Chemical tools to monitor and control human proteasome activities

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CHAPTER 7

Development of an inhibitor and activity-based probe selective for β5c

Introduction

The chymotryptic sites of mammalian proteasomes are often considered as the major proteolytic activities and are the predominant targets in proteasome-directed drug discovery programs. Indeed, the clinical drug bortezomib, while targeting also caspase-like proteasome activities, was initially developed to exclusively disable proteasome chymotryptic sites, and also the second-in-class clinical drug/proteasome inhibitor carfilzomib, has as its major target these activities. Despite the large body of evidence pointing towards the importance of the tryptic-like and caspase-like proteasome activities in protein turnover and (especially) in MHC class I directed antigen presentation, most studies reporting on the discovery of proteasome inhibitors present data on inhibition of chymotryptic activities only.

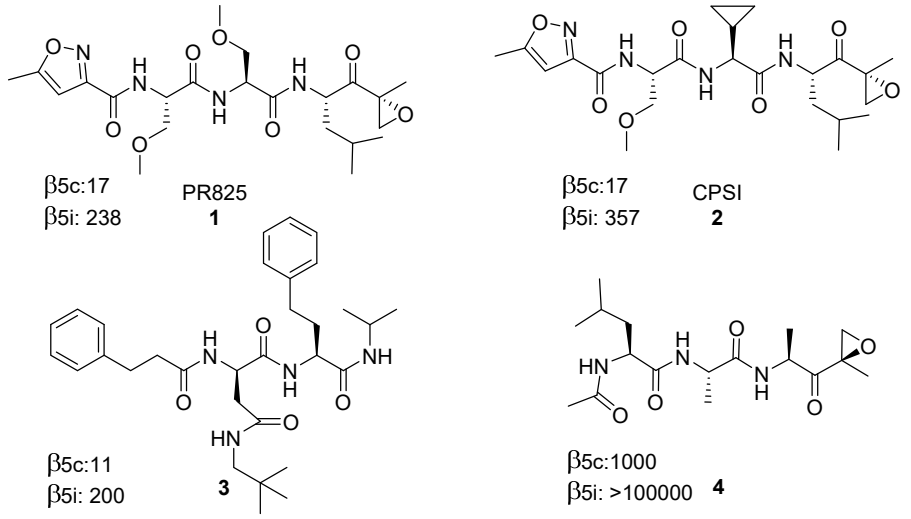


Figure 1. Structures and IC_{50} values (nM) of β5c selective inhibitors. IC_{50} values have been determined using different assays. 1/2: PROCISE assay (1. intact cells; 2. purified 20S), 3: fluorogenic substrates (purified 20S), 4: ABPP (cell lysate).

Given the predominant focus in proteasome inhibitor research on chymotryptic sites, it is perhaps surprising that only a few studies report on inhibitors able to discriminate between the respective chymotryptic activities of human constitutive proteasomes and immunoproteasomes, β5c and β5i. Of these studies, the majority focus on β5i selective inhibitors (see also chapter 5), and those few inhibitors reported as selective for β5c have only small selectivity windows. Currently known β5c selective inhibitors are shown in Figure 1. Both **1** and **2** have been identified in a medicinal chemistry effort aiming to develop orally available β5c/β5i selective proteasome inhibitors.[1] Inhibitors **1** and **2** differ at the P2 position only, and arguably these residues have limited interaction with the large and solvent exposed S2 pockets of both β5c and β5i active sites.[2] When the P1 Leu residue of **1** is replaced by Phe, β5c selectivity is completely abolished, resulting in equal activity against β5c and β5i, which can be explained by the preference of β5i for large P1 residues.[1] While β5i prefers small residues at P3, β5c favours large P3 residues, as can be deduced from mouse 20S (immuno)proteasome crystal structures.[2, 3] β5i selective inhibitors require a P3 Ala residue,[3] therefore, most likely, the relatively large P3 Ser(OMe) side chain is disfavoured by the β5i subunit, leading to β5c selectivity. As well, capped dipeptide **3** features a large P3 substituent, which is responsible for its β5c selectivity.[4] As is described in chapter 4, β5c has a smaller S1 pocket than β5i and this difference is caused by a different conformation adopted by Met45 in the respective active site pockets. For this reason, compound **4** (P1 Ala) displays high β5c selectivity, however, the potency of β5c inhibition is relatively low and the β2 subunits are also targeted.[5] Currently, there is no evidence that selective β5c inhibition could form the basis for the development of therapies against human diseases. However, from a more fundamental perspective, it is desirable to be able to selectively inhibit each individual proteasome subunit in order to determine the exact role of each proteasome subunit in protein turnover and antigen processing. Selective β5c inhibition by epoxyketone **2** has already shown that, unlike carfilzomib which inhibits both β5c and β5i, inhibition of β5c in haematological derived cancer cell lines without inhibition of β5i did not result in significant cytotoxicity.[1] Given the poor selectivity profiles of existing β5c selective inhibitors (compounds **1**, **2** and **3** are only 20-fold selective), it would be beneficial to have access to more selective β5c inhibitors. In this chapter the development of β5c inhibitors with both good potency and much higher selectivity windows compared to existing β5c inhibitors are described. In addition, these optimized β5c inhibitors form the basis for the development of an activity-based probe (ABP) that is selective for β5c over β5i.

Results and discussion

As stated above, β5c has a larger S3 pocket than β5i. Therefore, a series of compounds was designed varying in their (bulky, hydrophobic) P3 substituent: 1-naphthylalanine (1-Nap), 2-naphthylalanine (2- Nap), biphenylalanine (BiPhe), cyclohexylalanine (Cha), tryptophan (Trp), adamantylalanine (Ala(Ada), tert-butylalanine (Ala(tBu)), tert-butyl-O-Ser (Ser(OtBu)), and tert-butyl-O-Thr (Thr(OtBu)), see Figure 2. These amino acids were incorporated in the otherwise identical N_3Phe-XXX-Phe-Leu-epoxyketone (except for compound **10**, which contains a P2 Leu residue) scaffold using standard solution phase Boc/Fmoc chemistry and standard azide coupling of the peptide hydrazides and deprotected warheads, as described in the experimental part and in previous chapters. All inhibitors were evaluated for their inhibition activities against all six subunits at four different concentrations in Raji-lysates using the ABPP-assay described in chapter 3 (Figure 2). The compounds with aromatic substituents at the P3 sites (**5**, **6**, **7**, **9**) do not show any selectivity for β5c. Interestingly, compounds **5** (1-Nap), **6** (2-Nap) and **9** (Trp) do also inhibit β2 at higher concentrations whereas compound **7** (BiPhe) does not target β2, even not at 10 µM, making **7** a highly potent and selective β5c/β5i inhibitor. Compound **8** (Cha) shows almost complete inhibition of β5c at 10 nM, and some 10-fold selectivity for β5c over β5i. Incorporation of an adamantyl side chain (**10**) results in loss of potency and similar selectivity as **8**.

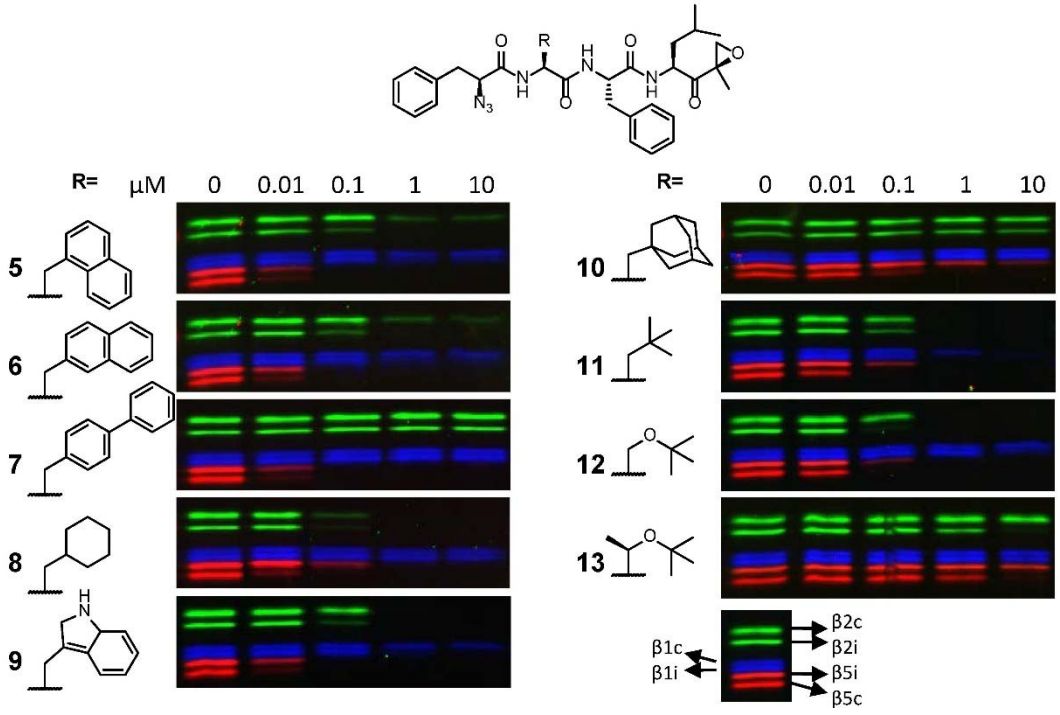


Figure 2. Structure and inhibition profiles in Raji lysates of a small library of compounds with different bulky hydrophobic P3 substituents. *P2=Leu

Compounds **11** (Ala(tBu)) and **12** (Ser(OtBu)) do not show selectivity for β5c, while compound **13** (Thr(OtBu)) is selective for β5c, however, only at high concentrations.

To further improve the selectivity towards β5c, a series of compounds was synthesized equipped with Ala at P1 and at P3 either one of the β5c-selectivity inducing amino acids Cha, Ala(Ada), Thr(OtBu) (compounds **14**, **15** and **16**) or BiPhe (compound **17**) (Figure 3). A very high selectivity for β5c over β5i was observed for compound **14**, which however also targets β2c/β2i. In contrast, while still being selective for β5c, Ala(Ada) (**15**) and Thr(OtBu) (**16**) at P3 in combination with Ala at P1 result in severe loss of potency. Compound **17** shows the highest potency for β5c and also a good selectivity over all other subunits. These results indicate the importance of both the P3 and P1 substituents for gaining β5c selectivity. Since compound **14** (P3 Cha) shows the highest selectivity for β5c over β5i, but no selectivity for β5c over β2c/β2i, bicyclohexylalanine (BiCha, as a 1:3 mixture of isomers, synthesized by reduction of BiPhe) was incorporated at P3. The resulting compound **18** shows similar selectivity for β5c over β5i as compound **14**, but does, due to the extra cyclohexyl group, no longer inhibit the β2 subunits.

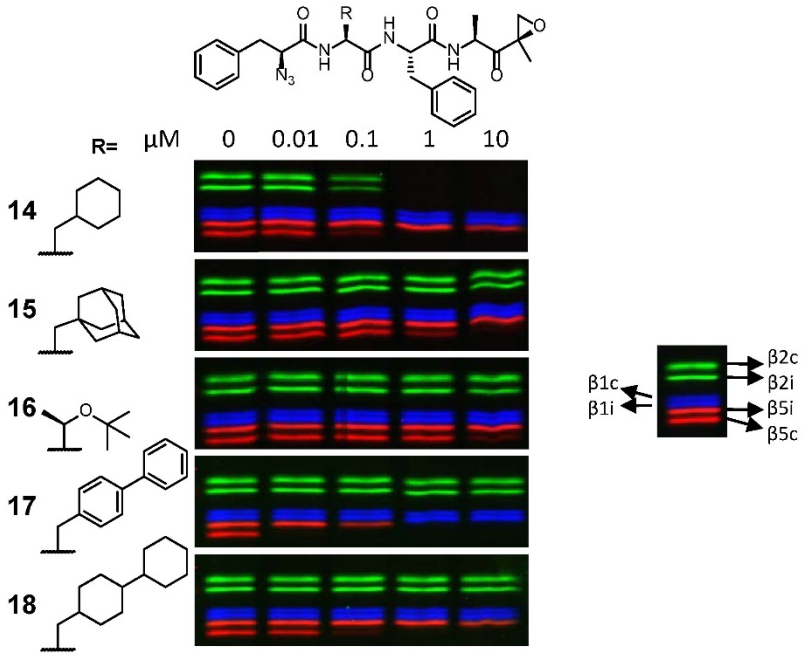
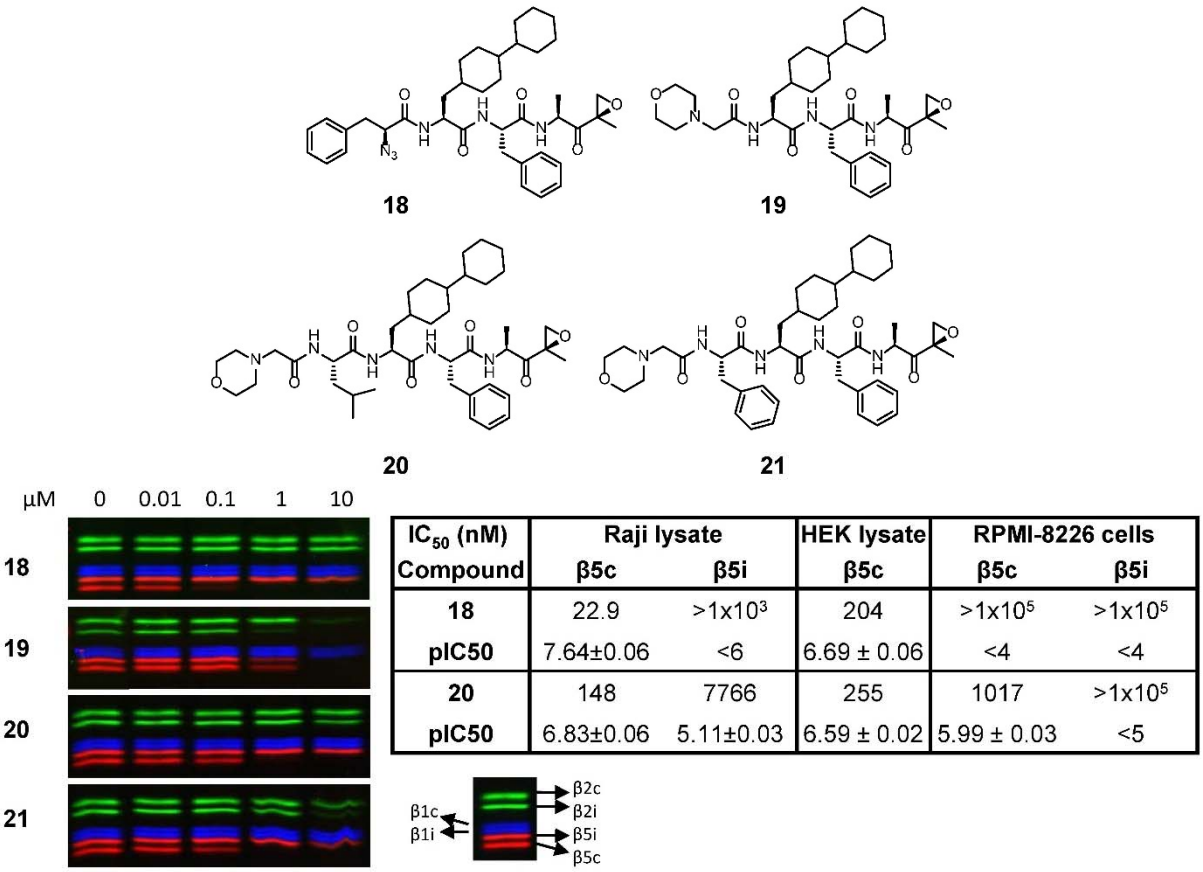


