Design of selective inhibitors for human immunoproteasomes

Dekker, P.M.

Chapter 4:

Probing the chemical space at the P' site in peptide epoxyketones towards new proteasome inhibitors

Introduction

The proteasome has proven to be a viable drug target in the treatment of the haematological cancers, multiple myeloma (MM) and mantle cell lymphoma (MCL).[1,2] The currently applied proteasome inhibitors, bortezomib, carfilzomib and ixazomib, while effective have shown to evoke detrimental adverse side effects including anaemia, thrombocytopenia, neutropenia, myocardial infarction, and polyneuropathy.[1–3] The introduction of this thesis and previous chapters outlined how the proteasome biology research field realized that in order to effectively treat MM and other haematological cancers, multiple active sites of the proteasomal core particle (CP) need to be inhibited. By virtue of their design bortezomib, carfilzomib and ixazomib are unable to distinguish between constitutive proteasomes core particles (cCPs) and immunoproteasomes core particles (iCPs) and this is what is considered to cause treatment ending side effects.[1,2,4,5] Additional experiments established that MM and the haematological cancers, B-cell acute lymphoblastic leukaemia (B-ALL), chronic lymphocytic leukaemia (CLL), acute myeloid leukaemia (AML) and T-cell acute lymphoblastic leukaemia (T-ALL), all predominantly express iCPs.[5,6] This has led to a shift in focus in the past years to the discovery and development of inhibitors that selectively inhibit all three iCP active sites over the corresponding cCP active sites.[7–9] Chapter three discussed the development of LU-005i derivatives featuring a hydroxymethyl, and ester derivatives thereof, C-terminal of the epoxyketone. A set of the LU-005i derivatives described in chapter three showed significant improvements in the iCP over cCP selectivity profile when

compared to LU-005i (**Figure 1**). In line with the work found in chapter three the work described in this chapter focused on further expanding and modifying the P' position. In one direction, the hydroxyl group of LU-005i-OH **1** was carbamoylated to link *O*-Me alanine through a carbamate moiety (compound **3**, **Figure 1**). Next, the chemical space for introducing and modifying the P' position was expanded by modifying two methodologies, introduced in previous chapters, used for the synthesis of epoxyketones. Specifically, the carbolithiation strategy used in chapter two was modified to facilitate carboxylate formation during the synthesis, which in turn could be extended through amide bond formation to give compound **4** (**Figure 1**).[5,10,11] Additionally, the Morita-Baylis-Hillman type reaction found in chapter three was repurposed for the introduction of a C-C bond, as in **5** (**Figure 1**).[12–15]

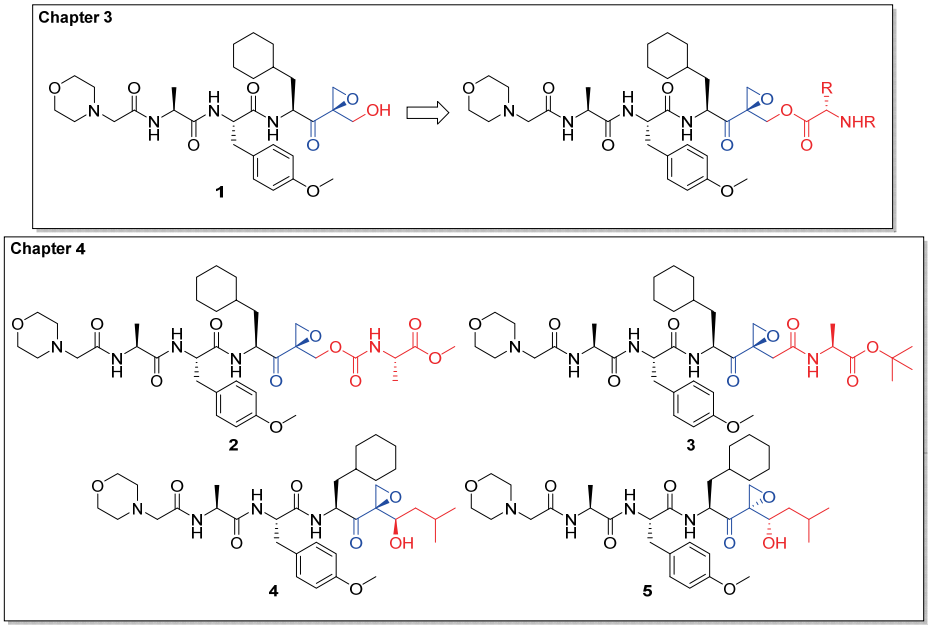
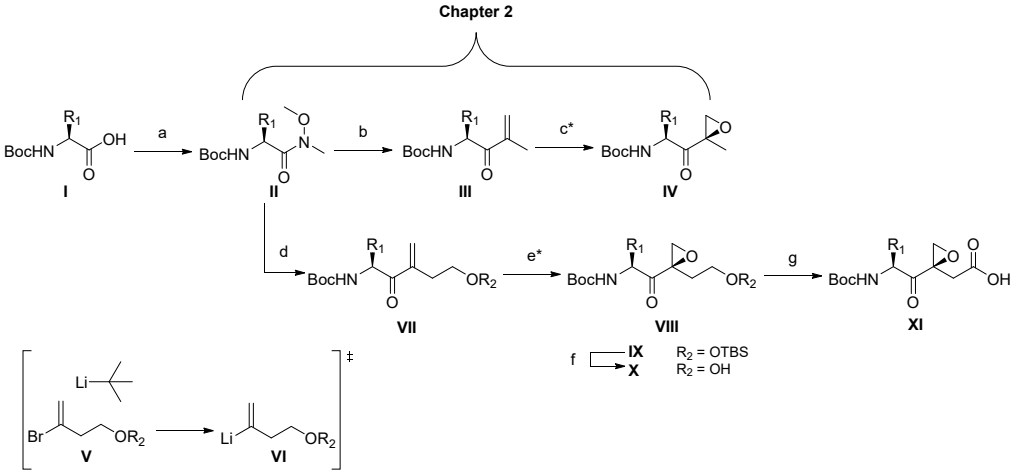


Figure 1. Chemical structures of the peptide epoxyketones **1**-**5** (where the epoxyketone is highlighted in blue and the P1' position is highlighted in red) subject of studies described in the previous chapter (top) and this chapter (bottom).

Results

Synthesis of three substituted peptide epoxyketones

As described in chapter two, *N*-Boc protected amino acid **I** can be condensed with *N,O*-dimethylhydroxylamine to give Weinreb amide **II**. Next, bromopropene is lithiated to Weinreb amide **II** giving unsaturated α,ß-ketone **III**, which can then be epoxidized directly into epoxyketone **IV** (**Scheme 1**).[5,10,11] Carbolithiation has proven to be a highly effective strategy for the synthesis of epoxyketones and thus it was proposed to modify and extend the lithiation substrate with a hydroxyethyl. The basic nature of tert-butyl lithium prevents successful carbolithiation when in the presence of acidic protons, thus it was decided to use the tert-butylsilyl (TBS) protecting group in the lithiation substrate **V**. *O*-TBS bromobutenol **V** is lithiated to form organolithium reagent **VI**, which is then added to Weinreb amide **II** forming the unsaturated α,ß-ketone **VII**. Enone **VII** can then be directly epoxidized using hydrogen peroxide and DiPEA affording both diastereomers of epoxide **VIII**, which can be separated by silica gel column chromatography. After desilylation of epoxide **VIII**, the newly afforded hydroxyl of epoxide **X** can then be oxidized to form the carboxylic acid **XI**. The newly formed carboxylic acid can then be used as a substrate for the formation of amide bonds (**Scheme 1**).[16]



Scheme 1. Synthetic strategy for the installation of the carboxyethyl functional group in *N*-Boc amino acids. Reagents and conditions; (a) HCl•NMeOMe, HCTU, DiPEA, DCM, rt; (b) i: bromopropene, t-BuLi, THF, -78 °C; (c) H_2O_2, benzonitrile, DiPEA, MeOH, 0 °C; (d) i: *O*-tert-butylsilyl 3-bromobutenol, t-BuLi, THF, -78 °C; (e) H_2O_2, benzonitrile, DiPEA, MeOH, 0 °C; (f) HF•NEt_3, THF, 0 °C; (g) TEMPO, BAIB, MeCN/H_2O 0 °C -> rt. * after work-up the epoxide diastereomers are separated by silica gel column chromatography.[10,11,16–18]

The synthesis of the hydroxy methyl epoxyketone moiety in LU-005i-OH **1** was adapted from the work of Kim *et al.* and featured a modified version of the Morita-Baylis-Hillman reaction. While chapter three featured a modified Morita-Baylis-Hillman reaction, here it is proposed to repurpose the reaction for the synthesis of novel enone moieties. Traditional use of the Morita-Baylis-Hillman reaction yields a non-invasive way of introducing a C-C bond. The initial reaction step involves the use of triethylenediamine (DABCO) as a catalyst to activate enone **XII** forming the zwitterionic aza-enolate **XIII**. Upon formation of aza-enolate **XIII**, an aldol addition with the chosen aldehyde takes place followed by an intramolecular proton shift generating the final Morita-Baylis-Hillman adduct **XIX**. The catalyst is then released via an E2 elimination forming target enone **XX** (**Figure 2**).[12,13]

