# Chemical tools to monitor and control human proteasome activities

Bruin, G. de

# CHAPTER 5

# Structure-based design of either β1i or β5i specific inhibitors of human immunoproteasomes*

## Introduction

Proteasomes are large, multi-catalytic complexes that partake in the degradation of cytosolic, nuclear and misfolded ER proteins in most kingdoms of life.[1] The 20S core particles (CP) in which proteolysis takes place are C2-symmetrical barrel-shaped multi-protein complexes composed of four stacked rings of seven subunits each: the two inner rings consisting of seven homologous yet distinct β-subunits (β1-β7) are flanked by two outer rings composed of the α-subunits 1-7.[2] The proteolytic activity resides within three catalytic subunits (one copy in each β-ring), namely β1c (also referred to as caspase-like, cleaving preferentially after acidic residues), β2c (trypsin-like, with a preference for basic residues) and β5c (chymotrypsin-like, preferring neutral, hydrophobic residues at the cleavage site[2-5]). Immune-competent cells express next to the constitutive proteasome (cCP) the interferon-γ-inducible subunits β1i (LMP2), β2i (MECL-1) and β5i (LMP7).[6] Upon cytokine stimulation these subunits replace their constitutive proteasome counterparts (β1c, β2c and β5c, respectively) to form immunoproteasomes (iCPs).

The substrate preference of cCP catalytic subunits considerably overlaps with their iCP analogues, though subtle differences have been observed.[7] In particular, whereas β1c prefers acidic residues at P1 (Asp or Glu; the amino acid residue residing at the C-terminus of the scissile peptide bond), β1i is biased to hydrophobic side chains at this position.[8] This divergence of substrate preference has been connected to processing and presentation of antigenic peptides from cytosolic/nuclear sources.

Proteasome inhibitors take up a prominent position both in fundamental and applied biomedical research. Based on the structural diversity of natural CP inhibitors, numerous

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synthetic compounds have been developed. Most of them are peptide-based and feature an electrophile that reacts with the N-terminal nucleophilic threonine hydroxyl group of catalytic proteasome subunits to form a covalent linkage. Depending on the nature of the electrophilic trap the covalent bond is transient (aldehydes, boronic acids), semi-permanent (beta-lactones) or permanent (vinyl sulfones, epoxyketones).[9, 10] Subunit specificity is largely governed by the nature of the peptide fragment (amino acid sequence) of a given inhibitor, though examples exist in which substitution of the electrophilic head group on an otherwise unaltered compound also impacts the proteasome inhibition profile.[11, 12] CP inhibitors are broadly used to study the proteolytic system in more detail and to modulate physiological processes, in particular cell proliferation. The finding that proteasomes are valid therapeutic targets in various cancers led to the development of the peptide boronic acid bortezomib (Velcade)[13-15] as a drug for the treatment of multiple myeloma (MM) and mantle cell lymphoma. This breakthrough was recently followed by the FDA approval of the peptide epoxyketone carfilzomib (Kyprolis)[16-18] as a second-generation CP inhibitor for the treatment of MM. Thus, both reversible and irreversible proteasome inhibitors are relevant candidates for drug development strategies. Currently, several proteasome inhibitors are in clinical trials, including marizomib (salinosporamide A, NPI-0052), delanzomib (CEP-18770)[19], ixazomib (MLN-9708)[20], and oprozomib (ONX-0912, PR-047).[21]

Although bortezomib and carfilzomib have been developed to primarily target the chymotrypsin-like active sites β5c and β5i, which are the most important ones for the anti-tumor effects, they also co-inhibit the subunits β1c/β1i as well as β2c/β2i at higher concentrations.[16, 22, 23] More recently, cell permeable ligands specific for one catalytic subunit ($\beta1^3$, $\beta2^4$ or $\beta5$[11, 24]) of the cCP together with its iCP counterpart have been developed. These compounds demonstrated that cytotoxicity in MM cell lines cannot be reached by exclusive blockage of β5c/β5i, but requires co-inhibition of either β1c/β1i or β2c/β2i.[3, 4] Thus, compounds specific for one out of the six catalytic subunits from which cCPs and iCPs are assembled, are highly desirable commodities for research and clinical applications nowadays. This certainly holds true for oncological research, given that in various lymphomas iCPs are the predominant proteasome species, where identification of the ideal target/dosage combination – which catalytic active site should be inhibited, and to what extent – needs to be established. Furthermore, this also applies to the immunological question of which proteolytic center is involved in the generation of a given antigenic peptide.

For many years X-ray diffraction data from yeast proteasomes (yCP) were used to clarify the mode of action and binding specificity of CP inhibitors, including approved drugs and drug candidates.[25-27] Recently, structure elucidation of the murine cCP and iCP complemented the yeast structures and highlighted the differences between the cCP and iCP.[28, 29] Perusal of the differences between the two structures and their inhibitor-bound counterparts provide

insight to structural features by which β1i and β5i may be targeted independently from β1c and β5c, respectively. Specifically, β5i appears to be able to accommodate a larger amino acid side chain at position P1 than β5c, while the opposite holds true for the P3 residue. On the other hand, the S1 pocket of β1i is more hydrophobic when compared to β1c, while the S3 pocket of β1i is more restricted in size and more polar compared to its counterpart β1c.

Here the discovery of inhibitors highly specific for either β5i or β1i in the presence of all constitutive proteasome and immunoproteasome active site is reported. The design of the β5i specific inhibitors is based on two compounds previously developed by ONYX Pharmaceuticals, namely ONX-0914[30, 31] (PR-957, **1**) and PR-924[30, 32, 33] (**2**) (Figure 1A). Using a structure-based design approach molecules were obtained with considerably improved selectivity for β5i over β5c. X-ray crystallographic analysis of the inhibitors in complex with the yCP provided important insights into the molecular basis of β5i selectivity. For the development of β1i selective inhibitors the previously reported β1c/β1i specific inhibitor NC-001[3] (**3**) (Figure 1B) was used as a starting point. In a first optimization round its key structural element, a proline residue at P3,[34] was modified and in a second step structural features at P1 were varied.

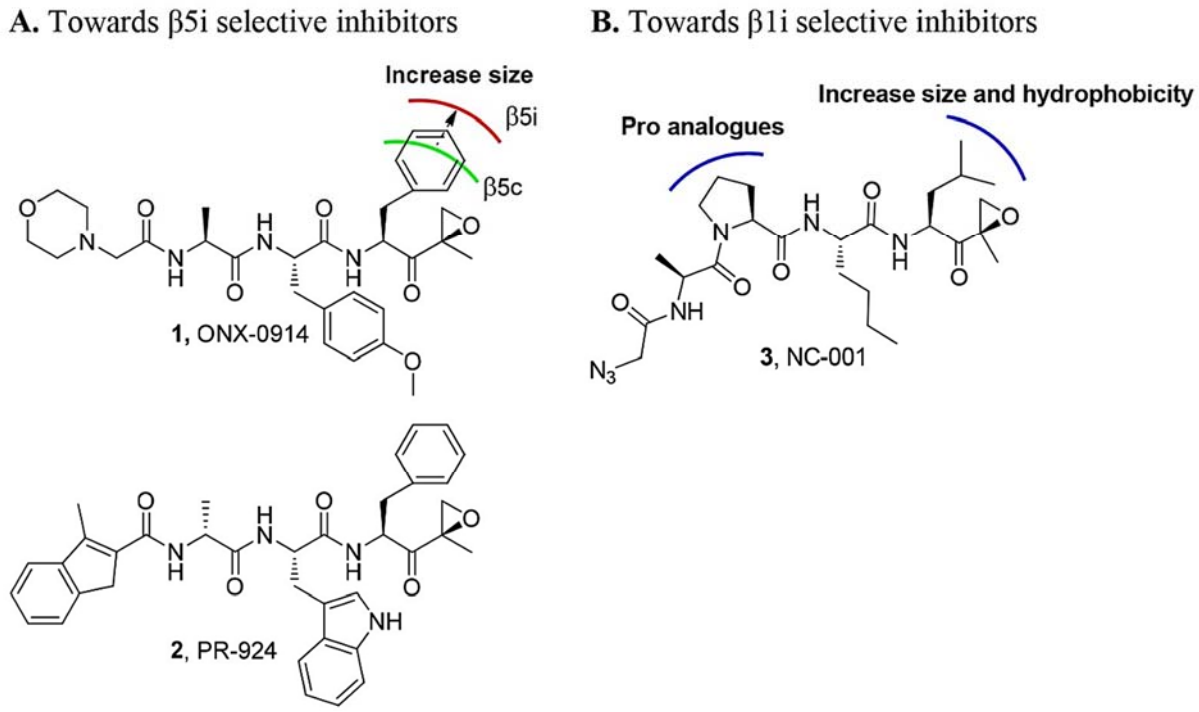



**Figure 1.** Design principles for immunoproteasome-specific inhibitors. A) Compounds with large hydrophobic P1 side chains display β5i selectivity. B) Proline analogues in P3 and bulky hydrophobic P1 residues are the prerequisites to design β1i selective inhibitors.

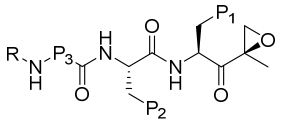
Altogether, this strategy led to identification of either β5i or β1i selective compounds with high subunit selectivity and potency. All synthesized inhibitors were proven to be cell-permeable and active in mammalian cancer cell lines and hence, provide an excellent tool kit for the systematic evaluation of the distinct proteasomal active sites.

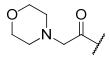
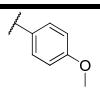
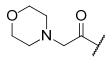
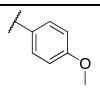
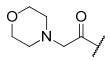
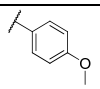
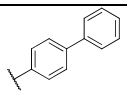
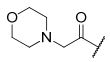
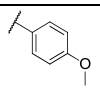
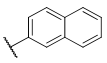
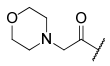
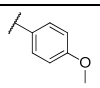
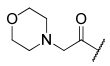
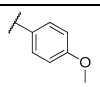
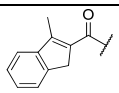
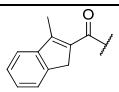
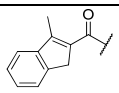
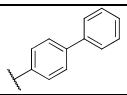
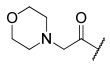
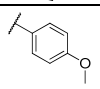
## Results

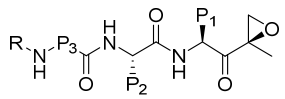
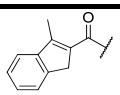
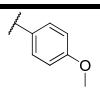
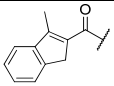
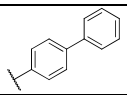
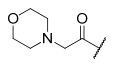
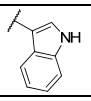
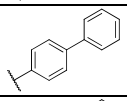
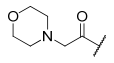
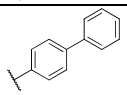
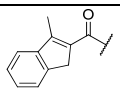
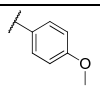
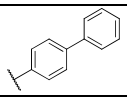
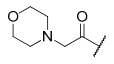
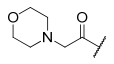
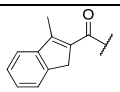
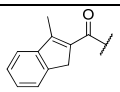
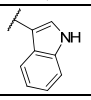
### Design, synthesis and evaluation of highly specific β5i inhibitors.

The X-ray structure of **1** covalently bound to the murine cCP and iCP indicates that the phenyl moiety at position P1 induces a major conformational change in the S1 pocket of β5c, but only minor alterations in β5i.[28] This difference in binding likely confers β5i selectivity to inhibitor **1** and it was reasoned that substitution at P1 of phenylalanine epoxyketone for larger hydrophobic P1 residues should lead to compounds with enhanced β5i selectivity (Figure 1A). To determine the optimal P1 residue for this purpose, epoxyketone analogues of **1** featuring an adamantyl- (**4**), biphenyl- (**5**), 2-naphthyl- (**6**), 1-naphthyl- (**7**) or cyclohexyl-alanine (**8**) in the P1 site were synthesized (Table 1). The compounds were evaluated for their potencies against β5c and β5i in Raji-cell lysates (B-cell lymphoma cell line) by competitive activity based protein profiling (ABPP) of the β5-subunits using the β5c/β5i specific probe BODIPY(TMR)-NC-005-VS (Table 1).[35, 36] In contrast to Muchamuel *et al.* who determined a 30-fold selectivity for β5i over β5c for **1**[31] using an ELISA-based assay in MOLT-4 cells, a nine-fold selectivity was observed by ABPP. Incorporation of adamantylalanine epoxyketone at P1 of the lead structure (**4**) resulted in complete loss of affinity. Interestingly, the bulky biphenyl side chain in **5** did not show increased selectivity but a two-to-three-fold enhanced potency for β5c and β5i. Furthermore, while the 2-naphthyl-side chain in **6** has both high affinity and increased selectivity for β5i, the 1-naphthyl derivative **7** is significantly less potent than compound **1**. Different orientations of the 1-naphthyl and 2-naphthyl substituents in the β5c/β5i active sites might explain this observation. The most prominent improvement in selectivity was noticed for **8**, featuring a cyclohexylalanine residue at P1. While the binding strength towards β5i remained similar, the affinity for β5c was significantly impaired compared to the lead structure ONX-0914 (**1**), hereby enhancing β5i selectivity almost five times. For further optimization three side chains were pursued: cyclohexyl (highest selectivity), biphenyl (highest potency) and phenyl (for comparison). Cyclohexyl (**9)** and biphenyl (**10)** analogues of the phenyl-bearing compound PR-924 (**2**) – another β5i selective inhibitor with 100-fold preference for β5i– were synthesized.[33, 37] **9** turned out to be 500-fold selective for β5i over β5c yet with threefold lower potency compared to **2**. Compound **10** appeared less selective than **2**, indicating that a biphenyl substituent at P1 is not suitable for optimal β5i selectivity.

**Table 1.** Optimization of β5i Selective Inhibitors Based on 1 (ONX-0914) and 2 (PR-924). $IC_{50}$ values were determined using ABPP in Raji cell lysates. >[conc] indicates less than 50% inhibition at indicated concentration.

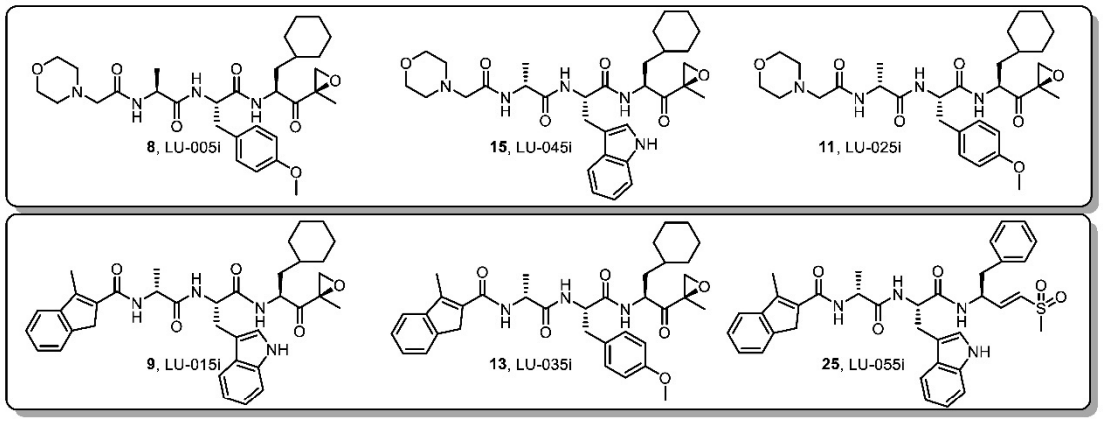


| | | | | | Apparent $IC_{50}$ (nM) | | Ratio |
|---|---|---|---|---|---|---|---|
| **Compound** | **R** | **P3** | **P2** | **P1** | **β5i** | **β5c** | **β5c/ β5i** |
| **1** ONX-0914 |  |  |  |  | 5.7 | 54 | 9 |
| **4** |  |  |  |  | >50x10$^3$ | >50x10$^3$ | - |
| **5** |  |  |  |  | 2.0 | 20 | 10 |
| **6** |  |  |  |  | 3.1 | 97 | 32 |
| **7** |  |  |  |  | 244 | 1.8 x 10$^3$ | 7 |
| **8** LU-005i |  |  |  |  | 6.6 | 287 | 43 |
| **2** PR-924 |  |  |  |  | 2.5 | 227 | 91 |
| **9** LU-015i |  |  |  |  | 8.3 | 4.6x10$^3$ | 553 |
| **10** |  |  |  |  | 5.0 | 79 | 16 |
| **11** LU-025i |  |  |  |  | 36 | 1.9 x 10$^3$ | 52 |
| **12** |  |  |  |  | 3.0 | 14.5 | 5 |
| **13** LU-035i |  |  |  |  | 11 | 5.5 x 10$^3$ | 500 |
| **14** |  |  |  |  | 5.0 | 6.0 | 1 |
| **15** LU-045i |  |  |  |  | 32 | 827 | 26 |
| **16** |  |  |  |  | 13 | 104 | 8 |
| **17** |  |  |  |  | 14 | 116 | 8 |

| |  | | | | Apparent $IC_{50}$ (nM) | | Ratio |
|---|---|---|---|---|---|---|---|
| **Compound** | **R** | **P3** | **P2** | **P1** | **β5i** | **β5c** | **β5c/ β5i** |
| **18** |  |  |  |  | 3 | 157 | 52 |
| **19** |  |  |  |  | 1.4 | 12.6 | 9 |
| **20** |  |  |  |  | 2.1 | 14 | 6 |
| **21** |  |  |  |  | 1.2 | 9.9 | 8 |
| **22** |  |  |  |  | 3.3 | 43 | 13 |
| **23** Vinyl Sulfone |  |  |  |  | 392 | 14 x $10^3$ | 37 |
| **24** Vinyl Sulfone |  |  |  |  | 5700 | >100 x $10^3$ | >18 |
| **25**, LU-055i Vinyl Sulfone |  |  |  |  | 53 | 25 x $10^3$ | 464 |
| **26** Vinyl Sulfone |  |  |  |  | 632 | 62 x $10^3$ | 98 |

Next, hybrid compounds featuring elements from both **1** and **2** with various P1 amino acid derivatives were evaluated (**11**-**22**, Table 1). From this focused set of compounds, several β5c/β5i inhibition and selectivity patterns emerge. First, differences in potency and selectivity between P2 residues such as 4-methyltyrosine (4MeTyr)- and tryptophan-derived epoxyketones are marginal. Second, the chirality (D/L) of the alanine in P3 can have significant influence on β5i selectivity and -potency. For example, the *N*-acylmorpholine capped compounds **8** and **11** show a similar selectivity profile, however **11** (featuring D-Ala in P3) is fivefold less active towards β5i and β5c compared to **8** (bearing L-Ala in P3). In contrast, the 3-methyl-*1H*-indene capped inhibitor **12** with L-Ala in P3 displays a significant decrease in selectivity but an increase in potency versus its D-Ala featuring analogue **13**. It can be concluded that L-Ala at P3 combines better with an *N*-acylmorpholine cap, whereas D-Ala at P3 requires a 3-methyl-*1H*-indene *N*-cap for optimal β5i selectivity. Compounds **9** and **13** show the highest β5i selectivity, indicating that D-Ala/3-methyl-*1H*-indene is preferred over L-Ala/*N*-acylmorpholine for β5i selectivity.

A



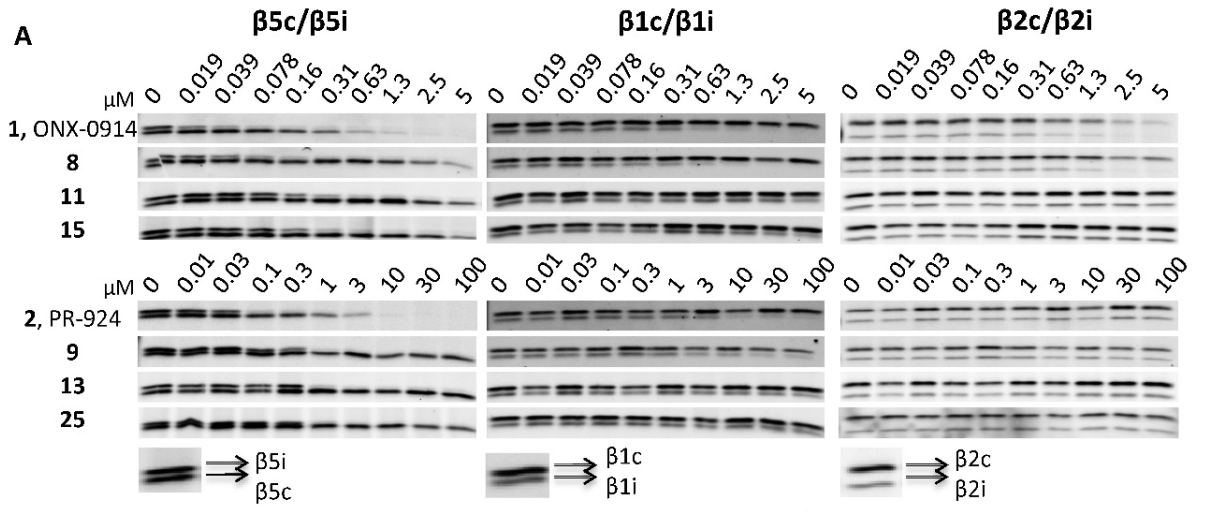

B

| | Apparent $IC_{50}$ (μM) | | | | | | Ratio $IC_{50}$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Compound** | **β5i** | **β5c** | **β1i** | **β1c** | **β2i** | **β2c** | **β5c/β5i** | **β1i/β5i** | **β2i/β5i** | **β2c/β5i** |
| **1,** ONX-0914 | 0.0057 | 0.054 | 0.46 | >10 | 0.59 | 1.1 | 9 | 81 | 103 | 192 |
| **8** | 0.0066 | 0.29 | 0.30 | >10 | 0.41 | 2.5 | 44 | 45 | 62 | 378 |
| **11** | 0.036 | 1.9 | >10 | >10 | >10 | >10 | 52 | >280 | >280 | >280 |
| **15** | 0.032 | 0.83 | >10 | >10 | >10 | >10 | 26 | >312 | >312 | >312 |
| **2,** PR-924 | 0.0025 | 0.23 | 1.84 | >10 | >10 | >10 | 92 | 740 | >4000 | >4000 |
| **9** | 0.0083 | 4.6 | 7.1 | >10 | >10 | >10 | 554 | 855 | >1200 | `>1200 |
| **13** | 0.011 | 5.5 | >10 | >10 | >10 | >10 | 500 | >900 | >900 | >900 |
| **25** | 0.053 | 25 | >100 | >100 | >100 | >100 | 471 | >1900 | >1900 | >1900 |

**Figure 2.** A) Chemical structures of the β5i inhibitors which show the largest improvement in β5i selectivity compared to **1** and **2**. B) Apparent $IC_{50}$ values of compounds in Raji cell lysates as determined by ABPP.

As previously reported, subunit selectivity of proteasome inhibitors can be influenced by the electrophilic trap.[11, 12] For example, exchanging the epoxyketone warhead by a vinyl sulfone in β5-selective inhibitors improves β5-selectivity.[11] Based on these results, epoxyketones **1**, **2**, **8** and **9** were compared with their vinyl sulfone counterparts **23**, **24**, **25** and **26** (Table 1). All tested peptide epoxyketones outperform their peptide vinyl sulfone analogues where it comes to β5i inhibition potency. Of the peptide vinyl sulfone series only **25** is a moderately potent β5i inhibitor, however with four times higher selectivity for β5i compared to **2**.

The six most specific and potent epoxyketone compounds, namely **8**, **9**, **11**, **13**, **15** and the vinyl sulfone **25** were evaluated for selectivity over the other subunits, i.e. β1c/β1i and β2c/β2i using ABPP in Raji cell lysates with β1c/β1i selective probe BODIPY(FL)-NC001 and pan-reactive probe BODIPY(TMR)-epoxomicin (Figure 2).[35] The obtained results disclose that **8** targets β1i and β2i with slightly more potency than **1**, while β1c is completely unaffected. Compound **2** and to a lesser extent **9** also co-inhibit β1i in the low micromolar range, whereas compounds **11**, **13**, **15** and **25** are highly specific for β5c/β5i and do not inhibit β1c/β1i and β2c/β2i at concentrations up to 10 μM.




B

| | Apparent $IC_{50}$ (μM) | | | | | | Ratio $IC_{50}$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | β5i | β5c | β1i | β1c | β2i | β2c | β5c/β5i | β1i/β5i | β2i/β5i | β2c/β5i |
| **1,** ONX-0914 | 0.018 | 0.18 | 0.34 | >5 | 0.59 | 1.1 | 10 | 19 | 33 | 61 |
| **8** | 0.044 | 4.2 | 0.37 | >5 | 0.90 | 4.9 | 95 | 8 | 20 | 111 |
| **11** | 0.10 | 4.3 | 7.3 | >10 | >10 | >10 | 43 | 73 | >100 | >100 |
| **15** | 0.059 | 2.9 | 6.2 | >10 | >10 | >10 | 49 | 105 | >169 | >169 |
| **2,** PR-924 | 0.025 | 2.9 | 3.5 | >100 | >100 | >100 | 116 | 140 | >400 | >400 |
| **9** | 0.23 | >100 | 16 | >100 | >100 | >100 | 435 | 70 | >435 | >435 |
| **13** | 0.37 | >100 | >100 | >100 | >100 | >100 | >270 | >270 | >270 | >270 |
| **25** | 0.39 | >100 | >100 | >100 | >100 | >100 | >256 | >256 | >256 | >256 |

C

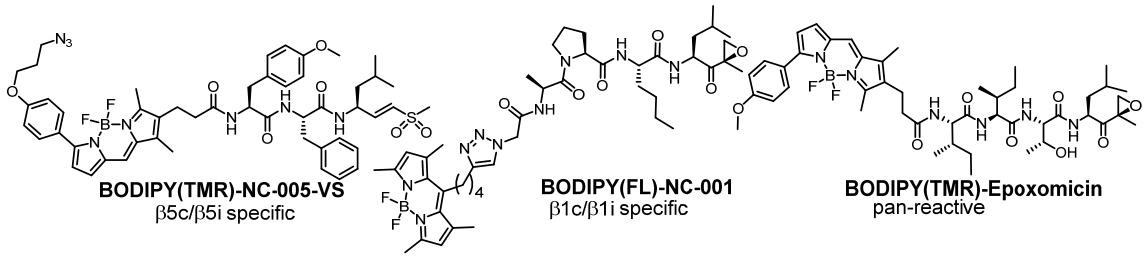



**Figure 3. Evaluation of selected β5i inhibitors in RPMI-8226 cells.** Inhibition profiles of β5i inhibitors (A), which are used to determine apparent $IC_{50}$ values (B). Cells were treated with the distinct compounds at indicated concentrations for 1 h, lysed and the occupancy of the remaining active sites was probed with BODIPY(TMR)-NC-005-VS (β5), BODIPY(TMR)-Epoxomicin (pan-reactive, used for β2) or BODIPY(FL)-NC-001 (β1) (C).

Next, the cellular potency and selectivity of **8**, **9**, **11**, **13**, **15** and **25** in RMPI-8226 (MM-cell line) was tested (Figure 3). All inhibitors proved to be cell-permeable and selectivity trends resemble those observed in Raji lysates. Compound **8** exhibits an increased selectivity for β5i over β5c compared to **1**. Notably, it also favours β1i and β2i over β5c, making it a broad-spectrum immunoproteasome inhibitor, when applied at a concentration of 1 μM. As far as known, this is the first proteasome inhibitor able to selectively inhibit all three iCP subunits over their constitutive counterparts. Although the potency of the D-alanine containing compounds **11** and **15** towards β5i is decreased by a factor of 3-5, they are 4 times more selective for β5i than **1**. Moreover, the analogues **9, 13** and **25** are >10 times less reactive

towards β5i compared to **2**. However, in case of **13** and **25** all other subunits remain fully active up to 100 µM (Figure 3). In conclusion, compounds **9**, **13** and **25** are the most selective β5i inhibitors known to date.