Figure 3. Structure and inhibition profiles of β5c selective compounds with P1 Ala and different P3 substituents. *P2=Leu

In order to verify whether compound **18** also can be used to selectively inhibit β5c in intact cells, the IC_{50} value of **18** for all subunits was determined in intact RMPI-8826 cells (a multiple myeloma cell line). Unfortunately, no inhibition could be observed, presumably due to the poor solubility since in living cells a lower amount of DMSO was used (1% vs. 10% in lysates) (Figure 4). In order to increase the solubility, the azido-Phe P4 residue of **18** was substituted for 2-morpholino acetate, providing compound **19**. 2-Morpholino acetate has also been used

to increase the solubility of YU-101[6], resulting in the FDA approved drug carfilzomib.[7] Interestingly, compound **19** shows decreased potency and almost no selectivity for β5c (Figure 4). It was reasoned that the incorporation of an extra amino acid could recover both the potency and selectivity, since carfilzomib also contains an extra amino acid. Both Leu (as in carfilzomib) and Phe (similar to P4 azido-Phe) were incorporated at P4 and both compounds **20** and **21** were evaluated for proteasome inhibition. Clearly, compound **20** is more selective for β5c than **21**, which also targets β2c/β2i at higher concentrations (Figure 4). More importantly, while being less potent than compound **18** in Raji lysates, compound **20** shows good activity and selectivity for β5c in living RPMI-8226 cells. Together, compounds **18** and **20** represent the most selective β5c inhibitors known to date.

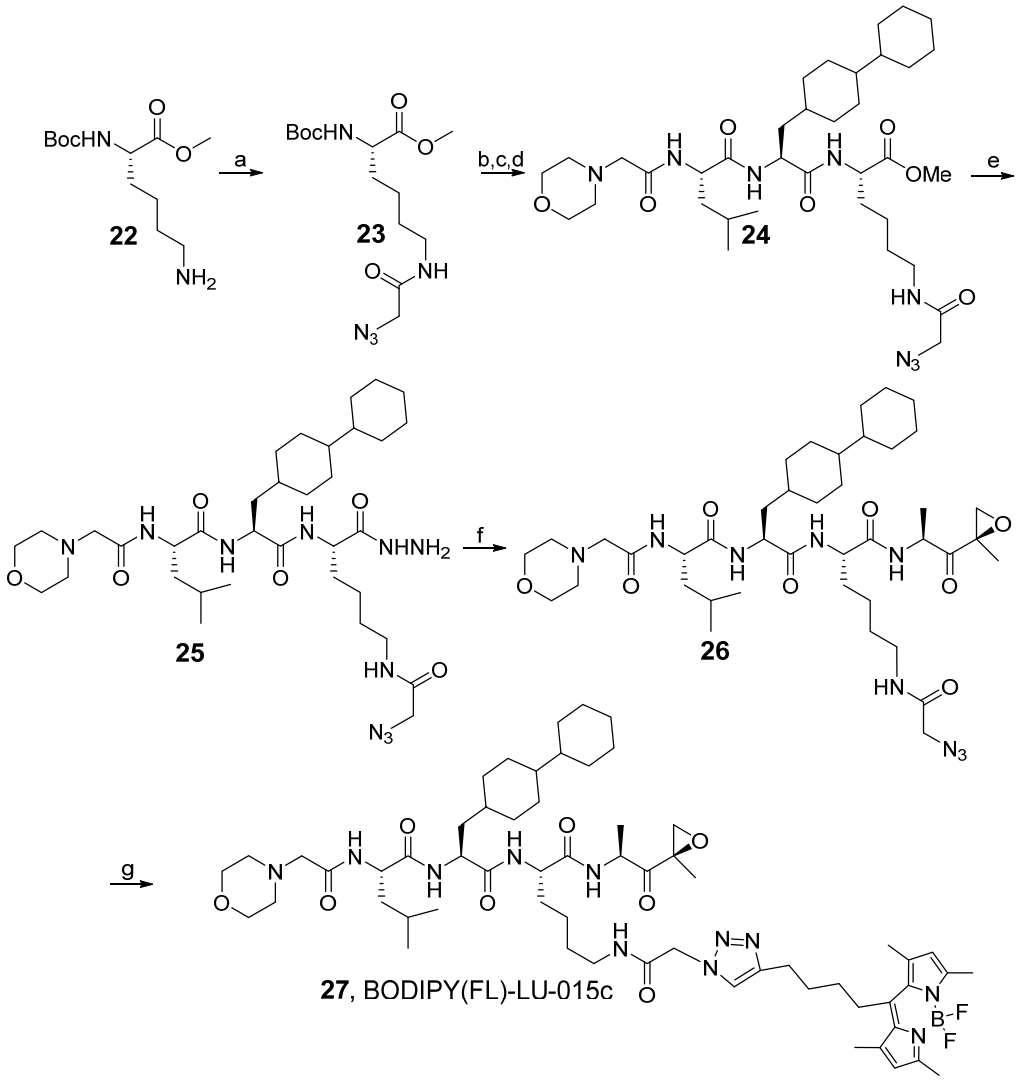


IC_{50} (nM)	Raji lysate		HEK lysate	RPMI-8226 cells	
Compound	β5c	β5i	β5c	β5c	β5i
18	22.9	>1x10^3	204	>1x10^5	>1x10^5
pIC50	7.64±0.06	<6	6.69 ± 0.06	<4	<4
20	148	7766	255	1017	>1x10^5
pIC50	6.83±0.06	5.11±0.03	6.59 ± 0.02	5.99 ± 0.03	<5

Figure 4. Structures and inhibition profiles of compound **18-20** in Raji lysates and apparent IC_{50} values of the optimized inhibitors **18** and **20**. Only the IC_{50} values for the β5 subunits are reported; no inhibition of other subunits was observed at concentrations up to 10 μM (in lysates) or 100 μM (in intact cells).

Currently, no selective ABPs for β5c are known. Therefore, the optimized β5c inhibitors were used to design β5c-probe **27** (Scheme 1). Given the fact that the P4 residue of β5c inhibitors appeared to be important for the selectivity and solubility of the inhibitors, it was decided to install the fluorophore at the P2 position, since the S2 pockets are solvent exposed.[5] It has already been shown that extension of the P2 side chain is a viable strategy towards subunit

selective proteasome ABPs.[8, 9] To ensure good solubility, it was decided to use compound **21** as starting point. Since for future studies a β5c probe equipped with a BODIPIY(FL) fluorophore was desired (see chapter 10) and because BODIPY(FL)-alkyne[10] was readily available, a Lys residue elongated with 2-azidoacetic acid was chosen as P2 residue. This enables straightforward attachment of the fluorophore using copper(I)-catalysed azide-alkyne cycloaddition (CuAAC). Starting from Boc-Lys-OMe, the azide tag was installed through peptide coupling with 2-azidoacetic acid, providing compound **23**. Standard solution phase Boc chemistry yielded capped tripeptide **24**. Hydrazinolysis of the methyl ester followed by a standard azide coupling with deprotected Ala epoxyketone provided compound **26**, which was converted to probe **27** by CuAAC with BODIPY(FL)-Alkyne.



Scheme 1. Synthesis of β5c selective ABP 27. Reagents and conditions: a. N_3AcOSu, DiPEA, DCM, 35%; b. 1. TFA; 2. Boc-BiCha-OH, HCTU, DiPEA, DCM, 70%; c. 1. TFA; 2. Boc-Leu-OH, HCTU, DiPEA, DCM, 80%; d. 1. TFA; 2. 2-morpholinoacetic acid, HCTU, DiPEA, DCM, 92%; e. $NH_2NH_2{\cdot}H_2O$, MeOH, 100%; f. 1. tBuONO, HCl, DMF. 2. H-Ala-EK, DiPEA, 55%; g. BODIPY(FL)-alkyne, $CuSO_4$, NaAsc, DMF/H_2O, 28%.

The potency and selectivity of ABP **27** was evaluated in Raji lysate. Because most probes do not show separate bands for β5c and β5i on SDS-PAGE, the samples were treated with BODIPY(TMR)-NC-005 (chemical structure: see chapter 3) after incubation with ABP **27** to verify the amount of inhibition for β5c and β5i (Figure 5). Labelling of β5c is already visible at 30 nM and complete labelling is achieved at 1 µM, while β5i remains untouched, however, significant labelling of both β2c and β2i is visible.

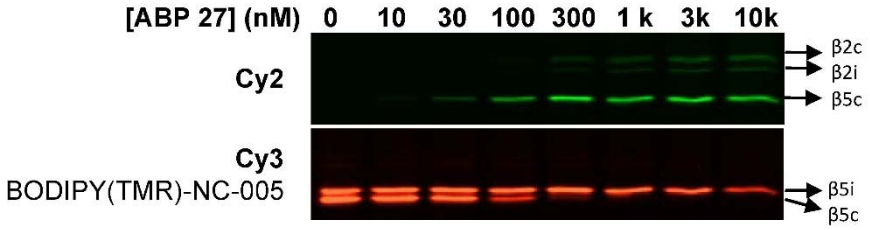


Figure 5. Evaluation of ABP **27** in Raji lysate. Lysates were treated with indicated concentrations of probe **27** for 1 h, followed by the addition of 100 nM BODIPY(TMR)-NC-005 (end concentration) for 1 h.

Conclusion

This chapter describes the design, synthesis and evaluation of new and improved β5c selective inhibitors. The nature of both P1 and P3 residues appears highly important to achieve good selectivity. A small P1 substituent (CH_3) in combination with a large, bulky P3 substituent (bicyclohexyl) proved to be optimal for selective and potent β5c inhibition. Compound **18** represents the most selective β5c inhibitor known to date and is the compound of choice when a high concentration of DMSO (at least 10%) can be used. In case lower DMSO concentrations are required, for instance, when β5c inhibition in living cells is desired, compound **21** can be used, which is more soluble due to the 2-morpholinoacetate *N*-cap. In addition, probe **27** was synthesized which allows selective labelling of β5c over β5i, however, significant labelling of the β2 subunits is observed. In case only β5c labelling is desired, a β2 selective inhibitor (for instance, LU-102[11]) can be used to block the β2 subunits. Since the BiCha building block used for these β5c inhibitors exist in an inseparable 1:3 mixture of isomers, further research is directed towards the synthesis of the pure isoforms which might lead to β5c inhibitors with improved activity and selectivity profiles. Together, these new β5c selective inhibitors and the ABP represent a valuable toolset which can be used to study the role of the β5c subunit in various biological processes and diseases, such as antigen presentation, auto-immune diseases and cancer.

Experimental

Synthetic procedures

General procedures

Acetonitrile (ACN), dichloromethane (DCM), N,N-dimethylformamide (DMF), methanol (MeOH),diisopropylethylamine (DiPEA) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased at Biosolve, and used as received. All general chemicals (Fluka, Acros, Merck, Aldrich, Sigma, Iris Biotech) were used as received. Column chromatography was performed on Screening Devices b.v. Silica Gel, with a particle size of 40-63 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 ^oC. ^1H- and ^{13}C-NMR spectra were recorded on a Bruker AV-400 (400 MHz), AV-600 (600 MHz) or AV-850 (850 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. Methods used are: xx→xx% MeCN, 15 min (0→0.5 min: 10% MeCN; 0.5→10.5 min: gradient time; 10.5→12.5 min: 90% MeCN; 12.5→15.0 min: 10% MeCN); 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. Boc-Ala(Ada)-OH was synthesized as described in chapter 11, Boc-Leu-EK and Boc-Ala-EK as, described in chapter 4. H-BiCha-Phe-OMe, compounds **10** and **20** were synthesized as described in chapter 3.

General procedure for azide couplings.

Compounds **5**-**21** were prepared via azide coupling of peptide hydrazides and properly deprotected epoxyketone amines. The appropriate hydrazide was dissolved in DMF or 1:1 DMF:DCM (v/v) or and cooled to -30 °C. *t*BuONO (1.1 equiv.) and HCl (4M solution in 1,4-dioxane, 2.8 equiv, 3.8 equiv in case of morpholino N-cap)) were added, and the mixture was stirred for 3 h at -30 °C after which TLC analysis (10% MeOH/DCM, v/v) showed complete consumption of the starting material. The epoxyketone 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 hours, 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 2-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 were treated with TFA (0.1 M) for 30 minutes, followed by co-evaporation with toluene (2x).