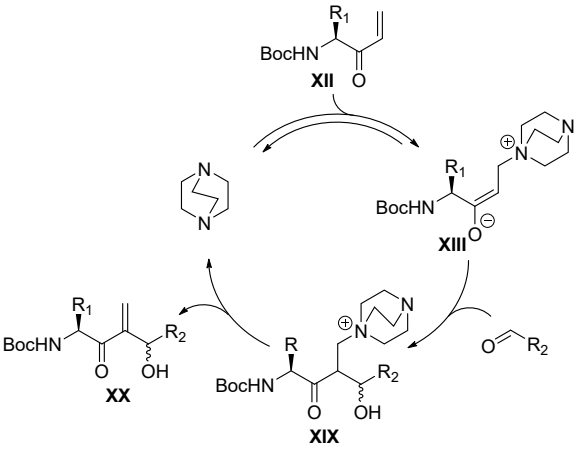
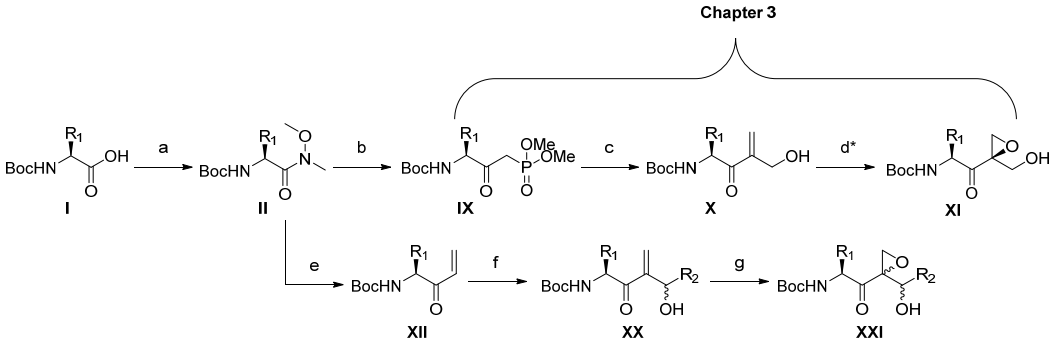


Figure 2. Generalized mechanism as proposed by Hill and Isaacs for the Morita-Baylis-Hillman reaction to give enone **XX**.[12,13]

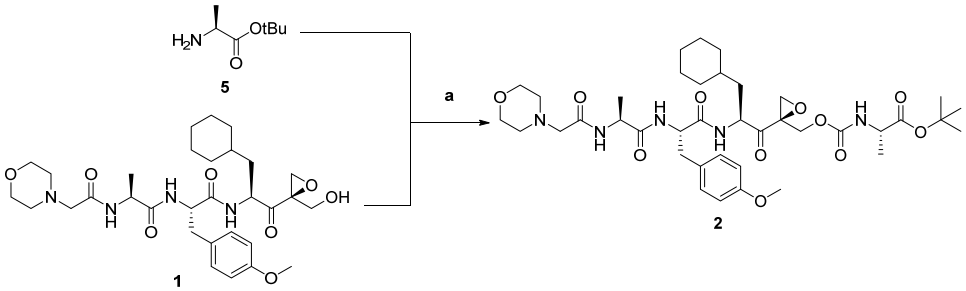
While chapter three used a modified Morita-Baylis-Hillman reaction to form the hydroxymethyl epoxyketone **XI**, here it is proposed to repurpose the synthesis found in chapter three and use the Morita-Baylis-Hillman reaction to form novel C-C bonds in amino acid epoxyketone derivatives.[14,15] As described in the previous chapters, Boc-protected amino acid **I**, with the desired and where relevant protected side chain residue, is condensed with *N,O*-dimethylhydroxylamine to Weinreb amide **II** (**Scheme 2**). Diverging from what was featured in chapter three a Grignard reaction is utilized to form the Morita-Baylis-Hillman substrate **XII**. α-ß Unsaturated ketone **XII** can then be transformed in to enone **XX** through a Morita-Baylis-Hillman reaction, which in turn can be epoxidized in to a diastereoselective manner using tert-butyl hydroperoxide and vanadyl acetylacetonate to afford

epoxide **XXI**. The Morita-Baylis-Hillman reaction yields both hydroxyl diastereomers, which can be separated by silica gel column chromatography. Identical to what was described in chapter two regarding diastereoselective epoxidations using the neighbouring hydroxyl as a chiral auxiliary, the newly formed hydroxyl in enone **XX** can be used for the final diastereoselective epoxidation (**Scheme 2**).[12,13]



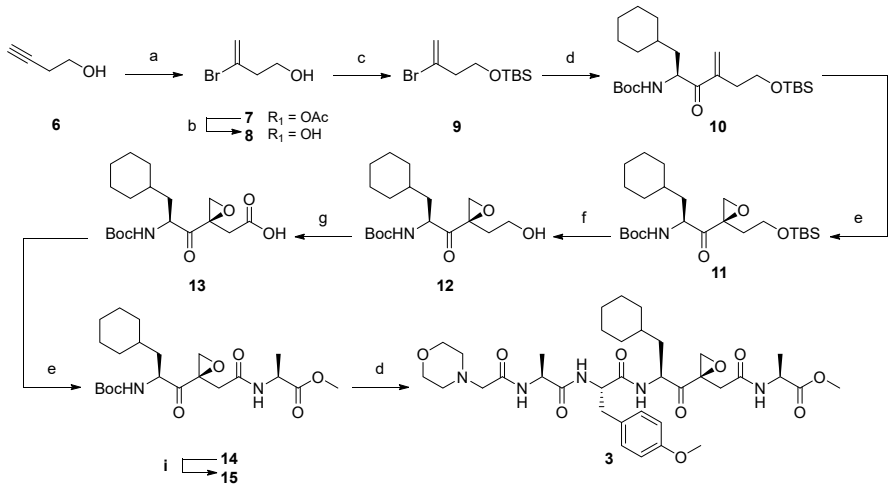
Scheme 2. Synthetic strategy for the formation of C-C bonds through the Morita-Baylis-Hillman reaction. Reagents and conditions; (a) HCl•NHMeOMe, HCTU, DiPEA, DCM, rt; (b) i: dimethylmethylphosphonate, n-BuLi, THF, -78 °C; ii: II, THF, -78 °C; (c) formaldehyde, K_2CO_3, H_2O/MeOH, 0 °C; (d) H_2O_2, benzonitrile, DiPEA, MeOH, 0 °C; (e) vinylmagnesiumbromide, THF, 0 °C; (f) HC=OR_2, DABCO, MeOH, rt; (g) t-BuOOH, Vo(acac)$_2$, 0 °C. * after work-up the epoxide diastereomers are separated by silica gel column chromatography.[13–15]

Chapter three described the synthesis of LU-005i-OH **1** which was used for the synthesis of carbamate **2**. The hydroxyl in **1** was activated using *N,N*-disuccinimidyl carbonate forming a succinimide carbonate intermediate, which after addition of O-tBu alanine **5** can be substituted to form target compound **2** (**Scheme 3**).



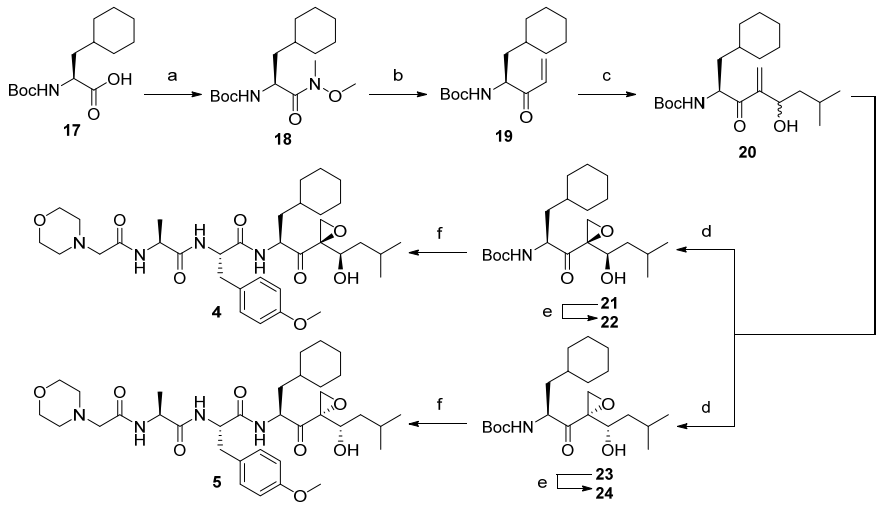
Scheme 3. Synthesis of carbamate **2**. Reagents and conditions: (a) i: N,N'-disuccinimidyl carbonate, NEt_3 , DMF, rt. ii: O-tBu alanine, 0 °C -> rt, 84%.