### X-ray structures of β5i specific inhibitors in complex with the yeast CP

The compounds **2**, **8**, **9**, **11** and **17** complexed with yCP were further analyzed by X-ray crystallography. Despite the high concentrations used for crystal soakings the D-Ala-featuring inhibitors **2**, **9**, **11** and **17** solely target subunit yβ5. In contrast, **1** (P3-L-Ala) was shown to inhibit all three active sites of the yCP[28] and its cyclohexyl analogue **8** (L-Ala) occupies at least the subunits yβ5 and yβ2. The P3-D-Ala residue hence confers selectivity for the chymotrypsin-like active sites, which is consistent with the ABPP results in Raji cell lysate and RPMI-8226 cell assays (Figures 2 and 3). Structural modelling by superposition with the murine substrate binding channels, which are built up of the two neighbouring subunits β1/2, β2/3 and β5/β6, respectively, provide explanations for this subunit preference (Sequence numbering according to Huber *et al*.[28]).

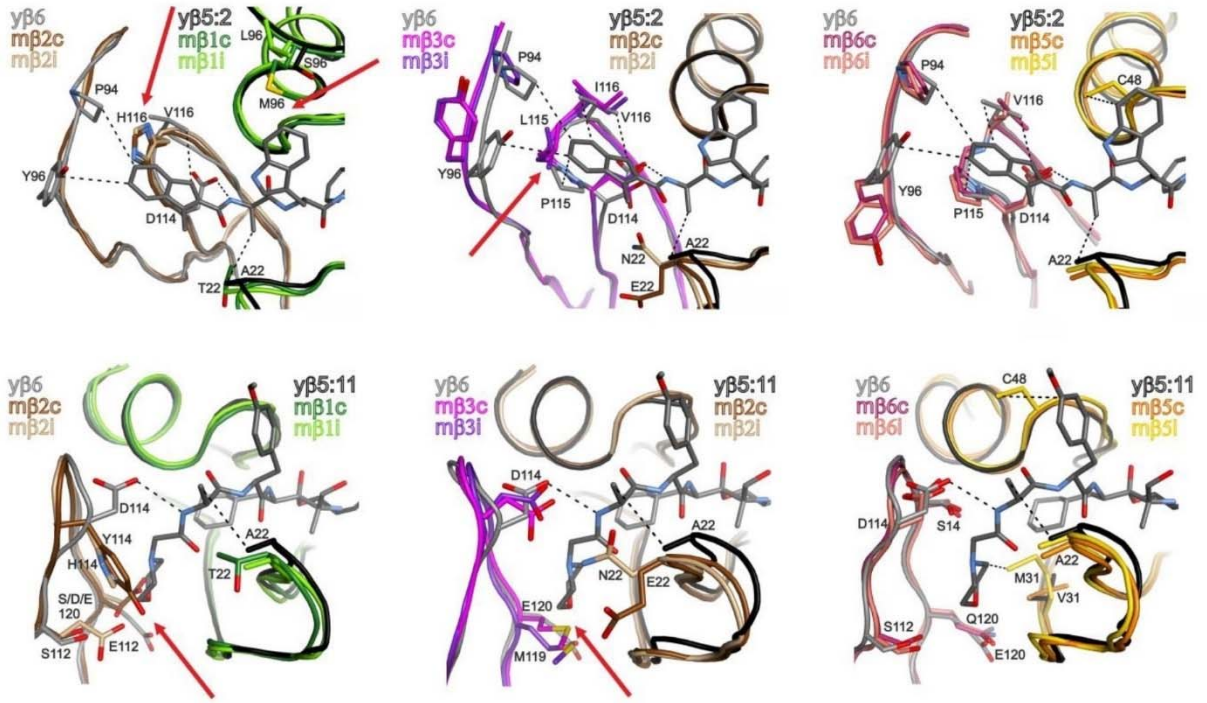



**Figure 4. Superposition of yβ5:ligand complexes onto murine β1 and β2 subunits.** Superposition's of the yβ5/6 substrate binding channel in complex with **2** and **11** onto the mβ1/2 (left panel), mβ2/3 (middle) and mβ5/6 (right) active sites of the murine cCP and iCP provide structural insights into the β5i selectivity of **2** and **11** (as well as **9** and **17**). Steric clashes with surrounding amino acids (red arrows) prevent/impair binding to the mβ1 and mβ2 subunits. Hydrophobic interactions and hydrogen bonds are marked by black dotted lines.

The compounds **11** and **17** do not properly fit into the β1/β2 substrate binding channels due to clashes of their N-caps with Glu112 (β2i; 1.8 Å), His114/Tyr114 (β2i/c; 2.7-3.1 Å) and Asp120 (β2c; direct contact) (Figure 4). Inhibition of the more polar caspase-like yβ1/β1c active sites is further disfavoured by the hydrophobicity of the ligand. In addition, modelling **11** into the β2/3 substrate binding channel depicts that the *N*-acylmorpholine cap clashes with Met119 (β3) (distance 1.3-2.3 Å), thereby preventing its binding.

Compound **2** and its derivative **9** preferentially bind to the subunits yβ5, β5c/i and β1i, a fact that is mostly due to the favourable interactions between their hydrophobic P1 sites and the respective protein S1 pockets. Compared to β5 subunits, the affinity for β1 active sites is reduced by a clash with His116.

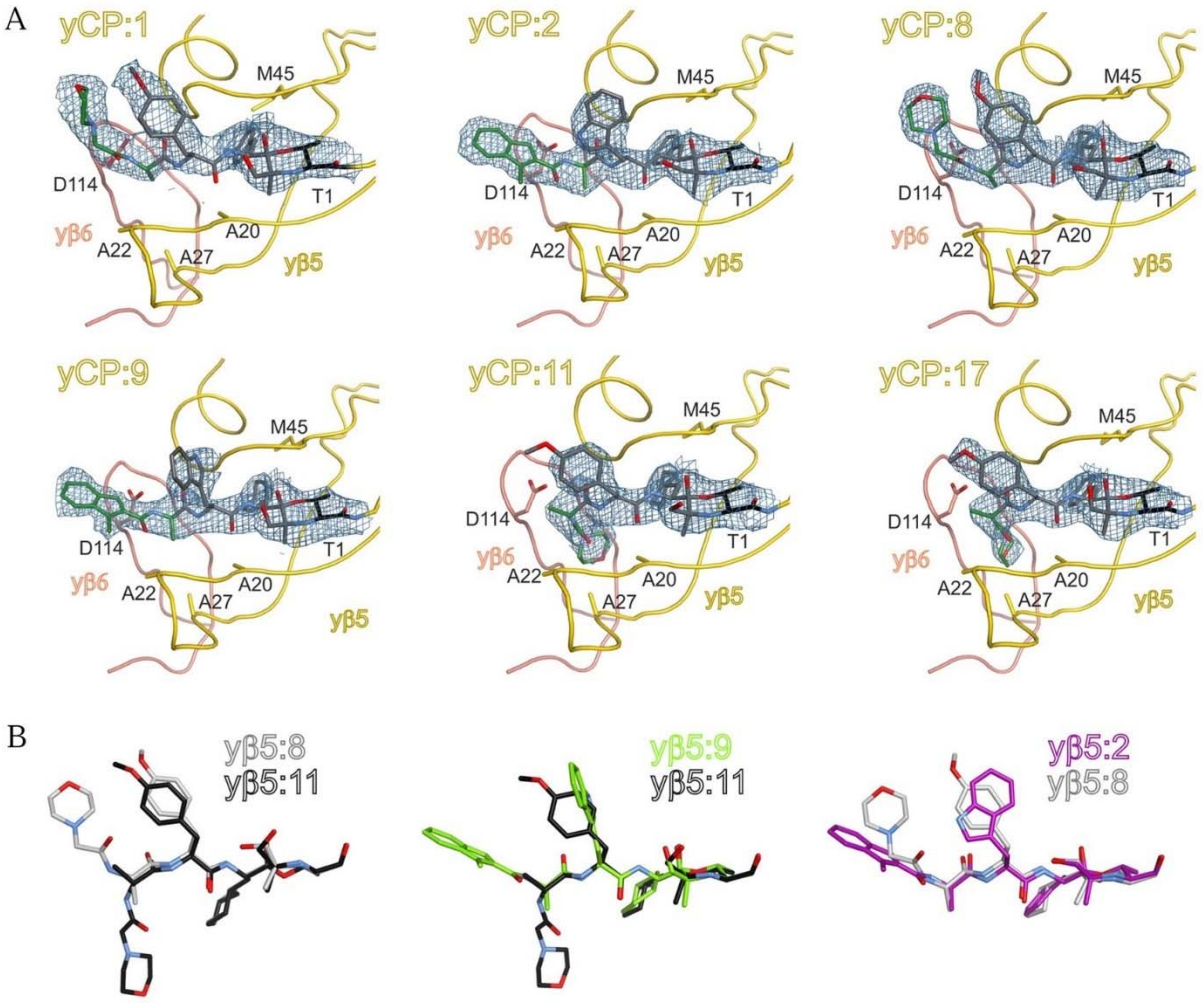


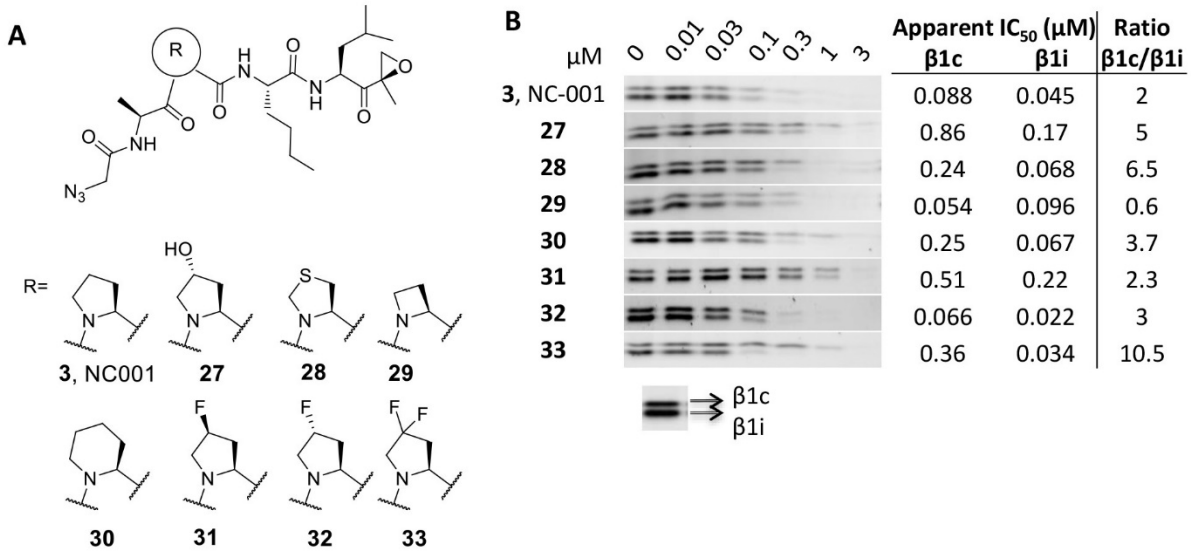

**Figure 5. Crystallographic analysis of yCP:ligand complexes.** A) Illustration of the yeast yβ5/6 substrate binding channel in complex with the indicated compounds. All inhibitors are covalently bound to Thr1 (black) of subunit yβ5 (yellow). Depending on the compound, the P3 site and the N-cap (marked in green) adopt different conformations. The $2F_O$-$F_C$ electron density map for the ligand (blue) is contoured at 1 σ. The inhibitor and Thr1 have been omitted prior to phasing. B) Inversion of the P3-chirality from L to D results in a flip of the N-acylmorpholine cap. While N-acylmorpholine capped compounds adopt a bended conformation, 3-methyl-*1H*-indene N-capped inhibitors bind in an extended form due to their restricted flexibility.

Polarity reasons and steric hindrance with Met96 (2.3 Å) further impair binding to the yβ1/β1c active sites. A clash of the 3-methyl-*1H*-indene cap with Leu115 (β3; 2.0-2.3 Å) as well as Asn22 (β2i; 2.6 Å) leads to reduced affinity also for the β2/3 substrate binding channel (Figure 4).
In agreement with the β5-selectivity of the investigated compounds, no clashes with residues from the β5/6 substrate binding channels in cCP or iCP were identified. Inspection of the $2F_O$-$F_C$ electron density maps, however, revealed pronounced differences in the binding mode of **1** and **8** compared to **11** and **17** as well as **2** and **9**. Compound **8** adopts a conformation, which is identical to **1**[28], whereas this does not apply to **11** and **17**, the P3-D-Ala analogues of **1** and **8** (Figure 5). The inverted chirality induces a sharp (almost 90°) turn in the peptide backbone of **11** and **17** and forces their P3 site as well as their *N*-acylmorpholine cap to undergo unique interactions. By contrast, binding of **2** and its analogue **9**, both featuring a D-Ala at P3 in combination with a 3-methyl-*1H*-indene cap is comparable to that of **1** and **8** (Figure 5).

**Development of β1i specific inhibitors**

The crystal structures of the murine cCP and iCP depict differences between the substrate binding channels of β1c and β1i to exist predominantly in the S1 and S3 pockets.[28] As a result of the substitutions T20V, T31F, R45L and T52A in β1i (relative to β1c), the cleavage preference for acidic residues at P1 is largely abolished, while processing after hydrophobic amino acids is enhanced.[5] Furthermore, the S1 and S3 pockets appear diminished in size when compared to β1c and the S3 pocket is more polar than in β1c.
For the development of β1i specific inhibitors, az-NC001[15] (**3**), a potent β1c/β1i -specific ligand was chosen as starting point (Figure 1B). Since the β1c/β1i selectivity of **3** appears to be governed by the turn-inducing P3-proline residue (see chapter 4), seven analogues of **3** (**27**-**33**) in which Pro was substituted for proline isosters were evaluated (Figure 6A). With the aim to increase the polarity of the P3 residue, hydroxyproline (Hyp) (**27**) and thioproline (Thz) (**28**) were selected. The optimal ring size was determined by comparing L-azetidine-2-carboxylic acid (Aze, 4-membered ring) (**29**) and L-pipecolic acid (Pip, 6-membered ring) (**30**). Fluorinated proline analogues **31**, **32** and **33** were included to probe the effect of altering *cis/trans* ratios around the peptide bonds.[38, 39]
The analogues **27**-**33** were evaluated by ABPP for blockage of β1c and β1i in Raji lysates (Figure 6B). As anticipated, **32** (P3 = *R*-4FPro) was found to be about tenfold more potent than **31** (P3 = *S*-4FPro), however, the affinity was not increased compared to **3**. Interestingly, **33** (P3 = 4,4-$F_2$Pro) showed a clear preference for β1i versus β1c, without loss of potency.

To further enhance β1i selectivity the P1 side chain was modified. During the evaluation of the β5i specific inhibitors, it was observed that compounds bearing a phenyl or cyclohexyl residue at P1 inhibit β1i much stronger than β1c (Figure 2B), confirming that the S1 pocket of β1i is more apolar than that of β1c.[28] In addition, neither β1c nor β1i is inhibited by compounds featuring a biphenyl substituent at P1 (data not shown), a finding that supports structural data showing that β1c/i have a smaller S1 pocket than β5c/i.[28] Hence, a series of analogues of **3** with phenyl or cyclohexylalanine in P1 and Pro or 4,4-$F_2$Pro in P3 were synthesized (Figure 7A). Compound **34** (P1 = Phe) binds to β1i without loss of potency compared to **3**, indicating that bulky residues in P1 are tolerated by β1i (Figure 7C). Substitution of the phenylalanine moiety for cyclohexylalanine (as in **35**) further increases the selectivity by a factor of four. Combining bulky residues in P1 with 4,4-$F_2$Pro in P3 resulted in the compounds **36** and **37**, of which the latter showed 250-fold selectivity for β1i over β1c. In RPMI-8226 cells as well as Raji lysate, **37** is selective for β1i and as potent as **3** (Figure 7D). Finally, **37** was compared to the epoxyketone UK-101 **(38)**[40, 41], the so far only β1i specific irreversible inhibitor (ML-604440[42] is the only other β1i inhibitor reported, which is equipped with the reversible boronic acid warhead) (Figure 7B). In Raji lysate, the potency of UK-101 (**38**) for β1i is comparable to **37**, however, it is about two-fold less specific for β1i and also targets β5c/β5i before β1c (Figure 7C). In conclusion, **37** is the most specific and cell-permeable non-reversible β1i inhibitor known to date.




| | Apparent $IC_{50}$ (µM) β1c | β1i | Ratio β1c/β1i |
|---|---|---|---|
| **3**, NC-001 | 0.088 | 0.045 | 2 |
| **27** | 0.86 | 0.17 | 5 |
| **28** | 0.24 | 0.068 | 6.5 |
| **29** | 0.054 | 0.096 | 0.6 |
| **30** | 0.25 | 0.067 | 3.7 |
| **31** | 0.51 | 0.22 | 2.3 |
| **32** | 0.066 | 0.022 | 3 |
| **33** | 0.36 | 0.034 | 10.5 |

**Figure 6. Inhibition profiles of P3 proline analogues of 3 in Raji lysates.** A) Chemical structures of compounds 27-33. B) After incubation of compounds with Raji lysates for 1h, β5 activity was blocked by NC-005 for an additional hour. Subsequently, the remaining β1 activity was probed by BOPIPY(FL)-NC-001.

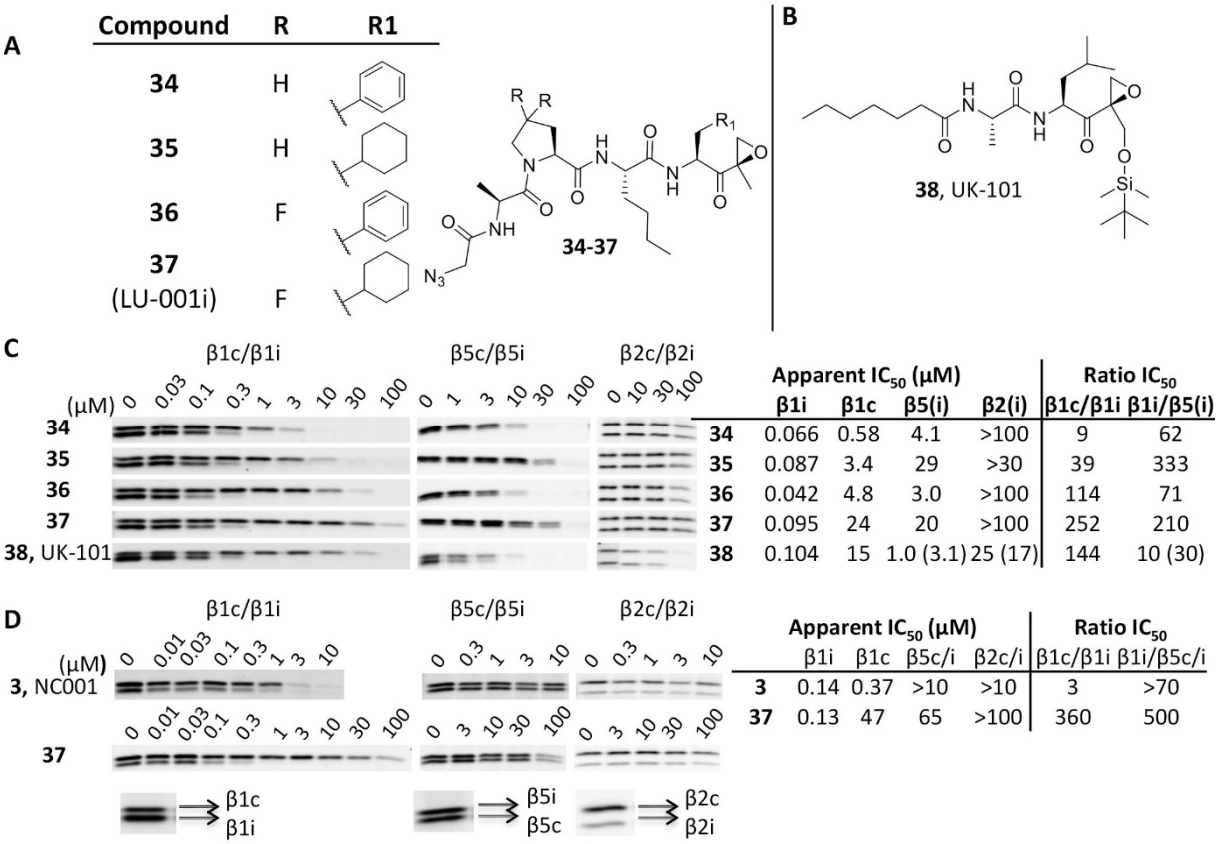



**Figure 7. Optimization of β1i selective inhibitors.** A) Chemical structures of compounds 34-37. B) Chemical structure of 38 (UK101). C) Raji lysates were incubated with the compounds for 1h, followed by cell lysis. Lysates were probed with BODIPY-NC005 (β5), BODIPY-NC001 (β1) or BODIPY-epoxomicin (pan-reactive, used for β2). D) Assay of the most selective β1i inhibitor **37**, compared to **3** in RPMI-8226-cells.

## Discussion/conclusion

This chapter describes the development of improved β5i inhibitors as well as a new class of β1i inhibitors, using design parameters derived from previously determined crystal structures.[2-5, 43] The co-crystallization of the β5i inhibitor ONX-0914 (**1**) with the murine cCP and iCP showed that the subunit-selectivity of **1** is mainly caused by the P1 phenyl ring.[28] This feature causes major structural rearrangements in the backbone and the S1 pocket of the β5c subunit, whereas β5i is able to accommodate the aromatic ring without pronounced conformational changes. Hence, it was hypothesized that larger P1 residues would further disfavour binding to β5c and enhance potency as well as selectivity for β5i. Indeed, large residues such as biphenyl (**5**) and 2-naphtyl (**6**) in P1 yielded more potent β5i inhibitors. Yet, the 1-naphthyl derivative (**7**) resulted in a 40-fold drop in activity and the adamantyl analogue (**4)** was not active at all. Interestingly, β5i selectivity is enhanced by the 2-naphthyl (**6**; three-fold) and cyclohexyl (**8**; four-fold) P1 side chains. Notably, this is due to the reduced affinity for subunit β5c. These observations indicate that only certain bulky P1 residues improve β5i

selectivity and that the difference in the S1 pockets between β5c and β5i are not in their linear depth but in their bottom. This is in agreement with the X-ray structures of the murine cCP in complex with ONX-0914 (**1**), which showed that Met45 forming the bottom of the S1 pocket is displaced upon ligand docking. From a structural point of view binding of both cyclohexyl and phenyl moieties to the S1 pocket of the yβ5 subunit is identical, yet, further analysis essentially requires crystal structures of the human cCP and iCP (in complex with the investigated compounds). Removing the aromatic system tends to increase the $IC_{50}$ values in both the β5c and β5i active sites, indicating that the π-system might interact with the sulphur of Met45 and that the strength of this contact depends on its orientation. Inverting the chirality of the P3 residue can also significantly impact β5-affinity and selectivity. Furthermore, the yCP complex structures provide detailed explanation for the β5-selectivity of the D-Ala/3-methyl-*1H*-indene compounds.

Based on the observation that the β5i inhibitors preferentially target β1i over β1c at higher concentrations, the P1 leucine epoxyketone of the β1c/β1i specific inhibitor NC-001 was substituted for either phenylalanine or cyclohexylalanine epoxyketone. The resulting compounds were indeed more specific for subunit β1i. Since the mouse cCP and iCP crystal structures show subtle differences between the S3 pockets of β1c and β1i, a P3 Pro analogue scan using **3** as a template was performed. This analysis revealed that the 4,4$F_2$-Pro (**33**) induces β1i selectivity without loss of activity. Combination of the cyclohexylalanine and the 4,4$F_2$-Pro ultimately led to compound **37**, which has a >300 fold binding preference for β1i over all other subunits in RPMI-8226 cells.

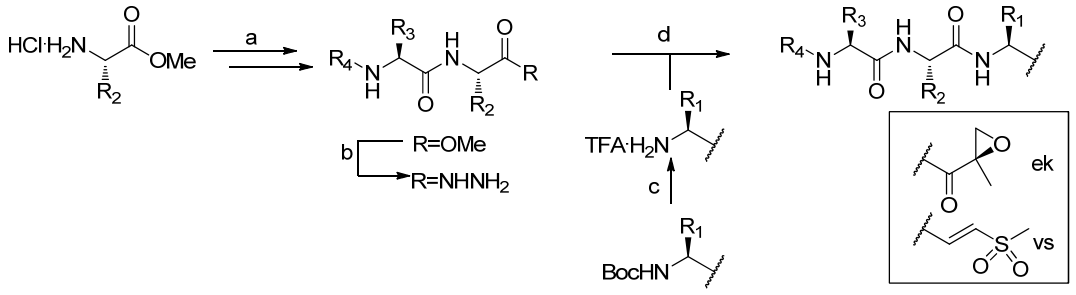
The set of improved subunit-specific and cell-permeable β5i and β1i inhibitors represent a useful tool to investigate the cytotoxic effect of subunit-specific proteasome inhibition in MM cells and disease models, for which controversial data exist.[33, 37]

# 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. Traces of water were removed from reagents used in reactions that require anhydrous conditions by co-evaporation with toluene. Solvents that were used in reactions were stored over 4 Å molecular sieves, except methanol and acetonitrile which were stored over 3 Å molecular sieves. 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. The eluents toluene, ethyl acetate and petroleum ether (40-60 °C boiling range) were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F254). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)4{\cdot}2H_2O$ (10 g/L) in 10% sulphuric acid, a solution of $KMnO_4$ (20 g/L) and $K_2CO_3$ (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 mL/L) in ethanol, where appropriate, followed by charring at ca. 150 °C. $^1H$ and $^{13}C$ NMR spectra were recorded on a Bruker AV-400 (400 MHz) or AV-600 (600 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane, $CD_3OD$ or $CDCl_3$ as internal standard. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile 50/50 (v/v) and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60,000 at m/z 400 (mass range m/z = 150-2,000) and dioctylphthalate (m/z = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). Optical rotations were recorded on a Propol automatic polarimeter. LCMS analysis was performed on a Finnigan Surveyor HPLC system with a Gemini C18 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 $H_2O$, MeCN and 1.0% TFA in $H_2O$ (0.1% TFA end concentration). Methods used are: 15 min (0→0.5 min: 10% MeCN; 0.5→10.5 min: 10% → 90% MeCN; 10.5→12.5 min: 90% MeCN; 12.5→15 min: 90% → 10% MeCN) or 12.5 min (0→0.5 min: 10% MeCN; 0.5→8.5 min: 10% → 90% MeCN; 8.5→10.5 min: 90% MeCN; 10.5→12.5 min: 90% → 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-Adamantyl-Ala-OH[44], Boc-Leu-EK, Boc-Phe-EK and Boc-Phe-VS[21, 30, 45] were synthesized according to literature procedures. All tested compounds are >95% pure on the basis of LC-MS and NMR.




**Scheme S1. General synthetic route towards peptide-epoxyketones and peptide vinylsulfones.** Reagent and conditions: (a). Sequential peptide coupling/Boc or Fmoc removal. Peptide coupling: HCTU, DiPEA, Boc-AA-OH/Fmoc-AA-OH, DCM. Boc-removal: TFA/DCM. Fmoc-removal: THF, DBU, EtSH; (b) $NH_2NH_2{\cdot}H_2O$, MeOH; (c) TFA; (d) i) tBuONO, HCl, DMF, DCM, -30°C; ii) amine (warhead), DiPEA, -30°C → RT.

**General procedure for azide couplings.**

Compounds **4-37** were prepared via azide coupling of properly protected tripeptide hydrazide and properly deprotected vinyl sulfone amines and epoxyketone amines. The appropriate hydrazide was dissolved in 1:1 DMF:DCM (v/v) and cooled to -30 °C. *t*BuONO (1.1 equiv.) and HCl (4M solution in 1,4-dioxane, 2.8 equiv.) were added, and the mixture was stirred for 3h at -30 °C after which TLC analysis (10% MeOH/DCM, v/v) showed complete consumption of the starting material. The epoxyketone or vinyl sulfone as a free amine was added to the reaction mixture as a solution in DMF. DiPEA (5 equiv.) was added to the reaction mixture, and this mixture was allowed to warm to RT slowly overnight. The mixture was diluted with EtOAc and extracted with $H_2O$ (3×). The organic layer was dried over $MgSO_4$ and purified by flash column chromatography (1-5% MeOH in DCM) and HPLC-purification (if necessary).