N_3Phe-1-Nal-Phe-Leu-EK (5)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (10→40% EtOAc/pent) provided the title compound (22.9 mg, 66%) as a white powder after lyophilisation. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 – 8.06 (m, 1H), 7.88 (dd, *J* = 8.0, 1.4 Hz, 1H), 7.78 (d, *J* = 8.2 Hz, 1H), 7.63 – 7.48 (m, 2H), 7.37 (dd, *J* = 8.3, 7.0 Hz, 1H), 7.31 – 7.18 (m, 4H), 7.19 – 7.10 (m, 5H), 6.93 – 6.85 (m, 2H), 6.84 (d, *J* = 6.9 Hz, 1H), 6.12 (d, *J* = 7.9 Hz, 1H), 6.01 (d, *J* = 7.4 Hz, 1H), 4.64 (d, *J* = 7.0 Hz, 1H), 4.55 – 4.35 (m, 1H), 3.94 (dd, *J* = 8.3, 4.0 Hz, 1H), 3.46 (d, *J* = 6.7 Hz, 1H), 3.35 (d, *J* = 7.2 Hz, 1H), 3.23 (d, *J* = 5.0 Hz, 1H), 3.12 (dd, *J* = 14.1, 4.0 Hz, 1H), 2.93 (d, *J* = 6.2 Hz, 1H), 2.87 (d, *J* = 4.9 Hz, 1H), 2.75 (dd, *J* = 14.1, 8.0 Hz, 2H), 1.50 (s, 3H), 1.48 – 1.39 (m, 2H), 1.23 (d, *J* = 21.5 Hz, 1H), 0.91 (dd, *J* = 13.1, 5.9 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 207.93, 170.03, 169.97, 168.87, 136.08, 135.81, 134.10, 132.19, 131.96, 129.51, 129.35, 129.09, 128.78, 128.67, 128.36, 127.89, 127.48, 127.10, 126.85, 126.19, 125.45, 123.67, 65.21, 59.15, 54.17, 54.14, 52.45, 50.23, 40.01, 38.45, 37.62, 34.81, 25.17, 23.50, 21.42, 16.81. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15.0 min): R_t (min): 10.91 (ESI-MS (m/z): 689.07 $[M+H]^+$). HRMS: calculated for $C_{40}H_{44}N_6O_5$ 689.34460 $[M+H]^+$; found 689.34479

N_3Phe-2-Nal-Phe-Leu-EK (6)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (10→40% EtOAc/pent) provided the title compound (16.8 mg, 49%) as a white powder after lyophilisation. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 – 7.78 (m, 1H), 7.78 – 7.72 (m, 2H), 7.52 – 7.43 (m, 3H), 7.32 – 7.06 (m, 9H), 6.99 – 6.90 (m, 2H), 6.72 (d, *J* = 7.6 Hz, 1H), 6.27 (d, *J* = 7.5 Hz, 1H), 6.05 (d, *J* = 7.9 Hz, 1H), 4.64 (q, *J* = 6.7 Hz, 1H), 4.55 (q, *J* = 6.8 Hz, 1H), 4.50 – 4.38 (m, 1H), 4.00 (dd, *J* = 8.0, 4.1 Hz, 1H), 3.22 – 3.12 (m, 3H), 3.03 (ddd, *J* = 13.9, 6.4, 3.3 Hz, 2H), 2.87 – 2.78 (m, 3H), 1.49 (s, 3H), 1.47 – 1.37 (m, 2H), 1.16 – 1.06 (m, 1H), 0.87 (dd, *J* = 15.6, 6.0 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 207.93, 170.16, 169.87, 168.77, 136.05, 135.75, 133.56, 133.37, 132.64, 129.57, 129.37, 128.81, 128.71, 128.66, 128.18, 127.82, 127.69, 127.51, 127.27, 127.14, 126.49, 126.09, 65.15, 59.16, 54.22, 54.04, 52.47, 50.35, 39.96, 38.42, 37.77, 37.69, 25.21, 23.44, 21.41, 16.82. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15.0 min): R_t (min): 10.86 (ESI-MS (m/z): 689.13 $[M+H]^+$). HRMS: calculated for $C_{40}H_{44}N_6O_5$ 689.34460 $[M+H]^+$; found 689.34473

N_3Phe-BiPhe-Phe-Leu-EK (7)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (10→40% EtOAc/pent) provided the title compound (23.7 mg, 66%) as a white powder after lyophilisation. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 – 7.53 (m, 2H), 7.50 (d, *J* = 8.1 Hz, 2H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.37 – 7.27 (m, 4H), 7.20 (q, *J* = 5.6 Hz, 5H), 7.10 (d, *J* = 8.1 Hz, 2H), 7.07 – 7.02 (m, 2H), 6.74 (d, *J* = 7.5 Hz, 1H), 6.41 (d, *J* = 7.6 Hz, 1H), 6.16 (d, *J* = 7.9 Hz, 1H), 4.61 (qd, *J* = 6.8, 3.0 Hz, 2H), 4.56 – 4.40 (m, 1H), 4.03 (dd, *J* = 8.0, 4.0 Hz, 1H), 3.22 (dt, *J* = 9.4, 4.2 Hz, 2H), 3.05 (td, *J* = 13.8, 6.3 Hz, 2H), 2.98 – 2.81 (m, 4H), 1.49 (s, 3H), 1.48 – 1.42 (m, 2H), 1.18 (t, *J* = 9.8 Hz, 1H), 0.87 (dd, *J* = 12.0, 5.9 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 207.99, 170.23, 169.88, 168.77, 140.60, 140.25, 136.13, 135.80, 134.88, 129.79, 129.62, 129.44, 128.93, 128.82, 128.71, 127.62, 127.54, 127.18, 127.08, 65.16, 59.14, 54.19, 54.05, 52.43, 50.31, 40.03, 38.43, 37.93, 37.18, 25.21, 23.43, 21.39, 16.80. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15.0 min): R_t (min): 11.10 (ESI-MS (m/z): 715.13 $[M+H]^+$. HRMS: calculated for $C_{42}H_{47}N_6O_5$ 715.36025 $[M+H]^+$; found 715.36047

N_3Phe-Cha-Phe-Leu-EK (8)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (10→40% EtOAc in pent) provided the title compound (3.9 mg, 12%) as a white powder after lyophilisation. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.13 (m, 10H), 6.70 (d, *J* = 7.8 Hz, 1H), 6.58 (d, *J* = 7.7 Hz, 1H), 6.35 (d, *J* = 8.0 Hz, 1H), 4.64 (q, *J* = 7.0 Hz, 1H), 4.53 (td, *J* = 10.1, 9.1, 2.8 Hz, 1H), 4.42 – 4.31 (m, 1H), 4.03 (dd, *J* = 7.9, 4.1 Hz, 1H), 3.27 (dd, *J* = 14.1, 4.0 Hz, 1H), 3.23 (d, *J* = 4.9 Hz, 1H), 3.10 – 2.95 (m, 3H), 2.87 (d, *J* = 4.9 Hz, 1H), 1.70 – 1.51 (m, 6H), 1.49 (s, 3H), 1.47 – 1.02 (m, 8H), 0.89 (dd, *J* = 11.6, 6.2 Hz, 6H), 0.86 – 0.73 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 208.01, 171.39, 170.53, 168.79, 136.40, 135.91, 129.58, 129.43, 128.82, 128.69, 127.45, 127.12, 65.14, 59.09, 54.08, 52.42, 51.11, 50.18, 40.20, 39.14, 38.37, 37.88, 33.95, 33.56, 32.60, 26.38, 26.11, 26.02, 25.19, 23.47, 21.42, 16.78. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15.0 min): R_t (min): 11.02 (ESI-MS (m/z): 645.07 $[M+H]^+$). HRMS: calculated for $C_{36}H_{49}N_6O_5$ 645.37590 $[M+H]^+$; found 645.37598

N_3Phe-Trp-Phe-Leu-EK (9)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by HPLC (C_{18}, 50→90% MeCN/H_2O, 0.1% TFA, 10 min gradient) provided the title compound (1.0 mg, 3%) as a white powder after lyophilisation. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.10 (s, 1H), 7.59 (d, *J* = 7.9 Hz, 1H), 7.40 (d, *J* = 8.1 Hz, 1H), 7.38 – 7.09 (m, 10H), 6.90 (d, *J* = 2.2 Hz, 1H), 6.84 (d, *J* = 7.0 Hz, 2H), 6.81 (d, *J* = 6.9 Hz, 1H), 6.13 (d, *J* = 8.0 Hz, 1H), 5.98 (d, *J* = 7.8 Hz, 1H), 4.60 – 4.49 (m, 3H), 3.98 (dd, *J* = 7.7, 4.1 Hz, 1H), 3.32 – 3.28 (m, 2H), 3.24 (dd, *J* = 14.2, 4.1 Hz, 1H), 3.05 – 2.93 (m, 3H), 2.91 (d, *J* = 5.0 Hz, 1H), 2.69 (dd, *J* = 13.9, 6.7 Hz, 1H), 1.54 (s, 3H), 1.49 – 1.38 (m, 2H), 1.24 – 1.17 (m, 1H), 0.91 (dd, *J* = 28.0, 6.3 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 207.89, 170.17, 168.67, 136.23, 135.91, 135.68, 129.54, 129.26, 128.69, 128.53, 127.39, 127.12, 126.92, 125.52, 123.13, 122.68, 120.01, 118.91, 111.31, 110.03, 65.00, 59.04, 53.89, 53.65, 52.32, 50.07, 39.83, 38.18, 37.18, 30.32, 27.26, 25.03, 23.33, 21.26, 16.69. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15.0 min): R_t (min): 9.94 (ESI-MS (m/z): 678.13 $[M+H]^+$). HRMS: calculated for $C_{38}H_{44}N_7O_5$ 678.33984 $[M+H]^+$; found 678.33984

N_3Phe-Ala(Ada)-Leu-Leu-EK (10)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→2% MeOH/DCM) provided the title compound (20.2 mg, 61%) as a white powder after lyophilisation. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.25 (m, 5H), 6.68 (d, *J* = 7.9 Hz, 1H), 6.50 (dd, *J* = 10.7, 8.2 Hz, 2H), 4.59 (ddd, *J* = 10.8, 8.0, 3.1 Hz, 1H), 4.53 – 4.34 (m, 2H), 4.23 (dd, *J* = 8.3, 4.0 Hz, 1H), 3.38 – 3.30 (m, 2H), 3.03 (dd, *J* = 14.1, 8.3 Hz, 1H), 2.91 (d, *J* = 5.0 Hz, 1H), 1.95 (s, 3H), 1.87 – 1.16 (m, 23H), 0.99 – 0.89 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 208.47, 172.01, 171.66, 168.46, 135.98, 129.65, 128.82, 127.48, 65.40, 59.24, 52.58, 51.84, 50.43, 49.47, 45.96, 42.47, 40.79, 40.15, 38.35, 36.83, 32.34, 29.84, 28.57, 25.30, 24.85, 23.57, 22.95, 22.26, 21.43, 16.89. LC-MS (linear gradient 50 → 90% MeCN, 0.1% TFA, 15.0 min): R_t (min): 9.29 (ESI-MS (m/z): 663.27 $[M+H]^+$). HRMS: calculated for $C_{37}H_{55}N_6O_5$ 663.42285 $[M+H]^+$; found 663.42291

N_3Phe-Ala(tBu)-Phe-Leu-EK (11)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (0→1% MeOH/DCM) followed by HPLC (C_{18}, 70→90% MeCN/H_2O, 0.1% TFA, 10 min gradient) provided the title compound (14.4 mg, 47%) as a white powder after lyophilisation. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.37 – 7.28 (m, 5H), 7.28 – 7.19 (m, 5H), 6.68 (d, *J* = 7.7 Hz, 1H), 6.50 (d, *J* = 7.6 Hz, 1H), 6.34 (d, *J* = 7.9 Hz, 1H), 4.64 (q, *J* = 7.2 Hz, 1H), 4.56 (ddd, *J* = 10.4, 8.1, 2.8 Hz, 1H), 4.28 (td, *J* = 8.0, 4.3 Hz, 1H), 3.97 (dd, *J* = 8.1, 3.9 Hz, 1H), 3.34 – 3.26 (m, 2H), 3.16 – 3.02 (m, 2H), 2.97 (dd, *J* = 14.1, 8.1 Hz, 1H), 2.91 (d, *J* = 5.0 Hz, 1H), 1.86 (dd, *J* = 14.6, 4.2 Hz, 1H), 1.53 (s, 3H), 1.52 – 1.45 (m, 2H), 1.33 – 1.22 (m, 2H), 0.93 (dd, *J* = 17.1, 6.2 Hz, 6H), 0.84 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 208.01, 171.56, 170.62, 168.55, 136.52, 135.82, 129.66, 129.43, 128.89, 128.71, 127.51, 127.10, 65.09, 59.16, 53.96, 52.49, 51.08, 50.29, 44.39, 40.09, 38.25, 37.49, 30.36, 29.67, 25.19, 23.51, 21.38, 16.83. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 9.28 (ESI-MS (m/z): 619.20 $[M+H]^+$). HRMS: calculated for $C_{34}H_{47}N_6O_5$ 619.36025 $[M+H]^+$; found 619.36023

N_3Phe-Ser(OtBu)-Phe-Leu-EK (12)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (0→1% MeOH/DCM) followed by (C_{18}, 70→90% MeCN/H_2O, 0.1% TFA, 10 min gradient) provided the title compound (9.6 mg, 30%) as a white powder after lyophilisation. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.37 – 7.24 (m, 8H), 7.20 (d, *J* = 6.9 Hz, 2H), 7.08 (d, *J* = 6.4 Hz, 1H), 6.95 (d, *J* = 8.1 Hz, 1H), 6.24 (d, *J* = 8.3 Hz, 1H), 4.70 – 4.64 (m, 1H), 4.60 (td, *J* = 10.3, 9.3, 2.5 Hz, 1H), 4.31 (ddd, *J* = 8.3, 6.5, 4.2 Hz, 1H), 4.17 (dd, *J* = 8.0, 4.0 Hz, 1H), 3.67 (dd, *J* = 9.1, 4.1 Hz, 1H), 3.32 (dd, *J* = 14.1, 4.0 Hz, 1H), 3.26 (d, *J* = 4.9 Hz, 1H), 3.21 (dd, *J* = 13.9, 5.8 Hz, 1H), 3.16 (t, *J* = 8.7 Hz, 1H), 3.06 (dd, *J* = 14.1, 8.0 Hz, 1H), 2.96 (dd, *J* = 13.9, 6.8 Hz, 1H), 2.91 (d, *J* = 4.9 Hz, 1H), 1.52 (s, 3H), 1.51 – 1.43 (m, 2H), 1.18 (dd, *J* = 19.8, 9.6 Hz, 1H), 1.14 (s, 9H), 0.95 (d, *J* = 6.0 Hz, 3H), 0.89 (d, *J* = 6.2 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 208.09, 170.36, 169.54, 168.68, 136.21, 135.90, 129.67, 129.53, 128.86, 128.77, 127.45, 127.32, 74.79, 65.32, 60.90, 59.04, 54.23, 53.12, 52.44, 50.06, 40.32, 38.59, 37.78, 27.42, 25.05, 23.45, 21.32, 16.78. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 9.25 (ESI-MS (m/z): 635.20 $[M+H]^+$).HRMS: calculated for $C_{34}H_{47}N_6O_6$ 635.35516 $[M+H]^+$; found 635.35504