Using the methodology depicted in **Scheme 1**, epoxyketone **15** was prepared as follows. 3-butynol **6** was brominated using HBr in acetic acid, simultaneously acetylating the hydroxyl forming the *O*-Ac bromopropene **7**. Bromopropene **7** was subsequently deacetylated using K_2CO_3 in methanol affording bromopropene **8**, which was then protected with a tertbutylsilyl (TBS) group using TBS-Cl and pyridine as a base to form the *O*-TBS bromopropene **9**.[16] *O*-TBS bromopropene **9** was lithiated and added to Weinreb amide **18** affording the α,ß-unsaturated ketone **10**. Nucleophilic epoxidation of enone **10** using hydrogen peroxide yielded both diastereomers of epoxide **11**, which after silica gel column chromatography were isolated to give epoxide **11**. Desilylation of *O*-TBS epoxide **11** using hydrogen fluoride in complex with triethylamine afforded epoxide **12**. Subsequently, the hydroxyl of epoxide **12** was oxidated using (2,2,6,6-tetramethylpiperidinyl)oxidanyl (TEMPO) and bisacetoxyiodobenzene (BAIB) yielding carboxylic acid **13**. Carboxylic acid **13** was then condensed with *O*-Me alanine to give epoxide **14**. The *N*-Boc protective group of **14** was then removed using TFA in DCM, resulting in epoxyketone building block **15**. Acyl hydrazide **16** was then converted into the acyl azide intermediate using tert-butyl nitrite under anhydrous acidic conditions, after which epoxyketone **15** was added onto the acyl azide intermediate to form compound **3** (**Scheme 4**).



Scheme 4. Synthesis of P' amide substituted LU005i derivative **3**. Reagents and conditions: (a) HBr in AcOH, DCM, 40 °C; (b) K_2CO_3, MeOH, rt; (c) TBS-Cl, pyridine, DCM, rt, 72% over three steps; (d) i: t-BuLi, **7**, THF, -78 °C, ii: **18**, THF, -78 °C, 42%; (e) H_2O_2, DiPEA, benzonitrile, 0 °C, 39%; (f) HF•TEA, THF, 0 °C; (g) TEMPO, BAIB, MeCN:H_2O, 0 °C, 60% over two steps; (h) PyBOP, *O*-Me alanine, NMM, DCM, 85%; (i) TFA in DCM, rt; (j) i: **16**, tBuONO, HCl in dioxane, -40 °C, ii: **15**, DiPEA. -40 °C -> rt, 32%.

The synthetic strategy depicted in **Scheme 2** was directly applied for the synthesis of compounds **4** and **5** (**Scheme 5**). Identical to previously syntheses *N*-Boc cyclohexylalanine **17** was condensed with Weinreb salt to afford Weinreb amide **18**. This was then treated with the Grignard reagent, vinylmagnesium bromide, to give α,β-unsaturated ketone **19**.[19] α,β-unsaturated ketone **19** was then subjected to the Morita-Baylis-Hillman reaction using DABCO as a catalyst forming the diastereomeric enones **20**, which were separated through silica gel column chromatography. Subsequently, diastereoselective epoxidation, using vanadium acetylacetonate as a chiral directing agent and tert-butyl hydroperoxide as an epoxidizing agent, of enone **20** yielded epoxyketone **21**. Finally, de-*N*-Bocylation using TFA in DCM yielded epoxyketone building block **22**. Acyl hydrazide **16** was converted into the acyl azide intermediate using tert-butyl nitrite under anhydrous acidic conditions. After full conversion, as monitored through LC-MS, the acidic conditions were neutralized with DiPEA, followed by addition of epoxyketone **22** forming target compound **4**. Target compound **5** was synthesized in an identical fashion as **4** (**Scheme 5**).



Scheme 5. Synthesis of LU-005i derivatives **4** and **5**. Reagents and conditions: (a) HNMeOMe•HCl, HCTU, DiPEA, DCM, rt, 90%; (b) vinylmagnesiumbromide, THF, 0 °C, 80%; (c) isovaleraldehyde, DABCO, MeOH, rt, 40%; (d) tBuOOH, $Vo(Acac)_2$, DCM, 0 °C, 39%; (e) TFA in DCM, rt; (f) i: **16**, tBuONO, HCl in dioxane, -40 °C, ii: **22**/**24**, DiPEA, -40 °C -> rt, **4**: 34%, **5**: 40%. * After work-up the epoxide diastereomers were separated through silica gel column chromatography. [19]

Competitive activity-based protein profiling proteasome inhibition assay

The potency and selectivity profiles, as proteasome inhibitors, for the four new compounds depicted in **Figure 1** were determined using a competitive ABPP assay in Raji cell lysates (non-Hodgkin B-cell lymphoma cell line that expresses both cCPs and iCPs). Compounds **2**-**5** were tested in a concentration series ranging from 0.003 µM to 30 µM to determine the apparent inhibitory concentrations (IC_{50}). The resulting apparent IC_{50} values are depicted in **Figure 3** shown below.[6]

None of the inhibitors showed any potency for ß2c, ß2i, ß1c, ß1i and ß5c. Additionally, **3**, **4** and **5** also did not show any potency for ß5i. Inhibitor **2** was the only inhibitor found in **Figure 3** that showed any potency toward one of the cCP/iCP active site subunits, namely ß5i. In contrast to what was found in chapter three, where inhibitors featured amino acid esters at the P' position, the inhibitors found in this chapter showed low to no potency for any of the proteasomal active sites. This suggests that the S' pocket is not as accepting of substituents as first thought when reviewing the apparent inhibition profiles of chapter three.

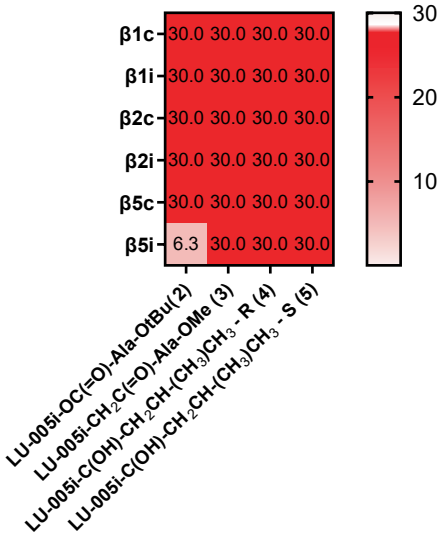


Figure 3. Inhibition profiles of LU-005i P1' analogues **2**-**5** in an IC_{50} (µM) heat map (white: potent; red: not potent). Apparent IC_{50} values in µM were determined by competitive ABPP in Raji lysates (N = 1). Chemical structures are shown in **Figure 1**.

Discussion

This chapter reports the synthesis of four new P1' featuring peptide epoxyketones, followed by the evaluation of their capacity in inhibiting the cCP/iCP active sites. Similar to the previous chapters of this thesis, the LU-005i scaffold was used as a lead and redesigned to feature novel P' moieties. Carbamoylation of LU-005i-OH **1** with *O*-tBu alanine yielded compound **2**, which when evaluated in a competitive activity-based protein profiling proteasome assay showed no potency for ß1c, ß1, ß2c, ß2i and ß5c. Compound **2** did however show potency for ß5i, be it rather weak when compared to inhibitors found in previous chapters. Additionally, the hydroxymethyl group found in LU-005i-OH **1** was homologated, oxidated, and condensed with *O*-Me alanine to yield inhibitor **3**, which showed no potency for either cCP- or iCP active sites. Finally, the Morita-Baylis-Hillman reaction featured in chapter three was successfully repurposed to give inhibitors **4** and **5**, which also did not show any potency for either cCP- or iCP active sites. Altogether and taking along the conclusions from the previous chapter, esterification of the secondary hydroxyl of LU005i-OH **1** appears the best way forward for the discovery of new, iCP-selective inhibitors. Further improvements may be found in merging structural features at P3 and P4 as identified in chapter two with the P'-modifications found in chapter three. The next chapter addresses such putative improvements in the search for iCP-selective inhibitors that target all three iCP active sites while leaving the cCP ones largely intact.

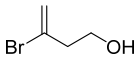
Acknowledgement

Dr. Elmer Maurits is kindly acknowledged for his contribution to the synthesis of the compounds featured in this chapter. Hans van Elst is kindly acknowledged for the HRMS measurements.

