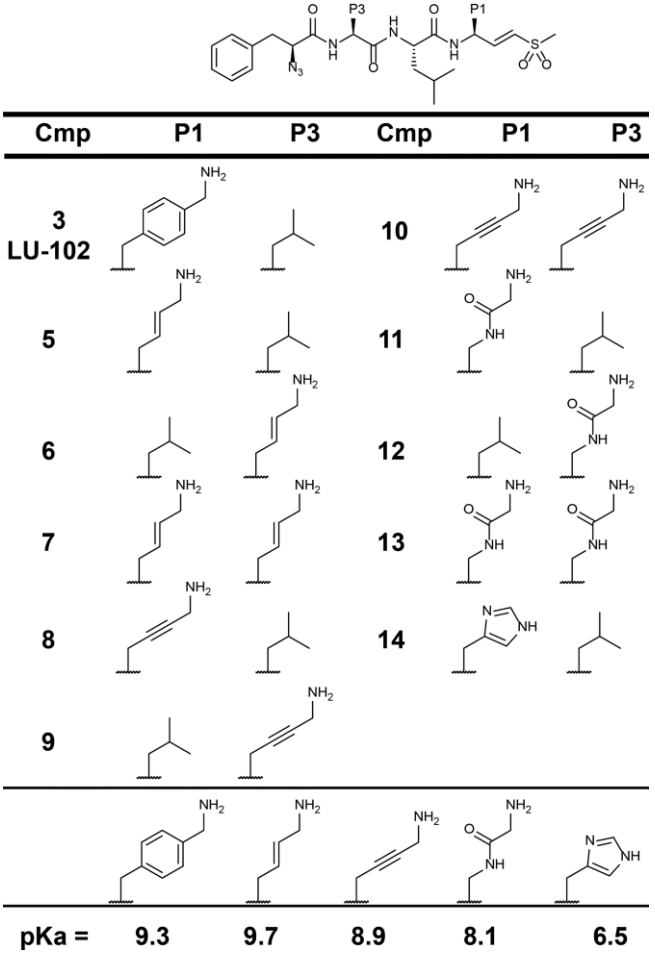


Figure 1. Structures of previously reported β2-selective inhibitors. IC_{50} against HeLa cell lysates: **3**: 0.084 μM; **4**: 0.022 μM, IC_{50} against HeLa cells: **3**: 2.7 μM; **4**: 50 μM. IC_{50} values [μM] were determined using HeLa cell lysates (1 h treatment) and intact HeLa cells (4 h treatment).

logical pH would be required. Possible amino acids with lower pK_a values include histidine, and the lysine analogues Lys(4-ene), Lys(4-yne), and diaminopropionic-acid-Gly [Dap(Gly)] (Table 1). The protonated ε-amine groups in these species show significantly lower pK_a values compared to lysine as a result of the electron-withdrawing properties of the alkene, alkyne, or amide moiety.[11]

Table 1. Structures of compounds synthesised in this study. pK_a values of conjugate acids are shown.

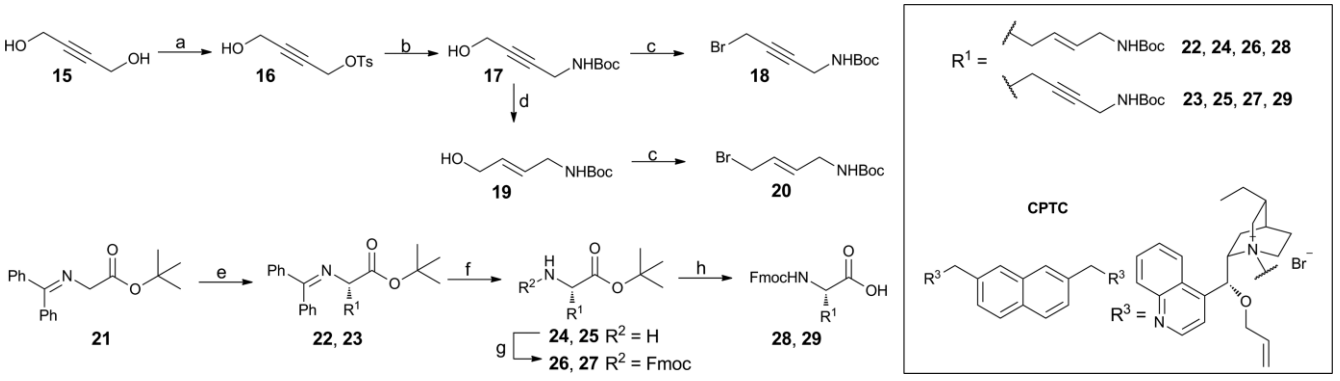


In this paper, we describe the synthesis of these lysine analogues and their incorporation into tetrapeptide vinylsulfones. We developed an enantioselective synthesis of both amino acids that includes as a key step the catalytic enantioselective phase-transfer alkylation of a glycine derivative. We also 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. Histidine and the lysine analogues were then used as building blocks, and were installed as P3 residues, and also converted into their corresponding vinyl sulfones at the P1 position All the inhibitors synthesised (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-yne)-Containing Inhibitors

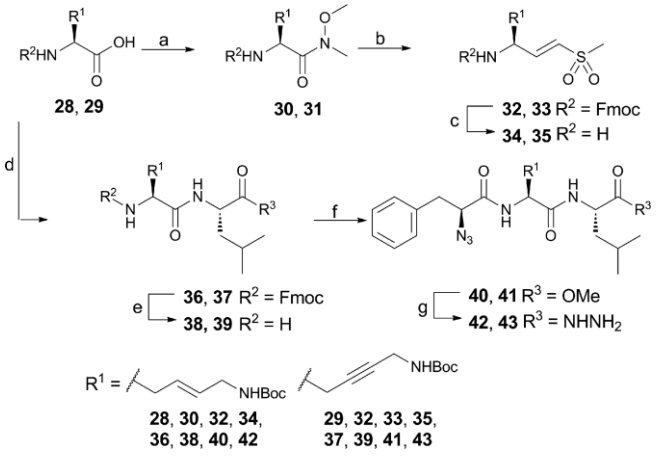
The synthesis of α-amino acids by catalytic enantioselective phase-transfer alkylation of a glycine derivative has been highly optimised by Park and coworkers.[12] In their procedure, a dimeric cinchona-derived chiral phase-transfer catalyst (CPTC; Scheme 1) is used in the synthesis of a wide range of α-amino acids, including allylglycine and propargylglycine. We envisioned that this method could also be used for the enantioselective synthesis of L-Lys(4-ene) and L-Lys(4-yne). 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 protecting-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** began with the monotosylation of diol **15**. Next, the OTs moiety of compound **16** was substituted by ammonia,[13] and subsequent Boc (*tert*-butoxycarbonyl) protection of the amine gave compound **17**. In order to obtain the *E* alkene, propargyl alcohol **17** was selectively reduced with $LiAlH_4$ to give allyl alcohol **19**.[14,15] The low yield in the reduction of alkyne **17** to alkene **19** is possibly caused by the harsh conditions (strong reducing agent and elevated



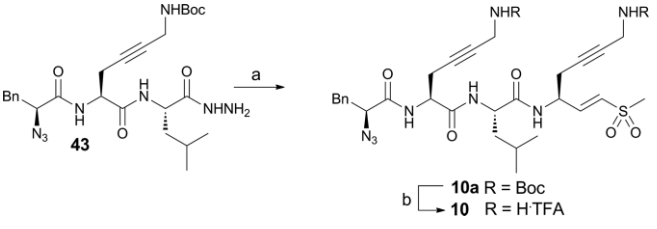
Scheme 1. Synthesis of Lys(4-ene) and Lys(4-yne) building blocks. Reagents and conditions: a) TsCl, pyridine, CH_2Cl_2, 63 %; b) 1) NH_3 (25 % aq.); 2) Boc_2O, Et_3N, THF, CH_2Cl_2, 42 %; c) PPh_3, CBr_4, CH_2Cl_2, 0 °C; **18**: 68 %, **20**: 58 %; d) $LiAlH_4$, THF, Δ, 38 %; e) **18** or **20**, CPTC, toluene/$CHCl_3$, KOH (50 % aq.); **22**: 84 %, 79 % *ee*; **23**: 84 %, 80 % *ee*; f) citric acid (15 % aq.), THF, 0 °C–r.t.; g) FmocOSu (Fmoc *N*-hydroxysuccinimide ester), DiPEA (diisopropylethylamine), CH_2Cl_2; **26**: 83 %, **27**: 89 % (over steps f and g); h) 1) TFA; 2) Boc_2O, DiPEA, MeCN; **28**: 82 %, **29**: 63 %.

temperature), which could result in partial Boc removal. Alcohols **17** and **19** were converted by an Appel reaction into bromides **18** and **20**. Although several of the steps leading to the bromides were rather low-yielding, the reactions were easily carried out on a 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] and compounds **22** and **23** were obtained in high yields with good enantiomeric excesses (79 % *ee* for **22**, and 80 % *ee* for **23**), as determined by chiral HPLC analysis. Mild acidic hydrolysis of the imine moieties provided amines **24** and **25**, which were Fmoc (fluorenylmethoxycarbonyl) protected to give compounds **26** and **27**. Subsequent removal of the Boc group and the *t*Bu ester by treatment with TFA (trifluoroacetic acid), followed by Boc protection of the ε-amine, gave building blocks **28** and **29** in good yields. For the synthesis of the corresponding vinyl sulfones, **28** and **29** were converted into 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**.

The Fmoc groups in **32** and **33** were removed by treatment with diethylamine to give free amines **34** and **35**. For the incorporation of L-Lys(4-ene) or L-Lys(4-yne) 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 with N_3-Phe-OH, and hydrazinolysis of the methyl ester provided hydrazides **42** and **43**. Standard azide couplings (see Scheme 3 for an example) between hydrazides **42**, **43**, or N_3-Phe-Leu-Leu-$NHNH_2$[10] 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-yne) vinyl sulfones and peptide hydrazides. Reagents and conditions: a) HCTU [*O*-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate], *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, CH_2Cl_2; **36**: 96 %, **37**: 76 %; e) piperidine, DMF; **38**: 94 %, **39**: 100 %; f) N_3-Phe-OH, HCTU, DiPEA, CH_2Cl_2; **40**: 60 %, **41**: 91 %; g) hydrazine monohydrate, MeOH, 100 %.

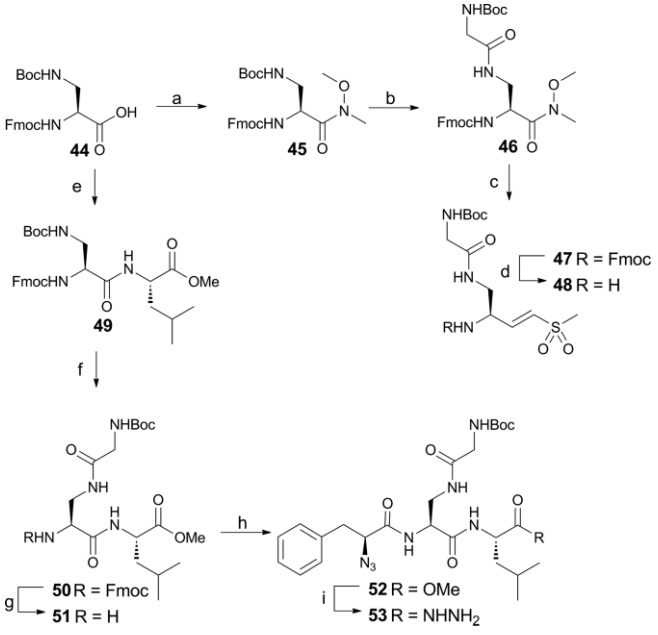


Scheme 3. Synthesis of compound **10** as example of an azide coupling followed by Boc removal. Reagents and conditions: a) 1) *t*BuONO, HCl, DMF; 2) H-Lys(4-yne)-VS, DiPEA; b) TFA, 79 % over two steps.