**General procedure for peptide couplings**

Free acid (1.2 equiv.), HCTU (1.2 equiv.) and free amine (1 equiv.) are dissolved in DCM (0.1 M), followed by the addition of DiPEA (3.5 equiv or 4.5 equiv in case of 2-morpholinoacetic acid HCl). After stirring overnight (or alternatively 1-3 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 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).

**Standard procedures amino acid epoxyketone synthesis**

**A. Boc-AA-N(OMe)Me**

To a solution of Boc-AA-OH (1 equiv.) in DCM are added HCTU (1.2 equiv.), N,O,-dimethylhydroxylamine (2 equiv.) and DiPEA (3.5 equiv.). After completion of the reaction (1 h to overnight), the solvent is removed. The residue is dissolved in EtOAc and washed with 1M HCl (2x), sat aq $NaHCO_3$ (2x), brine and dried over $Na_2SO_4$, filtered and concentrated. The crude product is purified by column chromatography (EtOAc /pent).

**B. Boc-AA-C($CH_3$)=$CH_2$**

To a solution of 2-bromopropene (3 equiv.) in $Et_2O$ at -78°C is added tBuLi (4.5 equiv, from 1.7 M in pent) in 10 in. After stirring for 15 min. at -78°C, the weinreb amide (1 equiv.) in $Et_2O$ is added slowly in 10 min. The reaction mixtures is stirred for 2-4 h, while warming up to max. -40°C. After TLC analysis revealed completion of the reaction, the reaction is quenched by the addition of sat. $NH_4Cl$ and warmed to RT. The mixture is transferred to a seperatory funnel and the water layer is extracted with EtOAc (3X). The combined organic layers are washed with brine, dried over $Na_2SO_4$, filtered and concentrated. The crude product is purified by column chromatography (EtOAc /pent mixtures).

**C. Boc-AA-OH-C($CH_3$)=$CH_2$**

To a solution of alkene **B** (1 equiv.) in MeOH is added $CeCl_3 \cdot 7H_2O$ (1.6 equiv.) and the mixture is stirred at RT. After the solution became clear, the mixture is cooled to 0°C and $NaBH_4$ (1.3 equiv.) is added in portion in 10 min. After TLC analysis showed completion of the reaction (about 30 min), the reaction is quenched by the addition of AcOH. The mixture is stirred for 15 min. followed by the addition of toluene and removal of the solvent. The residue is redissolved in a $H_2O$/ EtOAc mixture, which is then transferred to a seperatory funnel. The layers were separated and the aqueous layer was extracted with EtOAc (2X). The combined organic layers are washed with brine, dried over $Na_2SO_4$, filtered and concentrated. The crude product was purified (if necessary) by column chromatography (EtOAc /pent mixtures) .

**D. Boc-AA-EK**

To a solution of alcohol **C** in DCM at 0°C is added $VO(acac)_2$ (0.1 equiv.) followed by the addition of tBuOOH (5.5 M in decane, 3 equiv.). The reaction mixture is stirred at 0°C for 2-3 h. after which TLC analysis showed completion of the reaction. The reaction mixture is concentrated, redissolved in EtOAc and washed with 0.5 sat. $NaHCO_3$ (2x), $H_2O$ and brine. The organic layer is dried over $Na_2SO_4$, filtered and concentrated. The crude product is added as a solution in DCM to a solution of Dess-Martin-Periodane (1.5-3 equiv.) in DCM at 0°C. After TLC analysis revealed completion of the reaction, the reaction was quenched by the addition of sat. $NaHCO_3$. The mixture was transferred to a seperatory funnel and the layers were separated. The aqueous layer was extracted with DCM (1x) and the combined organics were washed with sat. $NaHCO_3$ (1x) and brine and dried over $Na_2SO_4$, filtered and concentrated. The crude product was purified by column chromatography (EtOAc /pent mixtures).

**MorphAc-Ala-Tyr(OMe)-Ala(Ada)-EK (4)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (2→4% MeOH in DCM) provided the title compound (21.62 mg, 68%) as a white powder after lyophilisation. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.43 (d, *J*= 7.6 Hz, 1H), 7.14 (d, *J*= 8.6 Hz, 2H), 6.92 – 6.66 (m, 3H), 6.14 (d, *J*= 7.5 Hz, 1H), 4.58 – 4.47 (m, 2H), 4.41 (p, *J*= 7.3 Hz, 1H), 3.76 (s, 3H), 3.68 (t, *J*= 4.6 Hz, 4H), 3.32 (d, *J*= 5.0 Hz, 1H), 3.00 – 2.91 (m, 3H), 2.89 – 2.83 (m, 2H), 2.44 (q, *J*= 4.2 Hz, 4H), 1.91 (s, 3H), 1.70 – 1.53 (m, 6H), 1.50 (s, 3H), 1.44 – 1.40 (m, 5H), 1.39 – 1.28 (m, 5H), 1.01 – 0.93 (m, 1H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.78, 172.02, 170.31, 170.23, 158.67, 130.54, 128.54, 114.08, 67.03, 61.72, 59.06, 55.33, 54.29, 53.86, 52.54, 48.32, 47.92, 44.66, 42.44, 36.83, 36.62, 32.91, 28.62, 17.66, 17.07. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 6.52 (ESI-MS (m/z): 639.6 ($M+H^+$)) HRMS: calculated for $C_{35}H_{50}N_4O_7$ 639.37523 $[M+2H]^{2+}$; found 639.37524

**MorphAc-Ala-Tyr(OMe)-BiPhe-EK (5)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (2→4% MeOH in DCM) provided the title compound (21.18 mg, 64.5%) as a white powder after lyophilisation. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.59 – 7.51 (m, 2H), 7.51 – 7.38 (m, 5H), 7.34 (d, *J* = 7.3 Hz, 1H), 7.09 (dd, *J* = 8.4, 3.2 Hz, 4H), 6.77 (d, *J* = 8.6 Hz, 2H), 6.68 (d, *J* = 7.5 Hz, 1H), 6.36 (d, *J* = 7.3 Hz, 1H), 4.77 (td, *J* = 7.9, 4.7 Hz, 1H), 4.50 (t, *J* = 7.1 Hz, 1H), 4.34 (t, *J* = 7.2 Hz, 1H), 3.73 (s, 3H), 3.67 (t, *J* = 4.5 Hz, 4H), 3.29 (d, *J* = 4.9 Hz, 1H), 3.12 (dd, *J* = 14.1, 4.6 Hz, 1H), 2.98 – 2.78 (m, 5H), 2.73 (dd, *J* = 14.1, 8.3 Hz, 1H), 2.44 – 2.38 (m, 4H), 1.51 (s, 3H), 1.26 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.02, 171.96, 170.52, 170.30, 158.69, 140.66, 140.05, 134.76, 130.52, 129.76, 128.89, 128.33, 127.44, 127.32, 127.09, 114.11, 67.02, 61.68, 59.36, 55.30, 54.32, 53.82, 52.75, 52.65, 48.39, 36.79, 36.65, 17.69, 16.69. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 7.05 (ESI-MS (m/z): 657.13 ($M+H^+$)). HRMS: calculated for $C_{37}H_{44}N_4O_7$ 657.32828 $[M+H]^+$; found 657.32831

**MorphAc-Ala-Tyr(OMe)-2-Nal-EK (6)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (2→4% MeOH in DCM) followed by purification by HPLC (20-70% MeCN, 0.1 % TFA, 10 min gradient) provided the title compound (13.75 mg, 43.5%) as a white powder after lyophilisation. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.82 – 7.70 (m, 3H), 7.49 – 7.34 (m, 4H), 7.18 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.06 (d, *J* = 8.6 Hz, 2H), 6.75 (d, *J* = 8.6 Hz, 2H), 6.61 (d, *J* = 7.5 Hz, 1H), 6.33 (d, *J* = 7.2 Hz, 1H), 4.88 – 4.78 (m, 1H), 4.46 (q, *J* = 7.0 Hz, 1H), 4.31 – 4.22 (m, 1H), 3.74 (s, 3H), 3.67 (t, *J* = 4.5 Hz, 4H), 3.34 – 3.20 (m, 2H), 2.96 – 2.75 (m, 6H), 2.45 – 2.37 (m, 4H), 1.50 (s, 3H), 1.15 (d, *J* = 7.1 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.03, 171.89, 170.48, 170.25, 158.68, 133.43, 133.21, 132.53, 130.53, 128.39, 128.34, 128.01, 127.80, 127.63, 127.25, 126.36, 125.95, 114.10, 67.01, 61.67, 59.42, 55.33, 54.31, 53.81, 52.74, 52.67, 48.38, 37.26, 36.82, 17.59, 16.69. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 6.60 (ESI-MS (m/z): 631.20 ($M+H^+$)). HRMS: calculated for $C_{35}H_{42}N_4O_7$ 631.31263 $[M+H]^+$; found 631.31262

**MorphAc-Ala-Tyr(OMe)-1-Nal-EK (7)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (2→4% MeOH in DCM) followed by purification by HPLC (20-70% MeCN, 0.1 % TFA, 10 min gradient) provided the title compound (9.51 mg, 30.1%) as a white powder after lyophilisation. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.17 (d, *J* = 8.3 Hz, 1H), 7.85 (d, *J* = 7.5 Hz, 1H), 7.74 (d, *J* = 8.3 Hz, 1H), 7.60 – 7.46 (m, 2H), 7.40 (d, *J* = 7.5 Hz, 1H), 7.30 (dd, *J* = 8.1, 7.1 Hz, 2H), 7.12 (d, *J* = 8.6 Hz, 2H), 6.97 (d, *J* = 6.7 Hz, 1H), 6.82 (d, *J* = 8.6 Hz, 2H), 6.54 (d, *J* = 7.3 Hz, 1H), 6.09 (d, *J* = 6.6 Hz, 1H), 4.83 (ddd, *J* = 9.8, 6.6, 4.7 Hz, 1H), 4.42 – 4.26 (m, 2H), 3.79 (s, 3H), 3.72 – 3.57 (m, 5H), 3.33 (d, *J* = 4.9 Hz, 1H), 2.97 – 2.78 (m, 6H), 2.43 (s, 4H), 1.51 (s, 3H), 1.24 (d, *J* = 7.1 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.37, 171.79, 170.29, 158.76, 134.01, 132.02, 131.85, 130.64, 129.03, 128.53, 128.32, 127.53, 126.71, 126.10, 125.25, 123.65, 114.14, 67.04, 61.71, 59.54, 55.39, 54.36, 53.84, 52.79, 52.08, 48.33, 37.10, 34.63, 17.80, 16.61. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 6.56 (ESI-MS (m/z): 631.13 ($M+H^+$)). HRMS: calculated for $C_{35}H_{42}N_4O_7$ 631.31263 $[M+H]^+$; found 631.31262

**MorphAc-Ala-Tyr(OMe)-Cha-EK (8)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (2→4% MeOH in DCM) provided the title compound (21.88 mg, 74.5%) as a white powder after lyophilization. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.45 (d, *J* = 7.5 Hz, 1H), 7.10 (d, *J* = 8.6 Hz, 2H), 6.85 – 6.69 (m, 3H), 6.27 (d, *J* = 7.7 Hz, 1H), 4.62 – 4.49 (m, 2H), 4.49 – 4.34 (m, 1H), 3.76 (s, 3H), 3.69 (t, *J* = 4.5 Hz, 4H), 3.25 (d, *J* = 5.0 Hz, 1H), 3.03 – 2.91 (m, 3H), 2.90 – 2.81 (m, 2H), 2.49 – 2.38 (m, 4H), 1.85 – 1.51 (m, 6H), 1.49 (s, 3H), 1.35 (d, *J* = 7.0 Hz, 3H), 1.25 – 1.06 (m, 5H), 0.88 (dd, *J* = 15.7, 7.9 Hz, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 208.24, 171.99, 170.71, 170.30, 158.66, 130.50, 128.35, 114.07, 67.03, 61.72, 59.15, 55.31, 54.39, 53.86, 52.51, 49.74, 48.43, 38.63, 36.81, 34.42, 34.02, 31.98, 26.42, 26.33, 26.05, 17.84, 16.83. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 6.62 (ESI-MS (m/z): 587.13 ($M+H^+$)). HRMS: calculated for $C_{31}H_{46}N_4O_7$ 587.34393 $[M+H]^+$; found 587.34393.

**3MeIndAc-D-Ala-Trp-Cha-EK (9)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→2% MeOH in DCM) provided the title compound (23.45 mg, 75%) as a white powder after lyophilization. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.53 – 8.48 (m, 1H), 7.66 (d, *J* = 7.8 Hz, 1H), 7.43 (t, *J* = 7.2 Hz, 2H), 7.38 – 7.28 (m, 3H), 7.17 – 7.02 (m, 4H), 6.61 (d, *J* = 6.9 Hz, 1H), 6.53 (d, *J* = 7.7 Hz, 1H), 4.77 (q, *J* = 7.0 Hz, 1H), 4.55 – 4.45 (m, 2H), 3.54 (s, 2H), 3.29 (dd, *J* = 14.7, 6.5 Hz, 1H), 3.24 – 3.11 (m, 2H), 2.80 (d, *J* = 5.0 Hz, 1H), 2.48 (s, 3H), 1.72 – 1.38 (m, 9H), 1.33 (d, *J* = 7.0 Hz, 3H), 1.20 – 0.97 (m, 5H), 0.80 (d, *J* = 11.4 Hz, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 208.40, 172.66, 171.31, 166.10, 148.10, 145.58, 142.28, 136.31, 131.61, 127.48, 127.39, 126.84, 123.91, 123.74, 122.22, 120.90, 119.75, 118.79, 111.38, 110.35, 59.15, 53.85, 52.53, 49.92, 49.27, 38.33, 34.31, 33.90, 31.99, 27.80, 26.38, 26.19, 25.98, 18.46, 16.83, 12.48. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 10.36 (ESI-MS (m/z): 625.13 ($M+H^+$)). HRMS: calculated for $C_{37}H_{44}N_4O_5$ 625.33845 $[M+H]^+$; found 625.33844.

**3MeIndAc-D-Ala-Trp-BiPhe-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 in DCM) provided the title compound (33.13 mg, 95.3%) as a white powder after lyophilisation. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.11 (s, 1H), 7.61 (d, *J* = 7.8 Hz, 1H), 7.57 – 7.51 (m, 2H), 7.46 – 7.28 (m, 9H), 7.20 (d, *J* = 8.0 Hz, 1H), 7.13 (t, *J* = 7.0 Hz, 1H), 7.10 – 6.99 (m, 3H), 6.89 (d, *J* = 2.2 Hz, 1H), 6.82 (d, *J* = 7.8 Hz, 1H), 6.72 (d, *J* = 7.5 Hz, 1H), 6.46 (d, *J* = 6.5 Hz, 1H), 4.80 – 4.67 (m, 2H), 4.42 (p, *J* = 6.9 Hz, 1H), 3.52 (dd, *J* = 7.3, 2.1 Hz, 2H), 3.35 – 3.22 (m, 2H), 3.13 – 2.98 (m, 2H), 2.84 (d, *J* = 4.9 Hz, 1H), 2.67 (dd, *J* = 13.8, 8.9 Hz, 1H), 2.48 (t, *J* = 2.1 Hz, 3H), 1.43 (s, 3H), 1.34 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.18, 172.58, 171.09, 166.32, 148.29, 145.55, 142.29, 140.51, 139.56, 136.11, 135.39, 131.49, 129.87, 129.03, 127.54, 127.48, 127.44, 127.11, 126.96, 126.86, 123.93, 123.56, 122.28, 120.95, 119.81, 118.72, 111.34, 110.02, 59.36,

53.56, 53.24, 52.64, 49.58, 38.33, 36.38, 27.47, 18.10, 16.65, 12.51. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 10.61 (ESI-MS (m/z): 695.07 ($M+H^+$)). HRMS: calculated for $C_{43}H_{42}N_4O_5$ 695.32280 $[M+H]^+$; found 695.32275

**MorphAc-D-Ala-Tyr(OMe)-Cha-EK (11)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→3% MeOH in DCM) provided the title compound (23.19 mg, 79.0%) as a white powder after lyophilisation. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.56 (d, *J* = 6.9 Hz, 1H), 7.11 (d, *J* = 8.5 Hz, 2H), 6.81 (d, *J* = 8.5 Hz, 2H), 6.58 (d, *J* = 8.1 Hz, 1H), 6.36 (d, *J* = 7.8 Hz, 1H), 4.65 – 4.48 (m, 2H), 4.34 (t, *J* = 7.1 Hz, 1H), 3.77 (s, 3H), 3.72 (t, *J* = 4.5 Hz, 4H), 3.27 (d, *J* = 5.0 Hz, 1H), 3.10 – 2.91 (m, 4H), 2.86 (d, *J* = 5.0 Hz, 1H), 2.60 – 2.46 (m, 4H), 1.65 (td, *J* = 38.0, 37.2, 13.3 Hz, 6H), 1.48 (s, 3H), 1.31 (d, *J* = 7.0 Hz, 3H), 1.27 – 1.10 (m, 5H), 0.93 – 0.83 (m, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 208.29, 171.77, 170.65, 158.79, 130.54, 128.18, 114.17, 67.06, 61.76, 59.15, 55.34, 54.18, 53.89, 52.46, 49.63, 48.86, 38.48, 37.10, 34.31, 34.02, 32.00, 26.44, 26.28, 26.01, 17.87, 16.81. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 6.80 (ESI-MS (m/z): 587.27 ($M+H^+$)) HRMS: calculated for $C_{31}H_{46}N_4O_7$ 587.34393 $[M+H]^+$; found 587.34393.

**3MeIndAc-Ala-Tyr(OMe)-Cha-EK (12)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→2% MeOH in DCM) provided the title compound (21.22 mg, 68.9%) as a white powder after lyophilisation. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.53 – 7.43 (m, 2H), 7.42 – 7.31 (m, 2H), 7.06 (d, *J* = 8.6 Hz, 2H), 6.91 (d, *J* = 7.8 Hz, 1H), 6.62 (d, *J* = 8.6 Hz, 2H), 6.44 (d, *J* = 7.9 Hz, 1H), 6.12 (d, *J* = 7.0 Hz, 1H), 4.68 – 4.53 (m, 3H), 3.56 – 3.48 (m, 2H), 3.47 (s, 3H), 3.28 (d, *J* = 5.0 Hz, 1H), 3.00 (d, *J* = 6.8 Hz, 2H), 2.88 (d, *J* = 5.0 Hz, 1H), 2.52 (t, *J* = 2.2 Hz, 3H), 1.84 – 1.53 (m, 5H), 1.50 (s, 3H), 1.43 (d, *J* = 7.0 Hz, 3H), 1.30 – 1.03 (m, 6H), 0.97 – 0.77 (m, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 208.32, 172.43, 170.89, 166.11, 158.63, 148.85, 145.56, 142.10, 131.03, 130.34, 128.32, 127.64, 127.03, 123.97, 121.04, 113.93, 59.18, 54.97, 54.38, 52.53, 49.72, 48.74, 38.68, 38.21, 36.74, 34.46, 34.02, 32.05, 26.45, 26.33, 26.08, 17.84, 16.85, 12.50. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 10.34 (ESI-MS (m/z): 616.13 ($M+H^+$)). HRMS: calculated for $C_{36}H_{45}N_3O_6$ 616.32811 $[M+H]^+$; found 616.32813

**3MeIndAc-D-Ala-Tyr(OMe)-Cha-EK (13)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→2% MeOH in DCM) provided the title compound (23.50 mg, 76.3%) as a white powder after lyophilization. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.45 (t, *J* = 5.7 Hz, 2H), 7.38 – 7.29 (m, 2H), 7.12 (d, *J* = 8.5 Hz, 2H), 6.94 (d, *J* = 8.0 Hz, 1H), 6.78 (d, *J* = 8.5 Hz, 2H), 6.49 (dd, *J* = 7.4, 2.7 Hz, 2H), 4.70 – 4.47 (m, 3H), 3.70 (s, 3H), 3.58 (s, 2H), 3.24 (d, *J* = 5.0 Hz, 1H), 3.01 (ddt, *J* = 22.1, 14.4, 7.6 Hz, 2H), 2.83 (d, *J* = 5.0 Hz, 1H), 2.51 (s, 3H), 1.78 – 1.45 (m, 5H), 1.43 (s, 3H), 1.38 (d, *J* = 7.0 Hz, 3H), 1.31 – 0.94 (m, 6H), 0.91 – 0.77 (m, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 208.31, 172.53, 170.87, 166.10, 158.74, 148.39, 145.57, 142.22, 131.44, 130.54, 128.30, 127.48, 126.89, 123.91, 120.94, 114.09, 59.14, 55.25, 54.38, 52.47, 49.74, 49.10, 38.55, 38.32, 37.00, 34.34, 33.97, 31.99, 26.39, 26.24, 25.98, 18.49, 16.79, 12.50. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 10.44 (ESI-MS (m/z): 616.13 ($M+H^+$)) HRMS: calculated for $C_{36}H_{45}N_3O_6$ 616.33811 $[M+H]^+$; found 616.33813.

**3MeIndAc -Ala-Tyr(OMe)-Phe-EK (14)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→2% MeOH in DCM) provided the title compound (15.31 mg, 50.2%) as a white powder after lyophilisation. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.53 – 7.42 (m, 2H), 7.42 – 7.30 (m, 2H), 7.24 (d, *J* = 7.3 Hz, 3H), 7.08 – 6.99 (m, 4H), 6.82 (d, *J* = 7.7 Hz, 1H), 6.62 (d, *J* = 8.6 Hz, 2H), 6.45 (d, *J* = 7.5 Hz, 1H), 6.08 (d, *J* = 7.1 Hz, 1H), 4.84 – 4.74 (m, 1H), 4.59 – 4.47 (m, 2H), 3.53 – 3.45 (m, 5H), 3.29 (d, *J* = 4.9 Hz, 1H), 3.09 (dd, *J* = 14.0,

5.0 Hz, 1H), 2.91 (dd, *J* = 13.0, 5.9 Hz, 3H), 2.71 (dd, *J* = 13.9, 8.2 Hz, 1H), 2.53 (t, *J* = 2.2 Hz, 3H), 1.48 (s, 3H), 1.36 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.21, 172.44, 170.60, 166.09, 158.64, 148.82, 145.58, 142.12, 135.77, 131.08, 130.34, 129.39, 128.65, 128.34, 128.06, 127.63, 127.22, 127.02, 123.97, 121.04, 120.46, 113.97, 59.34, 55.02, 54.34, 52.68, 52.60, 48.63, 38.21, 37.27, 36.75, 17.78, 16.63, 12.50. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 9.57 (ESI-MS (m/z): 610.13 ($M+H^+$)). HRMS: calculated for $C_{36}H_{39}N_3O_6$ 610.29116 $[M+H]^+$; found 610.29114

**MorphAc-D-Ala-Trp-Cha-EK (15)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→3% MeOH in DCM) provided the title compound (23.27 mg, 78.1%) as a white powder after lyophilisation.$^1$H NMR (400 MHz, $CDCl_3$) δ 8.35 (s, 1H), 7.67 (d, *J* = 7.8 Hz, 1H), 7.56 (d, *J* = 6.9 Hz, 1H), 7.36 (d, *J* = 8.1 Hz, 1H), 7.23 – 7.07 (m, 3H), 6.77 (d, *J* = 7.7 Hz, 1H), 6.28 (d, *J* = 7.7 Hz, 1H), 4.69 (q, *J* = 7.3 Hz, 1H), 4.53 – 4.43 (m, 1H), 4.36 (p, *J* = 7.1 Hz, 1H), 3.81 – 3.62 (m, 4H), 3.32 (dd, *J* = 14.6, 5.7 Hz, 1H), 3.23 – 3.07 (m, 2H), 2.95 (s, 2H), 2.82 (d, *J* = 5.0 Hz, 1H), 2.50 (tt, *J* = 11.7, 7.2 Hz, 4H), 1.73 – 1.57 (m, 4H), 1.45 (s, 5H), 1.31 (d, *J* = 7.0 Hz, 3H), 1.21 – 1.00 (m, 5H), 0.90 – 0.79 (m, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 208.33, 171.87, 171.00, 136.31, 127.49, 123.61, 122.40, 119.91, 118.87, 111.42, 110.35, 67.02, 61.74, 59.09, 53.85, 53.80, 52.52, 49.74, 48.85, 38.51, 34.23, 33.94, 31.99, 27.96, 26.41, 26.23, 26.01, 18.02, 16.81. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 6.85 (ESI-MS (m/z): 596.20 ($M+H^+$)) HRMS: calculated for $C_{32}H_{45}N_5O_6$ 596.34426 $[M+H]^+$; found 596.34418.