Experimental

General methods

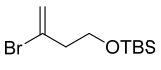
Solvents were purchased from Honeywell, VWR or Alfa Aesar. Water and oxygen sensitive reactions were performed under N_2 atmosphere. Solvents used in synthesis were, if necessary, dried, and stored over 4 å molecular sieves, except for pyridine, DIPEA and TEA which were stored over KOH pellets. Commercially available reagents were used without further purification, unless stated otherwise. TLC analysis was performed using aluminium sheets, pre-coated with silica gel (Merck, TLC silica gel 60 F254). Compounds were visualised by UV absorption (λ = 254 nm) or spraying with ninhydrin (0.7 g/L in ethanol) or cerium molybdate (25 g/L $(NH_4)_6Mo_7O_{24}$, 10 g/L $(NH_4Ce(SO_4)_4 \bullet H_2O$ in 10% H_2SO_4 solution), and where appropriate, followed by charring at 150 °C. Column chromatography was performed on screening devices b.v. silica gel (particle size 40-63 µm, pore diameter 60 å). Celite hyflo supercell (Merck) was used to impregnate reaction mixtures prior to silica gel chromatography when appropriate. ^1H, ^{13}C APT, ^1H Cosy and HSQC NMR spectra were recorded with a Bruker AV-400 (400/100 MHz) or AV-500 (500/125 MHz) spectrometer. Chemical shifts are reported in ppm (ð) and were referenced to an internal standard (TMS ð = 0.00 ppm) or the residual solvent peak. *J* couplings are reported in Hz. Liquid chromatography mass spectrometry analysis was performed on a Finnigan surveyor HPLC system with a nucleodur C18 Gravity 3 µm 50 x 4,60 mm column (detection at 200 – 500 nm) coupled to a Finnigan LCQ advantage max mass spectrometer with ESI or a Thermo LCQ Fleet ion mass spectrometer with ESI. The general method used was 10 to 90% with a total run time of 13.5 minutes. High resolution mass spectra were reported by direct injection (1,0 µM solution in H_2O/MeCN 1:1 and 0,1% formic acid) on a mass spectrometer (Thermo Fisher Exactive HF Orbitrap) equipped with an electrospray ion source in positive mode with resolution R = 240.000 at m/z 400 (mass range m/z = 160 – 2000) and an external lock mass. The high-resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).



3-bromo-3-butenol (8)

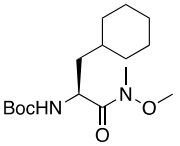
Tert-butyl ammonium bromide (20 mmol, 6.45 g, 2.0 eq) was dissolved in anhydrous DCM (0.3 M), cooled to 0 °C and purged with N_2. **HBr in AcOH** (33% volume percentage, 40 mmol, 6.58 ml, 4.0 eq) was added to this solution over a period of 15 min and the resulting reaction mixture was left stirring for 15 min. **But-3-ynol** (10 mmol, 0.757 ml, 1.0 eq) was added dropwise to the cooled solution, after which the reaction mixture was refluxed for 5 h under inert atmosphere. The resulting reaction mixture was cooled to 0 °C and quenched using $NaHCO_3$ (sat. aq.). The resulting mixture was

partitioned, washed with $NaHCO_3$ (sat. aq.), H_2O and brine (NaCl sat. aq.), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The resulting crude was dissolved in MeOH (0.1 M) and to this solution **K_2CO_3** (15 mmol, 2.073 g, 1.5 eq) was added. The resulting suspension was left stirring for 4 h. The suspended K_2CO_3 was filtered over a glass filter and the resulting MeOH was acidified using HCl (1.0 M, aq.). Subsequently, the aqueous layer was extracted with EtOAc three times. The resulting EtOAc layer was washed with H_2O and brine (NaCl sat. aq.), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The resulting crude was used without further purification.



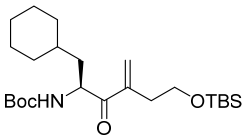
Br-C(CH_2CH_2OTBS)=CH_2 (9)

3-bromobut-3-enol (1.510 g, 10 mmol, 1.0 eq) was co-evaporated with toluene 3 times, dissolved in anhydrous DCM (0.1 M) and purged with N_2. The resulting solution was cooled to 0 °C and to this cooled solution were added **pyridine** (4.04 ml, 50 mmol, 5.0 eq) and **tert-butylsilyl chloride** (2.261 g, 15 mmol, 1.5 eq). The reaction mixture was left stirring at rt under N_2 atmosphere for 16 h. The reaction mixture was acidified with HCl (1.0 M aq.) and partitioned. The resulting DCM layer was washed with H_2O and brine (NaCl sat. aq.), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Silica gel column chromatography (PE -> 5:100 Et_2O:PE, v:v) yielded the title compound as a transparent liquid (7.20 mmol; 1.91 g; 72%). R_f: 0.4 in 100% PE. ^{1}H NMR (400 MHz, CDCl3) δ 5.64 (s, 1H), 5.46 (s, 1H), 3.80 (t, J = 6.3 Hz, 2H), 2.63 (t, J = 6.3 Hz, 2H), 0.92 (s, 8H), 0.09 (s, 7H). ^{13}C NMR (101 MHz, CDCl3) δ 131.0, 118.4, 60.9, 45.0, 18.4.



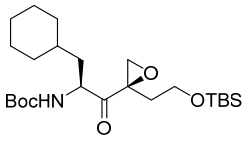
Boc-Cha-N(OCH_3)CH_3 (18)

Boc-Cha-OH (10 g, 36.9 mmol, 1 eq.) was dissolved in anhydrous DMF (0.1 M) and purged with N_2. To this solution **HCTU** (22.87 g, 55.3 mmol, 1.5 eq) and **N,O,-dimethylhydroxylamine** (7.19 g, 73.7 mmol, 2 eq.) were added. After reagents were fully dissolved **DiPEA** (25.7 ml, 147 mmol, 4 eq.) was added dropwise and the resulting yellow reaction mixture was left stirring for 16 hours. The reaction mixture was then acidified with HCl (1.0 M, aq.), washed with HCl (1.0 M, aq.), $NaHCO_3$ (sat., aq.) and brine (sat. NaCl, aq.), dried over $MgSO_4$ and concentrated *in vacuo*. Silica gel column chromatography (1:20 -> 1:4 EtOAc:PE) yielded the title compound as a transparent oil (10.43 g; 33.2 mmol; 90%). R_f: 0.3 in 1:4 EtOAc:PE. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.02 (d, *J* = 9.2 Hz, 1H), 4.87 – 4.55 (m, 1H), 3.78 (s, 3H), 3.20 (s, 3H), 1.91 (d, *J* = 12.6 Hz, 1H), 1.82 – 1.55 (m, 5H), 1.54 – 1.46 (m, 1H), 1.45 – 1.32 (m, 10H), 1.31 – 1.06 (m, 3H), 1.04 – 0.82 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.8, 155.8, 79.6, 61.7, 48.5, 40.7, 34.2, 34.1, 32.4, 32.3, 28.5, 26.6, 26.4, 26.2. LC-MS (linear gradient 10 to 90% MeCN/H2O, 0.1% TFA, 12.5 min) Rt (min): 8.40 min (ESI-MS (m/z): 314.80 ($M+H^+$)).



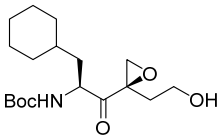
Boc-Cha-C(CH_2CH_2OTBS)=CH_2 (10)

O-TBS bromobutenol (7.96 g, 30.0 mmol, 3.0 eq.) was mixed with anhydrous THF (0.1 M) and cooled to -78 °C. To this cooled solution **t-BuLi** (1.7 M in pentane, 23.53 ml, 40.0 mmol, 4.0 eq.) was added dropwise over a period of 30 minutes. The resulting mixture was left stirring at -78 °C for 30 minutes. **Boc-Cha-N(OCH_3)CH_3** (3.14 g, 10.0 mmol, 1.0 eq.) was co-evaporated with toluene three times and dissolved in anhydrous THF (0.5 M) and purged with N_2. The solution containing Boc-Cha-N(OCH_3)CH_3 was added dropwise to the cooled carbolithiation reagent over a period of 30 minutes. The resulting mixture was left stirring for six hours at -78 °C. Following full conversion, the reaction mixture was quenched with NH_4Cl (sat., aq.) and left stirring overnight. The resulting suspension was diluted with EtOAc and extracted with EtOAc three times, washed with H_2O and brine (sat. NaCl, aq.) dried over $MgSO_4$ and concentrated *in vacuo*. Silica gel column chromatography (1:40 -> 1:8 Et_2O:PE) yielded the title compound as a transparent oil (2.86 g; 6.5 mmol; 65%). R_f: 0.3 in 1:20 Et_2O:PE. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.13 (s, 1H), 5.92 (s, 1H), 5.13 – 5.04 (m, 2H), 3.64 (t, *J* = 6.4 Hz, 2H), 2.59 – 2.49 (m, 1H), 2.41 (dt, *J* = 13.7, 6.7 Hz, 1H), 1.98 (d, *J* = 12.7 Hz, 1H), 1.74 – 1.46 (m, 7H), 1.41 (s, 13H), 1.29 – 1.04 (m, 6H), 0.85 (s, 15H), 0.00 (d, *J* = 3.2 Hz, 7H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 201.6, 155.7, 143.5, 127.5, 79.6, 61.7, 52.1, 41.8, 34.9, 34.4, 34.2, 32.0, 28.4, 26.5, 26.4, 26.2, 26.0, -5.2, -5.3.



