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Structure-Based Design and Synthesis of Potential Inhibitors of DPP III

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Für meinen Sohn Jakob

Abstract

Dipeptidyl-peptidase III (DPP III), an endogenous peptide degrading enzyme, has been found involved in important biological processes in the organism, such as pain signalling, inflammation, defence against oxidative stress and blood pressure regulation. However, the precise role is still unclear. For further research in an ongoing project in collaboration with biochemists and structural biologists, a specific inhibitor of DPP III is needed as a molecular tool compound. This work deals with the synthesis and characterization of new transition-state mimicking inhibitors of DPP III that are based on the structures of the neuropeptide enkephalin and the known inhibitor tynorphin. Four potential inhibitors have been synthesized in different synthetic routes. During the first synthetic approach, a new method for the reduction of *N*-protected α-amino acids to chiral α-amino aldehydes has been developed. The target molecules of the second synthetic strategy have successfully been demonstrated to be medium- to low-μM inhibitors of hDPP III *in vitro*.

Kurzfassung

Das endogene Enzym Dipeptidylpeptidase III (DPP III), welches Peptide abbauen kann, wurde in Zusammenhang mit wichtigen biologischen Prozessen wie Schmerzleitung, Entzündung, Abwehr von oxidativem Stress sowie Blutdruckregulierung gebracht. Die genaue Rolle ist jedoch noch immer unklar. Für weiterführende Forschung in einem laufenden Projekt in Kooperation mit Biochemikern und Strukturbiologen wird ein spezifischer Inhibitor von DPP III als molekulares Werkzeug benötigt. Diese Arbeit beschäftigt sich mit der Synthese und Charakterisierung von neuen Übergangszustandnachahmenden Inhibitoren von DPP III, welche auf den Strukturen des Neuropeptids Enkephalin sowie des bekannten Inhibitors Tynorphin basieren. Vier potentielle Inhibitoren wurden in unterschiedlichen Syntheserouten hergestellt. Während der ersten Synthese wurde eine neue Methode zur Reduktion von *N*-geschützten α-Aminosäuren zu chiralen α-Aminoaldehyden entwickelt. Es konnte erfolgreich *in vitro* gezeigt werden, dass die Zielmoleküle der zweiten Synthesestrategie Inhibitoren von hDPP III in mittel- bis niedrig-mikromolarer Konzentration sind.

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1 Introduction

The understanding of the exact role of specific enzymes in the organism is a major goal in modern life sciences. Many enzymes, beside cell surface receptors, nuclear hormone receptors, ion channels, transporters and DNA, are used as targets for various drugs. The large majority of those drugs work by inhibiting one specific enzyme, just a few inhibit more than one simultaneously.[1]

Most of the inhibitors are related to the naturally occurring ligand of the enzyme, which may be synthetic peptides, peptide mimetics or natural peptides. Stable molecules which are analogues of transition states of an enzyme-substrate complex, bind more tightly to the enzyme than the substrate itself. For many therapeutically important enzymes, such as ACE or HIV proteases, transition-state inhibitors are available.[1–4]

Enzyme inhibitors are not only limited for therapeutic use, but they are also used as pesticides and herbicides,[5,6] antiseptics,[7] and as molecular tools for research in biochemistry and pharmacology.[8]

Dipeptidyl-peptidase III, a zinc-dependent aminopeptidase, has been found in most tissues of mammals and also in insects.[9,10] The enzyme is able to hydrolyse dipeptides from the *N*-terminus of oligopeptides in an entropy-driven process.[11] Although no direct information about the biological role of DPP III has been encountered, there are indirect indications of its role in a variety of specific functions.[12–14] Over the past few decades, the involvement of hDPP III in pain signalling, inflammation, in the defence against oxidative stress, as well as in blood pressure regulation was reported. However, the precise function is still unclear.[9]

For further insight in the exact function of DPP III and its usage as a potential target for new drugs, a specific inhibitor is needed as a molecular tool. Although there are about 50 inhibitors of different classes known for DPP III, they are either nonselective, toxic, or unstable under physiological conditions.[9] Two important peptide inhibitors are the endogenous spinorphin (LVVYPWT), and its truncated synthetic analogue tynorphin (VVYPW). However, both of them are slowly degraded by DPP III.[14] In order to elude that problem, this work focuses on the development of new transition-state mimetics, which can be used for further *in vitro* and *in vivo* studies.

2 Theoretical background

2.1 Dipeptidyl-peptidase III – a peptide cutting enzyme

The enzyme dipeptidyl-peptidase III (DPP III, DPP3, EC 3.4.14.4), also known as *enkephalinase B* and *red cell angiotensinase*, was investigated for the first time together with other peptidases by S. Ellis and J. M. Nuenke during the 1960s and reported as *dipeptidyl arylamidase III*. It is the only known member of the M49 family of metallo-peptidases. DPP III shows the characteristic and unique catalytic motif HEXXGH and a second motif EEXRAE/D. These highly conserved motifs of the upper domain hold a zinc ion, which is a critical factor for its catalytic activity (Figure 1a).[9,14,15]

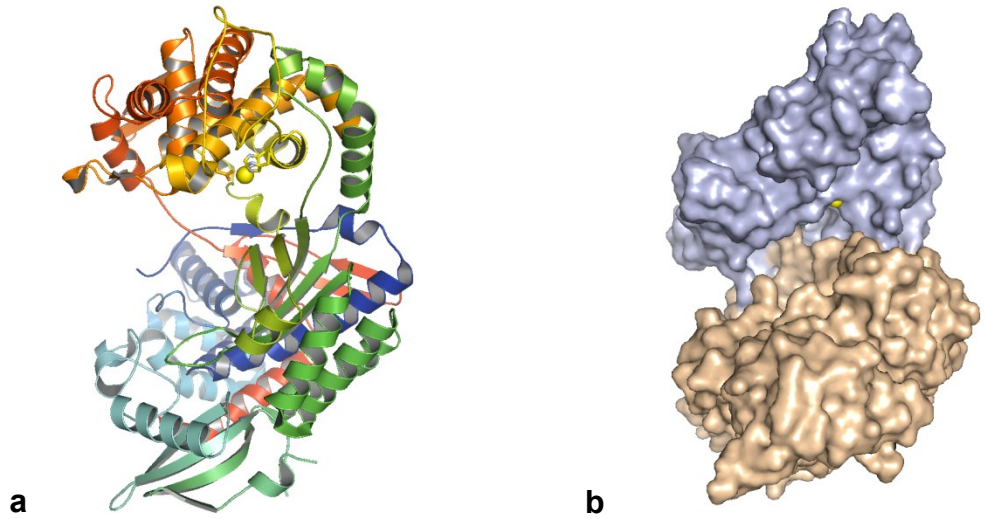


Figure 1: a) Overall ribbon diagram of hDPP III. The zinc ion, which is complexed by the amino acid residues His-450, His-455 (both yellow) and Glu-508 (orange) and a water molecule, is displayed by a yellow sphere. b) Surface representation of the two domains. (PDB: 3FVY, Dong *et al.*[16] The figures were drawn using PyMOL).

DPP III is able to hydrolyse dipeptides from the *N*-terminus of oligopeptides with 3–10 amino acids, however, substrates containing 4–8 amino acid residues are preferred by the enzyme.[9,14] A screening of different synthetic dipeptidyl-β-naphthylamides (βNA) revealed a high affinity to Arg-Arg-βNA, which gets hydrolysed to Arg-Arg and βNA.[15] Therefore, Arg-Arg-βNA has been used as a specific substrate for DPP III in biological assays and is still in use today.

DPP III contains two domains separated by a large cleft, where the substrate can bind to it. The first structural insights to the binding mode of yDPP III from *Saccharomyces cerevisiae* were made by Baral *et al.* in 2008 via X-ray crystallography (see chapter 2.4).[17]

DPP III has been isolated from a large variety of prokaryotic and eukaryotic species and tissues, which indicates the biological significance of the enzyme. Beside the ubiquitous distribution, a broad specificity for different oligopeptides indicates a general role in peptide catabolism.[9,14] However, many hints for more specific functions have been found. In the early 1980s, Lee and Snyder purified cytosolic and membrane-bound DPP III from rat brain, which showed the highest affinity for enkephalins (Leu-enkephalin and Met-enkephalin) and angiotensins (angiotensin II and III). Thus, the relevance of the enzyme in pain signalling and cardiovascular processes is high. In 2000, Hashimoto *et al.* reported DPP III activity in human neutrophils, indicating a role in the inflammatory mechanism.[18] Another publication reported that DPP III from *Drosophila melanogaster* may be involved in a major degradation pathway of proctolin, which is an insect neuropeptide.[19] Other studies revealed the association of overexpressed hDPP III in ovarian primary carcinoma[20] and other malignant gynaecological tissue,[21] which may protect tumour tissue against oxidative stress.[22] Therefore, DPP III seems to be a potential target for the development of new drugs in pain therapy, targeted therapy and therapy against cardiovascular diseases.

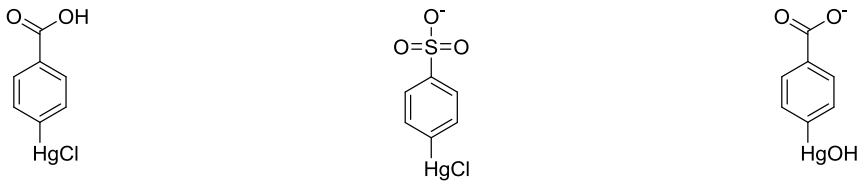
DPP III does not hydrolyse bradykinin (1–5), where the bond between Pro-Pro would have to be cleaved, neurotensin and dynorphin A-(1–13) (more than 10 amino acids), large peptides as well as proteins.[23,24]

2.2 Known inhibitors of DPP III

Inhibition of DPP III can be achieved with a large variety of different compounds. As a metallopeptidase, simple metal chelators such as EDTA can be used for inhibition; however, high concentrations and relatively long incubation times are necessary.[14,25] Interestingly, rat skin DPP III was not affected by EDTA, probably due to the low EDTA concentrations used.[9,23] Complete loss of functionality can be achieved by removing the zinc ion, which has a dissociation constant of 2.5×10^{-13} M at pH 7.4.[26] Dialyzing the apoenzyme against buffer containing various metal ions such as Co^{2+}, Ni^{2+}, Cu^{2+} can completely recover the enzyme activities; moreover, 10–500 µM Co^{2+} leads to an increased activity (2.5- to 11-fold).[14,27,28] As an interesting fact, high concentrations of these metal ions have inhibitory activity to the enzyme. The heavy metal ions Hg^{2+} and Cd^{2+} are potent inhibitors; a complete loss of activity could be achieved by a concentration of 0.04 mM Hg^{2+}.[25] Of course, these compounds cannot inhibit DPP III selectively.

Other inhibitors of DPP III can be found among serine peptidase-, cysteine peptidase- and aminopeptidase inhibitors within a large range of inhibition potency. Due to the highly reactive cysteine residues at the substrate binding sites, organomercury com-

pounds such as *p*CMB, *p*CMS and *p*HMB (IC_{50} = 3 × 10^{-9} µM; rat erythrocytes)[9] and other thiol reagents are very potent inhibitors of DPP III (Figure 2).[29]



***p*CMB** ***p*CMS** ***p*HMB**

Figure 2: Mercury containing compounds are potent inhibitors, but non-selective and irreversible.

Serine peptidase inhibitors also have inhibitory effect on DPP III, although no serine residue participates in catalysis. Only little or even no inhibition was achieved with different aminopeptidase inhibitors such as captopril, an important ACE inhibitor (Figure 10), or puromycin.[9] Fluostatins A and B (Figure 3), isolated from *Streptomyces* sp. TA-3391 by Akiyama *et al.*, inhibited placental hDPP III potently. The IC_{50} values have been reported with 0.44 and 0.24 µg/mL, respectively. Fluostatin A showed both a competitive and non-competitive inhibition with Leu-enkephalin as a substrate. Other dipeptidyl-peptidases had just slightly been affected by fluostatins A and B.[30]

fluostatin A **fluostatin B**

Figure 3: Fluostatins A and B, produced by *Streptomyces* sp. TA-3391, are potent inhibitors of hDPP III. Fluostatin A showed a mixed type inhibition.[30]

So far, the most potent inhibitor of all enkephalin-degrading peptidases, including DPP III, is kelatorphan (K_i = 1.4 nM),[31] and the most potent selective inhibitor reported yet is propioxatin A, an *N*-acylated dipeptide, which inhibits with K_i = 13 nM.[32–34] Both of them contain a hydroxamic acid moiety (Figure 4).

kelatorphan **propioxatin A**

Figure 4: Two nM inhibitors of DPP III. Unfortunately, kelatorphan (left; K_i = 1.4 nM) is non-selective for DPP III. Propioxatin A (right; K_i = 13 nM) was reported to be selective for DPP III and also very potent, but it needs an effortful isolation from *Kitasatosporia setae*.[33]

2.2.1 Peptide inhibitors

The fact, that peptide degrading enzymes can be inhibited by peptides, seems to be surprising at first glance. Those peptides are degraded slowly by the enzyme; therefore they can be considered as slowly-converted substrates as well as competitive inhibitors.[14] The only peptide inhibitor reported, which was not cleaved by DPP III at all is hisprophen (HPFHLlVY).[35] Dieptide fragments of the hydrolysed substrates itself may also inhibit the enzyme potently.[15,24] Table 1 summarizes some of the known peptide inhibitors of DPP III and their inhibitory potency.

Table 1: Known peptide inhibitors of DPP III. Table modified from S. C. Prajapati and S. S. Chauhan.[9]

Entry	Peptide inhibitor	K_i or IC_{50}	DPP III source	References
1	LVVYPWT (spinorphin)	IC_{50} = 6.67 ± 0.53 µM	rDPP III	[36]
2	VVYPW (tynorphin)	IC_{50} = 2.73 ± 0.44 µM	rDPP III	[13,36]
3	VVYPWTQ (valorphin)	K_i = 0.049 ± 0.006 µM	hDPP III	[37]
4	IVYPW	IC_{50} = 0.158 ± 0.022 µM	rDPP III	[36]
5	WVYPW	IC_{50} = 0.244 ± 0.022 µM	rDPP III	[36]
6	FVYPW	IC_{50} = 0.280 ± 0.032 µM	rDPP III	[36]
7	AVYPW	IC_{50} = 2.70 ± 0.36 µM	rDPP III	[36]
8	Tyr-Tyr	K_i = 5.8 ± 0.5 µM	rat brain DAP III	[24]
9	Tyr-Phe	K_i = 8.4 ± 3.6 µM	rat brain DAP III	[24]
10	Leu-Arg	K_i = 9.8 ± 0.1 µM	rat brain DAP III	[24]

2.2.2 Spinorphin and tynorphin – the lead structures for further research

The heptapeptide spinorphin (LVVYPWT), an endogenous inhibitor of DPP III, was isolated for the first time in 1993 from bovine spinal cord by K. Nishimura and T. Hazato. Spinorphin shows potent inhibitory activity against different enkephalin-degrading enzymes (aminopeptidase, dipeptidyl-aminopeptidase III, enkephalinase) as well as ACE.[38] Spinorphin shows an antinociceptive and anti-inflammatory effect *in vivo*.[12]

In search of more effective inhibitors, Yamamoto *et al.* reported tynorphin (VVYPW) as the more potent, truncated form of spinorphin. The inhibitory activity toward other enkephalin-degrading enzymes was not as high as for DPP III, so tynorphin was suggested to be a specific inhibitor.[13]

Unfortunately, both inhibitors are slowly hydrolysed by the enzyme,[13,14] which makes further research for a specific inhibitor necessary, that is stable under physiological conditions.

2.3 Assumed physiological importance of DPP III

2.3.1 Protein turnover

Although DPP III has been studied for 50 years now, and evidence for the involvement in various physiological and pathophysiological processes has been discovered, no specific role could be assigned. However, a general contributing role of DPP III as a peptide-degrading enzyme in intracellular catabolism is very likely. A variety of enzymes in the organism hydrolyse peptides (and proteins) of different lengths into smaller fragments in the cytosol. These resulting peptides are called peptidome and consist of 3–24 amino acids. The shorter peptides are subsequently hydrolysed to single amino acids that can be re-utilized for the synthesis of new peptides and proteins or catabolized to generate cellular energy. Peptide fragments of the peptidome consisting of 4–8 amino acids have the optimal substrate length for DPP III. Figure 5 summarizes the role of DPP III in the catabolic process.[9,39]

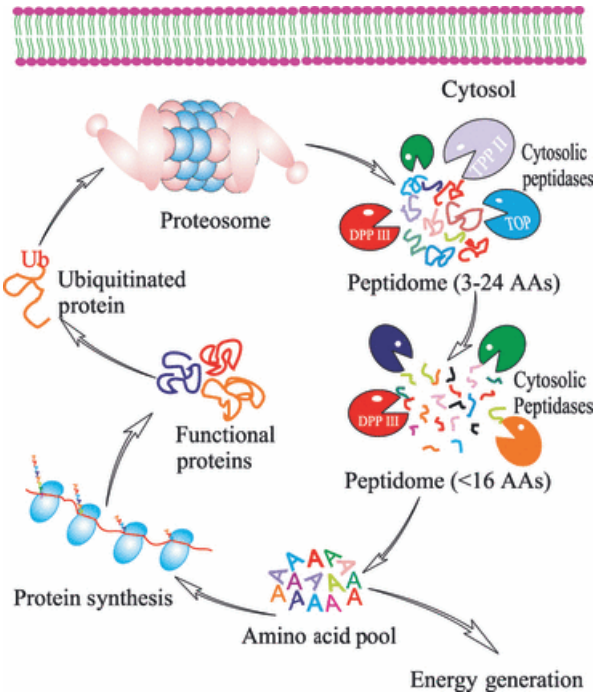


Figure 5: The role of DPP III in terminal stages of protein turnover. Copyright © 2011 by John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.: S. C. Prajapati, S. S. Chauhan, *FEBS Journal* **2011**, *278*, 3256–3276.[9]

DPP III may either hydrolyse peptides of the optimal length directly or peptide fragments from larger peptides that have been degraded by other enzymes such as tri-

peptidyl-peptidase II (TPP II; removes tripeptides from *N*-terminus of peptides >15 amino acids) or thimet oligopeptidase (TOP; cleaves peptides with hydrophobic amino acids from *C*-terminus in the range of 6–17 amino acids).[9,40,41]

2.3.2 Nociception and the role of enkephalin in pain signalling pathways

A pain signal caused by an injury or chronic inflammation causes the release of chemokines and is detected by peripheral nociceptive nerve endings (Figure 6; left box). The noxious message travels through the primary afferent neuron into spinal cord dorsal horn, where second-order neurons are activated by releasing chemokines (Figure 6; lower right box) to send the message through the ascending axon in upper brain areas. Activated interneurons as well as descending analgesic projections release endogenous opioid peptides (green circles) into synaptic cleft that promote an antinociceptive effect by activation of opioid receptors, which modulates nociceptive signalling.[42]

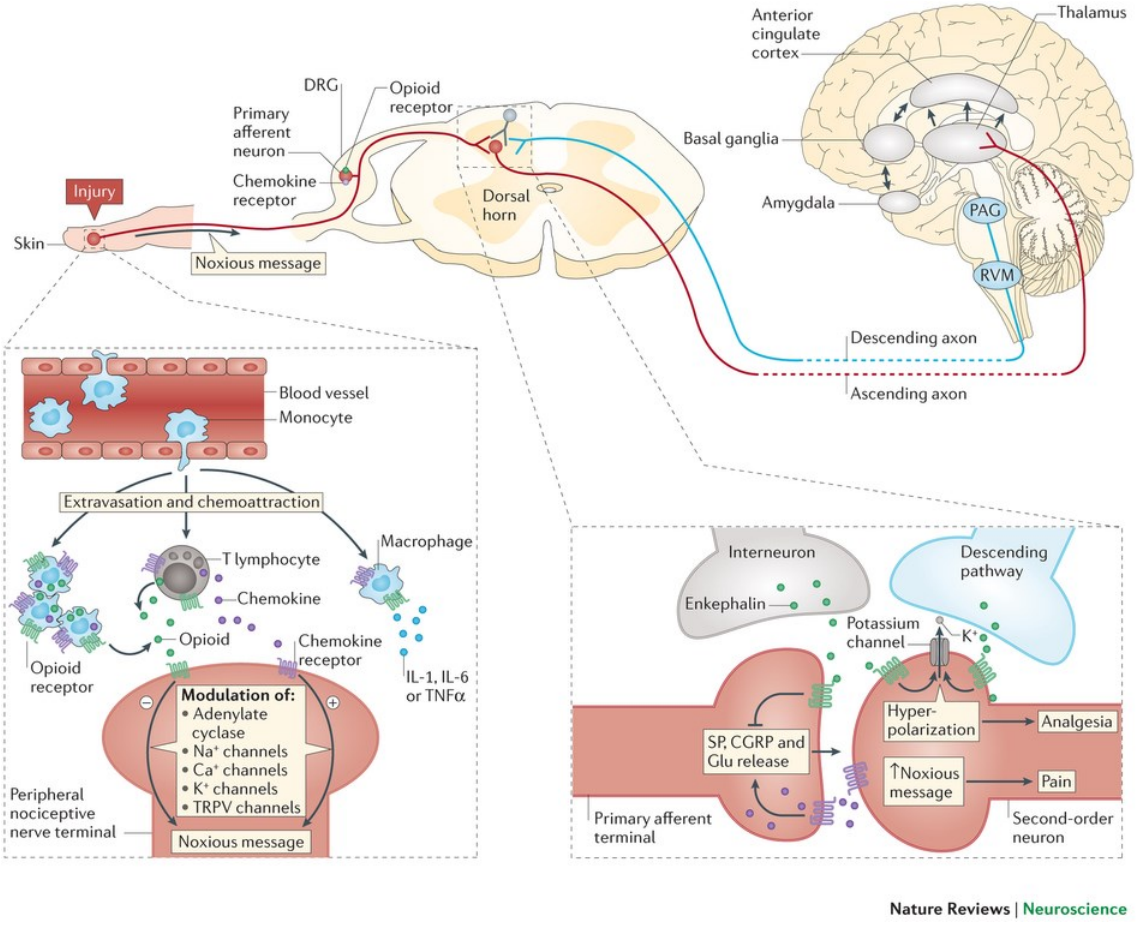


Figure 6: In nociceptive pathways analgesic effects can be mediated by releasing enkephalin and other endogenous opioids by activated interneurons. Inhibition of DPP III, which degrades enkephalin rapidly, promotes the analgesic effect. Reprinted by permission from Macmillan Publishers Ltd: S. Mélik Parsadaniantz *et al*. *Nat. Rev. Neurosci*. **2015**, *16*, 69–78, copyright 2015.[42]

Endogenous opioid peptides such as enkephalins (Tyr-Gly-Gly-Phe-Met/Leu), dynorphins, endomorphins and hemorphins are also involved in other physiological

functions including signal transduction, blood pressure regulation, immunomodulation, reproduction and behaviour.[9,18,43]

Enkephalin was found to have the same mode of action as morphine by docking to the same opioid receptor sites, which can be achieved by the similar structure elements (Figure 7).[44]

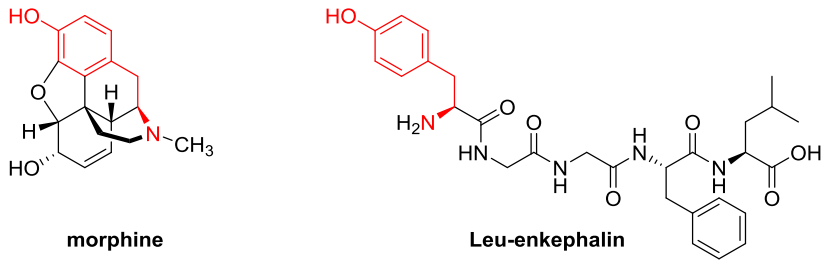


Figure 7: Comparison of the structures of morphine and Leu-enkephalin.

The lifetime of the released enkephalin is short, it immediately gets degraded by various enzymes including DPP III.[13,45] It is not surprising that inhibitors of DPP III have already been tested successfully as analgesic agents. H. Ueda *et al.* proved the analgesic effect of spinorphin and compared it to morphine-induced analgesia, with the result, that spinorphin was able to inhibit nociception resistant to morphine.[46]

2.3.3 Blood pressure regulation

A hint of participation of DPP III in blood pressure regulation is the high affinity to angiotensin II and III.[24] Angiotensin II can be seen as the main effector peptide of the complex renin-angiotensin system (RAS) by acting on specific receptors affecting vasoconstriction.[47] High concentration of DPP III in blood plasma may cause blood pressure lowering effects due to scavenging of angiotensins. The opposite effect, elevation of blood pressure, may be affected through hydrolysis of hemopressin by DPP III.[9,48]

2.3.4 Defence against oxidative stress

Significantly increased activity of DPP III has been observed in malignant gynaecological tissue compared to normal and benign tissues and correlates with aggressiveness of tumor growth.[20,21] Liu *et al.* have reported the cytoprotective effect of overexpressed DPP III against H_2O_2 and rotenone in human neuroblastoma cells, which may be a result of increased levels of detoxifying enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1) due to increased nuclear translocation of NF-E2-related factor 2 (NRF2).[49] Since H_2O_2 is a tumor-derived factor, which is produced in large amounts by human tumor cells, DPP III may help to attenuate the toxic effects and promote tumor growth.[9,50]

2.4 The binding mode and catalytic mechanism of DPP III

So far, crystal structures of DPP III from *Saccharomyces cerevisiae* and human DPP III have been determined, giving an insight in the binding mode and catalytic mechanism.[11,17,51]

Although the sequence identity of yDPP III and hDPP III is just 36 %, both structures are very similar and contain two domains that are very flexible and separated by a large cleft in unbound state.[51] The driving force of substrate binding to hDPP III has been shown by Bezerra *et al.* to be entropy increase driven by the release of water molecules of the binding cleft. The large movements of the lobes upon peptide binding is shown in Figure 8.[11]

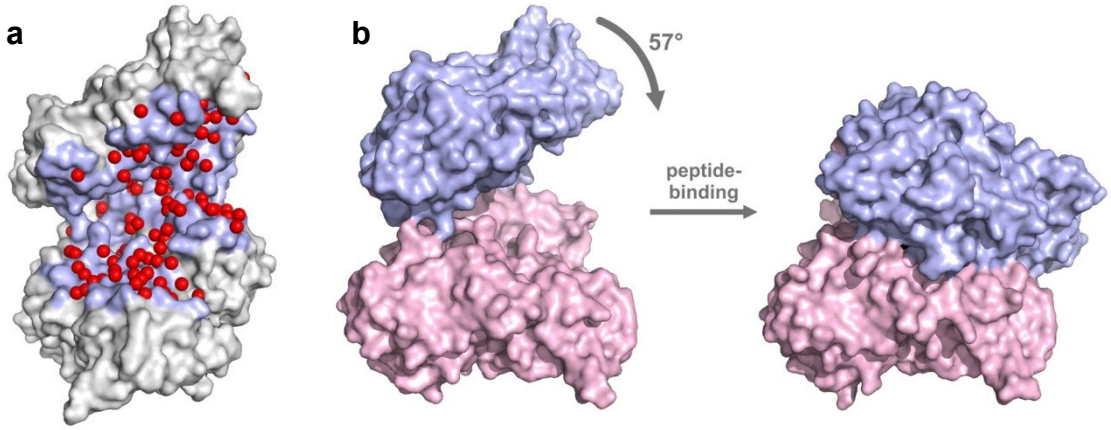


Figure 8: a) Illustrated water molecules in the nonbound hDPP III (red). b) Surface representation of the large domain motion upon tynorphin binding to hDPP III. Figures taken from Bezerra *et al.*[11]

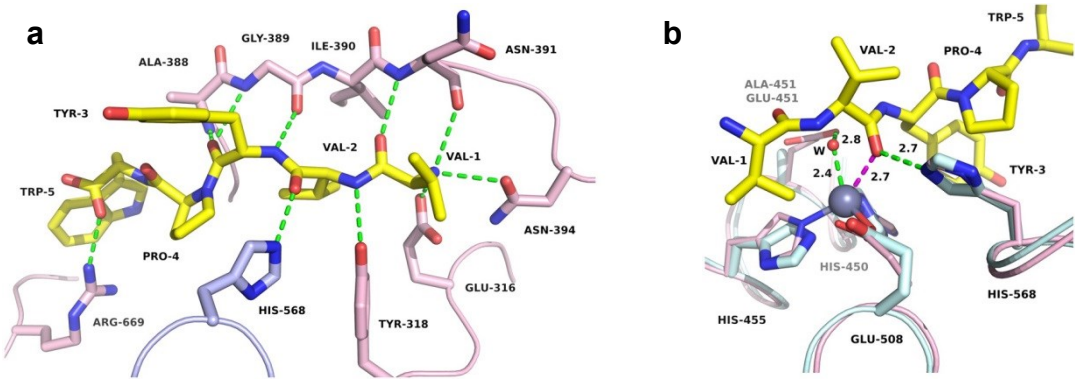
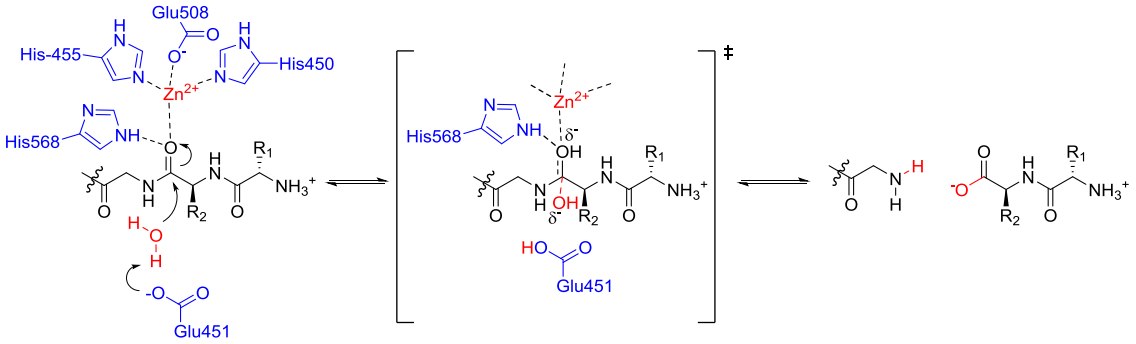


Figure 9: Structure of hDPP III in complex with tynorphin. a) Polar interactions via hydrogen bonds (green). b) Superposition of the nonbound enzyme (shown in light blue) and the hDPP III-tynorphin complex (pink). The pink dashed line represents the interaction of the zinc ion with the carbonyl group. Figures taken from Bezerra *et al.*[11]

The binding mode of substrates to DPP III occurs through backbone interactions of the peptide via hydrogen bonds, which is a common binding mode in enzyme catalysis and enables a broad substrate specificity. The domain motion is responsible for positioning

the scissile amide bond correctly to the catalytic centre. The zinc ion of hDPP III is tetrahedrally coordinated by His-450, His-455 (both from HELLGH motif), Glu-508 (from EECRAE motif) and a water molecule. A close view of the binding mode of hDPP III in complex with tynorphin is shown in Figure 9.[17,51]

A glutamate from HELLGH motif (Glu-451) has been proposed to deprotonate a water molecule, which attacks the peptide bond coordinated to the zinc ion and His-568. After a tetrahedral transition state, the peptide bond is cleaved (Scheme 1).[17,51]



Scheme 1: First proposed mechanism via a tetrahedral transition-state through activation of a water molecule, which attacks the carbonyl group.[17,51]

In a recent study, Kumar *et al.* analysed the structures of complexes of hDPP III with enkephalin (Leu/Met), angiotensin II, endomorphin-2 as well as IVYPW via X-ray crystallography and observed a difference in coordination of the carbonyl group of the scissile bond. In the complexes with Leu- and Met-enkephalin, the zinc ion does not interact directly with the carbonyl group; instead, a water molecule is coordinated to it. However, in the complex with IVYPW, the water molecule is missing, but Glu-451 has a smaller distance to the scissile peptide bond. Therefore, Glu-451 may also act directly to the bond as a nucleophile forming an acyl-enzyme-like intermediate.[51]

These two distinct mechanisms may be the explanation for the open question, why some peptides are good substrates whereas other peptides act as inhibitors.[51]

2.5 Transition-state mimicking inhibitors

The idea of using transition-state analogues as tight binding enzyme inhibitors goes back to the early 1970s. Initially, the majority of transition-state mimetics were natural products; synthetic examples started to predominate in the 1990s. There are some very prominent examples of drugs using transition-state mimetics as inhibitors: Captopril, which is used for the treatment of hypertension, inhibits the enzyme ACE (peptidyl-dipeptidase A; a zinc-dependent metallopeptidase as DPP III). Saquinavir and ritonavir, which are used for the treatment of AIDS, are both HIV-1 protease inhibitors.[1,52,53]

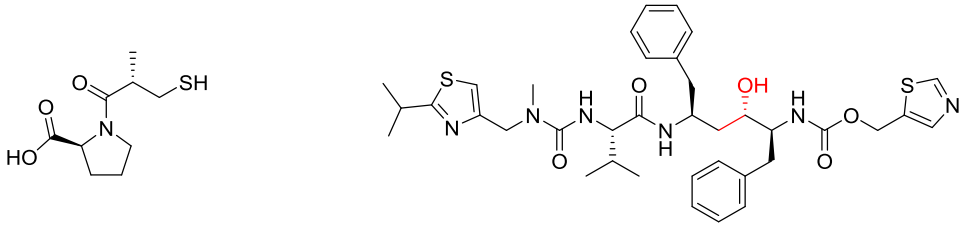


Figure 10: Two important examples of transition-state mimicking inhibitors. Ritonavir (right side) contains a hydroxyethylene moiety (red).

Ritonavir contains, as shown in Figure 10, a hydroxyethylene transition-state isostere that cannot be hydrolysed by HIV-1 protease thus inhibiting the enzyme.[53] The same principle has recently been demonstrated to successfully inhibit hDPP III with a non-cleavable isostere of tynorphin. While tynorphin gets hydrolysed within 24 hours by DPP III, the isostere remains intact in complex with wild-type hDPP III.[13,54]

Figure 11 illustrates the correlation of the hydroxyethylene moiety with the tetrahedral transition state of DPP III.

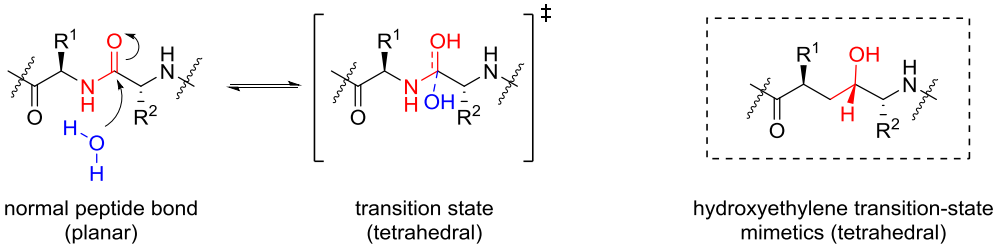


Figure 11: Transition-state of DPP III (middle) and hydroxyethylene transition-state mimetics (right).

3 Aims of this thesis

In 2011 Bezerra *et al*. described for the first time the X-ray crystal structure of (E451A inactive mutant) hDPP III in complex with the opioid peptide tynorphin and its large domain motion upon ligand binding. ITC analyses revealed an entropy-driven process as the major thermodynamic driving force. These results provided the fundament for the development of new specific inhibitors of DPP III.[11]

During his PhD thesis, Jakov Ivković developed a new transition-state mimicking inhibitor of DPP III in a well-established synthetic route, with an IC_{50} of 98 µM. That compound was also cocrystallized and used for obtaining the X-ray crystal structure of the (active wild type) hDPP III-***SHE*** complex, which revealed almost the same binding mode as tynorphin (with minor differences at the catalytic centre).[54]

Those great results opened up the possibility for designing new potential transition-state inhibitors of DPP III. Since ***SHE*** is a relatively big molecule and the synthesis with 14 steps takes some time, it was a logical consequence to develop smaller pseudopeptide analogues that are easier to synthesize. The new approach should be based on three considerations: Firstly, the enkephalin fragment Tyr-Gly-Gly-Phe can bind to DPP III; secondly, endomorphin I and II both contain an amide moiety on their *C*-terminus; and finally, an *N*-terminal isoleucine instead of valine may increase the inhibitory potency.[36,37] The same synthetic route as for ***SHE*** should be used, starting with an *N*-protected amino acid for the synthesis of an γ-lactone intermediate.[54] Comparison between three forms of the new inhibitor, (*S*)-hydroxyethylene, (*R*)-hydroxyethylene as well as ketomethylene should be made (Figure 12). The new design also has possibilities of further modification, e.g. introducing a hydroxy moiety at the *N*-terminus instead of the amine group.

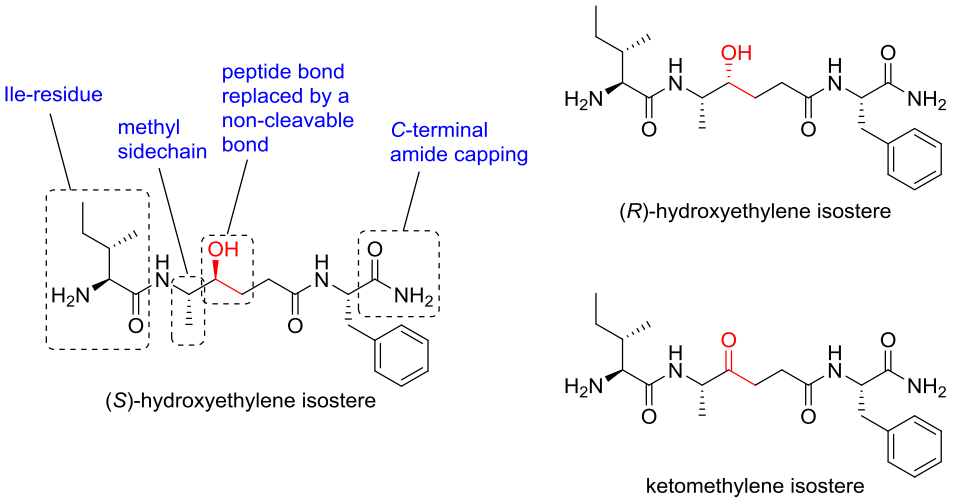


Figure 12: Design of new potential transition-state mimicking inhibitors of DPP III.

4 Results and discussion

4.1 Synthesis of a potential small molecule inhibitor

Based on previous work at our institute, where a promising inhibitor had been synthesized, we wanted to investigate whether smaller pseudopeptides, especially pseudotetrapeptides also can inhibit DPP III. Therefore we wanted to adapt the well-established synthesis route for the pseudopentapeptide ***SHE***, which had been synthesized in 14 steps by J. Ivković during his PhD thesis.[54]

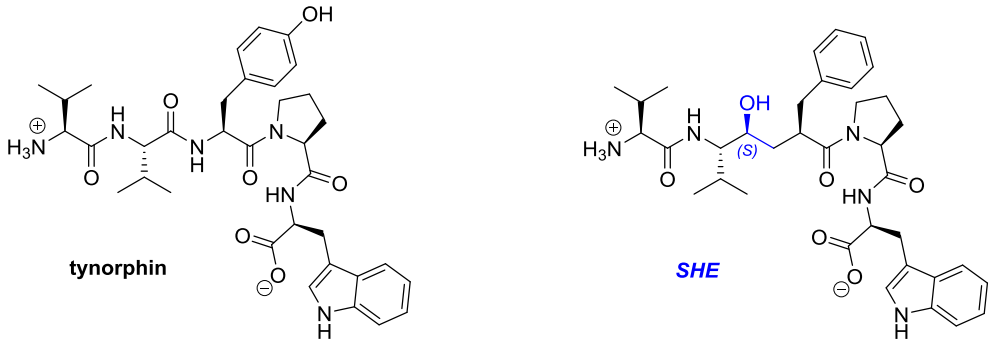


Figure 13: Two synthetic inhibitors of DPP III. Tynorphin[13] (left side) and ***SHE*** (right side), where the peptide bond has been replaced by a (*S*)-hydroxyethylene unit (blue).

The new inhibitor should contain Ile instead of Val on the *N*-terminus, because Chiba *et al.* showed increased inhibition for IVYPW in contrast to VVYPW.[36] We also wanted to vary the amino acid fragment next to Ile. By replacing Val by Ala we hoped to increase the inhibitory potency. The *C*-terminus of the new compound combines both an enkephalin fragment (Gly-Phe)[55] and a terminal amide group, since endomorphins are also *C*-terminally capped with such a $CONH_2$ group. Furthermore, the new compound contains a non-cleavable hydroxyethylene unit. The newly designed potential inhibitor is shown in Figure 14.

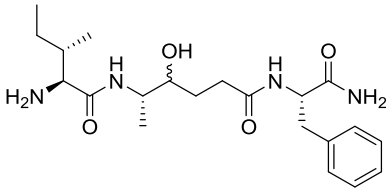
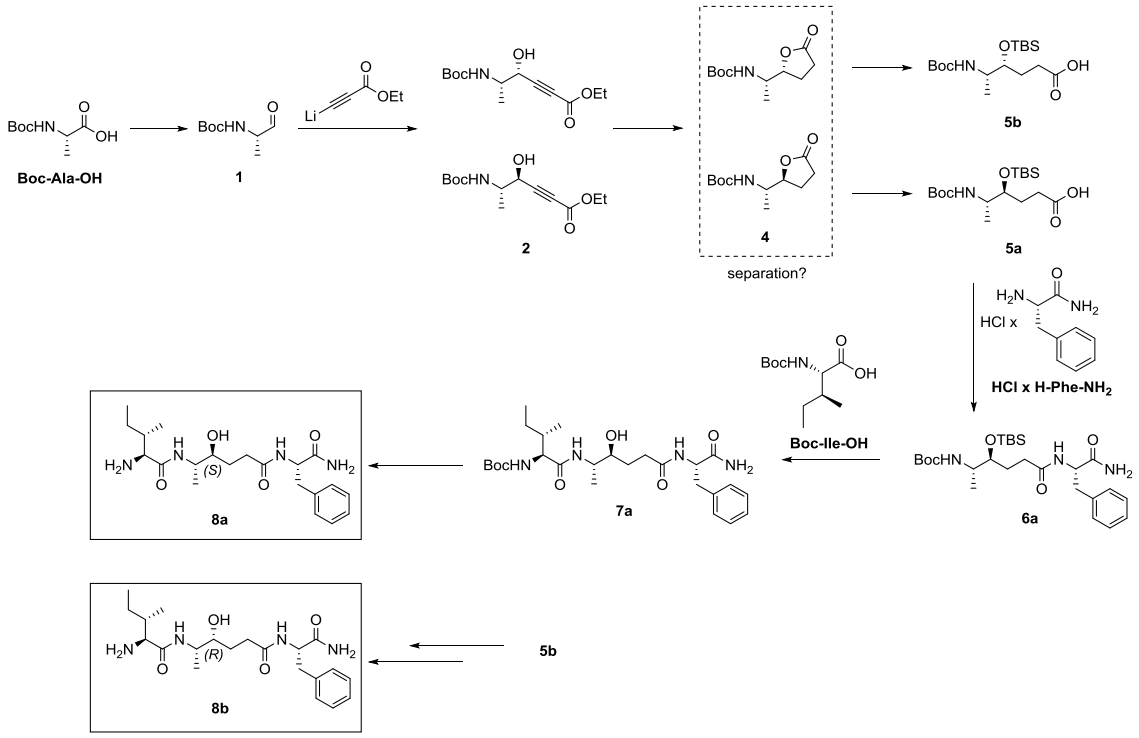


Figure 14: H-Ile-Ala-[Ψ](COH-CH_2)-Gly-Phe-NH_2, an enkephalin mimicking pseudotetrapeptide.

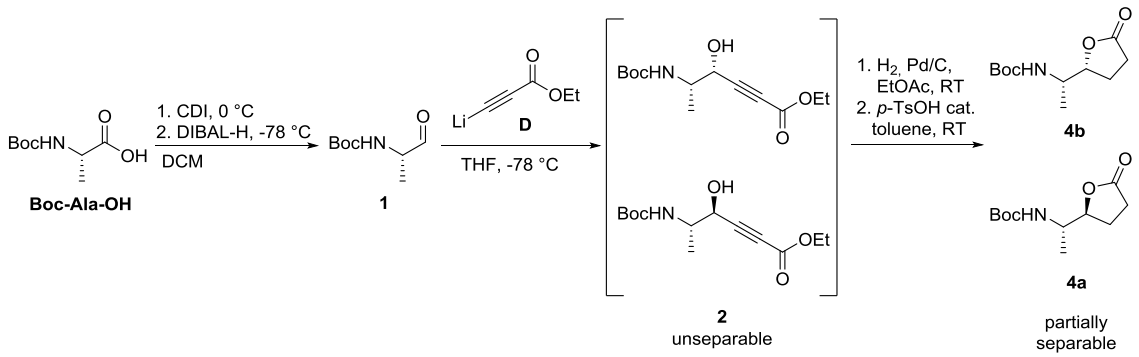
The synthetic route was based on the strategy of Ghosh *et al.* to separate the diastereomeric mixture of the γ-lactones **4**, obtained in two steps from Boc-Ala-OH and open them to the core dipeptide transition state mimicking units **5a** and **b**.[56,57] Elongation at

the *C*-terminus and *N*-terminus should provide compounds **8a** and **b** after a deprotection step (Scheme 2).



Scheme 2: Overview about the planned synthetic strategy from Boc-protected alanine towards the desired pseudotetrapeptides **8a** and **b**.

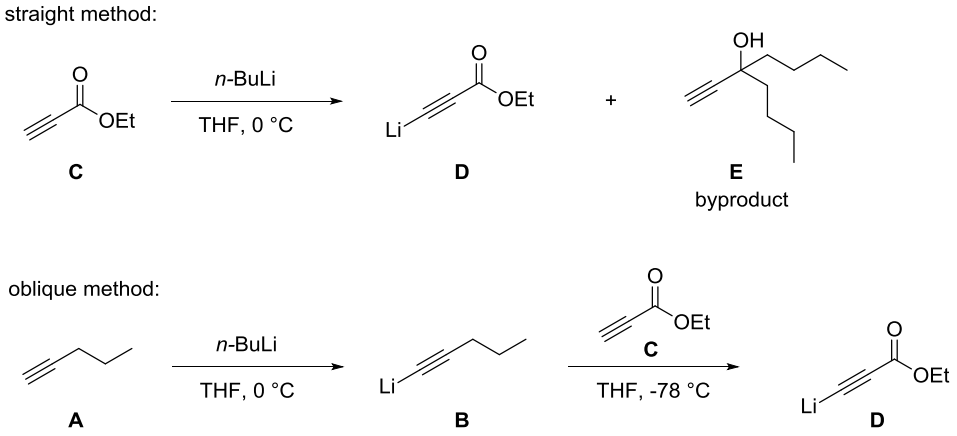
4.1.1 Synthesis of the γ-lactone



Scheme 3: Synthesis of the γ-lactone starting from commercially available Boc-L-alanine.

100 mmol (9.46 g) of the Boc-protected α-amino acid L-alanine was reduced to the chiral α-amino aldehyde **1** with a rapid and efficient one-pot method that was developed in our laboratory. 1,1'-Carbonyldiimidazole (CDI)[58] was used to activate the acid. Subsequent treatment of the resulting imidazolide with DIBAL-H provided the desired Boc-L-alaninal in 87 % yield, which could be used without further purification. This method, which we published in *Org. Biomol. Chem.*, will be described in detail in chapter 4.2.[59]

For the next step ethyl propiolate (**C**) was used to generate lithiated compound **D** *in situ* and allowed to react with aldehyde **1**.[56] First of all, *n*-Butyllithium was used to deprotonate 1-pentyne (**A)** at 0 °C to generate **B**. After cooling to –78 °C ethyl propiolate (**C**) was added to obtain lithiated ethyl propiolate (**D**). With this oblique method the formation of byproduct **E** was avoided which had been formed in significant amount when **C** was deprotonated directly with *n*-BuLi (Scheme 4).



Scheme 4: Comparison of two methods for *in situ* generation of lithiated ethyl propiolate (**D**).

By reacting **D** with aldehyde **1** the acetylenic alcohol **2** was obtained as an inseparable mixture of diastereomers with dr = 5:1 (*S*,*S*):(*R*,*S*). Catalytic hydrogenation of **2** with Pd/C and subsequent lactonization with a catalytic amount of *p*-toluenesulfonic acid in toluene at 50 °C led to the γ-lactone **4**.

In the synthetic route of ***SHE***, where Boc-Val-OH was used in the same way to generate the γ-lactones, these lactones could be separated via flash chromatography. Unfortunately, the diastereomers **4a** and **b** were just poorly separable and only 7 % of the major epimer could be obtained in a pure form (de = 98 %) after flash chromatography with a 100-fold excess of silica gel. Great efforts have been made to separate the diastereomers: Several different solvent mixtures were used, also with the acetylenic alcohols **2**, but unfortunately those approaches failed. In the next trial the acetylenic alcohols **2** were acetylated and an attempt to separate the resulting diastereomers **3** (see chapter 6.3.1.3) by flash chromatography was made, but also without success.

We decided to continue the synthesis with the mixture of diastereomers and separate them in a later step. Nevertheless, it was possible to identify the major epimer by X-ray crystallography when a small amount of single crystals was obtained by stereoselective crystallization from *n*-hexane (from the mixture of diastereomers). As expected, the major epimer was (*S*,*S*)-configurated (Figure 15).

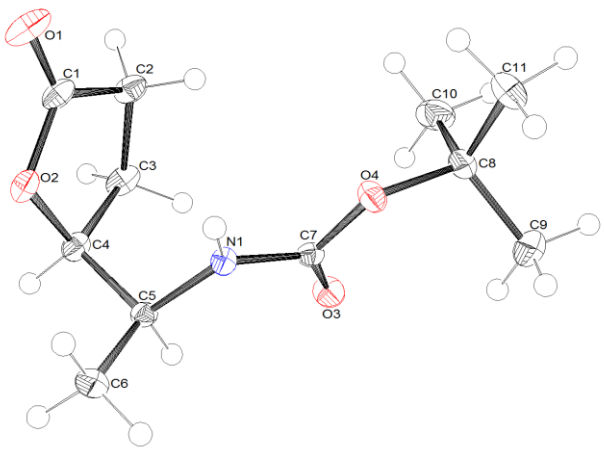
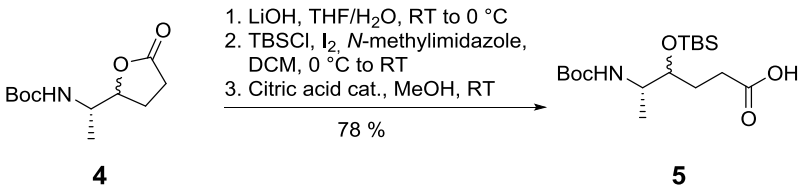


Figure 15: X-ray crystal structure (ORTEP) of the major (*S*,*S*)-configurated diastereomer. Thermal ellipsoids are drawn at 50 % probability level. Determined configuration of C-4 is based on the known configuration of C-5. We gratefully thank Prof. Roland Fischer for providing the X-ray data set.

4.1.2 Ring opening and silylation

The crucial step of the first synthesis route was the ring opening reaction of the γ-lactone **4** due to the property of the resulting γ-hydroxy acid to relactonize very easily again. Therefore the hydroxy group had to be protected immediately after careful workup, nevertheless lactonization was a problem.



4 **5**

Scheme 5: Hydrolysis of the γ-lactone and subsequent protection of the hydroxy moiety.

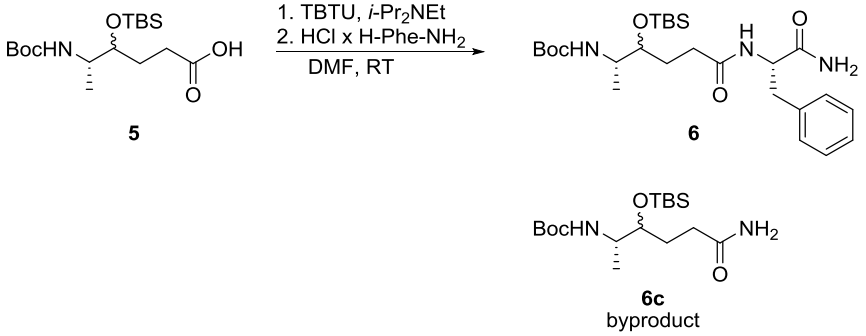
The first step was the saponification of lactone **4** with LiOH in a mixture of THF/H_2O = 2:3 (v/v) at RT. For the extractive workup, the reaction mixture had to be acidified to pH=4 although the γ-hydroxy acid is very sensitive to acidic conditions, so precautions against lactonization had to be taken: Acidification was carried out at 0 °C with 25 % aqueous citric acid and careful control of pH. After extraction with Et_2O the solvents were removed under reduced pressure at a temperature below 30 °C.

Immediately after drying of the crude intermediate the hydroxy group was protected with a silyl group according to a procedure of Bartoszewicz *et al.*[60,61] TBSCl in the presence of *N*-methylimidazole and iodine was used and the reaction mixture was stirred for 21 h at RT. A third reaction step was necessary after aqueous workup because of partial formation of the silyl ester. Therefore the crude product was stirred in presence of a catalytic amount of citric acid in methanol for 20 h (see also chapter 4.3.2.4).

Although some amount of relactonization could be observed, the overall yield of 78 % after three steps was quite acceptable.

4.1.3 Elongation of the core structure

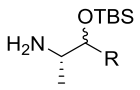
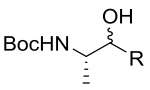
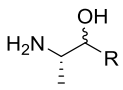
The core dipeptide transition state mimicking unit **5** was now ready to be elongated at both the *C*-terminal and *N*-terminus. We started with coupling of H-Phe-NH_2×HCl to the *C*-terminus using TBTU as a reliable coupling reagent[62] in presence of 4.0 eq of DIPEA as a base. Unfortunately, at this step the truncated byproduct **6c** was formed due to contamination of H-Phe-NH_2×HCl with ammonium chloride and could just be removed via flash chromatography after the last synthesis step.



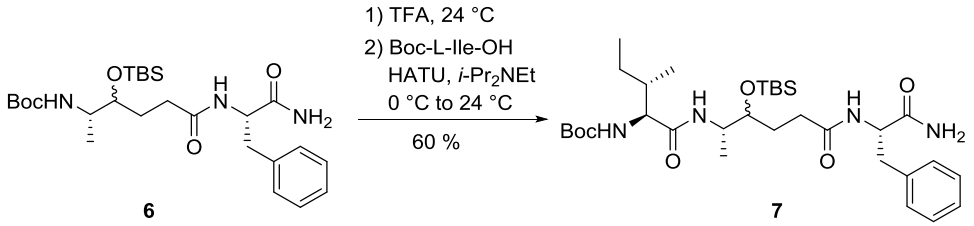
Scheme 6: Peptide coupling of core structure **5** with H-Phe-NH_2×HCl to **6**. Due to contamination with NH_4Cl a byproduct was formed, which could be removed via flash chromatography in the last step.

For elongation at the *N*-terminus some small scale deprotection experiments were performed. From the synthetic route of ***SHE*** it was known that $ZnBr_2$ in trifluoroethanol is able to cleave both the Boc- and the silyl group at RT.[54] During deprotection of compound **6**, Boc-deprotection could be observed rather than simultaneous deprotection of both protecting groups after 22 h, so a short screening was done (Table 2).

Table 2: Screening of different conditions for deprotection: 5.0 mg (9.8 µmol) compound **6** in 0.2 mL solvent were used for each experiment and the products were analysed by HPLC (results in area-%).

Entry	Conditions			
1	4 eq $ZnBr_2$, EtSH, CF_3CH_2OH, RT, 2 h	27.9	46.6	25.6
2	10 eq $ZnBr_2$, EtSH, CF_3CH_2OH, RT, 5 h	78.5	—	21.5
3	$ZnBr_2$, EtSH, MeCN, RT, 25 h	5.4	87.6	7.0
4	TFA, RT, 60 min	quant.	—	—
5	TFA, EtSH, RT, 60 min	quant.	—	—

Since it was not possible to cleave the Boc and TBS group in one step (Table 2, entries 1 and 2), we decided to cleave only the Boc group with TFA. An interesting fact was that by changing the solvent from trifluoroethanol to acetonitrile, a selective TBS deprotection could be achieved (entry 3).

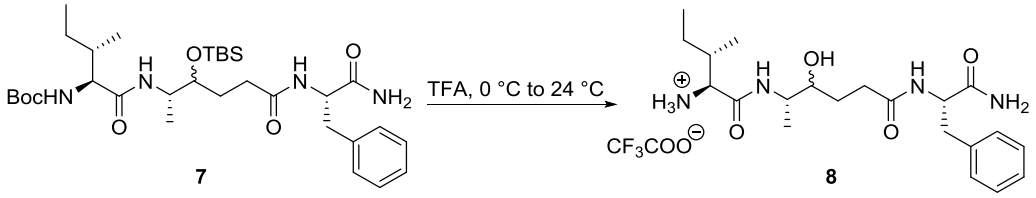


Scheme 7: Deprotection and subsequent peptide coupling with Boc-L-Ile-OH.