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 began with the conversion of commercially available Fmoc-Dap(Boc)-OH **44** into Weinreb amide **45**. Subsequent Boc removal and condensation with Boc-Gly-OH provided compound **46**. Conversion of the Weinreb amide into the vinyl sulfone by a procedure similar to that described above yielded **48**. For the synthesis of the peptide hydrazide, Fmoc-Dap(Boc)-OH **44** was first coupled with H-Leu-OMe, and then Boc removal and coupling with Boc-

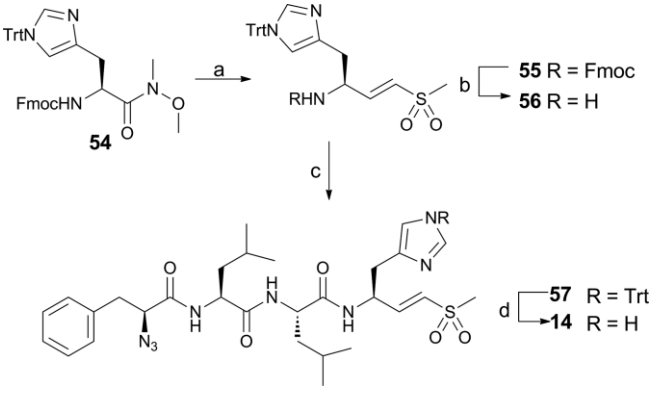
Gly-OH gave dipeptide **50**. Fmoc removal, peptide coupling with N_3-Phe-OH, and hydrazinolysis of the methyl ester resulted in peptide hydrazide **53**. The desired inhibitors with Dap(Gly) at P1 and/or P3 were obtained by standard azide couplings between the appropriate hydrazides and vinyl sulfones, 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) 1) TFA; 2) HCTU, Boc-Gly-OH, DiPEA, CH_2Cl_2, 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, CH_2Cl_2, 94 %; f) 1) TFA; 2) HCTU, Boc-Gly-OH, DiPEA, CH_2Cl_2, 100 %; g) piperidine, DMF, 100 %; h) N_3-Phe, HCTU, DiPEA, CH_2Cl_2, 68 %; i) hydrazine monohydrate, MeOH, 100 %.

Synthesis of a His-Containing Inhibitor

The synthesis of P1 His compound **14** is shown in Scheme 5. The appropriately protected His Weinreb amide[16] **54** was converted into vinyl sulfone **56**, using the procedures described above. Free amine **56** underwent an azide coupling reaction with N_3-Phe-Leu-Leu-$NHNH_2$ to provide tetrapeptide vinyl sulfone **57**. Subsequent trityl removal by treatment with TFA with the help of TIPS (triisopropylsilane) as a cation scavenger yielded compound **14**.



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_3-Phe-Leu-Leu-$NHNH_2$, *t*BuONO, HCl, DMF; 2) **56**, DiPEA, 53 %; d) TFA, TIPS, CH_2Cl_2, 38 %. Trt = trityl.

Biological Evaluation

The peptide vinyl sulfones were evaluated for proteasome inhibition in Raji cell lysates (a human B-cell lymphoma cell line expressing constitutive and immunoproteasomes), and were compared to LU-102 (**3**) in a competitive activity-based protein

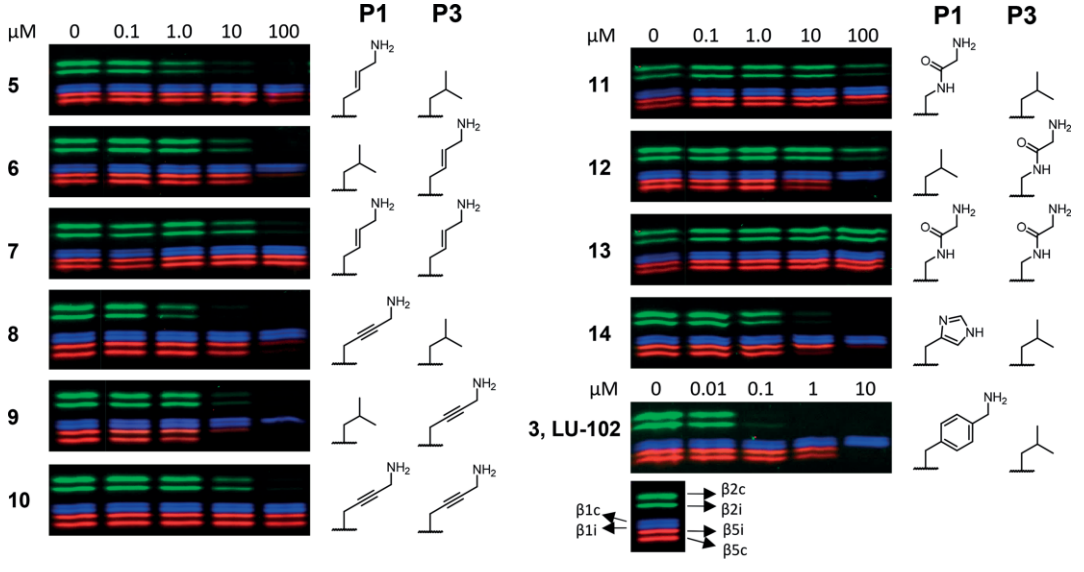


Figure 2. Inhibition profiles of compounds **5**–**14**, compared to **3** (LU-102) in Raji lysates. Lysates were incubated with compounds at the indicated concentration for 1 h, followed by labelling of residual proteasome activity with the ABP cocktail.

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 using the activity-based proteasome probe cocktail recently described by us.[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-yne)] both show some β2 selectivity, although a greater than 10-fold decrease in potency compared to LU-102 was observed. Incorporation of Lys(4-ene) or Lys(4-yne) at P3 or at P1 and P3 resulted in compounds with even lower potency (compounds **6**, **7**, **9**, and **10**). The compounds with Dap(Gly) at P1 and/or P3 (**11**, **12**, and **13**) were all very weak inhibitors, with almost no selectivity for β2 over β5. Finally, compound **14** (P1: His) inhibited β2 with a potency similar to compounds **5** and **8** (complete inhibition at 10 μM), although it showed poor selectivity over the β5 subunits. Since all the 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 show any inhibitory activity in living HeLa cells up to 100 μM (data not shown).

All the synthesised tetrapeptide vinyl sulfones showed decreased activity against β2 compared to LU-102 (**3**). The low activity of these inhibitors could originate from the lower basicity of the amine, a lack of interactions due to loss of the aromatic ring, or a lack of interactions between the side-chain amine and the β2 subunit as a result of the side-chains of the lysine analogues being shorter than that of 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 β2c subunit by hydrogen bonding (Glu53 in case of β2i).[10] Due to the 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 basic P1 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-yne)] have similar potencies, which indicates that the lower pK_a value of Lys(4-yne) compared to Lys(4-ene) does not result in any additional loss of activity, and suggests that both residues are able to interact with Asp53. In contrast, compound **6** shows a much lower activity, which might indicate that the lower basicity of the amine (pK_a 8) is too low for a strong interaction with Asp53 to be established. 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. An absence of interaction 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; the side-chains of the lysine analogues are one carbon atom shorter than that of the 4-(aminomethyl)-Phe residue of LU-102. This probably results in a greater distance between Asp53 and the P1 amine of the inhibitors, causing a weaker interaction and thus a lower potency of the compounds. In fact, in a previous study, a compound with a lysine residue at P1 also showed a 10-fold lower potency compared to LU-102;[10] this indicates that the low potency of compounds **5** and **8** is not a result of the lower basicity of the amine.

In the case of a His residue at P1 (compound **14**), the distance between Asp53 and the basic residue is even larger, probably resulting in the absence of an interaction between the imidazole moiety and Asp53. However, compound **14** is still moderately active, which indicates that the His at P1 might be stabilised 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 the Leu residue at P3 (disfavoured by β5i)[19] and the histidine at P1. The 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.

Superposition of the crystal structure of LU-112 in complex with yeast proteasome onto those of mammalian proteasomes (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 stabilised by several surrounding polar residues. These interactions can probably not be established with the shorter P3 residues of compounds **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 stabilised by several van der Waals interactions.[10] However, the P3 Leu moiety of LU-102 does not show any favourable interaction with the protein. Probably, compounds with lysine analogues at P3 do not benefit from van der Waals interactions with the protein either, and so do 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-yne) was developed, which gives access to amino-acid building blocks suitable for standard Fmoc chemistry. These amino acids were converted into the corresponding vinyl sulfones, and were incorporated as P1 and/or P3 residues into tetrapeptide vinyl sulfones. Moreover, a lysine analogue containing a peptide bond in the side-chain [Dap(Gly)] was incorporated as a P1 and/or P3 residue in potential proteasome inhibitors. Finally, histidine was explored as basic residue at P1. Evaluation by cABPP revealed that all the compounds targeted β2 with much lower potencies than LU-102. The low activity of the 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 Remarks: Acetonitrile (MeCN), dichloromethane (CH_2Cl_2), *N*,*N*-dimethylformamide (DMF), methanol (MeOH), diisopropylethylamine (DiPEA), and trifluoroacetic acid (TFA) were peptide-synthesis grade, purchased from Biosolve, and used as received. All general chemicals (Fluka, Acros, Merck, Aldrich, Sigma, Iris Biotech) were used as received. Column chromatography was carried out on Screening Devices b.v. silica gel, with a particle size of 40–63 μM and a pore diameter of 60 Å. TLC analysis was carried out on Merck aluminium sheets (silica gel 60 F254). Compounds were visualised 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 sulfuric acid (10 %), a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or a solution of 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 with Bruker AV-300 (300 MHz), AV-400 (400 MHz), or AV-600 (600 MHz) spectrometers. Chemical shifts are given in ppm (δ), and tetramethylsilane, CD_3OD, or $CDCl_3$ were used as internal standards. High-resolution mass spectra were recorded by direct injection [2 μL of a 2 μM solution in water/acetonitrile (50:50 v/v) with 0.1 % formic acid] with a Thermo Finnigan LTQ Orbitrap mass spectrometer 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 = 60000 at m/z = 400 (mass range m/z = 150–2000), and with dioctylphthalate (m/z = 391.28428) as a "lock mass". The high-resolution mass spectrometer was calibrated using a calibration mixture (Thermo Finnigan) before measurements were taken. LC–MS analysis was carried out with a Finnigan Surveyor HPLC system equipped 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 buffers used were H_2O, MeCN, and TFA (1.0 % aq.). 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 carried out with a Gilson HPLC system coupled to a Phenomenex Gemini 5 μM 250 × 10 mm column and a GX281 fraction collector. Enantiomeric excesses (*ee*) were determined by chiral HPLC analysis [Daicell Chiralcel OD column (250 × 5.4 mm), hexane/2-propanol (99:1), flow rate: 1 mL/min, detection: UV, 254 nm]. All tested compounds are >95 % pure on the basis of LC–MS and NMR spectroscopy.