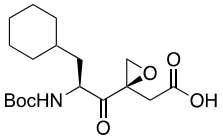
Boc-Cha-EK-CH_2CH_2OTBS (11)

Boc-Cha-C(CH_2CH_2OTBS)=CH_2 (0.59 g, 1.342 mmol, 1.0 eq) was dissolved in MeOH (0.1 M) and cooled to 0 °C. To this solution were added **H_2O_2** (30% in H_2O, 13.42 mmol, 0.822 ml, 10.0 eq), **DIPEA** (6.71 mmol, 1.172 ml, 5.0 eq) and **benzonitrile** (2.68 mmol, 0.274 ml, 2.0 eq) and the reaction was left stirring for 16 h. the reaction mixture was diluted with EtOAc, washed with HCl (1.0 M, aq.), H_2O and brine (NaCl sat., aq.), dried over $MgSO_4$, filtered and concentrated in *vacuo*. Column purification (1:20 -> 1:5 Et_2O:PE, v:v) yielded the both stereoisomers of the title compound as white crystals, (0.527 mmol; 0.24 g; 39%). R_f: 0.35 for F and 0.25 for B 1:20 EtO_2:PE. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.81 (d, *J* = 8.9 Hz, 1H), 4.29 (ddd, *J* = 11.3, 8.9, 2.7 Hz, 1H), 3.77 – 3.61 (m, 3H), 3.22 (d, *J* = 5.2 Hz, 1H), 2.96 (d, *J* = 5.1 Hz, 1H), 2.38 – 2.25 (m, 1H), 1.92 – 1.74 (m, 3H), 1.74 – 1.49 (m, 7H), 1.39 (s, 13H), 0.85 (s, 22H), 0.10 – -0.08 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 209.3, 155.8, 79.8, 59.9, 59.1, 51.4, 51.1, 38.8, 34.5, 34.3, 33.7, 31.9, 28.4, 26.5, 26.4, 26.1, 26.0, 18.4, -5.3.



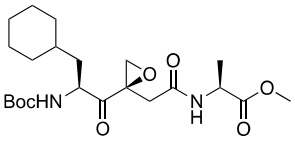
Boc-Cha-EK-CH_2CH_2OH (12)

Boc-Cha-EK-CH_2CH_2OTBS (0.137 g, 0.3 mmol, 1.0 eq) was co-evaporated with toluene three times, dissolved in anhydrous THF (0.1 M) and cooled to 0 °C. **$HF{\bullet}NEt_3$** (1.2 mmol, 0.195 ml, 4.0 eq) was added dropwise and the reaction mixture was left stirring for 16 h at 0 °C under inert atmosphere. To the reaction mixture was added $CaCO_3$, followed by diluting with $NaHCO_3$ (sat. aq.) and EtOAc, respectively. the resulting mixture was partitioned and the EtOAc layer was washed with $NaHCO_3$ (sat., aq.), H_2O and brine (NaCl sat., aq.), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The title compound was used crude in the following reaction.



Boc-Cha-EK-CH_2COOH (13)

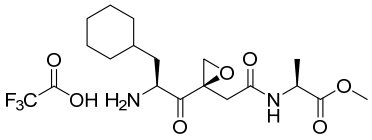
Boc-Cha-EK-CH_2CH_2OH (0.205 mmol, 0.070 g, 1.0 eq) was dissolved in a 1 to 1 ratio of MeCN and H_2O (0.2 M) and cooled to 0 °C. To this solution were added **TEMPO** (0.226 mmol, 0.035 g, 1.1 eq) and **BAIB** (0.226 mmol, 0.073 g, 1.1 eq) dan the resulting reaction mixture was left stirring for 12 hours at 0 °C. The reaction mixture was then acidified with HCl (1.0 M aq.) and partitioned, followed by extracting with EtOAc three times. The resulting organic layer was washed with HCl (1.0 M aq.), H_2O and brine (NaCl sat. aq.), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Column chromatography (1:10 -> 1:4 EtOAc:PE -> 1:8 -> 1:2 EtOAc:PE 1% AcOH, v:v) yielded the title compound as a white solid (0.123 mmol; 0.044 g; 60%). R_f: 0.5 1:6 EtOAc:PE + 1% AcOH. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.85 (d, *J* = 8.9 Hz, 1H), 4.33 (d, *J* = 9.8 Hz, 1H), 3.47 (d, *J* = 16.9 Hz, 1H), 3.34 (d, *J* = 5.0 Hz, 1H), 2.94 (d, *J* = 5.0 Hz, 1H), 2.30 (d, *J* = 17.0 Hz, 1H), 1.93 (dd, *J* = 32.1, 11.9 Hz, 3H), 1.82 – 1.47 (m, 11H), 1.41 (s, 13H), 1.35 – 1.05 (m, 20H), 0.98 (d, *J* = 8.7 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 208.8, 175.4, 155.8, 79.7, 65.5, 59.6, 50.9, 50.9, 38.7, 37.9, 37.1, 34.3, 34.3, 31.8, 29.8, 28.4, 27.7, 26.5, 26.4, 25.9, 20.2, 15.9.



Boc-Cha-EK-C(C=O)NH-Ala-OMe (14)

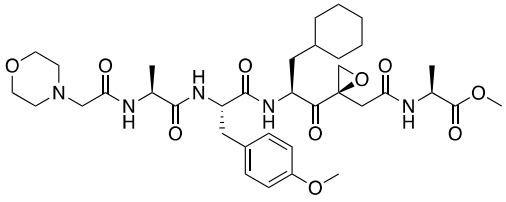
Boc-Cha-EK-CH_2COOH (0.098 mmol, 0.035 g) was co-evaporated with toluene three times and dissolved in anhydrous DCM (0.3 M). To this solution **NMM** (0.394 mmol, 0.043 ml, 4.0 eq) was added, followed by cooling the solution to 0 °C. Then **PyBOP** (0.118 mmol, 0.061 g, 1.2 eq) and **NH_2-Ala-OMe** (0.148 mmol, 0.029 ml, 1.5 eq) were added and the resulting reaction mixture was then left stirring until full conversion as monitored by LC-MS. The reaction mixture was then diluted with DCM, acidified with HCl (1.0 M aq.) and partitioned. The resulting DCM layer was washed with HCl (1.0 M aq.), $NaHCO_3$ (sat. aq.), H_2O and brine (NaCl sat. aq.), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Silica gel column chromatography (1:10 -> 4:1 EtOAc:PE, v:v)

yielded the title compound as a yellow oil (0.084 mmol; 0.037 g; 85%). LC-MS (linear gradient 10 to 90% MeCN/H_2O, 0.1% TFA, 12.5 min) Rt (min): 8.06 min (ESI-MS (m/z): 440.80 ($M+H^+$)).



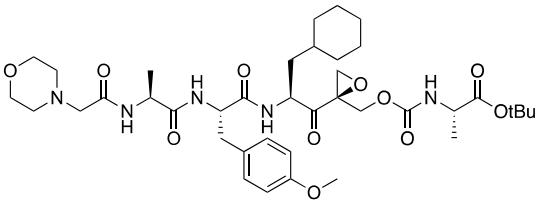
TFA●NH_2-Cha-EK-CH_2C(=O)NH-Ala-OMe (15)

Boc-Cha-EK-CH_2-C(=O)NH-Ala-OMe (0.037 g, 0.084 mmol) was dissolved in anhydrous DCM (2.0 ml), followed by dropwise addition of TFA (1 ml). The resulting reaction mixture was left stirring for two hours and upon full conversion was diluted with toluene and concentrated *in vacuo*, followed by co-evaporation with toluene three times. The resulting white solid was used crude in the next reaction.



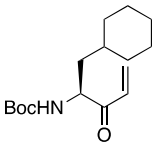
Morph-Ala-Tyr(OMe)-Cha-EK-C(=O)-Ala-OMe (2)

Morph-Ala-Tyr(OMe)-$NHNH_2$ (0.044 g, 0.108 mmol, 1.0 eq.) was co-evaporated with toluene two times, dissolved in anhydrous DMF (0.1 M), purged with N_2 and cooled to -40 °C. To this solution were added **tBuONO** (0.012 ml, 0.1 mmol, 1.2 eq.) and HCl (4.0 M in dioxane, 0.058 ml, 0.233 mmol, 2.8 eq.). The resulting reaction mixture was left stirring for four hours at -40 °C. After four hours the HCl was quenched with **DiPEA** (0.065 ml, 0.375 mmol, 5 eq.) and a solution of **NH_2-Cha-EK-C(=O)-Ala-OMe** (0.028 g, 0.083 mmol, 1.0 eq.) in anhydrous DMF (0.2 M) was added dropwise. The resulting reaction mixture was left stirring 16 hours, while over this period of time heating back to rt. The reaction mixture was diluted with H_2O and EtOAc, washed with $NaHCO_3$ (sat., aq.), H_2O and brine (sat. NaCl, aq.), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Silica gel column chromatography (0:100 -> 10:100 MeOH:DCM) yielded the title compound as a transparent oil (0.043 g; 0.60 mmol; 32%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (d, *J* = 7.4 Hz, 1H), 7.18 – 7.09 (m, 2H), 6.87 – 6.79 (m, 2H), 6.70 (d, *J* = 7.6 Hz, 1H), 6.38 (d, *J* = 7.5 Hz, 1H), 6.28 (d, *J* = 7.6 Hz, 1H), 4.66 – 4.48 (m, 3H), 4.43 (p, *J* = 7.1 Hz, 1H), 3.80 (s, 3H), 3.77 (s, 3H), 3.72 (t, *J* = 4.6 Hz, 4H), 3.42 (d, *J* = 4.9 Hz, 1H), 3.23 (d, *J* = 15.5 Hz, 1H), 3.06 (d, *J* = 4.9 Hz, 1H), 3.04 – 2.85 (m, 4H), 2.47 (dt, *J* = 10.5, 5.3 Hz, 5H), 1.85 – 1.52 (m, 10H), 1.40 (dd, *J* = 14.3, 7.1 Hz, 7H), 1.23 (d, *J* = 41.9 Hz, 11H), 1.08 – 0.79 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 207.2, 171.9, 170.9, 170.4, 167.7, 158.7, 130.6, 128.3, 114.1, 67.1, 61.7, 59.4, 55.3, 54.3, 53.9, 52.7, 49.9, 48.5, 48.2, 38.1, 36.7, 34.6, 34.0, 31.8, 29.8, 26.5, 26.1, 18.7, 17.7. HRMS (ESI) m/z: [$M+H^+$] calc for $C_{36}H_{53}N_5O_{10}$ 716.38652, found 716.38591