Deprotection with TFA was carried out at RT without harming the TBS group (30 min reaction time). After deprotection HATU was used as a coupling reagent to avoid the possibility of epimerization of isoleucine (Scheme 7).

4.1.4 Final reaction step and inhibition assay

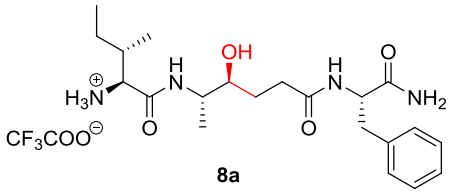
For the final deprotection of the Boc and TBS group two experiments have been performed in a small scale to test which order of deprotection was favourable. In the first experiment TBAF was used for desilylation, but no full conversion after 4 d could be achieved. In the second experiment where trifluoroacetic acid was used in the beginning, both protecting groups were surprisingly cleaved off after 40 min reaction time.



Scheme 8: Final simultaneous deprotection of Boc and TBS with trifluoroacetic acid.

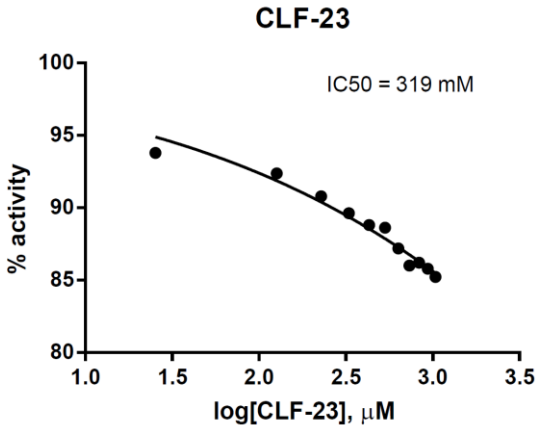
At this stage, the desired product **8a** ((*S*)-hydroxyethylene) could be isolated via preparative HPLC from the mixture of diastereomers with 31 % yield (de = 79 %) as a trifluoroacetate.

While we were thinking about an improved synthesis path for obtaining also the (*R*)-hydroxyethylene analogue and increased de for the (*S*)-hydroxyethylene pseudotetrapeptide, fluorescence-based inhibition assay revealed a very high IC_{50} value of the target molecule **8a** (Figure 16) and also for other shorter transition state peptidomimetics.[54] In consequence of this result we decided not to resynthesize this compound but focus on the synthesis of ***HER*** (see chapter 4.3).



8a

$CF_3COOH \times$ H-Ile-Ala-ψ[$COHCH_2$]-Gly-Phe-NH_2



$IC_{50} \approx 319$ mM

Figure 16: Fluorescence-based inhibition assay of compound **8a** revealed a very high IC_{50} value. Since this value was out of diagram range, it was estimated with approximately 319 mM. The inhibition assay was performed by S. Jha, Institute of Biochemistry, Graz University of Technology.

4.2 Dipeptide synthesis and reduction to peptide aldehydes using CDI/DIBAL-H

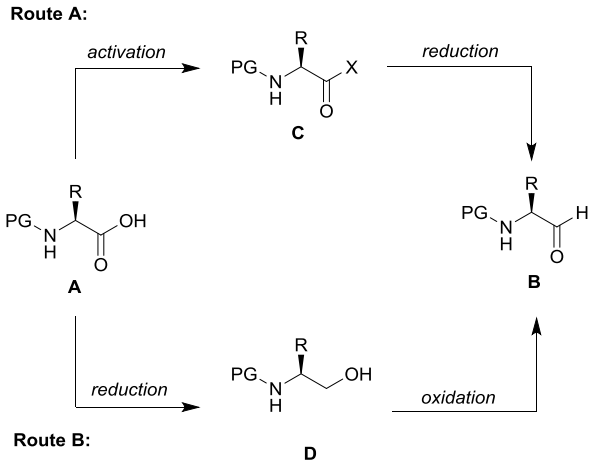
During the synthesis of transition state inhibitors we needed access to different Boc-protected α-amino aldehydes as building blocks. This requirement led to the development of a new method, which has been published in *Org. Biomol. Chem.* with the title: "*A rapid and efficient one-pot method for the reduction of N-protected α-amino acids to chiral α-amino aldehydes using CDI/DIBAL-H"*. Chapter 4.2 summarizes a part of that publication.[59]

4.2.1 Necessity of *N*-protected α-amino aldehydes and literature-known access

Chiral *N*-protected α-amino aldehydes are very important building blocks in organic chemistry with lots of applications.[63,64] Two typical routes can be used for their synthesis, starting from chiral *N*-protected α-amino acids (Scheme 9). In route A, the *N*-protected α-amino acid **A** is activated by conversion into derivative **C**, typically an ester[65,66] or a Weinreb amide,[67,68] and subsequently reduced to the aldehyde **B**. In route B the α-amino acid **A** is firstly reduced to the corresponding alcohol **D**, followed by oxidation to the aldehyde **B**.[69] In both cases racemization might be a problem, especially in presence of acid or base.[70]

The first attempt for the synthesis of Boc-valinal used by our group was a two-step method by Morwick *et al*. In this method Boc-L-valine was converted into the corresponding Weinreb amide using activation by CDI, followed by reduction of the isolated Weinreb amide with $LiAlH_4$.[71] Since the isolation of the Weinreb amide is a time-

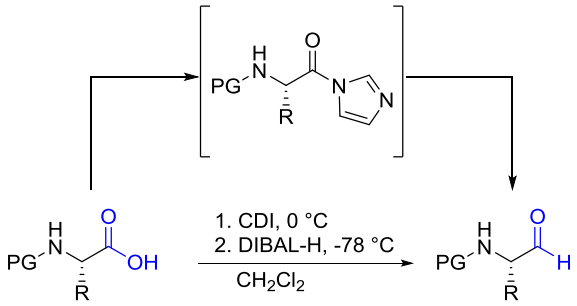
consuming nuisance, we found an alternative procedure by Stammer *et al*. where the isolation-step could be avoided by reducing the acyl imidazolide intermediate with DIBAL-H.[72] Although the reported ee was only 60 % for Cbz-leucinal, we were encouraged to optimize the reaction parameters and test this one-pot method also for synthesizing peptide aldehydes.



Scheme 9: Two typical routes for synthesizing *N*-protected α-amino aldehydes **B** from corresponding α-amino acids **A**. Scheme taken from Ivković *et al.*[59]

4.2.2 New CDI/DIBAL-H method and its limitations

As an optimized procedure for a milligram or even multigram scale the following parameters have been found: A solution of *N*-protected amino acid in DCM (c = 0.1 M to 0.15 M) was treated with 1.1 eq of CDI (freshly recrystallized from THF)[58] at 0 °C for 30–60 min. Subsequently 2.1 eq of DIBAL-H (1.0 M in toluene) were added dropwise at –78 °C and the reaction mixture was additionally stirred for 45 min at –78 °C.

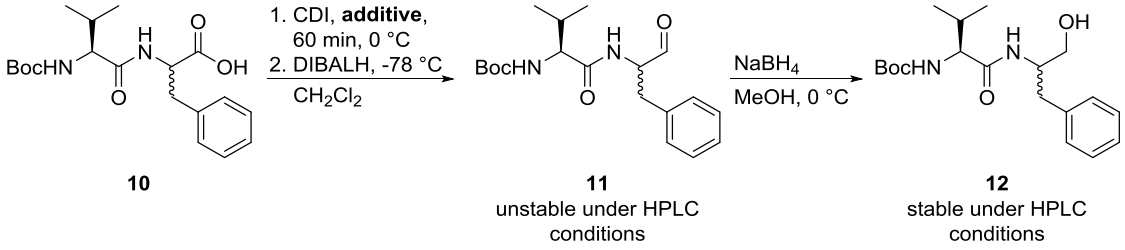


Scheme 10: One-pot synthesis of α-amino aldehydes using CDI and DIBAL-H. Scheme modified from Ivković *et al.*[59]

For the extractive workup we devised a rapid quenching method by using 25 % tartaric acid in H_2O instead of the commonly used Rochelle-salt solution,[73,74] which shortens complexation of aluminium salts to less than 20 min for multigram scale reactions. This method provides most of the tested aldehydes already in a pure form thus purification

via flash chromatography on silica gel accompanied by possible racemization can be avoided.

While most of the reduced *N*-protected α-amino acids provided the corresponding aldehyde with ee >99 % in excellent yields (for detailed results see Ivković *et al.*[59]), we wanted to investigate whether our method can also be used for the reduction of phenylglycine, which is a very racemisation-prone non-proteionogenic α-amino acid, and for peptides to their corresponding peptide aldehydes.[75]



Scheme 11: Overview about the synthesis of peptide aldehydes with CDI/DIBAL-H, followed by reduction to the corresponding peptide alcohol.

For that purpose Boc-L-Val-D-/L-Phe-OH, with the epimerization prone Phe at the *C*-terminus,[76] was synthesized as a suitable test substrate, and reduced by using our CDI/DIBAL-H method to the corresponding aldehydes **11a** and **11b** in good yields and purities (Table 3). Due to instability of aldehydes in HPLC conditions, they were reduced to the corresponding amino alcohols by using $NaBH_4$ (Scheme 11).[77]

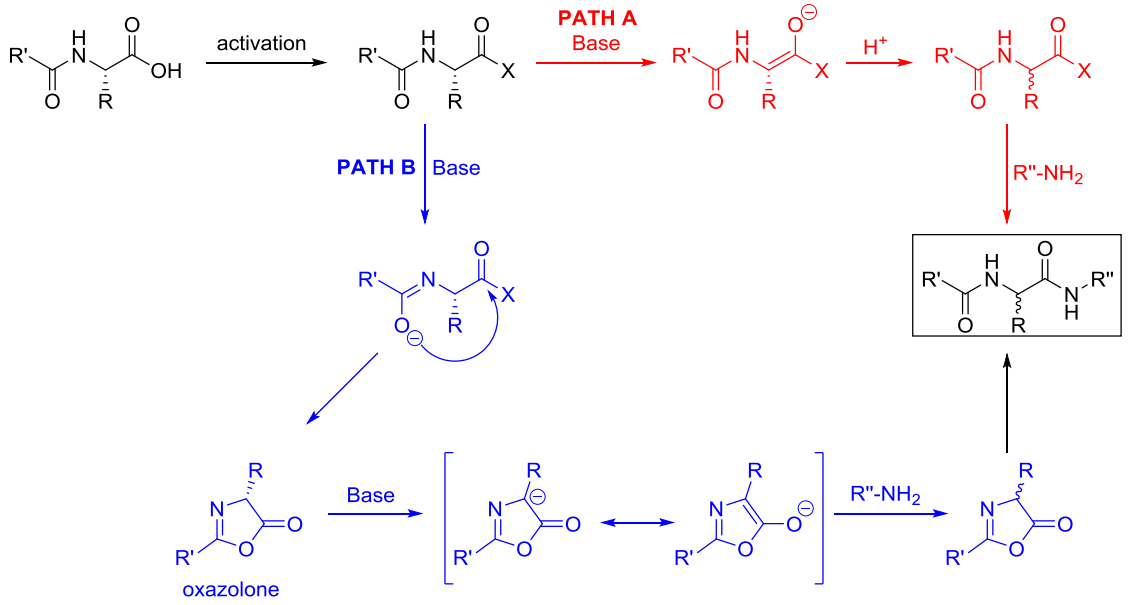
Table 3: Synthesis of peptide aldehydes using the CDI/DIBAL-H procedure.

Entry	Product	Additive	DIBAL-H	Yield	de
1	Boc-Val-L-Phe-H (**11a**)	—	3.1 eq	89 %	79 %
2	Boc-Val-D-Phe-H (**11b**)	—	3.1 eq	95 %	77 %
3	**11b**	$CuCl_2$, 1.0 eq	4.0 eq	52 %	81 %
4	**11b**	PPTS, 1.0 eq	3.5 eq	99 %	69 %
5	**11b**	HOBt, 1.0 eq	4.5 eq	93 %	90 %
6	**11b**	HOBt, 1.5 eq	4.5 eq	quant.	79 %
7	**11b**	HOBt, 1.5 eq, 4 Å mol. sieves	4.5 eq	84 %	85 %
8	**11b**	HOAt, 1.0 eq	4.5 eq	quant.	90 %

Due to instability of aldehydes in HPLC conditions, de was measured after reduction to the corresponding alcohols,[77] which were readily separated by reverse phase HPLC; PPTS: pyridinium *p*-toluenesulfonate; HOBt: 1-hydroxybenzotriazole; HOAt: 1-hydroxy-7-azabenzotriazole.

For determination of de of the peptide alcohols, RP-HPLC was used.

In contrast to the reduction of amino acids, increased amounts of DIBAL-H (3.1–4.5 eq) were necessary for complete conversion. Lower reaction concentrations were used, because of gelation of the reaction mixture at –78 °C. In the first two experiments both peptide aldehydes could be isolated in good yields but only with a de of 79 and 77 % (entries 1 and 2). As a next step, we tried to suppress epimerization by adding various additives developed for peptide coupling (entries 5–8),[78,79] mild acidic buffering with pyridinium *p*-toluenesulfonate (entry 4) or scavenging deprotonated imidazole by complexation with $CuCl_2$ (entry 3).[80,81]



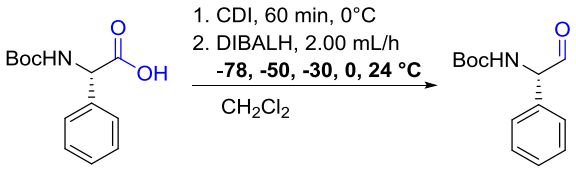
Scheme 12: Epimerization in the activation step via an oxazolone intermediate (Path B) is well known in peptide coupling.[82,83]

Almost each experiment provided good to excellent yields, but unfortunately it was not possible to increase optical purities to those of the α-amino aldehydes. By complexation with copper(II) chloride diastereoselectivity could slightly be increased from 77 % (entry 2) to 81 % de (entry 3), but with an isolated yield of just 52 %. Best results were achieved by addition of 1.0 eq HOBt (entry 5) and 1.0 eq HOAt (entry 8) with 90 % de each, judged by chiral GC. Addition of 1.5 eq HOBt or using 4 Å molecular sieves together with HOBt did not improve the results (entries 6 and 7). In summary it can be stated, therefore, that these results reflect the limitations of our CDI/DIBAL-H method.

4.2.3 Temperature-dependency of ee using the CDI/DIBAL-H method

The reduction of Boc-phenylglycine as a very challenging test substrate was used to determine the dependency of ee on different parameters such as the rate of addition of DIBAL-H or the addition of $CuCl_2$ as a scavenging agent for deprotonated imidazole.[80] In previous experiments the enantiopurity of Boc-phenylglycinal, reduced by our method was only 72 % ee and could be increased to 83 % ee by the addition of 0.5 eq

$CuCl_2$ during the activation step.[59] Now we wanted to investigate the dependence of ee on the temperature during the addition of DIBAL-H.



Scheme 13: Screening of temperature-dependency of ee during the addition of DIBAL-H.

For the temperature screening 50 mg (0.20 mmol, 1.0 eq) Boc-L-Phg-OH and 36 mg (0.22 mmol, 1.1 eq) CDI in 2.0 mL abs. DCM were used for each instance. After 60 min activation time 0.42 mL (0.42 mmol, 2.1 eq) DIBAL-H (1 M in toluene) were added at the rate of 2.0 mL/min and after stirring for 15 min at the appropriate temperature ee was determined by chiral GC-FID (see chapter 6.3.2.9). The results are summarized in Table 4 and Figure 17.

Table 4: Dependence of ee of Boc-phenylglycinal on the temperature during addition of DIBAL-H.

Entry	Temp.	CDI	DIBAL-H	ee
1	–78 °C	1.1 eq	2.1 eq	72 %
2	–50 °C	1.1 eq	2.1 eq	72 %
3	–30 °C	1.1 eq	2.1 eq	63 %
4	0 °C	1.1 eq	2.1 eq	59 %
5	+24 °C	1.1 eq	2.1 eq	37 %

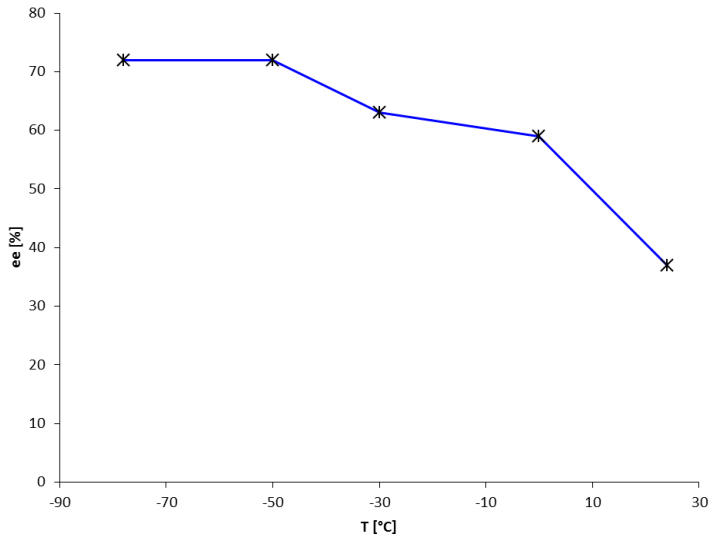


Figure 17: Dependence of ee of Boc-phenylglycinal on the temperature during addition of DIBAL-H.

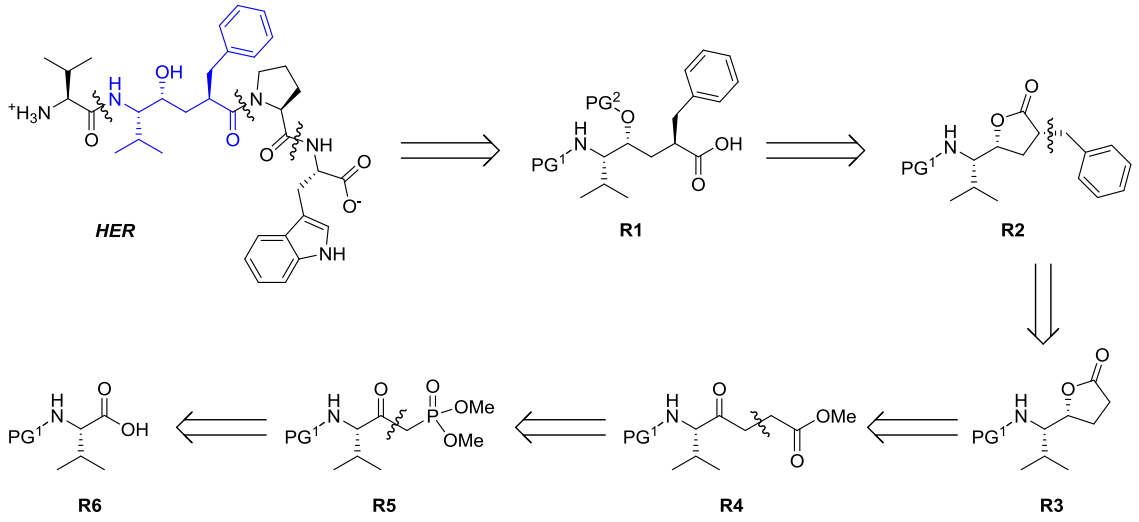
4.3 Alternative synthesis route to *HER* and derivatives

The transition-state mimicking pseudopentapeptide ***HER*** has been synthesized by J. Ivković at his PhD thesis work for the first time, and produced the best results at fluorescence-based inhibition assays of all tested peptidomimetics with an IC_{50} of 8.8 µM. Unfortunately, just 4.0 mg have been isolated which was too little for further studies.[54]

Chapter 4.3 focuses on the synthesis of ***HER*** with an option of developing new derivatives which are potentially more active than ***HER***. The new synthesis route was based on a publication of E. Haug and D. H. Rich, in which the synthesis of a Gln-Phe hydroxyethylene dipeptide isostere as a precursor for BoNT metalloprotease inhibitors has been reported.[84] Particular attention of the synthesis was given to the cost reduction with regard to the purification method, which may be a significant cost factor, especially for big batches.[85]

4.3.1 Retrosynthetic strategy

The desired final molecule ***HER*** can be divided into the Val-Phe (*R*)-hydroxyethylene pseudodipeptide core structure (blue), and elongation via peptide coupling to a pseudopentapeptide (Scheme 14).



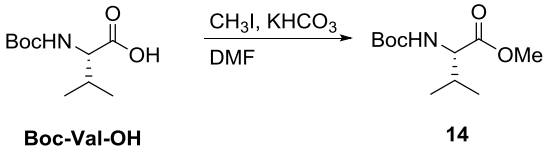
Scheme 14: New retrosynthetic strategy towards DPP III inhibitor ***HER***.

The *N*- and *O*-protected core structure **R1** can be obtained via lactone opening of **R2** and a protection step. Stereoselective benzylation of γ-lactone **R3** provides **R2**. In contrast to the former route, γ-lactone **R3** is obtained from **R4** after stereoselective reduction followed by cyclization with acid. A HWE-reaction can be used for the synthesis of **R4** from **R5** and methyl glyoxylate. Disconnection of **R5** leads to the *N*-protected α-amino acid L-valine, which is commercially available with several protecting groups.

4.3.2 Synthesis of the pseudodipeptide core structure

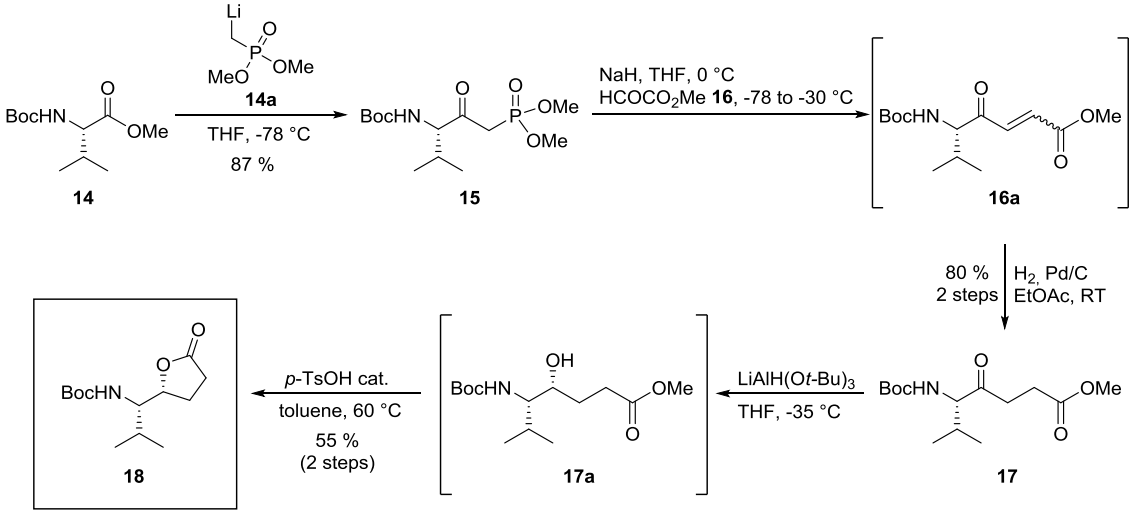
4.3.2.1 From Boc-L-valine to the keto ester

Our synthetic route started with *N*-protected L-valine using a Boc-protecting group as a reliable, base-resistant but easily cleavable moiety.[86] For the next step, also the carboxylic acid had to be protected by esterification. Therefore, it was converted into a methyl ester by using methyl iodide and potassium bicarbonate in DMF according to the procedure of Brenner *et al*. with 99 % yield and excellent purity even without further purification (Scheme 15).[87,88]



Scheme 15: Protection of the carboxylic acid moiety by esterification.[87,88]

In the next step, dimethyl methylphosphonate was lithiated with *n*-butyllithium to generate lithiated compound **14a**, which was allowed to react with **14** to β-keto phosphonate **15** at –78 °C.[89] Compound **15** was isolated as a pale yellow oil without the necessity of further purification since NMR showed no significant amount of impurities (Scheme 16).



Scheme 16: Synthetic route from protected L-valine towards the γ-lactone intermediate **18**.

For the following HWE reaction methyl glyoxylate (**16**) had to be freshly prepared and used within a day. An improved procedure of Schuda *et al.* was used to obtain the crude aldehyde, which appears to be a mixture of hydrated and non-hydrated forms, and was used without further purification after extensive drying.[84,90,91] Subsequently, the β-keto phosphonate **15** was converted with **16** to the intermediate **16a** at –30 °C in a HWE re-

action. The crude mixture of *cis*- and *trans*-isomers was then hydrogenated to produce keto ester **17** in 80 % yield over two steps (Scheme 16).

4.3.2.2 Unexpected side reaction – an "anti-Baldwin" cyclisation

When the HWE reaction was performed for the first time, a temperature rise to –7 °C instead of –30 °C occurred accidentally within a short period of time. After this incident, significant formation of a byproduct could be observed which was later identified as compound **31** via NOESY (Figure 18).

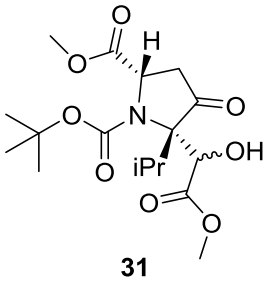
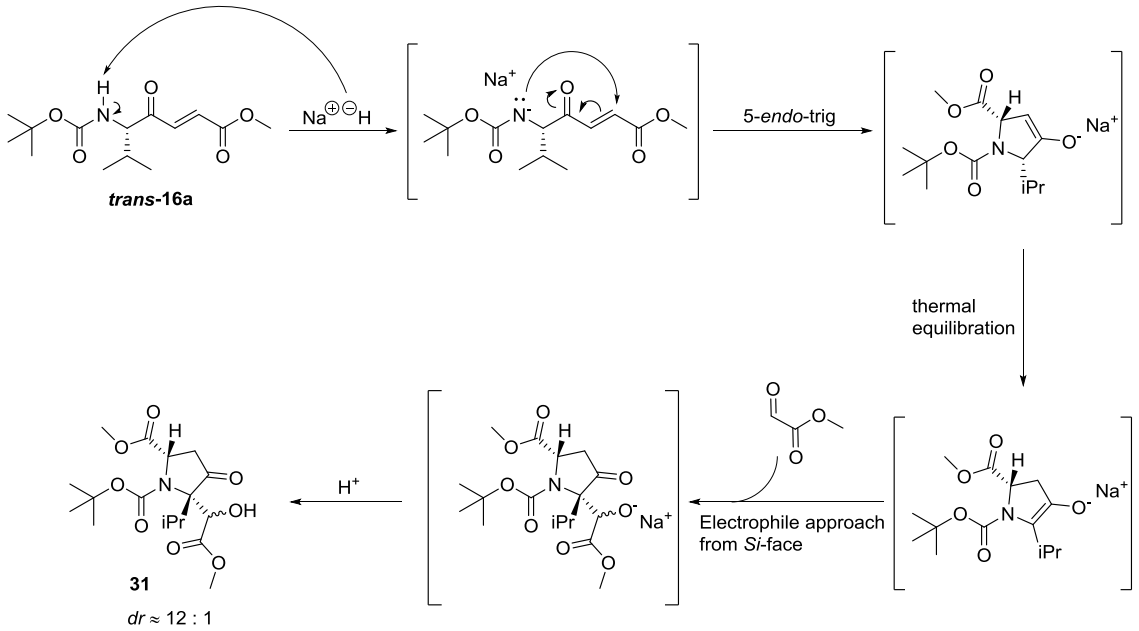


Figure 18: anti-Baldwin cyclisation product as a byproduct of the HWE reaction performed at –7 °C.

The Baldwin's Rules were published by J. E. Baldwin in 1976, focusing on the issue of favoured ring closure formations.[92] According to these rules, the 5-*endo*-trig ring closure reaction is disfavoured,[93] nevertheless there are couple of exceptions for this type of cyclisation.[94–98] The formation of byproduct **31** seems to include such a cyclisation although there are still open questions.



Scheme 17: Proposed mechanism of the formation of byproduct **31** during HWE reaction, which contains a 5-*endo*-trig cyclisation.

Scheme 17 shows the proposed mechanism of the formation of this cyclic byproduct starting with deprotonation of intermediate ***trans*-16a**, followed by a 5-*endo*-trig "anti-Baldwin" cyclisation. Thermal equilibration would be faster than the sterically demanding aldol reaction and explains why the second methyl glyoxylate residue has been found next to the isopropyl group. If the electrophile approached from the *Si*-face, it would explain the diastereomeric ratio of 12:1 found in ^{1}H-NMR spectrum. However, in the beginning the ***cis*-16a** intermediate also exists, which complicates the explanation why there is not a third diastereomer visible in NMR. Nevertheless, the observed reaction cascade is very interesting with great synthetic potential and would deserve closer attention.

4.3.2.3 Completion of the γ-lactone synthesis

When the side reaction was suppressed by careful temperature control and keto ester **17** was obtained after catalytic hydrogenation, the synthetic route was continued by stereoselective reduction with lithium tri-*tert*-butoxyaluminum hydride (Scheme 16). In this reaction step, which was originally carried out at –78 °C,[84] temperature adaption was necessary since the reaction was much slower with keto ester **17**. A temperature optimum between –40 °C to –30 °C was found; nevertheless the reaction required 20 h for completion and had to be monitored carefully during that time (Figure 19). Unfortunately, some formation of the unwanted (*S*,*S*)-diastereomer could not be avoided at higher temperatures. Transesterification from methyl ester to ethyl ester, which had been observed in the first approach, was avoided by replacing ethanol by tetrahydrofuran.

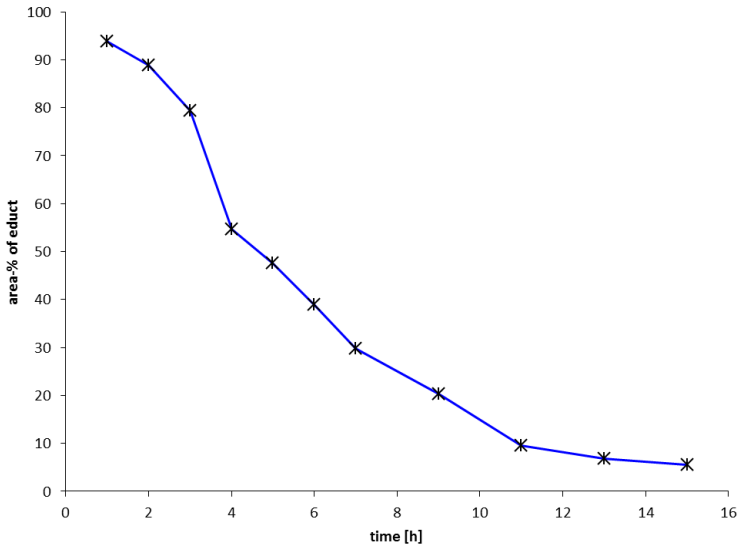


Figure 19: Decrease of keto ester **17** during the reduction with LiAlH(O*t*-Bu)$_3$, monitored by GC-MS.

The resulting amino alcohol intermediate **17a**, which is prone for lactonization even at room temperatures, could be easily lactonized by addition of a catalytic amount of *p*-toluenesulfonic acid in toluene and stirring at 60 °C for 12 h.

Major part of the desired (*R*,*S*)-γ-lactone **18** could be obtained in a very pure form just by precipitation of the crude oil in *n*-hexane, an additionally performed flash chromatography of the filtrate increased the yield to an acceptable 55 %.

For investigation of the relative configuration, a small amount of single crystals could be obtained by crystallization from *n*-hexane, like it had been done before for γ-lactone **4a**. Although the crystals had the shape of fine needles, it was possible to obtain the X-ray crystal structure of γ-lactone **18**, which is shown in the following Figure 20.

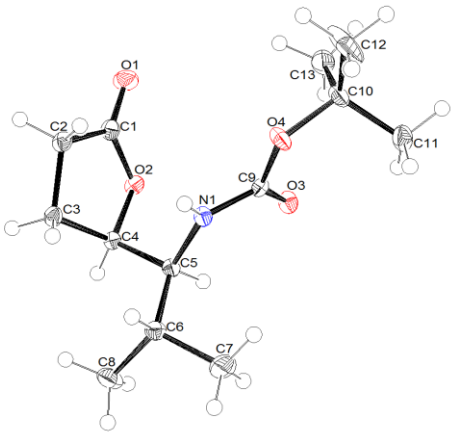
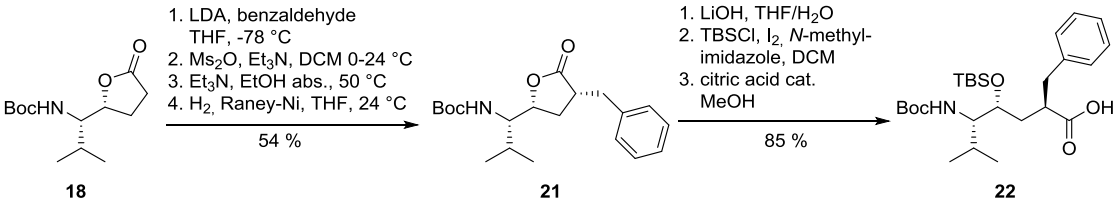


Figure 20: X-ray crystal structure (ORTEP) of the major (*R*,*S*)-configurated γ-lactone **18**. Thermal ellipsoids are drawn at 50 % probability level. Determined configuration of C-4 is based on the known configuration of C-5. We gratefully thank Prof. Roland Fischer for providing the X-ray data set.

4.3.2.4 Stereoselective alkylation, ring opening and silylation

Starting from the γ-lactone **18**, the *O*-protected pseudodipeptide core molecule **22** was synthesized by stereoselective alkylation, followed by opening of the lactone and immediate protection of the hydroxy moiety (Scheme 18).

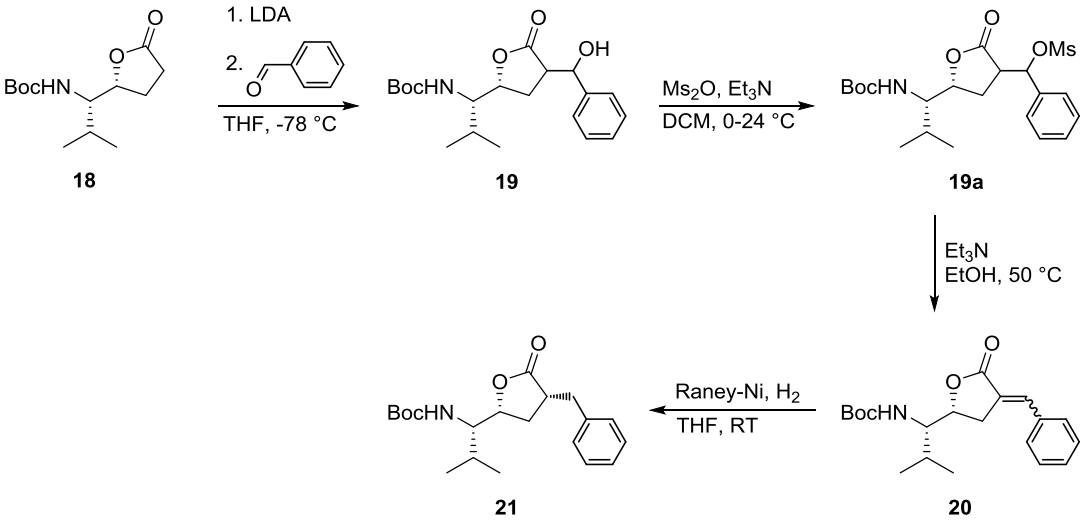


Scheme 18: From γ-lactone **18** towards the *O*-protected pseudodipeptide core structure **22**.

The stereoselective alkylation was carried out in a four-step sequence according to Nadin *et al.*, starting with an aldol reaction followed by a mesylation, elimination and a catalytic hydrogenation step (Scheme 19).[99]

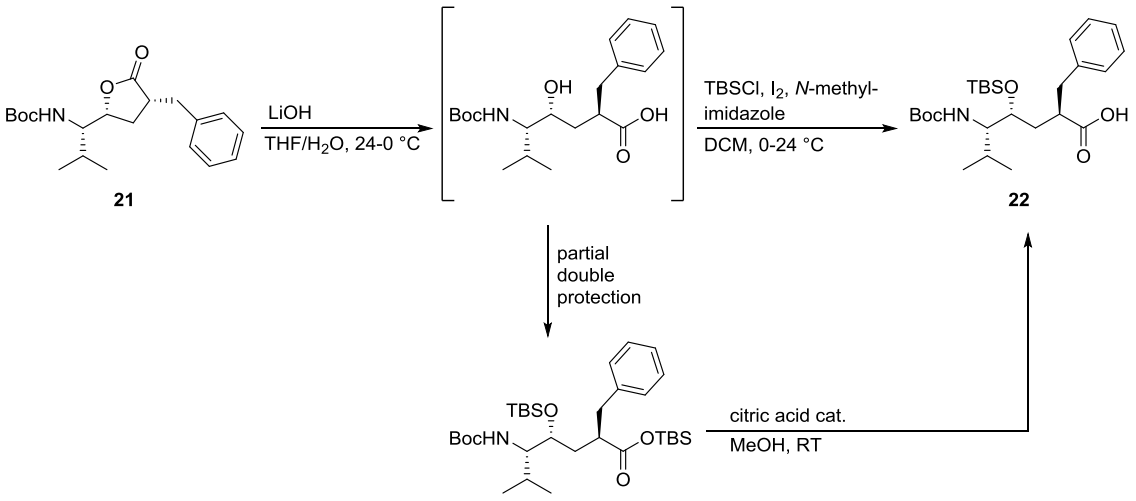
In the first step, the γ-lactone **18** was enolized by deprotonation with LDA at –78 °C in THF, and allowed to react with freshly distilled benzaldehyde to form the corresponding β-hydroxy lactone **19** as a complex mixture of diastereomers with 71 % yield. Subse-

quent treatment with methanesulfonic anhydride, followed by an elimination step of **19a**, resulted in formation of the α,β-unsaturated lactone **20**. The crude residue was then hydrogenated with Raney®-Nickel; hydrogenation in previous attempts with palladium on charcoal had failed.[54] Stereoselectivity of the hydrogenation could be achieved because of the bulky substituent of the unsaturated lactone, while the lactone itself is almost in a plane with the phenyl ring. Adsorption to the metal catalyst occurs with the less hindered face.[54,84]



Scheme 19: Detailed sequence of stereoselective alkylation of the γ-lactone **18**.

The next reaction step, ring opening of lactone **21** with a base, was the crucial step of the pseudodipeptide core structure synthesis. The same precautions had to be taken as described in chapter 4.1.2, due to the property of the originating γ-hydroxy acid to re-lactonize very easily.



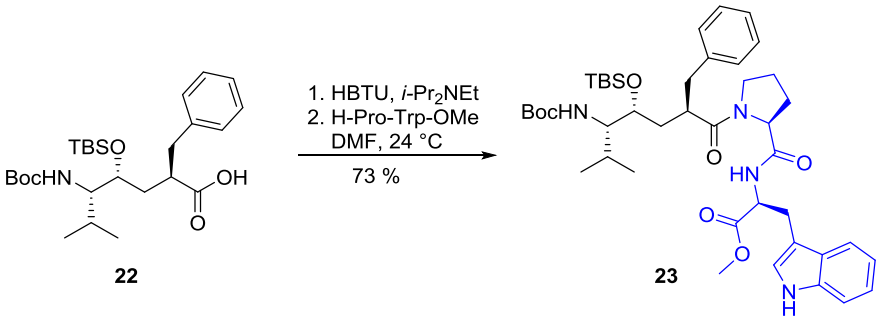
Scheme 20: Detailed reaction cascade for the lactone opening and protection of the hydroxy-moiety.

After opening of the lactone with LiOH in THF/H_2O = 2:3 (v/v) at RT, the reaction mixture was cooled to 0 °C and carefully acidified to pH=4. Again, after aqueous workup the solvents were removed carefully at a temperature below 30 °C to avoid re-lactonization. Immediately after drying of the crude product the hydroxy moiety was TBS-protected according to the procedure of Bartoszewicz *et al.*[60,61] Since also the TBS-ester had been formed in this step, a methanolysis was done afterwards (Scheme 20).[56] The desired pseudodipeptide core molecule **22** could be isolated in 85 % yield over 3 steps.

4.3.3 Elongation at the *C*- and *N*-terminus

Now the core structure was ready to be elongated at both the *C*- and *N*-terminus. The same well-established method for peptide coupling was used as in the synthetic route for the small molecule inhibitor (chapter 4.1.3). However, now a dipeptide was coupled, of which the Boc protecting group had to be removed in a first step with TFA.

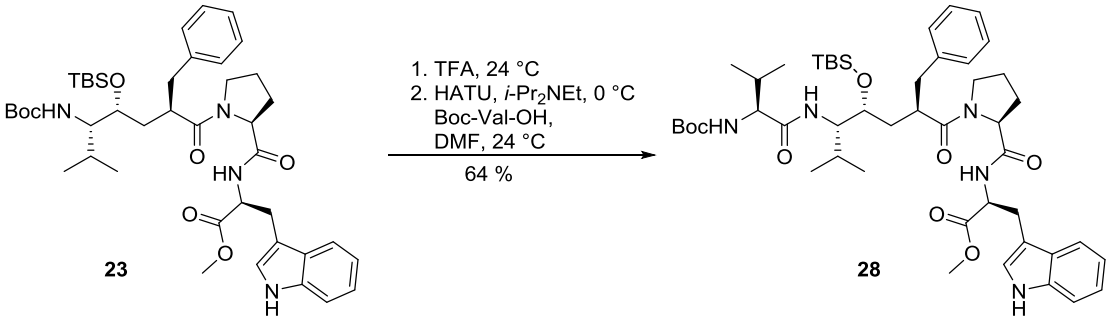
After purification of compound **23** via flash chromatography, a significant amount of tetramethylurea from peptide coupling remained, which could be removed by extensively washing with H_2O. Pseudopentapeptide **23** was isolated as a white solid in 73 % yield (794 mg), which gave us the opportunity to split the bulk for the synthesis of additional inhibitors.



Scheme 21: Peptide coupling of pseudodipeptide **22** to the Pro-Trp fragment (blue).

For the synthesis of ***HER***, the *N*-terminus of pseudotetrapeptide **23** was deprotected and elongated with Boc-L-valine (Scheme 22). In contrast to the initial synthesis route, where a simultaneous deprotection strategy of both the Boc and TBS protecting group had been pursued, the acid catalysed γ-lactone "backbite" was not of concern.[54]

For the peptide coupling itself, HATU was used as a coupling reagent to avoid the risk of racemization. Again, tetramethylurea had to be removed after flash chromatography by several H_2O washing steps. The desired target molecule **28** was finally isolated in 64 % yield.

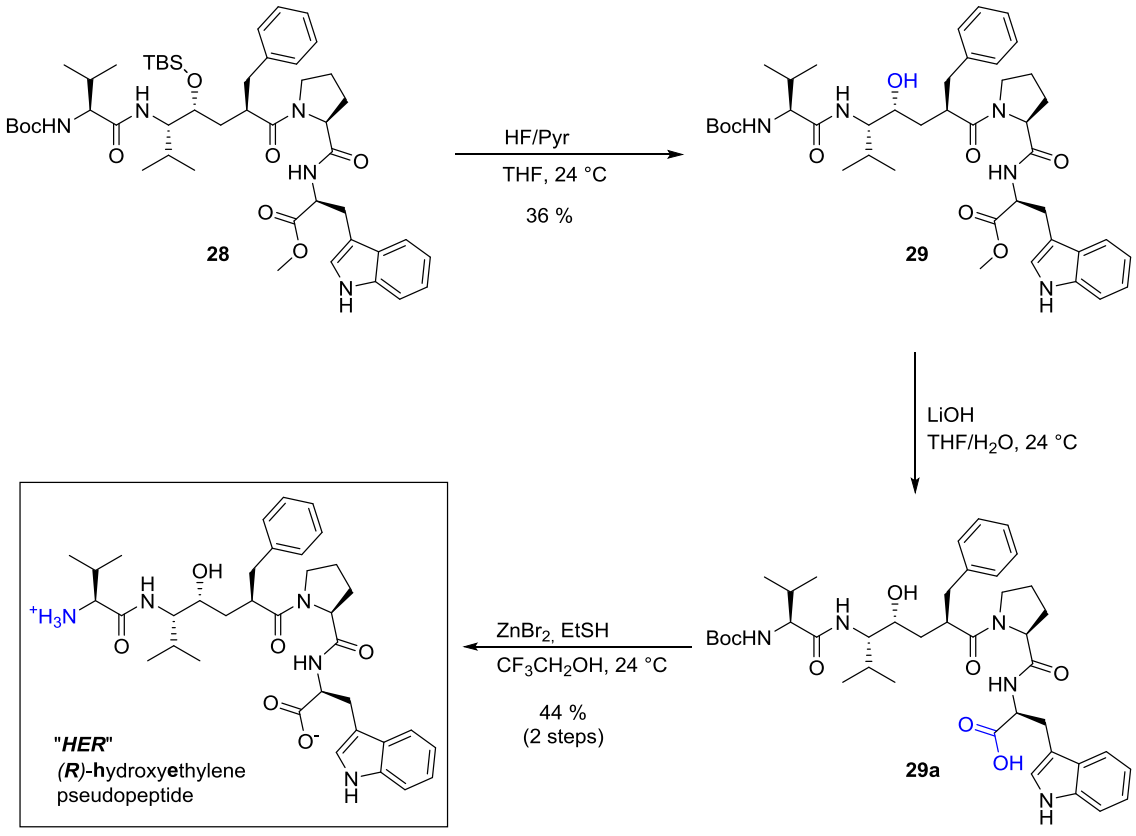


Scheme 22: Deprotection with TFA at the *N*-terminus and subsequent peptide coupling.

4.3.4 Final deprotection

Unfortunately, the final deprotection steps became more difficult than it had seemed at first sight. The result of the first approach, saponification of the methyl ester and subsequent simultaneously deprotection of both the Boc and TBS group with trifluoroacetic acid, resulted in acid catalyzed γ-lactone "backbite" and cleavage of the molecule. Hence, an alternative deprotection strategy had to be found.

Anhydrous hydrofluoric acid is widely used in solid phase peptide synthesis for the deprotection of acid labile protecting groups and also for the final cleavage of the peptide from the resin.[100] In pyridine, up to 70 % of HF can be dissolved to give a stable solution that can be used as a safe and efficient deprotecting agent.[101,102]



Scheme 23: Final deprotection of pseudopentapeptide ***HER*** has been realised in three single steps.

As an alternative to trifluoroacetic acid, HF/pyridine was now used as a milder deprotecting agent, which afforded the selective cleavage of TBS in a first step to obtain compound **29** in 36 % yield after flash chromatography. Despite careful reaction control, partial cleavage of the molecule due to the formation of the lactone could not be avoided.

Saponification with lithium hydroxide and subsequent treatment of crude **29a** with zinc bromide and ethanethiol in 2,2,2-trifluoroethanol provided the final target molecule "***HER***" in 44 % yield (2 steps) after preparative HPLC.

4.3.5 Synthesis of Ile-*HER* and B_3-*HER*

Two more potential inhibitors of DPP III have been synthesized (Figure 21). The first one, which we abbreviated as **Ile-*HER***, contains L-isoleucine instead of L-valine on the *N*-terminus, expecting that IC_{50} will be decreased due to higher similarity to the known DPP III inhibitor IVYPW.[36] The second one contains a niacin building block at the *N*-terminus, which achieved the lowest IC_{50} of all tested ketomethylene transition state mimetics in previous studies.[54]

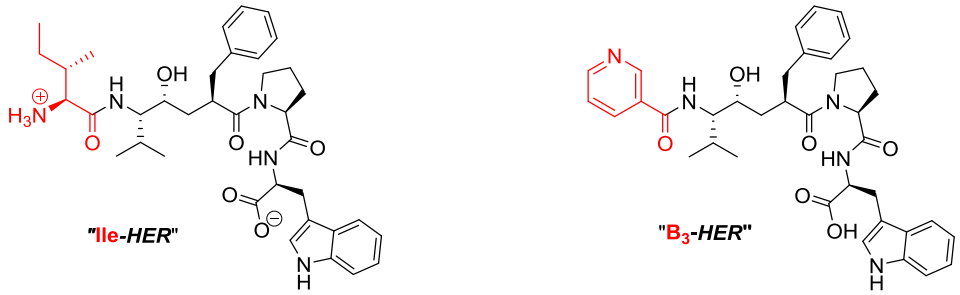
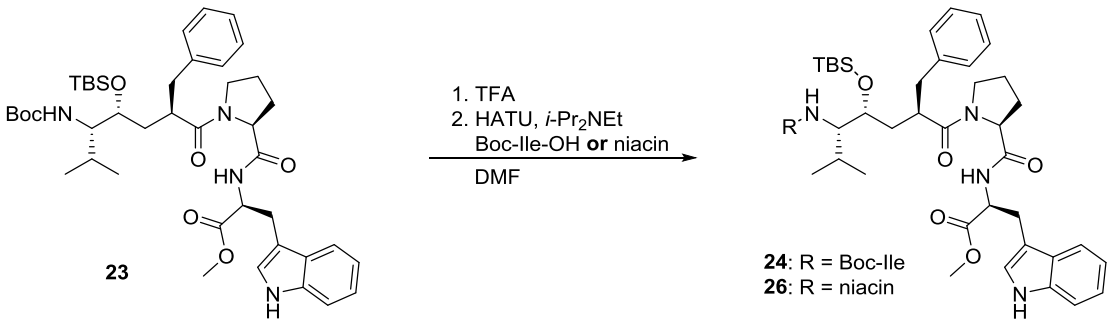


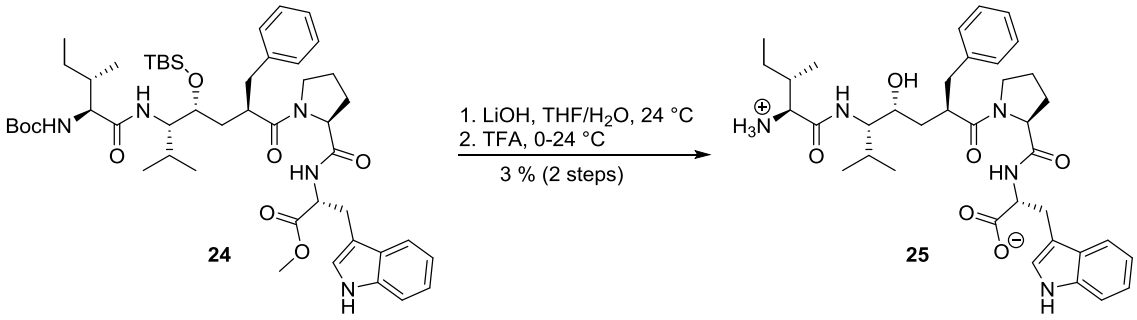
Figure 21: Two varieties of ***HER***, where valine has been replaced by isoleucine (left) and niacin (right).

The synthesis of both compounds started by elongation of pseudotetrapeptide **23** in the same way as in the synthesis of ***HER*** to give the fully protected pseudopentapeptides (Scheme 24).

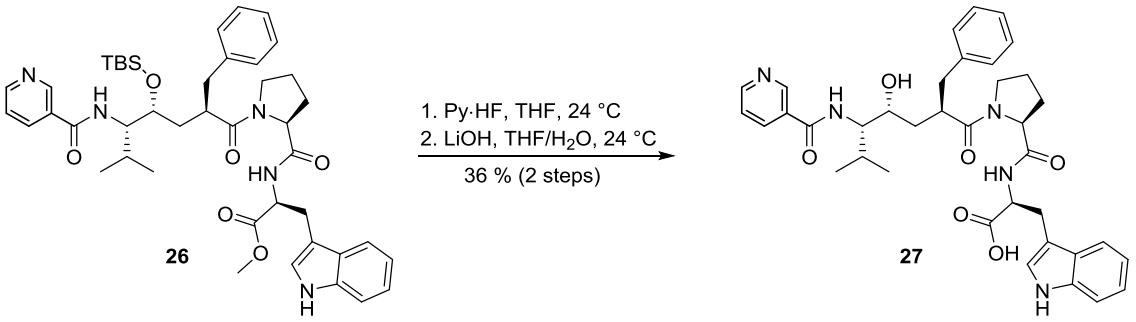


Scheme 24: Deprotection with TFA at the *N*-terminus and subsequent peptide coupling.

The final deprotection steps, which had been done earlier in the thesis project than the deprotection of ***HER***, were performed in a different order. While protected **Ile-*HER*** (**24**) was saponified with LiOH, followed by simultaneous Boc-deprotection and desilyation with TFA (Scheme 25), protected **B_3-*HER*** (**26**) was desilylated with HF/pyridine first, followed by saponification of the methyl ester (Scheme 26).



Scheme 25: Final deprotection steps of **Ile-*HER***, where most of the product has been destroyed due to acid catalyzed γ-lactone formation during treatment with TFA. Only 3 % of the desired product could be isolated via preparative HPLC.



Scheme 26: Final deprotection steps of **B_3-*HER*** with Py·HF followed by saponification with LiOH.

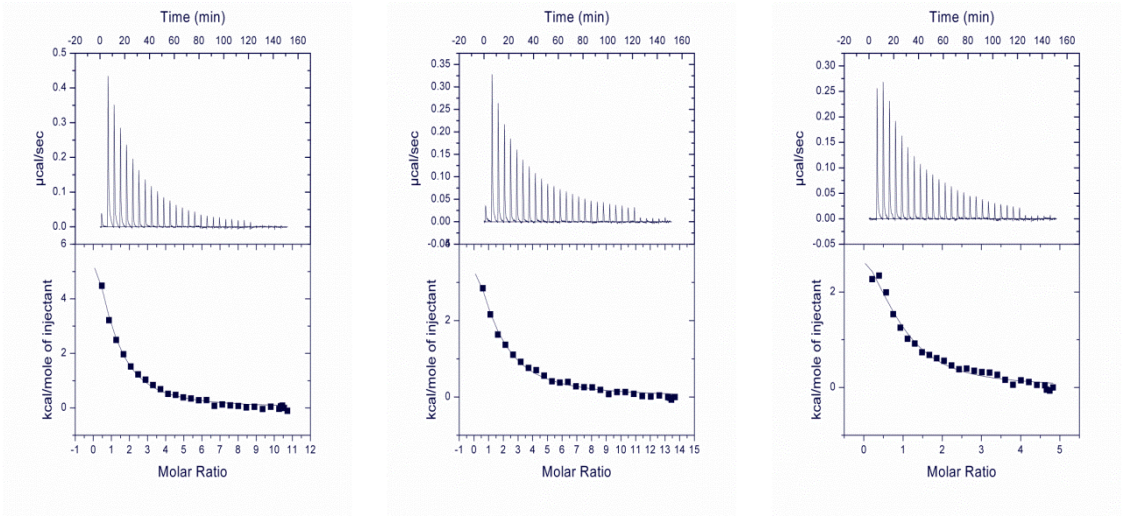
4.3.6 Biological assays of the inhibitors

The three synthesized inhibitors were used for a fluorescence-based inhibition assay and an isothermal microcalorimetry assay (only for ***HER***) to determine their biological activity. All experiments have been performed by Shalinee Jha at the Institute of Biochemistry, Graz University of Technology.

4.3.6.1 Isothermal titration calorimetry (ITC)

While an isothermal microcaloric titration with the inhibitor ***SHE*** had already been performed with the active wild type hDPP III enzyme, not enough amount of ***HER*** was available yet to perform the same experiment.[54] With the new batch of ***HER***, a triplicate ITC measurement with the active wild type hDPP III enzyme could be carried out as well. The results are shown in Figure 22.

1st replicate | 2nd replicate | 3rd replicate



K_d = 9.8 µM | K_d = 12.9 µM | K_d = 11.1 µM

Mean value: K_d = 11.3 ± 1.6 µM

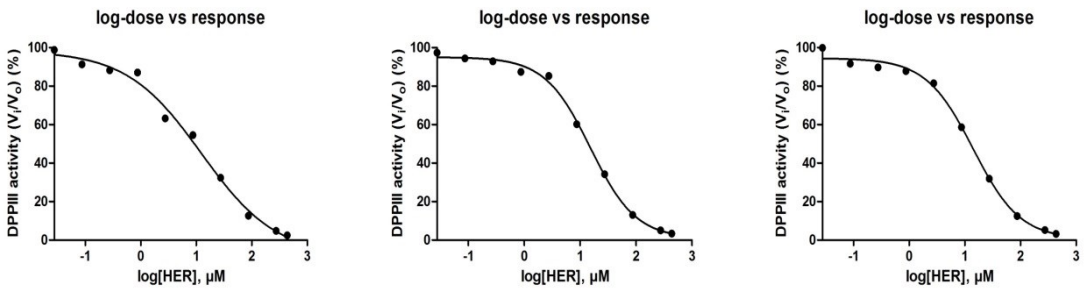
Figure 22: ITC thermograms at 298 K for ***HER***, performed with active wild type hDPP III. ITC was accomplished by S. Jha, Institute of Biochemistry, Graz University of Technology.

As Bezerra *et al*. showed for the ligand-hDPP III binding process with tynorphin, ***HER*** shows the same endothermic binding profile, which is a rare example for an entropy-driven binding mode among peptidases.[11,103] Compared with ***SHE***, where K_d = 23 ± 4 µM, ***HER*** binds more strongly to the enzyme.