N_3Phe-Thr(OtBu)-Phe-Leu-EK (13)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (0→1% MeOH/DCM) followed by HPLC (C_{18}, 70→90% MeCN/H_2O, 0.1% TFA, 10 min gradient) provided the title compound (5.9 mg, 18%) as a white powder after lyophilisation. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.34 – 7.22 (m, 9H), 7.15 (d, *J* = 6.9 Hz, 2H), 6.93 (d, *J* = 9.0 Hz, 1H), 6.26 (d, *J* = 8.3 Hz, 1H), 4.75 (dt, *J* = 9.0, 5.9 Hz, 1H), 4.61 (td, *J* = 9.4, 8.5, 2.6 Hz, 1H), 4.29 – 4.16 (m, 2H), 3.88 (dd, *J* = 6.5, 4.3 Hz, 1H), 3.32 (d, *J* = 5.0 Hz, 1H), 3.29 – 3.27 (m, 1H), 3.26 (d, *J* = 4.3 Hz, 1H), 3.12 (dd, *J* = 14.1, 7.2 Hz, 1H), 2.91 (d, *J* = 6.3 Hz, 1H), 2.90 – 2.87 (m, 1H), 1.48 (s, 3H), 1.45 – 1.38 (m, 2H), 1.13 – 1.10 (m, 10H), 0.92 (d, *J* = 5.9 Hz, 3H), 0.85 (d, *J* = 6.1 Hz, 3H), 0.73 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 208.09, 170.60, 168.52, 168.20, 136.12, 135.69, 129.78, 129.57, 128.93, 128.71, 127.46, 127.43, 75.92, 66.07, 65.11, 59.12, 57.35, 53.67, 52.45, 49.93, 40.26, 38.30, 37.56, 28.01, 24.84, 23.39, 21.25, 16.84, 16.77. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 9.67 (ESI-MS (m/z): 649.13 $[M+H]^+$). HRMS: calculated for $C_{35}H_{49}N_6O_6$ 649.37081 $[M+H]^+$; found 649.37079

N_3Phe-Cha-Phe-Ala-EK (14)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by HPLC (C_{18}, 70→80% MeCN/H_2O, 0.1% TFA, 10 min gradient) provided the title compound (5.70 mg, 19%) as a white powder after lyophilisation. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.36 – 7.15 (m, 10H), 6.69 (d, *J* = 7.7 Hz, 1H), 6.55 (d, *J* = 7.8 Hz, 1H), 6.42 (d, *J* = 7.0 Hz, 1H), 4.62 (q, *J* = 7.2 Hz, 1H), 4.47 (p, *J* = 7.1 Hz, 1H), 4.35 (td, *J* = 8.3, 6.1 Hz, 1H), 4.07 (dd, *J* = 8.0, 4.1 Hz, 1H), 3.28 (dd, *J* = 14.1, 4.1 Hz, 1H), 3.17 (d, *J* = 4.9 Hz, 1H), 3.05 (d, *J* = 7.1 Hz, 2H), 3.00 (dd, *J* = 14.1, 8.0 Hz, 1H), 2.89 (d, *J* = 4.9 Hz, 1H), 1.70 – 1.54 (m, 6H), 1.51 (s, 3H), 1.35 (ddd, *J* = 14.3, 8.8, 6.0 Hz, 1H), 1.24 (d, *J* = 7.1 Hz, 3H), 1.21 – 1.06 (m, 4H), 0.84 (s, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 207.79, 171.47, 170.16, 168.80, 136.47, 135.95, 129.59, 129.40, 128.84, 128.72, 127.47, 127.15, 65.19, 59.00, 54.20, 52.52, 51.10, 48.09, 39.06, 38.43, 38.02, 33.99, 33.55, 32.65, 26.40, 26.16, 26.06, 17.32, 16.91. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 9.08 (ESI-MS (m/z): 603.27 $[M+H]^+$). HRMS: calculated for $C_{33}H_{43}N_6O_5$ 603.32894 $[M+H]^+$; found 603.32874

N_3Phe-Ala(Ada)-Leu-Ala-EK (15)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→2% MeOH/DCM) provided the title compound (23.9 mg, 77%) as a white powder after lyophilisation. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.20 (m, 5H), 6.80 (dd, *J* = 7.2, 5.2 Hz, 2H), 6.68 (d, *J* = 8.2 Hz, 1H), 4.54 – 4.37 (m, 3H), 4.18 (dd, *J* = 8.4, 3.9 Hz, 1H), 3.33 – 3.27 (m, 1H), 3.23 (d, *J* = 4.9 Hz, 1H), 3.05 – 2.93 (m, 1H), 2.88 (d, *J* = 4.9 Hz, 1H), 1.91 (s, 3H), 1.72 – 1.54 (m, 9H), 1.51 (s, 3H), 1.50 – 1.37 (m, 6H), 1.27 (d, *J* = 7.1 Hz, 3H), 1.26 – 1.20 (m, 2H), 0.89 (dd, *J* = 13.1, 6.2 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 208.24, 172.09, 171.43,

168.45, 136.08, 129.60, 128.79, 127.43, 65.31, 59.13, 52.60, 51.68, 49.45, 48.06, 46.10, 42.45, 41.02, 38.32, 36.83, 32.37, 28.58, 24.82, 22.92, 22.27, 17.07, 16.93. LC-MS (linear gradient 50 → 90% MeCN, 0.1% TFA, 15.0 min): R_t (min): 7.35 (ESI-MS (m/z): 621.20 $[M+H]^+$). HRMS: calculated for $C_{34}H_{49}N_6O_5$ 621.37690 $[M+H]^+$; found 621.37592

N_3Phe-Thr(OtBu)-Phe-Ala-EK (16)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by HPLC (C_{18}, 60→90% MeCN/H_2O, 0.1% TFA, 10 min gradient) provided the title compound (1.79 mg, 6%) as a white powder after lyophilisation. ^{1}H NMR (850 MHz, $CDCl_3$) δ 7.32 – 7.28 (m, 3H), 7.27 – 7.23 (m, 6H), 7.15 (d, *J* = 7.1 Hz, 2H), 6.81 (d, *J* = 9.1 Hz, 1H), 6.55 (d, *J* = 7.1 Hz, 1H), 4.75 (dt, *J* = 9.1, 5.8 Hz, 1H), 4.54 (p, *J* = 7.1 Hz, 1H), 4.23 (ddd, *J* = 11.3, 6.6, 4.3 Hz, 2H), 3.91 – 3.84 (m, 1H), 3.30 – 3.25 (m, 3H), 3.13 (dd, *J* = 14.2, 7.3 Hz, 1H), 2.93 – 2.90 (m, 1H), 2.90 (d, *J* = 5.0 Hz, 1H), 1.50 (s, 3H), 1.17 (d, *J* = 7.1 Hz, 3H), 1.10 (s, 9H), 0.73 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (214 MHz, $CDCl_3$) δ 207.74, 170.42, 168.31, 168.20, 136.03, 135.71, 129.79, 129.57, 128.96, 128.71, 127.48, 127.47, 76.06, 66.19, 65.12, 59.12, 57.23, 53.45, 52.58, 47.92, 38.32, 37.69, 27.99, 17.24, 16.88, 16.68. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 8.87 (ESI-MS (m/z): 607.13 $[M+H]^+$). HRMS: calculated for $C_{32}H_{43}N_6O_6$ 607.32386 $[M+H]^+$; found 607.32385

N_3Phe-BiPhe-Phe-Ala-EK (17)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by HPLC (C_{18}, 60→80% MeCN/H_2O, 0.1% TFA, 10 min gradient) provided the title compound (9.02 mg, 27%) as a white powder after lyophilisation. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.57 – 7.52 (m, 2H), 7.48 (d, *J* = 8.2 Hz, 2H), 7.42 (t, *J* = 7.7 Hz, 2H), 7.37 – 7.28 (m, 3H), 7.29 – 7.24 (m, 1H), 7.24 – 7.15 (m, 5H), 7.12 – 7.04 (m, 4H), 6.75 (d, *J* = 7.8 Hz, 1H), 6.44 (d, *J* = 7.6 Hz, 1H), 6.25 (d, *J* = 7.0 Hz, 1H), 4.62 (q, *J* = 7.0 Hz, 1H), 4.56 (q, *J* = 7.0 Hz, 1H), 4.46 – 4.35 (m, 1H), 4.09 (dd, *J* = 8.0, 4.1 Hz, 1H), 3.23 (dd, *J* = 14.1, 4.1 Hz, 1H), 3.11 (d, *J* = 4.9 Hz, 1H), 3.04 – 2.96 (m, 2H), 2.96 – 2.87 (m, 3H), 2.85 (d, *J* = 4.9 Hz, 1H), 1.49 (s, 3H), 1.16 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 207.78, 170.01, 169.81, 168.71, 140.62, 140.18, 136.21, 135.87, 134.93, 129.81, 129.65, 129.38, 128.94, 128.83, 128.80, 128.71, 127.52, 127.50, 127.18, 127.06, 65.19, 58.98, 54.26, 54.14, 52.48, 48.09, 38.48, 38.12, 37.36, 17.19, 16.88. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 9.16 (ESI-MS (m/z): 673.27 $[M+H]^+$). HRMS: calculated for $C_{39}H_{41}N_6O_5$ 673.31329 $[M+H]^+$; found 673.31329

N_3Phe-BiCha-Phe-Ala-EK (18)

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by HPLC (C_{18}, 70→90% MeCN/H_2O, 0.1% TFA, 10 min gradient) provided the title compound (6.77 mg, 20%) as a white powder after lyophilisation. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.35 – 7.16 (m, 10H), 6.63 (t, *J* = 6.9 Hz, 1H), 6.49 (t, *J* = 7.5 Hz, 1H), 6.37 (d, *J* = 7.0 Hz, 1H), 4.60 (q, *J* = 7.2 Hz, 1H), 4.52 – 4.44 (m, 1H), 4.35 – 4.29 (m, 0.33H), 4.26 (td, *J* = 8.3, 6.1 Hz, 0.67H), 4.08 (dd, *J* = 7.7, 4.2 Hz, 0.67H), 4.07 – 4.04 (m, 0.33H), 3.27 (dt, *J* = 14.1, 3.6 Hz, 1H), 3.18 – 3.15 (m, 1H), 3.07 – 3.04 (m, 2H), 3.04 – 2.96 (m, 1H), 2.89 (d, *J* = 4.9 Hz, 1H), 1.77 – 1.53 (m, 8H), 1.51 (s, 3H), 1.42 – 1.25 (m, 7H), 1.24 (d, *J* = 7.1 Hz, 3H), 1.23 – 0.74 (m, 8H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 207.80, 207.76, 171.42, 170.12, 170.11, 168.79, 136.49, 135.84, 129.63, 129.61, 129.40, 128.85, 128.81, 128.73, 127.48, 127.45, 127.16, 65.20, 65.16, 59.02, 54.21, 52.53, 51.67, 51.23, 48.12, 43.37, 43.26, 39.01, 38.42, 38.36, 37.95, 37.91, 34.30, 33.79, 32.96, 30.61, 30.34, 30.01, 29.70, 29.62, 28.88, 26.97, 26.87, 25.48, 25.32, 17.32, 16.92. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 10.82 (ESI-MS (m/z): 685.33 $[M+H]^+$). HRMS: calculated for $C_{39}H_{53}N_6O_5$ 685.40720 $[M+H]^+$; found 685.40729

Morph-BiCha-Phe-Ala-EK (19)

This compound was obtained by the general protocol for azide coupling on a 100 µmol scale. Purification by column chromatography (1→2% MeOH/DCM) provided the title compound (36.0 mg, 56%) as a white powder after lyophilisation. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.12 (m, 6H), 6.82 (d, *J* = 6.3 Hz, 1H), 6.52 (d, *J* = 6.0 Hz,

1H), 4.58 (q, *J* = 7.2 Hz, 1H), 4.46 (q, *J* = 7.1 Hz, 1H), 4.34 (tt, *J* = 14.2, 7.3 Hz, 1H), 3.68 (s, 4H), 3.17 (d, *J* = 4.9 Hz, 1H), 3.08 – 2.82 (m, 5H), 2.45 (h, *J* = 7.5 Hz, 4H), 1.91 – 1.56 (m, 8H), 1.50 (s, 3H), 1.31 (ddd, *J* = 20.7, 8.1, 4.2 Hz, 7H), 1.22 (d, *J* = 7.1 Hz, 3H), 1.20 – 0.72 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 207.84, 171.94, 170.57, 170.24, 136.63, 129.42, 128.64, 127.02, 66.98, 66.25, 61.76, 58.98, 54.36, 53.90, 52.49, 51.45, 50.92, 48.03, 43.32, 43.22, 41.57, 40.25, 37.80, 35.07, 34.75, 33.96, 32.80, 31.45, 30.62, 30.30, 30.12, 29.71, 28.79, 26.91, 26.82, 25.68, 25.48, 17.25, 16.88. HRMS: calculated for $C_{36}H_{54}N_4O_5$ 639.41161 $[M+H]^+$; found 639.41162

Morph-Leu-BiCha-Phe-Ala-EK (20)

This compound was obtained by the general protocol for azide coupling on a 100 µmol scale. Purification by column chromatography (0→2% MeOH/DCM), followed by lyophilisation provided the product (52.7 mg, 69.2 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55 (s, 1H), 7.37 – 7.14 (m, 7H), 7.03 (s, 1H), 6.81 (s, 1H), 4.74 (q, *J* = 7.1 Hz, 1H), 4.55 – 4.42 (m, 2H), 4.35 (s, 1H), 3.80 (s, 4H), 3.22 (d, *J* = 5.0 Hz, 1H), 3.19 – 2.96 (m, 4H), 2.91 (d, *J* = 5.0 Hz, 1H), 2.60 (d, *J* = 28.7 Hz, 4H), 1.84 – 1.56 (m, 10H), 1.53 (s, 3H), 1.50 – 1.30 (m, 7H), 1.27 (d, *J* = 7.1 Hz, 3H), 1.16 (dt, *J* = 21.8, 12.3 Hz, 5H), 0.94 (dd, *J* = 12.3, 5.9 Hz, 6H), 0.90 – 0.78 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 207.77, 172.15, 172.06, 171.81, 170.40, 136.68, 59.05, 54.09, 52.50, 52.25, 47.94, 41.61, 40.61, 38.09, 34.53, 33.84, 31.14, 30.64, 30.13, 28.62, 26.94, 26.83, 25.67, 25.48, 25.07, 23.09, 22.18, 17.08, 16.93. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 13 min): R_t (min): 7.44 (ESI-MS (m/z): 752.20 $(M+H)^+$). HRMS: calculated for $C_{42}H_{65}N_5O_7$ 752.49568$[M+H]^+$; found 752.49561