General Procedure for Azide Couplings: Compounds **5**–**14** were prepared by azide coupling of peptide hydrazides and appropriately deprotected vinyl sulfone amines. The appropriate hydrazide was dissolved in DMF or DMF/CH_2Cl_2 (1:1 v/v), and the mixture was cooled to –30 °C. *t*BuONO (1.1 equiv.) and HCl (4 M solution in 1,4-dioxane; 2.8 equiv.) were added, and the mixture was stirred for 3 h at –30 °C. After this time, TLC analysis (MeOH/CH_2Cl_2, 10 % 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 warmed to room temp. slowly overnight. The mixture was then diluted with EtOAc and washed with H_2O (3 ×). The organic layer was dried with $MgSO_4$, and purified by flash column chromatography (MeOH/CH_2Cl_2, 1–5 %) and HPLC (if necessary).

General Procedure for Peptide Couplings: The free acid (1.2 equiv.), HCTU (1.2 equiv.), and the free amine (1 equiv.) were dissolved in CH_2Cl_2 (0.1 M), and then DiPEA (3.5 equiv., or 4.5 equiv. in the case of 2-morpholinoacetic acid hydrochloride) was added. The mixture was stirred overnight (or alternatively 1–3 h, until completion), then the mixture was concentrated. The residue was redissolved in EtOAc, and this solution was washed with HCl (1 N aq.; 2 ×), sat. $NaHCO_3$ (2 ×), and brine [for the morpholinoacetic acid coupling, no HCl (1 N aq.) washings]. The organic layer was dried with Na_2SO_4, filtered, and concentrated, and the residue was purified by column chromatography.

General Procedure for Boc Removal: Boc-protected compounds were treated with TFA (0.1 M) for 30 min, followed by coevaporation with toluene (2 ×).

General Procedure for Fmoc Removal: Fmoc-protected compounds were dissolved in piperidine (20 % solution in DMF) and the mixture was stirred until the reaction was complete (about 30 min). The reaction mixture was then concentrated, and the residue was purified by column chromatography.

N_3-Phe-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 (MeOH/CH_2Cl_2, 0→2 %) provided the Boc-protected compound. This was deprotected using the standard procedure for Boc removal. Purification by HPLC followed by lyophilisation gave compound **5** (6.1 mg, 8.5 μmol, 14 %). ^1H NMR (600 MHz, CD_3OD): δ = 7.39–7.24 (m, 5 H), 6.89–6.85 (m, 1 H), 6.84–6.67 (m, 1 H), 5.97–5.82 (m, 1 H), 5.82–5.63 (m, 1 H), 4.73 (dtd, J = 9.1, 5.2, 1.6 Hz, 1 H), 4.42–4.30 (m, 2 H), 4.30–4.20 (m, 1 H), 3.60–3.55 (m, 2 H), 3.27 (dd, J = 14.0, 4.9 Hz, 1 H), 3.05 (dd, J = 14.1, 8.6 Hz, 1 H), 3.03 (s, 3 H), 2.58 (dddd, J = 10.7, 9.5, 5.4, 2.7 Hz, 1 H), 2.51–2.37 (m, 1 H), 1.79–1.53 (m, 6 H), 1.08–0.89 (m, 12 H) ppm. ^{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 ppm. LC–MS (linear gradient 10→90 % MeCN, 0.1 % TFA; 13.0 min): t_R (min) = 6.15; ESI-MS: m/z = 604.06 $[M + H]^+$. HRMS: calcd. for $C_{29}H_{46}N_7O_5S$ $[M + H]^+$ 604.32756; found 604.32751.

N_3-Phe-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 (MeOH/CH_2Cl_2, 0→2 %) provided the Boc-protected compound. This was deprotected using the standard procedure for Boc removal. Purification by HPLC followed by lyophilisation gave compound **6** (7.04 mg, 9.7 μmol, 20 %). This compound was isolated with 15 % of the *cis* isomer. The peaks reported correspond to the *trans* isomer. ^1H NMR (600 MHz, CD_3OD): δ = 7.35–7.19 (m, 5 H), 6.80 (dd, J = 15.2, 5.3 Hz, 1 H), 6.60 (dd, J = 15.2, 1.6 Hz, 1 H), 5.83–5.72 (m, 1 H), 5.72–5.54 (m, 1 H), 4.61 (dtd, J = 10.3, 5.1, 1.6 Hz, 1 H), 4.41 (t, J = 7.1 Hz, 1 H), 4.34 (dd, J = 10.0, 5.2 Hz, 1 H), 4.14 (dd, J = 8.6, 5.2 Hz, 1 H), 3.49 (d, J = 6.5 Hz, 2 H), 3.20 (dd, J = 13.9, 5.3 Hz, 1 H), 3.02–2.95 (m, 1 H), 2.98 (s, 3 H), 2.52 (dd, J = 13.8, 7.4 Hz, 1 H), 2.44 (q, J = 6.8 Hz, 1 H), 1.77–1.41 (m, 6 H), 1.04–0.86 (m, 12 H) ppm. ^{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 ppm. LC–MS (linear gradient 10→90 % MeCN, 0.1 % TFA, 13.0 min): t_R (min) = 6.12; ESI-MS: m/z = 604.13 $[M + H]^+$. HRMS: calcd. for $C_{29}H_{46}N_7O_5S$ $[M + H]^+$ 604.32756; found 604.32758.

N_3-Phe-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 (MeOH/CH_2Cl_2, 0→2 %) provided the Boc-protected compound. This was deprotected using the standard procedure for Boc removal. Purification by HPLC followed by lyophilisation gave compound **7** (8.6 mg,

10.2 μmol, 20 %). ^{1}H NMR (600 MHz, CD_3OD): δ = 7.39–7.33 (m, 2 H), 7.33–7.24 (m, 3 H), 6.87 (dd, *J* = 15.2, 5.1 Hz, 1 H), 6.70 (dd, *J* = 15.2, 1.7 Hz, 1 H), 5.97–5.79 (m, 2 H), 5.77–5.64 (m, 2 H), 4.71 (dtd, *J* = 9.0, 5.6, 1.7 Hz, 1 H), 4.42 (dd, *J* = 8.0, 6.6 Hz, 1 H), 4.37–4.30 (m, 1 H), 4.23–4.15 (m, 1 H), 3.56 (dd, *J* = 19.1, 6.6 Hz, 4 H), 3.25 (dd, *J* = 14.0, 5.2 Hz, 1 H), 3.04 (s, 3 H), 3.07–3.01 (m, 1 H), 2.63–2.54 (m, 2 H), 2.54–2.42 (m, 2 H), 1.81–1.67 (m, 2 H), 1.58 (ddd, *J* = 13.8, 8.9, 4.6 Hz, 1 H), 1.04 (d, *J* = 6.5 Hz, 3 H), 0.99 (d, *J* = 6.5 Hz, 3 H) ppm. ^{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 ppm. LC–MS (linear gradient 10→90 % MeCN, 0.1 % TFA; 13.0 min): t_R (min) = 4.77/4.84 (different salt forms); ESI-MS: *m/z* = 617.07 $[M + H]^+$. HRMS: calcd. for $C_{29}H_{45}N_8O_5S$ $[M + H]^+$ 617.32281; found 617.32275.

N_3-Phe-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 ($MeOH/CH_2Cl_2$, 0→1.5 %) provided the Boc-protected compound. This was deprotected using the standard procedure for Boc removal. Lyophilisation gave compound **8** (20.4 mg, 28.5 μmol, 92 %) as a white powder. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.35–7.20 (m, 5 H), 6.87 (dd, *J* = 15.3, 5.0 Hz, 1 H), 6.73 (dd, *J* = 15.2, 1.5 Hz, 1 H), 4.81–4.71 (m, 1 H), 4.35 (dd, *J* = 10.1, 4.5 Hz, 2 H), 4.19 (tt, *J* = 8.6, 4.9 Hz, 1 H), 3.22 (dd, *J* = 14.1, 4.8 Hz, 1 H), 3.04–2.93 (m, 4 H), 2.77–2.56 (m, 2 H), 1.81–1.47 (m, 6 H), 1.03–0.80 (m, 12 H) ppm. ^{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 ppm. LC–MS (linear gradient 10→90 % MeCN, 0.1 % TFA; 12.5 min): t_R (min) = 6.84; ESI-MS: *m/z* = 602.70 $[M + H]^+$. HRMS: calcd. for $C_{29}H_{44}N_7O_5S$ $[M + H]^+$ 602.31191; found 602.31195.

N_3-Phe-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 ($MeOH/CH_2Cl_2$, 0→1.5 %) provided the Boc-protected compound. This was deprotected using the standard procedure for Boc removal, and lyophilisation gave compound **9** (34.2 mg, 48 μmol, 53 %) as a white powder. This compound was isolated with 10 % of the *cis* isomer. The peaks reported correspond to the *trans* isomer. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.40–7.15 (m, 5 H), 6.79 (dd, *J* = 15.2, 5.2 Hz, 1 H), 6.60 (dd, *J* = 15.2, 1.4 Hz, 1 H), 4.68–4.58 (m, 1 H), 4.52 (t, *J* = 7.1 Hz, 1 H), 4.38 (dd, *J* = 9.6, 5.3 Hz, 1 H), 4.15 (dd, *J* = 8.6, 5.2 Hz, 1 H), 3.74 (s, 2 H), 3.20 (dd, *J* = 13.9, 5.3 Hz, 1 H), 3.03–2.91 (m, 4 H), 2.74–2.46 (m, 2 H), 1.81–1.42 (m, 6 H), 1.06–0.85 (m, 12 H) ppm. ^{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 ppm. LC–MS (linear gradient 10→90 % MeCN, 0.1 % TFA; 13.0 min): t_R (min) = 6.78; ESI-MS: *m/z* = 602.7 $[M + H]^+$. HRMS: calcd. for $C_{29}H_{44}N_7O_5S$ $[M + H]^+$ 602.31191; found 602.31171.

N_3-Phe-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 ($MeOH/CH_2Cl_2$, 0→1.5 %) provided the Boc-protected compound. This was deprotected using the standard procedure for Boc removal, and lyophilisation gave compound **10** (29.2 mg, 34.7 μmol, 79 %) as a white powder. This compound was isolated with 10 % of the *cis* isomer. The peaks reported correspond to the *trans* isomer. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.36–7.20 (m, 5 H), 6.88 (dd, *J* = 15.2, 5.1 Hz, 1 H), 6.74 (dd, *J* = 15.2, 1.5 Hz, 1 H), 4.82–4.70 (m, 1 H), 4.53 (t, *J* = 7.2 Hz, 1 H), 4.37 (dd, *J* = 10.3, 4.9 Hz, 1 H), 4.16 (dd, *J* = 8.6, 5.3 Hz, 1 H), 3.81–3.69 (m, 4 H), 3.21 (dd, *J* = 13.9, 5.2 Hz, 1 H), 3.05–2.94 (m, 4 H), 2.79–2.56 (m, 2 H), 1.81–1.63 (m, 2 H), 1.63–1.51 (m, 1 H), 1.02–0.88 (m, 6 H) ppm. ^{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 ppm. LC–MS (linear gradient 10→90 % MeCN, 0.1 % TFA; 13.0 min): t_R (min) = 5.51; ESI-MS: *m/z* = 613.7 $[M + H]^+$. HRMS: calcd. for $C_{29}H_{41}N_8O_5S$ $[M + H]^+$ 614.29934; found 614.29935.