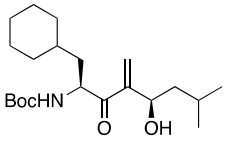
Morph-Ala-Tyr(OMe)-Cha-EK-$CH_2OC(=O)$NH-Ala-OMe (3)

LU-005i-OH (11,44 mg, 0.019 mmol) was dissolved in anhydrous DMF (0.05 M) and purged with N_2. Subsequently, **N,N'-disuccinimidyl carbonate** (5.84 mg, 0.022 mmol, 1.2 eq.) and **triethylamine** (13.2 µl, 0.095 mmol, 5.0 eq) were added. The resulting solution was cooled to 0C and left stirring until fully converted. After full conversion ***O*-tBu alanine** (10.35 mg, 0.057 mmol, 3.0 eq) was added and left stirring until fully converted. Subsequently, the reaction mixture was co-evaporated with heptane three times. Silica gel column chromatography (1:50 - > 1:33 MeOH:DCM) yielded the title compound as a white solid (12.24 mg; 15.83 µmmol; 84%).[1]H NMR (500 MHz, MeOD) δ 7.16 – 7.12 (m, 2H), 6.84 – 6.80 (m, 2H), 4.63 – 4.53 (m, 2H), 4.45 – 4.37 (m, 1H), 4.11 – 4.02 (m, 2H), 3.77 (s, 3H), 3.71 (t, *J* = 4.7 Hz, 5H), 3.28 (d, *J* = 5.0 Hz, 1H), 3.15 (d, *J* = 5.0 Hz, 1H), 3.10 – 2.94 (m, 6H), 2.89 – 2.79 (m, 3H), 2.52 – 2.44 (m, 4H), 1.87 – 1.60 (m, 8H), 1.52 – 1.44 (m, 13H), 1.38 – 1.28 (m, 15H), 1.00 – 0.87 (m, 3H). ^{13}C NMR (126 MHz, MeOD) δ 206.9, 174.9, 174.2, 173.5, 172.0, 159.9, 131.4, 130.1, 114.8, 67.9, 63.9, 62.4, 55.7, 54.7, 51.8, 51.2, 49.5, 38.1, 35.6, 35.1, 32.9, 30.8, 28.2, 27.6, 27.4, 27.1, 26.3, 18.7, 17.7.



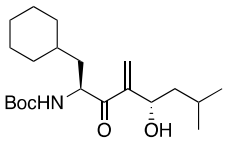
Boc-Cha-$CH=CH_2$ (19)

Boc-Cha-N(OMe)Me (1.57 g, 5.0 mmol, 1.0 eq) was co-evaporated with toluene two times, dissolved in anhydrous THF (0.5 M), purged with N_2 and cooled to 0 °C. To this cooled solution was added **vinylmagnesium bromide** (15.0 ml, 15.0 mmol, 3.0 eq) and the reaction mixture was left stirring for two hours. Upon full conversion the reaction mixture was poured to a cooled solution of HCl (2.0 M, aq.) and simultaneously diluted with EtOAc. The resulting biphasic system was poured to a separatory funnel and partitioned. The resulting HCl layer was extracted with EtOAc three times. The resulting combined EtOAc layers was washed with H_2O and brine (NaCl, sat., aq.), dried over $MgSO_4$, filtered and concentrated *in vacuo*. Silica gel column chromatography (1:30 -> 1:8 EtOAc:PE, v:v) yielded the title compound as a white solid (1.126 g; 4.0 mmol; 80%). R_f: 0.55 1:9 EtOAc:PE. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.50 – 6.26 (m, 2H), 5.82 (d, *J* = 10.1 Hz, 1H), 5.13 (d, *J* = 8.3 Hz, 1H), 4.64 (td, *J* = 9.1, 3.8 Hz, 1H), 1.89 (d, *J* = 12.0 Hz, 1H), 1.75 – 1.47 (m, 6H), 1.47 – 1.00 (m, 18H), 1.00 – 0.76 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 199.5, 155.6, 133.5, 129.8, 79.7, 55.1, 40.0, 34.2, 34.1, 32.6, 28.4, 26.4, 26.3, 26.1.



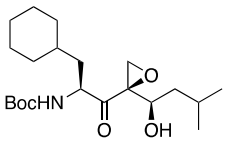
Boc-Cha-C(C-OH-$CH_2CH(CH_3)CH_3$)=CH_2 (20a)

Boc-Cha-CH=CH_2 (0.056 g, 0.2 mmol, 1.0 eq) was dissolved in MeOH (0.2 M), to this solution were added **DABCO** (0.011 g, 0.1 mmol, 0.5 eq) and **isovaleraldehyde** (0.065 ml, 0.6 mmol, 3.0 eq) and the resulting reaction mixture was left stirring until fully converted. After full conversion the reaction mixture was diluted with EtOAc, washed with HCl (1.0 M, aq.), H_2O and brine (NaCl, sat., aq.), dried over $MgSO_4$ and concentrated *in vacuo*. Silica gel column chromatography (1:20 -> 1:8 EtOAc:PE, v:v) yielded the title compound as a white solid (0.148 g; 0.05 mmol; 20%). R_f: 0.5 1:9 EtOAc:PE. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.19 (s, 1H), 6.07 (d, *J* = 1.0 Hz, 1H), 5.12 – 4.99 (m, 3H), 4.55 – 4.42 (m, 1H), 2.73 – 2.49 (m, 2H), 1.98 (d, *J* = 12.8 Hz, 2H), 1.91 – 1.48 (m, 16H), 1.48 – 1.33 (m, 24H), 1.33 – 1.04 (m, 22H), 1.03 – 0.68 (m, 18H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.3, 147.9, 126.3, 79.6, 70.4, 53.4, 45.4, 41.9, 34.4, 34.2, 32.5, 29.8, 28.4, 26.5, 26.4, 26.2, 24.9, 23.5, 21.9.



Boc-Cha-C(C-OH-$CH_2CH(CH_3)CH_3$)=CH_2 (20b)

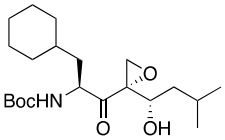
Boc-Cha-CH=CH_2 (0.043 g, 1.0 mmol, 2.0 eq) was dissolved in MeOH (0.2 M), to this solution were added **DABCO** (0.112 g, 1.0 mmol, 2.0 eq) and **isovaleraldehyde** (0.058 ml, 0.5 mmol, 1.0 eq) and the resulting reaction mixture was left stirring until fully converted. After full conversion the reaction mixture was diluted with EtOAc, washed with HCl (1.0 M, aq.), H_2O and brine (NaCl, sat., aq.), dried over $MgSO_4$ and concentrated *in vacuo*. Silica gel column chromatography (1:20 -> 1:8 EtOAc:PE, v:v) yielded the title compound as a white solid (0.074 g; 0.201 mmol; 20%). R_f: 0.3 1:9 EtOAc:PE. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.19 (s, 1H), 6.11 (s, 1H), 5.06 (s, 2H), 4.58 (dd, *J* = 9.4, 3.8 Hz, 1H), 1.98 (d, *J* = 12.3 Hz, 1H), 1.89 – 1.55 (m, 6H), 1.44 (s, 13H), 1.35 – 1.04 (m, 11H), 1.02 – 0.79 (m, 10H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.4, 155.7, 149.3, 125.1, 79.9, 69.4, 52.7, 45.7, 41.3, 34.4, 34.2, 32.4, 29.8, 28.4, 26.5, 26.4, 26.2, 24.9, 23.5, 21.8.