**MorphAc-D-Ala-Trp-Phe-EK (16)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→3% MeOH in DCM) provided the title compound (19.43 mg, 65.9%) as a white powder after lyophilisation.$^1$H NMR (400 MHz, $CDCl_3$) δ 8.15 (s, 1H), 7.64 (d, *J* = 7.9 Hz, 1H), 7.55 (d, *J* = 6.2 Hz, 1H), 7.35 (d, *J* = 8.1 Hz, 1H), 7.23 – 7.05 (m, 5H), 6.92 (d, *J* = 2.2 Hz, 1H), 6.88 (d, *J* = 6.4 Hz, 2H), 6.68 (d, *J* = 7.8 Hz, 1H), 6.42 (d, *J* = 7.5 Hz, 1H), 4.66 (p, *J* = 7.6 Hz, 2H), 4.30 (p, *J* = 7.0 Hz, 1H), 3.78 – 3.60 (m, 4H), 3.30 (dd, *J* = 14.7, 5.2 Hz, 1H), 3.22 (d, *J* = 4.9 Hz, 1H), 3.05 (dd, *J* = 14.6, 7.3 Hz, 1H), 2.97 – 2.89 (m, 3H), 2.84 (d, *J* = 4.9 Hz, 1H), 2.61 (dd, *J* = 13.8, 8.4 Hz, 1H), 2.56 – 2.38 (m, 4H), 1.42 (s, 3H), 1.31 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.22, 171.83, 170.77, 136.18, 136.12, 129.43, 128.60, 127.46, 126.93, 123.57, 122.43, 119.94, 118.86, 111.41, 110.03, 67.03, 61.67, 59.25, 53.82, 53.66, 52.93, 52.59, 48.87, 36.97, 27.70, 17.63, 16.57. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 6.25 (ESI-MS (m/z): 590.13 ($M+H^+$)). HRMS: calculated for $C_{32}H_{39}N_5O_6$ 590.29731 $[M+H]^+$; found 590.29730

**MorphAc-D-Ala-Tyr(OMe)-Phe-EK (17)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→3% MeOH in DCM) provided the title compound (21.60 mg, 74.4%) as a white powder after lyophilisation.$^1$H NMR (400 MHz, $CDCl_3$) δ 7.55 (d, *J* = 6.2 Hz, 1H), 7.23 (d, *J* = 6.8 Hz, 3H), 7.07 (d, *J* = 8.6 Hz, 2H), 7.01 (dd, *J* = 7.3, 1.8 Hz, 2H), 6.79 (d, *J* = 8.6 Hz, 2H), 6.61 (d, *J* = 8.0 Hz, 1H), 6.43 (d, *J* = 7.5 Hz, 1H), 4.72 (td, *J* = 7.9, 4.9 Hz, 1H), 4.52 (q, *J* = 6.7 Hz, 1H), 4.33 (p, *J* = 7.0 Hz, 1H), 3.77 (s, 3H), 3.74 – 3.67 (m, 4H), 3.27 (d, *J* = 5.0 Hz, 1H), 3.03 (dd, *J* = 13.9, 4.8 Hz, 1H), 2.97 (s, 2H), 2.93 (dd, *J* = 6.4, 4.7 Hz, 2H), 2.89 (d, *J* = 5.0 Hz, 1H), 2.71 (dd, *J* = 13.9, 8.2 Hz, 1H), 2.59 – 2.40 (m, 4H), 1.44 (s, 3H), 1.28 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.24, 171.83, 170.41, 158.78, 135.91, 130.50, 129.41, 128.65, 128.21, 127.17, 114.19, 67.05, 61.69, 59.28, 55.37, 54.28, 53.85, 52.83, 52.54, 48.74, 37.14, 37.04, 17.66, 16.56. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 6.17 (ESI-MS (m/z): 581.20 ($M+H^+$)) HRMS: calculated for $C_{31}H_{40}N_4O_7$ 581.29698 $[M+H]^+$; found 581.29694

**3MeIndAc-D-Ala-Tyr(OMe)-Phe-EK (18)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→2% MeOH in DCM) provided the title compound (23.89 mg, 78.3%) as a white powder after lyophilisation. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.46 (t, *J* = 6.3 Hz, 2H), 7.40 – 7.27 (m, 2H), 7.12 (dt, *J* = 26.3, 7.8 Hz, 5H), 6.99 (d, *J* = 6.9 Hz, 2H), 6.89 (d, *J* = 7.9 Hz, 1H), 6.75 (d, *J* = 8.6 Hz, 2H), 6.60 (d, *J* = 7.5 Hz, 1H), 6.43 (d, *J* = 6.9 Hz, 1H), 4.72 (td, *J* = 8.0, 5.0 Hz, 1H), 4.62 – 4.48 (m, 2H), 3.70 (s, 3H), 3.60 – 3.54 (m, 2H), 3.24 (d, *J* = 4.9 Hz, 1H), 3.04 – 2.93 (m, 3H), 2.85 (d, *J* = 4.9 Hz, 1H), 2.68 (dd, *J* = 13.8, 8.3 Hz, 1H), 2.53 (t, *J* = 2.1 Hz, 3H), 1.40 (s, 3H), 1.32 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.31, 172.54, 170.61, 166.15, 158.72, 148.43, 145.59, 142.27, 135.82, 131.45, 130.50, 129.34, 128.52, 128.31, 127.49, 127.09, 126.91, 123.94, 120.97, 114.10, 59.30, 55.27, 54.37, 52.86, 52.53, 49.05, 38.31, 37.19, 36.87, 18.33, 16.53, 12.52. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 9.62 (ESI-MS (m/z): 610.07 ($M+H^+$)). HRMS: calculated for $C_{36}H_{39}N_3O_6$ 610.29116 $[M+H]^+$; found 610.29114

**3MeIndAc-Ala-Tyr(OMe)-BiPhe-EK (19)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→2% MeOH in DCM) provided the title compound (18.21 mg, 53.1%) as a white powder after lyophilisation.$^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.58 – 7.28 (m, 11H), 7.12 (d, *J* = 8.2 Hz, 2H), 7.02 (d, *J* = 8.6 Hz, 2H), 6.85 (d, *J* = 7.8 Hz, 1H), 6.61 (d, *J* = 8.6 Hz, 2H), 6.55 (d, *J* = 7.5 Hz, 1H), 6.08 (d, *J* = 7.0 Hz, 1H), 4.81 (td, *J* = 8.1, 4.8 Hz, 1H), 4.60 – 4.49 (m, 2H), 3.55 – 3.37 (m, 5H), 3.32 (d, *J* = 4.9 Hz, 1H), 3.14 (dd, *J* = 14.0, 4.7 Hz, 1H), 2.95 (d, *J* = 6.9 Hz, 2H), 2.92 (d, *J* = 4.9 Hz, 1H), 2.76 (dd, *J* = 14.0, 8.4 Hz, 1H), 2.51 (t, *J* = 2.2 Hz, 3H), 1.51 (s, 3H), 1.34 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.01, 172.33, 170.61, 165.99, 158.52, 148.66, 145.44, 141.99, 140.57, 139.90, 134.77, 130.96, 130.22, 129.67, 128.75, 128.19, 127.93, 127.49, 127.27, 127.18, 126.97, 126.89, 123.84, 120.91, 120.34, 113.85, 59.25, 54.86, 54.18, 52.61, 52.52, 48.57, 38.08, 36.65, 36.59, 17.65, 16.56, 12.37. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 10.56 (ESI-MS (m/z): 686.13 ($M+H^+$)). HRMS: calculated for $C_{42}H_{43}N_3O_6$ 686.32246 $[M+H]^+$; found 686.32245

**MorphAc-D-Ala-Trp-BiPhe-EK (20)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→3% MeOH in DCM) provided the title compound (21.86 mg, 63.7%) as a white powder after lyophilisation. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.97 (s, 1H), 7.67 – 7.32 (m, 9H), 7.22 (d, *J* = 8.0 Hz, 1H), 7.16 (t, *J* = 7.3 Hz, 1H), 7.10 (t, *J* = 7.1 Hz, 1H), 6.97 (d, *J* = 8.0 Hz, 2H), 6.83 (d, *J* = 1.7 Hz, 1H), 6.64 (d, *J* = 7.9 Hz, 1H), 6.55 (d, *J* = 7.6 Hz, 1H), 4.79 – 4.63 (m, 2H), 4.28 (p, *J* = 7.0 Hz, 1H), 3.78 – 3.62 (m, 4H), 3.35 (dd, *J* = 14.7, 4.7 Hz, 1H), 3.29 (d, *J* = 4.9 Hz, 1H), 3.08 – 2.95 (m, 2H), 2.92 (s, 2H), 2.88 (d, *J* = 4.9 Hz, 1H), 2.68 (dd, *J* = 13.8, 8.7 Hz, 1H), 2.56 – 2.39 (m, 4H), 1.46 (s, 3H), 1.32 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.14, 174.77, 171.84, 170.82, 140.51, 139.50, 136.07, 135.40, 129.90, 129.10, 127.62, 127.51, 127.15, 126.99, 123.48, 122.39, 119.91, 118.82, 111.33, 109.86, 67.03, 61.61, 59.29, 53.79, 53.55, 52.99, 52.64, 49.01, 36.41, 27.48, 17.38, 16.63. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 7.33 (ESI-MS (m/z): 666.20 ($M+H^+$)). HRMS: calculated for $C_{42}H_{43}N_3O_6$ 666.32861 $[M+H]^+$; found 666.32861

**MorphAc-D-Ala-Tyr(OMe)-BiPhe-EK (21)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→3% MeOH in DCM) provided the title compound (25.08 mg, 746.3.4%) as a white powder after lyophilisation.$^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.60 – 7.38 (m, 7H), 7.34 (d, *J* = 7.4 Hz, 1H), 7.08 (d, *J* = 8.1 Hz, 4H), 6.78 (d, *J* = 8.6 Hz, 2H), 6.66 (d, *J* = 8.1 Hz, 1H), 6.51 (d, *J* = 7.5 Hz, 1H), 4.77 (td, *J* = 7.9, 4.6 Hz, 1H), 4.56 (q, *J* = 6.6 Hz, 1H), 4.33 (p, *J* = 7.0 Hz, 1H), 3.71 (s, 3H), 3.68 (d, *J* = 4.7 Hz, 4H), 3.29 (d, *J* = 4.9 Hz, 1H), 3.07 (dd, *J* = 13.9, 4.5 Hz, 1H), 3.03 – 2.86 (m, 5H), 2.78 (dd, *J* = 13.9, 8.2 Hz, 1H), 2.53 – 2.36 (m, 4H), 1.47 (s, 3H), 1.28 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.13, 171.85, 170.48, 158.80, 140.65, 139.93, 135.00, 130.53, 129.83, 128.92, 128.18, 127.45, 127.30, 127.09, 114.19, 67.03, 61.61, 59.30, 55.30, 54.24, 53.79, 52.87, 52.59,

48.76, 37.02, 36.65, 17.53, 16.61. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 7.26 (ESI-MS (m/z): 657.27 ($M+H^+$)). HRMS: calculated for $C_{37}H_{44}N_4O_7$ 657.32828 $[M+H]^+$; found 657.32831

**3MeIndAc-D-Ala-Tyr(OMe)-BiPhe-EK (22)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→3% MeOH in DCM) provided the title compound (26.38 mg, 79.2%) as a white powder after lyophilisation. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.54 – 7.49 (m, 2H), 7.47 – 7.27 (m, 9H), 7.08 (dd, *J* = 8.2, 5.8 Hz, 4H), 6.83 (d, *J* = 7.9 Hz, 1H), 6.73 (d, *J* = 8.6 Hz, 2H), 6.62 (d, *J* = 7.5 Hz, 1H), 6.36 (d, *J* = 6.9 Hz, 1H), 4.76 (td, *J* = 8.1, 4.7 Hz, 1H), 4.66 – 4.45 (m, 2H), 3.64 (s, 3H), 3.56 – 3.49 (m, 2H), 3.28 (d, *J* = 4.9 Hz, 1H), 3.10 – 2.90 (m, 3H), 2.90 – 2.86 (m, 1H), 2.72 (dd, *J* = 13.9, 8.5 Hz, 1H), 2.50 (t, *J* = 2.1 Hz, 3H), 1.43 (s, 3H), 1.33 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.17, 172.52, 170.72, 166.19, 158.74, 148.56, 145.55, 142.22, 140.61, 139.89, 134.99, 131.34, 130.51, 129.82, 128.88, 128.28, 127.50, 127.40, 127.22, 127.03, 126.90, 123.94, 120.99, 114.10, 59.34, 55.21, 54.30, 52.93, 52.58, 49.17, 38.27, 36.85, 36.66, 18.29, 16.60, 12.51. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 10.67 (ESI-MS (m/z): 686.13 ($M+H^+$)). HRMS: calculated for $C_{42}H_{43}N_3O_6$ 686.32246 $[M+H]^+$; found 686.32251

**MorphAc-Ala-Tyr(OMe)-Phe-VS (23)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→3% MeOH in DCM) provided the title compound (13.08 mg, 43.5%) as a white powder after lyophilisation. Isolated with 19% *cis* isomer. Peaks reported correspond to *trans* isomer. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.47 (s, 1H), 7.35 – 7.20 (m, 5H), 7.16 – 7.10 (m, 2H), 7.01 (d, *J* = 8.6 Hz, 2H), 6.85 – 6.75 (m, 2H), 6.57 (dd, *J* = 24.5, 6.5 Hz, 1H), 6.18 (dd, *J* = 15.1, 1.7 Hz, 1H), 4.98 (p, *J* = 7.1 Hz, 1H), 4.56 – 4.41 (m, 1H), 4.27 – 4.11 (m, 1H), 3.77 (s, 3H), 3.72 (t, *J* = 4.5 Hz, 4H), 3.08 – 2.75 (m, 9H), 2.47 (d, *J* = 32.6 Hz, 4H), 1.31 (d, *J* = 7.1 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.09, 170.45, 158.72, 146.23, 136.09, 130.37, 130.11, 129.48, 129.34, 128.82, 128.76, 128.07, 127.25, 114.35, 114.22, 66.88, 61.50, 55.36, 54.82, 53.86, 50.45, 49.44, 42.75, 39.81, 36.34, 17.27. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 4.98 (ESI-MS (m/z): 601.27 ($M+H^+$)). HRMS: calculated for $C_{30}H_{46}N_4O_7S$ 601.26905 $[M+H]^+$; found 601.26904

**MorphAc-Ala-Tyr(OMe)-Cha-VS (24)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→3% MeOH in DCM) provided the title compound (15.27 mg, 50.3%) as a white powder after lyophilisation. Isolated with 13% *cis* isomer. Peaks reported correspond to *trans* isomer. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.48 (d, *J* = 6.2 Hz, 1H), 7.11 (d, *J* = 8.6 Hz, 2H), 6.84 (d, *J* = 8.6 Hz, 2H), 6.72 (dd, *J* = 15.1, 4.5 Hz, 1H), 6.63 (d, *J* = 7.5 Hz, 1H), 6.45 (d, *J* = 8.3 Hz, 1H), 6.18 (dd, *J* = 15.1, 1.7 Hz, 1H), 4.72 (p, *J* = 7.4 Hz, 1H), 4.55 (q, *J* = 7.4 Hz, 1H), 4.28 (p, *J* = 7.0 Hz, 1H), 3.79 (s, 3H), 3.72 (t, *J* = 4.5 Hz, 4H), 3.15 (dd, *J* = 14.0, 6.1 Hz, 1H), 3.04 – 2.94 (m, 2H), 2.89 (s, 3H), 2.83 (d, *J* = 16.7 Hz, 1H), 2.48 (dq, *J* = 11.7, 7.1 Hz, 4H), 1.83 – 1.51 (m, 5H), 1.45 – 1.33 (m, 5H), 1.29 – 1.04 (m, 4H), 1.04 – 0.76 (m, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.14, 170.45, 158.78, 147.69, 130.48, 129.15, 128.14, 114.39, 66.95, 61.56, 55.35, 54.87, 53.89, 49.47, 47.41, 42.80, 41.30, 36.33, 34.05, 33.63, 32.46, 26.46, 26.31, 26.15, 17.45. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 5.44 (ESI-MS (m/z): 607.33 ($M+H^+$)). HRMS: calculated for $C_{30}H_{46}N_4O_7S$ 607.31600 $[M+H]^+$; found 607.31604

**3MeIndAc-Ala-Tyr(OMe)-Phe-VS (25)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→3% MeOH in DCM) provided the title compound (15.11 mg, 47.3%) as a white powder after lyophilization. Isolated with 16% *cis* isomer. Peaks reported correspond to *trans* isomer. $^1$H NMR (400 MHz, $CDCl_3$) δ 8.24 (s, 1H), 7.61 (d, *J* = 7.6 Hz, 1H), 7.45 (dd, *J* = 6.3, 2.3 Hz, 2H), 7.38 – 7.30 (m, 3H), 7.24 – 7.09 (m, 5H), 7.06 (dd, *J* = 7.3, 1.9 Hz, 2H), 6.95 (d, *J* = 8.6 Hz, 1H), 6.76 (d, *J* = 2.2 Hz, 1H), 6.68 (dd, *J* = 15.0, 4.4 Hz, 2H), 6.32 (d, *J* = 5.7 Hz, 1H), 6.15 (dd, *J* = 15.1, 1.7 Hz, 1H), 4.92 (dt, *J* = 12.1, 6.0 Hz, 1H), 4.81 – 4.68 (m, 1H),

4.23 (q, *J* = 6.7 Hz, 1H), 3.63 – 3.43 (m, 2H), 3.35 (dd, *J* = 14.6, 5.1 Hz, 1H), 3.22 – 3.09 (m, 1H), 2.76 (d, *J* = 7.6 Hz, 2H), 2.70 (s, 3H), 2.54 – 2.44 (m, 3H), 1.42 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 173.04, 171.06, 166.84, 148.99, 146.31, 145.34, 142.25, 136.68, 136.17, 130.83, 129.98, 129.79, 129.36, 128.75, 128.62, 127.76, 127.54, 126.99, 126.94, 124.03, 123.48, 122.50, 121.09, 119.97, 118.47, 111.70, 109.63, 54.34, 50.90, 50.30, 43.75, 42.65, 39.50, 38.36, 26.96, 17.43, 12.71. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 7.78 (ESI-MS (m/z): 639.13 ($M+H^+$)). HRMS: calculated for $C_{36}H_{38}N_4O_5S$ 639.26357 $[M+H]^+$; found 639.26355.

**3MeIndAc-Ala-Tyr(OMe)-Phe-VS (26)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→3% MeOH in DCM) provided the title compound (9.98 mg, 30.9%) as a white powder after lyophilisation. $^1$H NMR (400 MHz, $CDCl_3$) δ 8.52 (s, 1H), 7.66 (d, *J* = 7.7 Hz, 1H), 7.49 – 7.41 (m, 2H), 7.40 – 7.28 (m, 3H), 7.22 – 7.03 (m, 3H), 6.96 (d, *J* = 7.8 Hz, 1H), 6.63 – 6.59 (m, 1H), 6.57 (d, *J* = 4.6 Hz, 1H), 6.41 (d, *J* = 6.1 Hz, 1H), 6.05 (dd, *J* = 15.1, 1.4 Hz, 1H), 4.79 (tt, *J* = 10.1, 5.1 Hz, 1H), 4.68 (p, *J* = 7.0 Hz, 1H), 4.35 (q, *J* = 6.7 Hz, 1H), 3.58 – 3.34 (m, 3H), 3.24 (dd, *J* = 14.4, 7.7 Hz, 1H), 2.72 (s, 3H), 2.44 (t, *J* = 2.1 Hz, 3H), 1.67 – 1.50 (m, 5H), 1.46 (d, *J* = 7.0 Hz, 3H), 1.33 – 1.23 (m, 3H), 1.21 – 1.04 (m, 3H), 0.95 – 0.68 (m, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 173.02, 171.07, 166.62, 148.87, 147.57, 145.39, 142.22, 136.36, 130.91, 129.07, 127.68, 127.42, 126.96, 123.97, 123.69, 122.53, 121.04, 120.06, 118.58, 111.81, 109.99, 54.48, 50.03, 47.37, 42.68, 40.96, 38.33, 33.99, 33.35, 32.65, 27.51, 26.41, 26.17, 26.10, 17.80, 12.60. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 8.34 (ESI-MS (m/z): 645.20 ($M+H^+$)). HRMS: calculated for $C_{36}H_{44}N_4O_5S$ 645.31052 $[M+H]^+$; found 645.31055

**$N_3$Gly-Ala-Hyp-Nle-Leu-EK (27)**

This compound was obtained by the general protocol for azide coupling on a 64 µmol scale. Purification by column chromatography (0→1.5% MeOH in DCM) provided the product (7.6 mg, 12.5 µmol, 20%), which was next deprotected in 1:1 DCM:TFA (2 mL). After stirring for 30 min, the mixture was evaporated and co-evaporated with toluene (3x). Purification by HPLC (C18, linear gradient 20 → 70% MeCN: MeOH, 0.1% TFA) yielded the title compound (2.55 mg, 4.63 µmol, 7%). Complex NMR due to a 9:1 ratio of rotamers. Peaks of major rotamer are reported. $^1$H NMR (600 MHz, $CDCl_3$) δ 7.13 (dd, *J* = 18.5, 7.3 Hz, 2H), 6.40 (d, *J* = 8.0 Hz, 1H), 4.73 – 4.69 (m, 1H), 4.66 (t, *J* = 7.8 Hz, 1H), 4.59 (dt, *J* = 9.9, 5.5 Hz, 2H), 4.28 (q, *J* = 7.8 Hz, 1H), 3.99 (s, 2H), 3.85 (d, *J* = 11.1 Hz, 1H), 3.63 (dd, *J* = 11.1, 4.0 Hz, 1H), 3.29 (d, *J* = 5.0 Hz, 1H), 2.89 (d, *J* = 5.0 Hz, 1H), 2.44 – 2.36 (m, 1H), 2.18 – 2.10 (m, 1H), 1.86 – 1.76 (m, 1H), 1.66 – 1.60 (m, 1H), 1.54 (ddd, *J* = 13.2, 9.7, 3.2 Hz, 1H), 1.51 (s, 3H), 1.38 (d, *J* = 6.9 Hz, 3H), 1.35 – 1.22 (m, 7H), 0.93 (dd, *J* = 6.5, 4.5 Hz, 6H), 0.88 (t, *J* = 7.1 Hz, 3H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 208.56, 172.29, 171.71, 170.78, 166.79, 70.46, 59.21, 58.89, 55.47, 53.77, 52.59, 52.55, 50.50, 47.06, 40.21, 36.41, 31.79, 27.66, 25.34, 23.49, 22.47, 21.42, 17.97, 16.88, 14.03. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 6.10 (ESI-MS (m/z): 552.20 ($M+H^+$)). HRMS: calculated for $C_{25}H_{41}N_7O_7$ 552.31402 $[M+H]^+$; found 552.31403

**$N_3$Gly-Ala-Thz-Nle-Leu-EK (28)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (0→2% MeOH in DCM) provided the title compound (10.4 mg, 18.7 µmol, 37 as a white powder after lyophilisation. Complex NMR due to a 2:1 ratio of rotamers. $^1$H NMR (600 MHz, $CDCl_3$) δ 7.38 (m, 0.3H) 7.09 (d, *J* = 13.0 Hz, 0.7H), 7.01 – 6.70 (m, 1.4H), 6.27 (d, *J* = 6.8 Hz, 0.6H), 5.07 – 4.12 (m, 7H), 4.07 – 3.87 (m, 2H), 3.75 – 2.70 (m, 3H), 2.09 – 1.03 (m, 15H), 1.00 – 0.66 (m, 9H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 209.62, 208.41, 171.90, 171.61, 171.52, 171.40, 171.27, 168.95, 168.65, 168.54, 166.34, 68.33, 63.48, 63.08, 62.63, 59.20, 56.06, 53.55, 52.68, 52.56, 52.36, 50.62, 49.93, 49.61, 49.44, 49.11, 47.10, 40.75, 40.28, 35.09, 32.22, 31.34, 29.83, 27.45, 25.35, 23.49, 22.47, 22.22, 21.47, 18.54, 16.87, 13.99.

LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 7.12 (ESI-MS (m/z): 554.13 ($M+H^+$)). HRMS: calculated for $C_{24}H_{39}N_7O_6S$ 554.27553$[M+H]^+$; found 554.27545

**$N_3$Gly-Ala-Aze-Nle-Leu-EK (29)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→2% MeOH in DCM) provided the title compound (14.4 mg, 55%) as a white powder after lyophilisation. Complex NMR due to a 5:1 ratio of rotamers. Peaks of major rotamer are reported. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 7.7 Hz, 1H), 6.99 (d, *J* = 6.8 Hz, 1H), 6.42 (d, *J* = 8.0 Hz, 1H), 4.91 (dd, *J* = 9.3, 6.4 Hz, 1H), 4.62 – 4.48 (m, 2H), 4.32 (ddd, *J* = 12.6, 5.0, 2.8 Hz, 2H), 4.16 – 4.07 (m, 1H), 3.98 (d, *J* = 3.2 Hz, 2H), 3.31 (d, *J* = 5.0 Hz, 1H), 2.87 (d, *J* = 5.0 Hz, 1H), 2.80 – 2.66 (m, 1H), 2.57 – 2.40 (m, 1H), 1.93 – 1.71 (m, 1H), 1.68 – 1.50 (m, 3H), 1.49 (s, 3H), 1.39 – 1.17 (m, 8H), 0.92 (dd, *J* = 6.4, 3.5 Hz, 6H), 0.86 (t, *J* = 7.1 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 208.61, 174.09, 171.51, 170.25, 166.41, 62.12, 59.20, 53.58, 52.58, 50.43, 49.15, 44.70, 40.21, 31.67, 27.66, 25.36, 23.46, 22.44, 21.46, 18.68, 18.28, 16.86, 14.05. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 6.64 (ESI-MS (m/z): 522.20 ($M+H^+$)). HRMS: calculated for $C_{26}H_{43}N_7O_6$ 522.30346 $[M+H]^+$; found 522.30341

**$N_3$Gly-Ala-Pip-Nle-Leu-EK (30)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→2% MeOH in DCM) provided the title compound (18.6 mg, 67%) as a white powder after lyophilisation. Complex NMR due to a 1.5:1 ratio of rotamers. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.44 (d, *J* = 8.2 Hz, 0.4H), 7.38 (d, *J* = 7.3 Hz, 0.6H), 7.06 (d, *J* = 6.0 Hz, 0.4H), 6.84 (d, *J* = 8.7 Hz, 0.4H), 6.45 (d, *J* = 7.7 Hz, 0.6H), 6.28 (d, *J* = 7.9 Hz, 0.6H), 5.18 (d, *J* = 4.3 Hz, 0.6H), 4.96 (p, *J* = 6.9 Hz, 0.6H), 4.83 (p, *J* = 6.9 Hz, 0.4H), 4.67 – 4.48 (m, 2H), 4.38 – 4.25 (m, 1H), 3.98 (d, *J* = 5.4 Hz, 1.2H), 3.95 – 3.91 (m, 0.8H), 3.78 (d, *J* = 13.5 Hz, 0.6H), 3.28 (d, *J* = 5.0 Hz, 1H), 3.25 – 3.16 (m, 0.6H), 2.88 (d, *J* = 5.0 Hz, 0.6H), 2.85 (d, *J* = 4.9 Hz, 0.4H), 2.62 – 2.55 (m, 0.4H), 2.50 (dd, m, 0.4H), 2.22 – 2.14 (m, 0.6H), 1.86 – 1.06 (m, 20H), 0.95 – 0.83 (m, 9H). $^{13}$C NMR (101 MHz, CDCl3) δ 208.71, 208.68, 172.48, 172.40, 171.74, 171.59, 170.62, 169.25, 168.65, 166.25, 59.46, 59.26, 57.11, 55.76, 53.37, 52.99, 52.83, 52.69, 52.44, 50.84, 49.97, 46.44, 45.87, 44.06, 40.82, 40.56, 40.41, 32.51, 31.66, 28.60, 27.86, 26.85, 25.98, 25.66, 25.59, 25.51, 25.14, 23.79, 23.72, 22.73, 22.53, 21.64, 21.06, 20.53, 18.74, 17.29, 17.13, 17.04, 14.26. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 7.34 (ESI-MS (m/z): 550.07 ($M+H^+$)). HRMS: calculated for $C_{26}H_{43}N_7O_6$ 550.33476 $[M+H]^+$; found 550.33472

**$N_3$Gly-Ala-(4S)-FPro-Nle-Leu-EK (31)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (2→3% MeOH in DCM) followed by purification by HPLC (30-70% MeCN, 0.1 % TFA, 10 min gradient) provided the title compound (2.51 mg, 9%) as a white powder after lyophilisation. Complex NMR due to a 3:2 ratio of rotamers. $^1$H NMR (600 MHz, $CDCl_3$) δ 7.36 (d, *J* = 7.0 Hz, 0.4H), 7.05 (d, *J* = 7.2 Hz, 0.6H) 6.85 (d, *J* = 9.0 Hz, 0.4 H), 6.81 (d, *J* = 5.5 Hz, 0.4H), 6.73 (d, *J* = 7.7 Hz, 0.6H), 6.29 (d, *J* = 7.9 Hz, 0.6H), 5.43 – 5.24 (m, 1H), 4.79 – 4.64 (m, 1.6H), 4.60 – 4.54 (m, 1H), 4.48 (d, *J* = 9.4 Hz, 0.4H), 4.39 – 4.30 (m, 0.6H), 4.20 (m, 0.4H), 4.04 – 3.80 (m, 4H), 3.30 (d, *J* = 5.0 Hz, 0.6H), 3.27 (d, *J* = 4.9 Hz, 0.4H), 2.94 (t, *J* = 15.5 Hz, 0.4H), 2.88 (d, *J* = 5.0 Hz, 0.6H), 2.85 (d, *J* = 4.9 Hz, 0.4H), 2.83 – 2.74 (m, 0.6H), 2.40 – 2.16 (m, 1H), 1.90 – 1.16 (m, 15H), 1.10 (ddd, *J* = 14.0, 10.3, 4.5 Hz, 0.4H), 0.99 – 0.89 (m, 6H), 0.86 (t, *J* = 7.1 Hz, 3H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 209.58, 208.31, 172.97, 172.12, 171.95, 171.54, 170.09, 169.60, 168.52, 166.44, 92.20 (d, *J* = 179.4 Hz), 90.36 (d, *J* = 176.2 Hz),, 59.61, 59.49, 59.26, 58.89, 55.87, 54.38 (d, *J* = 24.0 Hz), 54.19 (d, *J* = 24.1 Hz), 53.21, 52.61, 52.57, 52.48, 52.36, 50.49, 49.26, 48.68, 46.93, 40.84, 40.14, 38.80 (d, *J* = 21.3 Hz), 34.60 (d, *J* = 21.4 Hz), 32.05, 31.23, 30.47, 27.99, 27.26, 25.32, 25.25, 23.59, 23.51, 22.46, 22.17, 21.57, 21.43, 18.40, 16.90, 16.70, 16.61, 14.00, 13.95 LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 7.78 (ESI-MS (m/z): 554.20 ($M+H^+$)). HRMS: calculated for $C_{25}H_{41}FN_7O_6$ $[M+H]^+$; 554.30969, found 554.30969.