Morph-Phe-BiCha-Phe-Ala-EK (21)

This compound was obtained by the general protocol for azide coupling on a 100 µmol scale. Purification by column chromatography (1→2% MeOH/DCM) provided the title compound (63.1 mg, 80%) as a white powder after lyophilisation. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.13 (m, 12H), 6.84 (s, 2H), 4.74 (q, *J* = 7.3 Hz, 2H), 4.51 (td, *J* = 7.0, 2.4 Hz, 1H), 4.35 (s, 1H), 3.60 (s, 4H), 3.24 (d, *J* = 4.9 Hz, 1H), 3.21 – 2.96 (m, 6H), 2.91 (d, *J* = 5.0 Hz, 1H), 2.33 (d, *J* = 28.3 Hz, 4H), 1.88 – 1.60 (m, 7H), 1.54 (s, 4H), 1.49 – 1.30 (m, 7H), 1.28 (d, *J* = 7.1 Hz, 3H), 1.15 (dt, *J* = 21.9, 12.2 Hz, 5H), 0.97 – 0.78 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 207.66, 177.52, 171.56, 171.22, 170.30, 136.68, 136.24, 129.31, 129.10, 128.82, 128.50, 127.19, 126.91, 58.93, 53.92, 52.39, 52.27, 47.79, 43.23, 37.79, 34.42, 31.01, 30.50, 30.20, 29.99, 26.80, 26.70, 25.52, 25.33, 17.01, 16.80. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 13.0 min): R_t (min): 7.47 (ESI-MS (m/z): 786.20 $[M+H]^+$). HRMS: calculated for $C_{45}H_{64}N_5O_7$ 786.48003 $[M+H]^+$; found 786.48022

Boc-Lys(*N*-N_3Ac)-OMe (23)

To a mixture of Boc-Lys-OMe·TFA **22** (373 mg, 1 mmol), N_3AcOSu (198 mg, 1 mmol, 1 equiv.) in DCM (10 mL) was added DiPEA (200 µl, 1.15 mmol, 1.15 equiv.). After stirring overnight, the reaction mixture was concentrated and dissolved in EtOAc and washed with 1N HCl (2x), sat. $NaHCO_3$ (2x) and brine (1x). The organic layer was dried over $NaSO_4$, filtered, concentrated and purified by column chromatography (50→80% EtOAc/pent) providing the title compound (120 mg, 0.35 mmol, 35%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.51 (s, 1H), 5.15 (d, *J* = 8.0 Hz, 1H), 4.33 – 4.18 (m, 1H), 3.95 (s, 2H), 3.72 (s, 3H), 3.26 (q, *J* = 6.8 Hz, 2H), 1.78 (dt, *J* = 11.3, 5.8 Hz, 1H), 1.69 – 1.59 (m, 1H), 1.54 (m, 2H), 1.42 (s, 9H), 1.40 – 1.30 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.23, 166.74, 155.51, 79.93, 53.19, 52.65, 52.33, 39.09, 32.35, 28.90, 28.32, 22.62.

Boc-BiCha-Lys(*N*-N_3Ac)-OMe (24a)

Boc-Lys(*N*-N_3Ac)-OMe **23** (120 mg, 0.35 mmol) was treated with TFA for 15 min, followed by co-evaporation with toluene (2x). The resulting product TFA·Lys(*N*-N_3Ac)-OMe was dissolved in DCM and Boc-BiCha-OH (148 mg, 0.42 mmol, 1.2 equiv.), HCTU (167 mg, 0.42 mmol, 1.2 equiv.) and DiPEA (206 µl, 1.23 mmol, 3.5 equiv.) were added. After stirring overnight, the reaction mixture was concentrated and redissolved in EtOAc and washed with 1N HCl (2x), sat. $NaHCO_3$ (2x) and brine (1x). The organic layer was dried over $NaSO_4$, filtered , concentrated and purified by column chromatography (50→70% EtOAc/pent) providing the title compound (143 mg, 0.25 mmol,

70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 (d, *J* = 7.3 Hz, 1H), 6.96 (d, *J* = 14.5 Hz, 1H), 5.24 (d, *J* = 8.3 Hz, 1H), 4.57 – 4.36 (m, 1H), 4.35 – 4.15 (m, 1H), 4.06 – 3.87 (m, 2H), 3.71 (s, 3H), 3.43 – 3.08 (m, 2H), 1.42 (s, 9H), 1.90 – 0.74 (m, 29H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.35, 172.48, 167.26, 155.92, 80.11, 52.81, 52.57, 52.39, 52.22, 43.29, 41.34, 39.73, 38.88, 36.53, 34.44, 33.97, 32.73, 31.54, 31.38, 30.60, 30.26, 29.95, 29.82, 29.70, 28.78, 28.49, 28.37, 26.87, 26.75, 25.80, 25.62, 22.69.

Boc-Leu-BiCha-Lys(*N*-N_3Ac)-OMe (24b)

Boc-BiCha-Lys(*N*-N_3Ac)-OMe **24a** (72 mg, 0.13 mmol) was treated with TFA for 15 min, followed by co-evaporation with toluene (2x). The resulting product TFA·BiCha-Lys(*N*-N_3Ac)-OMe was dissolved in DCM and Boc-Leu-OH (35 mg, 0.15 mmol, 1.2 equiv.), HCTU (62 mg, 0.15 mmol, 1.2 equiv.) and DiPEA (76 µl, 0.44 mmol, 3.5 equiv.) were added. After stirring overnight, the reaction mixture was concentrated and redissolved in EtOAc and washed with 1N HCl (2x), sat. $NaHCO_3$ (2x) and brine (1x). The organic layer was dried over $NaSO_4$, filtered , concentrated and purified by column chromatography (40→80% EtOAc/pent) providing the title compound (69 mg, 0.1 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.94 (d, *J* = 7.8 Hz, 1H), 6.88 – 6.73 (m, 2H), 5.09 (d, *J* = 5.9 Hz, 1H), 4.56 – 4.34 (m, 2H), 4.12 (d, *J* = 6.9 Hz, 1H), 4.07 – 3.92 (m, 2H), 3.73 (s, 3H), 3.44 – 3.30 (m, 1H), 3.23 – 3.11 (m, 1H), 1.85 (dt, *J* = 14.5, 8.7 Hz, 2H), 1.77 – 1.46 (m, 13H), 1.44 (s, 9H), 1.42 – 1.06 (m, 15H), 1.03 – 0.81 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.04, 172.43, 171.99, 167.40, 155.81, 80.32, 53.40, 52.66, 52.45, 52.05, 51.67, 51.24, 43.34, 43.30, 41.66, 41.17, 40.24, 38.72, 34.34, 31.28, 30.99, 30.61, 30.30, 30.00, 29.77, 28.74, 28.37, 26.91, 26.80, 25.63, 25.45, 24.83, 23.04, 22.28, 21.99.

Morph-Leu-BiCha-Lys(*N*-N_3Ac)-OMe (24c)

Boc-Leu-BiCha-Lys(*N*-N_3Ac)-OMe **24b** (69 mg, 0.1 mmol) was treated with TFA for 15 min, followed by co-evaporation with toluene (2x). The resulting product TFA·Leu-BiCha-Lys(*N*-N_3Ac)-OMe was dissolved in DCM and morpholino acetic acid·HCl (22 mg, 0.12 mmol, 1.2 equiv.), HCTU (50 mg, 0.12 mmol, 1.2 equiv.) and DiPEA (78 µl, 0.45 mmol, 4.5 equiv.) were added. After stirring for 1 hour, the reaction mixture was concentrated and redissolved in EtOAc and washed with sat. $NaHCO_3$ (2x) and brine (1x). The organic layer was dried over $NaSO_4$, filtered, concentrated and purified by column chromatography (0→20% MeOH/DCM) providing the title compound (66 mg, 0.92 mmol, 92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (d, *J* = 6.9 Hz, 1H), 6.90 (d, *J* = 7.9 Hz, 1H), 6.85 (d, *J* = 7.9 Hz, 1H), 6.80 – 6.71 (m, 1H), 4.52 – 4.31 (m, 3H), 4.05 – 3.88 (m, 2H), 3.83 – 3.60 (m, 7H), 3.43 – 3.26 (m, 1H), 3.23 – 3.10 (m, 1H), 3.02 (s, 2H), 2.52 (s, 4H), 1.82 (dt, *J* = 14.7, 8.2 Hz, 2H), 1.77 – 1.00 (m, 28H), 0.90 (dd, *J* = 12.9, 6.1 Hz, 6H), 0.87 – 0.74 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.46, 172.11, 171.94, 167.34, 66.84, 61.82, 53.86, 52.66, 52.46, 51.98, 51.76, 51.59, 51.37, 43.30, 41.53, 40.80, 40.09, 39.48, 38.68, 38.63, 35.73, 34.43, 33.83, 32.87, 31.33, 31.14, 30.60, 30.29, 30.03, 29.75, 28.74, 28.64, 26.87, 26.77, 25.70, 25.51, 25.03, 23.02, 22.25, 22.04.

Morph-Leu-BiCha-Lys(*N*-N_3Ac)-$NHNH_2$ (25)

To a solution of Morph-Leu-BiCha-Lys(*N*-N_3Ac)-OMe **24c** (66 mg, 0.92 mmol) in MeOH (1 mL) was added hydrazine-hydrate (135 µL, 2.7 mmol, 30 equiv.). After stirring for 3 h, the reaction mixture was concentrated and co-evaporated with toluene (3x) providing the product in a quantitative yield. ^{1}H NMR (400 MHz, MeOD/Chloroform- *d*) δ 4.48 (t, *J* = 7.0 Hz, 1H), 4.45 – 4.33 (m, 1H), 4.33 – 4.25 (m, 1H), 3.90 (s, 2H), 3.81 – 3.69 (m, 4H), 3.23 (tt, *J* = 13.4, 6.6 Hz, 2H), 3.07 (s, 2H), 2.56 (s, 4H), 1.87 – 1.05 (m, 30H), 0.96 (dd, *J* = 11.6, 5.4 Hz, 6H), 0.91 – 0.79 (m, 2H). ^{13}C NMR (101 MHz, MeOD, $CDCl_3$) δ 173.79, 173.59, 172.56, 171.60, 169.09, 67.41, 62.12, 54.25, 52.66, 52.51, 52.45, 52.18, 43.97, 42.22, 41.70, 40.78, 39.53, 38.84, 35.93, 35.01, 33.02, 32.11, 31.75, 31.16, 30.87, 30.64, 30.24, 29.25, 28.86, 27.39, 27.30, 26.29, 26.01, 25.54, 23.33, 23.30, 22.04.

Morph-Leu-BiCha-Lys(*N*-N_3Ac)-Ala-EK (26)

To a solution of Morph-Leu-BiCha-Lys(*N*-N_3Ac)-$NHNH_2$ **25** (72 mg, 0.1 mmol) in DMF at -35°C was added HCl (4N in dioxane, 95 µL, 3.8 equiv.) and *tert*-butyl nitrite (16 µL, 0.11 mmol, 1.1 equiv.). After stirring for 3 hours, TFA·Ala-EK (0.12 mmol, 1.2 equiv, obtained by treatment of Boc-Ala-EK with TFA for 30 min) and DiPEA (120 µL, 7 equiv.) were added and the reaction mixture was stirred overnight while warming up to room temperature. The reaction mixture was concentrated and purified by column chromatography (0→2% MeOH/DCM) providing the title compound as a white powder after lyophilization (35.4 mg, 0.42 mmol, 42%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 – 7.50 (m, 2H), 7.37 (d, *J* = 6.0 Hz, 1H), 6.85 (d, *J* = 6.4 Hz, 1H), 6.71 (t, *J* = 5.6 Hz, 1H), 4.73 – 4.58 (m, 1H), 4.53 (q, *J* = 7.5 Hz, 1H), 4.49 – 4.38 (m, 2H), 4.00 (q, *J* = 16.2 Hz, 2H), 3.82 – 3.67 (m, 4H), 3.39 (dq, *J* = 13.6, 6.8 Hz, 1H), 3.29 (d, *J* = 5.1 Hz, 1H), 3.18 (dq, *J* = 11.8, 5.8 Hz, 1H), 3.06 (dd, *J* = 13.6, 9.4 Hz, 2H), 2.91 – 2.84 (m, 1H), 2.55 (bs, 4H), 1.84 – 1.52 (m, 15H), 1.50 (s, 3H), 1.37 (dd, *J* = 19.6, 9.7 Hz, 10H), 1.27 (d, *J* = 7.1 Hz, 3H), 1.13 (dt, *J* = 22.4, 12.7 Hz, 5H), 0.90 (dd, *J* = 10.8, 5.9 Hz, 6H), 0.83 (d, *J* = 10.0 Hz, 2H). HRMS (m/z): calculated for $C_{41}H_{69}N_9O_8$ $[M+H]^+$ 816.53419, found 816.53406.