Boc-Cha-EK-C-OH-CH_2CH-$(CH_3)CH_3$ (21)

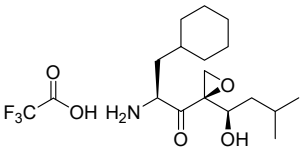
Boc-Cha-C(C-OH-$CH_2CH(CH_3)CH_3$)=CH_2 (0.059 g, 0.16 mmol, 1.0 eq) was co-evaporated with toluene two times, dissolved in anhydrous DCM (0.2 M), purged with N_2 and cooled to 0 °C. To this solution **VO(Acac)$_2$** (4.24 mg, 0.016 mmol, 0.1 eq) and **tBuOOH** (0.087 ml, 0.48 mmol, 3.0 eq) were added respectively. The resulting reaction mixture was left stirring for four hours. After full conversion the reaction mixture was diluted with DCM, washed with $NaHCO_3$ (sat., aq.), H_2O and brine (NaCl, sat., aq.), dried over $MgSO_4$ and concentrated *in vacuo*. Silica gel column chromatography (1:20 -> 1:8 EtOAc:PE) yielded the title compound as a white solid (0.06 g; 0.156 mmol; 65%). R_f: 0.2 1:9 EtOAc:PE. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.81 (d, *J* = 8.8 Hz, 1H), 4.36 – 4.25 (m, 1H), 3.81 (d, *J* = 8.3 Hz,

1H), 3.33 (d, *J* = 4.9 Hz, 1H), 3.05 (d, *J* = 4.8 Hz, 1H), 2.56 (d, *J* = 8.0 Hz, 1H), 1.95 – 1.80 (m, 3H), 1.78 – 1.51 (m, 15H), 1.40 (d, *J* = 13.6 Hz, 22H), 1.34 – 1.15 (m, 27H), 0.99 – 0.76 (m, 26H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 210.9, 165.5, 80.1, 69.7, 65.3, 62.8, 51.5, 50.5, 41.7, 38.1, 34.4, 33.8, 31.9, 30.6, 29.8, 28.4, 26.5, 26.4, 26.1, 24.4, 23.8, 21.6, 19.2, 13.8.



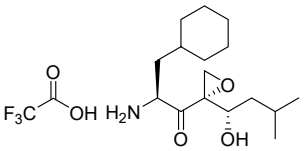
Boc-Cha-EK-C-OH-CH_2CH-(CH_3)CH_3 (23)

Boc-Cha-C(C-OH-CH_2CH(CH_3)CH_3)=CH_2 (0.074 mg, 0.201 mmol, 1.0 eq) was co-evaporated with toluene two times, dissolved in anhydrous DCM (0.2 M), purged with N_2 and cooled to 0 °C. To this solution **VO(acac)$_2$** (5.34 mg, 0.020 mmol, 0.1 eq) and **tBuOOH** (0.220 ml, 1.208 mmol, 6.0 eq) were added respectively. The resulting reaction mixture was left stirring for four hours. After full conversion the reaction mixture was diluted with DCM, washed with $NaHCO_3$ (sat., aq.), H_2O and brine (NaCl, sat., aq.), dried over $MgSO_4$ and concentrated *in vacuo*. Silica gel column chromatography (1:20 -> 1:8 EtOAc:PE) yielded the title compound as a white solid (0.06 g; 0.156 mmol; 78%). R_f: 0.2 1:9 EtOAc:PE. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.84 (d, *J* = 8.3 Hz, 1H), 4.66 – 4.50 (m, 1H), 4.03 (d, *J* = 9.8 Hz, 1H), 3.06 (d, *J* = 4.7 Hz, 1H), 2.94 (d, *J* = 4.7 Hz, 1H), 2.83 (s, 1H), 1.89 (ddt, *J* = 11.3, 4.6, 2.6 Hz, 2H), 1.79 – 1.53 (m, 7H), 1.50 – 1.04 (m, 27H), 1.04 – 0.68 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 209.3, 155.7, 80.4, 68.6, 53.5, 49.8, 41.2, 39.0, 34.2, 32.2, 29.8, 28.4, 26.5, 26.4, 26.1, 24.4, 23.8, 21.6.



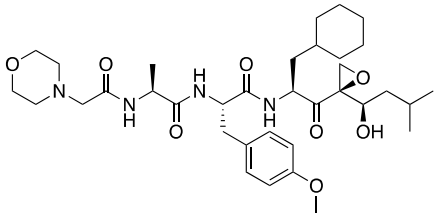
TFA●NH_2-Cha-EK-OH-CH_2CH-(CH_3)-CH_3 (22)

Boc-Cha-EK-OH-CH_2CH-(CH_3)-CH_3 (0.020 g, 0.123 mmol) was dissolved in anhydrous DCM (4 ml), followed by dropwise addition of TFA (1 ml). The resulting reaction mixture was left stirring for one hours and upon full conversion was diluted with Toluene and concentrated *in vacuo*, followed by co-evaporation with toluene three times. The resulting compound was used crude in the following reaction.



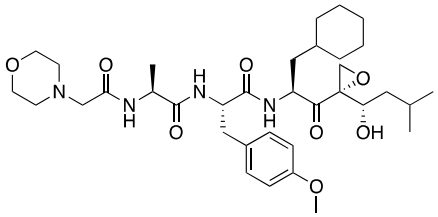
TFA●NH_2-Cha-EK-OH-CH_2CH-(CH_3)-CH_3 (24)

Boc-Cha-EK-OH-CH_2CH-(CH_3)-CH_3 (0.06 g, 0.16 mmol) was dissolved in anhydrous DCM (4 ml), followed by dropwise addition of TFA (1 ml). The resulting reaction mixture was left stirring for one hours and upon full conversion was diluted with Toluene and concentrated *in vacuo*, followed by co-evaporation with toluene three times. The resulting compound was used crude in the following reaction.



Morph-Ala-Tyr(OMe)-Cha-EK-OH-CH_2CH-$(CH_3)CH_3$ (4)

Morph-Ala-Tyr(OMe)-$NHNH_2$ (0.048 g, 0.119 mmol, 1.3 eq.) was co-evaporated with toluene two times, dissolved in anhydrous DMF (0.1 M), purged with N_2 and cooled to -40 °C. To this solution were added **tBuONO** (0.013 ml, 0.11 mmol, 1.2 eq.) and HCl (4.0 M in Dioxane, 0.064 ml, 0.256 mmol, 2.8 eq.). The resulting reaction mixture was left stirring for four hours at -40 °C. After four hours the HCl was quenched with **DiPEA** (0.072 ml, 0.411 mmol, 5 eq.) and a solution of **NH_2Cha-EK-OH-CH_2CH-(CH_3)-CH_3** (0.020 g, 0.123 mmol, 1.0 eq.) in anhydrous DMF (0.2 M) was added dropwise. The resulting reaction mixture was left stirring 16 hours, while over this period of time heating back to rt. The reaction mixture was diluted with H_2O and EtOAc, washed with $NaHCO_3$ (sat., aq.), H_2O and brine (sat. NaCl, aq.), dried over $MgSO_4$ and concentrated *in vacuo*. Silica gel column chromatography (0:100 -> 10:100 MeOH:DCM) yielded the title compound as a transparent oil (0.020 g; 0.031 mmol; 34%). R_f: 0.55 5:100 MeOH:DCM. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (d, *J* = 7.3 Hz, 1H), 7.11 (d, *J* = 8.7 Hz, 3H), 6.78 (dd, *J* = 13.9, 8.2 Hz, 4H), 6.43 (d, *J* = 7.8 Hz, 1H), 4.66 – 4.34 (m, 4H), 3.83 (d, *J* = 10.9 Hz, 1H), 3.77 (s, 4H), 3.70 (t, *J* = 4.7 Hz, 6H), 3.28 (d, *J* = 4.9 Hz, 1H), 3.05 (d, *J* = 5.0 Hz, 1H), 3.03 – 2.97 (m, 3H), 2.95 (s, 1H), 2.88 (s, 1H), 2.47 (dt, *J* = 11.0, 5.9 Hz, 5H), 2.18 (td, *J* = 7.0, 2.6 Hz, 1H), 1.93 – 1.82 (m, 1H), 1.82 – 1.50 (m, 9H), 1.50 – 1.39 (m, 3H), 1.36 (d, *J* = 7.1 Hz, 4H), 1.34 – 1.28 (m, 4H), 1.28 – 1.08 (m, 12H), 0.93 (dd, *J* = 12.8, 6.6 Hz, 10H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 209.2, 172.1, 170.9, 170.5, 158.7, 130.5, 128.3, 114.1, 69.4, 66.9, 63.0, 61.7, 55.3, 54.4, 53.9, 50.7, 50.4, 48.7, 41.6, 37.7, 36.7, 34.4, 34.0, 31.8, 29.8, 26.4, 26.4, 26.1, 24.4, 23.8, 21.6, 17.7. HRMS (ESI) m/z: [M+H$^+$] calc for $C_{35}H_{54}N_4O_8$ 659.40144, found 659.40086



Morph-Ala-Tyr(OMe)-Cha-EK-OH-CH_2CH-$(CH_3)CH_3$ (5)