**$N_3$Gly-Ala-(4R)-FPro-Nle-Leu-EK (32)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (1→2% MeOH in DCM) followed by purification by HPLC (30-70% MeCN, 0.1 % TFA, 10

min gradient) provided the title compound (2.95 mg, 11%) as a white powder after lyophilisation. Complex NMR due to a 95:5 ratio of rotamers. Peaks of major rotamer are reported. $^{1}$H NMR (600 MHz, $CDCl_3$) δ 7.21 (d, *J* = 6.9 Hz, 1H), 6.98 (d, *J* = 7.6 Hz, 1H), 6.23 (d, *J* = 8.0 Hz, 1H), 5.32 (d, *J* = 52.5 Hz, 1H), 4.76 (p, *J* = 6.9 Hz, 1H), 4.70 (t, *J* = 8.0 Hz, 1H), 4.62 – 4.56 (m, 1H), 4.30 (q, *J* = 7.7 Hz, 1H), 4.09 – 3.93 (m, 3H), 3.65 (ddd, *J* = 34.0, 12.3, 3.1 Hz, 1H), 3.29 (d, *J* = 4.9 Hz, 1H), 2.90 (d, *J* = 5.0 Hz, 1H), 2.65 – 2.49 (m, 1H), 2.43 (ddd, *J* = 22.6, 14.8, 8.2 Hz, 1H), 1.81 (dq, *J* = 13.3, 7.3, 6.6 Hz, 1H), 1.67 – 1.59 (m, 2H), 1.55 (ddd, *J* = 13.0, 9.7, 3.1 Hz, 1H), 1.51 (s, 3H), 1.48 – 1.42 (m, 1H), 1.36 (d, *J* = 6.9 Hz, 3H), 1.34 – 1.21 (m, 4H), 0.94 (t, *J* = 6.7 Hz, 6H), 0.89 (t, *J* = 7.1 Hz, 3H). $^{13}$C NMR (151 MHz, $CDCl_3$) δ 208.52, 171.99, 171.42, 169.92, 166.11, 91.74 (d, *J* = 180.5), 59.21, 58.63, 53.80, 53.69 (d, *J* = 16 Hz), 52.70, 52.59, 50.51, 47.05, 40.31, 34.25 (d, *J* = 21.7 Hz), 31.97, 27.61, 25.37, 23.50, 22.48, 21.45, 18.33, 16.88, 14.04. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 7.65 (ESI-MS (m/z): 554.20 ($M+H^+$)). HRMS: calculated for $C_{25}H_{41}FN_7O_6$ $[M+H]^+$; 554.30969, found 554.30969.

**$N_3$Gly-Ala-4,4-$F_2$Pro-Nle-Leu-EK (33)**

This compound was obtained by the general protocol for azide coupling on a 69 µmol scale. Purification by column chromatography (0→1.5% MeOH in DCM) provided the title compound (16.9 mg, 18.7 µmol, 24%) as a white powder after lyophilisation. Complex NMR due to a 3:1 ratio of rotamers. $^{1}$H NMR (600 MHz, $CDCl_3$) δ 7.56 (d, *J* = 7.2 Hz, 0.3H), 7.12 (d, *J* = 7.2 Hz, 0.7H), 7.03 (d, *J* = 7.7 Hz, 0.7H), 7.00 – 6.95 (m, 0.3H), 6.76 (d, *J* = 8.8 Hz, 0.3H), 6.36 (d, *J* = 8.0 Hz, 0.7H), 4.77 (dd, *J* = 9.2, 5.7 Hz, 0.8H), 4.72 – 4.63 (m, 1H), 4.59 (ddd, *J* = 10.8, 8.2, 3.0 Hz, 0.7H), 4.35 (q, *J* = 7.7 Hz, 1.5H), 4.29 – 4.05 (m, 1H), 4.00 (d, *J* = 4.9 Hz, 1.4H), 3.98 – 3.81 (m, 1.3H), 3.76 (m, 0.3H), 3.29 (d, *J* = 5.0 Hz, 0.7H), 3.26 (d, *J* = 4.4 Hz, 0.3H), 2.90 (d, *J* = 5.0 Hz, 0.7H), 2.88 – 2.77 (m, 1H), 2.71 (m, 0.3H), 2.58 (m, 1H), 1.92 – 1.16 (m, 15H), 0.97 – 0.82 (m, 9H). $^{13}$C NMR (151 MHz, $CDCl_3$, peaks of major rotamer) δ $^{13}$C NMR (151 MHz, $CDCl_3$) δ 208.55, 172.20, 171.37, 168.73, 166.41, 126.20 (t, *J* = 249.2 Hz), 59.21, 58.10, 53.76 (t, *J* = 23.0 Hz), 53.60, 52.55, 50.56, 46.84, 40.17, 39.73, 39.56, 35.77 (t, *J* = 24.2 Hz) 32.22, 27.42, 25.32, 23.46, 22.45, 21.38, 18.03, 16.86, 14.00. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 7.25 (ESI-MS (m/z): 572.20 ($M+H^+$)). HRMS: calculated for $C_{25}H_{39}F_2N_7O_6$ 572.30326 $[M+H]^+$; found 572.30323

**$N_3$Gly-Ala-Pro-Nle-Phe-EK (34)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (0→2% MeOH in DCM) provided the title compound (12.14 mg, 43%) as a white powder after lyophilisation. Complex NMR due to a 5:1 ratio of rotamers. Peaks of major rotamer are reported. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.32 – 7.20 (m, 3H), 7.17 – 7.12 (m, 3H), 7.02 (d, *J* = 7.6 Hz, 1H), 6.52 (d, *J* = 7.5 Hz, 1H), 4.81 (td, *J* = 7.9, 5.1 Hz, 1H), 4.74 (p, *J* = 7.0 Hz, 1H), 4.49 (dd, *J* = 8.1, 2.8 Hz, 1H), 4.23 (td, *J* = 7.9, 5.6 Hz, 1H), 3.97 (d, *J* = 4.7 Hz, 2H), 3.70 – 3.49 (m, 2H), 3.31 (d, *J* = 4.9 Hz, 1H), 3.13 (dd, *J* = 14.0, 5.0 Hz, 1H), 2.90 (d, *J* = 4.9 Hz, 1H), 2.79 (dd, *J* = 14.0, 8.1 Hz, 1H), 2.26 (ddd, *J* = 12.8, 6.6, 3.3 Hz, 1H), 2.17 – 2.06 (m, 1H), 2.01 (ddq, *J* = 16.4, 7.2, 4.4, 3.9 Hz, 1H), 1.89 (ddd, *J* = 18.2, 12.4, 7.8 Hz, 1H), 1.73 (dq, *J* = 13.4, 7.3 Hz, 2H), 1.48 (s, 3H), 1.36 (d, *J* = 6.9 Hz, 3H), 1.32 – 1.11 (m, 6H), 0.92 – 0.77 (m, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.56, 171.32 171.40, 170.81, 166.18, 135.79, 129.85, 129.45, 128.69, 128.54, 127.23, 60.01, 59.39, 53.34, 52.67, 52.65, 47.47, 46.80, 37.20, 31.58, 27.56, 27.26, 25.29, 22.44, 18.39, 16.68, 13.99. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 6.76 (ESI-MS (m/z): 570.13 ($M+H^+$)). HRMS: calculated for $C_{28}H_{39}N_7O_6$ 570.30346 $[M+H]^+$; found 570.30347

**$N_3$Gly-Ala-Pro-Nle-Cha-EK (35)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (0→2% MeOH in DCM) provided the title compound (11.35 mg, 39%) as a white powder after lyophilisation. Complex NMR due to a 5:1 ratio of rotamers. Peaks of major rotamer are reported. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.16 (d, *J* = 7.5 Hz, 1H), 7.08 (d, *J* = 7.7 Hz, 1H), 6.35 (d, *J* = 7.9 Hz, 1H), 4.76 (p, *J* = 7.0 Hz, 1H), 4.57 (dd, *J* = 8.1, 2.4 Hz, 2H), 4.29 (td, *J* = 7.8, 5.8 Hz, 1H), 3.97 (d, *J* = 5.0 Hz, 2H), 3.69 – 3.51 (m, 2H), 3.30 (d, *J* = 5.0 Hz, 1H), 2.87 (d, *J* = 5.0 Hz, 1H), 2.32 (ddd, *J* = 12.5, 6.7, 3.3 Hz, 1H), 2.18 – 1.87 (m, 3H), 1.84 – 1.53 (m, 10H),

1.50 (s, 3H), 1.38 (d, *J* = 6.9 Hz, 3H), 1.34 – 1.07 (m, 9H), 0.87 (t, *J* = 7.1 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$, all peaks reported) δ 208.63, 172.28, 171.59, 170.83, 166.18, 60.08, 59.23, 53.43, 52.67, 52.61, 49.82, 47.48, 46.82, 38.64, 34.54, 34.11, 32.04, 31.81, 27.60, 27.40, 26.47, 26.36, 26.08, 25.31, 22.50, 18.42, 16.89, 14.04. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 7.50 (ESI-MS (m/z): 576.27 ($M+H^+$)). HRMS: calculated for $C_{28}H_{45}N_7O_6$ 576.35041 $[M+H]^+$; found 576.35040

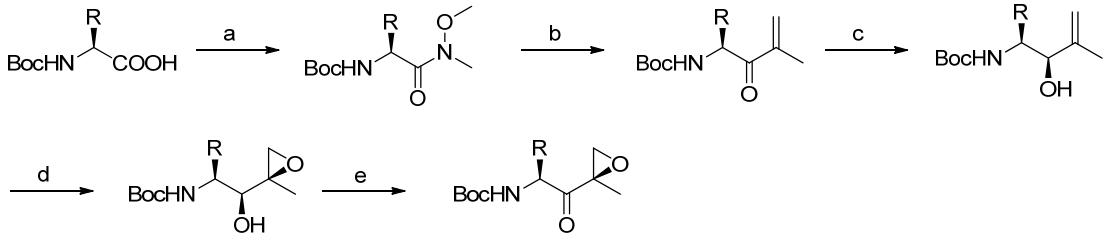
**$N_3$Gly-Ala-4,4-$F_2$Pro-Nle-Phe-EK (36)**

This compound was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (0→2% MeOH in DCM) provided the title compound (15.20 mg, 50%) as a white powder after lyophilisation. Complex NMR due to a 2:1 ratio of rotamers. Peaks of major rotamer are reported. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.20 (m, 3H), 7.16 – 7.11 (m, 3H), 6.89 (d, *J* = 7.7 Hz, 1H), 6.36 (d, *J* = 7.4 Hz, 1H), 4.81 (td, *J* = 7.8, 5.0 Hz, 1H), 4.72 – 4.62 (m, 2H), 4.28 – 4.21 (m, 1H), 4.14 (td, *J* = 12.3, 7.7 Hz, 1H), 3.99 (d, *J* = 3.7 Hz, 2H), 3.93 – 3.75 (m, 1H), 3.30 (d, *J* = 4.9 Hz, 1H), 3.14 (dd, *J* = 14.1, 5.0 Hz, 1H), 2.91 (d, *J* = 4.9 Hz, 1H), 2.78 (dt, *J* = 14.0, 7.0 Hz, 2H), 2.53 – 2.44 (m, 1H), 1.85 – 1.66 (m, 2H), 1.50 (s, 3H), 1.36 (d, *J* = 6.9 Hz, 3H), 1.23 (ddt, *J* = 26.4, 15.1, 5.0 Hz, 4H), 0.85 (td, *J* = 7.0, 3.8 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 207.45, 172.27, 171.05, 168.58, 166.35, 135.61, 129.92, 129.42, 128.76, 128.59, 127.34, 127.08, 126.18, 59.43, 58.00, 56.06, 53.70 (t, *J* = 31 Hz), 53.51, 52.79, 52.57, 52.14, 46.81, 37.10, 35.46 (t, *J* = 23 Hz), 31.87, 27.37, 22.43, 18.06, 16.71, 13.99.

LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 7.24 (ESI-MS (m/z): 606.20 ($M+H^+$)). HRMS: calculated for $C_{28}H_{37}F_2N_7O_6$ 606.28461 $[M+H]^+$; found 606.28467

**$N_3$Gly-Ala-4,4-$F_2$Pro-Nle-Cha-EK (37)**

Compound **37** was obtained by the general protocol for azide coupling on a 50 µmol scale. Purification by column chromatography (0→2% MeOH in DCM) provided the title compound (14.01 mg, 46%) as a white powder after lyophilization. Complex NMR due to a 2.5:1 ratio of rotamers. $^1$H NMR (400 MHz, $CDCl_3$, peaks of major rotamer) δ 7.08 (d, *J* = 7.3 Hz, 1H), 6.95 (d, *J* = 7.7 Hz, 1H), 6.13 (d, *J* = 7.9 Hz, 1H), 4.78 (dd, *J* = 9.3, 5.5 Hz, 1H), 4.67 (t, *J* = 7.1 Hz, 1H), 4.63 – 4.53 (m, 1H), 4.32 (td, *J* = 7.7, 5.6 Hz, 1H), 4.26 – 4.07 (m, 1H), 4.00 (d, *J* = 4.2 Hz, 2H), 3.91 – 3.79 (m, 1H), 3.28 (d, *J* = 5.0 Hz, 1H), 3.04 – 2.91 (m, 1H), 2.89 (d, *J* = 5.0 Hz, 1H), 2.65 – 2.48 (m, 1H), 1.87 – 1.54 (m, 10H), 1.51 (s, 3H), 1.39 (d, *J* = 6.9 Hz, 3H), 1.35 – 1.07 (m, 9H), 0.88 (t, *J* = 7.0 Hz, 3H). $^{13}$C NMR (151 MHz, $CDCl_3$, all peaks reported) δ 209.85, 208.58, 172.22, 171.73, 171.56, 171.28, 168.78, 168.67, 168.58, 166.34, 126.18 (t, *J* = 241.6 Hz), 59.24, 59.07, 59.03, 58.91, 58.06, 56.08, 53.73, 53.57, 52.59, 52.54, 52.50, 52.17, 49.95, 48.70, 47.79, 46.82, 38.58, 35.62 (t, *J* = 25.7 Hz), 34.48, 34.23, 34.09, 32.21, 32.02, 31.97, 31.32, 28.05, 27.37, 26.45, 26.42, 26.31, 26.03, 22.47, 22.23, 18.08, 16.89, 16.69, 16.65, 14.03, 13.97. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 7.91 (ESI-MS (m/z): 612.27 ($M+H^+$)). HRMS: calculated for $C_{28}H_{43}F_2N_7O_6$ 612.33157 $[M+H]^+$; found 612.33154.




**Scheme S2. Epoxyketone warhead synthesis.** Reagents and conditions: (a) NH(Me)OMe·HCl, HCTU, DiPEA, DCM; (b) 2-bromopropene, *t*BuLi, $Et_2O$, –78 °C; (c) $NaBH_4$, $CeCl_3{\cdot}7H_2O$, MeOH, 0 °C; (d) *t*BuOOH, $VO(Acac)_2$, DCM, 0 °C; (e) Dess-Martin periodinane, DCM. R= cycohexyl (compounds **38**-**41**), adamantly (compounds **42**-**45**), 2-naphtyl (compounds **46**-**49**), 1-naphtyl (compounds **50**-**53**), biphenyl (compounds **54**-**57**)

**(S)-*tert*-butyl(3-cyclohexyl-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)carbamate (39)**
This compound was synthesized according to the general procedure **A** described above on a 3.7 mmol scale and was isolated after column chromatography (10→30% EtOAc:pent) in a quantitative yield. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 5.03 (d, *J* = 9.3 Hz, 1H), 4.75 – 4.59 (m, 1H), 3.70 (s, 3H), 3.11 (s, 3H), 1.83 (d, *J* = 12.6 Hz, 1H), 1.71 – 1.47 (m, 4H), 1.46 – 1.24 (m, 12H), 1.23 – 0.96 (m, 3H), 0.85 (dt, *J* = 30.7, 11.6 Hz, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 173.93, 155.63, 79.35, 61.55, 48.27, 40.43, 34.01, 33.93, 32.15, 32.08, 28.31, 26.45, 26.24, 26.02. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 9.13 (ESI-MS (m/z): 314.80 ($M+H^+$)). HRMS: calculated for $C_{16}H_{30}N_2O_4$ 315.22783 $[M+2H]^{2+}$; found 315.22781. $[\alpha]_D^{20}$= -20 (C=1, $CHCl_3$)

**(S)-*tert*-butyl (1-cyclohexyl-4-methyl-3-oxopent-4-en-2-yl)carbamate (40)**
This compound was synthesized according to the general procedure **B** described above on a 3.7 mmol scale and was isolated after column chromatography (10→30% EtOAc:pent) (842 mg, 2.85 mmol, 77%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 6.00 (s, 1H), 5.79 (s, 1H), 5.15 (d, *J* = 8.6 Hz, 1H), 5.02 (dd, *J* = 12.4, 6.1 Hz, 1H), 1.91 (d, *J* = 12.0 Hz, 1H), 1.81 (s, 3H), 1.70 – 1.40 (m, 5H), 1.35 (s, 9H), 1.26 – 0.96 (m, 5H), 0.95 – 0.65 (m, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 201.59, 155.53, 142.21, 126.01, 79.43, 51.91, 41.62, 34.21, 34.06, 32.36, 28.30, 26.40, 26.26, 26.06, 17.84.

***tert*-butyl((S)-3-cyclohexyl-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)carbamate (41)**
This compound was synthesized according to the general procedure **C** described above on a 11.5 mmol scale and the crude (quant) was used directly in used in procedure **D** and was isolated after column chromatography (10→30% EtOAc:pent) (210 mg, 0.68 mmol, 49%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 4.83 (d, *J* = 8.6 Hz, 1H), 4.38 – 4.22 (m, 1H), 3.26 (d, *J* = 4.9 Hz, 1H), 2.86 (d, *J* = 5.0 Hz, 1H), 1.84 (d, *J* = 12.7 Hz, 1H), 1.74 – 1.52 (m, 5H), 1.49 (s, 3H), 1.39 (s, 9H), 1.27 – 1.03 (m, 5H), 1.03 – 0.80 (m, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 209.76, 155.73, 79.81, 59.12, 52.47, 50.86, 38.92, 34.36, 34.19, 32.01, 28.40, 26.48, 26.36, 26.04, 16.90. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 10.06 (ESI-MS (m/z): 311.60 ($M+H^+$)). HRMS: calculated for $C_{17}H_{29}NO_4$ 312.21693 $[M+H]^+$; found 312.21689. $[\alpha]_D^{20}$= 113.2 (C=0.5, $CHCl_3$)

***tert*-butyl((R)-3-((3R,5R,7R)-adamantan-1-yl)-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)carbamate (42)**
This compound was synthesized according to the general procedure **A** described above on a 1.55 mmol scale and was isolated after column chromatography (0→20% EtOAc:pent) (552 mg, 97%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 4.96 (d, *J* = 9.5 Hz, 1H), 4.77 (t, *J* = 8.4 Hz, 1H), 3.76 (s, 3H), 3.15 (s, 3H), 1.97 – 1.86 (m, 3H), 1.71 – 1.56 (m, 6H), 1.56 – 1.46 (m, 6H), 1.39 (s, 9H), 1.29 – 1.18 (m, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 174.56, 155.25, 79.48, 61.64, 47.04, 46.74, 42.57, 42.48, 37.02, 36.97, 32.69, 32.34, 28.75, 28.71, 28.47.

***tert*-butyl((R)-1-((3R,5R,7R)-adamantan-1-yl)-4-methyl-3-oxopent-4-en-2-yl)carbamate (43)**
This compound was synthesized according to the general procedure **B** described above on a 1 mmol scale and was isolated after column chromatography (0→20% EtOAc:pent) (239 mg, 69%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 6.06 (s, 1H), 5.81 (s, 1H), 5.11 (t, *J* = 8.5 Hz, 1H), 4.99 (d, *J* = 9.1 Hz, 1H), 1.91 (s, 3H), 1.85 (s, 3H), 1.69 – 1.46 (m, 13H), 1.38 (s, 9H), 1.10 (dd, *J* = 14.7, 9.6 Hz, 1H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 201.68, 154.96, 142.05, 125.76, 79.42, 49.88, 47.12, 42.46, 36.75, 32.94, 28.52, 28.27, 17.90.

***tert*-butyl((2R,3S)-1-((3R,5R,7R)-adamantan-1-yl)-3-hydroxy-4-methylpent-4-en-2-yl)carbamate (44)**
This compound was synthesized according to the general procedure **C** described above on a 0.69 mmol scale and the crude (quant) was used directly in the next step. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 5.02 (s, 1H), 4.93 (s, 1H), 4.71 (d, *J* = 8.4 Hz, 1H), 4.09 (s, 1H), 3.85 (dd, *J* = 17.4, 8.6 Hz, 1H), 2.43 (bs, 1H), 1.91 (s, 3H), 1.74 (s, 3H), 1.71 – 1.56 (m, 6H), 1.55 – 1.36 (m, 14H), 1.30 – 1.20 (m, 2H), 0.98 (dd, *J* = 14.7, 10.2 Hz, 1H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 155.61, 145.07, 111.36, 79.43, 79.04, 48.50, 42.77, 42.68, 42.49, 37.09, 31.90, 28.77, 28.60, 28.50, 19.67.

***tert*-butyl((R)-3-((3R,5R,7R)-adamantan-1-yl)-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)carbamate (45)**
This compound was synthesized according to the general procedure **D** described above on a 0.69 mmol scale and was isolated after column chromatography (0→20% EtOAc:pent) (88 mg, 0.24 mmol, 33%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 4.75 (d, *J* = 8.6 Hz, 1H), 4.37 (t, *J* = 8.4 Hz, 1H), 3.31 (d, *J* = 5.0 Hz, 1H), 2.87 (d, *J* = 5.0 Hz, 1H), 1.95 (s, 3H), 1.73 – 1.53 (m, 12H), 1.51 (s, 3H), 1.40 (s, 9H), 1.35 (d, *J* = 1.9 Hz, 1H), 0.96 (dd, *J* = 14.5, 9.7 Hz, 1H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 209.42, 155.35, 79.85, 58.96, 52.49, 49.02, 45.18, 42.68, 36.98, 33.03, 28.78, 28.50, 17.12. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 11.03 (ESI-MS (m/z): 363.80 HRMS: calculated for $C_{21}H_{33}NO_4$ 364.24824 $[M+H]^+$; found 364.24832. $[\alpha]_D^{20}$= 99.2 (C=0.5, $CHCl_3$)

***tert*-butyl(R)-(1-(methoxy(methyl)amino)-3-(naphthalen-2-yl)-1-oxopropan-2-yl)carbamate (46)**
This compound was synthesized according to the general procedure **A** described above on a 1.0 mmol scale and was isolated after column chromatography (10→50% EtOAc:pent) in a quantitative yield. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.79-7.77 (m, 3H), 7.61 (s, 1H), 7.46-7.39 (m, 2H), 7.32 (d, *J* = 8.0 Hz, 1H), 5.27(d, *J* = 8.0 Hz, 1H), 5.05 (m, 1H), 3.64 (s, 3H), 3.25-3.20 (m, 1H), 3.15 (s, 3H), 3.06-3.00 (m, 1H), 1.35 (s, 9H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.33, 155.26, 134.18, 133.47, 132.40, 128.10, 127.99, 127.67, 127.61, 126.00, 125.55, 79.63, 61.62, 51.50, 38.99, 32.12, 28.32. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 8.79 (ESI-MS (m/z): 358.73 ($M+H^+$)). HRMS: cald. for $C_{20}H_{26}N_2O_4$, 359.19666 $[M+H]^{1+}$; found 359.19653. $[\alpha]_D^{20}$= + 19.8 (C=1, $CHCl_3$)

***tert*-butyl(R)-(4-methyl-1-(naphthalen-2-yl)-3-oxopent-4-en-2-yl)carbamate (47)**
This compound was synthesized according to the general procedure **B** described above on a 1.0 mmol scale and was isolated after column chromatography (10→30% EtOAc:pent) (251 mg, 0.74 mmol, 74%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.73-7.71 (m, 3H), 7.50 (s, 1H), 7.44-7.39 (m, 2H), 7.21 (d, *J* = 8.4 Hz, 1H), 6.02 (s, 1H), 5.79 (s, 1H), 5.37-5.35 (m, 2H), 3.28-3.24 (m, 1H), 3.08-3.04 (m, 1H), 1.83 (s, 3H), 1.38 (s, 9H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 200.12, 155.12, 142.45, 133.74, 133.39, 132.38, 128.14, 128.04, 127.65, 126.69, 126.06, 125.62, 79.69, 54.95, 39.91, 29.73, 28.33, 17.76.

***tert*-butyl((2R,3S)-3-hydroxy-4-methyl-1-(naphthalen-2-yl)pent-4-en-2-yl)carbamate (48)**
This compound was synthesized according to the general procedure **C** described above on a 0.74 mmol scale and and was isolated after column chromatography (1→10% EtOAc:pent) (126 mg, 0.37 mmol, 50%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.78-7.76 (m, 3H), 7.60 (s, 1H), 7.44-7.39 (m, 2H), 7.32 (d, *J* = 1.2 Hz, 1H), 5.10 (s, 1H), 4.90 (s, 1H), 4.86 (d, *J* = 8.4 Hz, 1H), 4.21 (s, 1H), 4.10 (d, *J* = 7.2 Hz, 1H), 3.08-3.01 (m, 2H), 2.89 (t, *J*=10 Hz, *J*=13.2 Hz, 1H), 1.82 (s, 3H), 1.35(s, 9H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 155.90, 144.85, 136.13, 133.54, 132.20, 127.89, 127.62, 127.53, 125.91, 125.31, 112.36, 79.47, 53.75, 34.55, 28.26, 19.16.

***tert*-butyl((R)-1-((R)-2-methyloxiran-2-yl)-3-(naphthalen-2-yl)-1-oxopropan-2-yl)carbamate (49)**
This compound was synthesized according to the general procedure **D** described above on a 0.37 mmol scale and was isolated after column chromatography (10→30% EtOAc:pent) (75 mg, 0.21 mmol, 57%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.82-7.76 (m, 3H), 7.58 (s, 1H), 7.47-7.40 (m, 2H), 7.30 (d, *J* = 1.6 Hz, 1H), 5.03 (d, *J* = 8.0 Hz, 1H), 4.69 (m, 1H), 3.32-3.29 (m, 1H), 3.27 (d, *J* = 4.8 Hz, 1H), 2.90 (d, *J* = 4.8 Hz, 1H), 2.88-2.82 (m, 1H), 1.50 (s, 3H), 1.33 (s, 9H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 208.43, 155.33, 133.59, 133.45, 132.50, 128.31, 128.11, 127.76, 127.62, 127.49, 126.22, 125.78, 79.94, 59.35, 53.78, 52.55, 37.62, 28.30, 16.72. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 9.70 (ESI-MS (m/z): 355.67 ($M+H^+$)). HRMS: calculated for $C_{21}H_{25}NO_4$, 356.18557 $[M+H]^+$; found 356.18563. $[\alpha]_D^{20}$= 146.4 (C=1, $CHCl_3$)

***tert*-butyl(R)-(1-(methoxy(methyl)amino)-3-(naphthalen-1-yl)-1-oxopropan-2-yl)carbamate (50)**
This compound was synthesized according to the general procedure **A** described above on a 1.0 mmol scale and was isolated after column chromatography (10→50% EtOAc:pent) in a quantitative yield. $^{1}$H NMR(400 MHz, $CDCl_3$) δ 8.20 (d. *J*= 8.0 Hz, 1H), 7.79 (d, *J* = 8.0 Hz, 1H), 7.69 (d, *J* = 8.0 Hz, 1H), 7.52-7.29 (m, 4H), 5.58 (d, *J* = 9.0 Hz, 2H), 5.15-5.10 (m, 1H), 3.59-3.34 (m, 5H), 3.03 (s, 3H), 1.35 (s, 9H) $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.09, 154.95, 133.48, 132.68, 132.14, 128.60, 128.45, 128.18, 127.44, 127.30, 125.80, 125.25, 124.99, 123.33, 123.04, 79.02, 77.36, 61.02, 50.77, 35.75, 31.66, 28.05. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 8.56 (ESI-MS (m/z): 360.1 ($M+H^+$)). HRMS: calculated for $C_{20}H_{26}N_2O_4$, 359.19663 $[M]^+$; found 359.19653. $[\alpha]_D^{20}$= 8.4 (C=1, $CHCl_3$)

***tert*-butyl (R)-(4-methyl-1-(naphthalen-1-yl)-3-oxopent-4-en-2-yl)carbamate (51)**
This compound was synthesized according to the general procedure **B** described above on a 1.0 mmol scale and was isolated after column chromatography (10→30% EtOAc:pent) (200 mg, 0.59 mmol, 59%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.12 (d, *J* = 8.4 Hz, 1H), 7.81 (d, *J* = 8.0 Hz, 1H), 7.70 (d, *J* = 8.4 Hz, 1H), 7.54-7.50 (m, 1H), 7.46 (t, *J* = 7.2 Hz, 1H), 7.32 (t, *J* = 7.6 Hz, 1H), 7.17 (d, *J* = 7.2 Hz, 1H), 5.64 (s, 1H), 5.48-5.43 (m, 3H), 3.54 (m, 1H), 3.38-3.33 (m, 1H), 1.68 (s, 3H), 1.4 (s, 9H). $^{13}$C NMR (101 MHz, $CDCl_3$): δ ppm 200.92, 155.09, 142.78, 133.83, 132.66, 132.23, 129.05, 128.78, 128.15, 127.70, 126.55, 126.37, 125.66, 125.21, 123.69, 123.18, 79.66, 53.95, 37.42, 28.36, 17.41.