N_3-Phe-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 ($MeOH/CH_2Cl_2$, 0→4 %) provided the Boc-protected product. This was deprotected using the standard procedure for Boc removal. Purification by HPLC (30–50 % MeCN, 0.1 % TFA, 10 min gradient) followed by lyophilisation gave compound **11** (3.16 mg, 8.6 %) as a white powder. ^{1}H NMR (600 MHz, CD_3OD): δ = 7.34–7.22 (m, 5 H), 6.83 (dd, *J* = 15.3, 5.1 Hz, 1 H), 6.72 (dd, *J* = 15.3, 1.6 Hz, 1 H), 4.80 (dtd, *J* = 8.2, 5.3, 1.4 Hz, 1 H), 4.36 (dd, *J* = 9.4, 5.3 Hz, 1 H), 4.27 (dd, *J* = 10.2, 4.8 Hz, 1 H), 4.21 (dd, *J* = 8.4, 5.0 Hz, 1 H), 3.66 (d, *J* = 3.5 Hz, 2 H), 3.56 (dd, *J* = 13.7, 5.4 Hz, 1 H), 3.41 (dd, *J* = 13.8, 8.6 Hz, 1 H), 3.23 (dd, *J* = 14.0, 5.0 Hz, 1 H), 3.04–2.98 (m, 4 H), 1.75–1.64 (m, 2 H), 1.61–1.52 (m, 4 H), 1.02–0.88 (m, 12 H) ppm. ^{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 ppm. LC–MS (linear gradient 10→90 % MeCN, 0.1 % TFA; 13.0 min): t_R (min) = 5.97; ESI-MS: *m/z* = 621.33 $[M + H]^+$. HRMS: calcd. for $C_{28}H_{45}N_8O_6S$ $[M + H]^+$ 621.31773; found 621.31744.

N_3-Phe-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 ($MeOH/CH_2Cl_2$, 0→4 %) provided the Boc-protected product. This was deprotected using the standard procedure for Boc removal. Purification by HPLC (30–50 % MeCN, 0.1 % TFA, 10 min gradient) followed by lyophilisation gave compound **12** (7.71 mg, 21 %) as a white powder. This compound was isolated with 10 % of the *cis* isomer. The peaks reported correspond to the *trans* isomer. ^{1}H NMR (600 MHz, CD_3OD): δ = 7.33–7.23 (m, 5 H), 6.83 (dd, *J* = 15.2, 5.4 Hz, 1 H), 6.64 (dd, *J* = 15.2, 1.5 Hz, 1 H), 4.64 (ddt, *J* = 10.3, 5.2, 2.6 Hz, 1 H), 4.53 (t, *J* = 6.1 Hz, 1 H), 4.37 (t, *J* = 7.6 Hz, 1 H), 4.19–4.14 (m, 1 H), 3.67–3.64 (m, 2 H), 3.63–3.57 (m, 1 H), 3.53 (dd, *J* = 13.9, 5.6 Hz, 1 H), 3.23 (dd, *J* = 13.9, 5.2 Hz, 1 H), 3.00 (d, *J* = 8.9 Hz, 1 H), 2.99 (s, 3 H), 1.75–1.67 (m, 2 H), 1.67–1.60 (m, 3 H), 1.48 (ddd, *J* = 13.9, 9.0, 5.2 Hz, 1 H), 1.00 (d, *J* = 6.5 Hz, 3 H), 0.97 (d, *J* = 6.6 Hz, 3 H), 0.96–0.92 (m, 6 H) ppm. ^{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 ppm. LC–MS (linear gradient 10→90 % MeCN, 0.1 % TFA; 13.0 min): t_R (min) = 5.86; ESI-MS: *m/z* = 621.27 $[M + H]^+$. HRMS: calcd. for $C_{28}H_{45}N_8O_6S$ $[M + H]^+$ 621.31773; found 621.31757.

N_3-Phe-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 ($MeOH/CH_2Cl_2$, 0→4 %) provided the Boc-protected product. This was deprotected using the standard procedure for Boc removal. Purification by HPLC (10–50 % MeCN, 0.1 % TFA, 10 min gradient) followed by lyophilisation gave compound **13** (2.93 mg, 6.7 %) as a white powder. ^{1}H NMR (600 MHz, CD_3OD): δ = 7.36–7.22 (m, 5 H), 6.84 (dd, *J* = 15.3, 5.0 Hz, 1 H), 6.72 (dd, *J* = 15.3, 1.6 Hz, 1 H), 4.90–4.83 (m, 1 H), 4.48 (t, *J* = 6.8 Hz, 1 H), 4.30 (dd, *J* = 10.6, 4.6 Hz, 1 H), 4.17 (dd, *J* = 8.4, 5.5 Hz, 1 H), 3.79–3.67 (m, 5 H), 3.58 (dd, *J* = 13.7, 5.6 Hz, 1 H), 3.42

(dd, *J* = 7.5, 4.8 Hz, 1 H), 3.39 (dd, *J* = 7.5, 4.8 Hz, 1 H), 3.22 (dd, *J* = 13.9, 5.5 Hz, 1 H), 3.04–2.97 (m, 4 H), 1.76–1.63 (m, 2 H), 1.59 (ddd, *J* = 13.9, 9.4, 4.6 Hz, 1 H), 1.00 (d, *J* = 6.5 Hz, 3 H), 0.95 (d, *J* = 6.5 Hz, 3 H) ppm. ^{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 ppm. LC–MS (linear gradient 10→90 % MeCN, 0.1 % TFA; 13.0 min): t_R (min) = 4.66; ESI-MS: *m*/*z* = 651.27 $[M + H]^+$. HRMS: calcd. for $C_{28}H_{45}N_8O_6S$ $[M + H]^+$ 651.30314; found 651.30286.

N_3-Phe-Leu-Leu-His-VS (14): N_3-Phe-Leu-Leu-His(Trt)-VS **57** (45 mg, 53 µmol, 1 equiv.) was dissolved in CH_2Cl_2 (3 mL), and TFA (30 µL) and triisopropylsilane (TIPS; 75 µL) were added. After 30 min, TLC showed no formation of product, so further TFA (30 µL) was added. After 30 min, still no product formation was observed, so TFA (1 mL) and TIPS (75 µL) were added. After a further 1 h, TLC analysis revealed that the reaction was complete. The mixture was diluted with toluene and then concentrated. Purification by column chromatography (MeOH/CH_2Cl_2, 1–8 %) followed by lyophilisation gave the product (28 mg, 38 %) as a white powder. This compound was isolated with 7 % of the *cis* isomer. The peaks reported correspond to the *trans* isomer. ^{1}H NMR (600 MHz, CD_3OD): δ = 8.08 (s, 1 H), 7.35–7.19 (m, 5 H), 7.10 (s, 1 H), 6.84 (dd, *J* = 15.2, 5.1 Hz, 1 H), 6.63 (dd, *J* = 15.2, 1.5 Hz, 1 H), 4.90–4.85 (m, 1 H), 4.41–4.34 (m, 1 H), 4.28 (dd, *J* = 9.8, 5.1 Hz, 1 H), 4.18 (dd, *J* = 8.5, 5.0 Hz, 1 H), 3.21 (dd, *J* = 14.0, 4.9 Hz, 1 H), 3.09–3.03 (m, 1 H), 3.00 (dd, *J* = 14.3, 8.3 Hz, 2 H), 2.96 (s, 3 H), 1.70–1.47 (m, 6 H), 0.97 (d, *J* = 6.4 Hz, 3 H), 0.95–0.89 (m, 9 H) ppm. ^{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 ppm. LC–MS (linear gradient 10→90 % MeCN, 0.1 % TFA; 13.0 min): t_R (min) = 6.22; ESI-MS: *m*/*z* = 615.20 $[M + H]^+$. HRMS: calcd. for $C_{29}H_{43}N_8O_5S$ $[M + H]^+$ 615.30716; 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 CH_2Cl_2 (2000 mL) and pyridine (129 mL, 1.6 mol), and then 4-toluenesulfonyl chloride (76.2 g, 400 mmol, 0.5 equiv.) was added portionwise over 15 min. After 2 h, TLC (EtOAc/pentane, 50 %) confirmed that the reaction was complete. The mixture was washed with HCl (1 M aq.; 3 ×), and brine (3 ×), dried with Na_2SO_4, filtered, and concentrated. Purification by column chromatography yielded compound **16** (60.7 g, 253 mmol, 63 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (d, *J* = 8.2 Hz, 2 H), 7.34 (d, *J* = 8.1 Hz, 2 H), 4.69 (s, 2 H), 4.12 (s, 2 H), 3.12 (s, 1 H), 2.41 (s, 3 H) ppm. ^{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 ppm.

1-(*tert*-Butoxycarbonylamino)-4-hydroxy-2-butyne (17): Alcohol **16** (58.29 g, 243 mmol, 1 equiv.) was dissolved in ammonium hydroxide (25 % NH_3 in H_2O; 450 mL), resulting in the immediate formation of a white precipitate. After 1 h, TLC (EtOAc/pentane, 50 %) confirmed that the reaction was complete. The ammonium hydroxide was removed in vacuo, and the mixture was coevaporated with toluene (2 ×).

The resulting solid was dissolved in THF (950 mL), and di-*tert*-butyl dicarbonate (63.53 g, 291 mmol, 1.2 equiv.) was added. The solution was cooled to 0 °C, and then triethylamine (40.6 mL, 291 mmol, 1.2 equiv.) was added slowly over 20 min. The mixture was stirred overnight, then the reaction mixture was concentrated. The residue was redissolved in CH_2Cl_2 (500 mL) and washed with water (3 ×). The aqueous layer was back-extracted twice with CH_2Cl_2. The combined organic layers were washed with brine (1 ×), dried with Na_2SO_4, filtered, and concentrated. Purification by column chromatography (EtOAc/*n*-pentane, 10→25 %) yielded compound **17** (19.04 g, 102.8 mmol, 42 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.25 (s, 1 H), 4.26 (s, 2 H), 3.95 (s, 2 H), 3.71 (s, 1 H), 1.45 (s, 9 H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ = 155.76, 81.53, 80.12, 50.58, 30.59, 28.35 ppm.

1-(*tert*-Butoxycarbonylamino)-4-bromo-2-butyne (18): Alcohol **17** (9.26 g, 50 mmol, 1 equiv.) was dissolved in CH_2Cl_2 (79 mL), and the mixture was cooled to 0 °C. Triphenylphosphine (19.67 g, 75 mmol, 1.5 equiv.) was added, followed by the portionwise addition of tetrabromomethane (3.90 g, 11.8 mmol, 1.5 equiv.). The mixture was stirred for 2 h, then it was concentrated. Purification by column chromatography (EtOAc/pentane, 1→5 %) yielded compound **18** (8.50 g, 34.2 mmol, 68 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.00 (s, 1 H), 3.99 (s, 2 H), 3.92 (s, 2 H), 1.46 (s, 9 H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ = 155.31, 83.32, 80.02, 30.67, 28.35, 14.48 ppm.

***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 this solution was added dropwise over 15 min to a solution of $LiAlH_4$ (1.95 g, 51.2 mmol, 1.2 equiv.) in THF (400 mL) at 0 °C. After the addition was complete, the solution was heated to reflux and stirred for 2 h, after which TLC analysis (EtOAc/pentane, 50 %) confirmed that the reaction was complete. The reaction was quenched with KOH (3 M aq.) solution until no further gas evolution was observed. The mixture was then diluted with EtOAc (100 mL), washed with HCl (1 M aq.; 3 ×), $NaHCO_3$ (3 ×), and brine (1 ×), dried with Na_2SO_4, filtered, and concentrated. Purification by column chromatography (EtOAc/pentane, 10→30 %) yielded compound **19** (3.00 g, 16.05 mmol, 38 %) as the pure *E* isomer. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.72 (dt, 2 H), 4.89 (s, 1 H), 4.09 (dd, *J* = 4.9, 1.3 Hz, 2 H), 3.70 (d, *J* = 5.1 Hz, 2 H), 2.93 (s, 1 H), 1.42 (tt, *J* = 15.5, 5.2 Hz, 9 H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ = 156.03, 130.87, 128.04, 79.51, 62.65, 41.99, 28.44 ppm.