Morph-Ala-Tyr(OMe)-$NHNH_2$ (0.075 g, 0.185 mmol, 1.5 eq.) was co-evaporated with toluene two times, dissolved in anhydrous DMF (0.1 M), purged with N_2 and cooled to -40 °C. To this solution were added **tBuONO** (0.018 ml, 0.148 mmol, 1.2 eq.) and HCl (4.0 M in Dioxane, ml, mmol, 2.8 eq.). The resulting reaction mixture was left stirring for four hours at -40 °C. After four hours the HCl was quenched with **DiPEA** (0.097 ml, 0.556 mmol, 4.5 eq.) and a solution of **NH_2Cha-EK-OH-Me-Me_2** (0.035 g, 0.123 mmol, 1.0 eq.) in anhydrous DMF (0.2 M) was added dropwise. The resulting reaction mixture was left stirring 16 hours, while over this period of time heating back to rt. The reaction mixture

was diluted with H_2O and EtOAc, washed with $NaHCO_3$ (sat., aq.), H_2O and brine (sat. NaCl, aq.), dried over $MgSO_4$ and concentrated *in vacuo*. Silica gel column chromatography (0:100 -> 10:100 MeOH:DCM) yielded the title compound as a transparent oil (0.033 g; 0.050 mmol; 40%). R_f: 0. 5:100 MeOH:DCM. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (d, *J* = 7.2 Hz, 1H), 7.13 (d, *J* = 8.7 Hz, 2H), 6.81 (d, *J* = 8.7 Hz, 3H), 6.47 (d, *J* = 7.4 Hz, 1H), 4.72 (ddd, *J* = 10.9, 7.3, 3.8 Hz, 1H), 4.63 – 4.54 (m, 1H), 4.45 – 4.35 (m, 1H), 4.12 (dd, *J* = 10.2, 3.0 Hz, 1H), 3.77 (s, 4H), 3.71 (t, *J* = 4.6 Hz, 5H), 3.10 – 2.98 (m, 4H), 2.96 (d, *J* = 2.4 Hz, 2H), 2.89 (d, *J* = 5.6 Hz, 1H), 2.85 (d, *J* = 4.6 Hz, 1H), 2.47 (q, *J* = 4.3 Hz, 5H), 2.18 (s, 3H), 1.91 – 1.38 (m, 10H), 1.36 (d, *J* = 7.1 Hz, 5H), 1.20 (d, *J* = 42.9 Hz, 12H), 0.94 (dd, *J* = 6.7, 4.6 Hz, 10H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 207.1, 172.2, 170.8, 170.5, 158.7, 130.6, 128.3, 114.1, 67.8, 66.9, 64.2, 61.7, 55.3, 54.4, 53.9, 52.7, 49.5, 48.8, 40.7, 40.3, 38.4, 36.6, 34.2, 34.0, 32.1, 29.8, 26.4, 26.3, 26.1, 24.4, 23.8, 21.5, 17.8. HRMS (ESI) m/z: [M+H$^+$] calc for $C_{35}H_{54}N_4O_8$ 659.40144, found 659.40112

Biochemical methods

Lysates of cells were prepared by treating cell pellets with an addition of two volumes of lysis buffer, containing 250 mM sucrose, 50 mM Tris pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, 10% (v:v) glycerol, 2 mM ATP, 0.05% (w:v) digitonin and 25 U/ml benzonase, resting for 1 hour on ice, followed by centrifuging for 15 minutes at 15 °C on 22000 rcf. Protein concentrations were then determined using a Bradford assay, followed by diluting cell lysates with assay buffer.

Competitive activity-based protein profiling assay

Cell lysates were diluted to 2µg/µL total protein in an assay buffer containing 50 mM Tris pH 7.5, 2 mM DTT, 5 mM MgCl2, 10% glycerol and 2 mM ATP. Cell lysates were then exposed to an inhibitor dilution series (30 µM, 10 µM, 3 µM, 1 µM, 0.3 µM, 0.1 µM, 0.03 µM, 0.01 µM, 0.003 µM diluted in DMSO) for one hour at 37 °C. Additionally, inhibited cell lysates were exposed to a probe cocktail (Cy2: BODIPY(FL)-LU-112, Cy3: BODIPY(TMR)-NC-005VS, Cy5: Cy5-NC-001) for one hour at 37 °C. Next, cell lysates were denatured addition of a reducing gel loading buffer and boiling at 95 °C for four minutes. Subsequently, denatured cell lysates were loaded on a 12.5% SDS-PAGE gel and fractioned through gel electrophoresis for 90 minutes at 160 V. Directly after electrophoresis, multiplex fluorescent detection of residual activity-based probes was performed on a ChemiDoc™ MP System with Cy5, Cy3 and Cy2 channels. The gel was then fixed and stained in Coomassie Blue Stain overnight, followed by destaining in demineralized water for two days. Likewise, Coomassie blue detection was performed on the ChemiDoc™ MP system and transformed in Image Lab Adjusted intensities of

fluorescent bands were transformed with Image Lab. The normalized adjusted volume was plotted against inhibitor concentrations and then the IC_{50} values per inhibitor were calculated using GraphPad Prism 9.0 software (using a non-linear regression, [Inhibitor] vs. normalized response with a variable slope, model).

References

1. P. Morea, J. L. Harousseau. Proteasome inhibitors in multiple myeloma: 10 years later, *Blood.* **2012**, *120*, 947-959.
2. E. Scalzulli, S. Grammatico, F. Vozella, M. T. Petrucci. Proteasome inhibitors for the treatment of multiple myeloma, *Exp Opin Pharmacol.* **2018**, *19*, 375-386.
3. E. E. Manasanch, R. Z. Orlowski. Proteasome inhibitors in cancer therapy, *Nat. Rev. Clin. Oncol.* **2017**, *14*, 417-433.
4. A. Besse, L. Besse, M. Kraus, M. Garcia Lopez, C. Driessen. Proteasome inhibition in multiple myeloma: head-to-head comparison of currently available proteasome inhibitors, *Cell Chem. Biol.* **2019**, *26*, 340-351.
5. G. de Bruin, Chemical tools to monitor and control human proteasome activities. Thesis, Leiden University, **2016**.
6. G. de Bruin, H.S. Overkleeft. A set of activity-based probes to visualize human (immuno)proteasome activities, Angew. Chem. Int. Ed. **2016**, *55*, 4199-4203.
7. H. W. B. Johnson, D. L. McMinn. Required immunoproteasome subunit inhibition profile for anti-inflammatory efficacy and clinical candidate KZR-616 ((2 S,3 R)- N-((S)-3-(cyclopent-1-en-1-yl)-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)-3-hydroxy-3-(4-methoxyphenyl)-2-((S)-2-(2-morpholin, *J. Med. Chem.* **2018**, *61*, 11127-11143.
8. E. M. Huber, M. Groll. A nut for every bolt: Subunit-selective inhibitors of the immunoproteasome and their therapeutic potential, *Cells.* **2021**, *10*, 1929.
9. E. M. Huber, M. Groll. Inhibitors for the immuno- and constitutive proteasome: Current and future trends in drug development, Angew. Chem. Int. Ed. **2012**, *51*, 8708-8720.
10. G. de Bruin, H. S. Overkleeft. Structure-based design of β1i or β5i specific inhibitors of human immunoproteasomes, *J. Med. Chem.* **2014**, *57*, 6197-6209.
11. C. Maurits, Targeting human proteasomes: substrates, inhibitors and prodrugs. Thesis, Leiden University, **2020**.
12. N. S. Camilo, F. Coelho. An improved protocol for the morita-baylis-hillman reaction allows unprecedented broad synthetic scope, *Eur. J. Org. Chem.* **2022**, *e202101448*.
13. R. Robiette, V. K. Aggarwal, J. N. Harvey. Mechanism of the Morita-Baylis-Hillman reaction: A computational investigation, *J. Am. Chem. Soc.* **2007**, *129*, 15513-15525.
14. M. J. Lee, K. B. Kim. Development of novel epoxyketone-based proteasome inhibitors as a strategy to overcome cancer resistance to carfilzomib and bortezomib, *J. Med. Chem.* **2019**, *62*, 4444-4455.
15. A. Ho, K. Cyrus, K. B. Kim. Towards immunoproteasome-specific inhibitors: an improved synthesis of dihydroeponemycin, *Eur. J. Org. Chem.* **2005**, 4829-4834.
16. B. V. Subba Reddy, S. Gopal Reddy, M. Ramana Reddy, M. Pal Bhadra, A. V. S. Sarma. Tandem Prins/pinacol reaction for the synthesis of oxaspiro-decan-1-one scaffolds, *Org. Biomol. Chem.* **2015**, *12*, 7257-7260.
17. B. T. Xin, H. S. Overkleeft. Structure-based design of β5c selective inhibitors of human constitutive proteasomes, *J. Med. Chem.* **2016**, *59*, 7177-7187.

18. N. Sin, C. M. Crews. Total synthesis of the potent proteasome inhibitor epoxomicin: a useful tool for understanding proteasome biology, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2283-2288.
19. P. Wisse, The synthesis of chemical tools for studying sphingolipid metabolism. Thesis, Leiden University, **2018**.