4.3.6.2 Fluorescence-based inhibition assays

The IC_{50} value of ***HER*** has already been determined to be 8.8 µM by fluorescence-based inhibition assay in a single measurement. To confirm this result, we repeated a triplicate measurement with the new batch of ***HER***, which resulted in just a minor deviation of the new IC_{50} value of 13.8 µM (Figure 23).

1st replicate | 2nd replicate | 3rd replicate



IC_{50} = 12.3 µM | IC_{50} = 15.2 µM | IC_{50} = 14.0 µM

Mean value: IC_{50} = 13.8 ± 1.5 µM

Figure 23: Dose-response curves of fluorescence-based inhibition assay of ***HER***, performed by S. Jha.

Ile-*HER* and **B_3-*HER*** were tested both in a single fluorescence-based inhibition assay measurement. The results are shown in Figure 24.

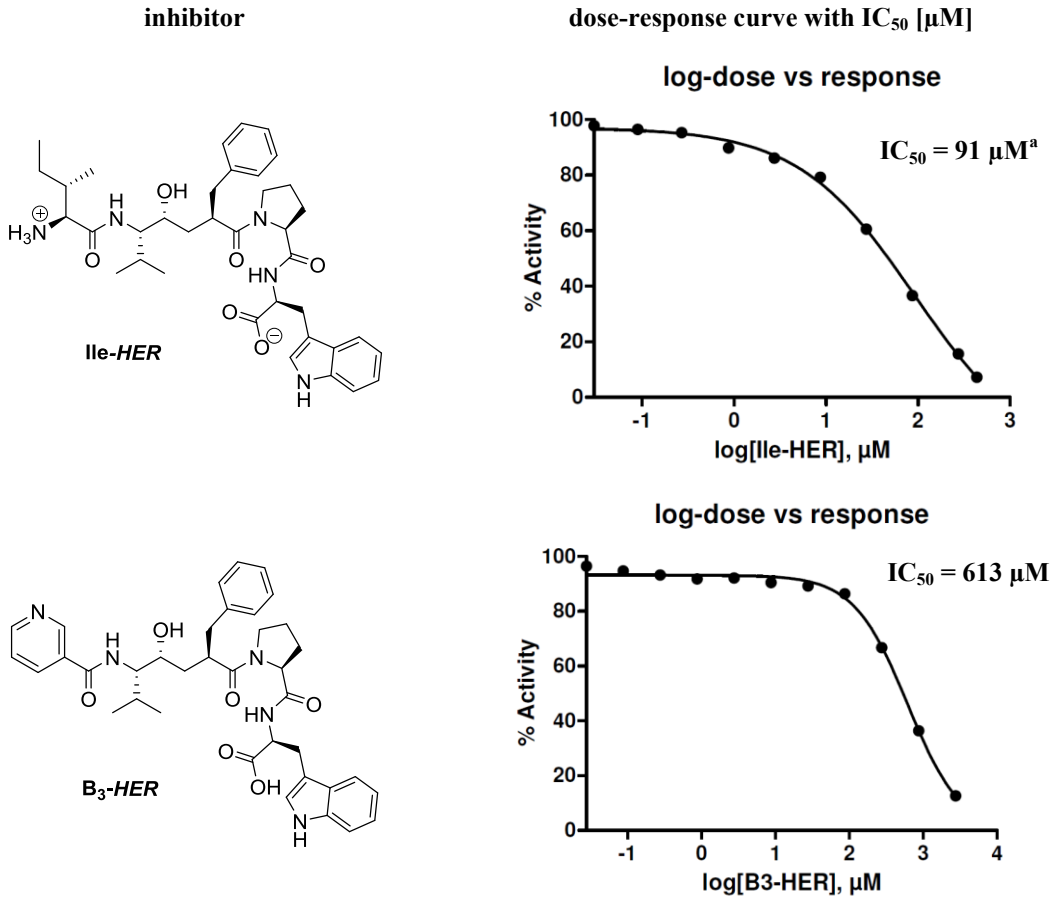


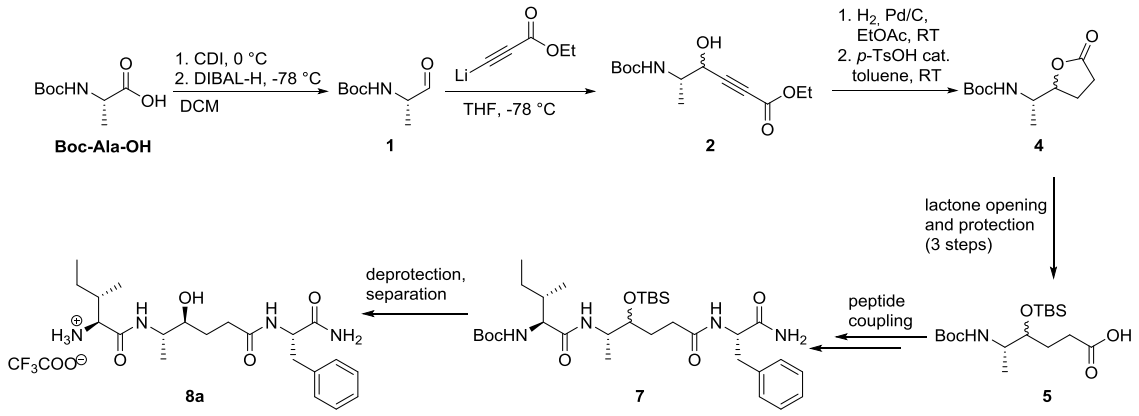
Figure 24: Fluorescence-based inhibition assays of degradation of Arg-Arg-βNA with hDPP III. [a]Due to the small isolated amount of **Ile-*HER*** its determined IC_{50} value is inaccurate. The inhibition assays were performed by S. Jha, Institute of Biochemistry, Graz University of Technology.

Due to the small isolated amount of **Ile-*HER*** of just 2.8 mg, the IC_{50} value of 91 µM is inaccurate and should be judged as a rough trend. Since it is unlikely that the result of a repeated assay would decrease one order of magnitude to an IC_{50} close to ***HER***, we did not pursue further studies on that molecule.

Unfortunately, the IC_{50} value of the promising molecule **B_3-*HER*** was calculated to be just 613 µM, which was far away from our expectations.

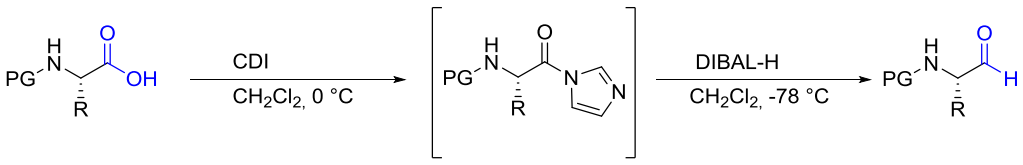
5 Summary and outlook

During this thesis, four inhibitors of DPP III were synthesized. The synthesis of the first potential small molecule inhibitor was performed by adapting a well-established synthetic route,[54,56] starting from the Boc-protected α-amino acid L-alanine to yield the γ-lactone **4** as a mixture of diastereomers in four steps. After hydrolytic lactone opening and protection of the hydroxy moiety, the desired core dipeptide **5** was elongated at both the *N*-terminus and *C*-terminus by peptide coupling and finally deprotected to provide the transition state mimicking inhibitor **8a** (Scheme 27).



Scheme 27: Summary of the first synthetic route towards the desired pseudotetrapeptide **8a**.

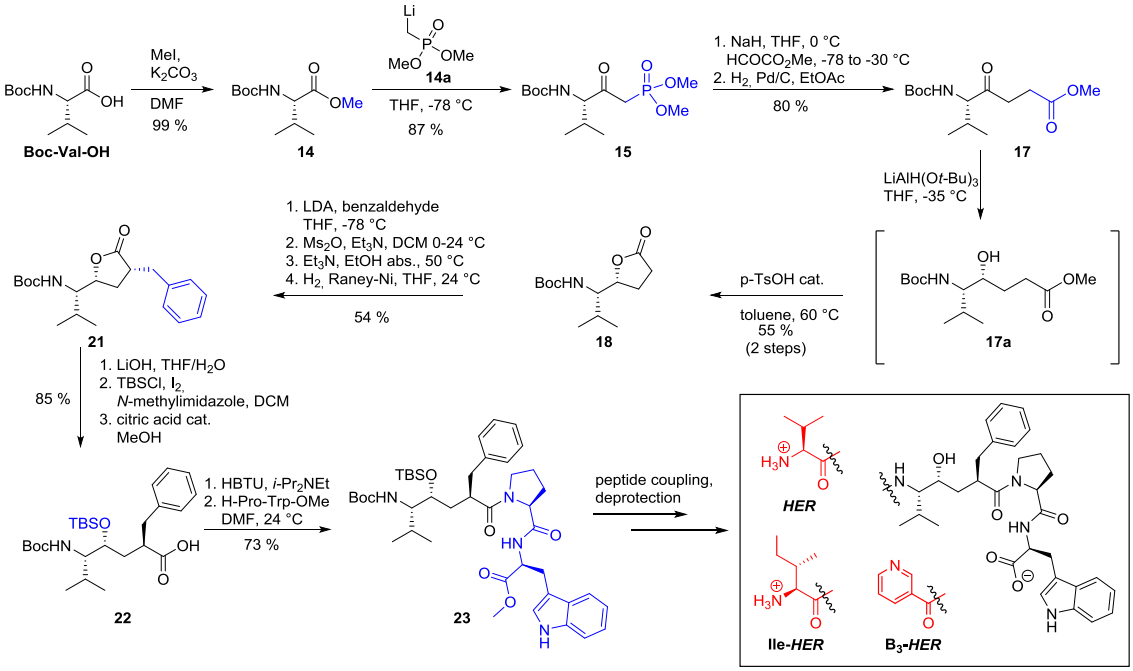
During the first synthetic route we needed access to different Boc-protected α-amino aldehydes as building blocks, such as Boc-alaninal (**1**). This requirement led to the development of a new method, which we published in *Org. Biomol. Chem*.[59]



Scheme 28: One-pot synthesis of α-amino aldehydes using CDI and DIBAL-H.[59]

With that efficient one-pot method, *N*-protected amino acids can be converted into chiral *N*-protected α-amino aldehydes by *in situ* activation with CDI, followed by reduction with DIBAL-H. While this method works excellently for proteinogenic amino acids, significant degree of epimerization could be observed in the reduction of peptides.[59]

For the synthesis of the three pseudopentapeptide transition state mimicking inhibitors ***HER***, **Ile-*HER*** and **B_3-*HER*** (***HER***: abbreviation for (***R***)-***h***ydroxy***e***thylene) an alternative route towards the γ-lactone intermediate was used to avoid the time-consuming separation of the diastereomers.



Scheme 29: Summary of the synthesis of ***HER***, **Ile-*HER*** and **B_3-*HER***.

The synthesis is based on a strategy of E. Haug and D. H. Rich,[84] using lithiated dimethyl methylphosphonate **14a** to generate keto phosphonate **15**, which was then utilized in a HWE reaction followed by a catalytic hydrogenation to give keto ester **17**. Stereoselective reduction with LiAlH(O*t*-Bu)$_3$ and subsequent lactonization gave γ-lactone **18**, which was alkylated stereoselectively. Hydrolytic lactone opening of **21** and protection of the hydroxy moiety provided the desired core dipeptide transition state mimicking unit **22**. The core unit was elongated at both the *N*-terminus and *C*-terminus by peptide coupling and finally deprotected in two different strategies to provide the DPP III inhibitors ***HER***, **Ile-*HER*** and **B_3-*HER***.

During the first HWE reaction, a byproduct was formed due to an accidental temperature rise, which could be identified as an anti-Baldwin cyclisation product.

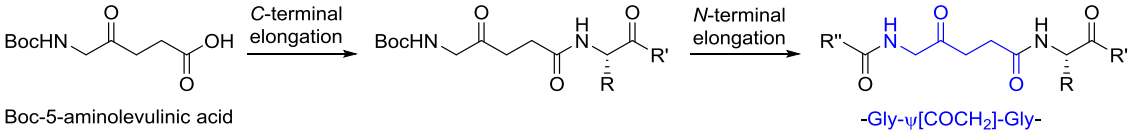
The synthesized inhibitors were tested in biological assays at the Institute of Biochemistry, confirming that ***HER*** inhibits hDPP III in low µM range, whereas the small molecule inhibitor H-Ile-Ala-ψ[COHCH$_2$]-Gly-Phe-NH$_2$ has an IC$_{50}$ of approximately 319 mM. All results are summarized in the following table.

Table 5: Summarized results of fluorescence-based inhibition assays and ITC.

Entry	Inhibitor	IC$_{50}$	K_d
1	H-Ile-Ala-ψ[COHCH$_2$]-Gly-Phe-NH$_2$	319 mM	N.A.
2	***HER***	13.8 µM	11.3 µM
3	**Ile-*HER***	91 µM	N.A.
4	**B_3-*HER***	613 µM	N.A.

For the ultimate goal, the access to very potent and specific inhibitors of DPP III which can be synthesized in rapid and easy routes, further research on this topic need to be performed. All tested molecules of this work and other works revealed that structures similar to the natural occurring peptide spinorphin (VVYPWT) are very potent inhibitors of DPP III. Since the truncated pentapeptide tynorphin showed a 16.5-fold inhibitory potency compared to spinorphin, it is likely to find also other and perhaps smaller inhibitors within nM range. [13,36,54]

Even though H-Ile-Ala-ψ[$COHCH_2$]-Gly-Phe-NH_2 did not inhibit DPP III, capability exists for the synthesis of shorter ketomethylene inhibitors, of which an easy access to the Gly-ψ[$COCH_2$]-Gly core through Boc-5-aminolevulinic acid has been discovered in our group and some derivatives have already been tested.[54]



Scheme 30: Suggestion for the synthesis of further DPP III inhibitors with non-cleavable ketomethylene Gly-Gly core.[54]

Also, it would be interesting to see whether other derivatives of ***HER*** (like **Ile-*HER*** and **B_3-*HER***) may give a boost to the inhibitory potential (Figure 25). For simplifying the synthetic route of ***HER***, the original tyrosine in tynorphin was replaced by pseudophenylalanine. Although the cocrystal structure of tynorphin with DPP III did not reveal the necessity of the hydroxy moiety, re-introducing could increase the affinity (since Tyr-Tyr is known as inhibitor) and make the inhibitor more specific for DPP III.[13,24,54]

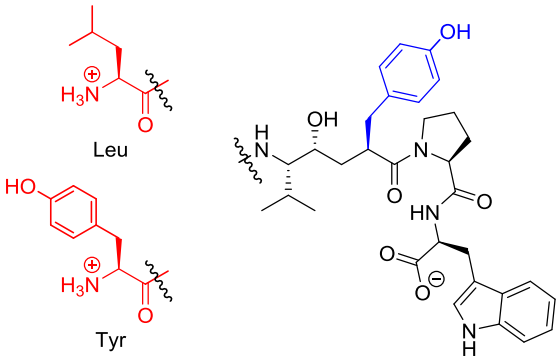


Figure 25: Examples for some additional (*R*)-hydroxyethylene pseudopentapeptide transition state mimicking inhibitors.

6 Experimental section

6.1 General methods of work

6.1.1 Organic reactions

All reactions with moisture sensitive reagents were carried out under inert atmosphere with standard Schlenk techniques and dry solvents (see chapter 6.1.3). Glassware for the experiments was dried using an oil pump vacuum (10^{-2} to 10^{-3} mbar) and heating with a heat gun. After cooling to room temperature the glassware was purged with nitrogen or argon to obtain oxygen- and moisture-free conditions. Solvents and reagents were added under argon or nitrogen counter-stream. HPLC, GC and TLC samples of the reaction mixture were taken using a glass pipette under argon or nitrogen counter-stream. Temperatures were measured externally unless otherwise stated. For each reaction a Teflon® coated magnetic stirring bar was used for stirring.

6.1.1.1 Safety for the workup of hydrogenation reactions

Hydrogenation catalysts were carefully removed under argon atmosphere by filtration through a Schlenk-frit containing a plug of Celite®. The plug was first rinsed with the solvent of the reaction, then with a water-miscible solvent and finally with H_2O. The remaining slurry was stored under water and disposed as hazardous waste.

6.1.2 Chemicals and reagents

All commercially available chemicals and reagents were obtained from the following companies: Acros Organics, Aldrich, Alfa Aesar, Fisher Scientific, Fluka, Merck, Novabiochem, Roth, Sigma-Aldrich and VWR. Reagents were used without further purification, unless otherwise stated.

Benzaldehyde: Benzaldehyde was obtained from Fluka and purified by vacuum distillation. The freshly prepared aldehyde was stored in a Schlenk flask under argon at 5 °C.

***n*-Butyllithium:** *n*-BuLi was obtained from Aldrich as a 2.5 M solution in hexanes. The exact concentration was determined by titration using the procedure of Kofron and Baclawski.[104] 90.0 mg (424 μmol) diphenylacetic acid were dissolved in 1 mL abs. THF in a dry 8 mL Schlenk tube with magnetic stirring bar under argon. To the stirred colorless solution, *n*-BuLi solution was added with a syringe through a septum until the color changed to yellow. The titration was repeated three times and the concentration was calculated using the mean value of the consumed *n*-BuLi solution.

1,1'-Carbonyldiimidazole: CDI was obtained from Aldrich and recrystallized from abs. THF using the procedure published by Staab and Wendel.[58] A dry 250 mL three-necked round bottom flask with a Schlenk adapter, a reflux condenser, a gas bubbler and a magnetic stirring bar (evacuated, heated, N_2-purged) was charged with a suspension of 25.0 g (154 mmol) CDI in 40 mL abs. THF and stirred. The suspension was heated to reflux (oil bath) and 20 mL abs. THF were added to enable full dissolution. Stirring was stopped and the pale yellow solution was allowed to cool down to RT for 60 min. Crystallization was completed by cooling to 0 °C (ice bath) for additional 30 min. The white crystals were collected by filtration through a glass frit under nitrogen atmosphere, washed with ice-cold abs. THF (15 mL) and dried *in vacuo*. 19.9 g (123 mmol, 80 %) of the recovered material was stored in a Schlenk flask under argon at –30 °C.

Molecular sieves: 3Å and 4Å molecular sieves (Sigma Aldrich, beads 8–12 mesh) were activated by heating them in a round bottom flask with a heat mantle (level 1) under high vacuum for 24 h. Activated molecular sieves were stored at RT under argon atmosphere.

6.1.3 Solvents

All solvents were purchased from the companies mentioned in chapter 6.1.2 and were used without further purification unless otherwise stated. For reactions where moisture and oxygen were excluded, absolute solvents were used. For that purpose the purchased solvents were dried by using the following methods and stored in brown 1 L Schlenk bottles under argon and over activated molecular sieves. For analytical applications solvents with analytical grade were purchased.

Chloroform: For purposes where complete dryness was not necessary, $CHCl_3$ was distilled using a rotary evaporator and stored in a brown glass bottle.

Dichloromethane: DCM (stabilized with EtOH) was first heated under reflux over P_4O_{10} for 12 h, then over CaH_2 for 2 d and distilled under argon atmosphere into a brown 1 L Schlenk bottle with activated 4Å molecular sieves.

Diethyl ether: Et_2O (for purposes where complete dryness was not necessary) was distilled using a rotary evaporator and stored in a brown bottle over KOH.

***N,N*-Dimethylformamide:** DMF was purchased in extra dry quality from Alfa Aesar and transferred into a brown 1 L Schlenk bottle with activated 3Å molecular sieves and stored under argon.

Ethanol: EtOH was purchased from Merck (99 %, containing 1 % methylethyl ketone) and heated under reflux together with sodium and diethyl phthalate in an inert distilla-

tion apparatus under argon for 2 h. Subsequently, the dry ethanol was distilled and stored over activated 3Å molecular sieves in a brown 1 L Schlenk bottle under argon.

Methanol: MeOH was heated under reflux over magnesium turnings and then distilled under argon atmosphere into a dry 1 L Schlenk bottle with activated 3Å molecular sieves.

Tetrahydrofuran: THF was heated under reflux over sodium for 21 h until the added benzophenone indicated dryness by turning color from green to blue. The dried THF was distilled into a 1 L Schlenk flask with activated 4Å molecular sieves and stored under argon.

Water: If water was used as a solvent in a reaction or for workup, deionized water from an ion exchanger was used.

6.2 Analytical methods

6.2.1 Thin-layer chromatography

Thin-layer chromatography was performed by using TLC-plates from Merck (TLC aluminium foil, silica gel 60, F_{254}). For detection of the spots a UV lamp with $\lambda = 254$ nm (fluorescence quenching) and/or staining with a reagent and subsequent development by heating with a heat gun was used. The following staining-reagents were used:

$KMnO_4$ (primarily used): 0.3 g $KMnO_4$ and 20 g K_2CO_3 were dissolved in 300 mL H_2O and 5 mL of 5 % aqueous NaOH were added.

CAM-solution: 2.0 g cerium(IV) sulfate, 50.0 g ammonium molybdate and 50 mL conc. H_2SO_4 in 400 mL water.

All used solvent mixtures, staining reagents and R_f values are stated in the experimental procedures.

6.2.2 Flash chromatography

Purification via flash chromatography was performed by using silica gel 60 from Acros Organics with a particle size of 35–70 µm. The mass of silica gel, depending on the separation problem, was a 30- to 100-fold amount (w/w) of the crude product. The length of the column was selected to get a pad of silica gel between 10 and 30 cm. All crude products were dissolved in a small amount of the mobile phase and applied in the dissolved form on the column. Solvent mixtures were chosen to adjust the R_f value of the product between $R_f = 0.15$ and $R_f = 0.40$. Solvents used for flash chromatography were purchased from Fisher Scientific as analytical grade solvents.

6.2.3 Gas chromatography

6.2.3.1 GC-MS

Analytical gas chromatography with mass detector was performed on an "Agilent Technologies 7890A GC System" with mass selective detector (EI, 70 eV; Agilent Technologies 5975C inert MSD with Triple-Axis Detector). Samples were injected by an autosampler (Agilent Technologies 7683B Series Autosampler) in split mode (20/1; inlet temperature 280 °C). Attached capillary column was a polar HP-5ms column (30 m × 0.25 mm, layer thickness 0.25 μm) with helium 5.0 as carrier gas. Following methods were used:

JI_M300_S: 50 °C 2 min, ramp 40 °C/min linear to 300 °C, 5 min

JI_DMFcut_S: 50 °C 2 min, ramp 40 °C/min linear to 300 °C, 5 min

For the calculation of conversion, the integrated peak areas were used. Since no internal or external standard was used, these values are not exact.

6.2.3.2 GC-FID

Analytical gas chromatography with flame ionization detector (FID) for the separation of enantiomers was performed on an "Agilent Technologies 6890N GC System" with a CP-Chiralsil Dex CB capillary column (25 m × 0.32 mm, layer thickness 0.25 μm) with nitrogen 5.0 as carrier gas. Samples were injected by an autosampler (CTC Analytics CombiPAL) in split mode (5/1; inlet temperature 200 °C). The following method was used:

AMAL_GCPAL.M: 80 °C 5 min, ramp 10 °C/min linear to 150 °C, 18 min

The integrated peak areas were used to determine the ee of chiral compounds.

6.2.4 High-performance liquid chromatography

6.2.4.1 Analytical HPLC-MS

Analytical high-performance liquid chromatography was performed on an "Agilent Technologies 1200 Series" HPLC system with 1260 HiP Degasser G4225A, binary pump SL G1312, autosampler HiP-ALS SL G1367C, thermostated column compartment TCC SL G1316B, multiple wavelength detector G1365C MWD SL with deuterium lamp (λ = 190–400 nm) and subsequent connected mass detector (Agilent Technologies 6120 Quadrupole LC/MS) with an electrospray ionization (ESI) source. The components were separated on a RP Agilent Poroshell 120 SB-C18 column (3.0 × 100 mm, 2.7 μm) with a Merck LiChroCART® 4-4 pre-column. Signals were detected at 210 nm or 280 nm. As mobile phase acetonitrile (VWR HiPerSolv, HPLC-MS grade) and water

(deionized and filtered through a 0.2 µm cellulose nitrate membrane filter) with 0.01 % formic acid were used. Following methods were used:

FAST_POROSHELL120_001HCOOH_8MINGRADIENT.M: 40 °C, flow rate 0.7 mL/min; 0.0–2.0 min MeCN/H_2O = 10:90 (v/v), 2.0–10.0 min linear increase to MeCN/H_2O = 95:5 (v/v), 10.0–16.0 min hold MeCN/H_2O = 95:5 (v/v).

LONG_POROSHELL120_001HCOOH_40PCISOCRAT: 40 °C, flow rate 0.7 mL/min; 0.0–2.0 min MeCN/H_2O = 10:90 (v/v), 2.0–6.0 min linear increase to MeCN/H_2O = 40:60 (v/v), 6.0–12.0 min hold MeCN/H_2O 40:60 (v/v), 12.0–16.0 min linear increase to MeCN/H_2O = 95:5 (v/v), 16.0–22.0 min hold MeCN/H_2O = 95:5 (v/v).

Integrated peak areas were used for the calculation of conversion. Since no internal or external standard was used, these values are not exact.

6.2.4.2 Preparative HPLC

Isolation of polar compounds were performed on a "Thermo Scientific Dionex UltiMate 3000" system with UltiMate 3000 pump, UltiMate 3000 autosampler, UltiMate 3000 column compartment, UltiMate 3000 diode array detector (deuterium lamp, λ = 190–380 nm) and a UltiMate 3000 automatic fraction collector. The components were separated on a RP Machery-Nagel 125/21 Nucleodur® 100-5 C18ec column (21 × 125 mm, 5.0 µm). Signals were detected at 210 nm and 280 nm. As mobile phase acetonitrile (VWR HiPerSolv, HPLC grade) and water (deionized and filtered through a 0.2 µm cellulose nitrate membrane filter) with 0.01 % formic acid or 0.01 % trifluoroacetic acid were used. The following methods were used:

JKV_NucleodurC18_001HCOOH_10to85: 24 °C, flow rate 15 mL/min; 0.0–3.0 min MeCN/H_2O = 10:90 (v/v), 3.0–11.0 min linear increase to MeCN/H_2O = 85:15 (v/v), 11.0–13.0 min hold MeCN/H_2O = 85:15 (v/v).

CHRISTIAN_NucleodurC18_001CF3COOH: 24 °C, flow rate 15 mL/min; 0.0–3.0 min MeCN/H_2O = 10:90 (v/v), 3.0–11.0 min linear increase to MeCN/H_2O = 85:15 (v/v), 11.0–13.0 min hold MeCN/H_2O = 85:15 (v/v).

CLF_NucleodurC18_001HCOOH_5to90hold60: 24 °C, flow rate 15 mL/min; 0.0–5.0 min MeCN/H_2O = 5:95 (v/v), 5.0–13.0 min linear increase to MeCN/H_2O = 60:30 (v/v), 13.0–14.0 min hold MeCN/H_2O = 60:30 (v/v), 14.0–18.0 min linear increase to MeCN/H_2O = 90:10 (v/v), 18.0–20.0 min hold MeCN/H_2O = 90:10 (v/v).

6.2.5 Nuclear magnetic resonance spectroscopy

All NMR spectra were recorded with a Bruker AVANCE III spectrometer with autosampler at 300.36 MHz (^1H) and 75.53 MHz (^{13}C) or a Varian Inova NB high resolu-

tion spectrometer at 499.88 MHz (^{1}H) and 125.70 MHz (^{13}C). Chemical shifts δ are referenced to residual protonated solvent signals as internal standard. Signal multiplicities *J* are abbreviated as s (singlet), bs (broad singlet), d (doublet), dd (doublet of doublet), t (triplet) and m (multiplet). When it was necessary, additional 1D and 2D techniques (APT, COSY, HSQC, HMBC, NOESY, TOCSY, ^{19}F, ^{31}P) were recorded to verify the structure.

Deuterated solvents for NMR spectroscopy were purchased from Euriso-top®.

6.2.6 Melting points

Melting points were measured on a MEL-TEMP® apparatus with integrated microscopical support from Electrothermal in open capillary tubes. Reported values are uncorrected.

6.2.7 Optical rotation

A Perkin Elmer Polarimeter 341 was used for the determination of the specific rotation at λ = 589 nm (sodium D-line). Measurements were recorded at 23 or 24 °C and repeated at least three times.

6.2.8 High-resolution mass spectroscopy

High-resolution mass spectra were recorded using MALDI-TOF on a Micromass® MALDI micro MX™ spectrometer. Dithranol (1,8-dihydroxy-9,10-dihydroanthracen-9-one) or α-cyano-4-hydroxycinnamic acid served as matrix and PEG as internal standard. The stated values are m/z.

6.2.9 X-Ray crystallography

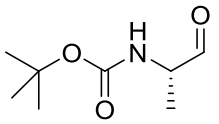
X-Ray crystallography was performed by Prof. Roland Fischer (Institute of Anorganic Chemistry, Graz University of Technology), using the following procedure:

All crystals suitable for single crystal X-ray diffractometry were removed from a vial and immediately covered with a layer of silicone oil. A single crystal was selected, mounted on a glass rod on a copper pin, and placed in the cold N_2 stream provided by an Oxford Cryosystems cryostream. XRD data collection was performed on a Bruker APEX II diffractometer with use of Mo-K_α radiation (Incoatec Microsource *Iμ50*, λ = 0.71073 Å) and a CCD area detector. Empirical absorption corrections were applied using SADABS.[105,106] The structures were solved with use of direct methods in SHELXS and refined by the full-matrix least-squares procedures in SHELXL.[107] The space group assignments and structural solutions were evaluated using PLATON.[108]

6.3 Experimental procedures and characterization

6.3.1 First synthetic route to a small molecule inhibitor

6.3.1.1 Boc-L-alaninal (1)[59]



1

A 1000 mL Schlenk flask with a magnetic stirring bar was dried under vacuum with a heat gun and purged with N_2. 9.46 g (50.0 mmol, 1.0 eq) Boc-L-Ala-OH were dissolved in 333 mL abs. dichloromethane. The solution was cooled to 0 °C (ice bath) and 8.92 g (55.0 mmol, 1.1 eq) 1,1'-carbonyldiimidazole (CDI) were added. After stirring for 30 min the mixture was cooled to –78 °C (dry ice bath) and 105 mL (105 mmol, 2.1 eq) DIBAL-H solution (1.0 mol/L in toluene) were added dropwise with a syringe through a septum within 3 h. After additional 45 min stirring at –78 °C, the reaction mixture was quenched by addition of 335 mL EtOAc, the dry ice bath removed and 335 mL tartaric acid solution (25 % in H_2O) added under vigorous stirring. After warming to RT, the layers were separated. The aqueous phase was extracted with EtOAc (335 mL) and the combined organic layers were washed with 1 M HCl (335 mL), saturated $NaHCO_3$ (335 mL) and brine (335 mL), dried over Na_2SO_4, filtrated and concentrated under reduced pressure. The crude product was dried under high vacuum and used without further purification.

Yield: 7.51 g (43.4 mmol, 87 %), white solid.

$C_8H_{15}NO_3$ [173.21 g/mol].

mp = 81–84 °C.

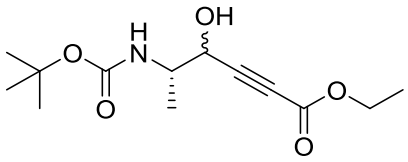
$[\alpha]_D^{24}$ = –39 (c = 1.0, MeOH); lit. $[\alpha]_D^{20}$ = –39 (c = 1, MeOH).[109]

R_f = 0.38 (cyclohexane/EtOAc = 2:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.55 (s, 1H, C<u>H</u>O), 5.11 (s, 1H, N<u>H</u>), 4.30-4.12 (m, 1H, C<u>H</u>CHO), 1.44 (s, 9H, H-Boc), 1.32 (d, ^3J = 7.4 Hz, 3H, C<u>H</u>$_3$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 199.9 (s, 1C, CHO), 155.4 (s, 1C, CONH), 80.2 (s, 1C, $(CH_3)_3$<u>C</u>O), 55.7 (s, 1C, CH_3<u>C</u>), 28.4 (s, 3C, (<u>C</u>H$_3$)$_3$CO), 15.0 (s, 1C, CH_3).

6.3.1.2 Ethyl (5*S*)-5-((*tert*-butoxycarbonyl)amino)-4-hydroxyhex-2-ynoate (2)



2

A 250 mL Schlenk flask with a magnetic stirring bar was dried under vacuum (heat gun), purged with N_2 and 5.13 mL (52.0 mmol, 2.6 eq) 1-pentyne were dissolved in 65 mL abs. THF. After cooling to 0 °C (ice bath) 20.0 mL (50.0 mmol, 2.5 eq) *n*-BuLi were added with a syringe within 10 min. The red solution was cooled to –78 °C (dry ice bath) and stirred for additional 15 min. 5.07 mL (50.0 mmol, 2.5 eq) ethyl propiolate were added dropwise with a syringe within 10 min, the mixture was stirred for 15 min and a solution of 3.46 g (20.0 mmol, 1 eq) Boc-L-alaninal (**1**) in 35 mL abs. THF was added slowly (25 min). After stirring for 2 h at –78 °C the yellow reaction mixture was quenched by addition of a solution of 10 mL acetic acid in 20 mL THF at –78 °C, warmed up to RT and extracted with 200 mL EtOAc. The organic phase was washed with $NaHCO_3$ (5 % in H_2O, 2×75 mL) and brine (35 mL), dried over Na_2SO_4, filtered, and the solvent was removed. The dark red oil was purified via flash chromatography (250 g SiO_2; cyclohexane/EtOAc = 4:1→2:1 v/v).

Yield: 4.20 g (15.5 mmol, 77 %), yellow oil, mixture of 2 diastereomers.

$C_{13}H_{21}NO_5$ [271.31 g/mol].

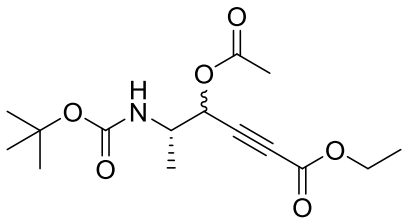
R_f = 0.18 (cyclohexane/EtOAc = 4:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$, *S*,*S*-diastereomer, based on COSY and HSQC): δ = 4.97 (d, ^3J = 8.1 Hz, 1H, NH), 4.47 (s, 1H, CHOH), 4.35 (s, 1H, OH), 4.16 (q, ^3J = 7.1 Hz, 2H, CH_2), 3.95–3.73 (m, 1H, CHNH), 1.37 (s, 9H, H-Boc), 1.29–1.14 (m, 6H, containing $CHCH_3$ and CH_2CH_3).

^{13}C NMR (75 MHz, $CDCl_3$, *S*,*S*-diastereomer, based on HSQC and HMBC): δ = 156.0 (s, 1C, C=O), 153.4 (s, 1C, HNCO), 85.8 (s, 1C, HOC≡C), 80.1 (s, 1C, Me_3C), 77.1 (solvent overlapped, 1C, C≡CCO_2), 65.4 (s, 1C, HCOH), 62.2 (s, 1C, CH_2), 50.4 (s, 1C, $CHCH_3$), 28.3 (s, 3C, $(CH_3)_3CO$), 15.8 (s, 1C, $CHCH_3$), 14.0 (s, 1C, CH_2CH_3).

HRMS (MALDI-TOF): Calcd. for $C_{13}H_{21}NO_5Na$ $[M+Na]^+$: 294.1317; found: 294.1324.

6.3.1.3 Ethyl (5*S*)-4-acetoxy-5-((*tert*-butoxycarbonyl)amino)hex-2-ynoate (3)[110]



3

A 15 mL Schlenk tube with magnetic stirring bar was dried under vacuum (heat gun) and purged with N_2. 194 µL (2.40 mmol, 6.5 eq) pyridine, 0.5 mg (4 µmol, 0.01 eq) DMAP and 38 µL (0.41 mmol, 1.1 eq) acetic anhydride were dissolved in 3.7 mL abs. dichloromethane and the solution was cooled to 0 °C (ice bath). 100 mg (369 µmol, 1.0 eq) compound **2** were added in one portion and the reaction mixture was stirred for 3 h at 0 °C. After addition of 6 mL 1 M HCl the ice bath was removed and the phases were separated. The organic phase was diluted with 2.5 mL EtOAc, washed with H_2O (2×3 mL) and brine (3 mL), dried over Na_2SO_4 and filtered. The solvents were removed under reduced pressure and the product was dried *in vacuo*. Purification via flash chromatography (12 g SiO_2; cyclohexane/EtOAc = 5:1 v/v) yielded compound **3** as a brown oil.

Yield: 67 mg (0.21 mmol, 58 %), brown oil.

$C_{15}H_{23}NO_6$ [313.35 g/mol].

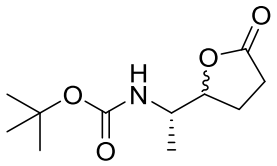
R_f = 0.58 (cyclohexane/EtOAc = 2:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$, mixture of diastereomers): δ = 5.51–5.42 (m, 1H, C<u>H</u>OAc), 4.7–4.54 (m, 1H, N<u>H</u>), 4.27–4.13 (m, 2H, C<u>H</u>$_2$), 4.10–3.91 (m, 1H, C<u>H</u>NH), 2.08 (s, 3H, C<u>H</u>$_3$CO$_2$), 1.41 (s, 9H, H-Boc), 1.32–1.18 (m, 6H, containing CHC<u>H</u>$_3$ and CH$_2$C<u>H</u>$_3$).

^{13}C NMR (75 MHz, $CDCl_3$, mixture of diastereomers, major diastereomer): δ = 169.3 (s, 1C, H_3C<u>C</u>=O), 154.9 (s, 1C, CC=O), 152.9 (s, 1C, HNC=O), 81.6 (s, 1C, AcOC<u>C</u>≡C), 80.1 (s, 1C, Me$_3$<u>C</u>), 77.9 (s, 1C, C≡<u>C</u>CO$_2$), 65.7 (s, 1C, H<u>C</u>OAc), 62.4 (s, 1C, CH$_2$), 48.3 (s, 1C, <u>C</u>HCH$_3$), 28.4 (s, 3C, (<u>C</u>H$_3$)$_3$CO), 20.7 (s, 1C, <u>C</u>H$_3$CO$_2$), 16.4 (s, 1C, CH<u>C</u>H$_3$), 14.0 (s, 1C, CH$_2$<u>C</u>H$_3$).

HRMS (MALDI-TOF): Calcd. for $C_{15}H_{23}NO_6Na$ $[M+Na]^+$: 336.1423; found: 336.1431.

6.3.1.4 *tert*-Butyl ((1*S*)-1-(5-oxotetrahydrofuran-2-yl)ethyl)carbamate (4)



4

Hydrogenation: A 50 mL round bottom flask with Schlenk adaptor and magnetic stirring bar was charged with 1.46 g (5.35 mmol, 1.0 eq) compound **2** dissolved in 21.5 mL EtOAc. After degassing/purging (3 × vacuum/N_2) 115 mg palladium on activated charcoal (5 % Pd/C) were added. The flask was evacuated and filled with H_2 three times and stirred under an atmosphere of H_2 (balloon) overnight. The catalyst was carefully removed under an argon atmosphere by filtration through a Schlenk-frit containing a plug of Celite® and the filtrate was concentrated under reduced pressure.

Lactonization: The residue was dissolved in 21.5 mL toluene, 10 mg (5.4 µmol, 0.01 eq) *p*-TsOH×H_2O were added and the mixture was heated to 50 °C for 1 h. After cooling to RT, the solution was washed with satd. $NaHCO_3$ (20 mL), brine (20 mL), dried over Na_2SO_4, filtered and the solvent was evaporated. The crude residue was purified via flash chromatography (120 g SiO_2; cyclohexane/EtOAc = 2:1 v/v) to yield the lactone **4** as a mixture of diastereomers (dr = 5:1 (*S*,*S*):(*R*,*S*)). Single crystals of the major diastereomer could be obtained by crystallization from *n*-hexane.

Yield: 841 mg (3.67 mmol, 68 %), yellow oil; 83 mg (0.36 mmol, 7 %), colorless crystals of the (major) *S*,*S*-diastereomer.

$C_{11}H_{19}NO_4$ [229.28 g/mol].

mp = 59 °C (*S*,*S*-diastereomer).

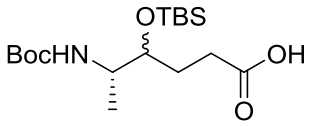
$[\alpha]_D^{23}$ = –29.6 (c = 0.92, $CHCl_3$); *S*,*S*-diastereomer.

R_f = 0.19 (cyclohexane/EtOAc = 2:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$, *S*,*S*-diastereomer, based on COSY and HSQC): δ = 4.68 (d, ^3J = 8.3 Hz, 1H, NH), 4.48–4.34 (m, 1H, CO_2CH), 3.91–3.73 (m, 1H, $CHCH_3$), 2.46 (dd, ^3J = 9.2 Hz, 7.6 Hz, 2H, CH_2CO_2), 2.26–1.94 (m, 2H, $CHCH_2$), 1.37 (s, 9H, H-Boc), 1.20 (d, 3H, $CHCH_3$).

^{13}C NMR (75 MHz, $CDCl_3$, *S*,*S*-diastereomer, based on HSQC): δ = 177.2 (s, 1C, C=O), 155.7 (s, 1C, HNC=O), 83.0 (s, 1C, CO_2CH), 79.7 (s, 1C, Me_3C), 48.6 (s, 1C, $CHCH_3$), 28.6 (s, 2C, CH_2CO_2), 28.3 (s, 3C, $(CH_3)_3CO$), 24.2 (s, 2C, $CHCH_2$), 18.6 (s, 1C, $CHCH_3$).

6.3.1.5 (5*S*)-5-((*tert*-Butoxycarbonyl)amino)-4-((*tert*-butyldimethylsilyl)-oxy)hexanoic acid (5)



5

Lactone opening: In a 50 mL round bottom flask with magnetic stirring bar 695 mg (3.03 mmol, 1.0 eq) lactone **4** were dissolved in 10.1 mL THF and a solution of 509 mg (12.1 mmol, 4.0 eq) $LiOH \times H_2O$ in 15.2 mL water was added under vigorous stirring at RT. After 30 min 15.2 mL Et_2O were added to the stirred solution and the mixture was cooled down to 0 °C (ice bath). After carefully adjusting to pH=4 with citric acid (25 % in H_2O), the phases were separated and the aqueous phase was extracted with Et_2O (2×15 mL). The combined organic layers were dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure with T ≤ 30 °C. The residue was transferred into a 50 mL Schlenk flask and dried under high vacuum.

Silylation: The crude product was dissolved in 12.1 mL abs. dichloromethane and the turbid solution was cooled to 0 °C (ice bath). 1.44 mL (18.2 mmol, 6.0 eq) *N*-methylimidazole were added and the colorless solution was stirred for 15 min at 0 °C. After the addition of 4.61 g (18.2 mmol, 6.0 eq) iodine in one portion the reaction mixture was stirred for additional 15 min and 1.37 g (9.09 mmol, 3.0 eq) TBSCl were added in small portions. When the addition was finished, the ice bath was removed and the resulting mixture was stirred at RT for 21 h. After this period 24 mL Et_2O were added and the mixture was treated with 34 mL aqueous $Na_2S_2O_3$ (1 M) until decoloration occurred. Citric acid (25 % in H_2O) was added to adjust to pH=4. The aqueous layer was extracted with Et_2O (2×12 mL) and the combined organic layers were washed with 25 % aqueous citric acid (12 mL) and brine (12 mL). The solvents were removed under reduced pressure and the crude product was dried *in vacuo*.

Ester hydrolysis: In a 100 mL round bottom flask with magnetic stirring bar the crude product was dissolved in 7.6 mL MeOH and 232 µL (0.30 mmol, 0.1 eq) citric acid (25 % in H_2O) were added. The solution was stirred at RT overnight. When the conversion was completed the solvents were removed under reduced pressure and the residue partitioned between EtOAc (24 mL) and H_2O (8 mL). The layers were separated and the solvents removed under reduced pressure to give a brown oil which was purified via flash chromatography (240 g SiO_2; cyclohexane/EtOAc = 4:1 (v/v) + 1 vol-% AcOH).

Yield: 852 mg (2.36 mmol, 78 %), colorless oil.

$C_{17}H_{35}NO_5Si$ [361.55 g/mol].

$[\alpha]_D^{23} = -5.2$ (c = 1.51, $CHCl_3$).

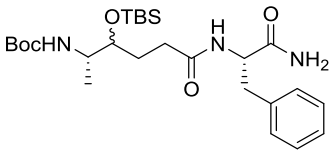
R_f = 0.19 (cyclohexane/EtOAc = 4:1 (v/v) + 1 vol-% AcOH; staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$, based on COSY): δ = 5.34 (s, 1H, $CO_2\underline{H}$), 4.62 (d, ^3J = 8.4 Hz, 1H, N$\underline{H}$), 3.80–3.68 (m, 1H, $CH_3C\underline{H}$), 3.68–3.56 (m, 1H, SiOC$\underline{H}$), 2.55–2.25 (m, 2H, CHC$\underline{H}_2$), 1.86–1.68 (m, 2H, C$\underline{H}_2CO_2H$), 1.44 (s, 9H, H-Boc), 1.14–1.04 (m, 3H, C$\underline{H}_3$CH), 0.90 (s, 9H, (C$\underline{H}_3$)$_3$OSi), 0.10–0.04 (m, 6H, $(CH_3)_2$Si).

^{13}C NMR (75 MHz, $CDCl_3$, based on HSQC): δ = 178.6 (d, 1C, CO_2H), 155.8 (s, 1C, HNCO), 79.5 (s, 1C, $Me_3\underline{C}$), 73.6 (d, 1C, SiOCH), 48.7 (s, 1C, $CH_3\underline{C}$H), 30.2 (s, 1C, CH$\underline{C}H_2$), 28.6 (s, 4C, containing $\underline{C}H_2CO_2H$ and ($\underline{C}H_3$)$_3$CO), 26.0 (s, 3C, ($\underline{C}H_3$)$_3$CSi), 18.3 (s, 1C, $(CH_3)_3\underline{C}$Si), 18.2 (s, 1C, CH$\underline{C}H_3$), –4.3 (s, 1C CH_3Si), –4.4 (s, 1C, CH_3Si).

HRMS (MALDI-TOF): Calcd. for $C_{17}H_{35}NO_5SiNa$ $[M+Na]^+$: 384.2182; found: 384.2170.

6.3.1.6 *tert*-Butyl ((2*S*)-6-(((*S*)-1-amino-1-oxo-3-phenylpropan-2-yl)amino)-3-((*tert*-butyldimethylsilyl)oxy)-6-oxohexan-2-yl)carbamate (6)



6

715 mg (1.98 mmol, 1 eq) compound **5** were dissolved in 13.2 mL abs. DMF in a dry 50 mL Schlenk flask with magnetic stirring bar. After the addition of 1.38 mL (7.91 mmol, 4.0 eq) DIPEA to the stirred solution 952 mg (2.97 mmol, 1.5 eq) TBTU were added in one portion at RT. After stirring for 30 min 476 mg (2.37 mmol, 1.2 eq) of H-Phe-NH_2×HCl were added and the mixture was stirred for 1.5 h. After this period the reaction mixture was quenched by addition of brine (13 mL) and extracted with EtOAc (40 mL). The organic phase was washed with H_2O (5 mL), brine (2×13 mL), 1 M HCl (5 mL) and brine (13 mL), dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure, the crude dried *in vacuo* and purified via flash chromatography (100 g SiO_2; cyclohexane/EtOAc = 1:1 (v/v); staining: $KMnO_4$).

Yield: 620 mg (1.22 mmol, 62 %), white solid.

$C_{26}H_{45}N_3O_5Si$ [507.75 g/mol].

mp = 62–66 °C.

$[\alpha]_D^{23}$ = –8.9 (c = 2.5, $CHCl_3$).

R_f = 0.18 (cyclohexane/EtOAc = 1:1 (v/v); staining: $KMnO_4$).

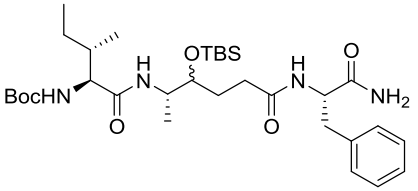
^{1}H NMR (300 MHz, $CDCl_3$, based on COSY): δ = 7.45–7.05 (m, 5H, 1H, Ar-H), 6.88 (d, ^3J = 7.3 Hz, 1H, N$\underline{H}$), 6.30 (d, ^2J = 19.3 Hz, 1H, CON$\underline{H}_2$), 6.06 (d, ^2J = 24.9 Hz, 1H,

$CONH_2$), 4.80–4.55 (m, 2H, containing OCONH and CH_2CHNH), 3.88–3.64 (m, 1H, H_3CCH), 3.64–3.48 (m, 1H, CH_2CH_2CH), 3.15–2.95 (m, 2H, $PhCH_2$), 2.38–2.10 (m, 2H, CH_2CH_2CONH), 1.95–1.55 (m, 2H, CH_2CH_2CONH), 1.45 (s, 9H, H-Boc), 1.16–1.00 (m, 3H, CH_3CH), 0.90 (s, 9H, $(CH_3)_3CSi$), 0.12–0.03 (m, 6H, $(CH_3)_2Si$).

^{13}C NMR (75 MHz, $CDCl_3$, based on HSQC): δ = 173.7 (s, 1C, C=O), 172.9 (s, 1C, C=O), 155.9 (s, 1C, CO_2NH), 136.9 (s, 1C, C_q-Ar), 129.4 (s, 2C, C-Ar), 128.7 (s, 2C, C-Ar), 127.0 (s, 1C, C-Ar), 79.4 (d, 1C, Me_3C), 74.1 (d, 1C, SiOCH), 54.2 (s, 1C, CH_2CH), 48.3 (s, 1C, CH_3CH), 38.3 (s, 1C, CH_2CH), 32.3 (s, 1C, CH_2CH_2CONH), 29.6 (s, 1C CH_2CH_2CONH), 28.6 (s, 3C, $(CH_3)_3CO$), 26.0 (s, 3C, $(CH_3)_3CSi$), 18.4 (s, 1C, $(CH_3)_3CSi$), 18.2 (d, 1C, $CHCH_3$), –4.2 (s, 1C CH_3Si), –4.6 (s, 1C, CH_3Si').

HRMS (MALDI-TOF): Calcd. for $C_{26}H_{45}N_3O_5SiNa$ $[M+Na]^+$: 530.3026; found: 530.3011.

6.3.1.7 *tert*-Butyl ((2*S*,3*S*)-1-(((2*S*)-6-(((*S*)-1-amino-1-oxo-3-phenylpropan-2-yl)amino)-3-((*tert*-butyldimethylsilyl)oxy)-6-oxohexan-2-yl)amino)-3-methyl-1-oxopentan-2-yl)carbamate (7)



7

Boc-deprotection: 390 mg (0.768 mmol, 1.0 eq) compound **6** were dissolved in 7.7 mL (100 mmol, 130 eq) trifluoroacetic acid in a 50 mL round bottom flask with magnetic stirring bar at RT. After stirring for 30 min the acid was removed under reduced pressure (Schlenk line with preceding cooling trap) and the crude product dried *in vacuo*.

Peptide coupling: A 10 mL Schlenk flask with magnetic stirring bar was dried under vacuum with a heat gun and purged with N_2. 213 mg (0.922 mmol, 1.2 eq) Boc-Ile-OH were dissolved in 3.7 mL abs. DMF and 161 µL (0.921 mmol, 1.2 eq) DIPEA were added to the stirred solution. After cooling to 0 °C (ice bath) 350 mg (0.922 mmol, 1.2 eq) HATU were added in one portion and the yellow solution was stirred for 5 min. In a second 50 mL round bottom flask with Schlenk adaptor the previously deprotected crude compound was dissolved in 4.0 mL abs. DMF and 376 µL (2.15 mmol, 2.8 eq) DIPEA were added. After cooling to 0 °C (ice bath) the preactivated Boc-Ile-OH solution was added with a syringe to the stirred mixture at 0 °C. After 5 min the ice bath was removed and the reaction mixture stirred at RT for 1 h. The mixture was quenched by the addition of 4 mL brine and extracted with EtOAc (16 mL). The layers were separated and the aqueous phase was extracted with EtOAc (2×16 mL). The combined organic layers were washed with brine (2×4 mL), dried over Na_2SO_4, concentrated under

reduced pressure and dried *in vacuo*. Purification via flash chromatography (100 g SiO_2; CH_2Cl_2/MeOH = 20:1 v/v) provided compound **7** as a white solid.

Yield: 288 mg (0.46 mmol, 60 %), white solid.

$C_{32}H_{56}N_4O_6Si$ [620.91 g/mol].

mp = 64–69 °C.

$[\alpha]_D^{23}$ = –15.5 (c = 2.0, $CHCl_3$).

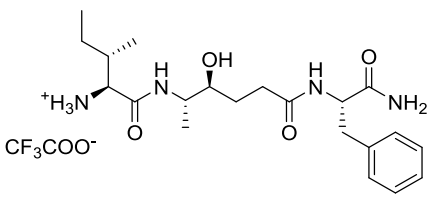
R_f = 0.56 (CH_2Cl_2/MeOH = 10:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$, based on COSY and HSQC): δ = 7.32–7.16 (m, 5H, Ar-H), 6.98 (d, ^3J = 7.0 Hz, 1H, CH_2CONH), 6.45–6.33 (m, 2H, containing CH_3CHNH) and 1 × $CONH_2$), 6.00 (s, 1H, $CONH_2$'), 5.25–5.00 (m, 1H, OCONH), 4.71 (q, ^3J = 7.2 Hz, 1H, H_2NCOCH), 4.16–4.01 (m, 1H, H_3CCHNH), 4.01–3.85 (m, 1H, OCONHCH), 3.70–3.55 (m, 1H, SiOCH), 3.20–2.98 (m, 2H, $PhCH_2$), 2.36–2.08 (m, 2H, CH_2CH_2CONH), 1.98–1.56 (m, 3H, containing CH_2CH_2CONH and CH_3CH_2CH), 1.43 (bs, 10H, containing H-Boc and 1 × CH_3CH_2CH), 1.19–1.04 (m, 4H, containing CH_3CHNH and 1 × CH_3CH_2CH), 0.90 (bs, 15H, containing $(CH_3)_3CSi$, CH_3CH_2 and H_3CCHCH_2), 0.11–0.04 (m, 6H, $(CH_3)_2Si$).

^{13}C NMR (75 MHz, $CDCl_3$, based on HSQC): δ = 173.8 (s, 1C, C=O), 172.8 (s, 1C, C=O), 171.2 (s, 1C, C=O), 156.0 (s, 1C, CO_2NH), 137.1 (s, 1C, C_q-Ar), 129.4 (s, 2C, C-Ar), 128.6 (s, 2C, C-Ar), 126.9 (s, 1C, C-Ar), 80.1 (s, 1C, Me_3C), 73.7 (d, 1C, SiOCH), 59.7 (s, 1C, OCONHCH), 54.3 (s, 1C, H_2NCOCH), 47.0 (s, 1C, H_3CCHNH), 38.1 (s, 1C, $PhCH_2$), 37.3 (s, 1C, CH_3CH_2CH), 31.9 (s, 1C, CH_2CH_2CONH), 29.9 (s, 1C, CH_2CH_2CONH), 28.4 (s, 3C, $(CH_3)_3CO$), 26.0 (d, 3C, $(CH_3)_3CSi$), 24.9 (s, 1C, CH_3CH_2CH), 18.3 (s, 1C, $(CH_3)_3CSi$), 18.1 (s, 1C, 1C, $HNCHCH_3$), 15.8 (s, 1C, H_3CCHCH_2), 11.7 (s, 1C, CH_3CH_2), –4.1 (s, 1C CH_3Si), –4.7 (s, 1C, CH_3Si').

HRMS (MALDI-TOF): Calcd. for $C_{32}H_{56}N_4O_6SiNa$ $[M+Na]^+$: 643.3867; found: 643.3878.

6.3.1.8 CF_3COOH × H-Ile-Ala-[Ψ]((*S*)COH-CH_2)-Gly-Phe-NH_2 (8a)



8a

A 25 mL round bottom flask containing 235 mg (0.378 mmol, 1 eq) compound **7** and a magnetic stirring bar was cooled to 0 °C (ice bath) and 7.6 mL (99 mmol, 261 eq) trifluoroacetic acid were added. The solution was stirred for 30 min at 0 °C, warmed up to

RT and stirred for additional 10 min. TFA was removed under reduced pressure (Schlenk line with preceding cooling trap) and the crude residue was dried *in vacuo*. Purification via flash chromatography (9 g SiO_2; CH_2Cl_2/MeOH = 10:1→8:1 v/v) and subsequent purification via preparative HPLC (*CHRISTIAN_NucleodurC18_001CF3-COOH*) provided the desired product as a trifluoroacetate.

Yield: 61.7 mg (119 µmol, 31 %), white solid, de = 79 %.

$C_{23}H_{35}F_3N_4O_6$ [520.55 g/mol].

mp = 93–98 °C.

$[\alpha]_D^{23}$ = +6.5 (c = 1.0, MeOH).