1-(*tert*-Butoxycarbonylamino)-4-bromo-2-butene (20): Alcohol **19** (3.00 g, 16.1 mmol, 1 equiv.) was dissolved in dry CH_2Cl_2 (160 mL), and triphenylphosphine (6.31 g, 24.08 mmol, 1.5 equiv.) was added. The solution was cooled to 0 °C, then tetrabromomethane (7.99 g, 24.1 mmol, 1.5 equiv.) was added slowly and portionwise. The mixture was stirred for 1 h, then it was concentrated. Purification by column chromatography (EtOAc/pentane, 0→10 %) yielded compound **20** (2.32 g, 9.28 mmol, 58 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.02–5.65 (m, 2 H), 4.66 (s, 1 H), 3.97 (d, *J* = 6.6 Hz, 2 H), 3.79 (s, 2 H), 1.47 (s, 9 H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ = 132.22, 127.71, 41.72, 32.15, 28.51 ppm.

***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 (7:3 v/v; 31.5 mL), and the solution was cooled to 0 °C. A KOH solution (50 % aq. w/w; 13.5 mL) that had been cooled to 4 °C was then added dropwise. The mixture was stirred over two nights at 4 °C, and the progress of the reaction was monitored by TLC (EtOAc/pentane, 15 %). The solution was then diluted with EtOAc, washed with water (1 ×), and brine (1 ×), dried with Na_2SO_4, filtered, and concentrated. Purification by column chromatography (EtOAc/pentane, 0→10 %) yielded compound **22** (2.98 g, 6.44 mmol, 84 %, 79.3 % *ee*) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.67–7.29 (m, 8 H), 7.16 (dd, *J* = 7.3, 2.4 Hz, 2 H), 5.60–5.42 (m, 2 H), 4.46 (s, 1 H), 3.98 (t, *J* = 6.4 Hz, 1 H), 3.65 (s, 2 H), 2.60 (t, *J* = 5.4 Hz, 2 H), 1.44 (s, 9 H), 1.41 (s, 9 H) ppm. ^{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 ppm.

***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 (7:3 v/v; 27 mL), and the solution was cooled to 0 °C. A KOH solution (50 % aq. w/w; 11.5 mL) that had been cooled to 4 °C was added dropwise. The mixture was stirred over two nights at 4 °C, and the progress of the reaction was monitored by TLC (EtOAc/pentane, 5 %). The solution was then diluted with EtOAc, washed with water (1 ×), and brine (1 ×), dried with Na_2SO_4, filtered, and concentrated. Purification by column chromatography (EtOAc/pentane, 1→10 %) yielded compound **23** (2.98 g, 6.44 mmol, 84 %, 80.2 % *ee*) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.75–7.19 (m, 10 H), 4.74 (s, 1 H), 4.16 (dt, *J* = 9.0, 4.5 Hz, 1 H), 3.92–3.77 (m, 2 H), 2.90–2.66 (m, 2 H), 1.46 (s, 9 H), 1.42 (s, 9 H) ppm. ^{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 ppm.

H-Lys(4-ene)(Boc)-O*t*Bu (24): Compound **22** (2.74 g, 5.90 mmol, 1 equiv.) was dissolved in THF (33 mL), and the solution was cooled to 0 °C. A citric acid solution (15 % aq. w/w; 38 mL) was added, and the precipitation of a white solid was observed. The ice bath was removed, and the reaction mixture was warmed to room temp. The mixture was stirred for 2 h, during which time the solution turned clear again. The reaction was monitored by TLC (EtOAc/pentane, 10 %), and it was quenched with a sat. aq. K_2CO_3 solution until no further gas evolution was observed (approx. 20 mL). The mixture was diluted with EtOAc, and washed with water (2 ×), and brine (1 ×). The organic layer was dried with Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography (MeOH/CH_2Cl_2, 0→5 %) to give compound **24** (yield given over two steps, see synthesis of **26**). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.71–5.40 (m, 2 H), 4.75 (s, 1 H), 3.70 (t, *J* = 5.5 Hz, 2 H), 3.40 (t, *J* = 6.0 Hz, 1 H), 2.58–2.18 (m, 2 H), 1.68 (s, 2 H), 1.46 (s, 9 H), 1.44 (s, 9 H) ppm. ^{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 ppm.

H-Lys(4-yne)(Boc)-O*t*Bu (25): Compound **23** (5.02 g, 10.9 mmol, 1 equiv.) was dissolved in THF (59 mL), and the solution was cooled to 0 °C. A citric acid solution (15 % aq. w/w; 69 mL) was added, and the precipitation of a white solid was observed. The ice bath was removed, and the reaction mixture was warmed to room temp. The mixture was stirred for 2 h, during which time the solution turned clear again. The reaction was monitored by TLC (MeOH/CH_2Cl_2, 5 %), and it was quenched with a sat. aq. K_2CO_3 solution until no further gas evolution was observed (approx. 20 mL). The mixture was diluted with EtOAc, and washed with water (2 ×), and brine (1 ×). The organic layer was dried with Na_2SO_4, filtered, and concentrated. The residue was purified by column chromatography (MeOH/CH_2Cl_2, 0→5 %) to give compound **25** (2.94 g, 9.87 mmol, 91 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.92 (s, 1 H), 3.89 (d, *J* = 5.3 Hz, 2 H), 3.48 (t, *J* = 5.5 Hz, 1 H), 2.65–2.50 (m, 2 H), 1.75 (s, 2 H), 1.47 (s, 9 H), 1.44 (s, 9 H) ppm. ^{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 ppm.

Fmoc-Lys(4-ene)(Boc)-O*t*Bu (26): H-Lys(4-ene)(Boc)-O*t*Bu **24** (5.90 mmol, 1 equiv.) was dissolved in dry CH_2Cl_2 (60 mL). FmocOSu (2.38 g, 7.08 mmol, 1.2 equiv.) was added, followed by the dropwise addition of DiPEA (1.2 mL, 7.08 mmol, 1.2 equiv.). The mixture was stirred overnight, then it was diluted with EtOAc, and washed with HCl (1 M aq.; 1 ×), sat. aq. $NaHCO_3$ (2 ×), and brine (1 ×). The organic layer was dried with Na_2SO_4, filtered, and concentrated. Purification by column chromatography (EtOAc/pentane, 0→20 %) yielded compound **26** (2.56 g, 4.91 mmol, 83 % over two steps) as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (d, *J* = 7.5 Hz, 2 H), 7.60 (d, *J* = 7.5 Hz, 2 H), 7.40 (t, *J* = 7.5 Hz, 2 H), 7.32 (t, *J* = 7.4 Hz, 2 H), 5.63–5.44 (m, 2 H), 5.39 (d, *J* = 8.1 Hz, 1 H), 4.56 (s, 1 H), 4.44–4.35 (m, 2 H), 4.22 (t, *J* = 7.0 Hz, 1 H), 3.69 (s, 2 H), 2.64–2.40 (m, 2 H), 1.47 (s, 9 H), 1.43 (s, 9 H) ppm. ^{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 ppm. $[\alpha]_D^{20}$ = 17.6 (*c* = 1, $CHCl_3$). LC–MS (linear gradient 10→90 % MeCN, 0.1 % TFA; 13.0 min): t_R (min) = 10.95; ESI-MS: *m*/*z* = 523.80 $[M + H]^+$. HRMS: calcd. for $C_{30}H_{38}N_2O_6$, $[M + H]^+$ 523.27579; found 523.27997.

Fmoc-Lys(4-yne)(Boc)-O*t*Bu (27): H-Lys(4-yl)(Boc)-O*t*Bu **25** (2.89 g, 9.68 mmol, 1 equiv.) was dissolved in dry CH_2Cl_2 (97 mL). FmocOSu (3.92 g, 11.6 mmol, 1.2 equiv.) was added, followed by the dropwise addition of DiPEA (2.0 mL, 11.6 mmol, 1.2 equiv.). The mixture was stirred overnight, then it was diluted with EtOAc, and washed with HCl (1 M aq.; 1 ×), sat. aq. $NaHCO_3$ (2 ×), and brine (1 ×). The organic layer was dried with Na_2SO_4, filtered, and concentrated. Purification by column chromatography (EtOAc/*n*-pentane, 0→20 %) yielded compound **27** (4.94 g, 9.48 mmol, 98 %) as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.80–7.74 (m, 2 H), 7.62 (d, *J* = 7.4 Hz, 2 H), 7.40 (t, *J* = 7.5 Hz, 2 H), 7.32 (tt, *J* = 7.4, 1.4 Hz, 2 H), 5.67 (d, *J* = 8.0 Hz, 1 H), 4.68 (s, 1 H), 4.44–4.34 (m, 3 H), 4.24 (t, *J* = 7.2 Hz, 1 H), 3.88 (s, 2 H), 2.73 (dt, *J* = 4.9, 2.3 Hz, 2 H), 1.49 (s, 9 H), 1.44 (s, 9 H) ppm. ^{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 ppm. $[\alpha]_D^{20}$ = 23.0 (*c* = 1, $CHCl_3$). LC–MS (linear gradient 10→90 % MeCN, 0.1 % TFA; 13.0 min): t_R (min) = 9.22; ESI-MS: *m*/*z* = 520.87 $[M + H]^+$. HRMS: calcd. for $C_{30}H_{37}N_2O_6$ $[M + H]^+$ 521.26461; found 521.26459.

Fmoc-Lys(4-ene)(Boc)-OH (28): Fmoc-L-Lys(4-ene)(Boc)-O*t*Bu **26** (2.48 g, 4.76 mmol, 1 equiv.) was dissolved in 100 % TFA (47.6 mL), and the mixture was stirred for 2 h. After this time, TLC analysis (EtOAc/pentane, 10 %) showed that the reaction was complete; TLC–MS and HPLC–MS analysis was used to ensure that ester hydrolysis was complete, and not only removal of the Boc group. The reaction mixture was concentrated and coevaporated with toluene (3 ×).

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. The mixture was stirred overnight, and then the reaction mixture was concentrated. The residue was dissolved in EtOAc, washed with HCl (0.1 M aq.; 2 ×), water (2 ×), and brine (2 ×), dried with Na_2SO_4, filtered, and concentrated. Purification by column chromatography (MeOH/CH_2Cl_2, 0→2 %) yielded compound **28** (1.82 g, 3.90 mmol, 82 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.74 (t, *J* = 7.9 Hz, 2 H), 7.56 (d, *J* = 7.9 Hz, 2 H), 7.37 (t, *J* = 7.7 Hz, 2 H), 7.31 (d, *J* = 8.2 Hz, 2 H), 6.94 (s, 1 H), 6.74 (d, *J* = 8.6 Hz, 1 H), 5.55 (dt, *J* = 15.8, 9.2 Hz, 2 H), 4.60 (s, 1 H), 4.43 (q, *J* = 12.0, 11.4 Hz, 2 H), 4.23 (t, 1 H), 4.13 (t, *J* = 6.6 Hz, 1 H), 3.63 (d, *J* = 48.0 Hz, 2 H), 2.79–2.46 (m, 2 H), 1.52 (s, 9 H) ppm. ^{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 ppm.

Fmoc-Lys-(4-yne)(Boc)-OH (29): Fmoc-Lys(4-yl)(Boc)-O*t*Bu **27** (4.93 g, 9.48 mmol, 1 equiv.) was dissolved in 100 % TFA (95 mL), and the mixture was stirred for 2 h. After this time, TLC analysis (EtOAc/pentane, 10 %) showed that the reaction was complete; TLC–MS and HPLC–MS analysis was used to ensure that ester hydrolysis was complete, and not only removal of the Boc group.

The reaction mixture was concentrated and coevaporated with toluene (3 ×).