Morph-Leu-BiCha-Lys(*N*-Ac-BODIPY(FL))-Ala-EK (27)

To a degassed solution of epoxyketone **26** (7.2 mg, 8.8 µmol) and BODIPY(Fl)-alkyne (4.4 mg, 24 µmol, 1.5 equiv.) in DMF (1 mL) under an argon atmosphere was added $CuSO_4 \cdot 5H_2O$ (0.5 equiv, 4.4 µmol (100 µL from degassed stock solution of 44 µmol/mL)) and NaAsc (0.75 equiv, 6.6 µmol (100 µL from degassed stock solution of 66 µmol/mL)). After stirring overnight, the reaction mixture immediately purified by HPLC (C_{18}, 55-65% MeCN, 0.1% TFA, 10 min gradient) provided the product as a blue powder after lyophilisation (2.81 mg, 2.5 µmol, 28%). ^{1}H NMR (850 MHz, $CDCl_3$) δ 7.59 (s, 1H), 7.31 (s, 1H), 7.07 (s, 1H), 6.96 (s, 1H), 6.79 (s, 1H), 6.05 (s, 2H), 5.08 (s, 2H), 4.51 – 4.46 (m, 1H), 4.42 – 4.30 (m, 3H), 3.82 (s, 4H), 3.70 (s, 2H), 3.36 (dt, *J* = 13.2, 6.6 Hz, 1H), 3.24 (d, *J* = 5.0 Hz, 1H), 3.15 (dd, *J* = 13.5, 5.5 Hz, 1H), 3.02 – 2.97 (m, 2H), 2.90 (dd, *J* = 4.9, 2.1 Hz, 1H), 2.86 (bs, 4H), 2.82 – 2.76 (m, 2H), 2.51 (s, 6H), 2.39 (s, 6H), 2.00 – 1.07 (m, 37H), 1.52 (s, 3H), 1.30 (d, *J* = 7.1 Hz, 3H), 0.94 – 0.90 (m, 3H), 0.87 (t, *J* = 5.5 Hz, 3H). ^{13}C NMR (214 MHz, $CDCl_3$) δ 208.61, 172.85, 172.78, 171.22, 165.91, 154.08, 147.98, 146.04, 140.40, 131.51, 125.67, 123.11, 121.82, 67.23, 59.21, 53.52, 52.95, 52.70, 52.68, 52.11, 51.71, 48.35, 43.38, 43.31, 40.65, 38.96, 34.55, 33.82, 32.74, 31.64, 30.98, 30.66, 30.46, 30.36, 30.34, 30.00, 29.83, 29.73, 28.51, 28.23, 28.14, 26.96, 26.86, 25.68, 25.59, 25.49, 25.09, 23.01, 22.96, 21.96, 21.93, 21.86, 16.98, 16.94, 16.91, 16.59, 14.61. LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 13.0 min):R_t (min): 8.30 (ESI-MS (m/z): 1144.27 $[M+H]^+$). HRMS (m/z): calculated for $C_{60}H_{92}BF_2N_{11}O_8$ $[M+H]^+$ 1144.72741, found 1144.72742.

Boc-1-Nap-Phe-OMe (28)

The title compound was prepared by the general procedure for peptide coupling on a 1 mmol scale. Column chromatography provided the product (423 mg, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, *J* = 8.0 Hz, 1H), 7.85 (d, *J* = 8.0 Hz, 1H), 7.75 (d, *J* = 8.2 Hz, 1H), 7.59 – 7.52 (m, 1H), 7.52 – 7.45 (m, 1H), 7.41 – 7.28 (m, 2H), 7.23 – 7.14 (m, 3H), 6.98 – 6.88 (m, 2H), 6.09 – 5.93 (m, 1H), 5.20 – 5.07 (m, 1H), 4.80 – 4.57 (m, 1H), 4.54 – 4.38 (m, 1H), 3.58 (s, 3H), 3.55 – 3.42 (m, 2H), 3.14 – 2.87 (m, 2H), 1.48 – 1.15 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.06, 170.87, 135.66, 134.03, 132.81, 132.06, 129.30, 128.90, 128.59, 127.92, 127.18, 126.62, 125.92, 125.50, 123.71, 55.58, 53.45, 52.32, 38.05, 35.86, 28.36.

N_3Phe-1-Nap-Phe-OMe (29)

Boc-1-Nap-Phe-OMe **28** (143 mg, 0.3 mmol) was deprotected using the standard procedure for Boc removal, followed by peptide coupling with N_3Phe-OH using the standard procedure for peptide couplings. Column chromatography provided the product in a quantitative yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 (d, *J* = 8.4 Hz, 1H), 7.84 – 7.78 (m, 1H), 7.71 (d, *J* = 8.2 Hz, 1H), 7.55 – 7.42 (m, 2H), 7.34 – 7.11 (m, 10H), 7.08 (d, *J* = 8.1 Hz, 1H), 6.86 (dd, *J* = 6.6, 2.9 Hz, 2H), 6.36 (d, *J* = 7.4 Hz, 1H), 4.90 (q, *J* = 8.1 Hz, 1H), 4.61 (q, *J* = 6.0 Hz, 1H), 3.89 (dd, *J* = 8.8, 3.9 Hz, 1H), 3.55 (s, 3H), 3.40 (dd, *J* = 13.9, 6.5 Hz, 1H), 3.28 (dd, *J* = 13.9, 8.3 Hz, 1H), 3.13 (dd, *J* = 14.0, 3.9 Hz, 1H), 2.96 (dd, *J* = 13.8, 5.7 Hz, 1H), 2.87 (dd, *J* = 13.8, 6.1 Hz, 1H), 2.70 (dd, *J* = 14.0, 8.8 Hz, 1H). ^{13}C NMR (101

MHz, $CDCl_3$) δ 170.78, 170.06, 168.38, 136.15, 135.54, 133.90, 132.27, 132.00, 129.54, 129.19, 128.84, 128.65, 128.46, 128.24, 127.96, 127.28, 127.12, 126.48, 125.81, 125.36, 123.71, 65.14, 53.65, 53.49, 52.22, 38.54, 37.95, 35.83.

N_3Phe-1-Nap-Phe-$NHNH_2$ (30)

N_3Phe-1-Nap-Phe-OMe **29** (0.3 mmol) was dissolved in 1:1 DCM/MeOH (6 mL), followed by the addition of $NH_2NH_2{\cdot}H_2O$ (872 µL, 18 mmol, 60 equiv.). The reaction mixture was stirred for 4 h, concentrated and co-evaporated with toluene (2x) thereby providing the product in a quantitative yield. Due to poor solubility of the product in MeOD, $CDCl_3$ and mixtures thereof, only a ^{1}H NMR could be measured. ^{1}H NMR (400 MHz, MeOD / $CDCl_3$) δ 8.07 (d, *J* = 8.4 Hz, 1H), 7.85 (d, *J* = 7.6 Hz, 1H), 7.76 (d, *J* = 8.2 Hz, 1H), 7.64 – 7.44 (m, 3H), 7.44 – 7.32 (m, 1H), 7.32 – 7.04 (m, 10H), 4.68 (t, *J* = 6.8 Hz, 1H), 4.52 – 4.40 (m, 1H), 4.02 (dd, *J* = 8.8, 4.3 Hz, 1H), 3.41 – 3.25 (m, 2H), 2.99 (t, *J* = 13.6 Hz, 2H), 2.81 (dd, *J* = 13.6, 7.9 Hz, 1H), 2.65 (dd, *J* = 14.0, 8.9 Hz, 1H).

Boc-2-Nap-Phe-OMe (31)

The title compound was prepared by the general procedure for peptide coupling on a 1 mmol scale. Column chromatography provided the product (465 mg, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 – 7.74 (m, 3H), 7.64 (s, 1H), 7.50 – 7.41 (m, 2H), 7.35 (d, *J* = 8.2 Hz, 1H), 7.20 – 7.11 (m, 3H), 6.95 – 6.88 (m, 2H), 6.33 (d, *J* = 6.8 Hz, 1H), 5.18 – 5.07 (m, 1H), 4.83 – 4.72 (m, 1H), 4.52 – 4.39 (m, 1H), 3.54 (s, 3H), 3.20 (d, *J* = 6.5 Hz, 2H), 3.02 (qd, *J* = 13.8, 6.0 Hz, 2H), 1.39 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.31, 170.85, 155.38, 135.61, 134.14, 133.53, 132.52, 129.22, 128.53, 128.46, 128.14, 127.71, 127.65, 127.48, 127.13, 126.23, 125.79, 80.26, 55.76, 53.37, 52.25, 38.59, 37.99, 28.30.

N_3Phe-2-Nap-Phe-OMe (32)

Boc-2-Nap-Phe-OMe **31** (143 mg, 0.3 mmol) was deprotected using the standard procedure for Boc removal, followed by peptide coupling with N_3Phe-OH using the standard procedure for peptide couplings. Column chromatography provided the product in a quantitative yield. ^{1}H NMR (400 MHz, MeOD) δ 7.83 (d, *J* = 8.9 Hz, 1H), 7.79 – 7.67 (m, 3H), 7.39 (tdd, *J* = 10.5, 9.2, 8.0, 3.6 Hz, 2H), 7.30 – 7.06 (m, 11H), 4.74 (t, *J* = 7.1 Hz, 1H), 4.63 (dd, *J* = 7.9, 6.0 Hz, 1H), 3.99 (dd, *J* = 8.8, 4.7 Hz, 1H), 3.48 (s, 3H), 3.16 (dd, *J* = 13.7, 6.3 Hz, 1H), 3.12 – 2.88 (m, 4H), 2.70 (dd, *J* = 14.0, 8.9 Hz, 1H). ^{13}C NMR (101 MHz, MeOD) δ 172.89, 172.43, 170.96, 137.55, 137.49, 135.04, 134.74, 133.79, 130.32, 130.25, 129.59, 129.51, 129.17, 129.11, 128.67, 128.63, 128.51, 128.08, 127.95, 127.60, 127.06, 126.67, 120.82, 110.60, 65.61, 55.20, 55.11, 52.88, 49.95, 49.74, 49.52, 49.31, 49.10, 48.88, 48.67, 39.29, 39.08, 38.57.

N_3Phe-2-Nap-Phe-$NHNH_2$ (33)

N_3Phe-2-Nap-Phe-OMe **32** (0.3 mmol) was dissolved in 1:1 DCM/MeOH (6 mL), followed by the addition of $NH_2NH_2{\cdot}H_2O$ (872 µL, 18 mmol, 60 equiv.). The reaction mixture was refluxed for 2 h, concentrated and co-evaporated with toluene (2x) thereby providing the product in a quantitative yield. Due to poor solubility of the product in MeOD, $CDCl_3$ and mixtures thereof, only a ^{1}H NMR could be measured.

Boc-BiPhe-Phe-OMe (34)

The title compound was prepared by the general procedure for peptide coupling on a 1 mmol scale. Column chromatography provided the product (497 mg, 99%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 (dd, *J* = 17.6, 7.7 Hz, 4H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.34 – 7.13 (m, 6H), 7.01 (d, *J* = 6.5 Hz, 2H), 6.58 (d, *J* = 7.6 Hz, 1H), 5.19 (d, *J* = 7.9 Hz, 1H), 4.86 – 4.75 (m, 1H), 4.51 – 4.38 (m, 1H), 3.60 (s, 3H), 3.18 – 2.97 (m, 4H), 1.40 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.26, 170.76, 155.18, 140.53, 139.58, 135.51, 129.65, 129.05, 128.58, 128.35, 127.06, 126.91, 126.78, 79.91, 55.43, 53.17, 52.08, 37.78, 28.10.

N_3Phe-BiPhe-Phe-OMe (35)

Boc-BiPhe-Phe-OMe **34** (143 mg, 0.28 mmol) was deprotected using the standard procedure for Boc removal, followed by peptide coupling with N_3Phe-OH using the standard procedure for peptide couplings. Column chromatography provided the product (155 mg, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 – 7.42 (m, 4H), 7.37 (t, *J* = 7.4 Hz, 2H), 7.34 – 7.14 (m, 9H), 7.11 (d, *J* = 8.1 Hz, 2H), 7.03 – 6.93 (m, 3H), 6.78 (d, *J* = 7.7 Hz, 1H), 4.90 (q, *J* = 6.9 Hz, 1H), 4.86 – 4.73 (m, 1H), 3.95 (dd, *J* = 8.3, 4.0 Hz, 1H), 3.61 (s, 3H), 3.23 (dd, *J* = 14.1, 4.0 Hz, 1H), 3.01 (dt, *J* = 13.8, 6.8 Hz, 3H), 2.91 (dt, *J* = 14.1, 7.8 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.22, 170.01, 168.45, 140.66, 139.98, 136.02, 135.62, 135.07, 129.94, 129.87, 129.62, 129.41, 129.27, 128.79, 128.68, 128.64, 128.54, 127.39, 127.35, 127.30, 127.27, 127.20, 127.01, 65.07, 53.74, 53.35, 52.35, 38.47, 38.16, 37.97.

N_3Phe-BiPhe-Phe-$NHNH_2$ (36)

N_3Phe-BiPhe-Phe-OMe **35** (0.25 mmol) was dissolved in 1:1 DCM/MeOH (6 mL), followed by the addition of $NH_2NH_2 \cdot H_2O$ (726 µL, 15 mmol, 60 equiv.). The reaction mixture was stirred at rt for 4 h, concentrated and co-evaporated with toluene (2x) thereby providing the product in a quantitative yield. Due to poor solubility of the product in MeOD, $CDCl_3$ and mixtures thereof, only a ^{1}H NMR could be measured. ^{1}H NMR (400 MHz, MeOD / $CDCl_3$) δ 7.60 (d, *J* = 6.1 Hz, 1H), 7.59 – 7.53 (m, 2H), 7.49 (d, *J* = 8.1 Hz, 2H), 7.41 (t, *J* = 7.6 Hz, 2H), 7.35 – 7.11 (m, 12H), 4.69 – 4.59 (m, 1H), 4.52 (t, *J* = 7.4 Hz, 1H), 4.08 (dd, *J* = 8.7, 4.6 Hz, 1H), 3.19 – 2.97 (m, 3H), 2.97 – 2.80 (m, 3H).

Boc-Cha-Phe-OMe (37)

The title compound was prepared by the general procedure for peptide coupling on a 1 mmol scale. Column chromatography provided the product (424 mg, 98%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 – 7.19 (m, 3H), 7.11 (d, *J* = 6.8 Hz, 2H), 6.66 (d, *J* = 7.2 Hz, 1H), 4.99 (d, *J* = 7.7 Hz, 1H), 4.84 (q, *J* = 6.1 Hz, 1H), 4.22 – 4.09 (m, 1H), 3.69 (s, 3H), 3.10 (qd, *J* = 13.8, 5.9 Hz, 2H), 1.85 – 1.53 (m, 8H), 1.44 (s, 9H), 1.33 – 1.04 (m, 5H), 1.04 – 0.77 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.20, 171.56, 155.40, 135.67, 129.17, 128.38, 126.93, 79.79, 53.04, 52.26, 52.13, 39.79, 37.78, 33.86, 33.45, 32.44, 28.16, 26.25, 26.06, 25.90.