R_f = 0.22 (CH_2Cl_2/MeOH = 5:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (500 MHz, methanol-d_4, based on COSY and HSQC): δ = 7.35–7.22 (m, 5H, Ar-H), 4.67 (dd, ^3J = 9.1, 5.5 Hz, 1H, $H_2NCOC\underline{H}$), 4.01–3.94 (m, 1H, $H_3CC\underline{H}NH$), 3.77 (d, ^3J = 5.4 Hz, 1 $COC\underline{H}CH$), 3.52–3.46 (m, 1H, $HOC\underline{H}$), 3.21 (dd, ^2J = 13.9 Hz, ^3J = 5.5 Hz, 1H, 1 × $PhC\underline{H}_2$), 2.94 (dd, ^2J = 13.9 Hz, ^3J = 9.2 Hz, 1H, 1 × $PhC\underline{H}_2$), 2.45–2.24 (m, 2H, $CH_2C\underline{H}_2CONH$), 2.00–1.90 (m, 1H, $CH_3C\underline{H}CH_2$), 1.72–1.57 (m, 3H, containing $C\underline{H}_2CH_2CONH$ and 1 × $CH_3C\underline{H}_2$), 1.31–1.19 (m, 4H, containing $C\underline{H}_3CHNH$ and 1 × $CH_3C\underline{H}_2$'), 1.08 (d, ^3J = 6.9 Hz, 3H, $C\underline{H}_3CHCH_2$), 1.01 (t, ^3J = 7.3 Hz, 3H, $C\underline{H}_3CH_2$).

^{13}C NMR (75 MHz, methanol-d_4, based on HSQC): δ = 176.3 (s, 1C, C=O), 175.6 (s, 1C, C=O), 169.2 (s, 1C, C=O), 138.6 (s, 1C, C_q-Ar), 130.2 (s, 2C, C-Ar), 129.4 (s, 2C, C-Ar), 127.7 (s, 1C, C-Ar), 73.9 (s, 1C, HCOH), 59.2 (s, 1C $CO\underline{C}HCH$), 55.7 (s, 1C, $H_2NCO\underline{C}H$), 51.0 (s, 1C, $H_3C\underline{C}HNH$), 39.0 (s, 1C, $Ph\underline{C}H_2$), 38.1 (s, 1C, $CH_3\underline{C}HCH_2$), 33.4 (s, 1C, $CH_2\underline{C}H_2CONH$), 30.7 (s, 1C, $\underline{C}H_2CH_2CONH$), 25.3 (s, 1C, $CH_3\underline{C}H_2$), 17.3 (s, 1C, $\underline{C}H_3CHNH$), 15.4 (s, 1C, $\underline{C}H_3CHCH_2$), 11.7 (s, 1C, $\underline{C}H_3CH_2$).

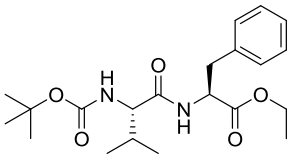
^{19}F NMR (470 MHz, methanol-d_4): δ = –76.90 ($C\underline{F}_3$).

HPLC/MS (FAST_POROSHELL120_001HCOOH_8MINGRADIENT.M): t_R = 4.73 min; m/z (ESI+) = 407.3 $[M+H]^+$ (free amine).

HRMS (MALDI-TOF): Calcd. for $C_{21}H_{34}N_4O_4Na$ $[M+Na]^+$: 429.2478; found: 429.2491.

6.3.2 Peptide synthesis and reduction

6.3.2.1 Ethyl (*tert*-butoxycarbonyl)-L-valyl-L-phenylalaninate (Boc-Val-Phe-OEt) (9a)[111]



9a

A 50 mL Schlenk flask with a magnetic stirring bar was dried under vacuum with a heat gun and purged with N_2. 261 mg (1.20 mmol, 1.0 eq) Boc-Val-OH were dissolved in 8 mL abs. DMF and 838 µL (4.80 mmol, 4.0 eq) Hünig's base were added to the stirred solution. After cooling to 0 °C (ice bath), 462 mg (1.44 mmol, 1.2 eq) TBTU were added in one portion. After 5 min of activation time H-Phe-OEt×HCl (304 mg, 1.32 mmol, 1.1 eq) was added, the ice bath removed and the reaction mixture was stirred for 50 min. The mixture was quenched by the addition of 8 mL brine and extracted with EtOAc (32 mL). The layers were separated and the aqueous phase was extracted with 16 mL EtOAc. The combined organic layers were washed with H_2O (2×8 mL) and brine (8 mL). The organic phase was dried over Na_2SO_4, concentrated under reduced pressure and purified via flash chromatography (25 g SiO_2; cyclohexane/EtOAc = 3:1 v/v).

Yield: 433 mg (1.10 mmol, 92 %), white solid.

$C_{21}H_{32}N_2O_5$ [392.50 g/mol].

mp = 112 °C, lit. 117–118 °C.[111]

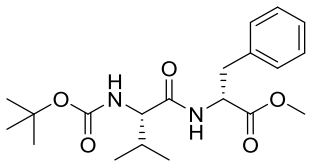
$[\alpha]_D^{23}$ = –24.7 (c = 0.99, EtOH), lit. $[\alpha]_D^{24}$ = –23 (c = 1, EtOH).[111]

R_f = 0.32 (cyclohexane/EtOAc = 3:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.33–7.08 (m, 5H, Ar-H), 6.39–6.27 (m, 1H, OCNH), 5.03 (d, ^3J = 7.3 Hz, 1H, O_2CNH), 4.85 (dd, ^3J = 13.6 Hz, 6.1 Hz, 1H, BnCH), 4.15 (q, ^3J = 7.1 Hz, 2H, CO_2CH_2), 3.90 (m, 1H, *i*-PrCH), 3.12 (d, ^3J = 5.8 Hz, 2H, $PhCH_2$), 2.16–2.02 (m, 1H, $CH(CH_3)_2$), 1.45 (s, 9H, $(CH_3)_3$), 1.22 (t, ^3J = 7.1 Hz, 3H, $CO_2CH_2CH_3$), 0.96–0.82 (m, 6H, $CH(CH_3)_2$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.4 (s, 1C, C=O), 171.3 (s, 1C, C=O), 155.8 (s, 1C, $HNCO_2$), 135.9 (s, 1C, C_q-Ar), 129.5 (s, 2C, C-Ar), 128.7 (s, 2C, C-Ar), 127.3 (s, 1C, C-Ar), 80.0 (s, 1C, Me_3C), 61.6 (s, 1C), 60.0 (s, 1C), 53.3 (s, 1C), 38.2 (s, 1C), 31.0 (s, 1C), 28.4 (s, 3C), 19.3 (s, 1C), 17.8 (s, 1C), 14.2 (s, 1C).

6.3.2.2 Methyl (*tert*-butoxycarbonyl)-L-valyl-D-phenylalaninate (Boc-Val-D-Phe-OMe) (9b)[112]



9b

A 50 mL Schlenk flask with a magnetic stirring bar was dried under vacuum with a heat gun and purged with N_2. 261 mg (1.20 mmol, 1.0 eq) Boc-Val-OH were dissolved in 8 mL abs. DMF and 838 µL (4.80 mmol, 4.0 eq) Hünig's base were added to the stirred solution. After cooling to 0 °C (ice bath) 462 mg (1.44 mmol, 1.2 eq) TBTU were added in one portion. After 5 min of activation time H-D-Phe-OMe×HCl (285 mg, 1.32 mmol, 1.1 eq) was added, the ice bath removed and the reaction mixture was stirred for 50 min. The mixture was quenched by the addition of 8 mL brine and extracted with EtOAc (32 mL). The layers were separated and the aqueous phase was extracted with 16 mL EtOAc. The combined organic layers were washed with H_2O (2×8 mL) and brine (8 mL), dried over Na_2SO_4, concentrated under reduced pressure and purified via flash chromatography (26 g SiO_2; cyclohexane/EtOAc = 3:1 (v/v).

Yield: 398 mg (1.05 mmol, 88 %), white solid.

$C_{20}H_{30}N_2O_5$ [378.47 g/mol].

mp. = 101–103 °C, lit. 104–105 °C.[112]

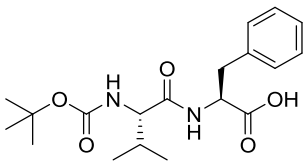
$[\alpha]_D^{23}$ = –35.7 (c = 1.0, $CHCl_3$), lit. $[\alpha]_D^{25}$ = +37.8 (c = 1.0, $CHCl_3$).[112]

R_f = 0.28 (cyclohexane/EtOAc = 3:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.35–7.18 (m, 3H, Ar-H), 7.17–7.05 (m, 2H, Ar-H), 6.44 (d, ^3J = 7.7 Hz, 1H, OCNH), 5.05–4.80 (m, 2H, O_2CNH and BnCH), 3.97 (m, 1H, *i*-PrCH), 3.71 (s, 3H, CO_2CH_3), 3.19–3.01 (m, 2H, $PhCH_2$), 2.19–2.03 (m, 1H, $CH(CH_3)_2$), 1.43 (s, 9H, $(CH_3)_3$), 0.88 (d, ^3J = 6.7 Hz, 3H, $CHCH_3CH_3$), 0.80 (d, ^3J = 6.8 Hz, 3H, $CHCH_3CH_3$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.0 (s, 1C, C=O), 171.4 (s, 1C, C=O), 155.9 (s, 1C, $HNCO_2$), 135.9 (s, 1C, C_q-Ar), 129.3 (s, 2C, C-Ar), 128.8 (s, 2C, C-Ar), 127.3 (s, 1C, C-Ar), 80.1 (s, 1C, Me_3C), 59.8 (s, 1C), 53.1 (s, 1C), 52.5 (s, 1C), 38.2 (s, 1C), 30.8 (s, 1C), 28.4 (s, 3C), 19.4 (s, 1C), 17.3 (s, 1C).

6.3.2.3 (*tert*-Butoxycarbonyl)-L-valyl-L-phenylalanine (Boc-Val-Phe-OH) (10a)[113,114]



10a

In a 50 mL round bottom flask 410 mg (1.04 mmol, 1 eq) **9a** were dissolved in 3.5 mL THF. A solution of 175 mg (4.18 mmol, 4.0 eq) LiOH×H_2O in 5.2 mL H_2O was added under vigorous stirring. After full conversion was indicated by TLC EtOAc (5 mL) was added and the pH adjusted with 25 % aqueous citric acid to pH=4. The mixture was poured into a separation funnel, the layers separated and the aqueous layer was extracted with EtOAc (5 mL). The combined organic extracts were washed with H_2O (2.5 mL) and brine (5 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified via silica gel filtration (9 g SiO_2; CH_2Cl_2/MeOH = 9:1 (v/v)) to obtain the desired product as a white solid.

Yield: 362 mg (0.99 mmol, 96 %), white solid.

$C_{19}H_{28}N_2O_5$ [364.44 g/mol].

mp = 123–128 °C, lit. 114–115 °C.[113]

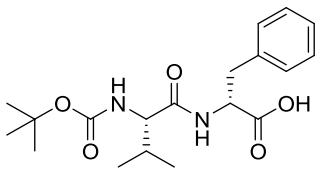
$[\alpha]_D^{23}$ = –15.2 (c = 1.0, MeOH), lit. $[\alpha]_D^{20}$ = +13.7 (c= 1.00, MeOH).[114]

R_f = 0.22 (cyclohexane/EtOAc = 1:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, methanol-d_4): δ = 7.30–7.15 (m, 5H, Ar-H), 4.68 (dd, ^3J = 7.9 Hz, 5.3 Hz, 1H, BnCH), 3.84 (d, ^3J = 7.0 Hz, 1H, *i*-PrCH), 3.19 (dd, ^2J = 13.8 Hz, ^3J = 4.9 Hz, 1H, PhCH_2), 2.99 (dd, ^2J = 13.8 Hz, ^3J = 8.5 Hz, 1H, PhCH_2), 2.03–1.86 (m, 1H, C$H(CH_3)_2$), 1.43 (s, 9H, (CH_3)$_3$), 0.92–0.80 (m, 6H, CH(CH_3)$_2$).

^{13}C NMR (75 MHz, methanol-d_4): δ = 174.3 (s, 1C, C=O), 174.2 (s, 1C, C=O), 157.8 (s, 1C, C=O), 138.3 (s, 1C, C_q-Ar), 130.3 (s, 2C, C-Ar), 129.4 (s, 2C, C-Ar), 127.7 (s, 1C, C-Ar), 80.5 (s, 1C, Me_3C), 61.5 (s, 1C), 54.8 (s, 1C), 38.5 (s, 1C), 32.1 (s, 1C), 28.7 (s, 3C), 19.7 (s, 1C), 18.5 (s, 1C).

6.3.2.4 (*tert*-Butoxycarbonyl)-L-valyl-D-phenylalanine (Boc-Val-D-Phe-OH) (10b)



10b

In a 50 mL round bottom flask 363 mg (0.96 mmol, 1 eq) **9b** were dissolved in 3.2 mL THF. A solution of 161 mg (3.84 mmol, 4.0 eq) LiOH×H_2O in 4.8 mL H_2O was added under vigorous stirring. After full conversion was indicated by TLC EtOAc (5 mL) was added and the pH adjusted with 25 % aqueous citric acid to pH=4. The mixture poured into a separation funnel, the layers separated and aqueous layer was extracted with additional EtOAc (5 mL). The combined organic extracts were washed with H_2O (2.5 mL) and brine (5 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified via silica gel filtration (SiO_2; CH_2Cl_2/MeOH = 9:1 (v/v)) to obtain the desired product as a white solid.

Yield: 315 mg (0.84 mmol, 90 %), white solid.

$C_{19}H_{28}N_2O_5$ [364.44 g/mol].

mp = 74–78 °C

$[\alpha]_D^{23}$ = –17.2 (c = 1.0, MeOH).

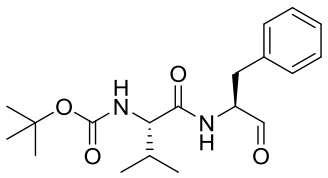
R_f = 0.23 (cyclohexane/EtOAc = 1:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, methanol-d_4): δ = 7.32–7.15 (m, 5H, Ar-H), 4.68 (dd, ^3J = 8.9 Hz, 4.3 Hz, 1H, BnCH), 3.91 (d, ^3J = 5.8 Hz, 1H, *i*-PrCH), 3.24 (dd, ^2J = 14.0 Hz, ^3J = 6.7 Hz, PhCHH), 2.96 (dd, ^2J = 14.0 Hz, ^3J = 9.6 Hz, PhCHH) 1.98–1.74 (m, 1H, CH$(CH_3)_2$), 1.43 (s, 9H, $(CH_3)_3$), 0.77 (d, ^3J = 6.8 Hz, 3H, CH$(CH_3)_2$), 0.70 (d, ^3J = 6.8 Hz, 3H, CH$(CH_3)_2$).

^{13}C NMR (75 MHz, methanol-d_4): δ = 174.5 (s, 1C, C=O), 174.2 (s, 1C, C=O), 157.9 (s, 1C, $HNCO_2$), 138.4 (s, 1C, C_q-Ar), 130.3 (s, 2C, C-Ar), 129.5 (s, 2C, C-Ar), 127.8 (s, 1C, C-Ar), 80.6 (s, 1C, Me_3C), 61.1 (s, 1C), 54.9 (s, 1C), 38.4 (s, 1C), 32.2 (s, 1C), 28.7 (s, 3C), 19.7 (s, 1C), 17.8 (s, 1C).

HRMS (MALDI-TOF): Calcd. for $C_{19}H_{28}N_2O_5Na$ $[M+Na]^+$: 387.1896; found: 387.1809.

6.3.2.5 *tert*-Butyl ((*S*)-3-methyl-1-oxo-1-(((*S*)-1-oxo-3-phenylpropan-2-yl)-amino)butan-2-yl)carbamate (Boc-Val-Phe-H) (11a)[115]



11a

A 50 mL Schlenk flask equipped with a glass stopper and a magnetic stirring bar was heated, dried under vacuum and purged with N_2. 146 mg (0.400 mmol, 1.0 eq) **10a** were dissolved in 8.0 mL abs. dichloromethane and the solution was cooled to 0 °C (ice bath). 78 mg (0.480 mmol, 1.2 eq) 1,1'-carbonyldiimidazole (CDI) were added and a gas bubbler was mounted instead of the glass stopper to allow for pressure relief. After stirring for 60 min the gas bubbler was removed and exchanged by a septum while maintaining a gentle counter flow of N_2. The heterogeneous reaction mixture was cooled to –78 °C (CO_2/acetone bath) for 15 min. Subsequently, 1.24 mL (1.24 mmol, 3.1 eq) DIBAL-H solution (1.0 mol/L in toluene) were added dropwise with a syringe through the septum at a rate of 2.0 mL/h. The reaction mixture was stirred at –78 °C until TLC indicated quantitative conversion (60 min). The reaction mixture was quenched by addition of 8.0 mL EtOAc. The acetone bath was removed, the gas bubbler was mounted, and 3.0 mL 25% aqueous tartaric acid solution were added to the mixture under vigorous stirring. The mixture was warmed up by immersing the vessel into a water bath at RT and stirred vigorously for 15 min. The stirring was stopped and the layers were separated. The aqueous phase was extracted with EtOAc (4.0 mL) and the combined organic extracts were washed with 1 M HCl (3.0 mL), 0.8 M $NaHCO_3$ (3.0 mL) and brine (3.0 mL), dried over Na_2SO_4, concentrated under reduced pressure and dried *in vacuo*.

Yield: 124 mg (0.356 mmol, 89 %), white solid.

$C_{19}H_{28}N_2O_4$ [348.44 g/mol].

mp = 128–131 °C, lit. 124–125 °C.[115]

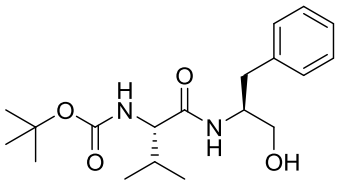
$[\alpha]_D^{23} = -3.0$ (c = 0.69, $CHCl_3$), lit. $[\alpha]_D^{20} = -55.4$ (c = 1.0, MeOH).[115]

R_f = 0.32 (cyclohexane/EtOAc = 1:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.61 (s, 1H, C<u>H</u>O), 7.39–7.10 (m, 5H, Ar-<u>H</u>), 6.52 (br s, 1H, <u>H</u>NCO), 5.07–4.90 (m, 1H, <u>H</u>NCO$_2$), 4.71 (d, ^3J = 6.7 Hz, 1H, BnC<u>H</u>), 4.01–3.88 (m, 1H, *i*-PrC<u>H</u>), 3.15 (d, ^3J = 6.1 Hz, 2H, PhC<u>H</u>$_2$), 2.20–2.01 (m, 1H, $(CH_3)_2$C<u>H</u>), 1.44 (s, 9H, $(C\underline{H}_3)_3$), 1.00–0.70 (m, 6H, $(C\underline{H}_3)_2$CH).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 198.6 (s, 1C, HC=O), 172.0 (s, 1C, HNC=O), 155.9 (s, 1C, $HNCO_2$), 135.6 (s, 1C, Ar-C_q), 129.4 (s, 2C, Ar-C), 129.0 (s, 2C, Ar-C), 127.4(s, 1C, Ar-C), 80.2 (s, 1C, $Me_3\underline{C}$), 60.1 (s, 1C), 59.8 (s, 1C), 35.4 (s, 1C), 30.7 (s, 1C), 28.4 (s, 3C), 19.4 (s, 1C), 17.7 (s, 1C).

6.3.2.6 *tert*-Butyl ((*S*)-1-(((*S*)-1-hydroxy-3-phenylpropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (Boc-Val-Phe-ol) (12a)[116]



12a

In a 5 mL glass vial 52 mg (0.15 mmol, 1.0 eq) **11a** were dissolved in 1.5 mL abs. MeOH, cooled to 0 °C (ice bath) and $NaBH_4$ (27 mg, 0.17 mmol, 1.1 eq) was added in one portion under vigorous stirring. After full conversion was indicated by TLC (20 min) 200 µL acetone were added. The ice bath was removed and the reaction mixture was stirred for 5 min. The solvents were removed under reduced pressure and the solid residue partitioned between EtOAc (3.0 mL) and 1.0 mL $NaHCO_3$ (0.8 M in H_2O). The organic layer was concentrated and dried under reduced pressure. The product was purified via flash chromatography (SiO_2; cyclohexane/EtOAc = 1:1 (v/v)) to obtain the desired product as a white solid.

Yield: 43 mg (0,12 mmol, 80 %), white solid.

$C_{19}H_{30}N_2O_4$ [350.46 g/mol].

mp = 134–138 °C, lit. 145.0–146.0 °C.[116]

$[\alpha]_D^{23}$ = –45.2 (c = 0.8, $CHCl_3$), lit. $[\alpha]_D^{24}$ = –49.4 (c = 1.0, MeOH).[116]

R_f = 0.26 (cyclohexane/EtOAc= 1:1 (v/v); staining: $KMnO_4$).

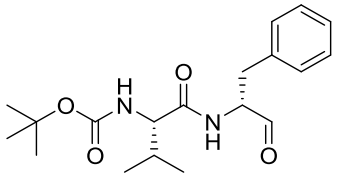
^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.37–7.13 (m, 5H, Ar-H), 6.47 (d, ^3J = 5.6 Hz, 1H, HNCO), 5.04 (d, ^3J = 6.8 Hz, 1H, $HNCO_2$), 4.19 (br s, 1H, BnCH), 3.90–3.79 (m, 1H, *i*-PrCH), 3.66 (dd, ^3J = 11.1 Hz, 3.5 Hz, 1H, CHHOH), 3.57 (dd, ^3J = 11.0 Hz, 4.7 Hz, 1H, CHHOH), 2.96–2.79 (m, 2H, $PhCH_2$), 2.68 (br s, 1H, OH), 2.17–2.01 (m, 1H, $(CH_3)_2CH$), 1.44 (s, 9H, $(CH_3)_3$), 0.99–0.70 (m, 6H, $(CH_3)_2CH$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.0 (s, 1C, HNC=O), 156.2 (s, 1C, $HNCO_2$), 137.8 (s, 1C, Ar-C_q), 129.3 (s, 2C, Ar-C), 128.7 (s, 2C, Ar-C), 126.7 (s, 1C, Ar-C), 80.4 (s, 1C, $Me_3\underline{C}$), 63.7 (s, 1C), 60.7 (s, 1C), 53.0 (s, 1C), 37.1 (s, 1C), 30.6 (s, 1C), 28.4 (s, 3C), 19.4 (s, 1C), 17.8 (s, 1C).

HPLC-ESI-MS: t_R(**12a**) = 10.66 min, 89.46%; t_R(**12b**) = 11.25 min, 10.54%; de = 78.92%; calcd. $[M+Na]^+$ = 373.2, $[M+K]^+$ = 389.2, found $[M+Na]^+$ = 372.9, $[M+K]^+$ = 388.9.

HRMS (MALDI-TOF): Calcd. for $C_{19}H_{30}N_2O_4Na$ $[M+Na]^+$: 373.2103; found: 373.2108.

6.3.2.7 *tert*-Butyl ((*S*)-3-methyl-1-oxo-1-(((*R*)-1-oxo-3-phenylpropan-2-yl)-amino)butan-2-yl)carbamate (Boc-Val-D-Phe-H) (11b)



11b

A 10 mL Schlenk flask equipped with a glass stopper and a magnetic stirring bar was heated, dried under vacuum and purged with N_2. 31 mg (0.08 mmol, 1.0 eq) **10b** were dissolved in 2.8 mL abs. dichloromethane, and the solution was cooled to 0 °C (ice bath). 13 mg (0.08 mmol, 1.0 eq) HOBt×H_2O were added, immediately followed by 16 mg (0.10 mmol, 1.1 eq) 1,1'-carbonyldiimidazole (CDI). A gas bubbler was mounted instead of the glass stopper to allow for pressure relief. After stirring for 60 min the gas bubbler was replaced with a septum while maintaining a gentle counter flow of N_2. The heterogeneous reaction mixture was cooled to –78 °C (CO_2/acetone bath) for 15 min. Subsequently, 378 µL (0.38 mmol, 4.5 eq) DIBAL-H solution (1.0 M in toluene) were added dropwise with a syringe through the septum at a rate of 2.0 mL/h. The reaction mixture was stirred at –78 °C until TLC indicated quantitative conversion (150 min). The reaction mixture was quenched by addition of 5.6 mL EtOAc. The acetone bath was removed, the gas bubbler was mounted, and 2.8 mL of 25% aqueous tartaric acid solution were added to the mixture under vigorous stirring. The mixture was warmed up by immersing the vessel into a water bath at RT and stirred vigorously for 15 min. The stirring was stopped and the layers were separated. The aqueous phase was extracted with EtOAc (2.8 mL) and the combined organic extracts were washed with 1 M HCl (2.8 mL), 0.8 M $NaHCO_3$ (2.8 mL) and brine (2.8 mL), dried over Na_2SO_4, concentrated under reduced pressure and dried *in vacuo*.

Yield: 27 mg (78 µmol, 93 %), white solid.

$C_{19}H_{28}N_2O_4$ [348.44 g/mol].

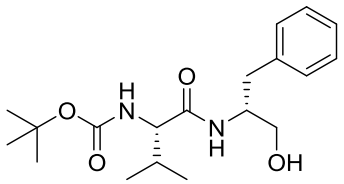
$[\alpha]_D^{23}$ = –3.0 (c = 0.69, $CHCl_3$).

R_f = 0.32 (cyclohexane/EtOAc = 1:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.61 (s, 1H, C<u>H</u>O), 7.38–7.09 (m, 5H, Ar-<u>H</u>), 6.49 (br s, 1H, <u>H</u>NCO), 5.04–4.89 (m, 1H, <u>H</u>NCO_2), 4.74 (dd, ^{3}J = 13.4 Hz, 6.6 Hz, 1H, BnC<u>H</u>), 4.04–3.88 (m, 1H, *i*-PrC<u>H</u>), 3.13 (d, ^{3}J = 6.7 Hz, 2H, PhC<u>H</u>$_2$), 2.19–2.00 (m, 1H, $(CH_3)_2$C<u>H</u>), 1.43 (s, 9H, $(C\underline{H}_3)_3$), 0.95–0.76 (m, 6H, $(C\underline{H}_3)_2$CH).

^{13}C NMR (75 MHz, $CDCl_3$, based on HSQC): δ = 198.8 (s, 1C, HC=O), 172.0 (s, 1C, HNC=O), 156.0 (s, 1C, $HNCO_2$), 135.6 (s, 1C, Ar-C_q), 129.4 (s, 2C, Ar-C), 129.0 (s, 2C, Ar-C), 127.4 (s, 1C, Ar-C), 80.2 (s, 1C, Me_3<u>C</u>), 60.0 (s, 1C), 59.7 (s, 1C), 35.3 (s, 1C), 30.7 (s, 1C), 28.4 (s, 3C), 19.4 (s, 1C), 17.6 (s, 1C).

6.3.2.8 *tert*-Butyl ((*S*)-1-(((*R*)-1-hydroxy-3-phenylpropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (Boc-Val-D-Phe-ol) (12b)[116]



12b

In a 5 mL glass vial 27 mg (78 µmol, 1.0 eq) **11b** were dissolved in 0.78 mL abs. MeOH, cooled to 0 °C (ice bath) and $NaBH_4$ (14 mg, 86 µmol, 1.1 eq) was added in one portion under vigorous stirring. After full conversion was indicated by TLC (20 min) 200 µL acetone were added. The ice bath was removed and the reaction mixture was stirred for 5 min. The solvents were removed under reduced pressure and the solid residue partitioned between EtOAc (3.0 mL) and 1.0 mL $NaHCO_3$ (0.8 M in H_2O). The organic layer was concentrated and dried under reduced pressure. The product was purified via flash chromatography (SiO_2; cyclohexane/EtOAc = 1:1 (v/v)) to obtain the desired product as a white solid.

Yield: 17 mg (49 µmol, 62 %), white solid.

$C_{19}H_{30}N_2O_4$ [350.46 g/mol].

mp = 125–127 °C, lit. 131–132 °C.[116]

$[\alpha]_D^{23}$ = +1.2 (c = 0.8, $CHCl_3$).

R_f = 0.28 (cyclohexane/EtOAc= 1:1 (v/v); staining: $KMnO_4$).

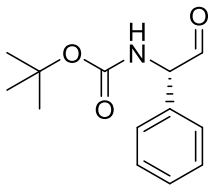
^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.34–7.12 (m, 5H, Ar-<u>H</u>), 6.36 (d, ^{3}J = 7.7 Hz, 1H, <u>H</u>NCO), 5.21 (d, ^{3}J = 7.9 Hz, 1H, <u>H</u>NCO_2), 4.25 (br s, 1H, BnC<u>H</u>), 3.81–3.62 (m, 2H, *i*-PrC<u>H</u> and C<u>H</u>HOH), 3.54 (dd, ^{3}J = 11.2 Hz, 4.9 Hz, 1H, CH<u>H</u>OH), 2.97–2.74 (m, 2H, PhC<u>H</u>$_2$), 2.02–1.81 (m, 1H, $(CH_3)_2$C<u>H</u>), 1.43 (s, 9H, $(C\underline{H}_3)_3$), 0.87–0.71 (m, 6H, $(C\underline{H}_3)_2$CH).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.3 (s, 1C, HNC=O), 156.4 (s, 1C, $HNCO_2$), 137.8 (s, 1C, Ar-C_q), 129.3 (s, 2C, Ar-C), 128.7 (s, 2C, Ar-C), 126.7 (s, 1C, Ar-C), 80.3 (s, 1C, $Me_3\underline{C}$), 64.0 (s, 1C), 60.9 (s, 1C), 52.9 (s, 1C), 37.2 (s, 1C), 30.8 (s, 1C), 28.5 (s, 3C), 19.2 (s, 1C), 18.1 (s, 1C).

HPLC-ESI-MS: t_R(**12a**) = 10.43 min, 5.34%; t_R(**12b**) = 10.98 min, 94.66%; de = 89.32%; calc. $[M+Na]^+$ = 373.2, $[M+K]^+$ = 389.2, found $[M+Na]^+$ = 372.9, $[M+K]^+$ = 388.9.

HRMS (MALDI-TOF): Calcd. for $C_{19}H_{30}N_2O_4Na$ $[M+Na]^+$: 373.2103; found: 373.2108.

6.3.2.9 *tert*-Butyl (*S*)-(2-oxo-1-phenylethyl)carbamate (Boc-Phg-H) (13)



13

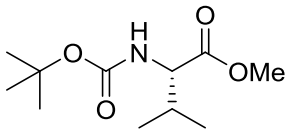
Five solutions of each 50 mg (0.20 mmol, 1.0 eq) Boc-L-Phg-OH in 2.0 mL abs. dichloromethane were treated with 36 mg (0.22 mmol, 1.1 eq) CDI at 0 °C, using the general procedure (see chapter 6.3.2.5). After 60 min of stirring, the temperatures were set to +24, 0, –30, –50 or –78 °C and every instance was treated with 0.42 mL (0.42 mmol, 2.1 eq) 1 M DIBAL-H, dropwise at the rate of 2.0 mL/min. The mixtures were stirred for 15 min at the respective temperature and quenched by addition of 4.0 mL EtOAc. After vigorous stirring with 1.0 mL 25% aqueous tartaric acid solution the crude extract was directly used to determine the temperature dependency of ee by GC-FID.

$C_{13}H_{17}NO_3$ [235.28 g/mol].

GC-FID (CP-Chiralsil Dex): t_R (*(S)*-enatiomer) = 14.2 min; t_R (*(R)*-enatiomer) = 14.6 min

6.3.3 Synthesis of *HER* and derivatives

6.3.3.1 Methyl (*tert*-butoxycarbonyl)-L-valinate (14)[87,117]



14

A 500 mL three-necked round bottom flask with magnetic stirring bar, fitted with a gas adapter and a septum with ballon was predried (evacuated, heated, N_2-purged) and charged with a solution of 21.7 g (100 mmol, 1.0 eq) Boc-Val-OH in 147 mL abs. DMF and 20.0 g (200 mmol, 2.0 eq) potassium hydrogen carbonate. 9.96 mL (160 mmol, 1.6 eq) methyl iodide were added dropwise to the stirred white suspension with a syringe (syringe pump, flowrate: 20 mL/h) over a period of 30 min. After the addition was finished the pale yellow reaction mixture was stirred for additional 3 h at RT and transferred into a 1 L separation funnel afterwards. The mixture was diluted with water (400 mL) and the product was extracted with a mixture of cyclohexane/EtOAc (1:1 v/v, 3×85 mL). The combined organic layers were washed with H_2O (2×85 mL), 5 % aqueous Na_2SO_4 (2×85 mL) and brine (85 mL). After drying over Na_2SO_4 the solvents were removed under reduced pressure and the residue was dried *in vacuo* to yield a colorless oil which was used without further purification.

Yield: 22.9 g (99.1 mmol, 99 %), colorless oil.

$C_{11}H_{21}NO_4$ [231.29 g/mol].

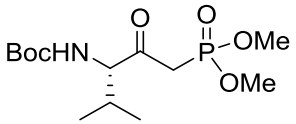
$[\alpha]_D^{24}$ = +13.2 (c = 1.91, $CHCl_3$); lit. $[\alpha]_D^{22}$ = +12.9 (c = 2.43, $CHCl_3$)[117].

R_f = 0.86 (cyclohexane/EtOAc = 1:4 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.01 (d, ^3J = 7,3 Hz, 1H, NH), 4.20 (dd, ^3J = 8.5, 4.6 Hz, 1H, HNCH), 3.71 (s, 3H, OCH_3), 2.18–1.98 (m, 1H, $(CH_3)_2CH$), 1.42 (s, 9 H, H-Boc), 0.93 (d, ^3J = 6.8 Hz, 3H, H_3CCHCH_3), 0.87 (d, ^3J = 6.9 Hz, 3H, H_3CCHCH_3).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.0 (s, 1C, C=O), 155.8 (s, 1C, OCONH), 79.8 (s, 1C, Me_3C), 58.7 (s, 1C, NHCH), 52.1 (s, 1C, OCH_3), 31.4 (s, 1C, $(CH_3)_2CH$), 28.4 (s, 3C, $(CH_3)_3C$), 19.1 (s, 1C, H_3CCHCH_3), 17.7 (s, 1C, H_3CCHCH_3).

6.3.3.2 *tert*-Butyl (*S*)-(1-(dimethoxyphosphoryl)-4-methyl-2-oxopentan-3-yl)carbamate (15)[84,118]



15

A 1 L three-necked round bottom flask with magnetic stirring bar, fitted with a gas valve and a septum was predried (evacuated, heated, N_2-purged) and charged with a solution of 17.0 mL (157 mmol, 3.3 eq) dimethyl methylphosphonate in 430 mL abs. THF. After cooling to –78 °C in a dry ice/acetone bath a 2.5 M *n*-butyllithium solution in hexane (60.8 mL, 152 mmol, 3.2 eq) was added with a syringe (syringe pump, flowrate: 2.0 mL/min) over a period of 30 min. The yellow solution was additionally stirred for 1 h at –78 °C at which point a –78 °C cold solution of the methyl ester **14** (11.0 g, 47.6 mmol, 1.0 eq) in 140 mL abs. THF was added. Stirring was continued for 3 h at –78 °C until TLC indicated full conversion and the reaction mixture was quenched by addition of 200 mL satd. NH_4Cl. The mixture was transferred into a separation funnel, the layers were separated and aqueous layer was extracted with EtOAc (2×200 mL). The combined organic phases were washed with H_2O (2×200 mL) and brine (100 mL), dried over Na_2SO_4, concentrated under reduced pressure and dried *in vacuo*. The resulting pale yellow oil was directly used for the next step without further purification.

Yield: 13.5 g (41.8 mmol, 87 %), pale yellow oil.

$C_{13}H_{26}NO_6P$ [323.33 g/mol].

$[\alpha]_D^{24}$ = +26.1 (c = 0.95, $CHCl_3$); lit. $[\alpha]_D^{22}$ = +16.8 (c = 1.35, $CHCl_3$)[118].

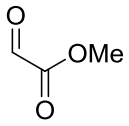
R_f = 0.31 (cyclohexane/EtOAc = 1:4 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$, based on COSY): δ = 5.31 (d, ^3J = 8.8 Hz, 1H, NH), 4.35–4.21 (m, 1H, HNC<u>H</u>), 3.79–3.70 (m, 6H, 2 × OC<u>H</u>$_3$), 3.35–2.95 (m, 2H, C<u>H</u>$_2$), 2.35–2.07 (m, 1H, $(CH_3)_2$C<u>H</u>), 1.39 (s, 9H, H-Boc), 0.95 (d, ^3J = 6.7 Hz, 3H, CH(C<u>H</u>$_3$)$_2$), 0.76 (d, ^3J = 6.7 Hz, 3H, CH(C<u>H</u>$_3$)$_2$').

^{13}C NMR (75 MHz, $CDCl_3$, based on HSQC): δ = 201.4 (d, 1C, C=O), 156.0 (s, 1C, OCONH), 79.9 (s, 1C, Me_3<u>C</u>), 65.1 (s, 1C, HNCH), 53.2 (dd, 2C, 2 × OCH_3), 39.6 and 37.8 (CH_2P), 29.2 (s, 1C, $(CH_3)_2$<u>C</u>H), 28.3 (s, 3C, (<u>C</u>H$_3$)$_3$C), 19.9 (s, 1C, CH(<u>C</u>H$_3$)$_2$), 16.8 (s, 1C, CH(<u>C</u>H$_3$)$_2$').

^{31}P NMR (202 MHz, $CDCl_3$) δ = 22.19 (s).

6.3.3.3 Methyl glyoxylate (16)[84,90]



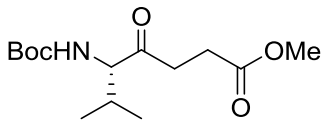
16

A dry 500 mL round bottom flask with magnetic stirring bar (evacuated, heated, N_2-purged) was charged with a suspension of 11.9 g (66.8 mmol, 1.0 eq) dimethyl tartrate in 134 mL Et_2O. 15.2 g (66.8 mmol, 1.0 eq) periodic acid were added to the stirred suspension in small portions over a period of 40 min. During the addition a fine, white precipitate was formed. After the addition was finished the reaction mixture was stirred for 20 min until the suspension turned to a clear solution with a white precipitate stuck on the glass wall of the flask. The suspension was filtered through a glass frit (por.4), the filter cake was washed with Et_2O (3×30 mL) and the filtrate was dried over Na_2SO_4 for 30 min. The solvent was removed under reduced pressure ($T \leq 35$ °C) and the oily residue dried in high vacuum for 15 min. The crude aldehyde was stored under Ar in the fridge and directly used without further purification on the next day.

R_f = 0.47 (cyclohexane/EtOAc = 1:1 (v/v); product stains immediately with $KMnO_4$).

$C_3H_4O_3$ [88.06 g/mol].

6.3.3.4 Methyl (*S*)-5-((*tert*-butoxycarbonyl)amino)-6-methyl-4-oxoheptanoate (17)[84]



17

Horner-Wadsworth-Emmons reaction: A 1000 mL three-necked round bottom flask with magnetic stirring bar was dried (evacuated, heated, N_2-purged) and equipped with a gas valve and a thermometer. The flask was charged with a solution of 13.5 g (41.8 mmol, 1.0 eq) ketophosphonate **15** in 417 mL abs. THF and cooled to 0 °C (ice bath). 3.34 g (83.5 mmol, 2.0 eq) NaH (60 % dispersion in mineral oil) were added in small portions under a slight stream of N_2 over a period of 30 min and the reaction mixture was additionally stirred for 25 min at 0 °C. After cooling to –78 °C (dry ice/acetone bath) a –78 °C cold solution of the freshly prepared aldehyde **16** in 104 mL abs. THF was added with a cannula and the yellow mixture was stirred for 30 min at –78 °C. The flask was warmed up to –30 °C and the mixture stirred for 1.5 h at that temperature (cooled in an ice/$CaCl_2$ slurry and liquid N_2) until TLC indicated full conversion. The reaction mixture was quenched by slow addition of 5 mL glacial acetic acid in 10 mL THF over a period of 1 h at –30 °C, warmed to RT and the solvent was removed under

reduced pressure. The oily residue was partitioned between EtOAc (300 mL) and H_2O (100 mL). The layers were separated and the organic phase washed with H_2O (100 mL), satd. $NaHCO_3$ (100 mL) and brine (100 mL), dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure and the resulting yellow oil dried *in vacuo*.

Hydrogenation: The crude oil (13.6 g) was dissolved in 250 mL THF in a 500 mL Schlenk flask with magnetic stirring bar. The flask was evacuated and purged with argon and 890 mg palladium on charcoal (5 % palladium; 1 mol-%) were added. The flask was evacuated and purged with H_2 three times (balloon) and the suspension was stirred at RT for 16 h. Subsequently, the catalyst was carefully removed under an argon atmosphere by filtration through a Schlenk-frit containing a plug of Celite® and the filtrate was concentrated under reduced pressure. Purification via flash chromatography (140 g SiO_2; cyclohexane/EtOAc = 1:0→1:1 (v/v); staining: $KMnO_4$) yielded 9.67 g of a wax-like white solid.

Yield: 9.67 g (33.6 mmol, 80 %, 2 steps), wax-like white solid.

$C_{14}H_{25}NO_5$ [287.36 g/mol].

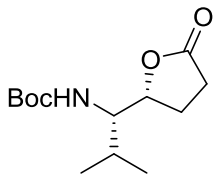
$[\alpha]_D^{23}$ = +34.0 (c = 1.50, $CHCl_3$).

R_f = 0.70 (cyclohexane/EtOAc = 1:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 5.09 (d, ^3J = 8.0 Hz, 1H, NH), 4.26 (dd, ^3J = 8.3 Hz, 3.9 Hz, 1H, NHCH), 3.66 (s, 3H, CO_2CH_3), 3.00–2.45 (m, 4H, 2 × CH_2), 2.30–2.10 (m, 1H, $(CH_3)_2CH$), 1.42 (s, 9H, H-Boc), 1.00 (d, ^3J = 6.8 Hz, 3H, H_3CCH), 0.79 (d, ^3J = 6.8 Hz, 3H, $H_3C'CH$).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 208.0 (s, 1C, C=O), 173.0 (s, 1C, C=O), 156.1 (s, 1C, OCONH), 79.8 (s, 1C, Me_3C), 64.0 (s, 1C, HNCH), 51.9 (s, 1C, OCH_3), 35.5 (s, 1C, CH_2), 30.4 (s, 1C, $(CH_3)_2CH$), 28.4 (s, 3C, $(CH_3)_3C$), 27.6 (s, 1C, CH_2'), 20.0 (s, 1C, H_3CCH), 16.8 (s, 1C, $H_3C'CH$).

6.3.3.5 *tert*-Butyl ((*S*)-2-methyl-1-((*R*)-5-oxotetrahydrofuran-2-yl)propyl)-carbamate (18)[84]



18

Stereoselective reduction: A 500 mL three-necked round bottom flask with magnetic stirring bar was dried (evacuated, heated, N_2-purged) and equipped with a gas valve, septum and thermometer. 9.00 g (31.3 mmol, 1.0 eq) ketoester **17** were dissolved in 313

mL abs. THF and the solution was cooled to –60 °C (dry ice/acetone bath). LiAlH(O*t*-Bu)$_3$ (23.9 g, 94.0 mmol, 3.0 eq) was added in one portion and the white suspension, which turned yellow after 30 min, was stirred for 20 h at a temperature between –40 to –30 °C using a dry ice/acetone bath. The reaction was quenched by addition of 25 % aqueous tartaric acid (300 mL), extracted with EtOAc (2×300 mL) and the combined organic phases were washed with H_2O (2×100 mL), satd. $NaHCO_3$ (100 mL) and brine (100 mL), dried over Na_2SO_4 and filtered. The solvents were evaporated under reduced pressure to a small volume and the crude residue was directly used for the next step without further purification.

Acid-catalyzed lactonization: The crude residue was dissolved in 150 mL toluene in a 250 mL round bottom flask equipped with a reflux condenser and 6.0 mg (32 µmol, 0.1 mol-%) *p*-TsOH×H_2O were added. The colorless solution was heated to 60 °C (oil bath) and stirred for 12 h. Subsequently, the solvent was removed under reduced pressure and the oily residue was dried in high vacuum for 1 h. The product was precipitated by dissolving the oil in 150 mL *n*-hexane. The product was collected by filtration through a glass frit (por.4), washed with *n*-hexane (3×30 mL) and dried *in vacuo* to obtain 3.28 g (12.8 mmol, 41 %) of the pure diastereomer **18** as a white powder. The filtrate was concentrated *in vacuo* and purified via flash chromatography (430 g SiO_2; cyclohexane/EtOAc = 3:1 (v/v); staining: $KMnO_4$) to obtain additional 1.11 g (4.31 mmol, 14 %) **18**. Single crystals (colorless needles) were obtained by crystallization from *n*-hexane.

Yield: 4.39 g (17.1 mmol, 55 %), white powder.

$C_{13}H_{23}NO_4$ [257.33 g/mol].

mp = 106–108 °C.

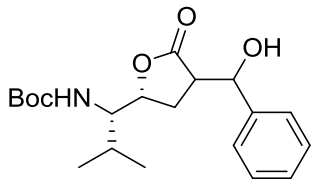
$[\alpha]_D^{23}$ = –9.0 (c = 1.02, $CHCl_3$).

R_f = 0.27 (cyclohexane/EtOAc = 2:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$, based on COSY and HSQC): δ = 4.41 (d, ^3J = 9.7 Hz, 1H, N<u>H</u>), 4.37-4.27 (m, 1H, CO_2C<u>H</u>), 3.72-3.53 (m, 1H, HNC<u>H</u>), 2.65-2.40 (m, 2H, CH_2C<u>H_2</u>CO_2), 2.35-2.20 (m, 1H, 1 × C<u>H_2</u>CH_2CO_2), 2.20-1.98 (m, 2H, containing 1 × C<u>H_2</u>CH_2CO_2 and $(CH_3)_2C$<u>H</u>), 1.43 (s, 9H, H-Boc), 0.94 (d, ^3J = 6.9 Hz, 3H, <u>H_3</u>CCH), 0.88 (d, ^3J = 6.9 Hz, 3H, <u>H_3</u>C'CH).

^{13}C NMR (75 MHz, $CDCl_3$, based on HSQC): δ = 177.0 (s, 1C, C=O), 156.2 (s, 1C, OCONH), 80.0 (s, 1C, Me_3<u>C</u>), 79.8 (s, 1C, CO_2<u>C</u>H), 57.8 (HNCH), 28.4 (s, 3C, (<u>C</u>H_3)$_3$C), 28.2 (s, 2C, containing CH_2<u>C</u>H_2CO_2 and $(CH_3)_2$<u>C</u>H), 25.1 (s, 1C, <u>C</u>$H_2CH_2CO_2$), 19.9 (s, 1C, H_3<u>C</u>CH), 15.7 (s, 1C, H_3<u>C</u>'CH).

6.3.3.6 *tert*-Butyl ((1*S*)-1-((2*R*)-4-(hydroxy(phenyl)methyl)-5-oxotetrahydrofuran-2-yl)-2-methylpropyl)carbamate (19)[84]



19

Aldol reaction: A 100 mL three-necked round bottom flask with magnetic stirring bar was dried (evacuated, heated, N_2-purged) and equipped with a gas valve, thermometer and a glass stopper. 1.29 g (5.00 mmol, 1.0 eq) starting lactone **18** were dissolved in 25 mL abs. THF and the colorless solution was cooled to –78 °C (dry ice/acetone bath). Under vigorous stirring 5.00 mL (10.0 mmol, 2.0 eq) LDA solution (2.0 M in THF) were added and stirring was continued for 35 min at –78 °C. Freshly distilled benzaldehyde (1.02 mL, 10.0 mmol, 2.0 eq) was added with a syringe within 5 min and the reaction mixture was stirred for additional 30 min until TLC indicated full conversion. The mixture was quenched by addition of 12.5 mL satd. NH_4Cl solution and 5 mL H_2O and stirring was continued until the emulsion warmed up to RT. The mixture was poured into a separation funnel and the product was extracted with EtOAc (2×12 mL). The combined organic layers were washed with 1 M HCl (20 mL), satd. $NaHCO_3$ (20 mL) and brine (10 mL), dried over Na_2SO_4 and filtered. The solvents were removed under reduced pressure and the oily residue dired *in vacuo*. Purification via flash chromatography (110 g SiO_2; cyclohexane/EtOAc = 3:1→2:1 (v/v); staining: $KMnO_4$) yielded 1.29 g of a mixture of diastereomers.

Yield: 1.29 g (3.54 mmol, 71 %), white amorphous solid.

$C_{20}H_{29}NO_5$ [363.45 g/mol].

R_f = 0.30 and 0.38 (cyclohexane/EtOAc = 2:1 (v/v); staining: $KMnO_4$).

Two diastereomers were characterized separately:

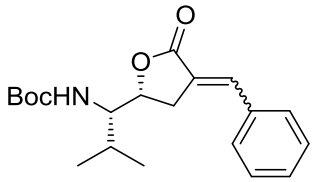
^{1}H NMR (300 MHz, $CDCl_3$, unidentified diastereomer A, based on COSY): δ = 7.42–7.23 (m, 5H, Ar-H), 4.80 (dd, ^3J = 8.6 Hz, 7.0 Hz, 1H, PhCH), 4.35–4.10 (m, 2H, containing NH and *i*-PrCHCH), 3.74–3.46 (m, 1H, *i*-PrCH), 3.23–2.80 (m, 1H, HOCHCH), 2.14–1.76 (m, 3H, containing $CH(CH_3)_2$ and CH_2), 1.45–1.29 (m, 9H, H-Boc), 0.97–0.78 (m, 6H, $CH(CH_3)_2$).

^{1}H NMR (300 MHz, $CDCl_3$, unidentified diastereomer B, based on COSY): δ = 7.40–7.21 (m, 5H, Ar-H), 5.35 (s, 1H, PhCH), 4.70–4.20 (m, 2H, containing NH and *i*-PrCHCH), 3.63–3.36 (m, 1H, *i*-PrCH), 3.14–2.88 (m, 1H, HOCHCH), 2.50–2.15 (m,

1H, 1 × C<u>H</u>$_2$), 2.12–1.90 (m, 1H, C<u>H</u>(CH$_3$)$_2$), 1.89–1.74 (m, 1H, 1 × C<u>H</u>$_2$), 1.50–1.20 (m, 9H, H-Boc), 0.98–0.76 (m, 6H, CH(C<u>H</u>$_3$)$_2$).

HRMS (MALDI-TOF): Calcd. for $C_{20}H_{29}NO_5Na$ $[M+Na]^+$: 386.1943; found: 386.1945.

6.3.3.7 *tert*-Butyl ((*S*)-1-((*R*)-4-benzylidene-5-oxotetrahydrofuran-2-yl)-2-methylpropyl)carbamate (20)



20

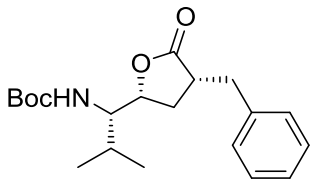
Mesylation: A 50 mL Schlenk flask was dried (evacuated, heated, N_2-purged) and loaded with a solution of 1.20 g (3.29 mmol, 1.0 eq) of compound **19** in 22 mL abs. DCM. 1.38 mL (9.88 mmol, 3.0 eq) triethylamine were added under stirring and the yellow solution was cooled to 0 °C (ice bath). 1.15 g (6.59 mmol, 2.0 eq) methanesulfonic anhydride were added in small portions within 45 min. Stirring was continued for 15 min at 0 °C, then 3.5 h at RT. Since no conversion could be observed, additional 0.69 mL (4.9 mmol, 1.5 eq) triethylamine and 0.57 g (3.3 mmol, 1.0 eq) methanesulfonic anhydride were added at RT. The reaction mixture was warmed to 30 °C (oil bath) and stirred at that temperature overnight. The reaction was quenched by addition of cold H_2O (11 mL) and the mixture was extracted with EtOAc (2×44 mL). The combined organic phases were washed with brine (11 mL), dried over Na_2SO_4 and filtered. Evaporation of the solvent and drying in vacuum yielded 1.75 g of the crude brown solid which was directly used without further purification.

Elimination: The crude was dissolved in 16.5 mL abs. EtOH in a 50 mL round bottom flask and 0.55 mL (3.95 mmol, 1.2 eq) triethylamine were added. The mixture was heated to 50 °C and stirred for 60 h. Subsequently, the solvent was evaporated under reduced pressure to a small volume and the residue partitioned between EtOAc (33 mL) and H_2O (15 mL). The phases were separated and the aqueous phase was extracted with EtOAc (16.5 mL). The combined organic layers were washed with brine (9 mL), concentrated *in vacuo* and directly used in the next step without further treatment.

R_f = 0.45 (cyclohexane/EtOAc = 2:1 (v/v); stains brown immediately with $KMnO_4$).

$C_{20}H_{27}NO_4$ [345.44 g/mol]

6.3.3.8 *tert*-Butyl ((*S*)-1-((2*R*,4*R*)-4-benzyl-5-oxotetrahydrofuran-2-yl)-2-methylpropyl)carbamate (21)



21

Crude compound **20** (3.29 mmol) was dissolved in 33 mL THF in a 100 mL Schlenk flask with magnetic stirring bar. The flask was evacuated and purged with argon and 57 mg (0.99 mmol, 0.3 eq) Raney®-Nickel (slurry in H_2O) were added. Argon was exchanged by hydrogen (3×evacuation/purging) and the reaction mixture was stirred vigorously under H_2-atmosphere (balloon) for 21 h. Subsequently, the catalyst was carefully removed under an argon atmosphere by filtration through a Schlenk-frit containing a plug of Celite® and the filter cake was washed with EtOAc (3×10 mL). The filtrate was dried over Na_2SO_4 and the solvent evaporated. Purification via flash chromatography (120 g SiO_2; cyclohexane/EtOAc = 6:1 (v/v); staining: $KMnO_4$) yielded compound **21** as a white solid.

Yield: 623 mg (1.79 mmol, 54 % over 3 steps), white solid.

$C_{20}H_{29}NO_4$ [347.46 g/mol]

mp = 57–58 °C.

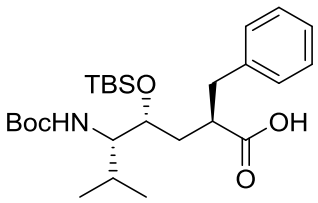
$[\alpha]_D^{23}$ = –60.8 (c = 1.24, $CHCl_3$).

R_f = 0.49 (cyclohexane/EtOAc = 2:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$, based on COSY and NOESY): δ = 7.33–7.14 (m, 5H, Ar-H), 4.31 (d, ^3J = 10.0 Hz, NH), 4.24–4.10 (m, 1H, CO_2CH), 3.73–3.54 (m, 1H, HNCH), 3.29 (dd, ^2J = 13.6 Hz, ^3J = 2.9 Hz, 1H, 1 × $PhCH_2$), 2.94–2.76 (m, 1H, BnCH), 2.75–2.60 (m, 1H, 1 × $PhCH_2$), 2.31–2.16 (m, 1H, 1 × $CHCH_2CH$) 2.14–1.96 (m, 1H, $(CH_3)_2CH$), 1.93–1.76 (m, 1H, 1 × $CHCH_2CH$), 1.42 (s, 9H, H-Boc), 0.93 (d, ^3J = 6.8 Hz, 3H, H_3CCH), 0.84 (d, ^3J = 6.9 Hz, 3H, $H_3C'CH$).

^{13}C NMR (75 MHz, $CDCl_3$, based on HSQC): δ = 177.8 (s, 1C, C=O), 156.1 (s, 1C, OCONH), 138.8 (s, 1C, C_q-Ar), 129.0 (s, 2C, C-Ar), 128.8 (s, 2C, C-Ar), 126.8 (s, 1C, C-Ar), 79.9 (s, 1C, Me_3C), 78.2 (s, 1C, HCOCO), 58.3 (s, 1C, $(CH_3)_2CH$), 42.7 (s, 1C, BnCH), 36.5 (s, 1C, $PhCH_2$), 32.2 (s, 1C, $CHCH_2CH$), 28.4 (s, 4C, containing $(CH_3)_2CH$ and 3 × $(CH_3)_3C$), 19.9 (s, 1C, H_3CCH), 16.0 (s, 1C, $H_3C'CH$).

6.3.3.9 (2*R*,4*R*,5*S*)-2-Benzyl-5-((*tert*-butoxycarbonyl)amino)-4-((*tert*-butyldimethylsilyl)oxy)-6-methylheptanoic acid (RGSA) (22)



22

Lactone opening: In a 50 mL round bottom flask with magnetic stirring bar 577 mg (1.66 mmol, 1.0 eq) lactone **21** were dissolved in 5.5 mL THF and a solution of 279 mg (6.64 mmol, 4.0 eq) LiOH×H_2O in 8.3 mL water was added under vigorous stirring at RT. After 30 min 16.6 mL Et_2O were added to the stirred solution and the mixture was cooled down to 0 °C (ice bath). After carefully adjusting to pH=4 with citric acid (25 % in H_2O), the phases were separated and the aqueous phase was extracted with Et_2O (2×11 mL). The combined organic layers were washed with H_2O (11 mL) and brine (11 mL), dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure with T ≤ 30 °C. The residue was transferred into a 10 mL Schlenk flask and dried under high vacuum.