***tert*-butyl((2R,3S)-3-hydroxy-4-methyl-1-(naphthalen-1-yl)pent-4-en-2-yl)carbamate (52)**
This compound was synthesized according to the general procedure **C** described above on a 0.59 mmol scale and was isolated after column chromatography (1→10% EtOAc:pent) (119 mg, 0.35 mmol, 60%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.01 (d, *J* = 8.0 Hz, 1H), 7.85 (d, *J*=7.6 Hz, 1H), 7.34 (d, *J* = 8.0 Hz, 1H), 7.52-7.44 (m, 2H), 7.40 (t, *J* = 7.2 Hz, 1H), 7.33 (d, *J* = 6.8 Hz, 1H), 5.21 (s, 1H), 5.08 (s, 1H), 4.76 (d, *J* = 8.8 Hz, 1H), 4.34 (s, 1H), 4.04-4.02 (m, 1H), 3.44 (d, *J*=14.0 Hz, 1H), 3.17 (t, *J*=10.8 Hz, 1H), 2.94 (s, 1H), 1.87 (s, 3H), 1.28 (s, 9H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 155.92, 144.97, 134.94, 133.97, 132.51, 128.87, 128.09, 127.34, 127.13, 125.99, 125.51, 123.64, 112.94, 112.47, 79.45, 77.66, 53.92, 30.92, 29.80, 28.31, 27.55, 19.47.

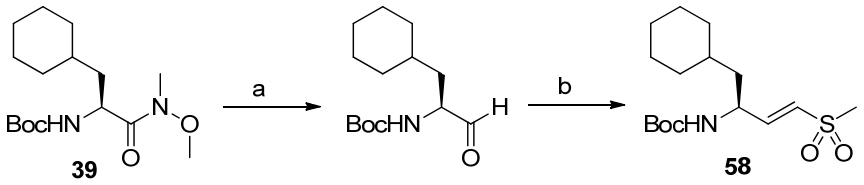
***tert*-butyl((2R)-1-hydroxy-1-((R)-2-methyloxiran-2-yl)-3-(naphthalen-1-yl)propan-2-yl)carbamate (53)**
This compound was synthesized according to the general procedure **D** described above on a 1.38 mmol scale and was isolated after column chromatography (10→30% EtOAc:pent) (117 mg, 0.33 mmol, 93%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.28 (d, *J* = 8.4 Hz, 1H), 7.86 (d, *J* = 8 Hz, 1H), 7.78 (d, *J* = 8.4 Hz, 1H), 7.60-7.56 (m, 1H), 7.52-7.48(m, 1H), 7.41 (t, *J* = 7.2 Hz, 1H), 7.27 (d, *J* = 7.6 Hz, 1H), 4.98 (d, *J*=7.6 Hz, 1H), 4.74-4.69 (m,1H), 3.66 (dd, *J* = 4.8 Hz, 1H), 3.34 (d, *J* = 4.8 Hz, 1H), 3.03-2.97 (m, 1H), 2.91 (d, *J* = 4.8 Hz, 1H), 1.48 (s, 3H), 1.30 (s, 9H).$^{13}$C NMR (101 MHz, $CDCl_3$): δ ppm 208.95, 208.90, 155.24, 133.99, 132.27, 128.91, 128.83, 128.53, 128.11, 127.80, 127.69, 126.56, 125.93, 125.58, 125.31, 125.15, 123.87, 123.74, 123.62, 79.91, 59.44, 53.91, 52.98, 52.62, 52.37, 51.59, 37.19, 35.17, 29.81, 28.44, 28.32, 27.66, 16.61. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 9.71 (ESI-MS (m/z): 355.53 ($M^+$)). HRMS: calculated for $C_{21}H_{25}NO_4$ 356.18563 $[M+H]^+$; found 356.18556.

***tert*-butyl(R)-(3-([1,1'-biphenyl]-4-yl)-1-(methoxy(methyl)amino)-1-oxopropan-2-yl)carbamate (54)**
This compound was synthesized according to the general procedure **A** described above on a 2 mmol scale and was isolated after column chromatography (10→50% EtOAc:pent) in a quantitative yield. $^{1}$H NMR (400 MHz, $CDCl_3$) δ ppm 7.56 (t, *J* = 7.2 Hz, 2H), 7.54 (d, *J* = 8.4 Hz, 2H), 7.49 (t, *J* = 7.6 Hz, 2H), 7.31-7.23 (m, 3), 5.36 (d, *J* = 8.8 Hz, 1H), 5.00-4.98 (m, 1H), 3.65 (s, 3H), 3.16 (s, 3H), 3.13-3.08 (d, *J* = 5.6 Hz, 2H), 2.93-2.88 (m, 1H), 1.39 (s, 9H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.14, 155.15, 140.76, 139.45, 135.67, 129.82, 128.65, 127.07, 126.91, 126.87, 79.42, 61.45, 51.43, 38.23, 31.96, 28.23. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 9.37 (ESI-MS (m/z): 385.73 ($M+H^+$)). HRMS: calculated for $C_{22}H_{28}N_2O_4$ 358.21218 $[M+H]^+$; found 385.21255. $[\alpha]_D^{20}$= +10 (C=1, $CHCl_3$)

***tert*-butyl (R)-(1-([1,1'-biphenyl]-4-yl)-4-methyl-3-oxopent-4-en-2-yl)carbamate (55)**
This compound was synthesized according to the general procedure **B** described above on a 2.0 mmol scale and was isolated after column chromatography (10→30% EtOAc:pent) (679 mg, 1.86 mmol, 93%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.56-7.54 (m, 2H), 7.49 (d, *J* = 8.0 Hz, 2H), 7.42 (t, *J* = 7.2 Hz, 2H), 7.32 (t, *J* = 7.2 Hz, 1H), 7.14 (d, *J*=8.0 Hz, 2H), 6.05 (s, 1H), 5.86 (s, 1H), 5.38-5.30 (m, 2H), 3.18-3.13 (m, 1H), 2.97-2.92 (m, 1H), 1.87 (s, 3H), 1.41 (s, 9H). $^{13}$C NMR (101 MHz, $CDCl_3$): δ ppm 200.02, 155.11, 142.41, 140.78, 139.70, 135.25, 130.10, 129.93, 129.90, 128.76, 127.22, 127.07, 126.99, 126.68, 79.70, 54.96, 39.39, 29.74, 28.36, 17.80.

***tert*-butyl((2R,3S)-1-([1,1'-biphenyl]-4-yl)-3-hydroxy-4-methylpent-4-en-2-yl)carbamate (56)**
This compound was synthesized according to the general procedure **C** described above on a 1.86 mmol scale and and was isolated after column chromatography (1→10% EtOAc:pent) (444 mg, 1.21 mmol, 65%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.57-7.55 (m, 2H), 7.51 (d, *J* = 8.0 Hz, 2H), 7.44-7.40 (m, 2H), 7.34-7.32 (m, 1H), 7.27-7.24 (m, 2H), 5.10 (s, 1H), 5.00 (s, 1H), 4.78 (d, *J* = 8.8 Hz, 1H), 4.21 (s, 1H), 4.04 (s, 1H), 2.95 (dd, *J*= 4.0, 3.6 Hz, 1H), 2.77-2.71 (m, 1H), 2.66 (s, 1H), 1.83 (s, 3H), 1.34 (s, 9H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 144.89, 141.15, 139.24, 137.71, 129.96, 128.84, 127.17, 127.15, 127.09, 112.41, 79.59, 77.26, 53.86, 34.13, 28.39, 19.21.

***tert*-butyl((R)-3-([1,1'-biphenyl]-4-yl)-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)carbamate (57)**
This compound was synthesized according to the general procedure **D** described above on a 1.21 mmol scale and was isolated after column chromatography (10→30% EtOAc:pent) (194 mg, 0.51 mmol, 42%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.59-7.52 (m, 5H), 7.45-7.41 (m, 2H), 7.35-7.31 (m, 1H), 7.24 (d, *J*=8.0 Hz, 1H), 5.00 (d, *J* = 8 Hz, 1H), 4.64-4.59 (m, 1H), 3.31 (d, *J* = 4.8 Hz, 1H), 3.18 (d, *J* = 4.4 Hz, 1H), 2.93 (d, *J*=6.2 Hz, 1H), 2.79-2.74 (m, 1H), 1.53 (s, 3H), 1.37 (s, 9H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 208.41, 155.36, 140.85, 139.98, 135.06, 129.95, 128.87, 127.36, 127.34, 127.13, 80.02, 59.32, 53.78, 52.06, 37.14, 28.38, 16.77. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 10.22 (ESI-MS (m/z): 381.40 ($M^+$)). HRMS: calculated for $C_{23}H_{27}NO_4$ 382.20125 $[M+H]^+$; found 382.20128. $[\alpha]_D^{20}$= +115.6 (C=1, $CHCl_3$)




**Scheme S3. Synthesis of Boc-Cha-VS (58).** Reagents and conditions: (a) $LiAlH_4$, $Et_2O$; (b) Diethyl((methylsulfonyl)methyl) phosphonate, NaH, THF, 0°C, 72% (over two steps).

**(S,E)-*tert*-butyl (1-cyclohexyl-4-(methylsulfonyl)but-3-en-2-yl)carbamate (58)**
Weinreb amide **38** (0.80 g, 2.60 mmol) was dissolved in $Et_2O$ (25 mL), put under an argon atmosphere and cooled to 0 °C. $LiAlH_4$ (1.5 equiv, 3,9 mmol, 3.9 mL of a 1 M solution in THF) was added slowly and the mixture was stirred at 0°C for 0.5 h after which TLC analysis indicated complete conversion of the starting compound. 1 M aq. HCl was slowly added and the layers were separated. The organic layer was extracted with 1 M HCl and brine, dried over $Na_2SO_4$ and concentrated. Diethyl((methylsulfonyl)methyl) phosphonate (1.5 equiv, 3.90 mmol, 0.90 g) was dissolved in THF (25 mL) and cooled to 0 °C under an argon atmosphere. NaH (1.5 equiv, 3.90 mmol, 156 mg, 60% w/w in mineral oil) was slowly added and the mixture was stirred at 0°C for 30 min. Next, the freshly obtained aldehyde (in THF (5 mL)) was slowly added and the mixture was stirred for 1.5 h while slowly warming it to RT. After this time TLC analysis indicated complete conversion of the aldehyde. EtOAc was added and the mixture was extracted with 1 M aq. HCl (2×) and brine, dried over $Na_2SO_4$ and concentrated. Column chromatography (10→30% EA:pent) yielded the title compound (617 mg, 1.86 mmol, 72%, contains 16% *Z*-isomer, based on NMR). $^{1}$H NMR (Peaks reported for *E*-isomer) (400 MHz, $CDCl_3$) δ 6.78 (dd, *J* = 15.1, 5.0 Hz, 1H),

6.45 (dd, *J* = 15.1, 1.5 Hz, 1H), 4.68 (d, *J* = 7.7 Hz, 1H), 4.40 (s, 1H), 2.89 (s, 3H), 1.75 (d, *J* = 12.8 Hz, 1H), 1.71 – 1.50 (m, 4H), 1.39 (m, 11H), 1.15 (m, 4H), 1.02 – 0.71 (m, 2H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 155.13, 149.30, 148.83, 128.93, 80.01, 48.80, 43.79, 42.92, 41.97, 34.07, 33.96, 33.87, 33.52, 32.61, 28.43, 28.38, 28.35, 26.40, 26.36, 26.27, 26.17, 26.02. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 7.94 (ESI-MS (m/z): 331.80 ($M+H^+$)). HRMS: calculated for $C_{16}H_{29}NO_4S$ 332.18901 $[M+H]^+$; found 332.18912. $[\alpha]_D^{20}$= -9.2 (C=1, $CHCl_3$)

***Synthesis of peptide hydrazides***

**MorphAc-Ala-Tyr(OMe)-OMe (60b)**

Boc-Ala-Tyr(OMe)-OMe was prepared by the general procedure for peptide coupling on a 1 mmol scale. Column chromatography provided (10→40% EtOAc:pent) the product (365 mg, 0.96 mmol, 96%). Boc-Ala-Tyr(OMe)-OMe (365 mg, 0.54 mmol, 1 equiv.) was deprotected using the standard procedure for Boc removal**.** MorphAc-Ala-Tyr(OMe)-OMe (60b) was prepared by the general procedure for peptide coupling on a 0.96 mmol scale. Column chromatography provided (1→3% MeOH:DCM) the product in a quantitative yield. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.53 (d, *J* = 7.9 Hz, 1H), 7.00 (d, *J* = 8.6 Hz, 2H), 6.77 (d, *J* = 8.6 Hz, 2H), 6.71 (d, *J* = 7.7 Hz, 1H), 4.74 (q, *J* = 6.8 Hz, 1H), 4.49 (p, *J* = 7.1 Hz, 1H), 3.74 (s, 3H), 3.70 (s, 3H), 3.69 – 3.64 (m, 4H), 3.12 – 2.87 (m, 4H), 2.52 – 2.40 (m, 4H), 1.34 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 171.89, 171.85, 169.91, 158.68, 130.29, 127.73, 114.01, 66.96, 61.77, 55.23, 53.80, 53.53, 52.46, 48.16, 36.89, 18.27.

**MorphAc-Ala-Tyr(OMe)-$NHNH_2$ (61)**

Methyl ester **60b** (390 mg, 0.96 mmol, 1 equiv.) was dissolved in MeOH (10 mL). Hydrazine hydrate (1500 µl, 29 mmol, 30 equiv.) was added and the mixture was stirred for 3 h before being co-evaporated with tol (3x). The residue was used without further purification. $^1$H NMR (400 MHz, MeOD) δ 7.07 (d, *J* = 8.5 Hz, 2H), 6.77 (d, *J* = 8.6 Hz, 2H), 4.46 (t, *J* = 7.3 Hz, 1H), 4.39 (d, *J* = 7.0 Hz, 1H), 3.73 (s, 3H), 3.71 – 3.65 (m, 4H), 3.06 – 2.77 (m, 4H), 2.50 – 2.42 (m, 4H), 1.29 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, MeOD) δ 173.82, 172.46, 171.73, 159.73, 131.41, 129.71, 115.00, 68.02, 62.55, 56.16, 54.85, 54.77, 38.43, 19.26.

**Fmoc-D-Ala-Trp(Boc)-OMe (62)**

The title compound was prepared by the general procedure for peptide coupling on a 1.53 mmol scale. Column chromatography (20→80% EtOAc:pent) provided the product in a quantitative yield.
$^1$H NMR (400 MHz, $CDCl_3$) δ 8.10 (s, 1H), 7.74 (d, *J* = 7.5 Hz, 2H), 7.56 (d, *J* = 6.9 Hz, 2H), 7.48 (d, *J* = 7.6 Hz, 1H), 7.44 – 7.34 (m, 3H), 7.31 – 7.25 (m, 3H), 7.22 (t, *J* = 7.4 Hz, 1H), 7.00 (d, *J* = 7.2 Hz, 1H), 5.68 (d, *J* = 7.4 Hz, 1H), 4.99 – 4.89 (m, 1H), 4.41 – 4.28 (m, 3H), 4.21 – 4.14 (m, 1H), 3.65 (s, 3H), 3.32 – 3.09 (m, 2H), 1.65 (s, 9H), 1.36 (d, *J* = 6.7 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.16, 171.86, 155.90, 149.50, 143.87, 143.74, 141.27, 141.25, 135.27, 130.33, 127.70, 127.05, 125.11, 124.60, 124.23, 122.61, 119.96, 118.72, 115.32, 114.79, 83.71, 67.08, 60.44, 52.51, 52.46, 50.38, 47.04, 28.18, 27.39, 19.02, 14.23.

**H-D-Ala-Trp(Boc)-OMe (63)**

To a solution of Fmoc-D-Ala-Trp(Boc)-OMe **62** (1.19 g, 1.53 mmol, 1 equiv.) in THF (15 mL) were added DBU (67 µL, 0.46 mmol, 0.3 equiv.) and ethanethiol (11 mL, 15.3 mmol, 10 equiv.). After 45 min. the reaction mixture was concentrated and co-evaporated with toluene. Column chromatography (50→100% EtOAc:pent →10% MeOH in EtOAc) yielded the title compound (575 mg, 1.38 mmol, 90%) $^1$H NMR (400 MHz, $CDCl_3$) δ 8.10 (s, 1H), 7.53 (d, *J* = 9.1 Hz, 1H), 7.43 (s, 1H), 7.32 (t, *J* = 7.3 Hz, 1H), 7.25 (t, *J* = 6.6 Hz, 1H), 4.84 (s, 1H), 3.73 (s, 3H), 3.49 – 3.13 (m, 3H), 1.68 (s, 9H), 1.24 (d, *J* = 5.2 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 176.17, 171.94, 130.07, 124.33, 123.68, 122.32, 118.41, 115.09, 114.93, 83.68, 55.14, 52.08, 52.05, 49.80, 49.55, 27.65, 26.78, 20.26. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 6.58 (ESI-MS (m/z): 389.93 ($M+H^+$)).

**3MeIndAc-D-Ala-Trp-OMe (64)**

The title compound was prepared by the general procedure for peptide coupling on a 0.4 mmol scale. Column chromatography (20→60% EtOAc:pent) provided the product (198 mg, 0.36 mmol, 90%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.16 – 8.03 (m, 1H), 7.51 (d, *J* = 7.7 Hz, 1H), 7.46 – 7.37 (m, 3H), 7.37 – 7.23 (m, 4H), 7.20 (t, *J* = 7.4 Hz, 1H), 6.60 (d, *J* = 7.3 Hz, 1H), 4.93 (q, *J* = 6.5 Hz, 1H), 4.75 (p, *J* = 6.9 Hz, 1H), 3.66 (s, 3H), 3.54 (s, 2H), 3.36 – 3.16 (m, 2H), 2.47 (s, 3H), 1.64 (s, 9H), 1.42 (d, *J* = 6.9 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.53, 171.86, 165.75, 147.51, 145.53, 142.22, 131.84, 130.34, 127.25, 126.74, 124.62, 124.27, 123.85, 122.64, 120.79, 118.75, 115.34, 114.97, 83.72, 52.63, 52.57, 48.63, 38.32, 28.23, 27.33, 18.97, 12.31. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 10.51 (ESI-MS (m/z): 545.93 ($M+H^+$)).

**3MeIndAc-D-Ala-Trp(Boc)-NHNH$_2$ (65)**

Methyl ester **64** (165 mg, 0.30 mmol, 1 equiv.) was dissolved in MeOH (4 mL). Hydrazine hydrate (523 µl, 10.5 mmol, 35 equiv.) was added and the mixture was stirred for 3 h before being co-evaporated with tol (3x). The residue was used without further purification (isolated as mixture of + and - Boc. $^{1}$H NMR (400 MHz, MeOD) δ 7.61 – 7.49 (m, 1H), 7.49 – 7.38 (m, 3H), 7.28 (dq, *J* = 26.3, 11.2, 9.3 Hz, 3H), 7.07 – 6.92 (m, 2H), 4.73 – 4.59 (m, 1H), 4.59 – 4.48 (m, 1H), 4.45 – 4.33 (m, 1H), 3.65 – 3.41 (m, 1H), 3.27 – 3.14 (m, 1H), 2.45 (d, *J* = 7.5 Hz, 2H), 1.42 (s, 6H (partially –Boc)), 1.30 – 1.15 (m, 3H). LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 6.16 (ESI-MS (m/z): 445.93 ($M+H^+$-Boc) and 7.76 (ESI-MS (m/z): 546.00 ($M+H^+$+Boc).

**Boc-D-Ala-Tyr(OMe)-OMe (66)**

The title compound was prepared by the general procedure for peptide coupling on a 1 mmol scale. Column chromatography (20→60% EtOAc:pent) provided the product (349 mg, 0.92 mmol, 92%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 6.99 (d, *J* = 8.6 Hz, 2H), 6.87 – 6.71 (m, 3H), 5.17 (d, *J* = 7.5 Hz, 1H), 4.85 – 4.70 (m, 1H), 4.29 – 4.11 (m, 1H), 3.73 (s, 3H), 3.67 (s, 3H), 3.01 (qd, *J* = 14.0, 5.9 Hz, 2H), 1.40 (s, 9H), 1.26 (d, *J* = 7.1 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.42, 171.97, 158.66, 155.45, 130.29, 127.74, 113.98, 79.98, 55.18, 53.27, 52.33, 49.94, 37.04, 28.34, 18.56.

**MorphAc-D-Ala-Tyr(OMe)-OMe (67)**

Boc-D-Ala-Tyr(OMe)-OMe (365 mg, 0.54 mmol, 1 equiv.) was deprotected using the standard procedure for Boc removal. The title compound was prepared by the general procedure for peptide coupling on a 0.54 mmol scale. Column chromatography (1→3% MeOH:DCM) provided the product (160 mg, 0.39 mmol, 79%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, *J* = 8.0 Hz, 1H), 7.01 (dd, *J* = 14.1, 8.4 Hz, 3H), 6.76 (d, *J* = 8.6 Hz, 2H), 4.73 (q, *J* = 7.3 Hz, 1H), 4.53 (p, *J* = 7.0 Hz, 1H), 3.71 (s, 3H), 3.65 (d, *J* = 4.8 Hz, 7H), 3.09 – 2.85 (m, 4H), 2.51 – 2.36 (m, *J* = 4.3 Hz, 4H), 1.24 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 171.82, 171.80, 169.78, 158.58, 130.25, 127.79, 113.85, 66.86, 61.74, 55.15, 53.69, 53.25, 52.28, 48.00, 36.92, 18.65.

**MorphAc-D-Ala-Tyr(OMe)-NHNH$_2$ (68)**

Methyl ester **67** (160 mg, 0.39 mmol, 1 equiv.) was dissolved in MeOH (4 mL). Hydrazine hydrate (567 µl, 11.7 mmol, 30 equiv.) was added and the mixture was stirred for 3 h before being co-evaporated with tol (3x). The residue was used without further purification. $^{1}$H NMR (400 MHz, MeOD) δ 7.10 (d, *J* = 8.6 Hz, 2H), 6.80 (d, *J* = 8.7 Hz, 2H), 4.54 (dd, *J* = 9.4, 5.5 Hz, 1H), 4.30 (q, *J* = 7.0 Hz, 1H), 3.74 (s, 3H), 3.70 (t, *J* = 4.6 Hz, 4H), 3.12 (dd, *J* = 14.0, 5.5 Hz, 1H), 2.98 (d, *J* = 3.6 Hz, 2H), 2.79 (dd, *J* = 14.0, 9.4 Hz, 1H), 2.56 – 2.39 (m, *J* = 4.3 Hz, 4H), 1.13 (d, *J* = 7.1 Hz, 3H). $^{13}$C NMR (101 MHz, MeOD) δ 173.59, 171.81, 171.34, 159.17, 130.68, 129.29, 114.37, 67.38, 61.89, 55.51, 54.14, 53.98, 37.50, 18.50. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 3.14 (ESI-MS (m/z): 408.13 ($M+H^+$)).

**3MeIndAc-D-Ala-Tyr(OMe)-OMe (69)**

Boc-D-Ala-Tyr(OMe)-OMe (365 mg, 0.50 mmol, 1 equiv.) was deprotected using the standard procedure for Boc removal. The title compound was prepared by the general procedure for peptide coupling on a 0.5 mmol scale. Column chromatography (10→80% EtOAc:pent) provided the product (159 mg, 0.36 mmol, 73%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.39 (d, *J* = 7.2 Hz, 2H), 7.31 (q, *J* = 7.1 Hz, 2H), 7.17 (d, *J* = 8.0 Hz, 1H), 7.06 (d, *J* = 8.5 Hz, 2H), 6.76 (d, *J* = 8.5 Hz, 2H), 6.66 (d, *J* = 7.0 Hz, 1H), 4.81 (q, *J* = 7.6 Hz, 1H), 4.67 (p, *J* = 6.9 Hz, 1H), 3.68 (s, 3H), 3.67 (s, 3H), 3.51 (s, 2H), 3.13 (dd, *J* = 14.0, 5.3 Hz, 1H), 3.00 (dd, *J* = 14.0, 7.5 Hz, 1H), 2.42 (s, 3H), 1.35 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.75, 172.32, 165.93, 158.66, 147.94, 145.41, 142.22, 131.49, 130.27, 127.85, 127.30, 126.72, 123.81, 120.77, 113.93, 55.11, 53.49, 52.48, 48.85, 38.17, 36.79, 18.57, 12.23.

**3MeIndAc-D-Ala-Tyr(OMe)-NHNH$_2$ (70)**

Methyl ester **69** (159 mg, 0.36 mmol, 1 equiv.) was dissolved in MeOH (4 mL). Hydrazine hydrate (523 µl, 10.8 mmol, 30 equiv.) was added and the mixture was stirred for 3 h before being co-evaporated with tol (3x). The residue was used without further purification. $^{1}$H NMR (400 MHz, MeOD) δ 7.47 – 7.25 (m, 4H), 7.08 (d, *J* = 8.6 Hz, 2H), 6.72 (d, *J* = 8.6 Hz, 2H), 4.61 – 4.52 (m, 1H), 4.41 (q, *J* = 7.1 Hz, 1H), 3.60 (d, *J* = 14.3 Hz, 5H), 3.11 (dd, *J* = 14.0, 5.4 Hz, 1H), 2.86 (dd, *J* = 14.0, 8.9 Hz, 1H), 2.46 (s, 3H), 1.25 (d, *J* = 7.1 Hz, 3H). $^{13}$C NMR (101 MHz, MeOD) δ 174.39, 171.92, 167.55, 159.10, 149.04, 145.83, 142.99, 131.82, 130.62, 129.01, 127.89, 127.22, 124.29, 121.26, 114.31, 55.36, 53.85, 49.98, 38.48, 37.28, 17.86, 12.45. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 5.88 (ESI-MS (m/z): 437.00 ($M+H^+$)).