N_3Phe-Cha-Phe-OMe (38)

Boc-Cha-Phe-OMe **37** (130 mg, 0.3 mmol) was deprotected using the standard procedure for Boc removal, followed by peptide coupling with N_3Phe-OH using the standard procedure for peptide couplings. Column chromatography provided the product (143 mg, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.20 (m, 8H), 7.12 – 7.08 (m, 2H), 6.97 (d, *J* = 7.9 Hz, 1H), 6.70 (d, *J* = 8.5 Hz, 1H), 4.88 (dt, *J* = 7.7, 6.0 Hz, 1H), 4.59 (td, *J* = 8.5, 6.5 Hz, 1H), 3.97 (dd, *J* = 8.2, 4.0 Hz, 1H), 3.71 (s, 3H), 3.29 (dd, *J* = 14.1, 4.0 Hz, 1H), 3.19 – 3.04 (m, 2H), 2.98 (dd, *J* = 14.1, 8.2 Hz, 1H), 1.73 – 1.52 (m, 6H), 1.45 – 1.32 (m, 1H), 1.24 – 1.05 (m, 4H), 1.00 – 0.73 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.64, 171.38, 168.43, 136.08, 135.83, 129.56, 129.37, 128.71, 128.59, 127.31, 127.20, 65.16, 53.21, 52.41, 50.76, 39.77, 38.43, 37.97, 33.86, 33.44, 32.77, 26.37, 26.11, 26.04.

N_3Phe-Cha-Phe-$NHNH_2$ (39)

N3Phe-Cha-Phe-OMe **38** (0.3 mmol) was dissolved in 1:1 DCM/MeOH (6 mL), followed by the addition of NH2-NH2.H2O (872 µL, 18 mmol, 60 equiv.). The reaction mixture was stirred at rt for 4 h, concentrated and co-evaporated with toluene (2x) thereby providing the product in a quantitative yield. Due to poor solubility of the product in MeOD, $CDCl_3$ and mixtures thereof, NMR analysis could not be performed.

Fmoc-Trp(Boc)-Phe-OMe (40)

The title compound was prepared by the general procedure for peptide coupling on a 2 mmol scale. Column chromatography (10→30% EtOAc/pent) provided the product (726 mg, 52%), which was directly used in the next step.

H-Trp(Boc)-Phe-OMe (41)

A solution of Fmoc-Trp(Boc)-Phe-OMe **40** (726 mg, 1.04 mmol) in 1:1 DCM/diethylamine (10 mL) was stirred for 1 h. After evaporation of the solvent, the residue does is washed with MeCN and DCM (product does not dissolve, Fmoc residues do) and evaporated to dryness providing the product with impurities left (293 mg, 0.63 mmol). LC-MS (linear gradient 10 → 90% MeCN/H2O, 0.1% TFA, 13.0 min):Rt (min): 7.89 (ESI-MS (m/z): 433.8 [M+ Na - tBu]+).

N_3Phe-Trp(Boc)-Phe-OMe (42)

The title compound was prepared by the general procedure for peptide coupling on a 0.63 mmol scale. Column chromatography provided the product (60 mg, 15%). LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 13.0 min):R_t (min): 10.82 (ESI-MS (m/z): 639.00 $[M+H]^+$)

N_3Phe-Trp(+-Boc)-Phe-$NHNH_2$ (43)

N_3Phe-Trp(Boc)-Phe-OMe (**42**) (60 mg, 0.1 mmol) was dissolved in MeOH (1 mL), followed by the addition of $NH_2NH_2{\cdot}H_2O$ (145 µL, 3 mmol, 30 equiv.). The reaction mixture was stirred for 3 h at rt, concentrated and co-evaporated with toluene (2x) thereby providing the product as a mixture of compounds (+ and – Boc). LC-MS (linear gradient 10 → 90% MeCN/H_2O, 0.1% TFA, 13.0 min):R_t (min): 7.06 (ESI-MS (m/z): 539.00 $[M+H]^+$, -Boc), 8.45. (ESI-MS (m/z): 639.07 $[M+H]^+$, +Boc)

Boc-Ala(Ada)-Leu-OMe (44)

The title compound was prepared by the general procedure for peptide coupling on a 0.31 mmol scale. Column chromatography (5-20% EtOAc/pent) provided the product (125 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.54 (d, *J* = 8.0 Hz, 1H), 4.83 (d, *J* = 8.3 Hz, 1H), 4.57 (td, *J* = 8.7, 4.8 Hz, 1H), 4.20 – 4.07 (m, 1H), 3.69 (s, 3H), 1.93 (s, 3H), 1.79 – 1.48 (m, 16H), 1.42 (s, 9H), 1.23 (dd, *J* = 14.5, 8.0 Hz, 1H), 0.90 (d, *J* = 6.0 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.20, 172.80, 155.52, 80.16, 52.30, 50.76, 50.63, 46.01, 42.53, 41.61, 36.96, 32.28, 28.67, 28.41, 24.78, 22.95, 21.92.

N_3Phe-Ala(Ada)-Leu-OMe (45)

Boc-Ala(Ada)-Leu-OMe **44** (124 mg, 0.39 mmol) was deprotected using the standard procedure for Boc removal, followed by peptide coupling with N_3Phe-OH using the standard procedure for peptide couplings. Column chromatography (10→20% EtOAc/pent), provided the product (149 mg, 100%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 (p, *J* = 7.2 Hz, 5H), 6.76 (d, *J* = 8.2 Hz, 1H), 6.62 (d, *J* = 8.1 Hz, 1H), 4.53 (td, *J* = 8.4, 5.4 Hz, 1H), 4.45 (td, *J* = 7.9, 4.8 Hz, 1H), 4.16 (dd, *J* = 8.4, 3.9 Hz, 1H), 3.70 (s, 3H), 3.29 (dd, *J* = 14.1, 3.9 Hz, 1H), 2.98 (dd, *J* = 14.1, 8.5 Hz, 1H), 1.91 (s, 3H), 1.76 – 1.48 (m, 10H), 1.44 (s, 6H), 1.32 – 1.18 (m, 1H), 0.90 (t, *J* = 5.9 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.00, 171.70, 168.22, 135.98, 129.49, 128.66, 127.29, 65.24, 52.26, 50.86, 49.22, 45.95, 42.30, 41.34, 38.24, 36.74, 32.23, 28.48, 24.87, 22.73, 21.99.

N_3Phe-Ala(Ada)-Leu-$NHNH_2$ (46)

N_3Phe-Ala(Ada)-Leu-OMe **45** (149 mg, 0.29 mmol) was dissolved in MeOH (2 mL), followed by the addition of $NH_2NH_2{\cdot}H_2O$ (350 µL, 8.6 mmol, 30 equiv.). The reaction mixture was stirred for 2 h at rt, followed by refluxing for 2 h, concentrated and co-evaporated with toluene (2x) thereby providing the product in a quantitative yield.^{1}H NMR (400 MHz, MeOD/$CDCl_3$) δ 7.35 – 7.21 (m, 5H), 4.54 – 4.28 (m, 2H), 4.16 (dd, *J* = 8.6, 4.6 Hz, 1H), 3.22 (dd, *J* = 14.1, 4.6 Hz, 1H), 3.00 (dd, *J* = 14.0, 8.6 Hz, 1H), 1.95 (d, *J* = 10.7 Hz, 3H), 1.77 – 1.33 (m, 17H), 0.93 (dd, *J* = 15.5, 6.1 Hz, 6H). ^{13}C NMR (101 MHz, MeOD/$CDCl_3$) δ 173.69, 172.90, 170.14, 136.92, 129.94, 129.15, 127.69, 65.17, 50.99, 50.39, 46.63, 42.88, 41.42, 38.32, 37.35, 32.95, 29.26, 25.28, 23.09, 22.15.

Boc-Ala(tBu)-Phe-OMe (47)

The title compound was prepared by the general procedure for peptide coupling on a 1 mmol scale. Column chromatography (10→20% EtOAc/pent) provided the product (400 mg, 100%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.20 (m, 3H), 7.11 (d, *J* = 6.8 Hz, 2H), 6.71 (d, *J* = 7.5 Hz, 1H), 4.94 (d, *J* = 7.9 Hz, 1H), 4.82 (q, *J* = 6.0 Hz, 1H), 4.20 – 4.09 (m, 1H), 3.69 (s, 3H), 3.10 (qd, *J* = 13.8, 6.0 Hz, 2H), 1.81 (d, *J* = 14.4 Hz, 1H), 1.43 (s, 9H), 1.37 (dd, *J* = 14.5, 8.8 Hz, 1H), 0.93 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.66, 171.69, 155.33, 135.87, 129.35, 128.54, 127.09, 80.02, 53.30, 52.26, 45.60, 37.95, 30.42, 29.67, 28.38.

N_3Phe-Ala(tBu)-Phe-OMe (48)

Boc-Ala(tBu)-Cha-Phe-OMe **47** (130 mg, 0.3 mmol) was deprotected using the standard procedure for Boc removal, followed by peptide coupling with N_3Phe-OH using the standard procedure for peptide couplings. Column chromatography provided the product (445 mg, 93%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36 – 7.24 (m, 8H), 7.24 – 7.12 (m, 3H), 6.79 (d, *J* = 8.6 Hz, 1H), 4.91 (q, *J* = 6.2 Hz, 1H), 4.64 (td, *J* = 8.2, 4.7 Hz, 1H), 3.96 (dd, *J* = 8.6, 3.8 Hz, 1H), 3.73 (s, 3H), 3.34 (dd, *J* = 14.0, 3.7 Hz, 1H), 3.14 (qd, *J* = 14.0, 6.1 Hz, 2H), 2.97 (dd, *J* = 14.0, 8.7 Hz, 1H), 1.85 (dd, *J* = 14.5, 4.6 Hz, 1H), 1.40 (dd, *J* = 14.5, 7.9 Hz, 1H), 0.89 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.69, 171.62, 168.10, 136.08, 135.89, 129.55, 129.32, 128.66, 128.49, 127.24, 127.10, 65.05, 53.20, 52.27, 50.49, 45.23, 38.23, 37.93, 30.34, 29.59.

N_3Phe-Ala(tBu)-Phe-$NHNH_2$ (49)

N_3Phe-Ala(tBu)-Cha-Phe-OMe **48** (445 mg, 0.93 mmol) was dissolved in MeOH (10 mL), followed by the addition of $NH_2NH_2{\cdot}H_2O$ (1.36 mL, 28 mmol, 30 equiv.). The reaction mixture was stirred at rt for 4 h, concentrated and co-evaporated with toluene (2x) thereby providing the product in a quantitative yield. ^{1}H NMR (400 MHz, MeOD) δ 7.37 – 7.10 (m, 10H), 4.60 (t, *J* = 7.4 Hz, 1H), 4.46 (dd, *J* = 8.5, 4.1 Hz, 1H), 4.08 (dd, *J* = 8.8, 4.4 Hz, 1H), 3.16 (dd, *J* = 14.1, 4.4 Hz, 1H), 3.09 (dd, *J* = 13.7, 6.8 Hz, 1H), 3.00 – 2.87 (m, 2H), 1.60 (dd, *J* = 14.5, 4.2 Hz, 1H), 1.43 (dd, *J* = 14.5, 8.6 Hz, 1H), 0.87 (s, 9H). ^{13}C NMR (101 MHz, MeOD) δ 173.36, 171.69, 170.11, 137.23, 136.97, 129.94, 129.82, 129.19, 129.00, 127.67, 127.38, 65.11, 53.94, 53.85, 51.81, 51.71, 45.70, 38.64, 38.38, 30.86, 29.82.

Fmoc-Ser(OtBu)-Phe-OMe (50)

The title compound was prepared by the general procedure for peptide coupling on a 2 mmol scale. Column chromatography (10→50% EtOAc/pent) provided the product (960 mg, 88%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.78 (d, *J* = 7.5 Hz, 2H), 7.62 (d, *J* = 7.3 Hz, 2H), 7.42 (t, *J* = 7.4 Hz, 8H), 7.15 (d, *J* = 6.5 Hz, 2H), 5.80 (s, 1H), 4.93 (q, *J* = 5.8 Hz, 1H), 4.41 (d, *J* = 6.9 Hz, 2H), 4.25 (t, *J* = 7.0 Hz, 2H), 3.84 (dd, *J* = 8.1, 3.4 Hz, 1H), 3.72 (s, 3H), 3.41 (t, *J* = 8.4 Hz, 1H), 3.15 (t, *J* = 5.0 Hz, 2H), 1.20 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.48, 170.01, 143.93, 143.77, 141.32, 135.82, 129.31, 128.62, 127.76, 127.19, 127.11, 125.18, 120.03, 74.38, 67.22, 61.71, 54.23, 53.43, 52.28, 47.17, 38.08, 27.35.

H-Ser(OtBu)-Phe-OMe (51)

Fmoc-Ser(OtBu)-Phe-OMe **50** (547 mg, 1 mmol) was dissolved in THF (10 mL), followed by the addition of EtSH (0.72 mL, 10 mmol, 10 equiv.) and DBU (15 µL, 0.1 mmol, 0.1 equiv.). After stirring for 90 min, the reaction mixture was concentrated and purified by column chromatography (10→100% EtOAc/pent) yielding the product (260 mg, 81%). ^{1}H NMR (300 MHz, MeOD) δ 7.34 – 7.05 (m, 5H), 4.78 (t, *J* = 6.2 Hz, 1H), 3.69 (s, 3H), 3.49 – 3.34 (m, 3H), 3.08 (qd, *J* = 13.8, 6.2 Hz, 2H), 1.13 (s, 9H).

N_3Phe-Ser(OtBu)-Phe-OMe (52)

The title compound was prepared by the general procedure for peptide coupling on a 0.81 mmol scale. Column chromatography (10→50% EtOAc/pent) provided the product (230 mg, 58%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37 – 7.18 (m, 9H), 7.16 – 7.03 (m, 3H), 4.87 (q, *J* = 6.0 Hz, 1H), 4.45 – 4.32 (m, 1H), 4.31 – 4.21 (m, 1H), 3.71 (s, 3H), 3.63 (dd, *J* = 8.4, 3.9 Hz, 1H), 3.31 (dd, *J* = 14.0, 3.9 Hz, 1H), 3.20 – 3.01 (m, 4H), 1.14 (s, 9H). ^{13}C NMR (75 MHz,

$CDCl_3$) δ 171.40, 169.54, 168.41, 135.89, 135.70, 129.57, 129.23, 128.56, 127.21, 127.14, 74.40, 65.19, 60.86, 53.32, 52.43, 52.23, 38.40, 37.95, 27.24.