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. The pH was adjusted until it was basic by the addition of DiPEA (1 mL). The mixture was stirred overnight, then it was concentrated. The residue was dissolved in EtOAc, and this solution was washed with HCl (0.1 M aq.; 2 ×), water (2 ×), and brine (2 ×), dried with Na_2SO_4, filtered, and concentrated. Purification by column chromatography ($MeOH/CH_2Cl_2$, 0→1 %) yielded compound **29** (2.80 g, 6.02 mmol, 63 %). ^1H NMR (400 MHz, $CDCl_3$): δ = 7.73 (d, J = 7.5 Hz, 2 H), 7.57 (dd, J = 17.7, 7.4 Hz, 2 H), 7.48 (d, J = 4.1 Hz, 1 H), 7.36 (d, J = 7.5 Hz, 2 H), 7.28 (d, J = 7.2 Hz, 2 H), 4.72–4.60 (m, 1 H), 4.43–4.33 (m, 1 H), 4.30–4.13 (m, 2 H), 3.93–3.72 (m, 2 H), 2.89 (d, J = 4.2 Hz, 2 H), 1.51 (s, 9 H) ppm. ^{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 ppm.

Fmoc-Lys(4-ene)(Boc)-N(OMe)Me (30): Compound **30** was prepared by the general procedure for peptide coupling on a 0.5 mmol scale. Purification by column chromatography (EtOAc/pentane, 10→40 %) yielded compound **30** (quantitative yield). ^1H NMR (400 MHz, $CDCl_3$): δ = 7.76 (d, J = 7.5 Hz, 2 H), 7.60 (t, J = 7.0 Hz, 2 H), 7.40 (t, J = 7.6 Hz, 2 H), 7.31 (tdd, J = 7.4, 3.0, 1.2 Hz, 2 H), 5.64–5.51 (m, 3 H), 4.80 (q, J = 7.0 Hz, 1 H), 4.69–4.50 (m, 1 H), 4.47–4.27 (m, 2 H), 4.22 (t, J = 7.2 Hz, 1 H), 3.77 (s, 3 H), 3.71–3.63 (m, 2 H), 3.22 (s, 3 H), 2.57–2.44 (m, 1 H), 2.44–2.32 (m, 1 H), 1.43 (s, 9 H) ppm. ^{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 ppm.

Fmoc-Lys(4-yl)(Boc)-N(OMe)Me (31): Compound **31** was prepared by the general procedure for peptide coupling on a 3.0 mmol scale. Purification by column chromatography (EtOAc/pentane, 10→40 %) yielded compound **31** (1.29 g, 2.54 mmol, 85 %) as a white foam. ^1H NMR (400 MHz, $CDCl_3$): δ = 7.73 (d, J = 7.5 Hz, 2 H), 7.60 (t, J = 7.3 Hz, 2 H), 7.37 (td, J = 7.6, 1.4 Hz, 2 H), 7.32–7.25 (m, 2 H), 6.13 (d, J = 8.8 Hz, 1 H), 5.04 (br. s, 1 H), 4.90 (br. s, 1 H), 4.35 (dd, J = 7.4, 1.8 Hz, 2 H), 4.21 (t, J = 7.1 Hz, 1 H), 3.86 (br. s, 2 H), 3.72 (s, 3 H), 3.21 (s, 3 H), 2.79–2.55 (m, 2 H), 1.40 (s, 9 H) ppm. ^{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 ppm. LC–MS (linear gradient 10→90 % MeCN, 0.1 % TFA; 13.0 min): t_R (min) = 8.20; ESI-MS: m/z = 507.8 $[M + H]^+$. HRMS: calcd. for $C_{28}H_{33}N_3O_6$ $[M + H]^+$ 508.24421; 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 the solution was cooled to –30 °C. $LiAlH_4$ (2 M in THF; 0.25 mL, 0.5 mmol, 1 equiv.) was added dropwise. After 1 h, TLC analysis indicated that the reaction was complete. The reaction was quenched with HCl (1 M aq.; approx. 2 mL), and the mixture was diluted with EtOAc, and washed with HCl (1 M aq.; 2 ×) and brine (2 ×). The organic layer was dried with Na_2SO_4, filtered, and concentrated, and the crude product was 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 the solution was cooled to 0 °C. NaH (60 % w/w in mineral oil; 24 mg, 0.6 mmol, 1.2 equiv.) was then added. The mixture was stirred for 30 min, then a solution of the freshly obtained aldehyde in THF (5 mL) was added dropwise to the reaction mixture. After 2.5 h, the mixture was diluted with EtOAc, and washed with HCl (1 M aq.; 1 ×) and brine (1 ×). The organic layer was dried with Na_2SO_4, filtered, and concentrated. NMR spectroscopic analysis of the crude product indicated significant amounts of aldehyde remaining. Therefore, the reaction was repeated with diethyl[(methylsulfonyl)-methyl]phosphonate (0.7 equiv.) and NaH (0.5 equiv.). Purification by column chromatography (EtOAc/*n*-pentane, 10→40 %) yielded compound **32** (105 mg, 0.2 mmol, 40 %). ^1H NMR (400 MHz, $CDCl_3$): δ = 7.76 (d, J = 7.4 Hz, 2 H), 7.58 (dd, J = 7.3, 3.2 Hz, 2 H), 7.40 (t, J = 7.3 Hz, 2 H), 7.36–7.28 (m, 2 H), 6.83 (dd, J = 15.2, 4.6 Hz, 1 H), 6.44 (d, J = 15.1 Hz, 1 H), 5.65–5.40 (m, 2 H), 5.31–5.10 (m, 1 H), 4.80–4.59 (m, 1 H), 4.54–4.30 (m, 3 H), 4.20 (q, J = 6.6, 5.9 Hz, 1 H), 3.68 (q, J = 7.1, 6.0 Hz, 2 H), 2.91 (s, 3 H), 2.46–2.22 (m, 2 H), 1.43 (s, 9 H) ppm. ^{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 ppm.

Fmoc-Lys(4-yne)(Boc)-VS (33): Weinreb amide **31** (1.29 g, 2.54 mmol, 1 equiv.) was dissolved in dry Et_2O (26 mL), and the solution was cooled to –30 °C. $LiAlH_4$ (2 M in THF; 1.3 mL, 2.54 mmol, 1 equiv.) was added dropwise. After <10 min, TLC analysis indicated that the reaction was complete. The reaction was quenched with HCl (1 M aq.; approx. 10 mL), and the mixture was diluted with EtOAc, and washed with HCl (1 M aq.; 2 ×) and brine (2 ×). The organic layer was dried with Na_2SO_4, filtered, and concentrated, and the crude product was 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 the solution was cooled to 0 °C. NaH (60 % w/w in mineral oil; 0.12 g, 3.05 mmol, 1.2 equiv.) was then added. The mixture was stirred for 30 min, then a solution of the freshly obtained aldehyde in THF (10 mL) was added dropwise to the reaction mixture. After 1 h, TLC ($MeOH/CH_2Cl_2$, 2.5 %) indicated that the reaction was complete. The mixture was diluted with EtOAc, and washed with HCl (1 M aq.; 1 ×) and brine (1 ×). The organic layer was dried with Na_2SO_4, filtered, and concentrated. Purification by column chromatography (EtOAc/*n*-pentane, 10→40 %) yielded compound **33** (0.94 g, 1.79 mmol, 70 %). ^1H NMR (400 MHz, $CDCl_3$): δ = 7.76 (d, J = 7.5 Hz, 2 H), 7.60 (t, J = 7.3 Hz, 2 H), 7.40 (td, J = 7.5, 1.2 Hz, 2 H), 7.36–7.29 (m, 2 H), 6.88 (dd, J = 15.1, 4.8 Hz, 1 H), 6.55 (dt, J = 15.1, 1.6 Hz, 1 H), 5.58 (d, J = 8.6 Hz, 1 H), 4.93 (s, 1 H), 4.60 (s, 1 H), 4.44 (d, J = 7.0 Hz, 2 H), 4.21 (t, J = 6.7 Hz, 1 H), 3.87 (s, 2 H), 2.94 (s, 3 H), 2.75–2.41 (m, 2 H), 1.44 (s, 9 H) ppm. ^{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 ppm.

H-Lys(4-ene)(Boc)-VS (34): Diethylamine (0.85 mL) was added to a solution of vinyl sulfone **32** (86 mg, 0.16 mmol, 1 equiv.) in MeCN (2 mL). After 1 h, TLC analysis ($MeOH/CH_2Cl_2$, 2.5 %) showed that the reaction was complete. The mixture was diluted with toluene, evaporated to dryness, and coevaporated with toluene (2 ×). Purification by column chromatography ($MeOH/CH_2Cl_2$, 0→5 %) yielded compound **34** (37 mg, 0.12 mmol, 75 %) as a yellow oil. ^1H NMR (400 MHz, $CDCl_3$): δ = 6.94 (dd, J = 15.0, 4.8 Hz, 1 H), 6.60 (dd, J = 15.0, 1.3 Hz, 1 H), 5.67–5.47 (m, 2 H), 4.63 (s, 1 H), 3.76–3.61 (m, 3 H), 2.95 (s, 3 H), 2.43–2.31 (m, 1 H), 2.25–2.10 (m, 1 H), 1.57 (s, 2 H), 1.45 (s, 9 H) ppm. ^{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 ppm.

H-Lys(4-yne)(Boc)-VS (35): Diethylamine (8 mL) was added dropwise to a solution of vinyl sulfone **33** (0.94 g, 1.79 mmol, 1 equiv.) in MeCN (18 mL). After 1 h, TLC analysis ($MeOH/CH_2Cl_2$, 2.5 %) showed that the reaction was complete. The mixture was diluted with toluene (30 mL), evaporated to dryness, and coevaporated

with toluene (2 ×). Purification by column chromatography (MeOH/CH_2Cl_2, 0→5 %) yielded compound **35** (0.26 g, 0.84 mmol, 47 %) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.94 (dd, *J* = 15.3, 4.8 Hz, 1 H), 6.68 (d, *J* = 15.1 Hz, 1 H), 5.11 (br. s, 1 H), 3.89 (s, 2 H), 3.76 (d, *J* = 5.9 Hz, 1 H), 2.98 (s, 3 H), 2.46 (qd, *J* = 16.8, 5.9 Hz, 2 H), 1.76 (s, 2 H), 1.45 (s, 9 H) ppm. ^{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 ppm.

Fmoc-Lys(4-ene)(Boc)-Leu-OMe (36): Compound **36** was prepared by the general procedure for peptide coupling on a 0.5 mmol scale. Column chromatography (EtOAc/pentane, 0→30 %) gave compound **36** (285 mg, 0.48 mmol, 96 %). The product was isolated with 10 % of the minor diastereomer. The peaks reported are for the major diastereomer. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.74 (d, *J* = 7.5 Hz, 2 H), 7.57 (d, *J* = 6.3 Hz, 2 H), 7.41–7.35 (m, 2 H), 7.32–7.26 (m, 2 H), 6.86 (d, *J* = 8.1 Hz, 1 H), 5.81 (d, *J* = 8.2 Hz, 1 H), 5.65–5.49 (m, 2 H), 5.00 (t, *J* = 5.8 Hz, 1 H), 4.65–4.53 (m, 1 H), 4.46–4.24 (m, 3 H), 4.19 (t, *J* = 7.1 Hz, 1 H), 3.71 (s, 3 H), 3.69–3.56 (m, 2 H), 2.57–2.40 (m, 2 H), 1.71–1.50 (m, 3 H), 1.42 (s, 9 H), 0.88 (d, *J* = 4.2 Hz, 6 H) ppm. ^{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 ppm.