Silylation: The crude product was dissolved in 6.6 mL abs. dichloromethane and the turbid solution was cooled to 0 °C (ice bath). 790 µL (9.96 mmol, 6.0 eq) *N*-methylimidazole were added and the colorless solution was stirred for 10 min at 0 °C. After the addition of 2.53 g (9.96 mmol, 6.0 eq) iodine in one portion the reaction mixture was stirred for additional 15 min and 751 mg (4.98 mmol, 3.0 eq) TBSCl were added in small portions. When the addition was finished, the ice bath was removed and the resulting mixture was stirred at RT for 18 h. After this period 12 mL Et_2O were added and the mixture was treated with 24 mL aqueous $Na_2S_2O_3$ (1 M) until decoloration occurred. The mixture was poured into a separation funnel and the phases were separated. The aqueous layer was extracted with Et_2O (2×12 mL) and the combined organic layers were washed with 25 % aqueous citric acid (12 mL) and brine (12 mL), dried over Na_2SO_4 and filtered. The solvents were removed under reduced pressure and the yellowish oil was dried *in vacuo*.

Ester hydrolysis: In a 10 mL glass vial with magnetic stirring bar the crude product was dissolved in 4.2 mL MeOH and 128 µL (0.17 mmol, 0.1 eq) citric acid (25 % in H_2O) were added. The solution was stirred at RT overnight. When the conversion was completed the solvents were removed under reduced pressure and the residue partitioned between EtOAc (12 mL) and H_2O (3 mL). The layers were separated and the solvents removed under reduced pressure. Purification via flash chromatography (85 g SiO_2;

CH_2Cl_2/MeOH = 100:1 (v/v) + 0.5 vol-% AcOH) yielded compound **22** as a colorless oil.

Yield: 679 mg (1.42 mmol, 85 %), colorless oil.

$C_{26}H_{45}NO_5Si$ [479.73 g/mol]

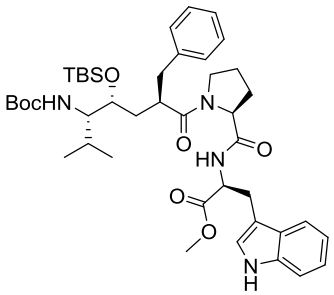
$[\alpha]_D^{23} = -18.0$ (c = 0.31, $CHCl_3$).

R_f = 0.15 (CH_2Cl_2/MeOH = 100:1 (v/v) + 0.5 vol-% AcOH; product stains white immediately with $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.34–7.15 (m, 5H, Ar-H), 7.85 and 4.59 (d, ^3J = 10.2 Hz, 1H, NH), 3.85–3.69 (m, 1H, SiOC<u>H</u>), 3.52–3.24 (m, 1H, HNC<u>H</u>), 3.08–2.94 (m, 1H, 1 × PhC<u>H</u>$_2$), 2.84–2.66 (m, 2H, containing 1 × PhC<u>H</u>$_2$ and PhCH$_2$C<u>H</u>), 1.95–1.78 (m, 1H, 1 × CHC<u>H</u>$_2$CH), 1.73–1.50 (m, 2H, containing 1 × CHC<u>H</u>$_2$CH and (CH$_3$)$_2$C<u>H</u>), 1.42 (s, 9H, H-Boc), 0.97–0.70 (m, 15H, containing (C<u>H</u>$_3$)$_3$CSi and (C<u>H</u>$_3$)$_2$CH), 0.12–0.03 (m, 6H, (C<u>H</u>$_3$)$_2$Si).

^{13}C NMR: See PhD thesis of J. Ivković.[54]

6.3.3.10 Methyl ((2*R*,4*R*,5*S*)-2-benzyl-5-((*tert*-butoxycarbonyl)amino)-4-((*tert*-butyldimethylsilyl)oxy)-6-methylheptanoyl)-L-prolyl-L-tryptophanate (Boc-RGSA-Pro-Trp-OMe) (23)



23

Boc-deprotection of Boc-Pro-Trp-OMe: A 10 mL round bottom flask with magnetic stirring bar was charged with 700 mg (1.68 mmol, 1.2 eq) Boc-Pro-Trp-OMe and 1.05 mL (14.0 mmol, 10 eq) ethanethiol. The heterogeneous mixture was dissolved in 3.24 mL (42.1 mmol, 30 eq) trifluoroacetic acid and stirred at RT for 60 min. Subsequently, the volatile componds were removed under reduced pressure and the residue was partitioned between EtOAc (13 mL) and 25 % aqueous NH_3 (3.2 mL). The layers were separated and the aqueous layer was extracted with EtOAc (3×13 mL). The combined organic phases were washed with brine (13 mL), dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure and the crude product was dried *in vacuo*. The deprotected H-Pro-Trp-OMe was used without further purification.

Peptide coupling: In a 50 mL round bottom flask with magnetic stirring bar 673 mg (1.40 mmol, 1.0 eq) acid **22** were dissolved in 6.3 mL abs. DMF and 245 µL (1.40 mmol, 1.0 eq) DIPEA were added to the stirred solution. After cooling to 0 °C (ice bath) 639 mg (1.68 mmol, 1.2 eq) HBTU were added in one portion. After 5 min of activation time a solution of the crude H-Pro-Trp-OMe (1.68 mmol, 1.2 eq) and 490 µL (2.81 mmol, 2.0 eq) DIPEA in 3.1 mL DMF was added and the ice bath was removed. The reaction mixture was stirred at RT for 90 min until TLC indicated full conversion. The mixture was quenched by the addition of 15 mL brine, transferred into a separation funnel and the product was extracted with EtOAc (45 mL). The organic phase was washed with brine (3×15 mL), dried over Na_2SO_4, filtered and the solvents were removed under reduced pressure. Purification via flash chromatography (110 g SiO_2; CH_2Cl_2/MeOH = 100:3 v/v), followed by H_2O washing (5×100 mL; product dissolved in 150 mL Et_2O) to remove remaining tetramethylurea, yielded compound **23** as an off-white solid.

Yield: 794 mg (1.02 mmol, 73 %), off-white solid.

$C_{43}H_{64}N_4O_7Si$ [777.09 g/mol].

mp = 62–67 °C.

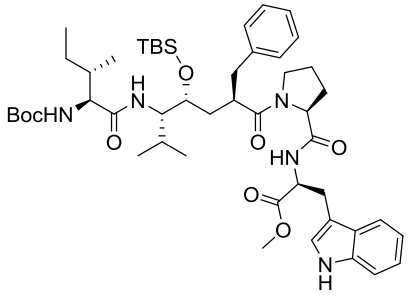
$[\alpha]_D^{23}$ = –5.9 (c = 1.00, $CHCl_3$).

R_f = 0.80 (CH_2Cl_2/MeOH = 10:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 8.13 (bs, 1H, NH indole), 7.53 (d, ^3J = 7.1 Hz, 1H, H-Ar indole), 7.34 (d, ^3J = 7.1 Hz, 1H, NH amide), 7.29–6.97 (m, 9H, H-Ar), 4.81–4.71 (m, 1H, CHCO$_2$), 4.62–4.46 (m, 2H, containing prolyl-CH and NH carbamate), 3.67 (s, 4H, containing CO$_2$CH$_3$ and SiOCH), 3.55–3.40 (m, 1H, 1 × prolyl-CH$_2$), 3.55–3.40 (m, 3H, containing *i*-PrCH and CH$_2$-tryptophan), 2.99–2.88 (m, 1H, 1 × prolyl-CH$_2$), 2.80–2.68 (m, 1H, BnCH), 2.67–2.45 (m, 2H, PhCH), 2.28–2.15 (m, 1H, 1 × prolyl-CH$_2$), 1.95–1.52 (m, 6H, containing (CH$_3$)$_2$CH, SiOCHCH$_2$ and 3 × prolyl-CH$_2$), 1.40 (s, 9H, H-Boc), 0.94–0.75 (m, 15H, containing (CH$_3$)$_3$CSi and (CH$_3$)$_2$CH), 0.15–0.03 (m, 6H, (CH$_3$)$_2$Si).

^{13}C NMR: See PhD thesis of J. Ivković.[54]

6.3.3.11 Methyl ((2*R*,4*R*,5*S*)-2-benzyl-5-((2*S*,3*S*)-2-((*tert*-butoxycarbonyl)-amino)-3-methylpentanamido)-4-((*tert*-butyldimethylsilyl)oxy)-6-methylheptanoyl)-L-prolyl-L-tryptophanate (protected Ile-*HER*) (24)



24

Boc-deprotection: 170 mg (0.219 mmol, 1.0 eq) compound **23** were dissolved in 1.0 mL (13.1 mmol, 60 eq) trifluoroacetic acid in a 10 mL round bottom flask with magnetic stirring bar at RT. After stirring for 15 min the acid was removed under reduced pressure (Schlenk line with preceding cooling trap) and the crude product dried *in vacuo*.

Peptide coupling: A 10 mL Schlenk flask with magnetic stirring bar was dried under vacuum with a heat gun and purged with N_2. 61 mg (0.262 mmol, 1.2 eq) Boc-Ile-OH were dissolved in 1.1 mL abs. DMF and 38.3 µL (0.219 mmol, 1.0 eq) DIPEA were added to the stirred solution. After cooling to 0 °C (ice bath) 100 mg (0.262 mmol, 1.2 eq) HATU were added in one portion and the yellow solution was stirred for 5 min. In a second 10 mL round bottom flask with Schlenk adaptor the previously deprotected crude compound was dissolved in 1.1 mL abs. DMF and 115 µL (0.658 mmol, 3.0 eq) DIPEA were added. After cooling to 0 °C (ice bath) the solution was added to the preactivated Boc-Ile-OH solution with a syringe. After 5 min the ice bath was removed and the reaction mixture was stirred at RT for 90 min. Since no full conversion could be observed, additional 76.5 µL (0.438 mmol, 2.0 eq) DIPEA and 50 mg (0.13 mmol, 0.6 eq) HATU were added at 0 °C. The mixture was stirred for additional 30 min at RT, subsequently quenched by the addition of 2.2 mL brine and extracted with EtOAc (8 mL). The layers were separated and the aqueous phase was extracted with EtOAc (8 mL). The combined organic layers were washed with with a 1:2 mixture of H_2O/brine (10×16 mL) and brine (16 mL), dried over Na_2SO_4 and filtered. The product was concentrated under reduced pressure and dried *in vacuo*. Purification via flash chromatography (22 g SiO_2; cyclohexane/EtOAc = 6:4 v/v) provided compound **24** as a white solid.

Yield: 119 mg (0.134 mmol, 61 %), white solid.

$C_{49}H_{75}N_5O_8Si$ [890.25 g/mol].

mp = 72–77 °C.

$[\alpha]_D^{23}$ = –9.75 (c = 1.52, $CHCl_3$).

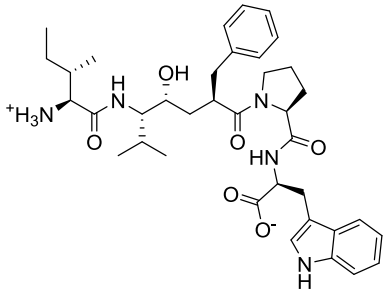
R_f = 0.36 (cyclohexane/EtOAc = 1:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$, based on COSY and HSQC): δ = 8.61 (bs, 1H, NH indole), 7.50 (d, ^3J = 7.6 Hz, 1H, Ar-H), 7.38–6.92 (m, 10H, Ar-H and amide-NH from Trp), 6.21 (d, ^3J = 8.6 Hz, 1H, *i*-PrCHNH), 4.99 (d, ^3J = 9.1 Hz, 1H, NH carbamate), 4.81–4.71 (m, 1H, $CHCO_2$), 4.52 (d, ^3J = 6.1 Hz, 1H, prolyl-CH), 3.92–3.82 (m, 1H, HNCH from Ile), 3.79–3.68 (m, 2H, containing *i*-PrCH, and SiOCH), 3.67 (s, 3H, CO_2CH_3), 3.57–3.43 (m, 1H, 1 × prolyl-CH_2N), 3.27 (d, ^3J = 5.7 Hz, 2H, CH_2-tryptophan), 3.10–3.00 (m, 1H, 1 × prolyl-CH_2N), 2.83–2.61 (m, 2H, containing BnCH and 1 × PhCH_2), 2.52–2.39 (m, 1H, 1 × PhCH_2), 2.29–2.14 (m, 1H, 1 × $CHCH_2$ from Pro), 1.95–1.74 (m, 4H, containing 1 × SiOCHCH_2, 1 × $CHCH_2$ from Pro, 1 × $CH_2CH_2CH_2$ and CH_3CH_2CH), 1.72–1.48 (m, 3H, containing 1 × SiOCHCH_2, 1 × $CH_2CH_2CH_2$ and $(CH_3)_2CH$), 1.44 (s, 9H, H-Boc), 1.18–1.00 (m, 2H, CH_3CH_2), 0.99–0.74 (m, 21H, containing $(CH_3)_2CH$, $(CH_3)_3CSi$, CH_3CH_2CH and CH_3CHCH_2), 0.11 (s, 3H, $SiCH_3$), 0.07 (s, 3H, $SiCH_3$').

^{13}C NMR (75 MHz, $CDCl_3$, based on HSQC): δ = 174.8 (s, 1C, C_2NC=O), 172.4 (s, 1C, C=O), 171.6 (s, 1C, C=O), 171.1 (s, 1C, C=O), 156.2 (s, 1C, C=O carbamate), 139.1 (s, 1C, C_q from Ph), 136.3 (s, 1C, HNC_q from indole), 129.0 (s, 2C, C-Ar from Ph), 128.6 (s, 2C, C-Ar from Ph), 127.7 (C_q from indole), 126.6 (s, 1C, C-Ar from Ph), 123.8 (s, 1C, HNCH from indole), 122.0 (s, 1C, C-Ar indole), 119.5 (s, 1C, C-Ar indole), 118.6 (s, 1C, C-Ar indole), 111.3 (s, 1C, C-Ar indole), 109.8 (s, 1C, C_qCH_2 indole), 80.1 (s, 1C, Me_3CO), 71.8 (s, 1C, SiOCH), 60.0 (s, 2C, containing HNCH from Ile and prolyl-CH), 57.1 (s, 1C, *i*-PrCH), 53.1 (s, 1C, $CHCO_2$), 52.4 (s, 1C, CO_2CH_3), 47.2 (s, 1C, prolyl-CH_2N), 41.8 (s, 1C, BnCH), 38.8 (s, 1C, PhCH_2), 36.8 (s, 1C, CH_3CHCH_2), 34.7 (s, 1C, BnCHCH_2), 28.5 (s, 4C, $(CH_3)_3CO$ and $(CH_3)_2CH$), 27.5 (s, 2C, CH_2 from Trp and $CHCH_2$ from Pro), 26.1 (s, 3C, $(CH_3)_3CSi$), 24.9 (s, 1C, $CH_2CH_2CH_2$), 24.6 (s, 1C, CH_3CH_2CH), 21.3 (s, 1C, 1 × $(CH_3)_2CH$), 18.8 (s, 1C, 1 × $(CH_3)_2CH$), 18.1 (s, 1C, Me_3CSi), 15.9 (s, 1C, CH_3CHCH_2), 11.4 (s, 1C, CH_3CH_2CH), –3.8 (s, 1C, $SiCH_3$), –4.5 (s, 1C, $SiCH_3$').

HRMS (MALDI-TOF): Calcd. for $C_{49}H_{75}N_5O_8SiNa$ $[M+Na]^+$: 912.5283; found: 912.5244.

6.3.3.12 ((2*R*,4*R*,5*S*)-5-((2*S*,3*S*)-2-Ammonio-3-methylpentanamido)-2-benzyl-4-hydroxy-6-methylheptanoyl)-L-prolyl-L-tryptophanate (Ile-*HER*) (25)



25

Saponification: 115 mg (129 µmol, 1.0 eq) compound **24** were dissolved in 0.6 mL THF in a 5 mL flask with magnetic stirring bar. 22 mg (52 µmol, 4.0 eq) LiOH×H_2O were dissolved in 0.6 mL H_2O and added to the solution under vigorous stirring at RT. The emulsion was stirred for 60 min at RT until TLC indicated full conversion. The mixture was acidified with 25 % aqueous citric acid to pH=4 and EtOAc (4 mL) was added. The layers were separated and the aqueous phase was extracted with EtOAc (2×2 mL). The combined organic phases were washed with brine (2 mL), dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure and the product was dried *in vacuo*.

Boc- and TBS-deprotection: The crude residue was dissolved in 0.6 mL cold trifluoroacetic acid in a 5 mL glass vial with magnetic stirring bar. The reaction mixture was stirred for 60 min at RT until HPLC indicated full conversion. The acid was removed under reduced pressure (Schlenk line with preceding cooling trap) and the crude residue was dried *in vacuo*. Purification via preparative HPLC (*JKV_NucleodurC18_001-HCOOH_10to85*) provided only 2.8 mg (3 %) of the desired product.

Yield: 2.8 mg (4.2 µmol, 3 %), colorless solid.

$C_{37}H_{51}N_5O_6$ [661.84 g/mol].

mp = 96–102 °C.

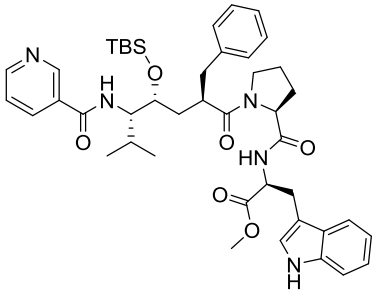
$[\alpha]_D^{23}$ = –3 (c = 0.125, MeOH).

NMR: Due to the minor yield, no decent NMR spectra could be obtained.

HPLC/MS (FAST_POROSHELL120_001HCOOH_8MINGRADIENT.M): t_R = 6.76 min; m/z (ESI+) = 662.4 $[M+H]^+$.

HRMS (MALDI-TOF): Calcd. for $C_{37}H_{51}N_5O_6Na$ $[M+Na]^+$: 684.3737; found: 684.3732.

6.3.3.13 Methyl ((2*R*,4*R*,5*S*)-2-benzyl-4-((*tert*-butyldimethylsilyl)oxy)-6-methyl-5-(nicotinamido)heptanoyl)-L-prolyl-L-tryptophanate (protected B_3-*HER*) (26)



26

Boc-deprotection: 170 mg (0.219 mmol, 1.0 eq) compound **23** were dissolved in 1.0 mL (13.1 mmol, 60 eq) ice cold trifluoroacetic acid in a 10 mL round bottom flask with magnetic stirring bar. The ice bath was removed and the brown reaction mixture was stirred for 10 min at RT. The acid was removed under reduced pressure (Schlenk line with preceding cooling trap) and the crude product dried *in vacuo*.

Peptide coupling: A 10 mL Schlenk flask with magnetic stirring bar was dried under vacuum with a heat gun and purged with N_2. 32 mg (0.262 mmol, 1.2 eq) nicotinic acid were dissolved in 1.1 mL abs. DMF and 38.3 µL (0.219 mmol, 1.0 eq) DIPEA were added to the stirred solution. After cooling to 0 °C (ice bath) 100 mg (0.262 mmol, 1.2 eq) HATU were added in one portion and the yellow solution was stirred for 2 min. In a second 10 mL round bottom flask with Schlenk adaptor the previously deprotected crude compound was dissolved in 1.1 mL abs. DMF and 153 µL (0.874 mmol, 4.0 eq) DIPEA were added. After cooling to 0 °C (ice bath) the preactivated nicotinic acid solution was added with a syringe. After 5 min the ice bath was removed and the reaction mixture was stirred at RT for 45 min. The mixture was quenched by the addition of 2.2 mL brine and extracted with EtOAc (3×8 mL). The combined organic layers were washed with with a 1:2 mixture of H_2O/brine (8×10 mL) and brine (10 mL), dried over Na_2SO_4 and filtered. The product was concentrated under reduced pressure and dried *in vacuo*. Purification via flash chromatography (18 g SiO_2; EtOAc = 1), followed by H_2O washing (5×4 mL; product dissolved in 20 mL EtOAc) to remove remaining tetramethylurea, provided compound **26** as a white solid.

Yield: 118 mg (151 µmol, 69 %), white solid.

$C_{44}H_{59}N_5O_6Si$ [782.07 g/mol].

mp = 63–67 °C.

$[\alpha]_D^{23}$ = +22.1 (c = 1.97, $CHCl_3$).

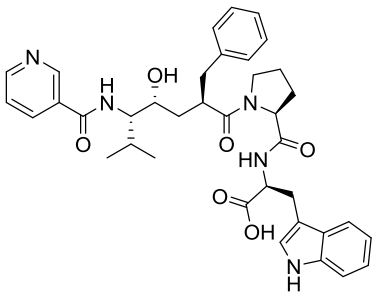
R_f = 0.20 (EtOAc = 1; staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$, based on COSY and HSQC): δ = 9.06 (s, 1H, NC<u>H</u> from Py), 8.74 (s, 1H, N<u>H</u> indole), 8.63 (s, 1H, NC<u>H</u>' from Py), 8.14 (d, ^3J = 7.7 Hz, 1H, Ar-H from Py), 7.51 (d, ^3J = 7.4 Hz, 1H, Ar-H from indole), 7.43–6.96 (m, 11H, Ar-H and amide-N<u>H</u> from Trp), 6.75 (d, ^3J = 8.6 Hz, 1H, *i*-PrCHN<u>H</u>), 4.86–4.77 (m, 1H, C<u>H</u>CO_2), 4.48 (d, ^3J = 4.9 Hz, 1H, prolyl-C<u>H</u>), 3.96–3.77 (m, 2H, containing *i*-PrC<u>H</u>, and SiOC<u>H</u>), 3.66 (s, 3H, CO_2CH_3), 3.61–3.48 (m, 1H, 1 × prolyl-C<u>H</u>$_2$N), 3.30 (d, ^3J = 5.6 Hz, 2H, C<u>H</u>$_2$-tryptophan), 3.24–3.14 (m, 1H, 1 × prolyl-C<u>H</u>$_2$N), 2.87–2.68 (m, 2H, containing BnC<u>H</u> and 1 × PhC<u>H</u>$_2$), 2.56–2.42 (m, 1H, 1 × PhC<u>H</u>$_2$), 2.25–2.01 (m, 1H, containing 1 × SiOCHC<u>H</u>$_2$ and 1 × CHC<u>H</u>$_2$ from Pro), 1.98–1.54 (m, 5H containing 1 × CHC<u>H</u>$_2$ from Pro, $CH_2CH_2CH_2$, $(CH_3)_2CH$ and 1 × SiOCHC<u>H</u>$_2$), 1.05–0.68 (m, 15H, containing $(CH_3)_3$CSi and $(CH_3)_2$CH), 0.07 (s, 3H, $SiCH_3$), 0.01 (s, 3H, $SiCH_3$').

^{13}C NMR (75 MHz, $CDCl_3$, based on HSQC): δ = 174.8 (s, 1C, C_2NC=O), 172.5 (s, 1C, C=O), 171.4 (s, 1C, C=O), 165.2 (s, 1C, Py<u>C</u>=O), 151.0 (s, 1C, NCH from Py), 147.1 (s, 1C, NCH' from Py), 139.0 (s, 1C, C_q from Ph), 136.3 (s, 1C, C-Ar form Py), 136.2 (s, 1C, HNC_q from indole), 131.2 (s, 1C, C_q from Py), 129.1 (s, 2C, C-Ar from Ph), 128.6 (s, 2C, C-Ar from Ph), 127.7 (C_q from indole), 126.7 (s, 1C, C-Ar from Ph), 123.9 (s, 1C, HNCH from indole), 123.7 (C-Ar from Py), 122.0 (s, 1C, C-Ar indole), 119.4 (s, 1C, C-Ar indole), 118.6 (s, 1C, C-Ar indole), 111.4 (s, 1C, C-Ar indole), 109.9 (s, 1C, <u>C</u>$_q$CH$_2$ indole), 70.8 (s, 1C, SiOCH), 60.2 (s, 1C, prolyl-<u>C</u>H), 58.0 (s, 1C, *i*-Pr<u>C</u>H), 53.3 (s, 1C, <u>C</u>HCO_2), 52.4 (s, 1C, CO_2<u>C</u>H_3), 47.4 (s, 1C, prolyl-<u>C</u>H$_2$N), 41.6 (s, 1C, Bn<u>C</u>H), 39.0 (s, 1C, Ph<u>C</u>H$_2$), 33.7 (s, 1C, BnCH<u>C</u>H$_2$), 28.7 (s, 1C, $(CH_3)_2$<u>C</u>H), 27.9 (s, 1C, CH<u>C</u>H$_2$ from Pro), 27.4 (s, 1C, CH$_2$ from Trp), 25.9 (s, 3C, (<u>C</u>H$_3$)$_3$CSi), 25.0 (s, 1C, CH_2<u>C</u>H_2CH_2), 21.2 (s, 1C, 1 × (<u>C</u>H$_3$)$_2$CH), 19.0 (s, 1C, 1 × (<u>C</u>H$_3$)$_2$CH), 18.0 (s, 1C, Me_3<u>C</u>Si),–4.0 (s, 1C, $SiCH_3$), –4.7 (s, 1C, $SiCH_3$').

HRMS (MALDI-TOF): Calcd. for $C_{44}H_{59}N_5O_6SiNa$ $[M+Na]^+$: 804.4132; found: 804.4121.

6.3.3.14 ((2*R*,4*R*,5*S*)-2-Benzyl-4-hydroxy-6-methyl-5-(nicotinamido)-heptanoyl)-L-prolyl-L-tryptophan (B$_3$-*HER*)[49,101]



27

TBS-deprotection: 105 mg (135 µmol, 1.0 eq) compound **26** were dissolved in 1.3 mL THF in a 3 mL polypropylene vial with magnetic stirring bar. 350 µL (13.4 mmol, 100

eq) HF/pyridine (70 % HF) were added to the solution and the reaction mixture was stirred at RT for 25 min. Since no full conversion could be observed, additional 350 µL (13.4 mmol, 100 eq) HF/pyridine were added at RT and the mixture was stirred for additional 20 min. The reaction was quenched by transferring to 6.5 mL ice cold satd. $NaHCO_3$ solution and the product was extracted with EtOAc (2×20 mL). The combined organic layers were washed with H_2O (10 mL) and brine (10 mL), dried over Na_2SO_4 and filtered. The solvents were removed under reduced pressure and the crude yellow solid was used without further purification.

Saponification: The residue was dissolved in 0.4 mL THF in a 5 mL glass vial with magnetic stirring bar and a solution of 22.6 mg (538 µmol, 4.0 eq) $LiOH \times H_2O$ in 0.7 mL H_2O was added under vigorous stirring at RT. After 30 min the reaction mixture was carefully acidified with 1 M HCl to pH=6. The solvents were removed under reduced pressure and the product was dried *in vacuo*. Purification by preparative HPLC (*CLF_NucleodurC18_001HCOOH_5to90hold65*) yielded compound **27** as a white powder.

Yield: 31 mg (48 µmol, 36 %), white powder.

$C_{37}H_{43}N_5O_6$ [653.78 g/mol].

mp = 139–146 °C.

$[\alpha]_D^{23}$ = –18.7 (c = 1.44, MeOH).

^{1}H NMR (300 MHz, $CDCl_3$, based on COSY and HSQC, mixture of 2 rotamers in 2:1 ratio, major rotamer): δ = 8.98 (s, 1H, NC<u>H</u> from Py), 8.66 (s, 1H, NC<u>H</u>' from Py), 8.24 (d, ^3J = 7.8 Hz, 1H, C_qC<u>H</u> from Py), 7.60–7.42 (m, 2H, Ar-H), 7.33–6.90 (m, 9H, Ar-H), 4.69–4.54 (m, 1H, C<u>H</u>CO_2H), 4.10–4.00 (m, 1H, *i*-PrC<u>H</u>), 3.97–3.77 (m, 1H, C<u>H</u>OH), 3.59–3.35 (m, 2H, containing 1 × C<u>H_2</u>-tryptophan and prolyl-C<u>H</u>), 3.25–3.00 (m, 2H, containing 1 × C<u>H_2</u>-tryptophan and 1 × prolyl-C<u>H_2</u>N), 2.99–2.82 (m, 1H, 1 × prolyl-C<u>H_2</u>N), 2.82–2.51 (m, 3H, containing BnC<u>H</u> and PhC<u>H_2</u>), 2.32–2.07 (m, 1H, $(CH_3)_2$C<u>H</u>), 2.04–1.83 (m, 1H, 1 × C<u>H_2</u>CHOH), 1.77–1.65 (m, 1H, 1 × C<u>H_2</u>CHOH), 1.62–1.46 (m, 1H, 1 × CHC<u>H_2</u> from Pro), 1.32–1.12 (m, 1H, 1 × CH_2C<u>H_2</u>CH_2), 1.10–0.77 (m, 7H, containing 1 × CHC<u>H_2</u> from Pro and (C<u>H_3</u>)$_2$CH), 0.74–0.49 (m, 1H, 1 × CH_2C<u>H_2</u>CH_2).

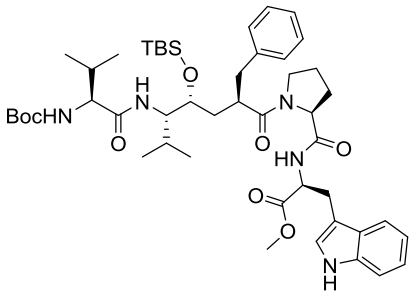
^{13}C NMR (75 MHz, $CDCl_3$, based on HSQC, mixture of 2 rotamers in 2:1 ratio, major rotamer): δ = 177.8 (s, 1C, C_2NC=O), 175.7 (s, 1C, C=O), 173.9 (s, 1C, C=O), 168.8 (s, 1C, C=O), 152.5 (s, 1C, NCH from Py), 149.2 (s, 1C, NCH' from Py), 140.3 (s, 1C, C_q from Ph), 138.1 (s, 1C, HNC_q from indole), 137.3 (s, 1C, C-Ar form Py), 132.6 (s, 1C, C_q from Py), 130.1 (s, 2C, C-Ar from Ph), 129.6 (s, 2C, C-Ar from Ph), 128.5 (s, 1C, C_q from indole), 127.7 (s, 1C, C-Ar from Ph), 125.1 (s, 1C, HNCH from indole), 124.5 (s, 1C, C-Ar from Py), 122.5 (s, 1C, C-Ar indole), 120.0 (s, 1C, C-Ar indole), 119.2 (s, 1C,

C-Ar indole), 112.3 (s, 1C, C-Ar indole), 111.5 (s, 1C, $\underline{C}_qCH_2$ indole), 71.3 (s, 1C, CHOH), 62.1 (s, 1C, prolyl-$\underline{C}$H), 61.4 (s, 1C, *i*-Pr$\underline{C}$HCOH), 55.2 (s, 1C, $\underline{C}HCO_2H$), 47.3 (s, 1C, prolyl-$\underline{C}H_2N$), 45.7 (s, 1C, Bn$\underline{C}$H), 40.8 (s, 1C, Ph$\underline{C}H_2$), 38.0 (s, 1C, BnCH$\underline{C}H_2$), 32.0 (s, 1C, CH$\underline{C}H_2$ from Pro), 29.6 (s, 1C, $(CH_3)_2\underline{C}H$), 27.6 (s, 1C, $\underline{C}H_2$ from Trp), 22.4 (s, 1C, $CH_2\underline{C}H_2CH_2$), 21.1 (s, 1C, 1 × $(\underline{C}H_3)_2CH$), 17.7 (s, 1C, 1 × $(\underline{C}H_3)_2CH$).

HPLC/MS (FAST_POROSHELL120_001HCOOH_8MINGRADIENT.M): t_R = 7.41 min; m/z (ESI+) = 654.3 $[M+H]^+$.

HRMS (MALDI-TOF): Calcd. for $C_{37}H_{43}N_5O_6Na$ $[M+Na]^+$: 676.3111; found: 676.3129.

6.3.3.15 Methyl ((2*R*,4*R*,5*S*)-2-benzyl-5-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-4-((*tert*-butyldimethylsilyl)oxy)-6-methylheptanoyl)-L-prolyl-L-tryptophanate (fully protected *HER*) (28)



28

Boc-deprotection: 429 mg (0.552 mmol, 1.0 eq) compound **23** were dissolved in 2.55 mL (33.1 mmol, 60 eq) ice cold trifluoroacetic acid in a 50 mL round bottom flask with magnetic stirring bar. The ice bath was removed and the brown reaction mixture was stirred for 5 min at RT. The acid was removed under reduced pressure and the crude product dried *in vacuo* overnight.

Peptide coupling: A 10 mL Schlenk flask with magnetic stirring bar was dried under vacuum with a heat gun and purged with N_2. 144 mg (0.662 mmol, 1.2 eq) Boc-Val-OH were dissolved in 2.75 mL abs. DMF and 96.4 µL (0.552 mmol, 1.0 eq) DIPEA were added to the stirred solution. After cooling to 0 °C (ice bath) 252 mg (0.662 mmol, 1.2 eq) HATU were added in one portion and the yellow solution was stirred for 2 min. In a second 10 mL round bottom flask with Schlenk adaptor the previously deprotected crude compound was dissolved in 2.75 mL abs. DMF and 386 µL (2.21 mmol, 4.0 eq) DIPEA were added. After cooling to 0 °C (ice bath) the preactivated Boc-Val-OH solution was added with a syringe. After 5 min the ice bath was removed and the reaction mixture was stirred at RT for 45 min. The mixture was quenched by the addition of 5.5 mL brine and extracted with EtOAc (3×20 mL). The combined organic layers were

washed with with a 1:1 mixture of H_2O/brine (6×20 mL) and brine (2×10 mL), dried over Na_2SO_4 and filtered. The product was concentrated under reduced pressure and dried *in vacuo*. Purification via flash chromatography (54 g SiO_2; cyclohexane/EtOAc = 1:1 v/v), followed by H_2O washing (5×10 mL; product dissolved in 60 mL EtOAc) to remove remaining tetramethylurea, provided compound **28** as a white solid.

Yield: 309 mg (353 µmol, 64 %), white solid.

$C_{48}H_{73}N_5O_8Si$ [876.22 g/mol].

mp = 82–88 °C.

$[\alpha]_D^{23}$ = –3.5 (c = 0.47, $CHCl_3$).

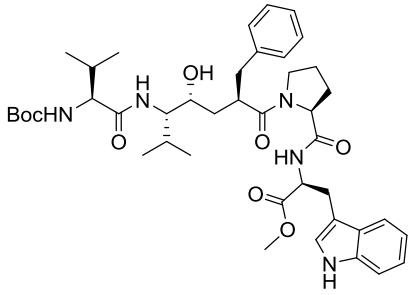
R_f = 0.29 (cyclohexane/EtOAc = 1:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$, based on COSY and HSQC): δ = 8.73 (bs, 1H, NH indole), 7.49 (d, ^3J = 7.6 Hz, 1H, Ar-H), 7.39–6.90 (m, 10H, Ar-H and amide-NH from Trp), 6.21 (d, ^3J = 8.6 Hz, 1H, *i*-PrCHNH), 5.00 (d, ^3J = 9.1 Hz, 1H, NH carbamate), 4.82–4.71 (m, 1H, CHCO$_2$), 4.52 (d, ^3J = 8.6 Hz, 1H, prolyl-CH), 3.92–3.80 (m, 1H, HNCH from Val), 3.79–3.69 (m, 2H, containing *i*-PrCH, and SiOCH), 3.66 (s, 3H, CO$_2$CH$_3$), 3.57–3.40 (m, 1H, 1 × prolyl-CH$_2$N), 3.27 (d, ^3J = 5.6 Hz, 2H, CH$_2$-tryptophan), 3.14–2.98 (m, 1H, 1 × prolyl-CH$_2$N), 2.82–2.61 (m, 2H, containing BnCH and 1 × PhCH$_2$), 2.54–2.38 (m, 1H, 1 × PhCH$_2$), 2.28–2.15 (m, 1H, 1 × CHCH$_2$ from Pro), 2.15–2.02 (m, 1H, (CH$_3$)$_2$CH from Val), 1.95–1.71 (m, 4H, containing 1 × SiOCHCH$_2$, 1 × CHCH$_2$ from Pro and CH$_2$CH$_2$CH$_2$), 1.70–1.51 (m, 2H, containing 1 × SiOCHCH$_2$, and (CH$_3$)$_2$CHCHCOSi), 1.44 (s, 9H, H-Boc), 1.01–0.71 (m, 21H, containing (CH$_3$)$_2$CHCOSi, (CH$_3$)$_3$CSi and (CH$_3$)$_2$CH from Val), 0.10 (s, 3H, SiCH$_3$), 0.06 (s, 3H, SiCH$_3$').

^{13}C NMR (75 MHz, $CDCl_3$, based on HSQC): δ = 174.8 (s, 1C, C$_2$NC=O), 172.4 (s, 1C, C=O), 171.5 (s, 1C, C=O), 171.1 (s, 1C, C=O), 156.2 (s, 1C, C=O carbamate), 139.1 (s, 1C, C$_q$ from Ph), 136.3 (s, 1C, HNC$_q$ from indole), 129.0 (s, 2C, C-Ar from Ph), 128.6 (s, 2C, C-Ar from Ph), 127.7 (s, 1C, C$_q$ from indole), 126.6 (s, 1C, C-Ar from Ph), 123.8 (s, 1C, HNCH from indole), 122.0 (s, 1C, C-Ar indole), 119.4 (s, 1C, C-Ar indole), 118.6 (s, 1C, C-Ar indole), 111.4 (s, 1C, C-Ar indole), 109.8 (s, 1C, C$_q$CH$_2$ indole), 80.1 (s, 1C, Me$_3$CO), 71.7 (s, 1C, SiOCH), 60.5 (s, 1C, HNCH from Val), 60.0 (s, 1C, prolyl-CH), 57.1 (s, 1C, *i*-PrCHCOSi), 53.3 (s, 1C, CHCO$_2$), 52.4 (s, 1C, CO$_2$CH$_3$), 47.2 (s, 1C, prolyl-CH$_2$N), 41.8 (s, 1C, BnCH), 38.7 (s, 1C, PhCH$_2$), 34.6 (s, 1C, BnCHCH$_2$), 30.6 (s, 1C, (CH$_3$)$_2$CH from Val), 28.4 (s, 4C, (CH$_3$)$_3$CO and (CH$_3$)$_2$CHCHCOSi), 27.5 (s, 2C, CH$_2$ from Trp and CHCH$_2$ from Pro), 26.0 (s, 3C, (CH$_3$)$_3$CSi), 24.9 (s, 1C, CH$_2$CH$_2$CH$_2$), 21.2 (s, 1C, 1 × (CH$_3$)$_2$CHCHCOSi), 19.7 (s, 1C, 1 × (CH$_3$)$_2$CH from Val), 18.9 (s, 1C, 1 × (CH$_3$)$_2$CHCHCOSi), 18.1 (s, 1C, 1 × (CH$_3$)$_2$CH from Val), 17.8 (s, 1C, Me$_3$CSi), –3.9 (s, 1C, SiCH$_3$), –4.6 (s, 1C, SiCH$_3$').

HRMS (MALDI-TOF): Calcd. for $C_{48}H_{73}N_5O_8SiNa$ $[M+Na]^+$: 898.5126; found: 898.5116.

6.3.3.16 Methyl ((2*R*,4*R*,5*S*)-2-benzyl-5-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-4-hydroxy-6-methylheptanoyl)-L-prolyl-L-tryptophanate (protected *HER*) (29)[49,101]



29

309 mg (353 µmol, 1.0 eq) compound **28** were dissolved in 3.5 mL THF in a 15 mL polypropylene vial with magnetic stirring bar. 917 µL (35.3 mmol, 100 eq) HF/pyridine (70 % HF) were added to the solution and the reaction mixture was stirred at RT for 25 min. Since no full conversion could be observed, additional 917 µL (35.3 mmol, 100 eq) HF/pyridine were added at RT and the mixture was stirred for additional 20 min. The reaction was quenched by pouring into 60 mL ice cold satd. $NaHCO_3$ solution and the product was extracted with EtOAc (2×60 mL). The combined organic layers were washed with H_2O (30 mL) and brine (30 mL), dried over Na_2SO_4 and filtered. The solvents were removed under reduced pressure and the product was dried *in vacuo*. Purification via flash chromatography (24 g SiO_2; CH_2Cl_2/MeOH = 20:1 v/v) provided compound **29** as an off-white solid.

Yield: 96 mg (0.13 mmol, 36 %), white solid.

$C_{42}H_{59}N_5O_8$ [761.96 g/mol].

mp = 93–96 °C.

$[\alpha]_D^{23}$ = –25.7 (c = 1.56, $CHCl_3$).

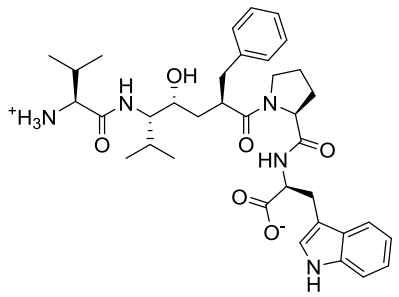
R_f = 0.20 (CH_2Cl_2/MeOH = 20:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$, mixture of 2 rotamers in 4:1 ratio, major rotamer): δ = 9.03 (bs, 1H, NH indole), 7.48 (d, ^3J = 7.6 Hz, 1H, Ar-H), 7.38–6.86 (m, 10H, Ar-H and amide-NH from Trp), 6.07 (d, ^3J = 9.7 Hz, 1H, HOCHCHNH), 5.02 (d, ^3J = 8.7 Hz, 1H, NH carbamate), 4.69–4.55 (m, 1H, CHCO$_2$), 3.93–3.80 (m, 1H, *i*-PrCH from Val), 3.79–3.53 (m, 5H, containing *i*-PrCH, CHOH and CO_2CH_3), 3.43–3.13 (m, 3H, containing CH_2-tryptophan and prolyl-CH), 3.10–2.95 (m, 1H, 1 × prolyl-CH_2N), 2.87–2.72 (m, 1H, 1 × prolyl-CH_2N), 2.70–2.52 (m, 2H, PhCH_2), 2.51–2.34 (m, 1H, BnCH), 2.30–

1.59 (m, 5H, containing $(CH_3)_2C\underline{H}$), $(CH_3)_2C\underline{H}$'), $C\underline{H_2}CHOH$) and 1 × $CHC\underline{H_2}$ from Pro), 1.47–1.36 (m, 9H, H-Boc), 1.35–1.25 (m, 1H, 1 × $CH_2C\underline{H_2}CH_2$), 1.08–0.70 (m, 14H, containing 1 × $CHC\underline{H_2}$ from Pro, 1 × $CH_2C\underline{H_2}CH_2$, $(C\underline{H_3})_2CH$ and $(C\underline{H_3})_2CH$').

^{13}C NMR (75 MHz, $CDCl_3$, mixture of 2 rotamers in 4:1 ratio, major rotamer): δ = 175.3 (s, 1C, $C_2NC{=}O$), 172.8 (s, 1C, C=O), 172.7 (s, 1C, C=O), 172.4 (s, 1C, C=O), 156.2 (s, 1C, C=O carbamate), 138.8 (s, 1C, C_q from Ph), 136.3 (s, 1C, HNC_q from indole), 129.2 (s, 2C, C-Ar from Ph), 128.6 (s, 2C, C-Ar from Ph), 127.6 (s, 1C, C_q from indole), 126.8 (s, 1C, C-Ar from Ph), 123.3 (s, 1C, HNCH from indole), 122.3 (s, 1C, C-Ar indole), 119.9 (s, 1C, C-Ar indole), 118.0 (s, 1C, C-Ar indole), 111.8 (s, 1C, C-Ar indole), 109.9 (s, 1C, $\underline{C_q}CH_2$ indole), 80.4 (s, 1C, $Me_3\underline{C}O$), 71.0 (s, 1C, SiOCH), 61.2 (s, 1C, HN$\underline{C}$H from Val), 60.7 (s, 1C, prolyl-$\underline{C}$H), 59.2 (s, 1C, *i*-Pr$\underline{C}$HCOH), 53.7 (s, 1C, $\underline{C}HCO_2$), 52.6 (s, 1C, $CO_2\underline{C}H_3$), 46.2 (s, 1C, prolyl-$\underline{C}H_2N$), 43.9 (s, 1C, Bn$\underline{C}$H), 39.9 (s, 1C, Ph$\underline{C}H_2$), 37.1 (s, 1C, BnCH$\underline{C}H_2$), 31.0 (s, 1C, $CH\underline{C}H_2$ from Pro), 30.0 (s, 1C, $(CH_3)_2\underline{C}$H from Val), 28.5 (s, 3C, $(\underline{C}H_3)_3CO$), 28.1 (s, 1C, $(CH_3)_2\underline{C}HCHCOH$), 26.2 (s, 1C, $\underline{C}H_2$ from Trp), 21.8 (s, 1C, $CH_2\underline{C}H_2CH_2$), 20.6 (s, 1C, 1 × $(\underline{C}H_3)_2CHCHCOH$), 20.0 (s, 1C, 1 × $(\underline{C}H_3)_2$CH from Val), 18.2 (s, 1C, 1 × $(\underline{C}H_3)_2CHCHCOH$), 16.8 (s, 1C, 1 × $(\underline{C}H_3)_2$CH from Val).

6.3.3.17 ((2*R*,4*R*,5*S*)-5-((*S*)-2-Ammonio-3-methylbutanamido)-2-benzyl-4-hydroxy-6-methylheptanoyl)-L-prolyl-L-tryptophanate (*HER*) (30)



30

Saponification: 94.8 mg (124 µmol, 1.0 eq) compound **29** were dissolved in 0.4 mL THF in a 10 mL round bottom flask with magnetic stirring bar and a solution of 20.9 mg (498 µmol, 4.0 eq) $LiOH{\times}H_2O$ in 0.6 mL H_2O was added under vigorous stirring at RT. After 50 min the reaction mixture was carefully acidified with 1 M HCl to pH=5. The solvents were removed under reduced pressure and the yellow solid mass was dried *in vacuo*.

Boc-deprotection: The reaction was carried out in two equal batches. The crude saponified product and 38 µL (0.50 mmol, 4.0 eq) ethanethiol were dissolved in 1.2 mL 2,2,2-trifluoroethanol in a 5 mL glass vial with magnetic stirring bar. 224 mg (996 µmol, 8.0 eq) zinc(II) bromide were added and the turbid yellowish solution was stirred at RT for

7 h. The solvent was evaporated and the product was purified by preparative HPLC (*JKV_NucleodurC18_001HCOOH_10to85*) to yield compound **30** as a white powder.

Yield: 35 mg (54 µmol, 44 %), white powder.

$C_{36}H_{49}N_5O_6$ [647.82 g/mol].

mp = 148–152 °C.

$[\alpha]_D^{23}$ = –20.8 (c = 1.54, MeOH).

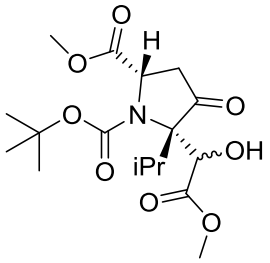
^{1}H NMR (300 MHz, $CDCl_3$, based on COSY and HSQC, mixture of 2 rotamers in 3:1 ratio, major rotamer): δ = 8.41 (bs, 1H, NH indole), 7.54 (d, ^3J = 7.7 Hz, 1H, Ar-H), 7.32–6.86 (m, 9H, Ar-H), 4.60–4.47 (m, 1H, CHCO$_2^-$), 3.83–3.59 (m, 3H, containing H_3N^+CH, *i*-PrCH and CHOH), 3.45–3.30 (m, 2H, containing 1 × CH_2-tryptophan and prolyl-CH), 3.21–3.09 (m, 1H, 1 × CH_2-tryptophan), 3.09–2.95 (m, 1H, 1 × prolyl-CH_2N), 2.95–2.79 (m, 1H, 1 × prolyl-CH_2N), 2.77–2.45 (m, 3H, containing BnCH and $PhCH_2$), 2.30–2.17 (m, 1H, $(CH_3)_2CH$), 2.17–2.04 (m, 1H, $(CH_3)_2CH$'), 2.03–1.88 (m, 1H, 1 × CH_2CHOH), 1.67–1.53 (m, 1H, 1 × CH_2CHOH), 1.53–1.39 (m, 1H, 1 × $CHCH_2$ from Pro), 1.20–0.74 (m, 14H, containing 1 × $CHCH_2$ from Pro, 1 × $CH_2CH_2CH_2$, $(CH_3)_2CH$ and $(CH_3)_2CH$'), 0.73–0.54 (m, 1H, 1 × $CH_2CH_2CH_2$).

^{13}C NMR (75 MHz, $CDCl_3$, based on HSQC, mixture of 2 rotamers in 3:1 ratio, major rotamer): δ = 177.7 (s, 2C, C=O), 173.3 (s, 1C, C=O), 170.4 (s, 1C, C=O), 140.2 (s, 1C, C_q from Ph), 138.0 (s, 1C, HNC_q from indole), 130.1 (s, 2C, C-Ar from Ph), 129.6 (s, 2C, C-Ar from Ph), 129.0 (s, 1C, C_q from indole), 127.7 (s, 1C, C-Ar from Ph), 124.3 (s, 1C, HNCH from indole), 122.4 (s, 1C, C-Ar indole), 119.8 (s, 1C, C-Ar indole), 119.4 (s, 1C, C-Ar indole), 112.2 (s, 1C, C_qCH_2 indole), 112.0 (s, 1C, C-Ar indole), 71.1 (s, 1C, CHOH), 62.2 (s, 1C, prolyl-CH), 61.2 (s, 1C, *i*-PrCHCOH), 60.2 (s, 1C, H_3N^+CH), 56.6 (s, 1C, $CHCO_2^-$), 47.3 (s, 1C, prolyl-CH_2N), 45.4 (s, 1C, BnCH), 40.7 (s, 1C, $PhCH_2$), 37.7 (s, 1C, $BnCHCH_2$), 31.9 (s, 1C, $CHCH_2$ from Pro), 31.6 (s, 1C, $(CH_3)_2CH$ from Val), 29.3 (s, 1C, $(CH_3)_2CHCHCOH$), 28.3 (s, 1C, CH_2 from Trp), 22.4 (s, 1C, $CH_2CH_2CH_2$), 21.0 (s, 1C, 1 × $(CH_3)_2CHCHCOH$), 19.6 (s, 1C, 1 × $(CH_3)_2CH$ from Val), 18.1 (s, 1C, 1 × $(CH_3)_2CHCHCOH$), 17.5 (s, 1C, 1 × $(CH_3)_2CH$ from Val).

HPLC/MS (FAST_POROSHELL120_001HCOOH_8MINGRADIENT.M): t_R = 6.73 min; m/z (ESI+) = 648.4 $[M+H]^+$.

HRMS (MALDI-TOF): Calcd. for $C_{36}H_{49}N_5O_6Na$ $[M+Na]^+$: 670.3580; found: 670.3586.

6.3.3.18 1-(*tert*-Butyl) 2-methyl (2*S*,5*S*)-5-(1-hydroxy-2-methoxy-2-oxo-ethyl)-5-isopropyl-4-oxopyrrolidine-1,2-dicarboxylate (31)



31

A 1000 mL three-necked round bottom flask with magnetic stirring bar was dried (evacuated, heated, N_2-purged) and equipped with a gas inlet and a thermometer. The flask was charged with a solution of 10.9 g (33.7 mmol, 1.0 eq) ketophosphonate **15** in 337 mL abs. THF and cooled to 0 °C (ice bath). 2.70 g (67.4 mmol, 2.0 eq) NaH (60 % dispersion in mineral oil) were added in small portions under a slight stream of N_2 over a period of 20 min and the reaction mixture was additionally stirred for 25 min at 0 °C. After cooling to –78 °C (dry ice/acetone bath) a –78 °C cold solution of the freshly prepared aldehyde **16** in 84 mL abs. THF was added with a cannula and the yellow mixture was stirred for 30 min at –78 °C. The flask was warmed up to –7 °C and the mixture stirred for 24 h at that temperature (cryostat). The reaction mixture was quenched by slow addition of 3.4 mL glacial acetic acid in 5 mL THF over a period of 1 h at –7 °C, warmed to RT and the solvent was removed under reduced pressure. The oily residue was partitioned between EtOAc (300 mL) and H_2O (100 mL). The layers were separated and the organic phase washed with H_2O (100 mL), satd. $NaHCO_3$ (100 mL) and brine (100 mL), dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure and the resulting yellow oil dried *in vacuo*. Purification via flash chromatography (590 g SiO_2; cyclohexane/EtOAc = 4:1→1:1 v/v) provided compound **31** as a pale yellow sticky gum which solidified after drying under high vacuum for several days.

Yield: 3.61 g (9.68 mmol, 29 %), pale yellow solid.

$C_{17}H_{27}NO_8$ [373.40 g/mol].

mp = 77–83 °C.

$[\alpha]_D^{23}$ = –0.2 (c = 1.34, $CHCl_3$).

R_f = 0.60 (cyclohexane/EtOAc = 1:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$, mixture of diastereomers in 12:1 ratio, major diastereomer): δ = 4.98–4.82 (m, 2H, containing NC<u>H</u> and O<u>H</u>), 4.74 (s, 1H, C<u>H</u>OH), 3.72 (s,

6H, 2 × $CO_2C\underline{H}_3$), 2.86 (dd, ^2J = 16.4 Hz, ^3J = 3.3 Hz, 1H, 1 × $C\underline{H}_2$), 2.61 (dd, ^2J = 16.5 Hz, ^3J = 8.4 Hz, 1H, 1 × $C\underline{H}_2$), 1.38 (s, 9H, H-Boc), 1.08–0.88 (m, 6H, $(C\underline{H}_3)_2CH$).

^{13}C NMR (75 MHz, $CDCl_3$, mixture of diastereomers in 12:1 ratio, major diastereomer): δ = 210.0 (s, 1C, $H_2C\underline{C}$=O), 170.3 (s, 1C, C=O), 169.9 (s, 1C, C=O), 155.2 (s, 1C, C=O carbamate), 80.8 (s, 1C, $Me_3\underline{C}$), 79.1 (s, 1C, HOCH), 75.8 (s, 1C, NCH), 66.4 (s, 1C, NC_q), 52.4 (s, 1C, $CO_2\underline{C}H_3$), 52.3 (s, 1C, $CO_2\underline{C}H_3$'), 35.5 (s, 1C, CH_2), 34.0 (s, 1C, $(CH_3)_2\underline{C}H$), 28.3 (s, 3C, $(\underline{C}H_3)_3CO$), 18.0 (s, 1C, $(\underline{C}H_3)_2CH$), 16.7 (s, 1C, $(\underline{C}H_3)_2CH$').

HRMS (MALDI-TOF): Calcd. for $C_{17}H_{27}NO_8Na$ $[M+Na]^+$: 396.1634; found: 396.1650.

6.4 Biological assays

All biological assays were performed by Shalinee Jha at the Institute of Biochemistry, Graz University of Technology, in the research group of Prof. Peter Macheroux. The following two protocols were used:

6.4.1 Isothermal microcalorimetry

The wild type recombinant hDPP III expressed in *E. coli* was used for the microcalorimetric analysis. The titrations were performed in a buffer with pH = 8.0 containing 50 mM Tris-HCl and 100 mM NaCl. Both the purified enzyme and ligand were dissolved in exactly the same buffer, and all solutions were degassed using an ultrasonic bath immediately before the measurements. The measurements were performed on VP-ITC microcalorimeter (MicroCal, Northampton, MA, USA).

In each measurement run the temperature was equilibrated at 298 K. A 500 µM solution of ligand in the syringe was titrated into a 20 µM solution of hDPP III in the measurement cell. In a typical experiment, under constant stirring at 270 rpm, a total of one aliquot of 2 µL and 29 aliquots of 10 µL of the ligand solution were injected at a rate of 0.5 µL/s into 1.421 mL of the enzyme solution. Every injection was carried out over a period of 20 s with a spacing of 300 s between the injections. The heats of binding were determined by integration of the observed peaks. The heat values were plotted against the ratio of peptide vs. protein concentration in the cell to generate the binding isotherm. Nonlinear least-squares fitting using Origin® version 7.0 (MicroCal®) was used to obtain association constants (K_a), heats of binding (ΔH) and stoichiometries. Dissociation constant (K_d) values were calculated according to the simple reciprocal equation:

$$K_d = \frac{1}{K_a}$$

6.4.2 Fluorescence-based inhibition assays

The enzyme activity of hDPP III was measured by fluorometrically measuring (excitation, 332 nm; emission, 420 nm) the liberation of 2-naphthylamine at 37 °C in a mixture containing 25 µL of 200 µM Arg-Arg-2-naphthylamide as substrate in 50 mM Tris-HCl buffer, pH 8.0, 0.05-0.1 µM of enzyme in a total reaction mixture of 235 µL (White, Tissue Culture treated Krystal 2000 96-well plate from Porvair sciences, Norfolk, UK). The activity assay was performed by continuous measurement of fluorescence of 2-naphthylamide for 30 min (Fluorescent plate reader from Molecular Devices, Sunnyvale CA, USA). For inhibition assay, the inhibitors were added to the mixture without the substrate and incubated for 10 min at room temperature. The reaction was started by the addition of the substrate.