N_3Phe-Ser(OtBu)-Phe-$NHNH_2$ (53)

N_3Phe-Ser(OtBu)-Phe-OMe **52** (445 mg, 0.93 mmol) was dissolved in MeOH (5 mL), followed by the addition of $NH_2NH_2{\cdot}H_2O$ (670 µL, 14 mmol, 30 equiv.). The reaction mixture was stirred for 30 min at rt, concentrated and co-evaporated with toluene (2x) thereby providing the product in a quantitative yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37 – 7.11 (m, 10H), 7.07 (d, *J* = 6.9 Hz, 1H), 6.79 (d, *J* = 8.5 Hz, 1H), 4.71 (dt, *J* = 8.3, 6.4 Hz, 1H), 4.32 (td, *J* = 7.9, 4.4 Hz, 1H), 4.19 (dd, *J* = 7.7, 4.1 Hz, 1H), 3.56 (dd, *J* = 9.0, 4.4 Hz, 1H), 3.28 (dd, *J* = 14.1, 4.1 Hz, 1H), 3.19 (dd, *J* = 13.8, 6.2 Hz, 1H), 3.14 – 2.97 (m, 3H), 1.12 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.84, 169.52, 168.70, 135.90, 135.71, 129.53, 129.19, 128.81, 128.59, 127.28, 74.81, 65.00, 61.06, 53.04, 53.01, 38.31, 37.59, 27.18.

Fmoc-Thr(OtBu)-Phe-OMe (54)

The title compound was prepared by the general procedure for peptide coupling on a 2 mmol scale. Column chromatography (10→50% EtOAc/pent) provided the product (420 mg, 38%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.71 (d, *J* = 7.6 Hz, 1H), 7.61 (d, *J* = 7.4 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.35 – 7.19 (m, 5H), 7.13 (d, *J* = 6.4 Hz, 2H), 6.00 (d, *J* = 4.2 Hz, 1H), 4.88 (q, *J* = 6.3 Hz, 1H), 4.38 (d, *J* = 7.1 Hz, 2H), 4.23 (t, *J* = 7.2 Hz, 1H), 4.19 – 4.04 (m, 2H), 3.73 (s, 3H), 3.13 (qd, *J* = 14.0, 6.1 Hz, 2H), 1.21 (s, 9H), 1.08 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.65, 169.22, 156.11, 143.79, 141.38, 135.98, 129.22, 128.71, 127.80, 127.26, 127.14, 125.25, 120.08, 75.63, 67.07, 66.75, 58.43, 53.53, 52.32, 47.26, 38.05, 28.14, 16.44.

H-Thr(OtBu)-Phe-OMe (55)

Fmoc-Thr(OtBu)-Phe-OMe **54** (370 mg, 0.66 mmol) was dissolved in 20% piperidine/DMF). After stirring for 90 min, the reaction mixture was concentrated and purified by column chromatography (0→20% MeOH/EtOAc) yielding the product (185 mg, 83%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.95 (d, *J* = 7.8 Hz, 1H), 7.33 – 7.10 (m, 5H), 4.83 (q, *J* = 6.4 Hz, 1H), 4.01 (qd, *J* = 6.2, 3.3 Hz, 1H), 3.69 (s, 3H), 3.14 (d, *J* = 3.3 Hz, 1H), 3.08 (t, *J* = 6.6 Hz, 2H), 1.96 (bs, 2H), 1.14 – 1.05 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.39, 172.05, 136.28, 129.43, 129.25, 128.55, 127.05, 74.11, 67.91, 59.75, 53.11, 52.18, 38.19, 29.72, 28.90, 28.51, 19.47.

N_3Phe-Thr(OtBu)-Phe-OMe (56)

The title compound was prepared by the general procedure for peptide coupling on a 0.55 mmol scale. Column chromatography (5→20% EtOAc/pent) provided the product (126 mg, 45%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.62 (d, *J* = 7.5 Hz, 1H), 7.39 – 7.18 (m, 9H), 7.09 (d, *J* = 6.6 Hz, 2H), 4.81 (q, *J* = 6.5 Hz, 1H), 4.23 (dt, *J* = 7.6, 3.9 Hz, 2H), 3.91 (dd, *J* = 6.3, 4.3 Hz, 1H), 3.71 (s, 3H), 3.28 (dd, *J* = 14.0, 4.0 Hz, 1H), 3.08 (ddt, *J* = 20.2, 14.0, 6.4 Hz, 3H), 1.20 (s, 9H), 0.76 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.59, 168.93, 168.23, 135.94, 135.83, 129.72, 129.20, 128.72, 128.66, 127.36, 127.27, 75.70, 66.08, 65.23, 57.20, 53.50, 52.32, 38.36, 37.96, 28.11, 16.33.

N_3Phe-Thr(OtBu)-Phe-$NHNH_2$ (57)

N_3Phe-Thr(OtBu)-Phe-OMe **56** (126 mg, 0.25 mmol) was dissolved in MeOH (2.5 mL), followed by the addition of $NH_2NH_2{\cdot}H_2O$ (362 µL, 7.4 mmol, 30 equiv.). The reaction mixture was stirred for 2.5 h at rt, concentrated and co-evaporated with toluene (2x) thereby providing the product in a quantitative yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36 – 7.17 (m, 10H), 7.18 – 7.11 (m, 2H), 6.59 (d, *J* = 9.2 Hz, 1H), 4.77 (dt, *J* = 9.2, 5.9 Hz, 1H), 4.31 – 4.18 (m, 2H), 3.85 (dt, *J* = 10.9, 5.5 Hz, 1H), 3.36 – 3.20 (m, 2H), 3.11 (dd, *J* = 14.1, 7.1 Hz, 1H), 2.95 (dd, *J* = 13.7, 6.3 Hz, 1H), 1.96 (s, 2H), 1.10 (s, 9H), 0.68 (d, *J* = 6.4 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.41, 168.65, 136.16, 136.04, 130.13, 129.77, 129.42, 129.07, 127.93, 127.83, 76.56, 66.64, 65.38, 57.69, 53.15, 38.62, 38.01, 28.28, 21.40, 17.08.

Morph-BiCha-Phe-OMe (58)

The title compound was prepared by the general procedure for peptide coupling on a 0.5 mmol scale. Column chromatography (20→100% EtOAc/pent; followed by 5% MeOH/DCM) provided the product (209 mg, 77%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40 (d, *J* = 8.1 Hz, 1H), 7.34 – 7.20 (m, 3H), 7.13 (d, *J* = 6.8 Hz, 2H), 6.79 – 6.69 (m, 1H), 4.82 (q, *J* = 6.7 Hz, 1H), 4.53 – 4.36 (m, 1H), 3.73 (s, 3H), 3.72 – 3.66 (m, 4H), 3.17 (dd, *J* = 13.9, 5.5 Hz, 1H), 3.05 (dd, *J* = 14.2, 6.5 Hz, 1H), 2.97 (d, *J* = 3.9 Hz, 2H), 2.53 – 2.44 (m, 4H), 1.93 – 0.76 (m, 23H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.35, 171.26, 169.78, 135.51, 128.88, 128.14, 126.66, 66.49, 61.34, 53.41, 52.89, 51.98, 50.71, 50.23, 42.84, 41.10, 39.76, 38.66, 37.36, 35.04, 34.20, 33.49, 32.40, 30.92, 30.15, 29.83, 29.62, 29.26, 28.39, 26.44, 26.34, 25.23, 25.03.

Morph-BiCha-Phe-$NHNH_2$ (59)

Morph-BiCha-Phe-$NHNH_2$ **58** (209 mg, 0.41 mmol) was dissolved in MeOH (5 mL), followed by the addition of $NH_2NH_2{\cdot}H_2O$ (550 µL, 12 mmol, 30 equiv.). The reaction mixture was stirred for 3 h at rt, concentrated and co-evaporated with toluene (2x) thereby providing the product in a quantitative yield. ^{1}H NMR (400 MHz, MeOD) δ 7.35 – 7.11 (m, 5H), 4.57 (ddt, *J* = 8.7, 6.4, 3.3 Hz, 1H), 4.50 – 4.34 (m, 1H), 3.74 – 3.63 (m, 4H), 3.08 (dd, *J* = 13.7, 6.3 Hz, 1H), 3.00 (s, 2H), 2.97 – 2.87 (m, 1H), 2.47 (d, *J* = 4.4 Hz, 4H), 1.86 – 0.75 (m, 23H). ^{13}C NMR (101 MHz, MeOD) δ 172.87, 172.82, 171.00, 136.90, 129.07, 128.11, 126.45, 66.51, 61.16, 53.43, 53.27, 51.29, 50.83, 43.33, 41.67, 40.27, 39.36, 37.79, 35.61, 34.43, 33.73, 32.29, 31.16, 30.38, 30.07, 29.89, 29.71, 29.50, 28.15, 26.61, 26.53, 25.44, 25.15.

Boc-Phe-BiCha-Phe-OMe (60)

The title compound was prepared by the general procedure for peptide coupling on a 0.75 mmol scale. Column chromatography (20→40% EtOAc/pent) provided the product (473 mg, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.17 (m, 9H), 7.14 (d, *J* = 7.1 Hz, 2H), 6.96 (d, *J* = 7.3 Hz, 1H), 6.84 – 6.74 (m, 1H), 4.84 (q, *J* = 6.3 Hz, 1H), 4.50 (dt, *J* = 13.7, 7.7 Hz, 2H), 3.70 (s, 3H), 3.20 – 2.95 (m, 4H), 1.41 (s, 9H), 1.87 – 0.73 (m, 23H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.65, 171.48, 171.43, 171.40, 155.49, 136.66, 135.89, 129.38, 129.29, 128.56, 127.08, 126.84, 80.05, 55.46, 53.50, 53.42, 52.26, 51.64, 51.23, 43.23, 43.17, 41.61, 40.26, 37.90, 35.83, 34.13, 33.61, 30.71, 30.49, 30.19, 29.79, 29.62, 29.54, 28.91, 28.27, 26.83, 26.72, 25.44, 25.29.

Morph-Phe-BiCha-Phe-OMe (61)

Boc-Phe-BiCha-Phe-OMe **60** (473 mg, 0.7 mmol) was deprotected using the standard procedure for Boc removal, followed by peptide coupling with 2-morpholinoacetic acid using the standard procedure for peptide couplings. Column chromatography (2% MeOH/DCM), provided the product (503 mg, 100%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 (d, *J* = 7.8 Hz, 1H), 7.33 – 7.15 (m, 9H), 7.15 – 7.10 (m, 2H), 7.00 (d, *J* = 7.8 Hz, 1H), 6.75 (d, *J* = 7.8 Hz, 1H), 4.82 (q, *J* = 6.2 Hz, 1H), 4.73 (td, *J* = 8.5, 5.7 Hz, 1H), 4.48 – 4.32 (m, 1H), 3.70 (s, 3H), 3.55 (t, *J* = 4.2 Hz, 4H), 3.23 – 2.95 (m, 5H), 2.38 – 2.27 (m, 2H), 2.27 – 2.17 (m, 2H), 1.88 – 0.71 (m, 23H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.67, 171.41, 171.08, 170.99, 170.34, 136.43, 135.86, 129.28, 129.12, 128.67, 128.53, 127.08, 127.01, 66.81, 61.58, 53.52, 53.33, 53.23, 52.30, 51.82, 51.40, 43.20, 43.11, 41.47, 40.06, 39.31, 38.61, 37.74, 37.09, 37.00, 35.51, 34.33, 33.63, 32.84, 30.96, 30.49, 30.20, 29.92, 29.64, 28.62, 26.78, 26.68, 25.54, 25.36.

Morph-Phe-BiCha-Phe-$NHNH_2$ (62)

Morph-Phe-BiCha-Phe-$NHNH_2$ **58** (203 mg, 0.7 mmol) was dissolved in MeOH (7.5 mL), followed by the addition of $NH_2NH_2{\cdot}H_2O$ (1 mL, 21 mmol, 30 equiv.). The reaction mixture was stirred for 3 h at rt, concentrated and co-evaporated with toluene (2x) thereby providing the product in a quantitative yield. ^{1}H NMR (400 MHz, $CDCl_3$, Methanol-*d*) δ 7.36 – 7.14 (m, 10H), 4.72 – 4.68 (m, 1H), 4.57 (t, *J* = 7.4 Hz, 1H), 4.40 – 4.27 (m, 1H), 3.65 – 3.54 (m, 4H), 3.20 – 3.07 (m, 2H), 3.08 – 2.82 (m, 4H), 2.36 (dd, *J* = 10.8, 6.4 Hz, 2H), 2.25 (dt, *J* = 11.1, 4.3 Hz, 2H), 1.87 – 0.70 (m, 23H). ^{13}C NMR (101 MHz, $CDCl_3$, MeOD) δ 173.52, 173.03, 171.82, 171.67, 137.43, 137.23, 129.84, 129.25, 129.10, 127.65, 127.48, 67.43, 61.99, 54.31, 54.12, 54.06, 52.93, 52.50, 49.64, 49.43, 49.21, 49.00, 48.79, 48.57, 48.36, 44.06, 42.42, 41.08, 39.86, 38.41, 38.24, 35.97, 34.95, 33.20, 31.57, 31.21, 30.95, 30.68, 29.00, 27.46, 27.38, 26.22, 25.94.

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. 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°C. Probe cocktail consist 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)-LU-112, BODIPY(FL)-epoxomicin and BODIPY(FL)-NC-001, Cy3 settings to detect BODIPY(TMR)-NC-005-VS and BODIPY(TMR)-epoxomicin and Cy5 settings to detect Cy5-NC-001.

Competition experiments in cell lysate

Cell lysates (diluted to 10-15 µg total protein in 9 µL buffer) were exposed to the inhibitors (10x stock in DMSO) at indicated concentrations for 1 h at 37 °C, followed by addition of probe cocktail (10x stock, 1.1 µL) and SDS-PAGE as described above. For IC_{50} determinations, intensities of bands were measured by fluorescent densitometry and divided by the intensity of bands in mock-treated lysates. Average values of three independent experiments were plotted against inhibitor concentrations. IC_{50} values were calculated using GraphPad Prism software.

Competition experiments in living RPMI-8226 cells

RPMI-8226 were cultured in RPMI-1640 media supplemented with 10% fetal calf serum, GlutaMAX™, penicillin, streptomycin in a 5% CO_2 humidified incubator. 5-8 × 10^5 cells/mL were exposed to inhibitors for 1 h at 37 °C. Cells were harvested and washed twice with PBS. Cell pellets were treated with lysis buffer on ice for 15 min, followed by centrifugation at 14000 rpm for 5 min. Proteasome inhibition in the obtained cell lysates was determined using the method described above. Intensities of bands were measured by fluorescent densitometry and divided by the intensity of bands in mock-treated cells. Average values of three independent experiments were plotted against inhibitor concentrations. IC_{50} values were calculated using GraphPad Prism software.

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