**MorphAc-D-Ala-Trp(Boc)-OMe (71)**

The title compound was prepared by the general procedure for peptide coupling on a 0.43 mmol scale. Column chromatography (1→3% MeOH:DCM) provided the product (349 mg, 0.92 mmol, 92%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 8.08 (d, *J* = 6.9 Hz, 1H), 7.60 (d, *J* = 8.0 Hz, 1H), 7.45 (d, *J* = 7.7 Hz, 1H), 7.38 (s, 1H), 7.27 (t, *J* = 7.0 Hz, 1H), 7.20 (t, *J* = 7.5 Hz, 1H), 7.05 (d, *J* = 7.8 Hz, 1H), 4.85 (q, *J* = 6.0 Hz, 1H), 4.54 (p, *J* = 7.0 Hz, 1H), 3.72 – 3.62 (m, 7H), 3.20 (qd, *J* = 14.8, 5.9 Hz, 2H), 2.98 – 2.85 (m, 2H), 2.49 – 2.37 (m, *J* = 4.5 Hz, 4H), 1.63 (s, 9H), 1.29 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 171.92, 171.80, 169.86, 149.51, 130.30, 124.61, 124.26, 122.62, 118.76, 115.31, 114.84, 83.73, 66.93, 61.74, 53.73, 53.37, 52.51, 52.34, 48.11, 28.22, 27.32, 18.52. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 6.68 (ESI-MS (m/z): 517.07 ($M+H^+$)).

**MorphAc-D-Ala-Trp(Boc)-NHNH$_2$ (72)**

Methyl ester **71** (234 mg, 0.43 mmol, 1 equiv.) was dissolved in MeOH (6 mL). Hydrazine hydrate (640 µl, 12.9 mmol, 30 equiv.) was added and the mixture was stirred for 3 h before being co-evaporated with tol (3x). The residue was used without further purification (isolated as mixture of + and - Boc. $^{1}$H NMR (400 MHz, MeOD) δ 7.55 (d, *J* = 8.7 Hz, 1H), 7.32 (d, *J* = 8.1 Hz, 1H), 7.09 (t, *J* = 7.1 Hz, 1H), 7.07 – 6.97 (m, 2H), 4.67 – 4.55 (m, 2H), 4.31 (q, *J* = 7.0 Hz, 1H), 3.67 (hept, *J* = 6.9, 5.9 Hz, 4H), 3.26 (dd, *J* = 14.7, 6.1 Hz, 1H), 3.13 (dd, *J* = 14.7, 7.6 Hz, 1H), 2.91 (d, *J* = 4.6 Hz, 2H), 2.51 – 2.34 (m, 4H), 1.42 (s, 5H (Boc, partially removed)), 1.15 (d, *J* = 7.1 Hz, 3H). $^{13}$C NMR (101 MHz, MeOD) δ 173.36, 172.21, 171.28, 137.02, 127.87, 123.96, 122.07, 119.47, 118.62, 111.90, 109.76, 67.27, 61.81, 54.16, 54.03, 53.31, 49.14, 28.53, 28.29, 18.26. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 3.56 (ESI-MS (m/z): 417.07 ($M+H^+$)).

**3MeIndAc-Ala-Tyr(OMe)-OMe (73)**

The title compound was prepared by the general procedure for peptide coupling on a 0.21 mmol scale. Column chromatography (10→50% EtOAc:pent) provided the product (66 mg, 0.15 mmol, 75%). $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.45 (t, *J* = 7.9 Hz, 2H), 7.34 (p, *J* = 7.0 Hz, 2H), 7.01 (d, *J* = 8.5 Hz, 2H), 6.87 (d, *J* = 7.9 Hz, 1H), 6.68 (d, *J* = 8.5 Hz, 2H), 6.36 (d, *J* = 7.4 Hz, 1H), 4.81 (q, *J* = 7.1 Hz, 1H), 4.69 (p, *J* = 7.0 Hz, 1H), 3.73 (s, 3H), 3.57 (s, 3H), 3.54 (s, 2H), 3.11 (dd, *J* = 14.1, 5.4 Hz, 1H), 2.99 (dd, *J* = 14.1, 7.0 Hz, 1H), 2.52 (s, 3H), 1.43 (d, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.28, 171.92, 165.81, 158.69, 148.13, 145.59, 142.17, 131.53, 130.25, 127.74, 127.42,

126.88, 123.91, 120.89, 113.96, 55.05, 53.57, 52.47, 48.49, 38.27, 37.02, 18.27, 12.39. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 8.42 (ESI-MS (m/z): 437.00 ($M+H^+$)).

**3MeIndAc-Ala-Tyr(OMe)-$NHNH_2$ (74)**

Methyl ester **73** (66 mg, 0.15 mmol, 1 equiv.) was dissolved in MeOH (1.5 mL) and $CHCl_3$ (0.5 mL). Hydrazine hydrate (218 µl, 4.5 mmol, 30 equiv.) was added and the mixture was stirred for 3 h before being co-evaporated with tol (3x). The residue was used without further purification. No NMR-analysis was performed due to poor solubility of the product in MeOD and $CDCl_3$ and mixtures thereof. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 6.14 (ESI-MS (m/z): 437.00 ($M+H^+$)).

**Fmoc-Hyp(tBu)-Nle-OMe (75)**

The title compound was prepared by the general procedure for peptide coupling on a 3 mmol scale. Column chromatography (30→60% EtOAc:pent) provided the product (1.75 g, 3.00 mmol, 100%). Complex NMR due to a presence of rotamers. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.72 (d, *J* = 7.4 Hz, 2H), 7.63 – 7.48 (m, 2H), 7.36 (t, *J* = 7.3 Hz, 2H), 7.32 – 7.22 (m, 2H), 7.17 (d, *J* = 7.5 Hz, 0.7H), 6.46 (d, *J* = 7.7 Hz, 0.3H), 4.62 – 4.08 (m, 6H), 3.69 (s, 3H), 3.54 (s, 1H), 3.44 – 3.20 (m, 1H), 2.48 – 2.34 (m, 0.8H), 2.19 (s, 0.8H), 2.06 – 1.88 (m, 1H), 1.86 – 1.72 (m, 0.8H), 1.65 (dd, *J* = 14.3, 7.2 Hz, 0.6H), 1.33 – 1.07 (m, 13H), 0.79 (q, *J* = 9.2, 7.8 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.64, 171.83, 171.18, 155.81, 154.77, 144.00, 143.76, 143.67, 141.16, 127.61, 126.92, 73.94, 69.56, 68.43, 67.79, 60.25, 59.45, 58.88, 54.03, 53.04, 52.36, 52.11, 51.76, 47.00, 39.02, 36.09, 32.02, 31.70, 28.20, 27.30, 22.11, 13.75.

**H-Hyp(tBu)-Nle-OMe (76)**

Fmoc-Hyp(tBu)-Nle-OMe **75** (1745 mg, 3.00 mmol, 1 equiv.) was dissolved in THF (30 mL). After addition of ethanethiol (2.22 mL, 30 mmol, 10 equiv.) and DBU (45 µL, 0.3 mmol, 0.1 equiv.), the reaction mixture was stirred for 1 h. The reaction mixture was then concentrated and co-evaporated with toluene (3x). Purification by column chromatography (10→100% EtOAc: Pent) yielded the title compound (540 mg, 1.72 mmol, 57%, not completely pure). The product was used without further purification.

**Fmoc-Ala-Hyp(tBu)-Nle-OMe (77)**

The title compound was prepared by the general procedure for peptide coupling on a 3 mmol scale. Column chromatography (30→60% EtOAc:pentyielded the product (211 mg, 0.348 mmol, 48%, not completely pure) which was used without further purification.

**H-Ala-Hyp(tBu)-Nle-OMe (78)**

Fmoc-Ala-Hyp(tBu)-Nle-OMe **77** (639 mg, 1.05 mmol, 1 equiv.) was dissolved in THF (11 mL). After addition of ethanethiol (0.78 mL, 10.5 mmol, 10 equiv.) and DBU (32 µL, 0.21 mmol, 0.2 equiv.) the reaction mixture was stirred for 1h. the reaction mixture was concentrated and co-evaporated with toluene (3x). Purification by column chromatography (4:6:0→9:0:1 EtOAc: Pent: MeOH) yielded the title compound (247 mg, 0.64 mmol, 61%), which was directly used in the next step.

**$N_3$Gly-Ala-Hyp(tBu)-Nle-OMe (79)**

Ala-Hyp(tBu)-Nle-OMe (247 mg, 0.64 mmol, 1 equiv.) was dissolved in DMF (7 mL) before adding $(ClAc)_2O$ (131 mg, 0.77 mmol, 1.2 equiv.) and DiPEA (0.45 mL, 2.56 mmol, 4 equiv.). The reaction mixture was stirred for 1h before $NaN_3$ (166 mg, 2.56 mmol, 4 equiv.) was added. The reaction mixture was then stirred overnight followed by addition of EtOAc (50 mL). The mixture was washed with 1M HCl (2x), sat. aq. $NaHCO_3$ (2x) and brine (1x). The organic layer was dried over $Na_2SO_4$, filtered and concentrated. Purification by column chromatography (0→3% MeOH: DCM) yielded the title compound (114 mg, 0.29, 46%). $^1$H NMR (400 MHz, $CDCl_3$) δ 7.29 (d, *J* = 3.7 Hz, 1H), 7.14 (d, *J* = 7.8 Hz, 1H), 4.65 – 4.51 (m, 2H), 4.51 – 4.22 (m, 2H), 3.91 (s, 2H), 3.73 – 3.51 (m, 4H), 3.49 –

3.22 (m, 1H), 2.48 – 2.18 (m, 1H), 1.87 (m, 1H), 1.80 – 1.65 (m, 1H), 1.65 – 1.47 (m, 1H), 1.36 – 0.94 (m, 16H), 0.81 (t, *J* = 6.9 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.70, 171.68, 170.48, 166.06, 74.14, 69.82, 58.56, 53.53, 52.28, 52.25, 52.20, 46.51, 35.49, 31.74, 28.13, 27.28, 22.11, 18.01, 13.77. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 6.62 (ESI-MS (m/z): 469.07 ($M+H^+$)).

**$N_3$Gly-Ala-Hyp(tBu)-Nle-$NHNH_2$ (80)**

Azido-Ac-Ala-Hyp(tBu)-Nle-OMe **79** was dissolved in MeOH (3 mL). After addition of hydrazine (0.42 mL, 8.7 mmol, 30 equiv.) the reaction mixture was stirred for approximately 2 hours at room temperature. The reaction mixture was concentrated before being co-evaporated with MeOH (2x). This yielded the title compound in a quantitative yield. $^1$H NMR (400 MHz, MeOD) δ 4.66 (q, *J* = 6.9 Hz, 1H), 4.56 (t, *J* = 7.1 Hz, 1H), 4.52 – 4.41 (m, 1H), 4.24 (dd, *J* = 8.4, 5.9 Hz, 1H), 3.90 (d, *J* = 9.7 Hz, 2H), 3.82 (dd, *J* = 10.3, 5.6 Hz, 1H), 3.70 – 3.19 (m, 2H), 2.21 – 2.00 (m, 2H), 1.86 – 1.57 (m, 2H), 1.51 – 1.06 (m, 16H), 0.93 (t, *J* = 6.6 Hz, 3H). $^{13}$C NMR (101 MHz, MeOD) δ 173.99, 173.45, 173.22, 169.59, 75.42, 71.34, 71.14, 69.27, 60.39, 55.46, 54.95, 53.59, 52.51, 49.66, 49.45, 49.23, 49.02, 48.81, 48.60, 48.42, 48.38, 48.12, 38.29, 32.82, 28.99, 28.55, 23.37, 17.09, 14.30. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 4.77 (ESI-MS (m/z): 469.13 ($M+H^+$)).

**Boc-Thz-Nle-OMe (81)**

The title compound was prepared by the general procedure for peptide coupling on a 3 mmol scale. Column chromatography (10→50% EtOAc:pent) provided the product (989 mg, 2.74 mmol, 91%). $^1$H NMR (400 MHz, $CDCl_3$) δ 7.11 (bs, 1H), 6.62 (bs, 1H), 4.92 – 4.46 (m, 3H), 4.46 – 4.15 (m, 1H), 3.71 (s, 3H), 3.54 – 2.95 (m, 2H), 1.96 – 1.73 (m, 1H), 1.73 – 1.55 (m, 1H), 1.47 (s, 9H), 1.36 – 1.09 (m, 4H), 0.99 – 0.69 (m, 3H). $^{13}$C NMR (101 MHz, CDCl3) δ 172.63, 169.94, 80.04, 52.43, 52.41, 52.23, 32.28, 28.30, 28.25, 27.31, 27.27, 22.34, 22.31, 13.97, 13.95.

**Boc-Ala-Thz-Nle-OMe (82)**

Boc-Thz-Nle-OMe **81** (989 mg, 2.74 mmol) was deprotected using the standard procedure for Boc removal. The title compound was prepared by the general procedure for peptide coupling on a 2.74 mmol scale. Column chromatography (40→60% EtOAc:pent) provided the product (747 mg, 1.73 mmol, 63%). $^1$H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, *J* = 6.3 Hz, 0.3H), 7.50 (d, *J* = 7.9 Hz, 0.2H), 6.97 (d, *J* = 5.9 Hz, 0.5H), 5.36 (d, *J* = 6.6 Hz, 0.6H), 5.16 (m, 0.4H), 5.01 (m, 0.5H), 4.81 (d, *J* = 9.3 Hz, 0.6H), 4.74 – 4.62 (m, 0.4H), 4.61 – 4.35 (m, 2.5H), 4.35 – 4.20 (m, 0.6H), 4.16 (dd, *J* = 7.4, 4.2 Hz, 0.3H), 3.94 (d, *J* = 9.9 Hz, 0.2H), 3.78 – 3.57 (m, 3H), 3.52 – 3.29 (m, 1H), 3.21 – 2.98 (m, 1H), 2.02 – 1.70 (m, 1H), 1.70 – 1.50 (m, 1H), 1.50 – 1.04 (m, 16H), 0.84 (d, *J* = 5.5 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.70, 170.67, 168.89, 80.04, 66.01, 62.89, 62.21, 53.65, 53.24, 52.50, 52.44, 52.40, 52.22, 52.06, 49.38, 49.19, 48.05, 35.37, 34.55, 32.16, 31.95, 31.81, 30.28, 28.39, 28.17, 27.48, 27.21, 22.27, 18.82, 16.54, 13.93.

**$N_3$Gly-Ala-Thz-Nle-OMe (3) (83)**

Boc-Ala-Thz-Nle-OMe **82** (349 mg, 0.81 mmol) was deprotected using the standard procedure for Boc removal. The crude TFA salt was dissolved in DMF (8 mL) before adding $(ClAc)_2O$ (164 mg, 0.97 mmol, 1.2 equiv.) and DiPEA (0.56 mL, 3.24 mmol, 4 equiv.). The reaction mixture was stirred for 1 h before $NaN_3$ (210 mg, 3.24 mmol, 4 equiv.) was added. After stirring overnight. EtOAc (50mL) was added to the reaction mixture before being washed with 1M HCl (2x), sat. aq. $NaHCO_3$ (2x) and brine (1x). The organic layer was dried over $Na_2SO_4$, filtered and concentrated. Purification by column chromatography (80→100% EtOAc:Tol) yielded the title compound (213 mg, 0.51 mmol, 64%). Complex NMR due to a presence of rotamers. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, *J* = 7.4 Hz, 0.2H), 7.30 (d, *J* = 9.1 Hz, 0.8H), 7.14 (d, 0.3H), 7.00 (d, *J* = 7.6 Hz, 0.7H), 4.97 – 4.30 (m, 5H), 3.91+3.84 (2xs, 2H), 3.68+3.62 (2xs, 3H), 3.37 (dd, *J* = 11.7, 4.1 Hz, 1H), 3.18 (dd, *J* = 11.6, 7.3 Hz, 1H), 1.92 – 1.71 (m, 1H), 1.71 – 1.51 (m, 1H), 1.48 – 1.04 (m, 7H), 0.84 (t, *J* = 6.8 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.73, 171.27, 168.83, 166.44, 62.83, 62.29, 53.14, 52.43, 52.37, 52.29, 52.21, 51.73, 49.51, 49.31, 48.61, 46.89, 34.79, 32.19, 31.85, 30.47, 29.62, 28.03, 27.17, 22.19, 21.99, 18.27, 16.62, 13.83.

**$N_3$Gly-Ala-Thz-Nle-$NHNH_2$ (84)**

Azido-Ac-Ala-Thz-Nle-OMe **83** (213 mg, 0.51 mmol) was dissolved in MeOH (5 mL). After addition of hydrazine hydrate (0.75 mL, 15.4 mmol, 30 equiv.) the reaction mixture was stirred for 2 h. The reaction mixture was then concentrated before being co-evaporated with MeOH (3x). This yielded the title compound in a quantitative yield. Complex NMR due to a presence of rotamers. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.72 – 7.44 (m, 2H), 7.37 (d, *J* = 8.1 Hz, 1H), 5.15 – 4.22 (m, 5H), 3.92 (s, 2H), 3.84 – 3.40 (m, 3H), 3.40 – 3.05 (m, 2H), 1.84 – 1.47 (m, 2H), 1.47 – 1.05 (m, 7H), 0.82 (t, *J* = 6.5 Hz, 3H).$^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.02, 171.48, 169.47, 169.21, 166.87, 62.93, 62.72, 53.22, 52.19, 52.12, 51.80, 49.71, 49.16, 48.17, 47.08, 34.96, 32.66, 32.40, 31.46, 30.31, 29.66, 27.93, 27.58, 22.30, 21.18, 17.98, 17.37, 13.93. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 4.11 (ESI-MS (m/z): 415.00 ($M+H^+$)).

**Boc-Aze-Nle-OMe (85)**

The title compound was prepared by the general procedure for peptide coupling on a 1.0 mmol scale. Column chromatography (30→60% EtOAc:pent) provided the product (270 mg, 0.82 mmol, 82%).
$^1$H NMR (300 MHz, $CDCl_3$) δ 4.75-4.48 (m, 2H), 3.95-3.75 (m, 2H), 3.75 (s, 3H, $OCH_3$), 2.52-2.35 (m, 2H), 1.88-1.70 (m, 2H), 1.65-1.22 (m, 4H), 1.48 (s, 9H), 0.91 (m, 3H). $^{13}$C NMR (300 MHz, $CDCl_3$) δ 172.46, 171.28, 80.87, 62.01, 52.12, 51.94, 47.01, 31.93, 29.59, 28.12, 27.29, 22.20, 13.20.

**Boc-Ala-Aze-Nle-OMe (86)**

Boc-Aze-Nle-OMe **85** (270 mg, 0.82 mmol, 1 equiv.) was deprotected using the standard procedure for Boc removal. The title compound was prepared by the general procedure for peptide coupling on a 2.74 mmol scale. Column chromatography (50→100% EtOAc:pent) provided the product (280 mg, 0.7 mmol, 85%). Complex NMR due to a 7:1 ratio of rotamers. Peaks of major rotamer are reported. $^1$H NMR (300 MHz, $CDCl_3$) δ 8.00 (d, *J* = 7.5 Hz, 1H), 5.24 (d, 7.5 Hz), 4.94 (q, *J* = 6.3 Hz, 1H), 4.48 (q, *J* = 7.5 Hz, 1H), 4.26 (m, 2H), 4.08 (m, 1H), 3.71 (s, 3H), 2.76-2.69 (m, 1H), 2.51-2.42 (m, 1H), 1.82-1.61 (m, 2H), 1.41 (s, 9H), 1.24 (m, 7H), 0.87 (t, *J*=7.2 Hz, 3H). $^{13}$C NMR (300 MHz, $CDCl_3$) δ 174.89, 172.44, 170.10, 155.10, 79.77, 61.79, 52.34, 52.22, 48.82, 45.50, 31.60, 28.27, 27.29, 22.14, 18.41, 18.34, 13.84.

**$N_3$Gly-Ala-Aze-Nle-OMe (87)**

Boc-Ala-Aze-Nle-OMe **86** (256 mg, 0.64 mmol, 1 equiv.) was deprotected using the standard procedure for Boc removal. The obtained TFA-salt was dissolved in DMF (6 mL) and $(ClOAc)_2O$ (348 mg, 2.0 mmol, 1.2 equiv.) and DiPEA (1.18 mL, 6.8 mmol, 4 equiv.) were added. After 1.5 h, $NaN_3$ (0.27 g, 4.1 mmol) was added and the resulting mixture was stirred overnight. The reaction mixture was diluted with EtOAc, washed with 1M HCl, sat. aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated. Purification by column chromatography (1→3% MeOH:DCM) yielded the title compound (144 mg, 0.38 mmol, 59%). Complex NMR due to a 7.5:1 ratio of rotamers. Peaks of major rotamer are reported. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.91 (d, *J* = 7.7 Hz, 1H), 7.26 (d, *J* = 7.3 Hz, 1H), 4.95 (dd, *J* = 9.3, 6.3 Hz, 1H), 4.58 – 4.49 (m, 2H), 4.35 (q, *J* = 8.8 Hz, 1H), 4.19 – 4.08 (m, 1H), 3.98 (s, 2H), 3.74 (s, 3H), 2.80 – 2.69 (m, 1H), 2.57 – 2.43 (m, 1H), 1.83 (dq, *J* = 14.2, 5.5, 4.8 Hz, 1H), 1.76 – 1.59 (m, 1H), 1.41 – 1.19 (m, 7H), 0.89 (q, *J* = 7.2 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 173.83, 172.45, 169.77, 166.48, 61.80, 52.29, 52.25, 52.18, 49.00, 44.39, 31.63, 27.24, 22.12, 18.46, 17.99, 13.82. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 5.44 (ESI-MS (m/z): 383.07 ($M+H^+$)).

**$N_3$Gly-Ala-Aze-Nle-$NHNH_2$ (88)**

Methyl ester **87** (144 mg, 0.38 mmol, 1 equiv.) was dissolved in MeOH (4 mL) before adding hydrazine hydrate (582 µL, 11.4 mmol, 30 equiv.). After stirring for 4 h at RT, TLC analysis showed complete conversion. The reaction mixture was concentrated and co-evaporated with toluene to give the title compound in a quantitative yield. No NMR-analysis was performed due to poor solubility of the product in MeOD and $CDCl_3$ and mixtures thereof. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 3.67 (ESI-MS (m/z): 383.07 ($M+H^+$)).

**Boc-Ala-Pip-Nle-OMe (89)**

Boc-Pip-Nle-OMe was prepared by the general procedure for peptide coupling on a 3 mmol scale. Column chromatography (50→80% EtOAc:pent) provided the product (1.09 g, 2.79 mmol, 93%). Boc-Pip-Nle-OMe was deprotected using the standard procedure for Boc removal. The title compound was prepared by the general procedure for peptide coupling on a 3 mmol scale. Column chromatography (50→80% EtOAc:pent) provided the product in a quantitative yield. Complex NMR due to a 2:1 ratio of rotamers. 1H NMR (400 MHz, $CDCl_3$) δ 7.88 (d, *J* = 7.5 Hz, 0.3H), 6.47 (d, *J* = 7.5 Hz, 0.7H), 5.55 (d, *J* = 7.8 Hz, 0.7H), 5.26 (d, *J* = 6.2 Hz, 0.4H), 5.17 (d, *J* = 4.8 Hz, 0.7H), 4.65 (q, *J* = 6.9 Hz, 0.6H), 4.55 (d, *J* = 13.5 Hz, 0.4H), 4.51 – 4.39 (m, 1.4H), 4.34 (ddd, *J* = 9.3, 7.7, 5.5 Hz, 0.4H), 3.75 (d, *J* = 12.8 Hz, 0.6H), 3.67 (s, 2H), 3.63 (s, 1H), 3.13 (t, *J* = 12.2 Hz, 0.6H), 2.44 (q, *J* = 12.9 Hz, 0.8H), 2.16 (d, *J* = 13.6 Hz, 0.6H), 1.88 – 1.40 (m, 6H), 1.38 (s, 6H), 1.33 (s, 3H), 1.28 – 1.12 (m, 7H), 0.85 – 0.77 (m, 3H). $^{13}C$ NMR (101 MHz, $CDCl_3$) δ 172.93, 172.74, 172.15, 170.37, 169.83, 156.28, 155.03, 80.19, 79.58, 52.13, 46.50, 43.65, 39.92, 31.96, 30.44, 27.40, 26.36, 25.59, 25.33, 24.82, 22.21, 22.11, 20.67, 20.21.

**$N_3$Gly-Ala-Pip-Nle-OMe (90)**

1:1 DCM/TFA (10 mL) was added to **89** (726 mg, 1.7 mmol, 1 equiv.) and after 30 min, the reaction mixture was concentrated and co-evaporated with toluene to give the deprotected peptide, which was dissolved in DMF (14 mL). $(ClAc)_2O$ (348 mg, 2.0 mmol, 1.2 equiv.) and DiPEA (1.18 mL, 6.8 mmol, 4 equiv.) were added. After 1.5 h, $NaN_3$ (0.27 g, 4.1 mmol) was added and the resulting mixture was stirred overnight. The reaction mixture was diluted with EtOAc, washed with 1M HCl, sat. aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated. Purification by column chromatography (1→3% MeOH:DCM) yielded the title compound (631 mg, 1.53 mmol, 90%). Complex NMR due to a 3.5:1 ratio of rotamers. $^1H$ NMR (400 MHz, $CDCl_3$) δ 7.57 (d, *J* = 7.6 Hz, 0.2H), 7.40 (d, *J* = 7.3 Hz, 0.8H), 7.04 (d, *J* = 5.8 Hz, 0.2H), 6.42 (d, *J* = 7.6 Hz, 0.8H), 5.21 (d, *J* = 4.7 Hz, 0.7H), 4.96 (p, *J* = 6.9 Hz, 0.7H), 4.70 (p, *J* = 6.7 Hz, 0.7H), 4.57 (d, *J* = 13.4 Hz, 0.7H), 4.50 (m, 1H), 4.47 – 4.36 (m, 0.3H), 3.96 (d, *J* = 5.2 Hz, 1.3H), 3.77 (d, *J* = 13.2 Hz, 0.8H), 3.72 (s, 2.3 H), 3.69 (s, 0.7H), 3.28 – 3.18 (m, 0.8H), 2.54 (t, *J* = 13.3 Hz, 0.5H), 2.21 (d, *J* = 13.7 Hz, 0.8H), 1.88 – 1.40 (m, 8H), 1.35 (d, *J* = 6.9 Hz, 3H), 1.33 – 1.14 (m, 4H), 0.85 (t, *J* = 7.1 Hz, 3H). $^{13}C$ NMR (101 MHz, $CDCl_3$) δ 172.87, 172.07, 170.16, 169.49, 165.96, 52.69, 52.30, 52.14, 43.78, 40.26, 32.12, 30.77, 28.26, 27.48, 26.64, 25.83, 25.38, 24.87, 22.32, 22.17, 20.72, 20.22. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 6.36 (ESI-MS (m/z): 410.93 ($M+H^+$)).

**$N_3$Gly-Ala-Pip-Nle-$NHNH_2$ (91)**

Methyl ester **90** (631 mg, 1.53 mmol, 1 equiv.) was dissolved in MeOH (15 mL) before adding hydrazine hydrate (2.2 mL, 45.9 mmol, 30 equiv.). After stirring for 4 h at RT, TLC analysis showed complete conversion. The reaction mixture was concentrated and co-evaporated with toluene to give the title compound in a quantitative yield. No NMR-analysis was performed due to poor solubility of the product in MeOD and $CDCl_3$ and mixtures thereof. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 4.35 (ESI-MS (m/z): 411.00 ($M+H^+$)).

**Boc-(4S)FPro-Nle-OMe (92)**

The title compound was prepared by the general procedure for peptide coupling on a 1 mmol scale. Column chromatography (50% EtOAc:pent) provided the product in a quantitative yield. $^1H$ NMR (400 MHz, $CDCl_3$): δ 6.17 (s, 1H), 4.83 (d, *J* = 48.2 Hz, 1H), 4.26 (s, 1H), 4.08 (s, 1H), 3.68 – 3.23 (m, 5H), 2.69 – 1.96 (m, 2H), 1.68 (s, 1H), 1.52 (s, 1H), 1.38 (s, 9H), 1.17 (m, 4H), 0.80 (t, *J* = 6.0 Hz, 3H). $^{13}C$ NMR (101 MHz, $CDCl_3$): δ 172.47, 92.45, 90.73, 81.37, 59.89, 53.94, 52.11, 37.47, 32.15, 28.07, 26.80, 22.15, 13.71; LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 7.60 (ESI-MS (m/z): 360.93 ($M+H^+$)).