The concentration of an inhibitor that gave 50 % inhibition (IC_{50}) was determined through a series of assays with a fixed substrate concentration but with various inhibitor concentrations. Percent activity in the presence of increasing concentrations of inhibitor was calculated:

$$\text{Percent activity} = 100 \times (\Delta\text{fluorescence} / \Delta\text{fluorescence of control})$$

Percent activity against concentration of inhibitor (log scale for inhibitor concentration (x-axis) and linear scale for percent activity (y-axis)) was plotted. Percent activity vs. log of concentration was fitted to a sigmoidal dose-response curve using the four parameter logistic equation entitled “log (inhibitor) vs. response – Variable slope” in GraphPad Prism.

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Appendix A: Abbreviations

$[\alpha]_D^{24}$	specific rotation
Å	Ångström (10^{-10} m)
AAs	amino acids
abs.	absolute
Ac	acetyl
ACE	angiotensin-converting enzyme
AcOH	acetic acid
AIDS	acquired immune deficiency syndrome
APT	attached proton test
Ar	aryl
B_3	niacin (vitamin B_3)
Bn	benzyl
βNA	2-naphthylamide
Boc	*tert*-butoxycarbonyl
BoNT	botulinum neurotoxin
bs	broad singlet
Bu	butyl
°C	degree Celsius
c	concentration
calcd.	calculated
Cbz	benzyloxycarbonyl
CCD	charge-coupled device
CDI	1,1'-carbonyldiimidazole
CGRP	calcitonin gene-related peptide
cm	centimeter
COSY	correlation spectroscopy
C_q	quaternary carbon

δ	delta (chemical shift in ppm)
d	day(s) or doublet
DAP III	dipeptidyl-aminopeptidase III (DPP III)
DCM	dichloromethane
dd	doublet of doublet
de	diastereomeric excess
DIBAL-H	diisobutylaluminum hydride
DIPEA	*N*,*N*-diisopropylethylamine (Hünig's base)
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DPP III	dipeptidyl-peptidase III
DRG	dorsal root ganglia
E451A	glutamate-451 exchanged by alanine
EDTA	ethylenediaminetetraacetic acid
ee	enantiomeric excess
e.g.	*exempli gratia*
EI	electron impact
eq	equivalent(s)
ESI	electrospray ionization
Et	ethyl
Et_2O	diethyl ether
EtOAc	ethyl acetate (ethyl ethanoate)
EtOH	ethanol
eV	electron volt
FID	flame ionization detector
g	gram
GC	gas chromatography
h	hour(s)
HATU	1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate

HBTU	*O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
hDPP III	human dipeptidyl-peptidase III
HER	(*R*)-hydroxyethylene pseudopeptide
HIV	human immunodeficiency virus
HOAt	1-hydroxy-7-azabenzotriazole (3-hydroxytriazolo[4,5-*b*]pyridine)
HOBt	hydroxybenzotriazole (benzotriazol-1-ol)
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
HSQC	heteronuclear single quantum coherence
HWE	Horner-Wadsworth-Emmons (reaction)
Hz	Hertz
IC_{50}	half maximal inhibitory concentration
IL	interleukin
i-Pr	isopropyl
i-Pr_2NEt	*N*,*N*-diisopropylethylamine (Hünig's base)
ITC	isothermal titration calorimetry
J	coupling constant
K_a	association constant
K_d	dissociation constant
K_i	inhibitory constant
λ	lambda (wavelength)
LC	liquid chromatography
LDA	lithium diisopropylamide
lit.	literature
M	molar (mol/L)
m	meter or multiplet
MALDI	matrix-assisted laser desorption/ionization
mbar	millibar
Me	methyl

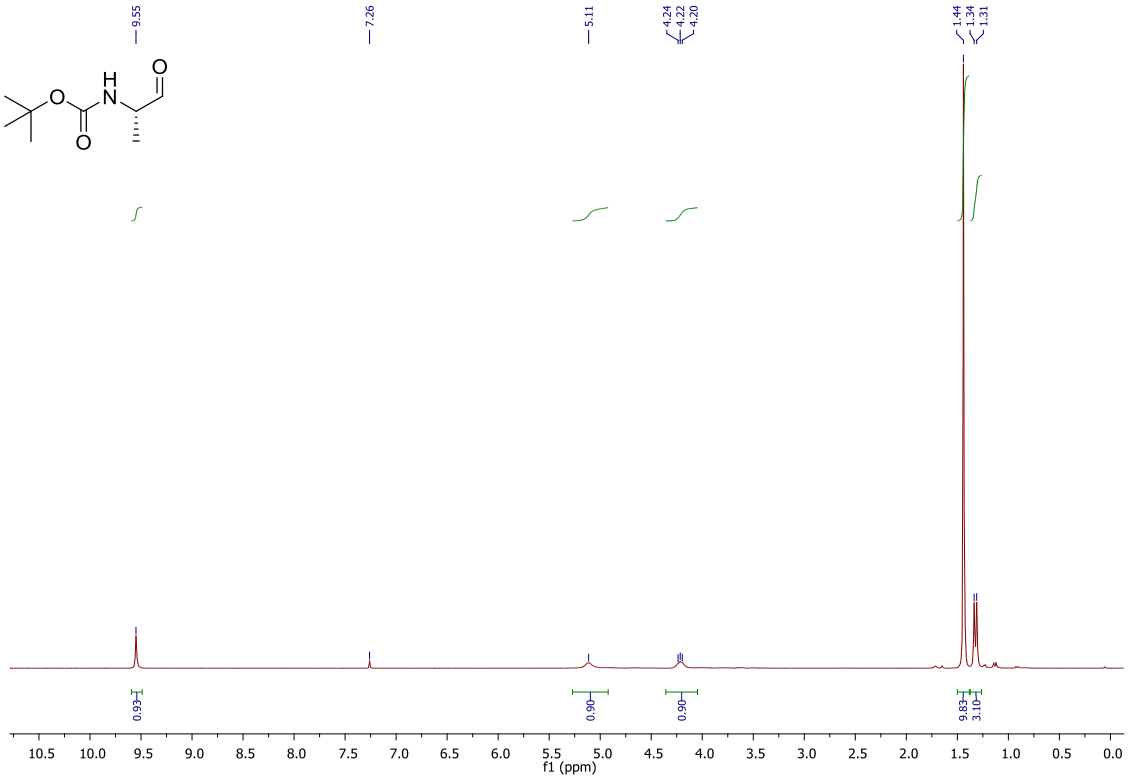
MeCN	acetonitrile
MeOH	methanol
methanol-d_4	deuterated methanol
mg	milligram
MHz	megahertz
min	minute(s)
mL	milliliter
µL	microliter
µm	micrometer
mmol	millimole
mol	mole
mol/L	mole(s) per liter
mp	melting point
MS	mass spectrometry
MWD	multiple wavelength detector
m/z	mass/charge
N.A.	not analysed
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NF-E2	nuclear factor erythroid-derived 2
nm	nanometer
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
n-BuLi	*n*-butyllithium
ORTEP	Oak Ridge Thermal Ellipsoid Plot
p-TsOH	*para*-toluenesulfonic acid (4-methylbenzenesulfonic acid)
PAG	periaqueductal grey
*p*CMB	*p*-chloromercuribenzoic acid
*p*CMS	*p*-chloromercuriphenylsulfonate
PEG	polyethylene glycol
Ph	phenyl

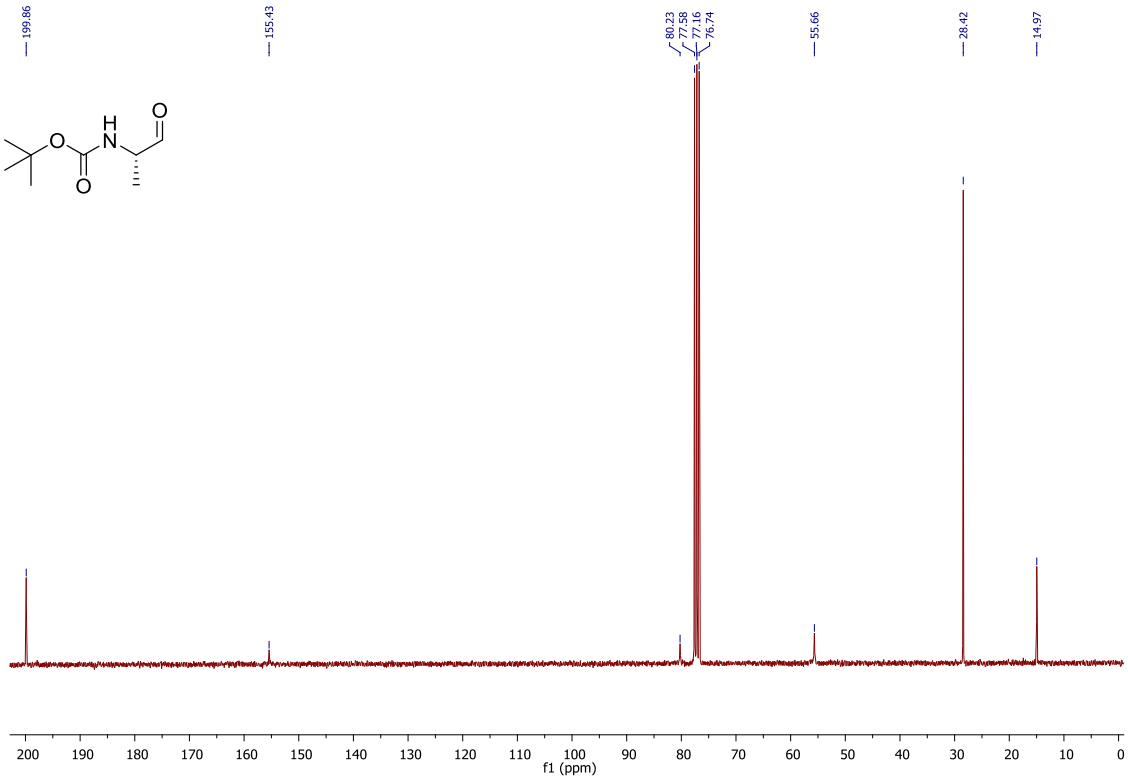
Phg	phenylglycine
*p*HMB	*p*-hydroxymercuribenzoate
ppm	parts per million
PDB	protein data bank
Py	pyridyl
rDPP III	rat dipeptidyl peptidase III
R_f	retardation factor (retention factor)
RGSA	(*R*)-γ-siloxy acid
RP	reversed phase
RT	room temperature
RVM	rostral ventromedial medulla
s	singlet
satd.	saturated
SHE	(*S*)-hydroxyethylene pseudopeptide
SiO_2	silica gel
SP	substance P
sp.	species
t	triplet
TBAF	tetrabutylammonium fluoride
TBS	*tert*-butyldimethylsilyl
TBSCl	*tert*-butyldimethylsilyl chloride
TBTU	*O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate
tert	tertiary
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TNFα	tumour necrosis factor-alpha
TOP	thimet oligopeptidase
TPP II	tripeptidyl-peptidase II
t_R	retention time

TRPV	transient receptor potential subfamily V member 1 receptor
TOF	time-of-flight (mass spectrometry)
UV	ultraviolet
v/v	volume to volume ratio
w/w	weight to weight ratio
XRD	X-ray diffraction

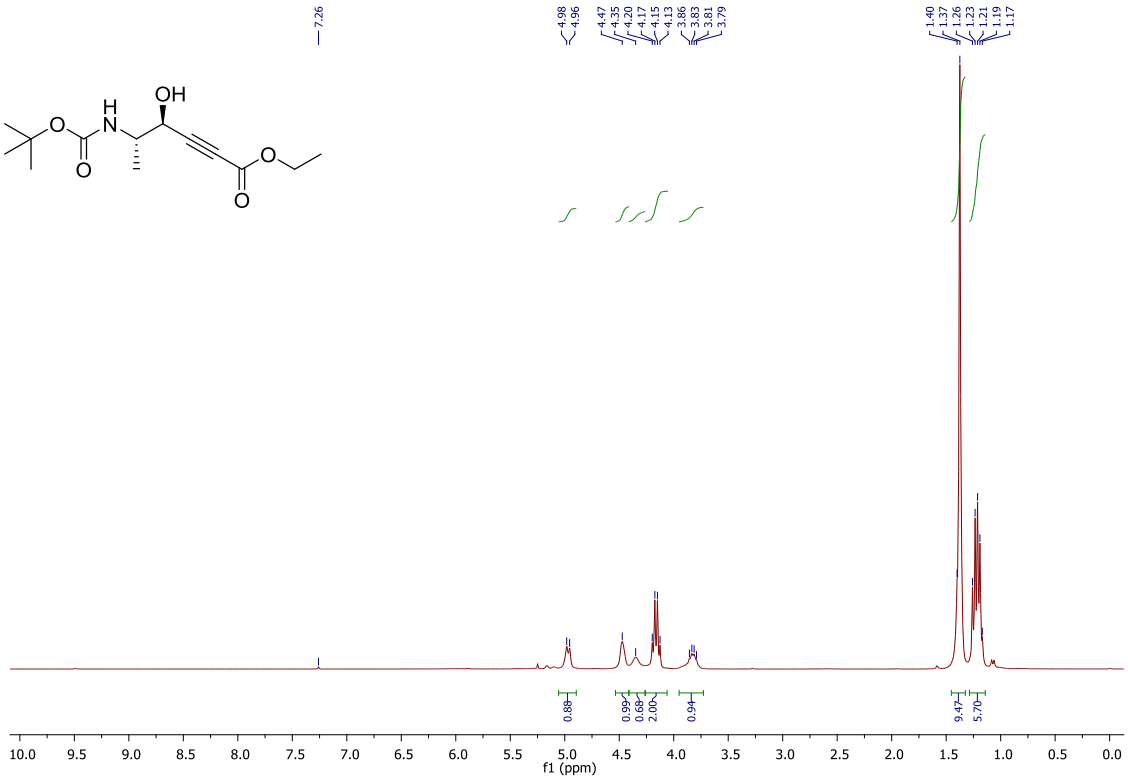
Appendix B: NMR Spectra

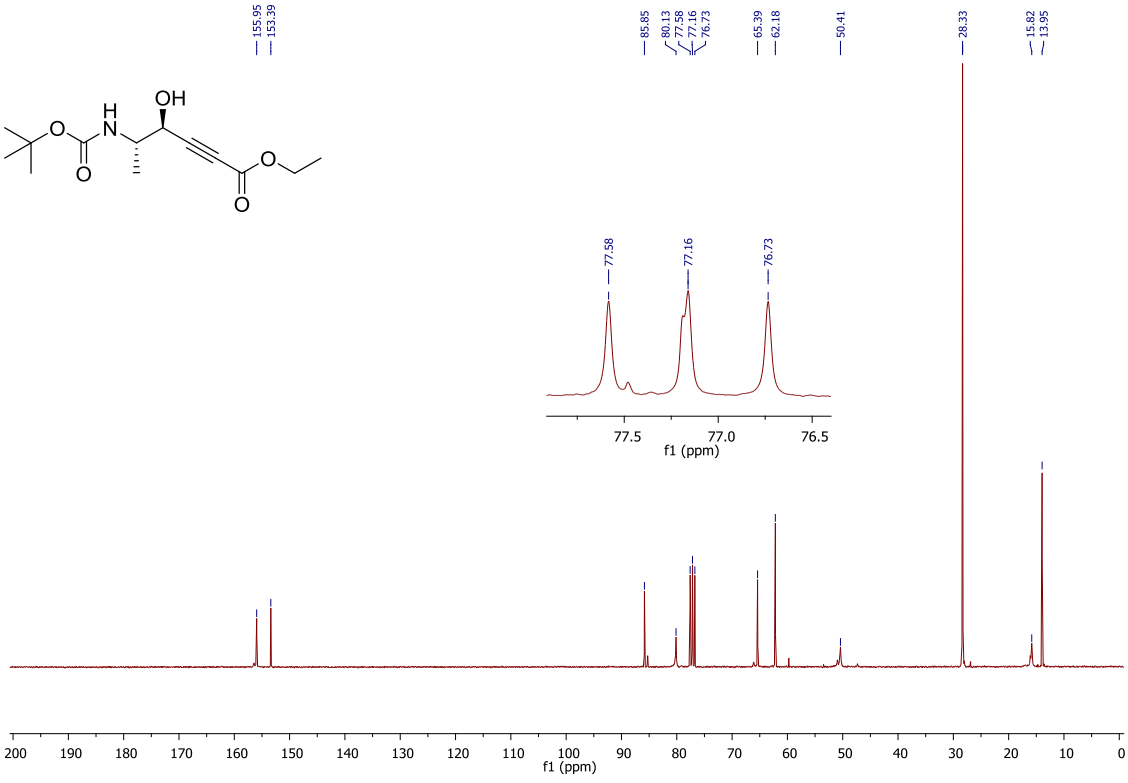
^{1}H and ^{13}C NMR spectra of Boc-L-alaninal (1)



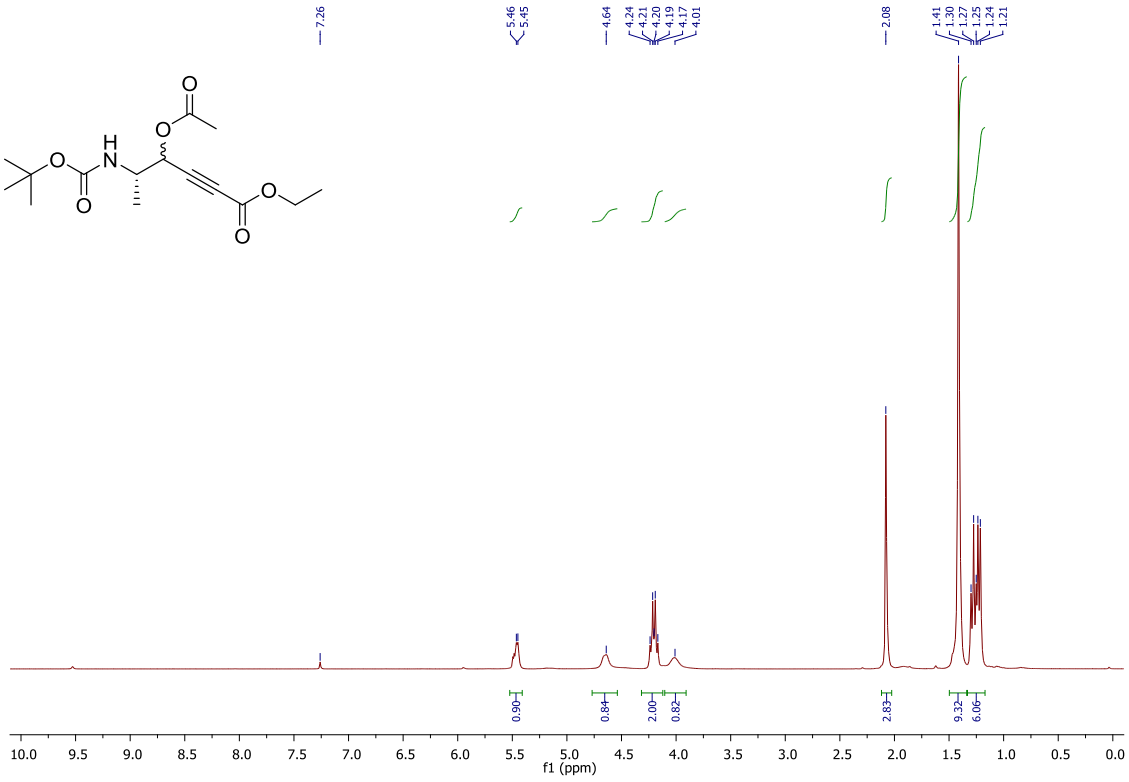


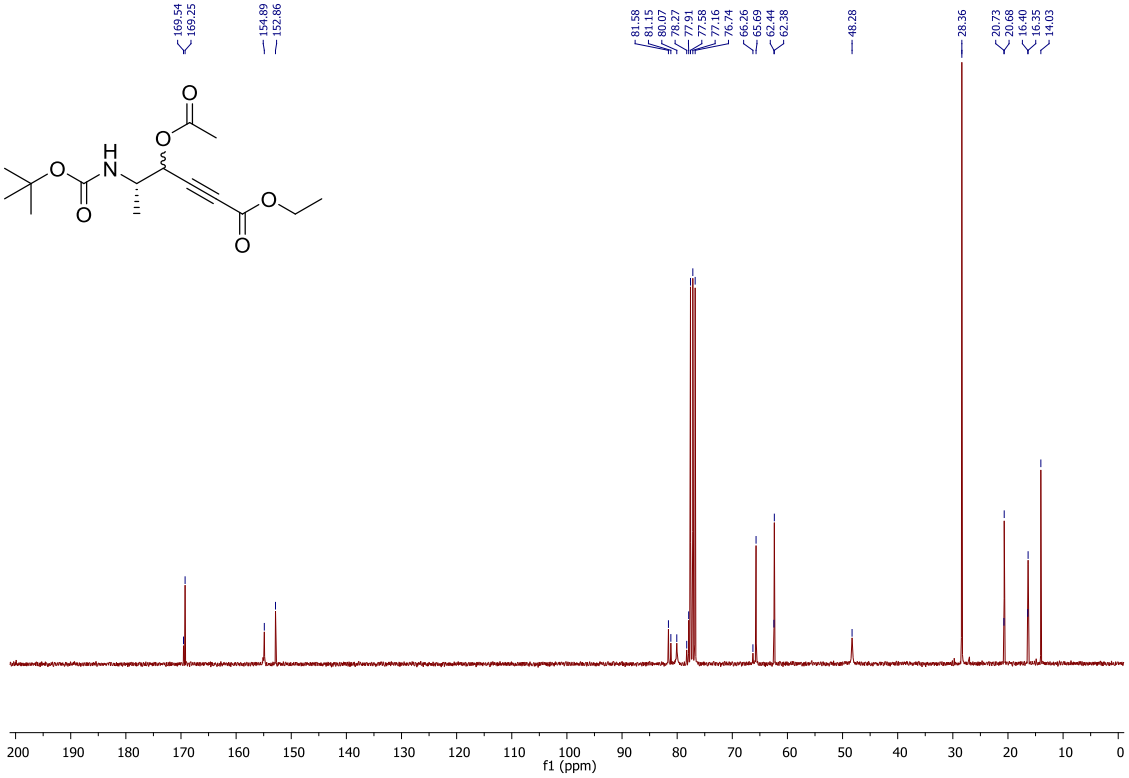
^{1}H and ^{13}C NMR spectra of Ethyl (4*S*,5*S*)-5-((*tert*-butoxycarbonyl)amino)-4-hydroxyhex-2-ynoate (2a)



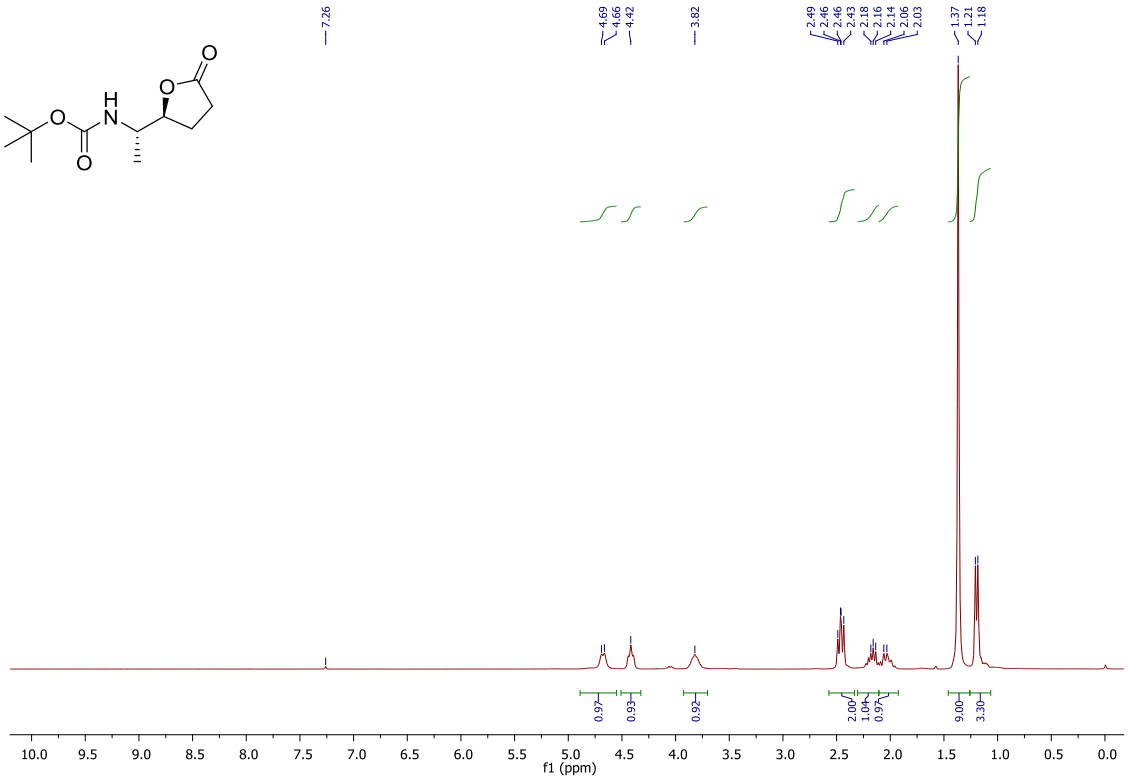


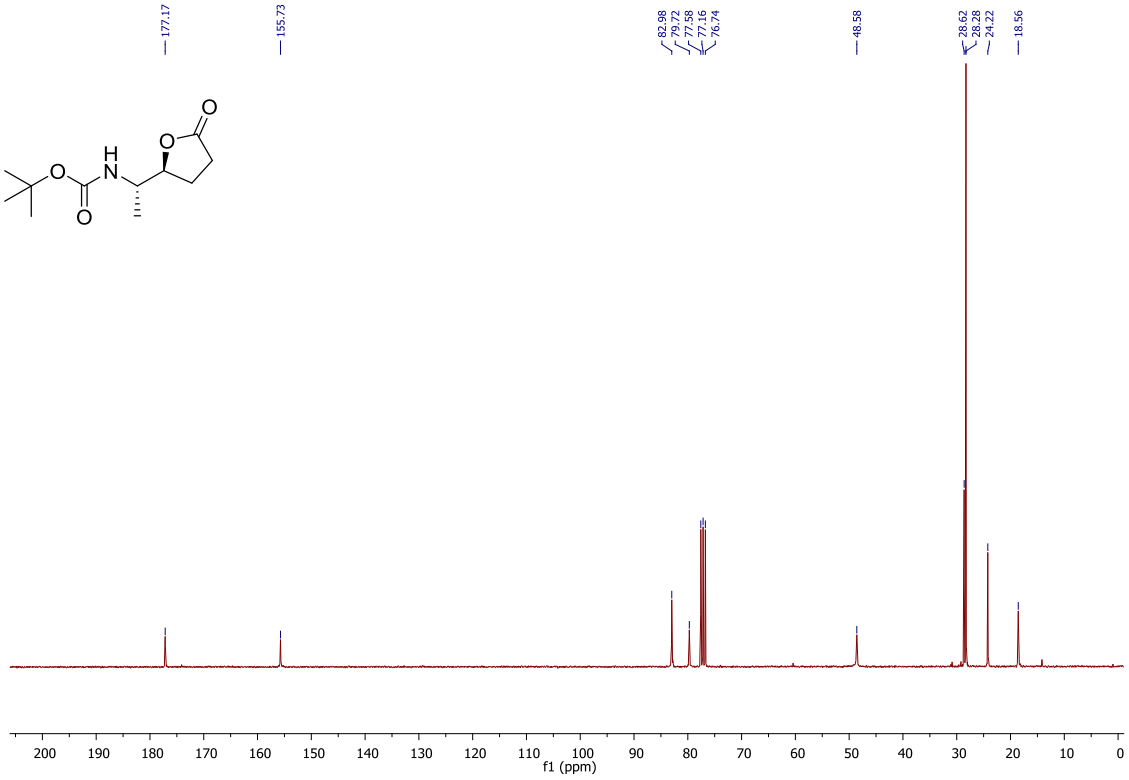
^{1}H and ^{13}C NMR spectra of Ethyl (5*S*)-4-acetoxy-5-((*tert*-butoxycarbonyl)-amino)hex-2-ynoate (3)



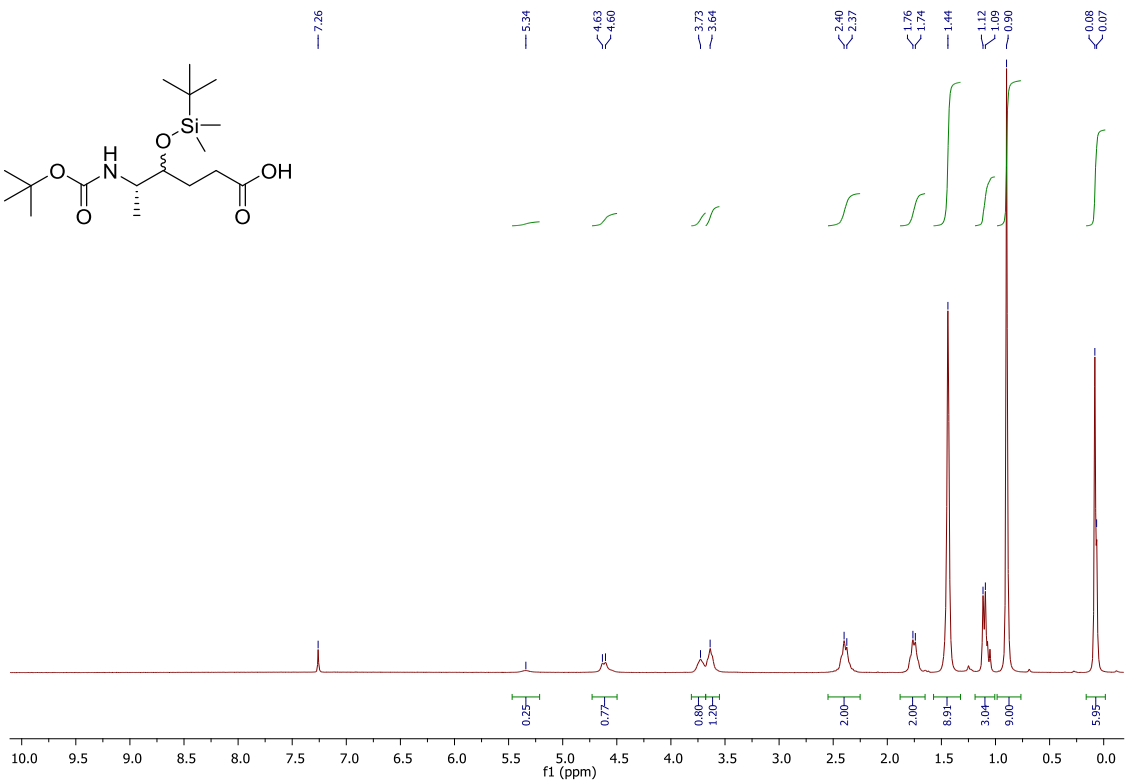


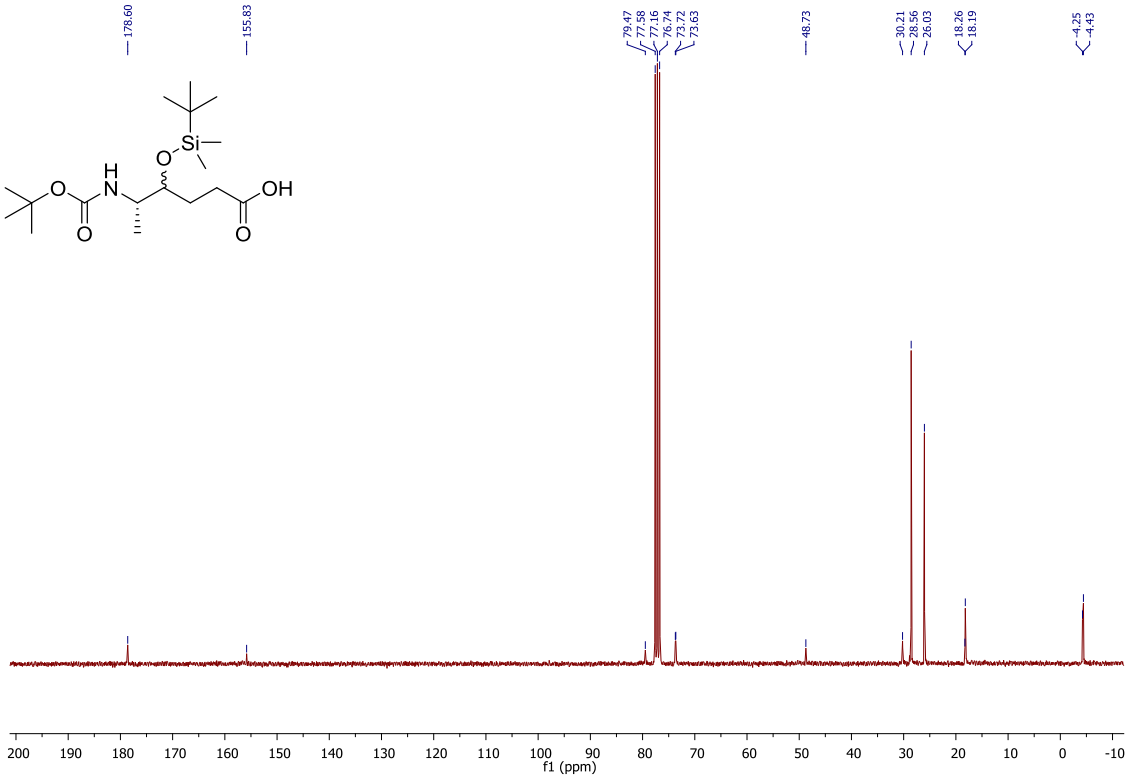
^{1}H and ^{13}C NMR spectra of *tert*-Butyl ((*S*)-1-((*S*)5-oxotetrahydrofuran-2-yl)ethyl)carbamate (4)



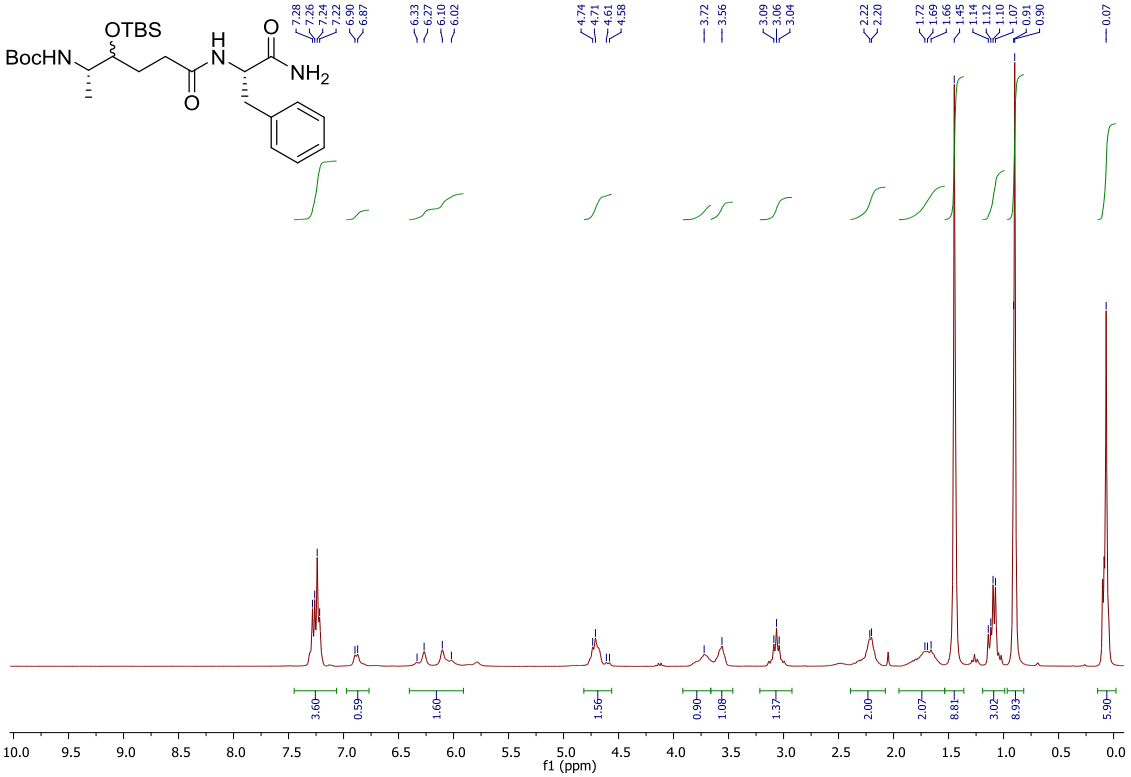


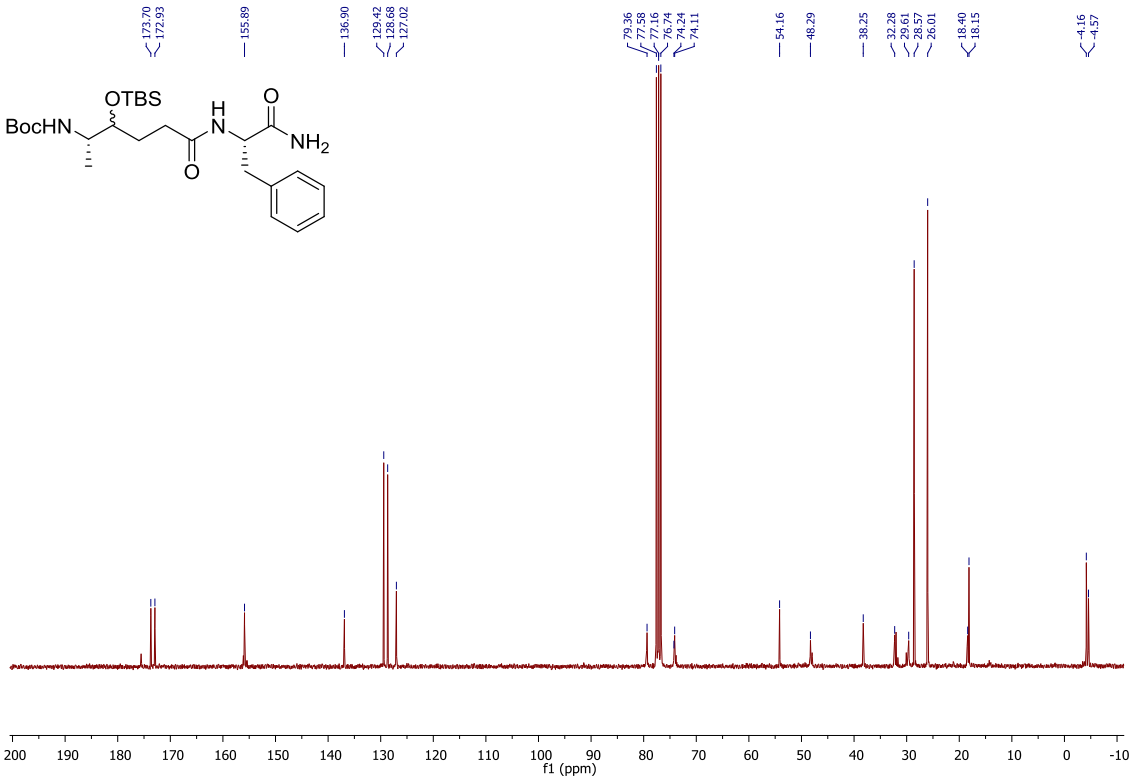
^{1}H and ^{13}C NMR spectra of (5*S*)-5-((*tert*-Butoxycarbonyl)amino)-4-((*tert*-butyldimethylsilyl)oxy)hexanoic acid (5)



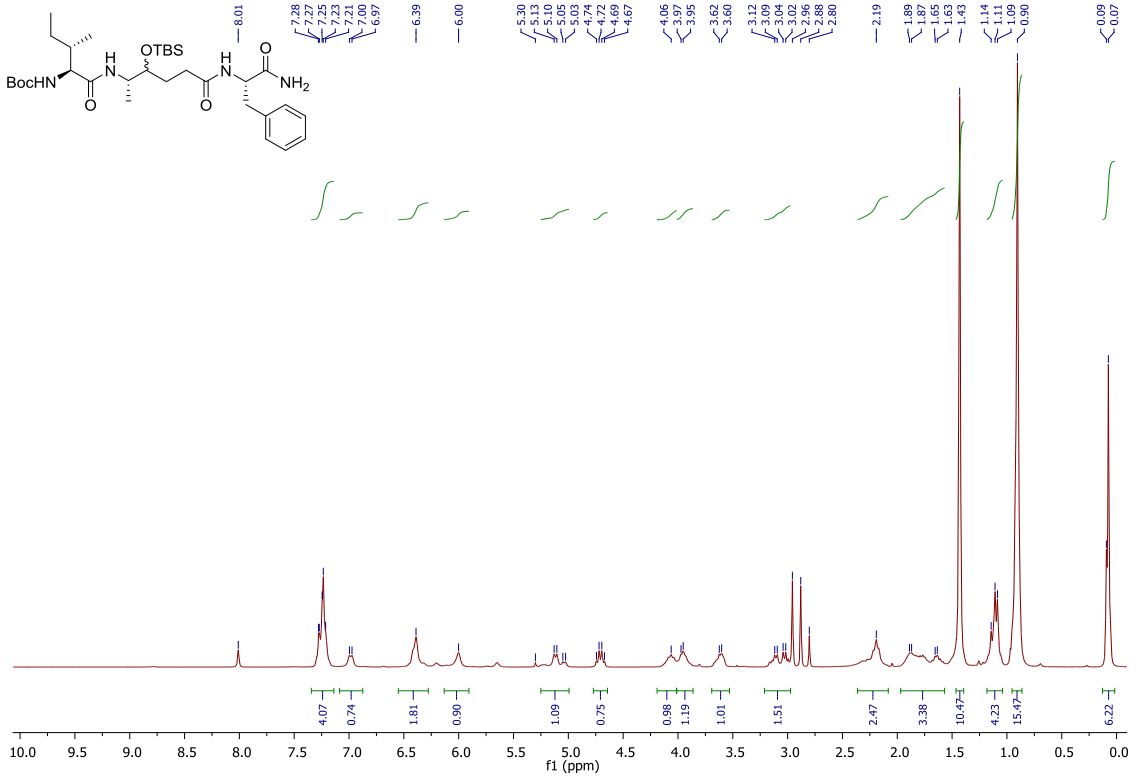


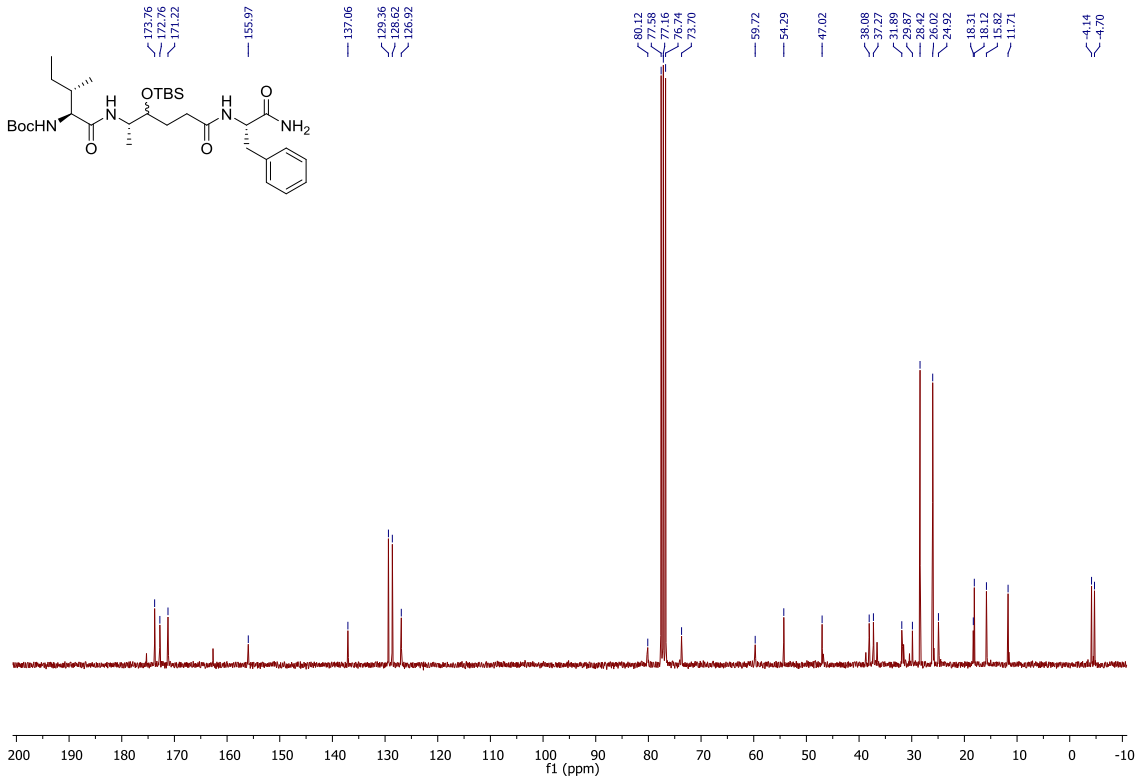
^{1}H and ^{13}C NMR spectra of *tert*-Butyl ((2*S*)-6-(((*S*)-1-amino-1-oxo-3-phenylpropan-2-yl)amino)-3-((*tert*-butyldimethylsilyl)oxy)-6-oxohexan-2-yl)carbamate (6)



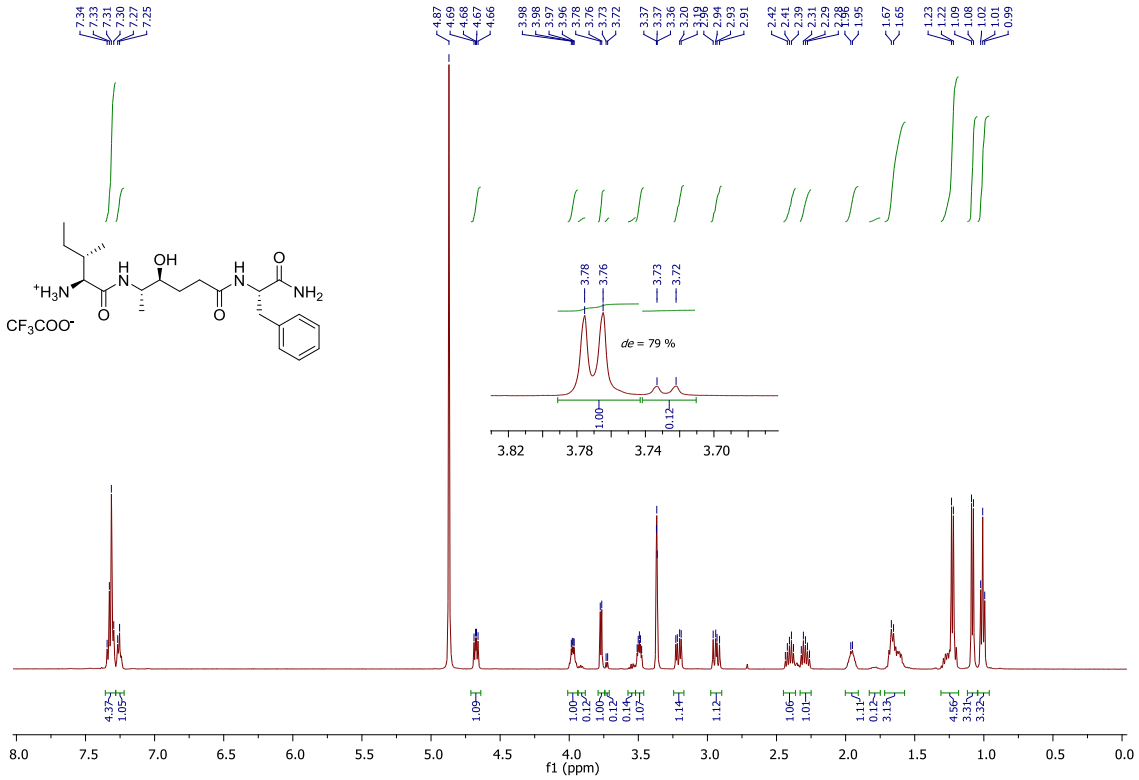


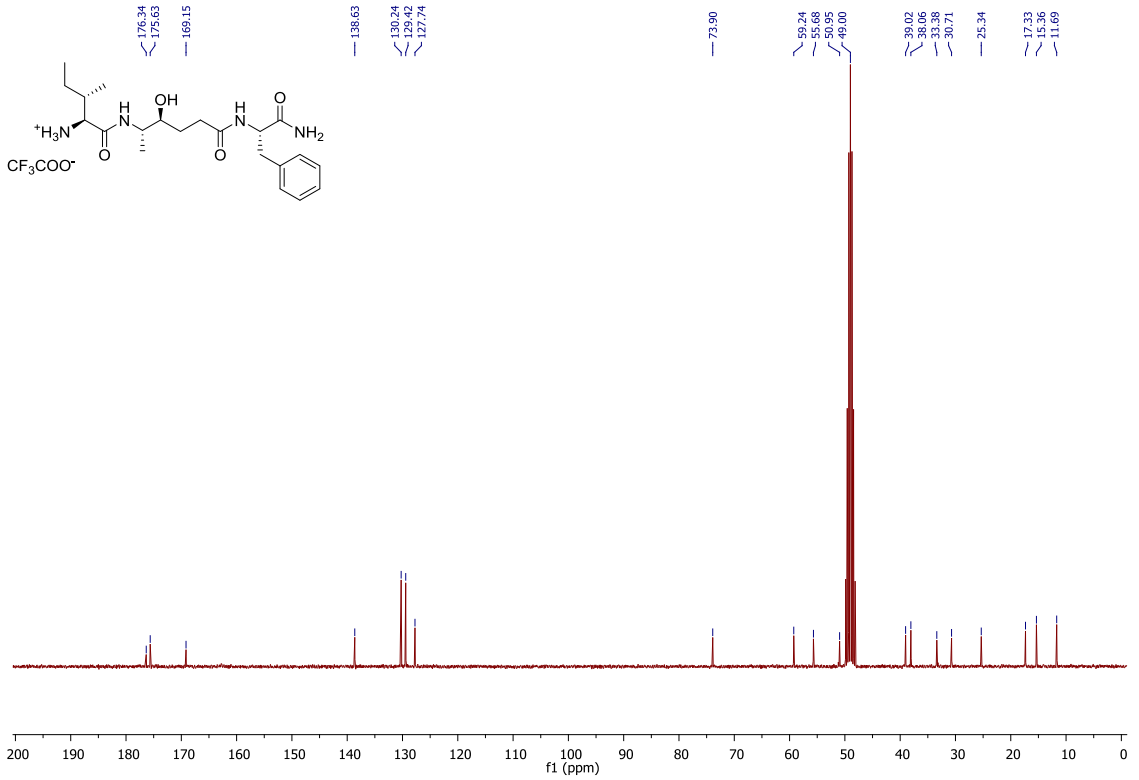
^{1}H and ^{13}C NMR spectra of *tert*-Butyl ((2*S*,3*S*)-1-(((2*S*)-6-(((*S*)-1-amino-1-oxo-3-phenylpropan-2-yl)amino)-3-((*tert*-butyldimethylsilyl)oxy)-6-oxohexan-2-yl)amino)-3-methyl-1-oxopentan-2-yl)carbamate (7)



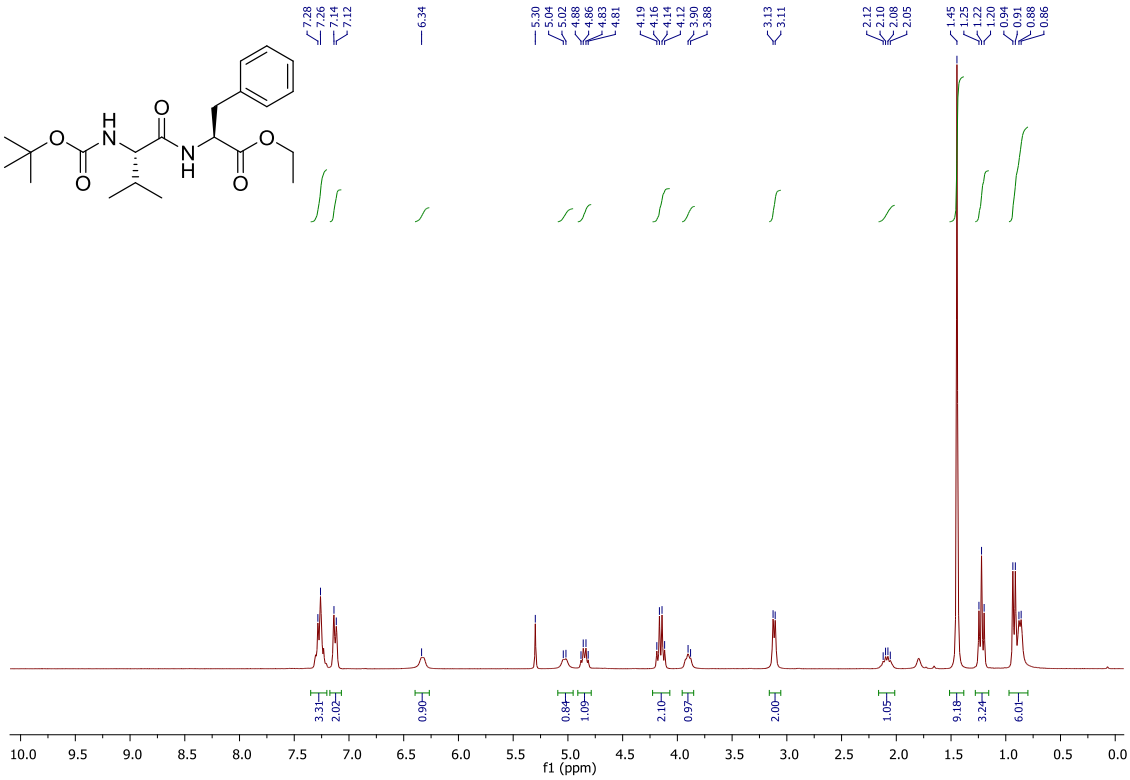


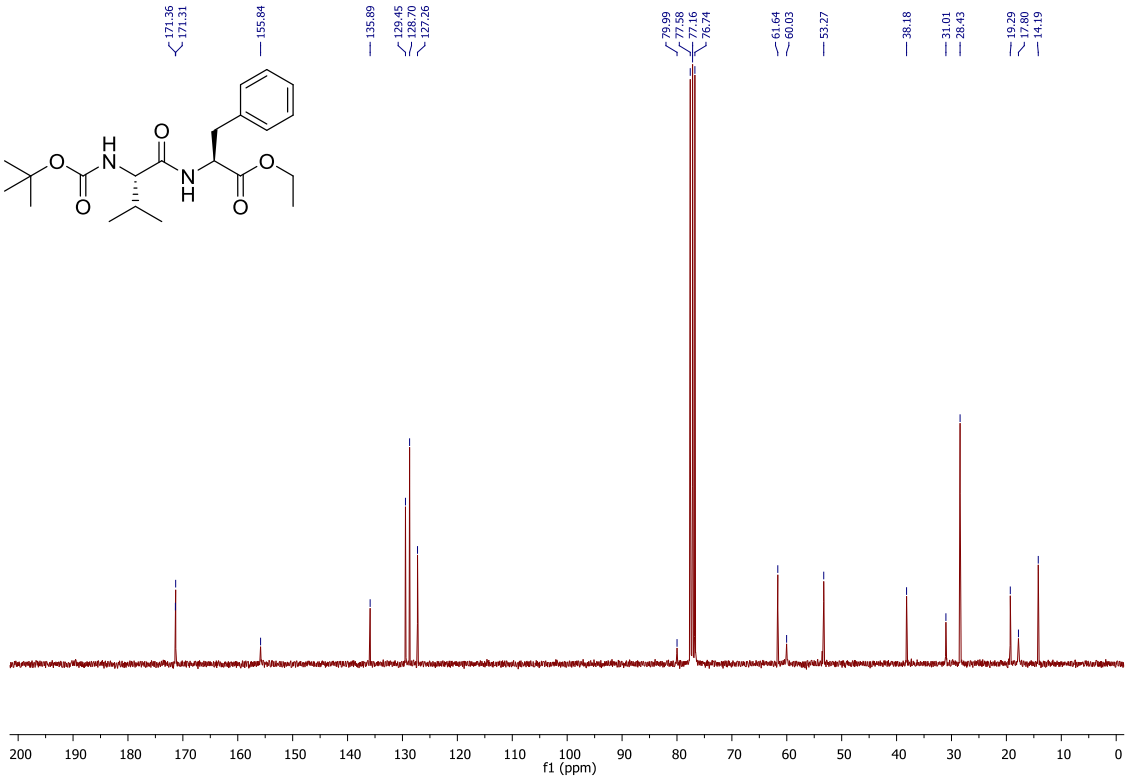
^{1}H (500 MHz) and ^{13}C (75 MHz) NMR spectra of CF_3COOH × H-Ile-Ala-[Ψ](COH-CH_2)-Gly-Phe-NH_2 (8a)