Fmoc-Lys(4-yne)(Boc)-Leu-OMe (37): Compound **37** was prepared by the general procedure for peptide coupling on a 0.6 mmol scale. Column chromatography (EtOAc/pentane, 10→30 %) gave compound **37** (220 mg, 0.38 mmol, 76 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.73 (d, *J* = 7.5 Hz, 2 H), 7.57 (dd, *J* = 7.7, 2.9 Hz, 2 H), 7.37 (t, *J* = 7.5 Hz, 2 H), 7.32–7.24 (m, 2 H), 6.03 (d, *J* = 7.9 Hz, 1 H), 5.28 (s, 1 H), 4.73–4.58 (m, 1 H), 4.51–4.27 (m, 3 H), 4.19 (t, *J* = 7.1 Hz, 1 H), 3.99–3.77 (m, 2 H), 3.71 (d, *J* = 6.8 Hz, 3 H), 2.89–2.40 (m, 2 H), 1.73–1.53 (m, 3 H), 1.42 (s, 9 H), 0.90 (t, *J* = 5.2 Hz, 6 H) ppm. ^{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 ppm.

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. Purification by column chromatography (MeOH/CH_2Cl_2, 0→3 %) gave compound **38** (161 mg, 0.43 mmol, 94 %). The product was isolated with 10 % of the minor diastereomer. The peaks reported are for the major diastereomer. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.64 (d, *J* = 8.6 Hz, 1 H), 5.58–5.49 (m, 2 H), 4.80 (s, 1 H), 4.57 (td, *J* = 8.8, 4.7 Hz, 1 H), 3.71 (s, 3 H), 3.69–3.61 (m, 2 H), 3.40 (dd, *J* = 8.3, 4.3 Hz, 1 H), 2.53 (dt, *J* = 13.6, 5.0 Hz, 1 H), 2.24 (dt, *J* = 13.5, 7.9 Hz, 1 H), 1.74 (s, 2 H), 1.68–1.52 (m, 3 H), 1.41 (s, 9 H), 0.92 (dd, *J* = 6.1, 3.4 Hz, 6 H) ppm. ^{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 ppm.

H-Lys(4-yne)(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. Purification by column chromatography (MeOH/CH_2Cl_2, 0→3 %) gave compound **39** (189 mg, 100 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.74 (d, *J* = 8.7 Hz, 1 H), 4.95 (s, 1 H), 4.70–4.51 (m, 1 H), 3.88 (s, 2 H), 3.74 (s, 3 H), 3.51 (t, *J* = 5.9 Hz, 1 H), 2.77–2.52 (m, 2 H), 1.89 (s, 2 H), 1.72–1.55 (m, 3 H), 1.44 (s, 9 H), 1.00–0.89 (m, 6 H) ppm. ^{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 ppm.

N_3-Phe-Lys(4-ene)(Boc)-Leu-OMe (40): Compound **40** was prepared by the general procedure for peptide coupling on a 0.38 mmol scale. Column chromatography (EtOAc/pentane, 0→50 %) provided compound **40** (124 mg, 0.23 mmol, 60 %). The product was isolated with 10 % of the minor diastereomer. The peaks reported are for the major diastereomer. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.39–7.24 (m, 5 H), 6.99 (d, *J* = 7.3 Hz, 1 H), 6.54 (d, *J* = 7.5 Hz, 1 H), 5.53–5.44 (m, 2 H), 4.88 (s, 1 H), 4.64–4.53 (m, 1 H), 4.38 (q, *J* = 7.3 Hz, 1 H), 4.24 (dd, *J* = 7.8, 4.2 Hz, 1 H), 3.77 (s, 3 H), 3.73–3.59 (m, 2 H), 3.32 (dd, *J* = 14.1, 4.2 Hz, 1 H), 3.08 (dd, *J* = 14.0, 7.8 Hz, 1 H), 2.46–2.37 (m, 1 H), 2.37–2.26 (m, 1 H), 1.64 (dtd, *J* = 16.3, 11.9, 10.4, 6.8 Hz, 3 H), 1.46 (s, 9 H), 0.95 (t, *J* = 5.3 Hz, 6 H) ppm. ^{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 ppm.

N_3-Phe-Lys(4-yne)(Boc)-Leu-OMe (41): Compound **41** was prepared by the general procedure for peptide coupling on a 0.23 mmol scale. Column chromatography (EtOAc/pentane, 0→40 %) provided compound **41** (111 mg, 0.21 mmol, 91 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.44–7.15 (m, 5 H), 7.00 (d, *J* = 7.8 Hz, 1 H), 5.22 (s, 1 H), 4.68–4.57 (m, 1 H), 4.57–4.46 (m, 1 H), 4.22 (dd, *J* = 8.0, 4.3 Hz, 1 H), 3.99–3.78 (m, 2 H), 3.75 (s, 3 H), 3.30 (dd, *J* = 14.0, 4.3 Hz, 1 H), 3.06 (dd, *J* = 14.1, 8.0 Hz, 1 H), 2.67–2.55 (m, 1 H), 2.37 (dd, *J* = 16.7, 8.7 Hz, 1 H), 1.75–1.56 (m, 3 H), 1.43 (s, 9 H), 1.00–0.89 (m, 6 H) ppm. ^{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 ppm.

N_3-Phe-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 dropwise. After 3 h, TLC analysis showed that the reaction was complete. The reaction mixture was evaporated to dryness, and coevaporated with toluene (3 ×) to give compound **42** (quantitative yield). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.38–7.19 (m, 5 H), 5.57–5.34 (m, 2 H), 4.43–4.32 (m, 2 H), 4.15 (dd, *J* = 8.6, 5.0 Hz, 1 H), 3.60 (qd, *J* = 16.1, 15.4, 4.4 Hz, 2 H), 3.19 (dd, *J* = 14.0, 5.1 Hz, 1 H), 2.97 (dd, *J* = 13.9, 8.6 Hz, 1 H), 2.50–2.26 (m, 2 H), 1.71–1.50 (m, 3 H), 1.43 (s, 9 H), 0.94 (dd, *J* = 16.7, 6.4 Hz, 6 H) ppm. ^{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 ppm.

N_3-Phe-Lys(4-yne)(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 dropwise. The solution was then heated at reflux at 80 °C for 1 h. The reaction mixture was evaporated to dryness, and coevaporated with toluene (3 ×) to give compound **43** (quantitative yield). NMR spectroscopic analysis could not be carried out due to poor solubility in chloroform, methanol, and mixtures thereof.

Fmoc-Dap(Boc)-N(OMe)Me (45): Compound **45** was prepared by the general procedure for peptide coupling on a 0.47 mmol scale, using *N*,*O*-dimethylhydroxylamine (2 equiv.). Column chromatography (EtOAc/pentane, 20–40 %) provided compound **45** (211 mg, 96 %). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.76 (d, *J* = 7.4 Hz, 2 H), 7.67–7.55 (m, 2 H), 7.40 (t, *J* = 7.4 Hz, 2 H), 7.31 (t, *J* = 7.6 Hz, 2 H), 5.93 (d, *J* = 6.1 Hz, 1 H), 4.96–4.83 (m, 1 H), 4.83–4.69 (m, 1 H), 4.39 (d, *J* = 7.0 Hz, 2 H), 4.21 (t, *J* = 6.9 Hz, 1 H), 3.78 (s, 3 H), 3.57 (s, 1 H), 3.44 (d, *J* = 13.2 Hz, 1 H), 3.22 (s, 3 H), 1.43 (s, 9 H) ppm. ^{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 ppm.

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 (EtOAc/pentane, 60→100 %) provided compound **46** (267 mg, 100 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.75 (d, J = 7.5 Hz, 2 H), 7.65–7.56 (m, 2 H), 7.39 (t, J = 7.4 Hz, 2 H), 7.35–7.27 (m, 2 H), 6.72 (s, 1 H), 6.12 (d, J = 6.9 Hz, 1 H), 5.20 (s, 1 H), 4.85 (s, 1 H), 4.37 (d, J = 7.0 Hz, 2 H), 4.20 (t, J = 7.0 Hz, 1 H), 3.77 (s, 3 H), 3.84–3.51 (m, 4 H), 3.20 (s, 3 H), 1.42 (s, 9 H) ppm. ^{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 ppm.

Fmoc-Dap(Gly-Boc)-VS (47): Fmoc-Dap(Gly-Boc)-N(OMe)Me **46** (157 mg, 0.3 mmol) was dissolved in THF (4 mL), and the solution was cooled to –20 °C. $LiAlH_4$ (2 M in THF; 150 µL, 1 equiv.) was added dropwise over 10 min. TLC analysis (MeOH/CH_2Cl_2, 3 %) revealed that the reaction was complete. The reaction was quenched by the addition of HCl (1 M aq.). EtOAc was added, and the layers were separated. The organic layer was washed with brine, dried with Na_2SO_4, filtered, and concentrated to give the crude aldehyde, which was directly used in the next step.

Diethyl[(methylsulfonyl)methyl]phosphonate (104 mg, 0.45 mmol, 1.5 equiv.) was dissolved in THF (4 mL), and the solution was cooled to 0 °C under an argon atmosphere. NaH (60 % w/w in mineral oil; 15.6 mg, 0.39 mmol, 1.3 equiv.) was slowly added, and the mixture was stirred at 0 °C for 30 min. Next, a solution of the freshly obtained aldehyde in THF (5 mL) was slowly added. The mixture was stirred for 1 h while slowly warming to room temp. After this time, TLC analysis indicated the complete conversion of the aldehyde. EtOAc was added, and the mixture was washed with HCl (1 M aq.; 2 ×) and brine, dried with Na_2SO_4, and concentrated. Column chromatography (EtOAc/pentane, 20→100 %) yielded compound **47** (105 mg, 43 %); the product contained 0.4 equiv. of diethyl[(methylsulfonyl)methyl] phosphonate, based on NMR spectroscopy. ^{1}H NMR (400 MHz, CD_3OD): δ = 7.79 (d, J = 7.5 Hz, 2 H), 7.65 (d, J = 7.2 Hz, 2 H), 7.40 (t, J = 7.4 Hz, 2 H), 7.33 (t, J = 7.3 Hz, 2 H), 6.82 (dd, J = 15.2, 5.1 Hz, 1 H), 6.59 (d, J = 15.3 Hz, 1 H), 4.55–4.39 (m, 3 H), 4.30–4.18 (m, 1 H), 3.69 (d, J = 4.9 Hz, 2 H), 3.43 (ddd, J = 42.5, 13.5, 7.0 Hz, 2 H), 2.99 (s, 3 H), 1.45 (s, 9 H) ppm.

H-Dap(Gly-Boc)-VS (48): Fmoc-Dap(Gly-Boc)-VS **47** (105 mg, 0.13 mmol) was dissolved in MeCN (2 mL), and the solution was cooled to 0 °C. Diethylamine (2 mL) was added, and the mixture was stirred for 1 h. The mixture was then concentrated, and the residue was purified by column chromatography (MeOH/CH_2Cl_2, 0→40 %) to give compound **48** (0.13 mmol, 100 %). ^{1}H NMR (400 MHz, CD_3OD): δ = 6.87 (dd, J = 15.2, 5.4 Hz, 1 H), 6.67 (d, J = 15.2 Hz, 1 H), 3.85–3.69 (m, 3 H), 3.50–3.23 (m, 2 H), 3.00 (s, 3 H), 1.45 (s, 9 H) ppm. ^{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 ppm.