**Boc-Ala-(4S)-FPro-Nle-OMe (93)**

Boc-Pip-Nle-OMe was deprotected using the standard procedure for Boc removal. The title compound was prepared by the general procedure for peptide coupling on a 1.9 mmol scale. Column chromatography (40:20 EtOAc:pent) provided the product (755 mg, 1.8 mmol, 93%). Complex NMR due to rotamers. $^1H$ NMR (400 MHz,

$CDCl_3$) δ 8.10 (d, *J* = 8.0 Hz, 0.4 H), 7.01 (d, *J* = 7.5 Hz, 0.6 H), 5.53 – 5.03 (m, 2H), 4.80 (d, *J* = 9.8 Hz, 0.6 H), 4.63 – 4.21 (m, 2.4 H), 4.06 – 3.76 (m, 2H), 3.72 (s, 2H), 3.66 (s, 1H), 3.04 – 2.82 (m, 1H), 2.41 – 2.07 (m, 1H), 1.98 – 1.71 (m, 1H), 1.71 – 1.52 (m, 1H), 1.52 – 1.33 (m, 10H), 1.34 – 1.08 (m, 6H), 0.85 (q, *J* = 6.9 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 173.97, 172.82, 172.74, 172.64, 170.58, 169.52, 155.94, 155.26, 93.15, 91.37, 91.19, 89.44, 80.24, 79.93, 59.30, 59.15, 54.12, 53.88, 52.61, 52.32, 52.22, 52.08, 48.66, 47.83, 38.61, 38.35, 38.14, 34.32, 34.11, 31.94, 30.14, 29.68, 28.37, 28.32, 28.22, 27.77, 26.81, 22.24, 22.00, 18.57, 16.58, 13.83. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 7.28 (ESI-MS (m/z): 431.93 ($M+H^+$)).

**$N_3$Gly-Ala-(4S)-FPro-Nle-OMe (94)**

TFA (6 mL) was added to **93** (1.75 mmol, 1 equiv.). After 30 min, the reaction mixture was concentrated and co-evaporated with toluene to give the deprotected peptide, which was dissolved in DMF (18 mL). $(ClOAc)_2O$ (0.36 g, 2.1 mmol) and DiPEA (0.9 mL, 5.3 mmol) were added and the reaction mixture turned deep red. After 1.5 h $NaN_3$ (0.34 g, 5.2 mmol) was added and the resulting mixture was stirred overnight. The reaction mixture was diluted with EtOAc, washed with 1M HCl, sat. aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated. Purification by column chromatography (9:1 EA:pent → 1:9 MeOH:EA) yielded the title compound (425 mg, 1.0 mmol, 59%).%). Complex NMR due to rotamers. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.9 Hz, 0.3 H), 7.14 (d, *J* = 6.9 Hz, 0.7 H), 6.93 (d, *J* = 7.4 Hz, 0.7 H), 6.73 (d, *J* = 4.6 Hz, 0.3 H), 5.32 (m, 1H), 4.86 – 4.65 (m, 1H), 4.67 – 4.33 (m, 2H), 4.15 – 3.85 (m, 4H), 3.76 (s, 2H), 3.71 (s, 1H), 2.93 – 2.75 (m, 1H), 2.42 – 2.04 (m, 1H), 1.95 – 1.60 (m, 2H), 1.55 (d, *J* = 6.9 Hz, 2H), 1.42 (d, *J* = 6.9 Hz, 1H), 1.37 – 1.07 (m, 4H), 0.88 (t, *J* = 7.0 Hz, 3H).

**$N_3$Gly-Ala-(4S)-FPro-Nle-$NHNH_2$ (95)**

Methyl ester **94** (1.0 mmol, 1 equiv.) was dissolved in MeOH (10 mL) before adding hydrazine hydrate (1.0 mL, 21 mmol, 21 equiv.). After stirring for 4 h at rt TLC analysis showed incomplete conversion and the reaction mixture was refluxed for 45 min to achieve full consumption of the starting material. The reaction mixture was concentrated and co-evaporated with toluene to give the title compound in a quantitative yield. No NMR-analysis was performed due to poor solubility of the product in MeOD and $CDCl_3$ and mixtures thereof. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 4.49 (ESI-MS (m/z): 415.07 ($M+H^+$)).

**Boc-(4R)-FPro-Nle-OMe (96)**

The title compound was prepared by the general procedure for peptide coupling on a 1 mmol scale. Column chromatography (50% EtOAc:pent) provided the product (288 mg, 0.80 mmol, 80%). Complex NMR due to rotamers. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.33 (d, *J* = 6.3 Hz, 0.6H), 6.50 (s, 0.4H), 5.16 (d, *J* = 53.0 Hz, 1H), 4.57 – 4.20 (m, 2H), 4.15 – 3.74 (m, 1H), 3.69 (s, 3H), 3.56 – 3.26 (m, 1H), 2.72 – 2.18 (m, 2H), 1.85 – 1.72 (m, 1H), 1.72 – 1.53 (m, 1H), 1.43 (s, 9H), 1.23 (d, *J* = 17.3 Hz, 4H), 0.83 (t, *J* = 5.5 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.72, 170.71, 92.88, 91.13, 80.99, 59.42, 58.06, 53.54, 53.31, 52.50, 52.31, 52.05, 37.81, 34.68, 34.47, 32.38, 31.96, 29.73, 28.31, 27.35, 22.31, 13.87.

**Boc-Ala-(4R)-FPro-Nle-OMe (97)**

Boc-(4R)FPro-Nle-OMe (**96**) was deprotected using the standard procedure for Boc removal. The title compound was prepared by the general procedure for peptide coupling on a 1.43 mmol scale. Column chromatography (50→60% EA:pent) provided the product (592 mg, 1.40 mmol, 98%). $^1$H NMR (400 MHz, $CDCl_3$): δ 7.23 (s, 1H), 5.53 (d, *J* = 6.8 Hz, 1H), 5.33 (d, *J* = 52.6 Hz, 1H), 4.77 (t, *J* = 7.8 Hz, 1H), 4.51 (m, 2H), 4.21 – 4.03 (m, 1H), 3.74 (s, 3H), 3.71 – 3.54 (m, 1H), 2.46 (m, 2H), 1.90 – 1.56 (m, 2H), 1.44 (s, 9H), 1.31 (m, 7H), 0.88 (t, *J* = 5.6 Hz, 3H). $^{13}$C NMR (101 MHz, CDCl3): δ 172.76, 169.94, 155.06, 92.68, 90.89, 79.82, 58.25, 53.60, 53.37, 52.55, 52.39, 47.93, 34.22, 33.99, 31.90, 28.39, 27.36, 22.26, 18.68, 13.91; LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 7.15 (ESI-MS (m/z): 431.93 ($M+H^+$)).

**$N_3$Gly-Ala-(4R)-FPro-Nle-OMe (98)**

TFA (4.6 mL) was added to **97** (592 mg, 1.40 mmol, 1 equiv.) and after 30 min, the reaction mixture was concentrated and co-evaporated with toluene to give the deprotected peptide, which was dissolved in DMF (14 mL). $(ClOAc)_2O$ (0.28 g, 1.60 mmol, 1.1 equiv.) and DiPEA (0.9 mL, 5.3 mmol, 3.8 equiv.) were added and the reaction mixture turned deep red. After 1.5 h $NaN_3$ (0.27 g, 4.1 mmol) was added and the resulting mixture was stirred overnight. The reaction mixture was diluted with EtOAc, washed with 1M HCl, sat. aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated. Purification by column chromatography (1:1 EA:pent) yielded the title compound (383 mg, 0.92 mmol, 67%). $^1$H NMR (400 MHz, $CDCl_3$): δ 7.34 (d, *J* = 7.2 Hz, 1H), 7.18 (d, *J* = 7.7 Hz, 1H), 5.33 (d, *J* = 52.6 Hz, 1H), 4.76 (dt, *J* = 12.3, 7.5 Hz, 2H), 4.57 – 4.46 (m, 1H), 4.17 – 3.59 (m, 7H), 2.53 (ddt, *J* = 46.6, 17.0, 6.2 Hz, 2H), 1.84 (ddd, *J* = 13.5, 10.2, 6.1 Hz, 1H), 1.70 (ddd, *J* = 13.6, 8.7, 4.7 Hz, 1H), 1.39 – 1.25 (m, 7H), 0.89 (t, *J* = 6.7 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$): δ 172.76, 171.65, 169.87, 166.13, 92.70, 90.91, 58.36, 53.73, 53.50, 52.55, 52.47, 52.39, 46.90, 34.54, 34.32, 31.88, 27.38, 22.26, 18.14, 13.90; LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 5.82 (ESI-MS (m/z): 415.00 ($M+H^+$)).

**$N_3$Gly-Ala-(4R)-FPro-Nle-$NHNH_2$ (99)**

Methyl ester **98** (383 mg, 0.92 mmol, 1 equiv.) was dissolved in MeOH (9.2 mL) before adding hydrazine hydrate (0.9 mL, 18.4 mmol, 20 equiv.). After stirring for 4 h at RT, TLC analysis showed incomplete conversion and the reaction mixture was refluxed for 45 min to achieve full consumption of the starting material. The reaction mixture was concentrated and co-evaporated with toluene to give the title compound in a quantitative yield. No NMR-analysis was performed due to poor solubility of the product in MeOD and $CDCl_3$ and mixtures thereof. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 15 min): $R_t$ (min): 4.20 (ESI-MS (m/z): 415.07 ($M+H^+$)).

**Boc-4,4-$F_2$Pro-Nle-OMe (100)**

The title compound was prepared by the general procedure for peptide coupling on a 1 mmol scale. Column chromatography (10→30% EtOAc:pent) provided the product (352 mg, 0.93 mmol, 93%). $^1$H NMR (400 MHz, $CDCl_3$) δ 4.57 (bs, 2H), 4.03 – 3.57 (m, 2H), 3.75 (s, 3H), 3.04 – 2.45 (m, 2H), 1.85 (m, 1H), 1.68 (m, 1H), 1.49 (s, 9H), 1.29 (m, 4H), 0.89 (t, *J* = 7.0 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.64, 53.59, 52.42, 32.11, 28.25, 27.23, 22.34, 13.89.

**Boc-Ala-4,4-$F_2$Pro-Nle-OMe (101)**

Boc-4,4-$F_2$Pro-Nle-OMe (**100**) (302 mg, 0.80 mmol, 1 equiv.) was deprotected using the standard procedure for Boc removal. The title compound was prepared by the general procedure for peptide coupling on a 0.93 mmol scale. Column chromatography (30→50% EA:pent) provided the product (167 mg, 0.37 mmol, 46%). Complex NMR due to a presence of rotamers. $^1$H NMR (400 MHz, $CDCl_3$) δ 8.21 (d, *J* = 8.1 Hz, 0.3H), 7.13 (d, *J* = 7.3 Hz, 0.7H), 5.38 (d, *J* = 7.6 Hz, 0.7H), 5.21 (bs, 0.3H), 4.79 (dd, *J* = 9.2, 5.3 Hz, 0.7H), 4.60 – 3.72 (m, 3.3H), 3.65 (2xs, 3H), 3.36 – 2.11 (m, 2H), 1.98 – 1.50 (m, 2H), 1.43 – 1.13 (m, 16H), 0.82 (t, *J* = 6.8 Hz, 3H).

**$N_3$Gly-Ala-4,4-$F_2$Pro-Nle-OMe (102)**

Boc-Ala-4,4-$F_2$Pro-Nle-OMe (167 mg, 0.37 mmol, 1 equiv.) **101** was dissolved in 1:1 TFA: DCM (10 mL). After 30 min the reaction mixture was concentrated before being co-evaporated with toluene (3x). The crude TFA salt was dissolved in DMF (4 mL) before adding $(ClAc)O_2$ (76 mg, 0.45 mmol, 1.2 equiv.) and DiPEA (0.26 mL, 1.49 mmol, 4 equiv.). The reaction mixture was stirred for one hour at room temperature and under argon atmosphere before $NaN_3$ (97 mg, 1.49 mmol, 4 equiv.) was added. The reaction mixture was then stirred overnight at room temperature and under argon atmosphere. EtOAc (50 mL) was then added to the reaction mixture before being washed with 1M HCl (2x), sat. aq. $NaHCO_3$ (2x) and brine (1x).The organic layer was dried over $Na_2SO_4$, filtered and concentrated. Purification by column chromatography (0→3% MeOH:DCM) yielded the title compound (117 mg, 0.27 mmol, 73%). Complex NMR due to a presence of rotamers. $^1$H NMR (400 MHz, $CDCl_3$) δ 8.01 (d, *J* = 8.0 Hz, 0.2H), 7.18 (d, *J* = 7.1 Hz, 0.8H), 7.06 (d, *J* = 7.4 Hz, 0.8H), 6.97 (s, 0.2H), 4.76 (dd, *J* =

9.1, 5.7 Hz, 1H), 4.64 (p, *J* = 6.9 Hz, 1H), 4.58 – 4.37 (m, 1H), 4.15 (td, *J* = 12.1, 7.1 Hz, 1H), 3.94 (s, 2H), 3.91 – 3.75 (m, 1H), 3.67 (d, *J* = 22.4 Hz, 3H), 3.01 – 2.43 (m, 2H), 1.79 (m, 1H), 1.63 (m, 1H), 1.34 (d, *J* = 6.8 Hz, 3H), 1.24 (dd, *J* = 18.0, 6.8 Hz, 4H), 0.83 (t, *J* = 6.8 Hz, 3H). $^{13}$C NMR (101 MHz, $CDCl_3$) δ 172.83, 172.70, 172.10, 171.42, 169.35, 168.64, 167.67, 166.45, 128.72, 126.24, 123.75, 59.02, 57.86, 53.99, 53.68, 53.34, 53.00, 52.55, 52.44, 52.37, 52.26, 51.79, 47.37, 46.70, 35.99, 35.74, 35.49, 31.88, 30.38, 27.97, 27.21, 22.22, 21.96, 17.87, 16.76, 13.84.

**Azido-Ac-Ala-4,4- $F_2$Pro-Nle-$NHNH_2$ (103)**

Azido-Ac-Ala-4,4-$F_2$-Nle-OMe (117 mg, 0.27 mmol) **102** was dissolved in MeOH (3 mL). After addition of hydrazine hydrate (0.41 mL, 8.31 mmol, 30 equiv.) the reaction mixture was stirred for 2 h. The reaction mixture was then concentrated before being co-evaporated with MeOH (3x). This yielded the title compound in a quantitative yield. Complex NMR due to a presence of rotamers. $^1$H NMR (400 MHz, MeOD) δ 4.69-4.63 (m, 1H), 4.54 (q, *J* = 7.0 Hz, 1H), 4.42 – 4.14 (m, 2H), 4.14 – 3.92 (m, 1H), 3.88-3.82 (m, 2H), 2.89 – 2.57 (m, 1H), 2.56 – 2.26 (m, 1H), 1.85 – 1.49 (m, 2H), 1.30 (m, 7H), 0.87 (t, *J* = 6.9 Hz, 3H). $^{13}$C NMR (101 MHz, MeOD) δ 173.60, 173.24, 171.93, 169.90, 130.30, 127.83, 125.37, 60.02, 59.22, 54.99, 54.66, 54.34, 53.57, 52.42, 52.12, 48.36, 48.06, 38.18, 37.94, 37.69, 32.97, 32.37, 29.12, 28.84, 23.33, 23.20, 17.23, 16.75, 14.23. LC-MS (linear gradient 10 → 90% MeCN, 0.1% TFA, 12.5 min): $R_t$ (min): 4.24 (ESI-MS (m/z): 433.00 ($M+H^+$)).

## Biochemical methods

**Competition Assays in Cell Lysate.**

Lysates of Raji cells were prepared by sonication in 3 volumes of lysis buffer containing 50 mM Tris pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 250 mM sucrose, 2 mM ATP, and 0.025% digitonin. Protein concentration was determined by the Bradford assay. Cell lysates (diluted to 10-15 µg total protein in buffer containing 50 mM Tris pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, 10% glycerol, 2 mM ATP) were exposed to the inhibitors for 1 h at 37 °C prior to incubation with AzidoBODIPY-MeTyr-Phe-Leu-VS (BODIPY-NC005; 0.1-0.5 µM; to probe β5), BODIPY-epoxomicin (0.5 µM; to probe all subunits, used for β2-profiling) or BODIPY-FL-Ala-Pro-Nle-Leu-EK (BODIPY-NC001; 1 µM; to probe β1 activity. This probe does bind slightly to β5i, so lysates were treated for 1 h with NC005VS[11] (5 µM), prior to probe incubation for an additional 1 h at 37 °C, followed by 3 min boiling with a reducing gel-loading buffer and fractionation on 12.5% SDS-PAGE. In-gel detection of residual proteasome activity was performed in the wet gel slabs directly on a ChemiDoc™ MP System using Cy3 settings to detect BODIPY-NC005 and BODIPY-epoxomicin and Cy2 settings to detect BODIPY-NC001. Intensities of bands were measured by fluorescent densitometry and normalized to the intensity of bands in mock-treated extracts. Average values of at least two or three independent experiments were plotted against inhibitor concentrations. $IC_{50}$ (inhibitor concentrations giving 50% inhibition) values were calculated using GraphPad Prism software.

**Competition Assays in living RPMI-8226 cells.**

RPMI-8226 were cultured in RPMI-1640 media supplemented with 10% fetal calfs 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 with twice with PBS. Cell pellets were treated with lysis buffer (50 µL: 50 mM Tris pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, 10% glycerol, 2 mM ATP, 0.05% digitonin) on ice for 1.5 h, followed by centrifugation at 14000 rpm for 15 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 extracts. Gels were stained by Coomassie Brilliant Blue, which was used to correct for gel loading differences. Average values of two or three independent experiments were plotted against inhibitor concentrations. $IC_{50}$ (inhibitor concentrations giving 50% inhibition) values were calculated using GraphPad Prism software.

**Crystallographic analysis.**

yCP crystals were grown by hanging drop vapour diffusion as previously described.[46] Crystal drops were incubated for 12 h with 5 µl of cryo protectant and 0.5 µl of inhibitor (concentration: 50 mM in DMSO). Diffraction datasets were collected using synchrotron radiation of λ=1.0 Å at the beamline X06SA, Swiss Light Source (SLS), Villigen, Switzerland. Structure determination was performed as previously reported.[46] For model building the programs SYBYL, MAIN[47] and COOT[48] were used. Refinement with REFMAC5[49] yielded excellent R factors as well as r.m.s.d. bond and angle values. Coordinates were confirmed to fulfill the Ramachandran plot. Figures were prepared with PyMOL. (For X-ray data collection and refinement statistics, PDB codes and all structures: see[50]).

## References


1. Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425-79 (1998).
2. Lowe, *J*. et al. Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4 A resolution. *Science* **268**, 533-539 (1995).
3. Britton, M. et al. Selective inhibitor of proteasome's caspase-like sites sensitizes cells to specific inhibition of chymotrypsin-like sites. *Chem. Biol.* **16**, 1278-1289 (2009).
4. Geurink, P.P. et al. Incorporation of non-natural amino acids improves cell permeability and potency of specific inhibitors of proteasome trypsin-like sites. *J. Med. Chem.* **56**, 1262-1275 (2013).
5. Groll, M. et al. Structure of 20S proteasome from yeast at 2.4A resolution. *Nature* **386**, 463-471 (1997).
6. Tanaka, K. Role of proteasomes modified by interferon-gamma in antigen processing. *J. Leukoc. Biol.* **56**, 571-5 (1994).
7. Strehl, B. et al. Antitopes define preferential proteasomal cleavage site usage. *J. Biol. Chem.* **283**, 17891-17897 (2008).
8. Orlowski, M., Cardozo, C. & Michaud, C. Evidence for the presence of five distinct proteolytic components in the pituitary multicatalytic proteinase complex. Properties of two components cleaving bonds on the carboxyl side of branched chain and small neutral amino acids. *Biochemistry* **32**, 1563-1572 (1993).
9. Kisselev, Alexei F., van der Linden, W.A. & Overkleeft, Herman S. Proteasome inhibitors: an expanding army attacking a unique target. *Chem. Biol.* **19**, 99-115 (2012).
10. Beck, P., Dubiella, C. & Groll, M. Covalent and non-covalent reversible proteasome inhibition. *Biol. Chem.* **393**, 1101 (2012).
11. Screen, M. et al. Nature of pharmacophore influences active site specificity of proteasome inhibitors. *J. Biol. Chem.* **285**, 40125-40134 (2010).
12. Stein, M.L. et al. Systematic comparison of peptidic proteasome inhibitors highlights the α-ketoamide electrophile as an auspicious reversible lead motif. *Angew. Chem. Int. Ed.* **53**, 1679-1683 (2014).
13. Adams, J. et al. Potent and selective inhibitors of the proteasome: Dipeptidyl boronic acids. *Bioorg. Med. Chem. Lett.* **8**, 333-338 (1998).
14. Adams, J. et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Res.* **59**, 2615-2622 (1999).
15. Adams, J. The proteasome: a suitable antineoplastic target. *Nat. Rev. Cancer* **4**, 349-60 (2004).
16. Demo, S.D. et al. Antitumor Activity of PR-171, a Novel Irreversible Inhibitor of the Proteasome. *Cancer Res.* **67**, 6383-6391 (2007).
17. Elofsson, M., Splittgerber, U., Myung, J., Mohan, R. & Crews, C.M. Towards subunit-specific proteasome inhibitors: synthesis and evaluation of peptide α', β'-epoxyketones. *Chem. Biol.* **6**, 811-822 (1999).
18. O'Connor, O.A. et al. A phase 1 dose escalation study of the safety and pharmacokinetics of the novel proteasome inhibitor carfilzomib (PR-171) in patients with hematologic malignancies. *Clin. Canc. Res.* **15**, 7085-7091 (2009).

19. Dorsey, B.D. et al. Discovery of a potent, selective, and orally active proteasome inhibitor for the treatment of cancer. *J. Med. Chem.* **51**, 1068-1072 (2008).
20. Kupperman, E. et al. Evaluation of the proteasome inhibitor MLN9708 in preclinical models of human cancer. *Cancer Res.* **70**, 1970-1980 (2010).
21. Zhou, H.-J. et al. Design and synthesis of an orally bioavailable and selective peptide epoxyketone proteasome inhibitor (PR-047). *J. Med. Chem.* **52**, 3028-3038 (2009).
22. Kuhn, D.J. et al. Potent activity of carfilzomib, a novel, irreversible inhibitor of the ubiquitin-proteasome pathway, against preclinical models of multiple myeloma. *Blood* **110**, 3281-3290 (2007).
23. Kisselev, A.F., Callard, A. & Goldberg, A.L. Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate. *J. Biol. Chem.* **281**, 8582-8590 (2006).
24. Geurink, P.P. et al. Incorporation of fluorinated phenylalanine generates highly specific inhibitor of proteasome's chymotrypsin-like sites. *J. Med. Chem.* **53**, 2319-2323 (2010).
25. Borissenko, L. & Groll, M. 20S Proteasome and its Inhibitors: crystallographic knowledge for drug development. *Chem. Rev.* **107**, 687-717 (2007).
26. Groll, M., Kim, K.B., Kairies, N., Huber, R. & Crews, C.M. Crystal structure of epoxomicin:20S proteasome reveals a molecular basis for selectivity of α',β'-epoxyketone proteasome inhibitors. *J. Am. Chem. Soc.* **122**, 1237-1238 (2000).
27. Groll, M., Huber, R. & Potts, B.C.M. Crystal structures of salinosporamide A (NPI-0052) and B (NPI-0047) in complex with the 20S proteasome reveal important consequences of β-lactone ring opening and a mechanism for irreversible binding. *J. Am. Chem. Soc.* **128**, 5136-5141 (2006).
28. Huber, Eva M. et al. Immuno- and constitutive proteasome crystal structures reveal differences in substrate and inhibitor specificity. *Cell* **148**, 727-738 (2012).
29. Huber, E.M. & Groll, M. Inhibitors for the immuno- and constitutive proteasome: current and future trends in drug development. *Angew. Chem. Int. Ed.* **51**, 8708-8720 (2012).
30. Shenk, K.D.P., F.; Zhou, H.- J.; Sylvain, C.; Smyth, M.S.; Bennet, M.K.; Laidig, G. J., *US/20070293465,* (2007).
31. Muchamuel, T. et al. A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat. Med.* **15**, 781-787 (2009).
32. Kuhn, D. J. et al. Targeted inhibition of the immunoproteasome is a potent strategy against models of multiple myeloma that overcomes resistance to conventional drugs and nonspecific proteasome inhibitors. *Blood* **113**, 4667-4676 (2009).
33. Parlati, F. et al. Carfilzomib can induce tumor cell death through selective inhibition of the chymotrypsin-like activity of the proteasome. *Blood* **114**, 3439-3447 (2009).
34. Myung, J., Kim, K.B., Lindsten, K., Dantuma, N.P. & Crews, C.M. Lack of proteasome active site allostery as revealed by subunit-specific inhibitors. *Mol. Cell* **7**, 411-420 (2001).
35. Verdoes, M. et al. A panel of subunit-selective activity-based proteasome probes. *Org. Biomol. Chem.* **8**, 2719-2727 (2010).
36. Li, N. et al. Relative quantification of proteasome activity by activity-based protein profiling and LC-MS/MS. *Nat. Protocols* **8**, 1155-1168 (2013).
37. Singh, A.V. et al. PR-924, a selective inhibitor of the immunoproteasome subunit LMP-7, blocks multiple myeloma cell growth both in vitro and in vivo. *Br. J.Haematol.* **152**, 155-163 (2011).
38. Renner, C. et al. Fluoroprolines as tools for protein design and engineering. *Ang. Chem. Int. Ed.* **40**, 923-925 (2001).
39. Staas, D.D. et al. Discovery of potent, selective 4-fluoroproline-based thrombin inhibitors with improved metabolic stability. *Bioorg. Med. Chem.* **14**, 6900-6916 (2006).
40. Ho, Y.K., Bargagna-Mohan, P., Wehenkel, M., Mohan, R. & Kim, K.-B. LMP2-specific inhibitors: chemical genetic tools for proteasome biology. *Chem. Biol.* **14**, 419-430 (2007).
41. Wehenkel, M. et al. A selective inhibitor of the immunoproteasome subunit LMP2 induces apoptosis in PC-3 cells and suppresses tumour growth in nude mice. *Br. J. Cancer* **107**, 53-62 (2012).

42. Basler, M. et al. Why the structure but not the activity of the immunoproteasome subunit low molecular mass polypeptide 2 rescues antigen presentation. *J. Immunol.* **189**, 1868-1877 (2012).
43. Unno, M. et al. Structure determination of the constitutive 20S proteasome from bovine liver at 2.75 A resolution. *J. Biochem.* **131**, 171-3 (2002).
44. Augeri, D. J. et al. Discovery and preclinical profile of saxagliptin (BMS-477118): a highly potent, long-acting, orally active dipeptidyl peptidase IV inhibitor for the treatment of type 2 diabetes. *J. Med. Chem.* **48**, 5025-5037 (2005).
45. Thompson, S.A., Andrews, P.R. & Hanzlik, R.P. Carboxyl-modified amino acids and peptides as protease inhibitors. *J. Med. Chem* **29**, 104-111 (1986).
46. Groll, M. & Huber, R. in Methods in Enzymology (ed. Raymond, J.D.) 329-336 (Academic Press, 2005).
47. Turk, D. Improvement of a Programme for Molecular Graphics and Manipulation of Electron Densities and Its Application for Protein Structure Determination. *PhD thesis, Technische Univ. München* (1992).
48. Emsley, P., Lohkamp, B., Scott, W.G. & Cowtan, K. Features and development of Coot. *Acta crystallographica. Section D, Biological crystallography* **66**, 486-501 (2010).
49. Vagin, A.A. et al. REFMAC5 dictionary: organization of prior chemical knowledge and guidelines for its use. *Acta Crystallographica Section D* **60**, 2184-2195 (2004).
50. de Bruin, G. et al. Structure-based design of β1i or β5i specific inhibitors of human immunoproteasomes. *J. Med. Chem.* **57**, 6197-209 (2014).