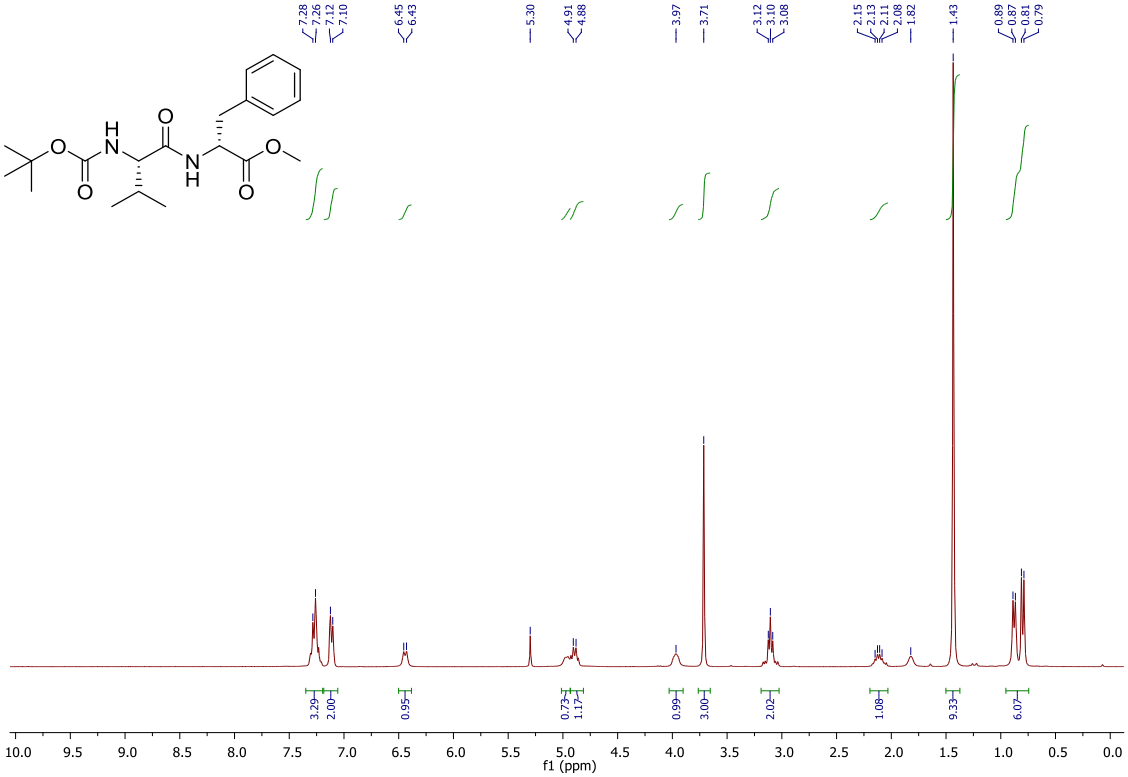


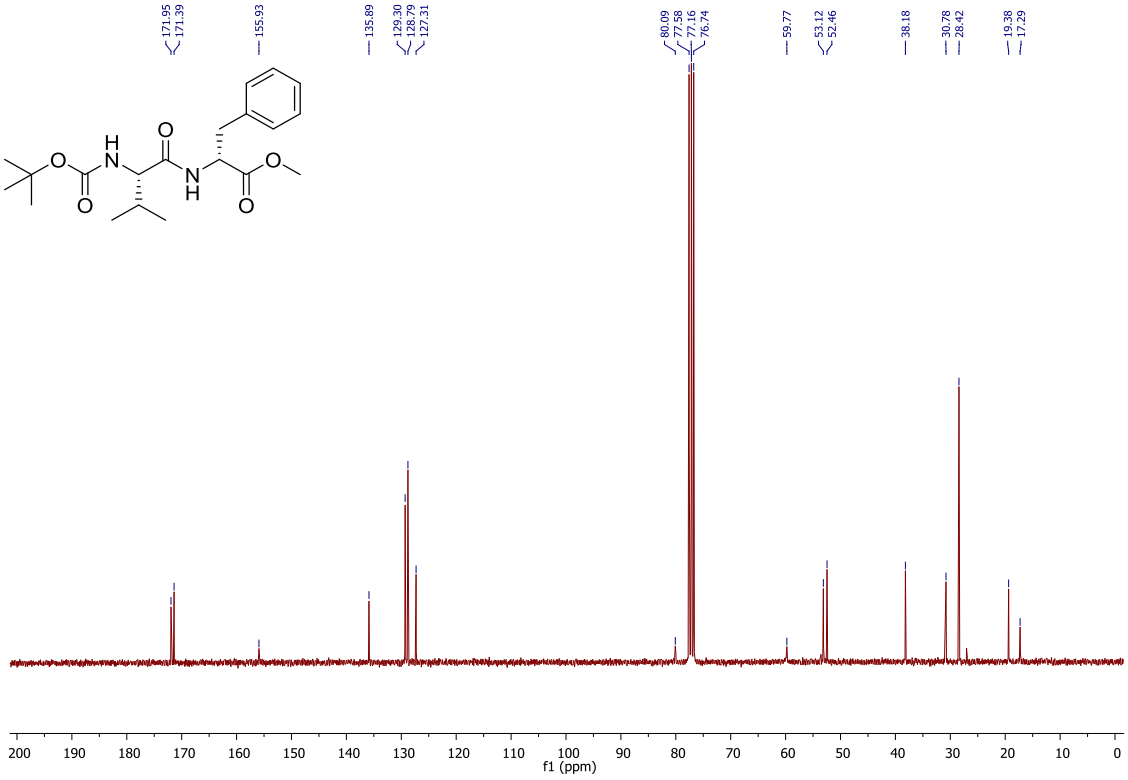
^{1}H and ^{13}C NMR spectra of Ethyl (*tert*-butoxycarbonyl)-L-valyl-L-phenylalaninate (Boc-Val-Phe-OEt) (9a)



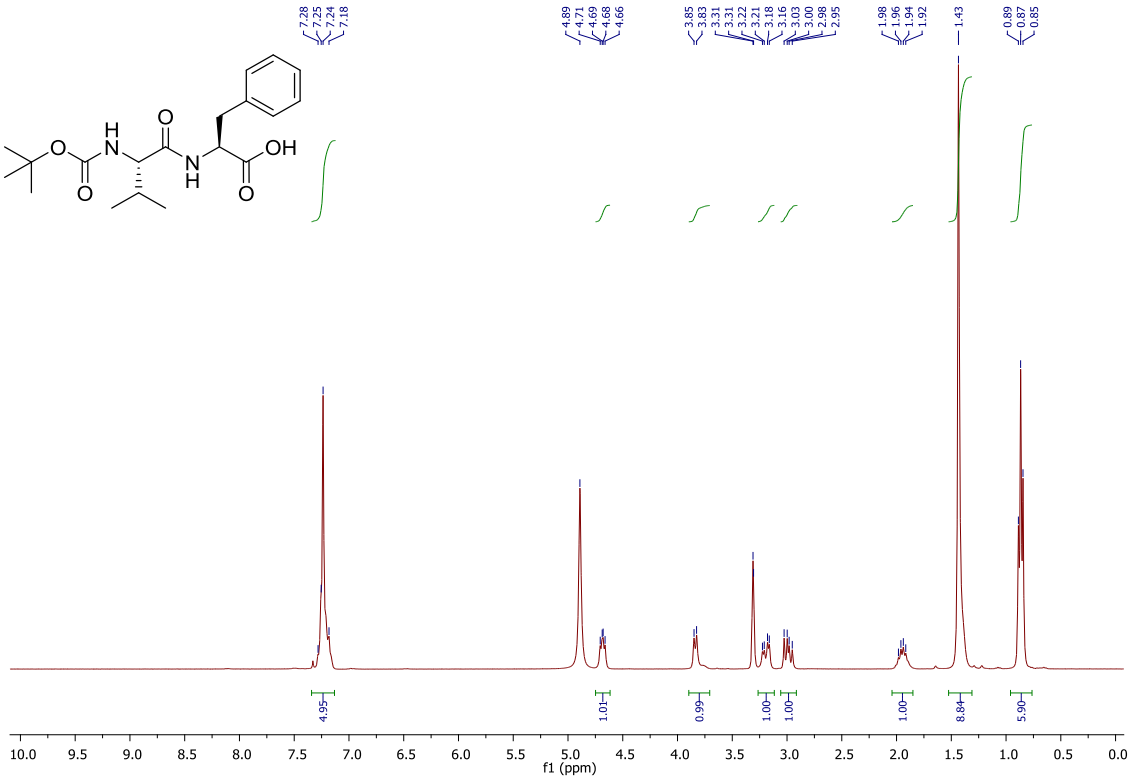


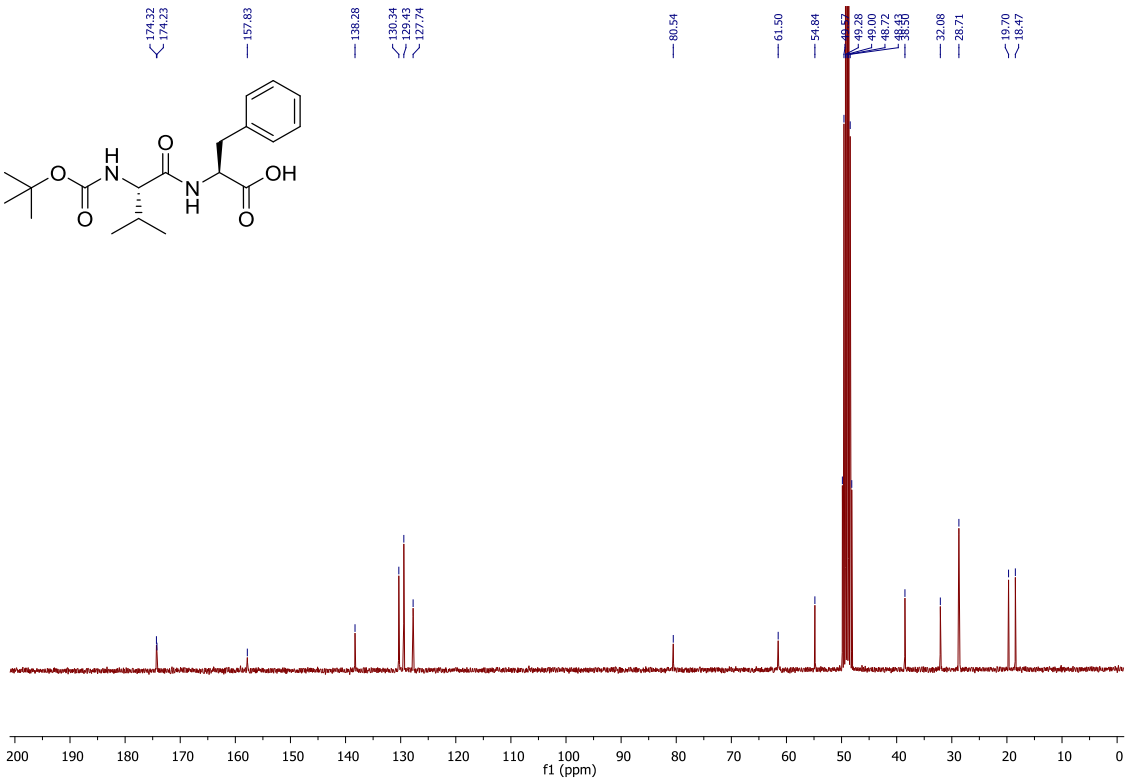
^{1}H and ^{13}C NMR spectra of Methyl (*tert*-butoxycarbonyl)-L-valyl-D-phenyl-alaninate (Boc-Val-D-Phe-OMe) (9b)



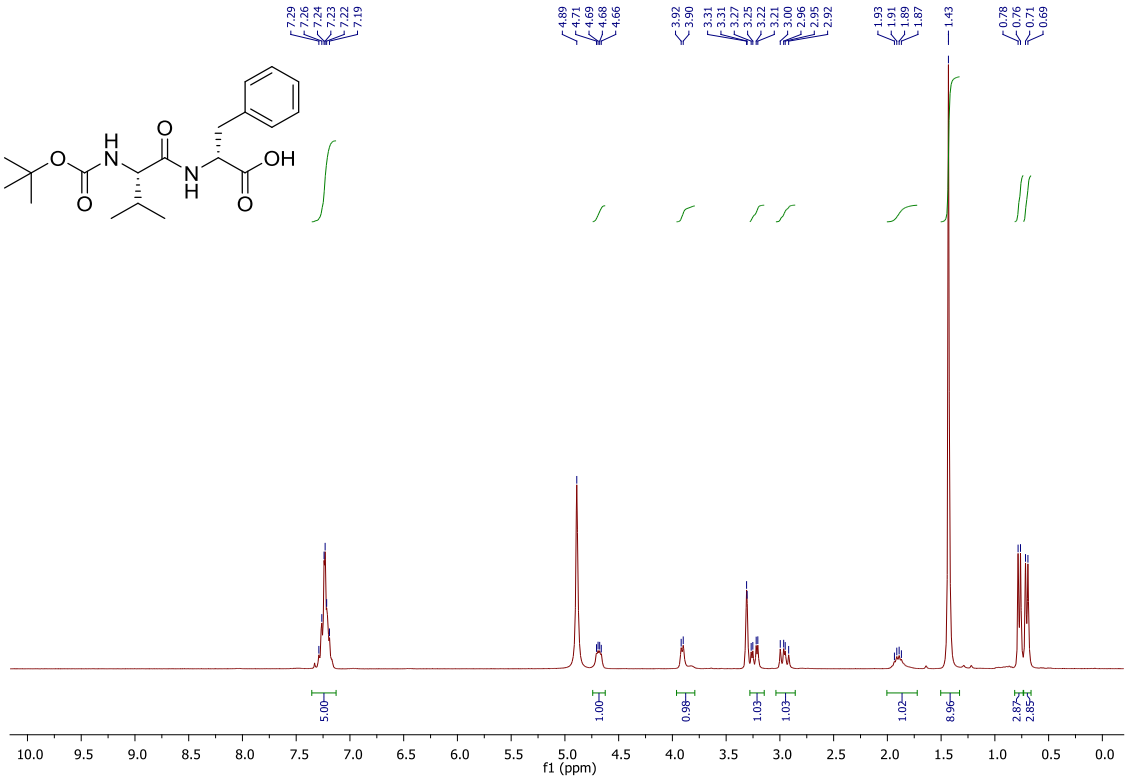


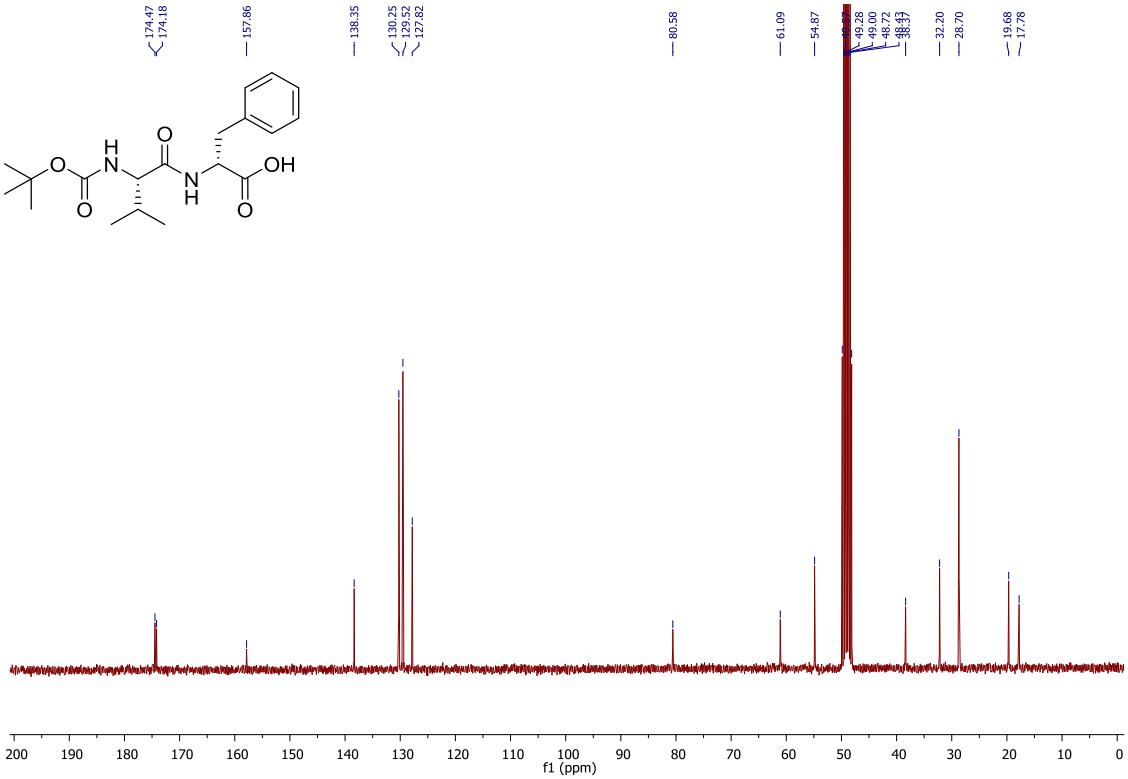
^{1}H and ^{13}C NMR spectra of (*tert*-Butoxycarbonyl)-L-valyl-L-phenylalanine (Boc-Val-Phe-OH) (10a)



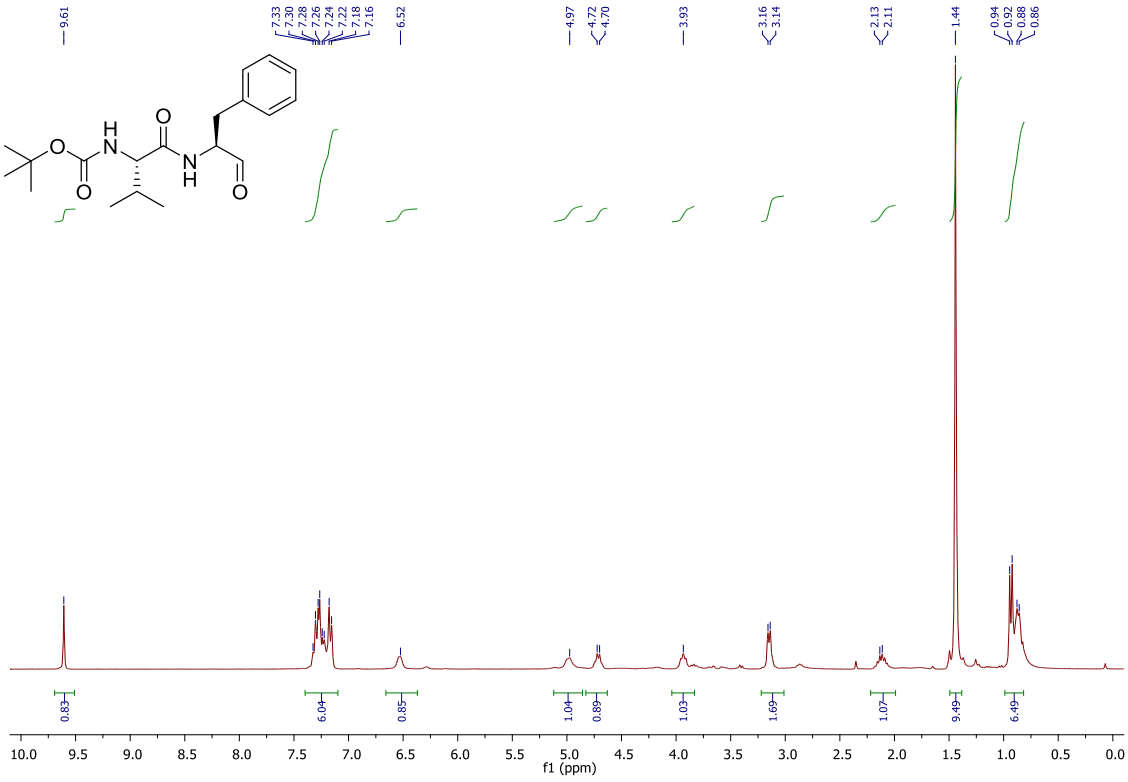


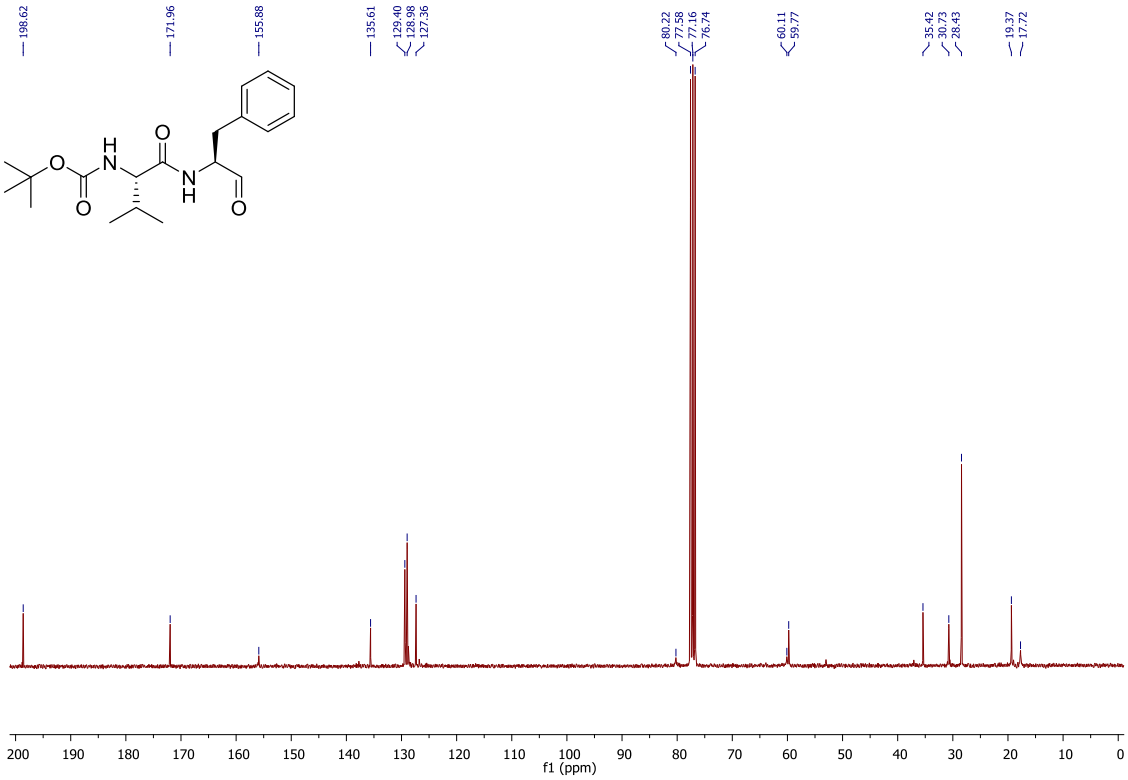
^{1}H and ^{13}C NMR spectra of (*tert*-Butoxycarbonyl)-L-valyl-D-phenylalanine (Boc-Val-D-Phe-OH) (10b)



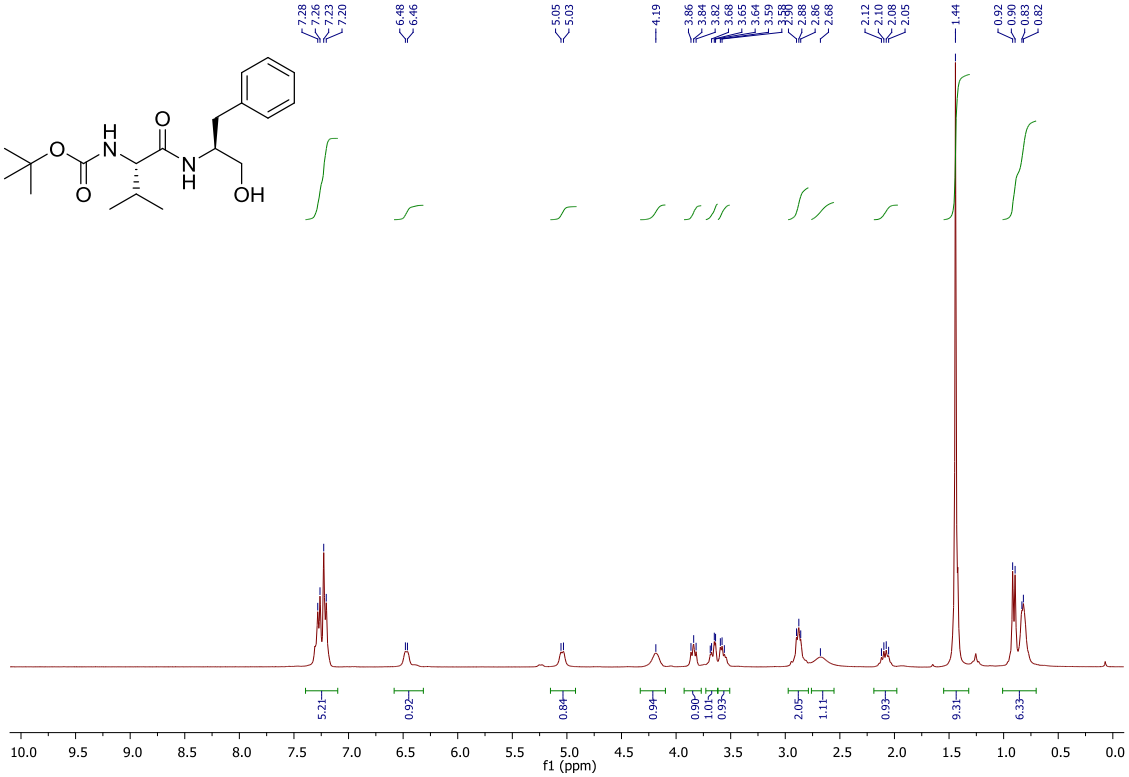


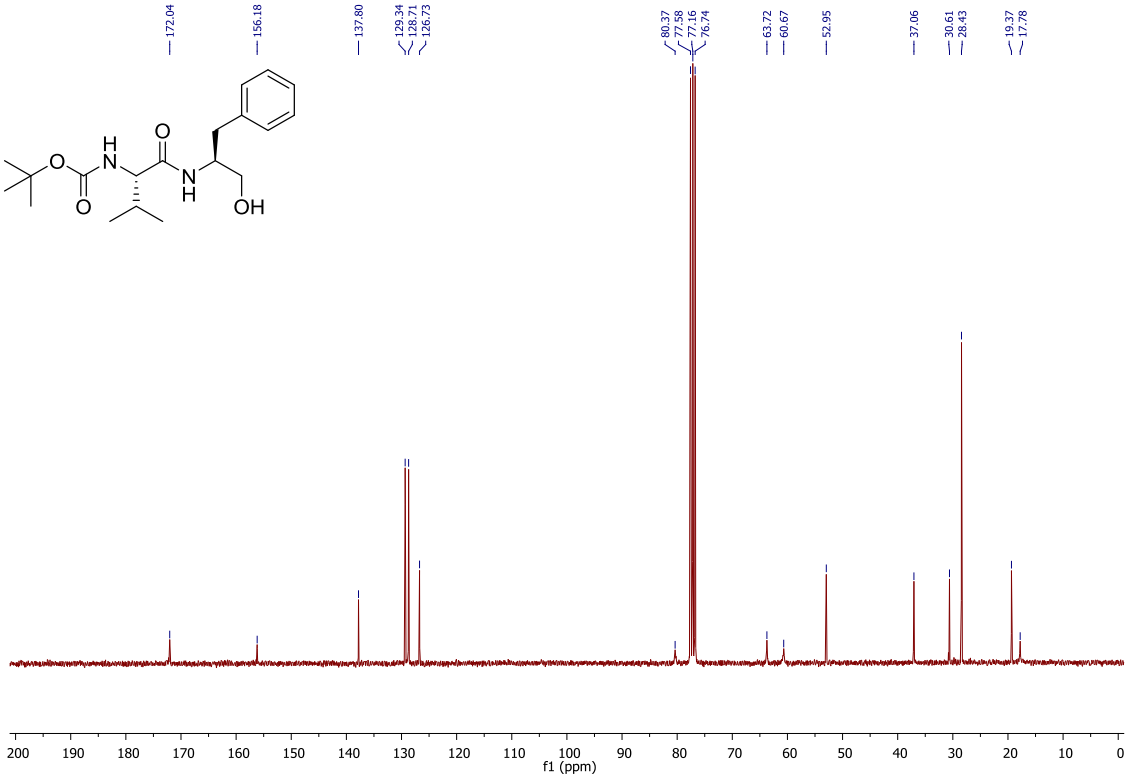
^{1}H and ^{13}C NMR spectra of *tert*-Butyl ((*S*)-3-methyl-1-oxo-1-(((*S*)-1-oxo-3-phenylpropan-2-yl)amino)butan-2-yl)carbamate (Boc-Val-Phe-H) (11a)



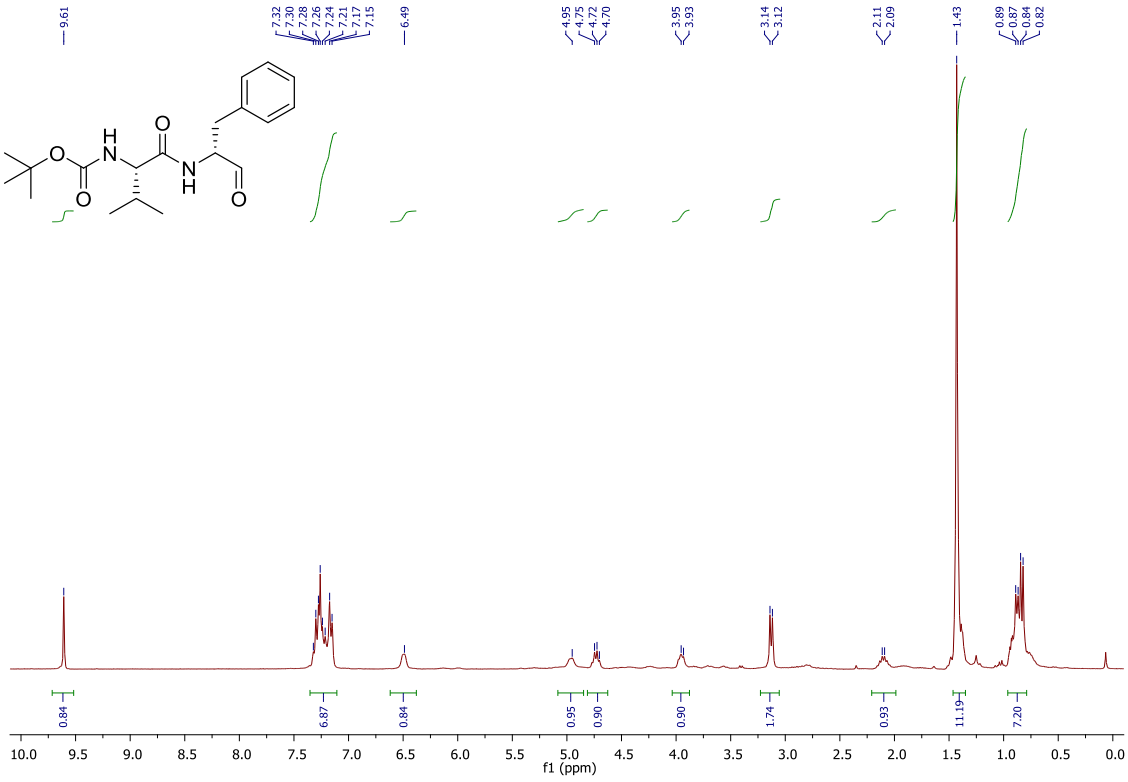


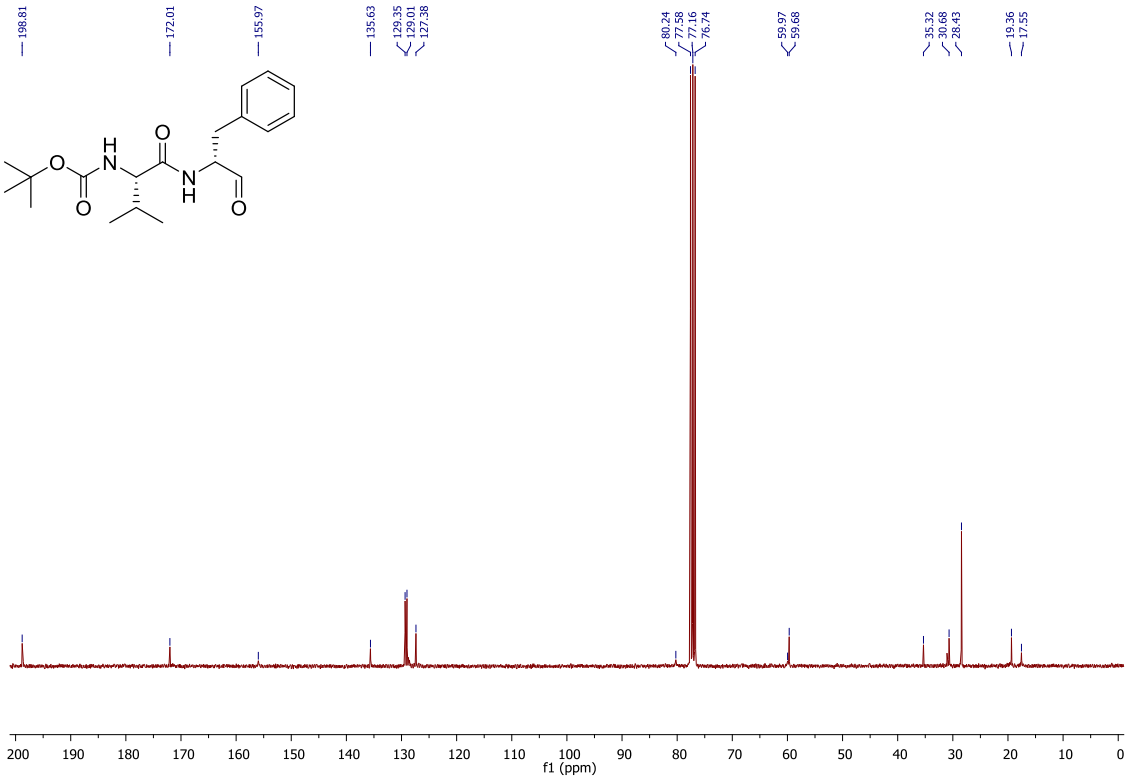
^{1}H and ^{13}C NMR spectra of *tert*-Butyl ((*S*)-1-(((*S*)-1-hydroxy-3-phenyl-propan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (Boc-Val-Phe-ol) (12a)



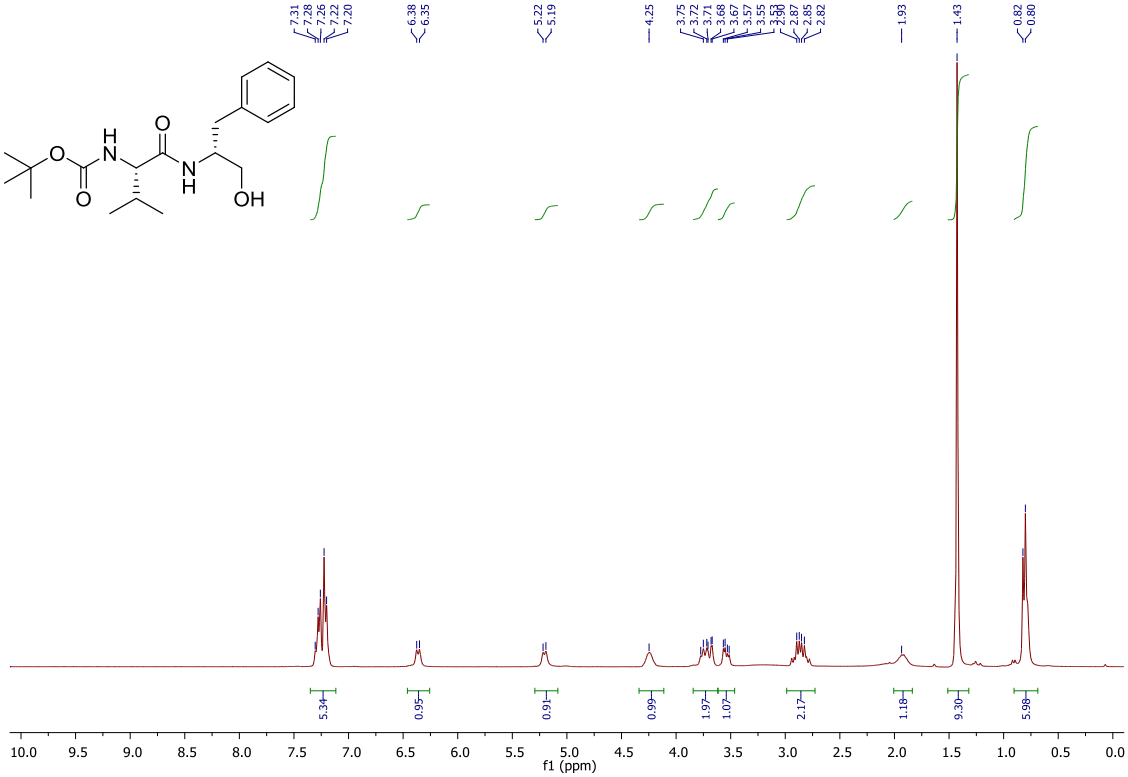


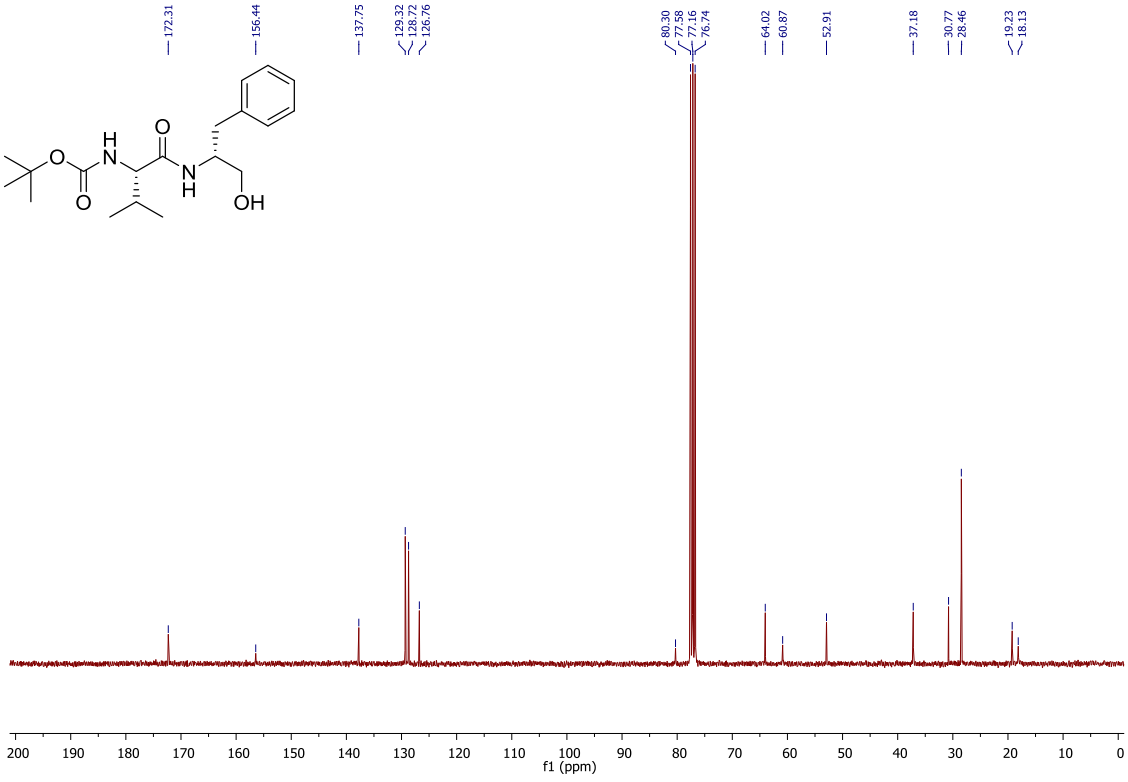
^{1}H and ^{13}C NMR spectra of *tert*-Butyl ((*S*)-3-methyl-1-oxo-1-(((*R*)-1-oxo-3-phenylpropan-2-yl)amino)butan-2-yl)carbamate (Boc-Val-D-Phe-H) (11b)



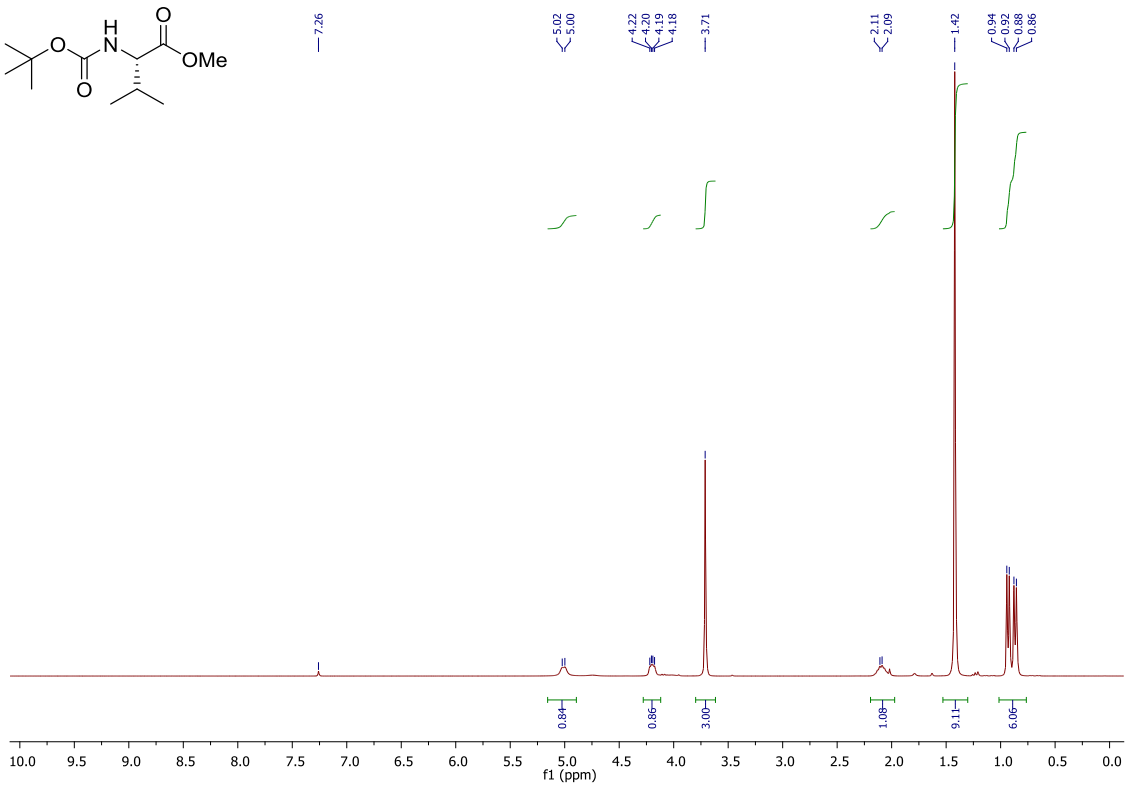


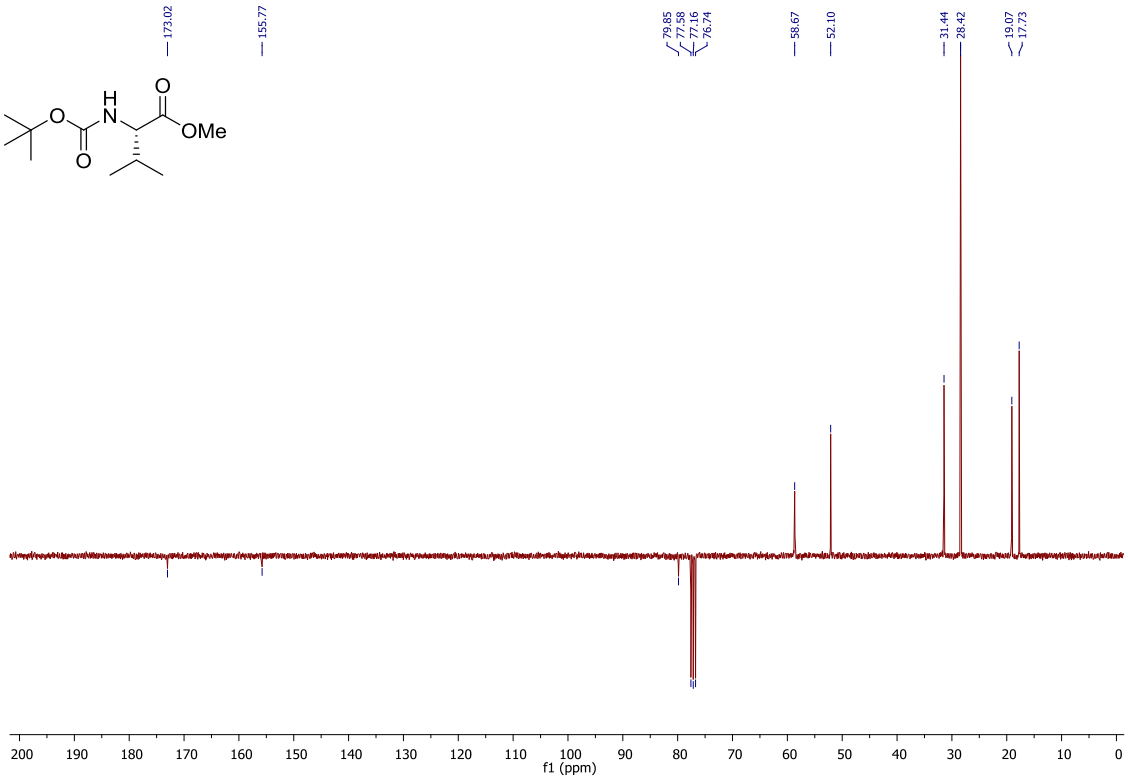
^{1}H and ^{13}C NMR spectra of *tert*-Butyl ((*S*)-1-(((*R*)-1-hydroxy-3-phenylpropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (Boc-Val-D-Phe-ol) (12b)



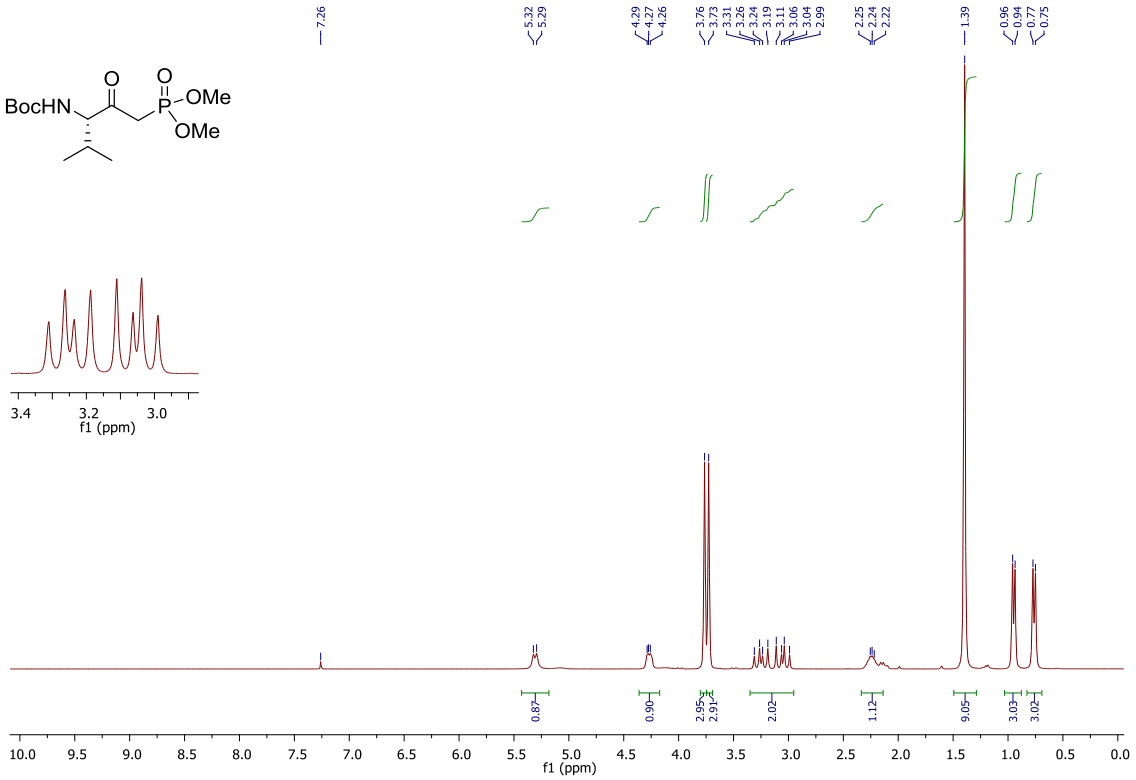


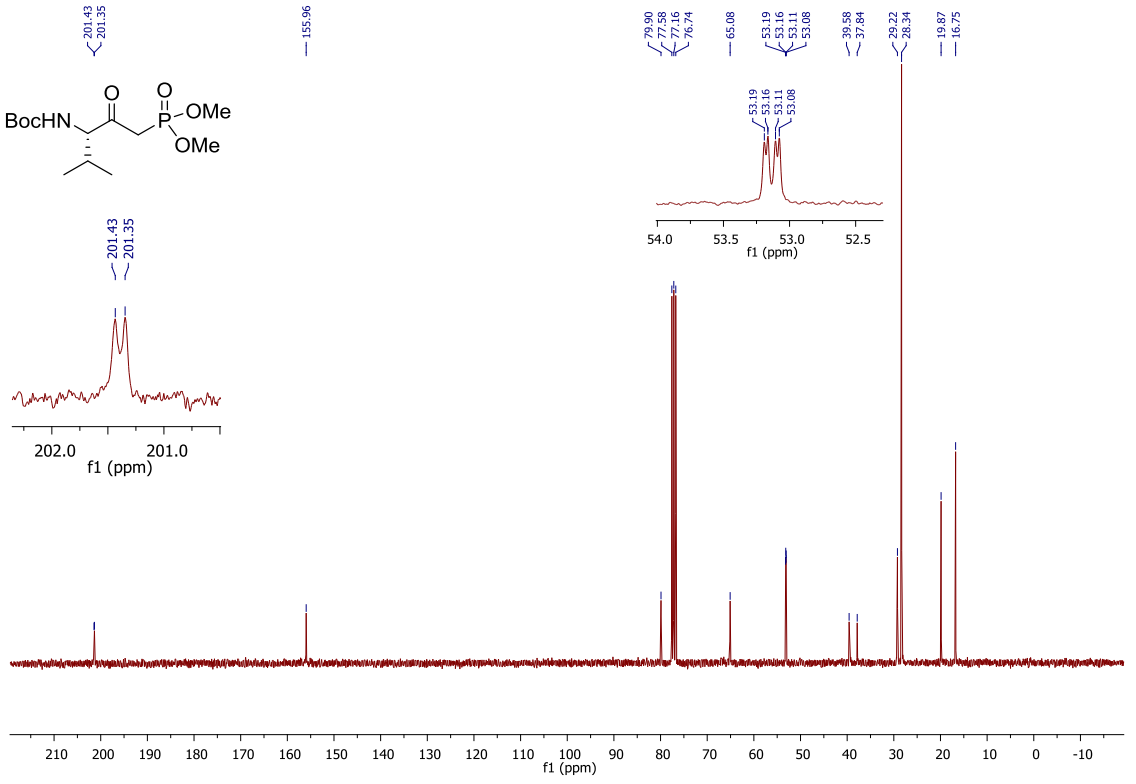
^{1}H and ^{13}C NMR-APT spectra of Methyl (*tert*-butoxycarbonyl)-L-valinate (14)



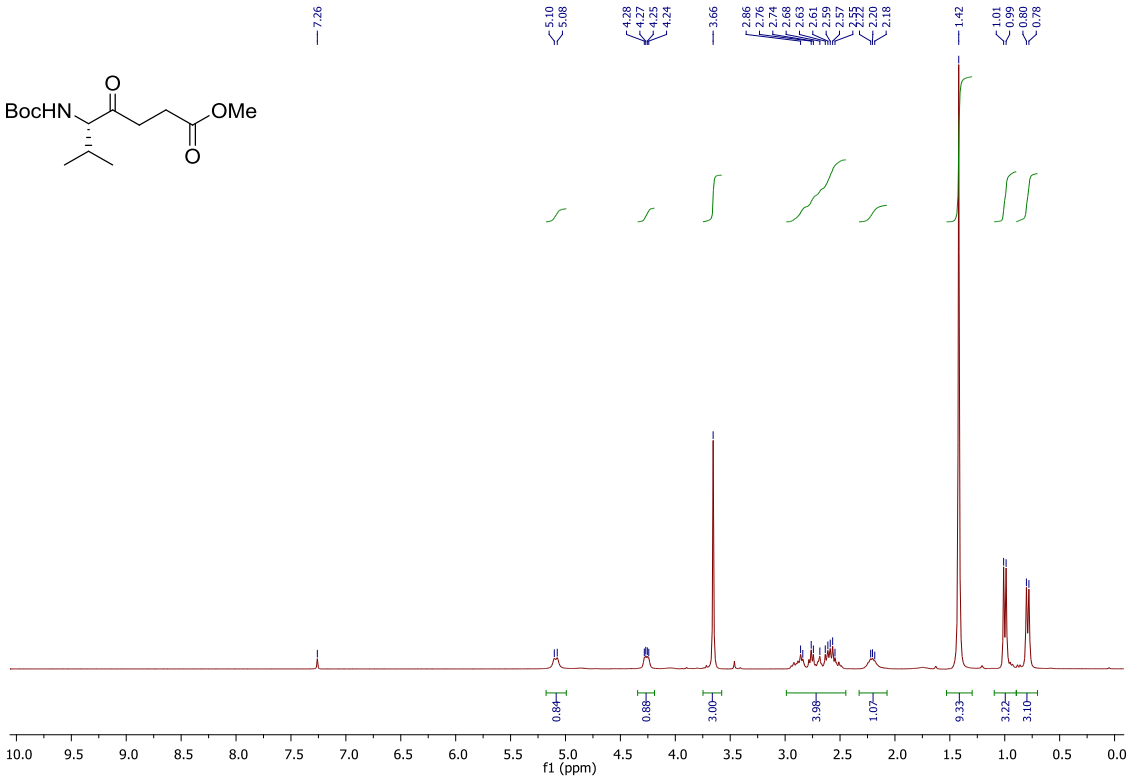


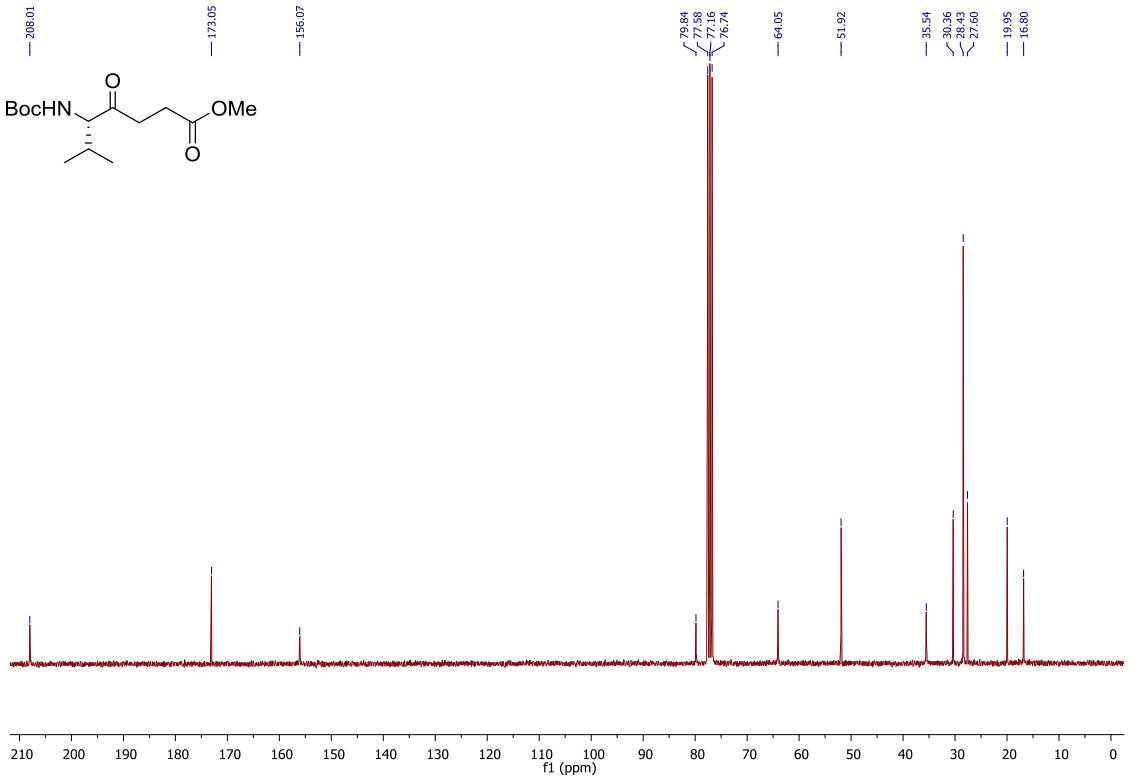
^{1}H and ^{13}C NMR spectra of *tert*-Butyl (*S*)-(1-(dimethoxyphosphoryl)-4-methyl-2-oxopentan-3-yl)carbamate (15)