Fmoc-Dap(Boc)-Leu-OMe (49): Compound **49** was prepared by the general procedure for peptide coupling on a 0.5 mmol scale. Column chromatography (EtOAc/pentane, 10→50 %) provided compound **49** (259 mg, 94 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.72 (d, J = 7.5 Hz, 2 H), 7.56 (d, J = 7.2 Hz, 2 H), 7.35 (t, J = 7.4 Hz, 2 H), 7.30–7.21 (m, 2 H), 7.15 (s, 1 H), 6.38 (s, 1 H), 5.38 (s, 1 H), 4.54 (s, 1 H), 4.32 (d, J = 7.0 Hz, 3 H), 4.18 (t, J = 7.2 Hz, 1 H), 3.67 (s, 3 H), 3.49 (s, 2 H), 1.58 (dq, J = 16.8, 8.7, 6.7 Hz, 3 H), 1.40 (s, 9 H), 0.87 (d, J = 5.5 Hz, 6 H) ppm. ^{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 ppm.

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 (EtOAc/pentane, 20→100 %) provided compound **50** (quantitative yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.73 (d, J = 7.5 Hz, 2 H), 7.56 (d, J = 7.4 Hz, 3 H), 7.47–7.40 (m, 1 H), 7.37 (t, J = 7.4 Hz, 2 H), 7.28 (d, J = 7.5 Hz, 2 H), 6.08 (d, J = 6.7 Hz, 1 H), 5.73 (t, J = 5.7 Hz, 1 H), 4.66–4.48 (m, 1 H), 4.44–4.26 (m, 3 H), 4.17 (t, J = 7.0 Hz, 1 H), 3.93–3.84 (m, 1 H), 3.82 (d, J = 5.8 Hz, 1 H), 3.75–3.62 (m, 4 H), 3.31–3.19 (m, 1 H), 1.75–1.58 (m, 3 H), 1.40 (s, 9 H), 0.88 (d, J = 5.7 Hz, 6 H) ppm. ^{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 ppm.

H-Dap(Gly-Boc)-Leu-OMe (51): Fmoc-Dap(Gly-Boc)-Leu-OMe **50** was deprotected using the standard procedure for Fmoc removal. Purification by column chromatography (EtOAc/pentane, 50 %, followed by MeOH/EtOAc, 0→10 %) gave compound **50** (189 mg, 100 %). Complex NMR spectra due to the presence of rotamers. Peaks of the major rotamer are reported. ^{1}H NMR (400 MHz, $CDCl_3$, CD_3OD): δ = 4.51 (t, J = 7.3 Hz, 1 H), 3.79–3.72 (m, 5 H), 3.58 (t, J = 6.3 Hz, 1 H), 3.55–3.46 (m, 1 H), 3.46–3.33 (m, 1 H), 1.70–1.51 (m, 3 H), 1.45 (s, 9 H), 1.01–0.91 (m, 6 H) ppm. ^{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 ppm.

N_3-Phe-Dap(Gly-Boc)-Leu-OMe (52): Compound **52** was prepared by the general procedure for peptide coupling on a 0.5 mmol scale. Column chromatography (EtOAc/pentane, 20→80 %) provided compound **52** (192 mg, 68 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.71 (d, J = 7.8 Hz, 1 H), 7.40 (d, J = 6.9 Hz, 2 H), 7.31–7.20 (m, 5 H), 5.83 (t, J = 5.7 Hz, 1 H), 4.65–4.46 (m, 2 H), 4.17 (dd, J = 8.4, 4.0 Hz, 1 H), 3.84 (dd, J = 16.6, 5.9 Hz, 1 H), 3.72 (s, 3 H), 3.77–3.66 (m, 2 H), 3.27 (dd, J = 14.1, 4.0 Hz, 1 H), 3.20–3.07 (m, 1 H), 2.99 (dd, J = 14.0, 8.6 Hz, 1 H), 1.64 (d, J = 6.6 Hz, 3 H), 1.40 (s, 9 H), 0.97–0.82 (m, 6 H) ppm. ^{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 ppm.

N_3-Phe-Dap(Gly-Boc)-Leu-$NHNH_2$ (53): N_3-Phe-Dap(Gly-Boc)-Leu-OMe **52** (0.34 mmol) was dissolved in MeOH (3 mL), and $NH_2NH_2{\cdot}H_2O$ (497 µL, 10 mmol, 30 equiv.) was added. The reaction mixture was stirred at room temp. for 4 h, concentrated and coevaporated with toluene (2 ×) thereby providing compound **53** (quantitative yield). ^{1}H NMR (400 MHz, CD_3OD): δ = 7.27 (qd, J = 8.6, 7.7, 4.0 Hz, 5 H), 4.51 (t, J = 6.3 Hz, 1 H), 4.41 (dd, J = 9.5, 5.5 Hz, 1 H), 4.18 (dd, J = 9.0, 4.8 Hz, 1 H), 3.83–3.65 (m, 2 H), 3.52 (tt, J = 13.8, 6.8 Hz, 2 H), 3.22 (dd, J = 14.0, 4.7 Hz, 1 H), 2.96 (dd, J = 13.9, 9.0 Hz, 1 H), 1.75–1.51 (m, 3 H), 1.44 (s, 9 H), 0.95 (d, J = 6.4 Hz, 3 H), 0.91 (d, J = 6.4 Hz, 3 H) ppm. ^{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 ppm.

Fmoc-His(Trt)-VS (55): Fmoc-His(Trt)-N(OMe)Me **54** (1.99 g, 3 mmol) was dissolved in in THF (30 mL), and the solution was cooled to 0 °C. $LiAlH_4$ (2 M in THF; 1.8 mL, 3.6 mmol, 1.2 equiv.) was added dropwise over 10 min. After 2 h, TLC analysis (MeOH/CH_2Cl_2, 3 %) revealed that the reaction was complete, and the reaction was quenched by the addition of HCl (1 M aq.). EtOAc was added, and the layers were separated. The organic layer was washed with brine,

dried with Na_2SO_4, filtered, and concentrated to give 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 the solution was cooled to 0 °C under an argon atmosphere. NaH (60 % w/w in mineral oil; 44 mg, 1.1 mmol, 1.3 equiv.) was slowly added, and the mixture was stirred at 0 °C for 45 min. Next, a solutioin of the freshly obtained aldehyde (510 mg, 0.85 mmol, 1 equiv.) in THF (3 mL) was slowly added. The mixture was stirred for 3 h while slowly warming to room temp. After this time, TLC analysis indicated the complete conversion of the aldehyde. EtOAc was added, and the mixture was washed with HCl (1 M aq.; 2 ×) and brine, dried with Na_2SO_4, and concentrated. Column chromatography (twice, EtOAc/pentane, 10→80 %) yielded compound **55** (378 mg, 65 %); the product contained 0.2 equiv. of diethyl[(methylsulfonyl)methyl] phosphonate, based on NMR spectroscopy. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.78 (d, J = 7.5 Hz, 2 H), 7.64 (dt, J = 11.8, 5.8 Hz, 2 H), 7.50–7.26 (m, 13 H), 7.19–7.11 (m, 7 H), 7.06 (d, J = 7.8 Hz, 1 H), 6.85 (dd, J = 15.0, 4.6 Hz, 1 H), 6.66 (s, 1 H), 6.47 (d, J = 15.0 Hz, 1 H), 4.78 (d, J = 5.3 Hz, 1 H), 4.47–4.33 (m, 2 H), 4.33–4.20 (m, 1 H), 3.04 (dd, J = 14.7, 4.7 Hz, 1 H), 2.90 (s, 3 H), 2.85 (dd, J = 14.7, 5.2 Hz, 1 H) ppm.

H-His(Trt)-VS (56): Fmoc-His(Trt)-VS (266 mg, 0.39 mmol) was dissolved in MeCN/diethylamine (1:1; 5 mL), and the solution was cooled to 0 °C. The mixture was stirred for 1 h, then it was concentrated. The residue was purified by column chromatography (EtOAc, followed by MeOH/CH_2Cl_2, 0→10 %) to give compound **56** (115 mg, 64 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.42 (d, J = 1.2 Hz, 1 H), 7.36 (dd, J = 5.1, 1.7 Hz, 10 H), 7.19–7.10 (m, 5 H), 6.98 (dd, J = 15.0, 4.8 Hz, 1 H), 6.65 (s, 1 H), 6.62 (dd, J = 15.1, 1.6 Hz, 1 H), 4.02 (ddt, J = 6.9, 5.3, 2.7 Hz, 1 H), 2.91 (s, 3 H), 2.85 (dd, J = 14.3, 5.3 Hz, 1 H), 2.67 (dd, J = 14.3, 7.8 Hz, 1 H) ppm. ^{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 ppm.

N_3-Phe-Leu-Leu-His(Trt)-VS (57): Compound **57** was obtained by the general protocol for azide coupling on a 100 µmol scale. Purification by column chromatography (MeOH/CH_2Cl_2, 0→2 %) provided compound **57** (45 mg, 53 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.28 (d, J = 8.1 Hz, 1 H), 7.37 (dd, J = 5.2, 1.8 Hz, 9 H), 7.34–7.27 (m, 4 H), 7.27–7.22 (m, 2 H), 7.16–7.05 (m, 6 H), 6.86 (d, J = 7.7 Hz, 1 H), 6.79 (dd, J = 15.1, 4.6 Hz, 1 H), 6.68–6.62 (m, 2 H), 6.45 (dd, J = 15.1, 1.5 Hz, 1 H), 5.01–4.91 (m, 1 H), 4.56–4.46 (m, 1 H), 4.43–4.34 (m, 1 H), 4.31 (dd, J = 7.5, 4.1 Hz, 1 H), 3.30 (dd, J = 14.1, 4.1 Hz, 1 H), 3.08 (dd, J = 14.1, 7.6 Hz, 1 H), 2.98–2.91 (m, 1 H), 2.90 (s, 3 H), 2.80 (dd, J = 14.7, 5.7 Hz, 1 H), 1.76 (ddd, J = 13.6, 8.9, 4.7 Hz, 1 H), 1.67–1.55 (m, 2 H), 1.55–1.42 (m, 2 H), 1.41–1.21 (m, 1 H), 0.93 (d, J = 6.5 Hz, 6 H), 0.79 (d, J = 6.6 Hz, 3 H), 0.76 (d, J = 6.5 Hz, 3 H) ppm. ^{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 ppm.

Biochemical Experiments

General Remarks: Lysates of cells were prepared by treating cell pellets with 4 volumes of lysis buffer containing tris (pH 7.5; 50 mM), DTT (dithiothreitol; 2 mM), $MgCl_2$ (5 mM), glycerol (10 %), ATP (adenosine 5′-triphosphate; 2 mM), and digitonin (0.05 %) for 60 min on ice. Protein concentration was determined using a Qubit® protein assay kit (Thermofisher). All cell-lysate-labelling experiments were carried out in assay buffer containing Tris (pH 7.5; 50 mM), DTT (2 mM), $MgCl_2$ (5 mM), glycerol (10 %), and ATP (2 mM). Cell-lysate-labelling and competition experiments were carried out at 37 °C. The probe cocktail consisted of: Cy5-NC-001 (100 nM), BODIPY(FL)-LU-112 (30 nM), and BODIPY(TMR)-NC-005-VS (100 nM), used as a premixed 10 × concentrated cocktail in DMSO that was incubated with the 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 7.5 × 10 cm (L × W) gels were run for 15 min at 80 V, followed by 120 min at 130 V. In-gel detection of (residual) proteasome activity was carried out in the wet gel slabs directly with 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 (10 × stock in DMSO) at the indicated concentrations for 1 h at 37 °C, followed by addition of the probe cocktail (10 × stock, 1.1 µL) and SDS-PAGE as described above.

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Keywords: Asymmetric synthesis · Amino acids · Inhibitors · Proteasomes · Peptidomimetics · Structure-activity relationships

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