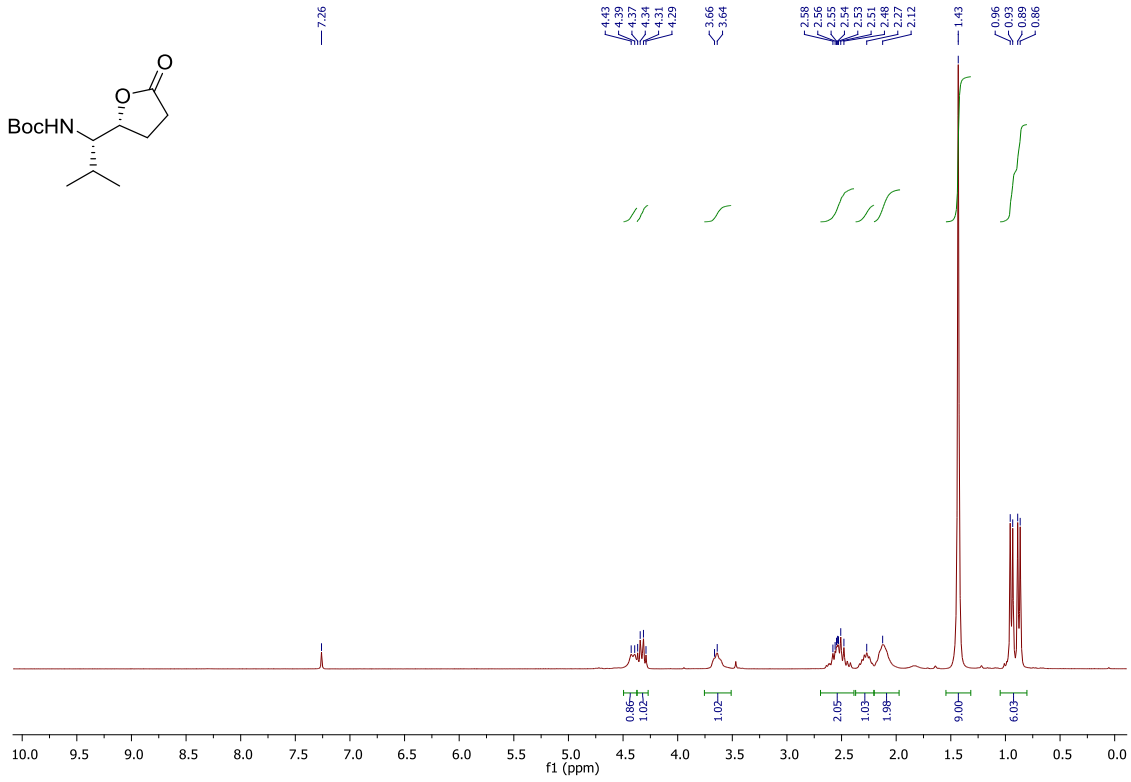


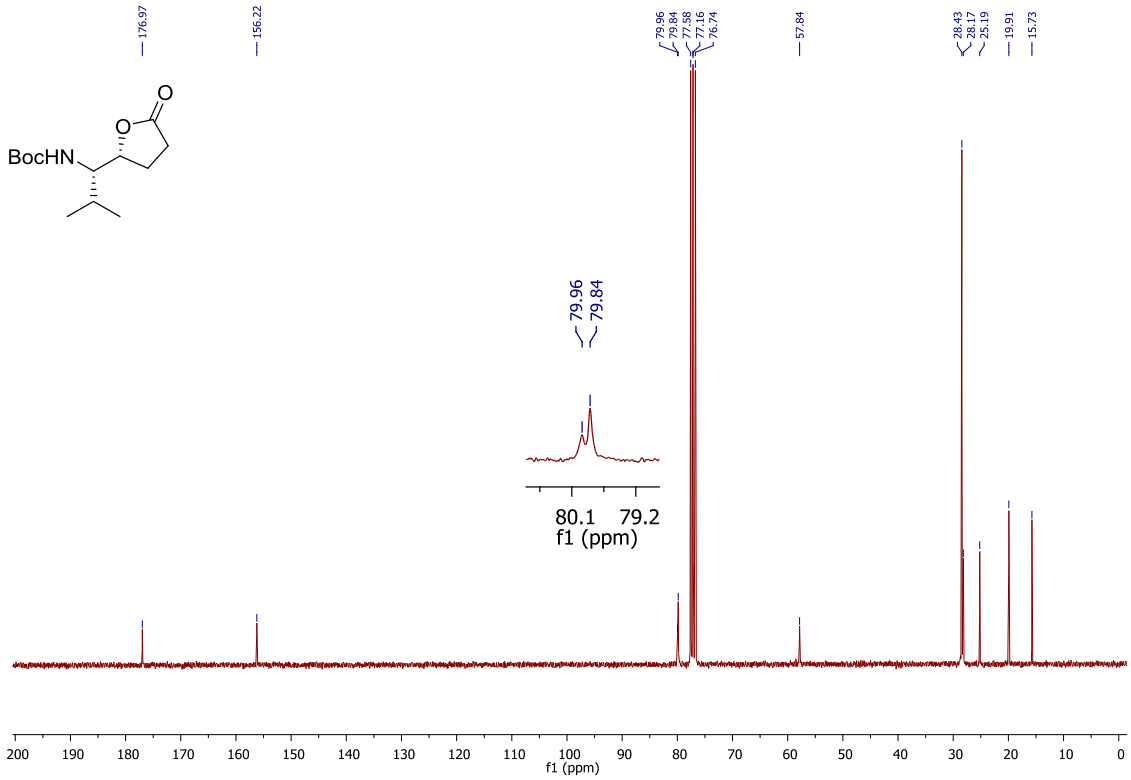
^{1}H and ^{13}C NMR spectra of Methyl (*S*)-5-((*tert*-butoxycarbonyl)amino)-6-methyl-4-oxoheptanoate (17)



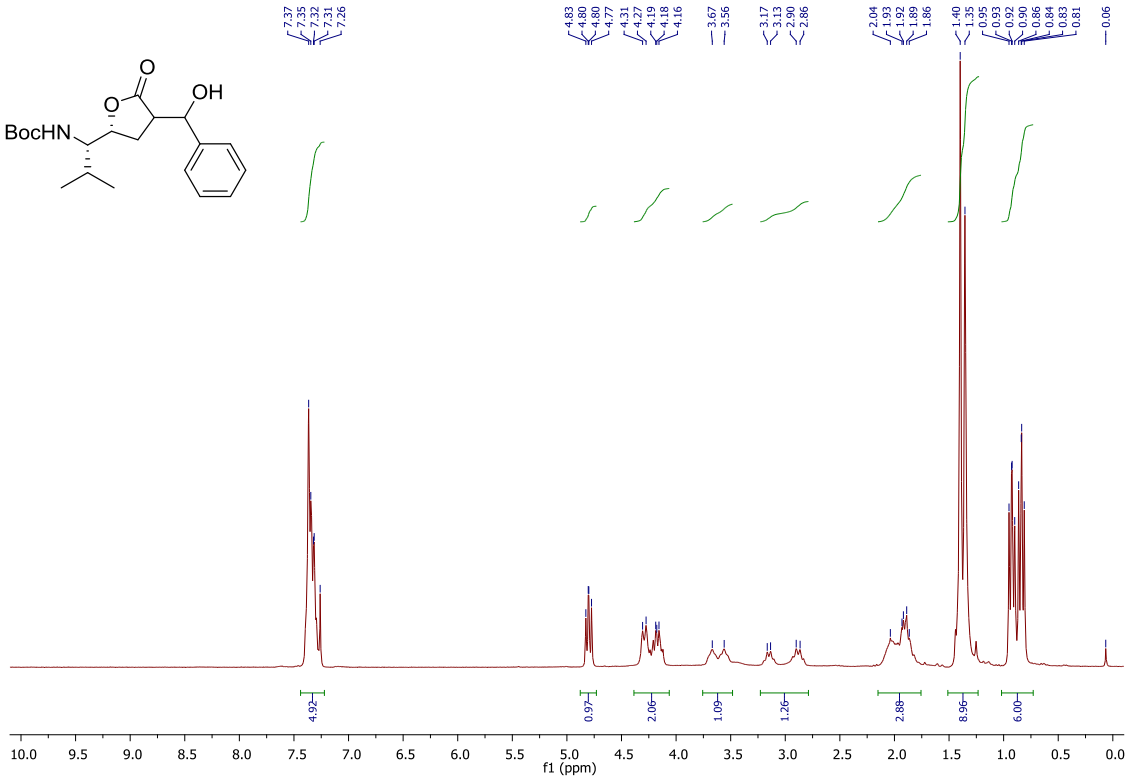


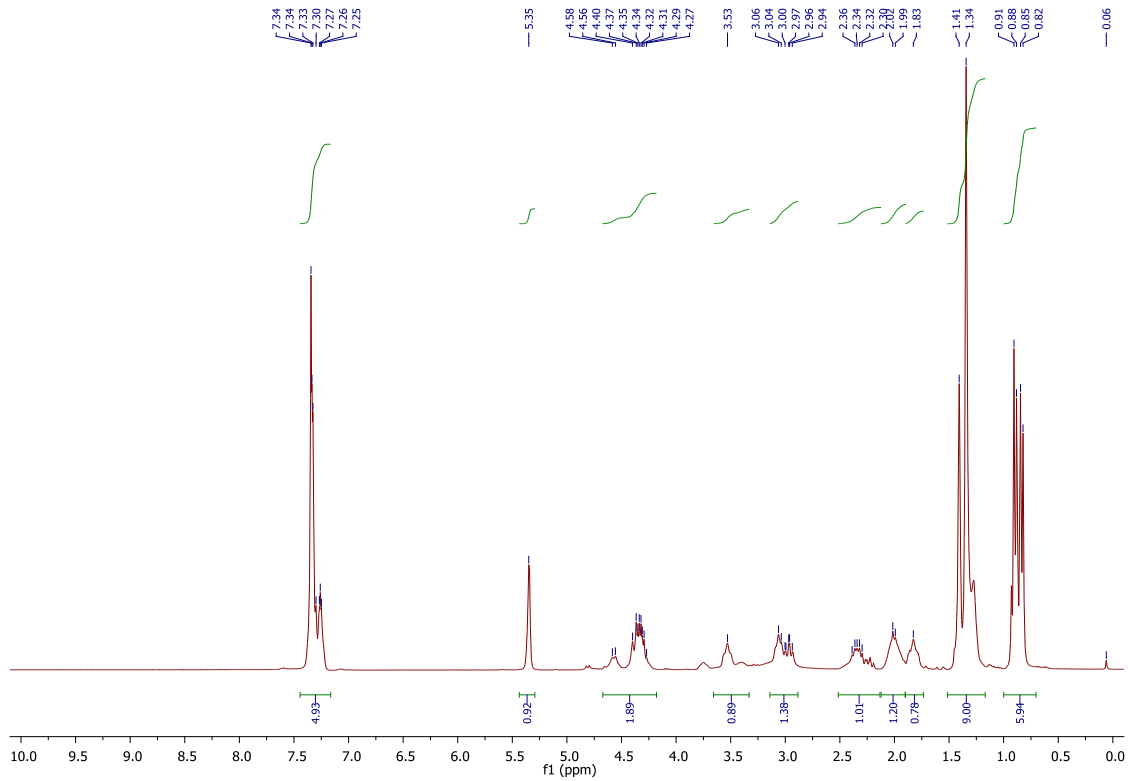
^{1}H and ^{13}C NMR spectra of *tert*-Butyl ((*S*)-2-methyl-1-((*R*)-5-oxotetrahydrofuran-2-yl)propyl)carbamate (18)



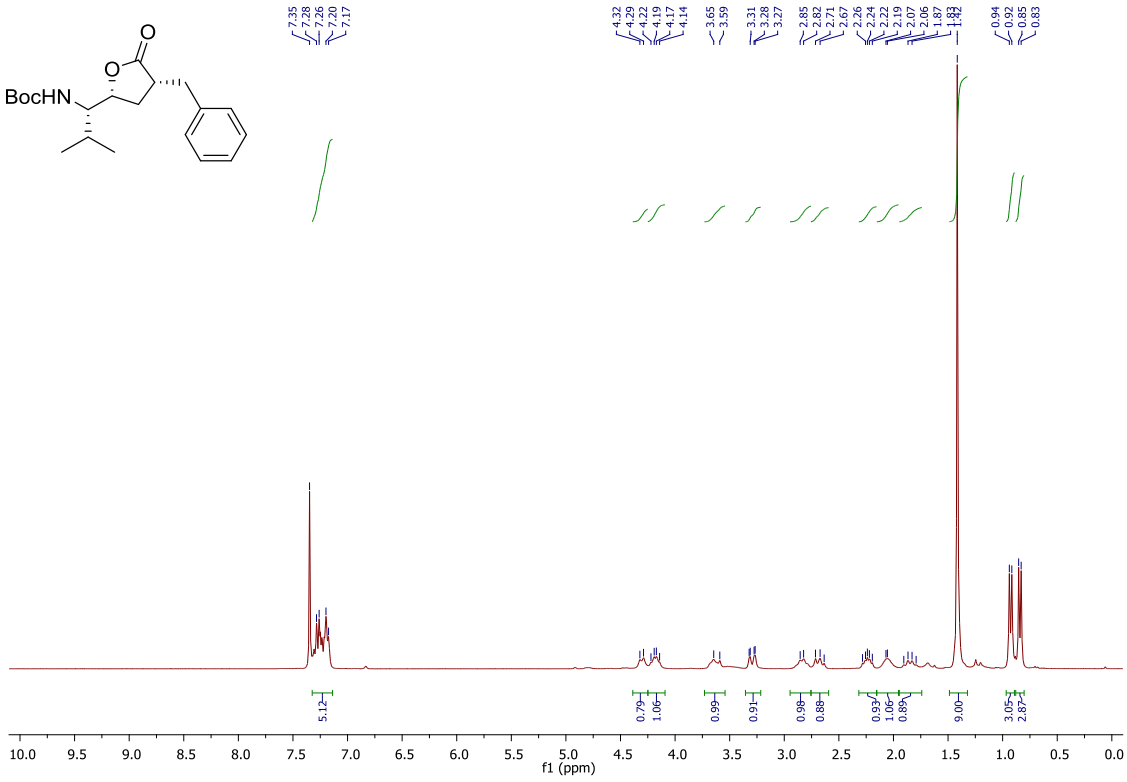


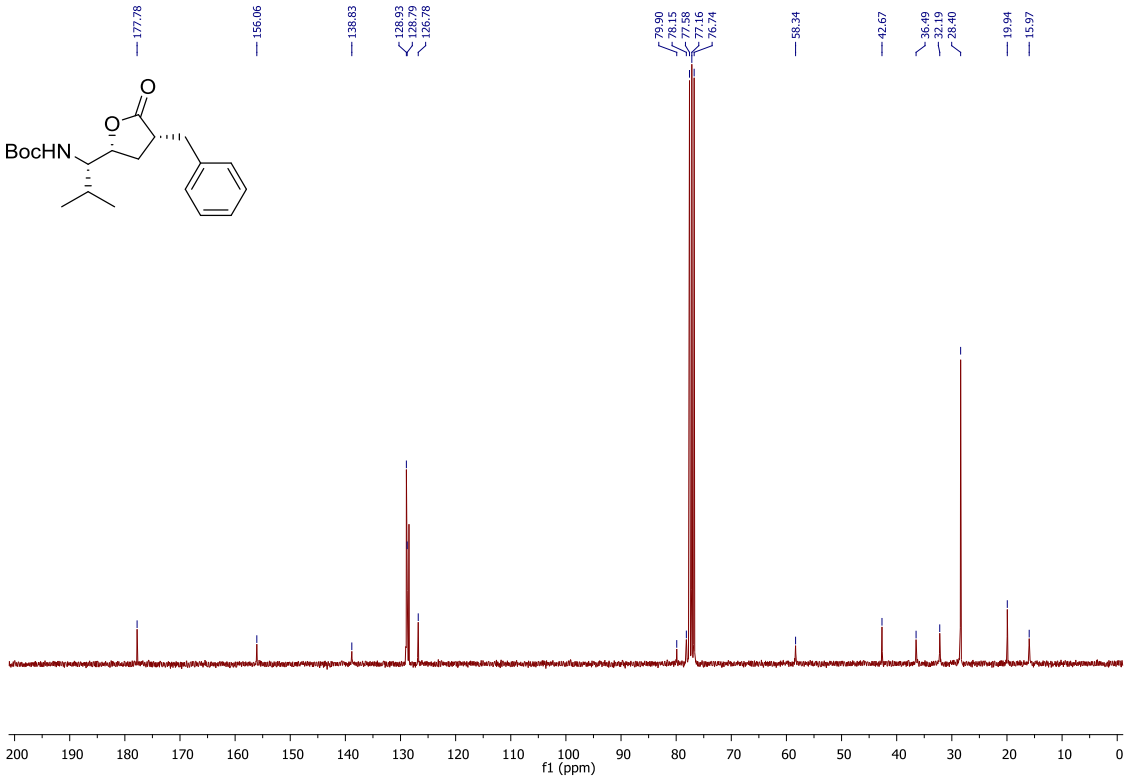
^{1}H NMR spectra of diastereomers of *tert*-butyl ((1*S*)-1-((2*R*)-4-(hydroxyl-(phenyl)methyl)-5-oxotetrahydrofuran-2-yl)-2-methylpropyl)carbamate (19)



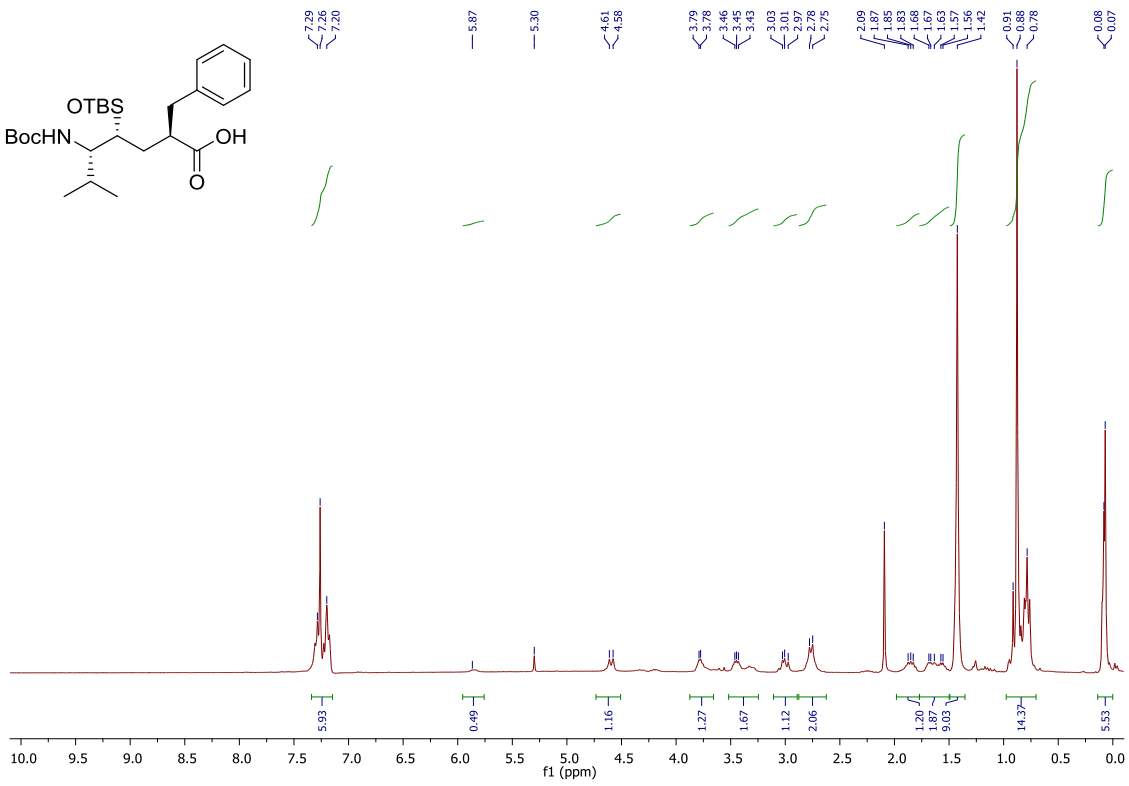


^{1}H and ^{13}C NMR spectra of *tert*-Butyl ((*S*)-1-((2*R*,4*R*)-4-benzyl-5-oxotetra-hydrofuran-2-yl)-2-methylpropyl)carbamate (21)

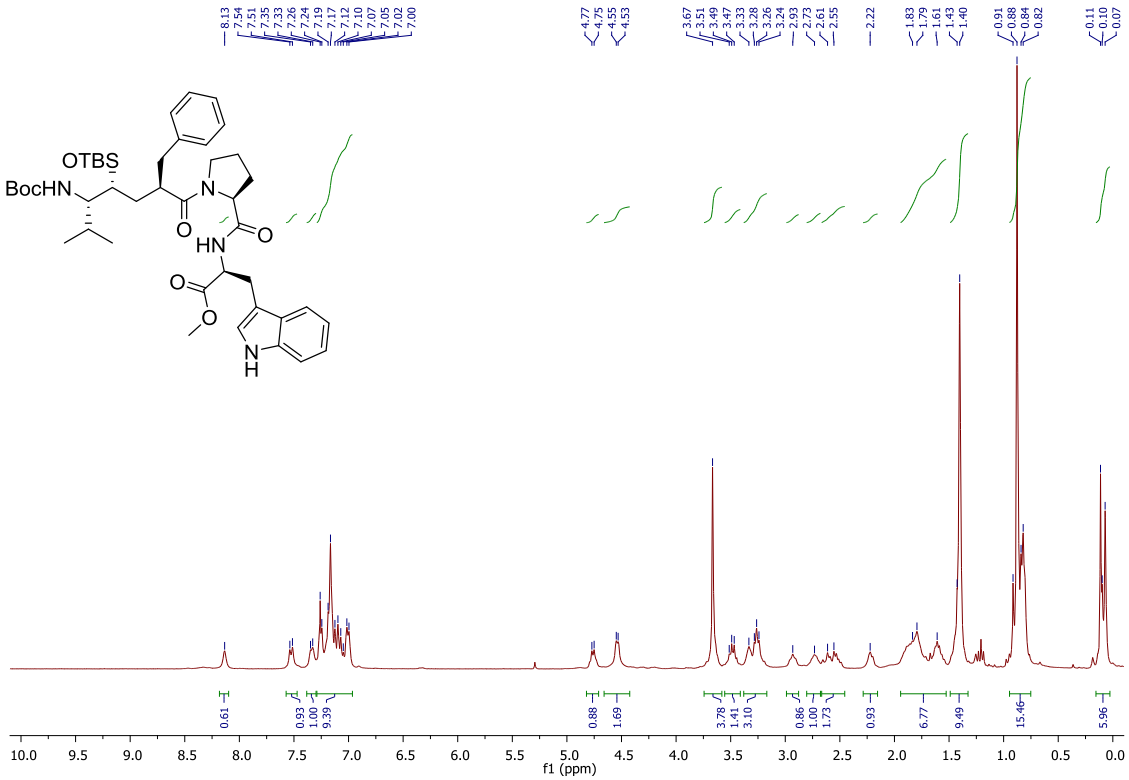




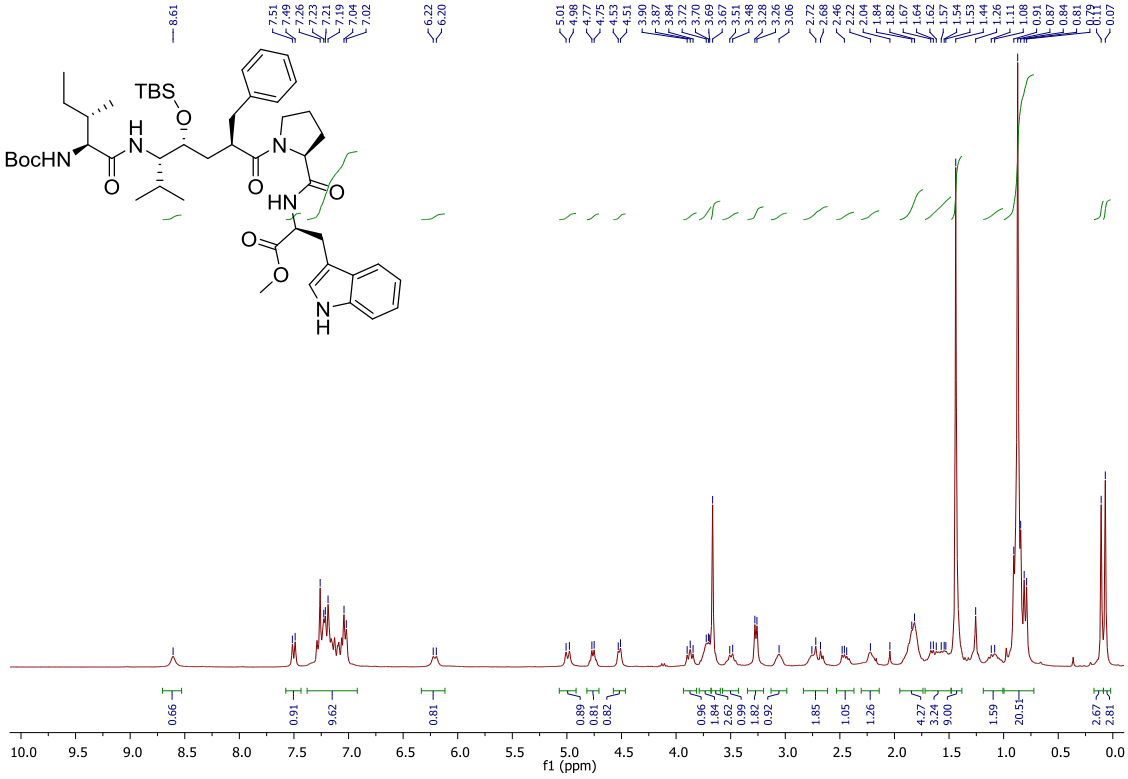
^{1}H NMR spectrum of (2*R*,4*R*,5*S*)-2-Benzyl-5-((*tert*-butoxycarbonyl)amino)-4-((*tert*-butyldimethylsilyl)oxy)-6-methylheptanoic acid (RGSA) (22)

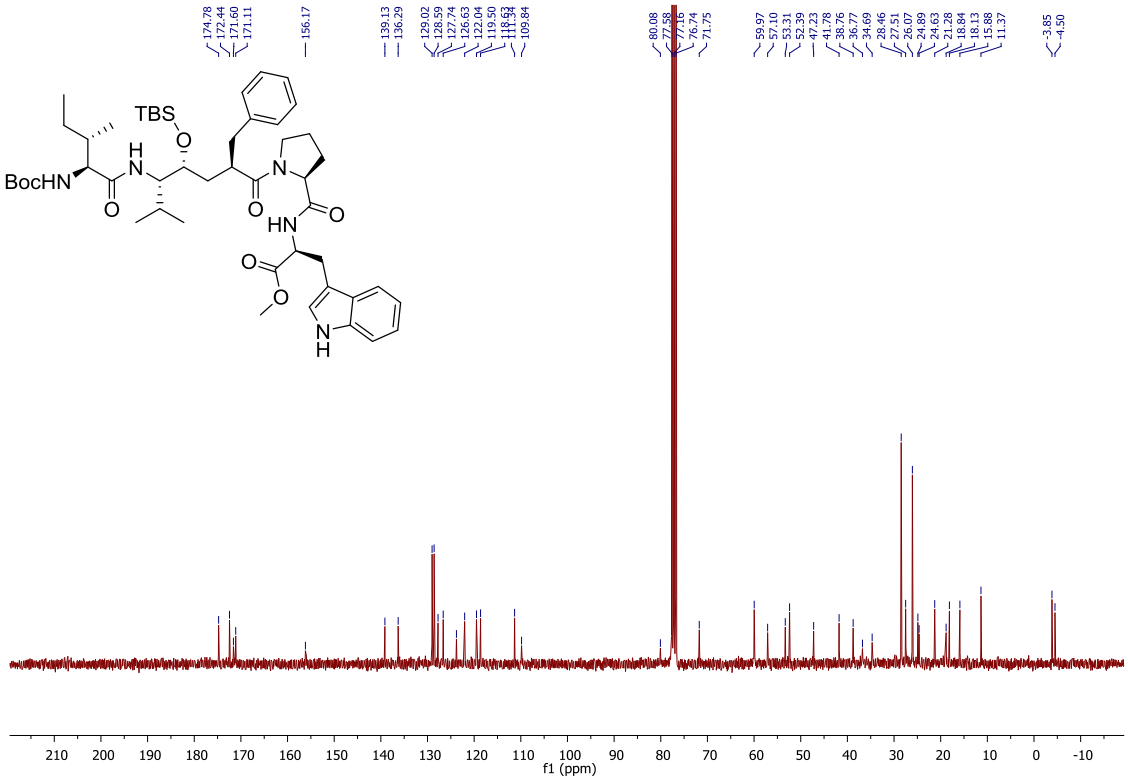


^{1}H NMR spectrum of Methyl ((2*R*,4*R*,5*S*)-2-benzyl-5-((*tert*-butoxycarbonyl)-amino)-4-((*tert*-butyldimethylsilyl)oxy)-6-methylheptanoyl)-L-prolyl-L-tryptophanate (Boc-RGSA-Pro-Trp-OMe) (23)

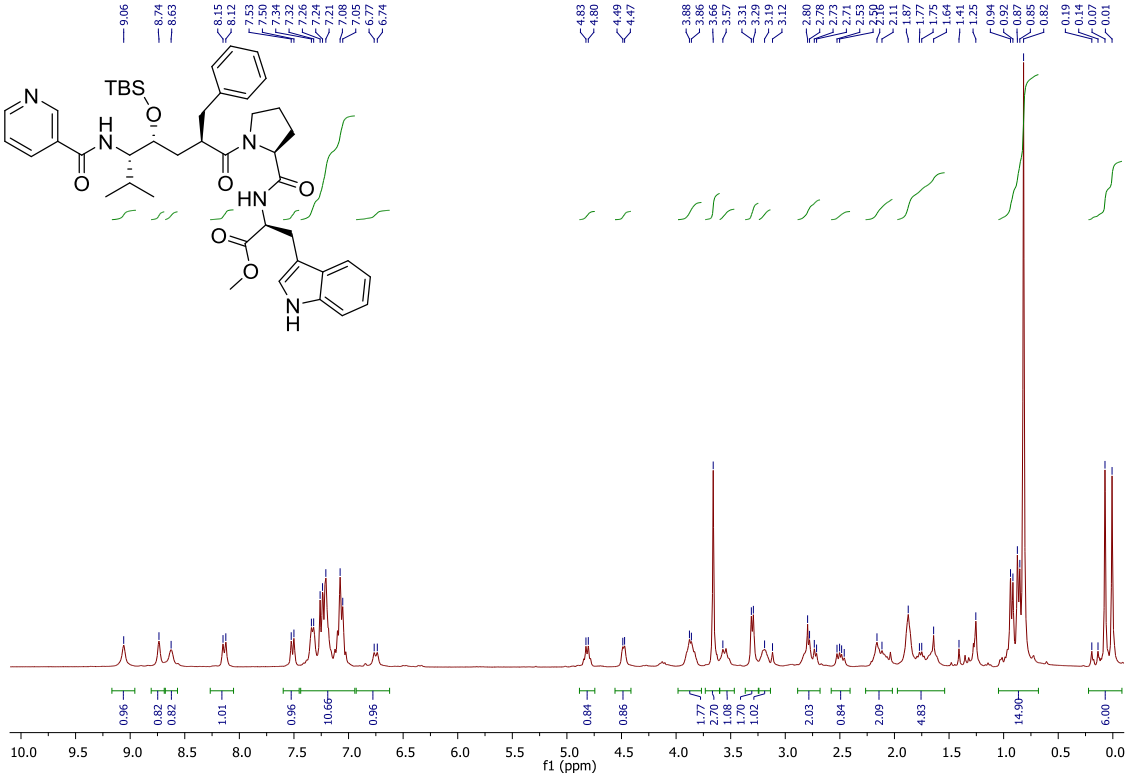


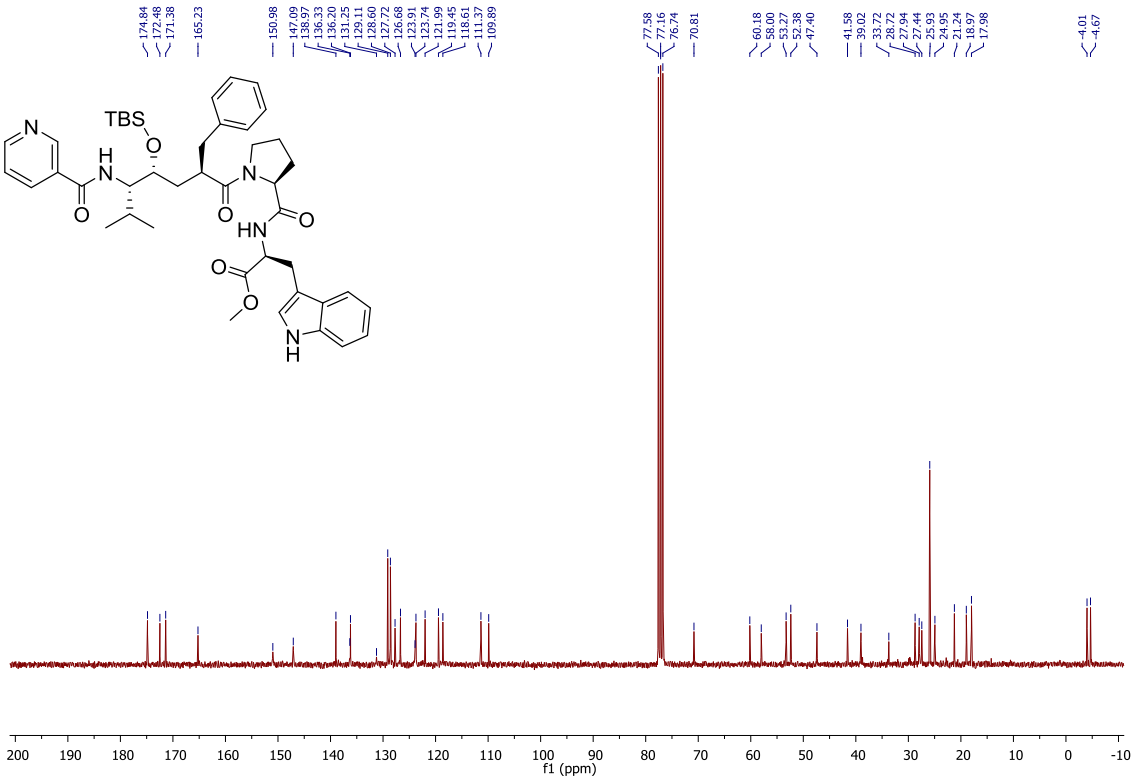
^{1}H and ^{13}C NMR spectra of Methyl ((2*R*,4*R*,5*S*)-2-benzyl-5-((2*S*,3*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylpentanamido)-4-((*tert*-butyldimethylsilyl)-oxy)-6-methylheptanoyl)-L-prolyl-L-tryptophanate (protected Ile-*HER*) (24)



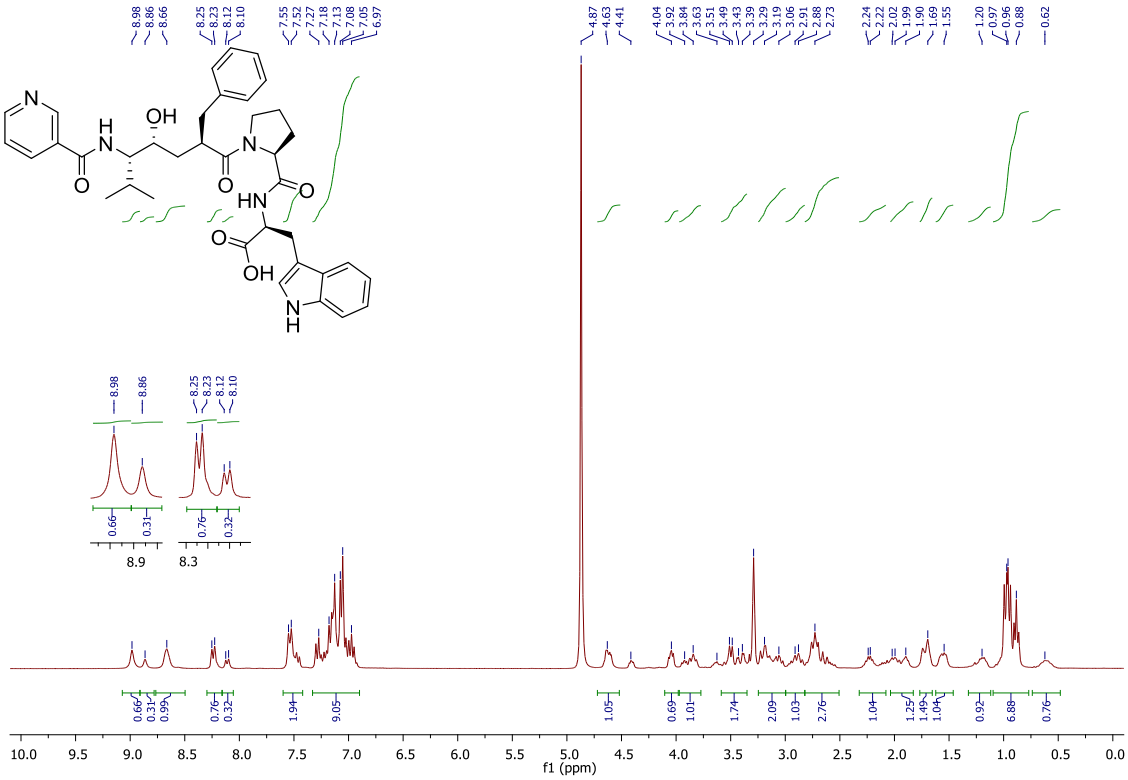


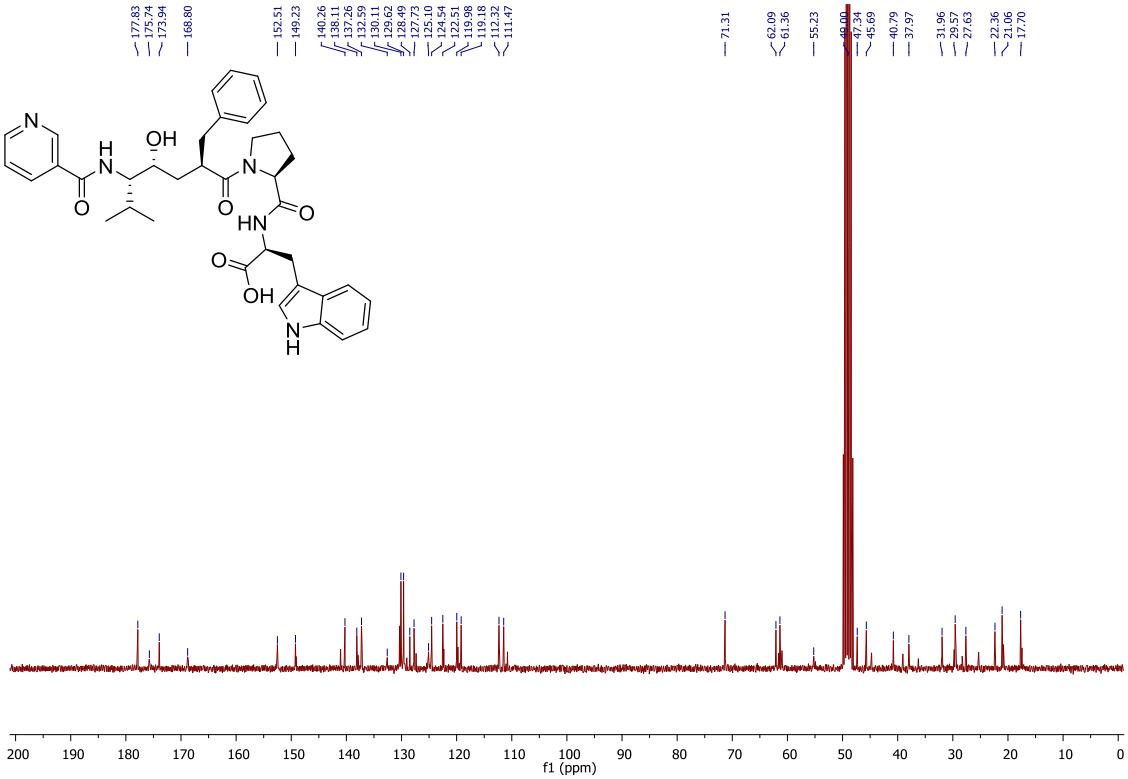
^{1}H and ^{13}C NMR spectra of Methyl ((2*R*,4*R*,5*S*)-2-benzyl-4-((*tert*-butyl-dimethylsilyl)oxy)-6-methyl-5-(nicotinamido)heptanoyl)-L-prolyl-L-tryptophanate (protected B$_3$-*HER*) (26)



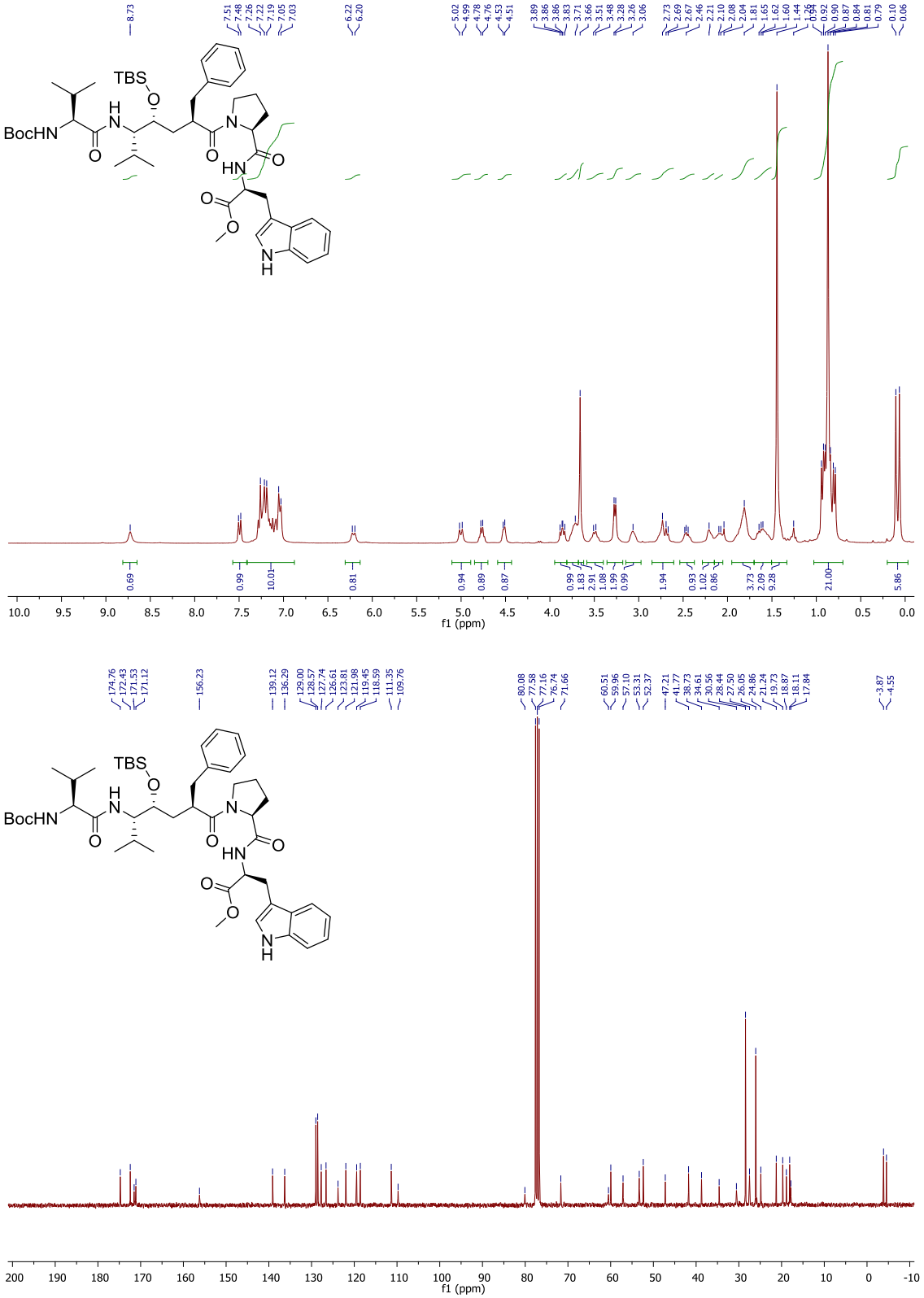


^{1}H and ^{13}C NMR spectra of ((2*R*,4*R*,5*S*)-2-Benzyl-4-hydroxy-6-methyl-5-(nicotinamido)heptanoyl)-L-prolyl-L-tryptophan (B_3-*HER*)

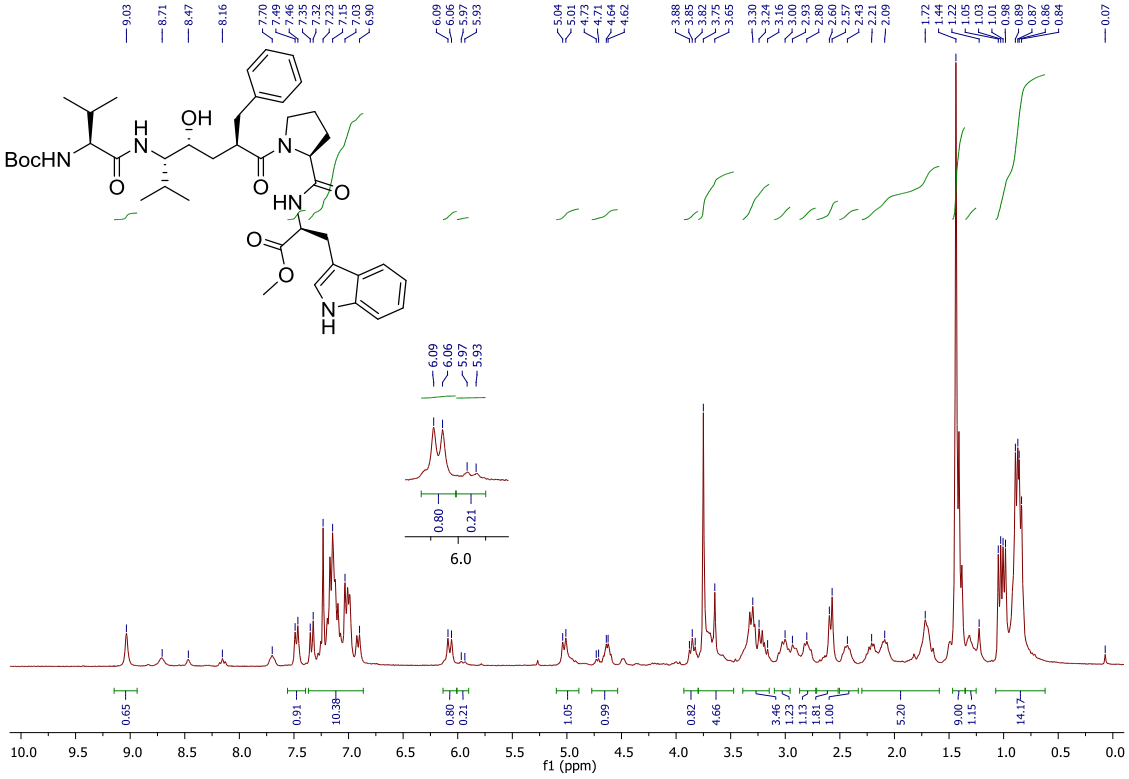


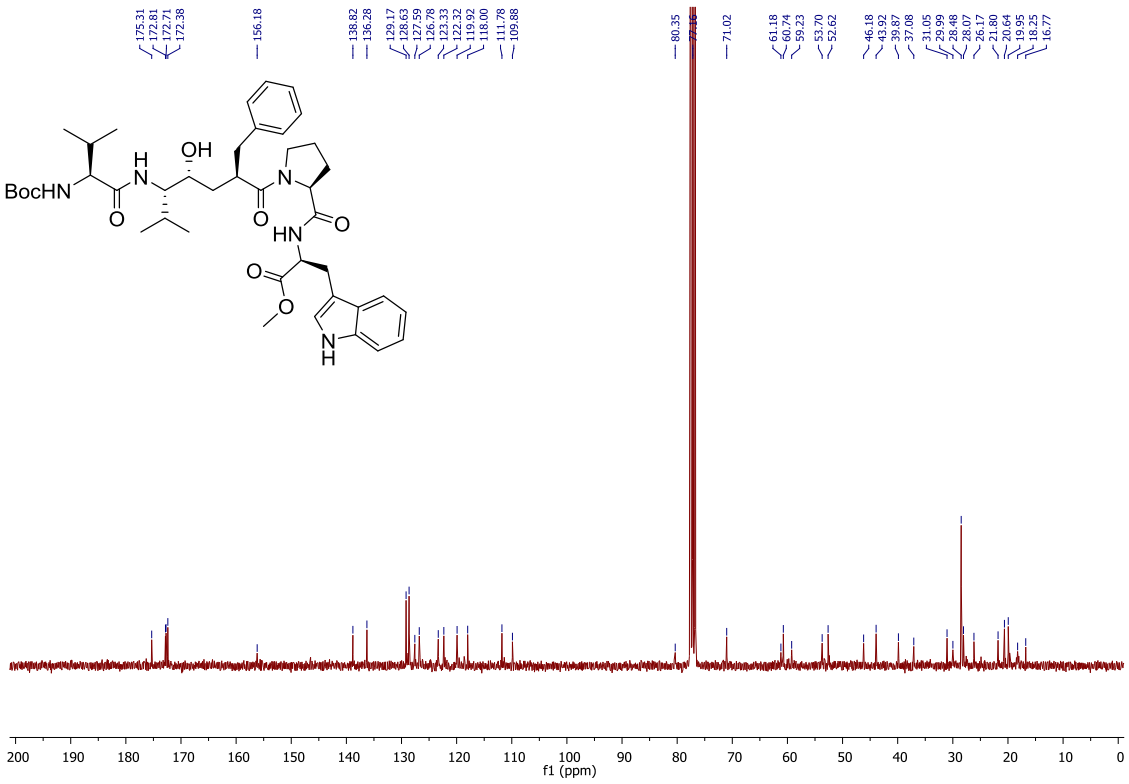


^{1}H and ^{13}C NMR spectra of Methyl ((2*R*,4*R*,5*S*)-2-benzyl-5-((*S*)-2-(((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-4-((*tert*-butyldimethylsilyl)-oxy)-6-methylheptanoyl)-L-prolyl-L-tryptophanate (fully protected *HER*) (28)

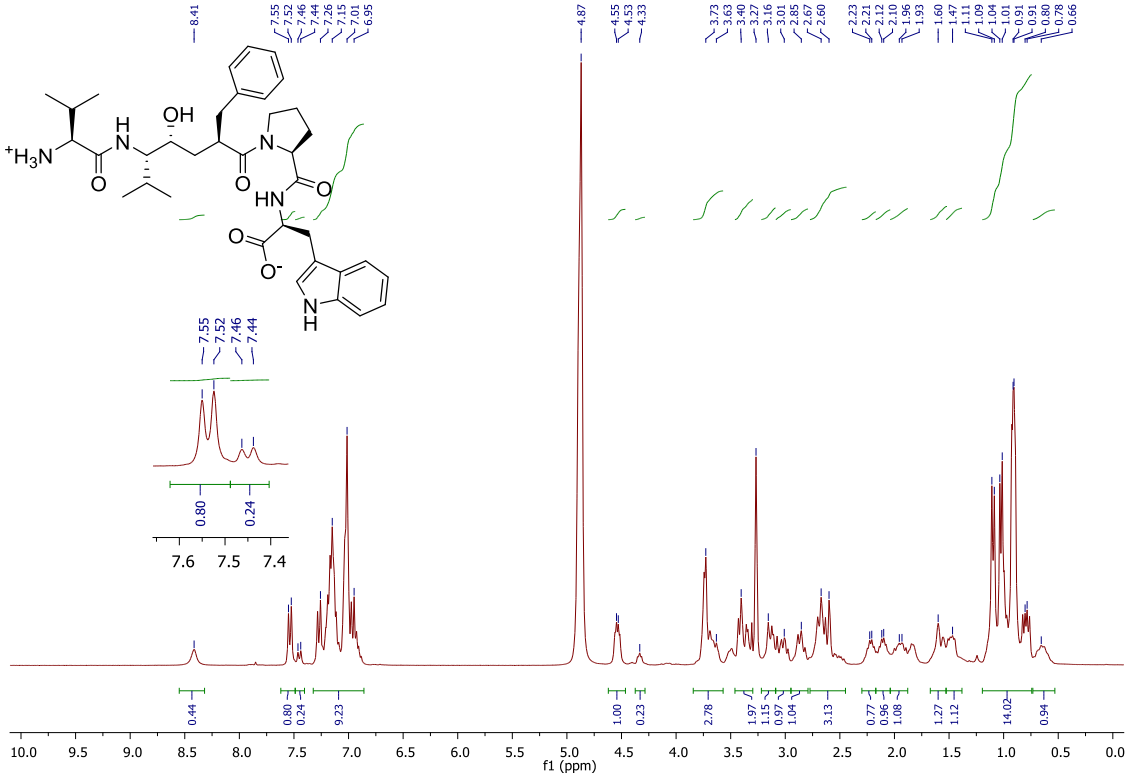


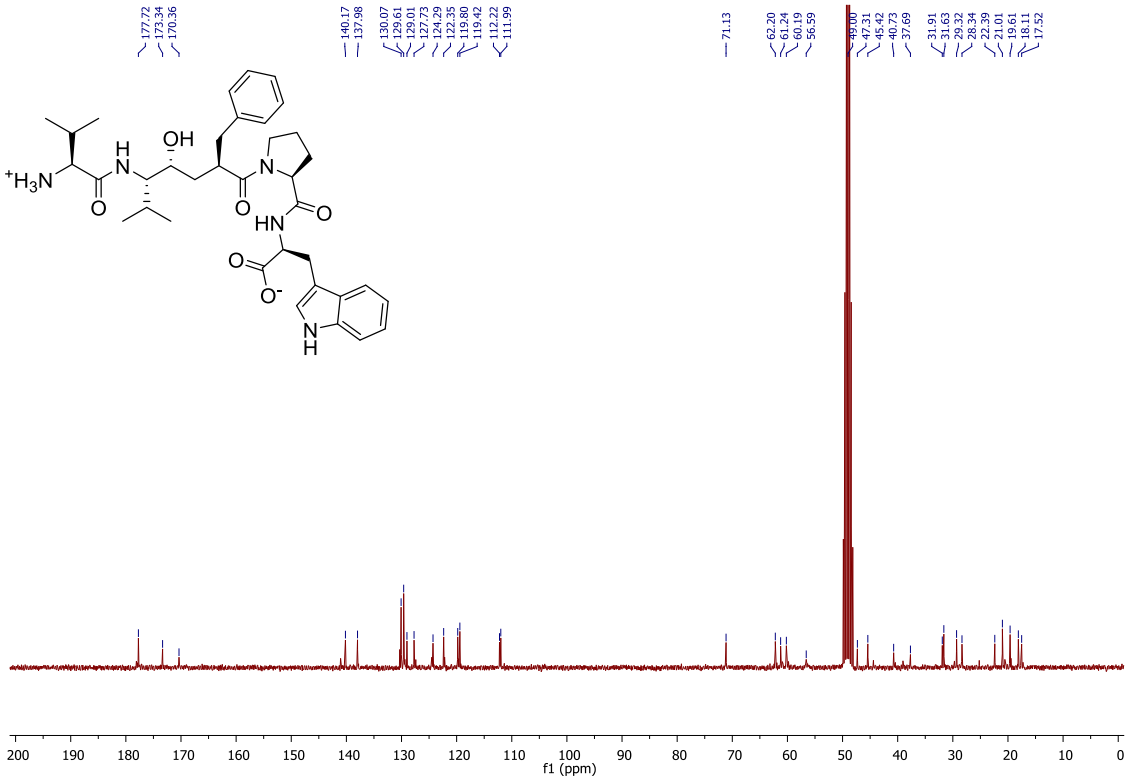
^{1}H and ^{13}C NMR spectra of Methyl ((2*R*,4*R*,5*S*)-2-benzyl-5-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-4-hydroxy-6-methylheptanoyl)-L-prolyl-L-tryptophanate (protected *HER*) (29)



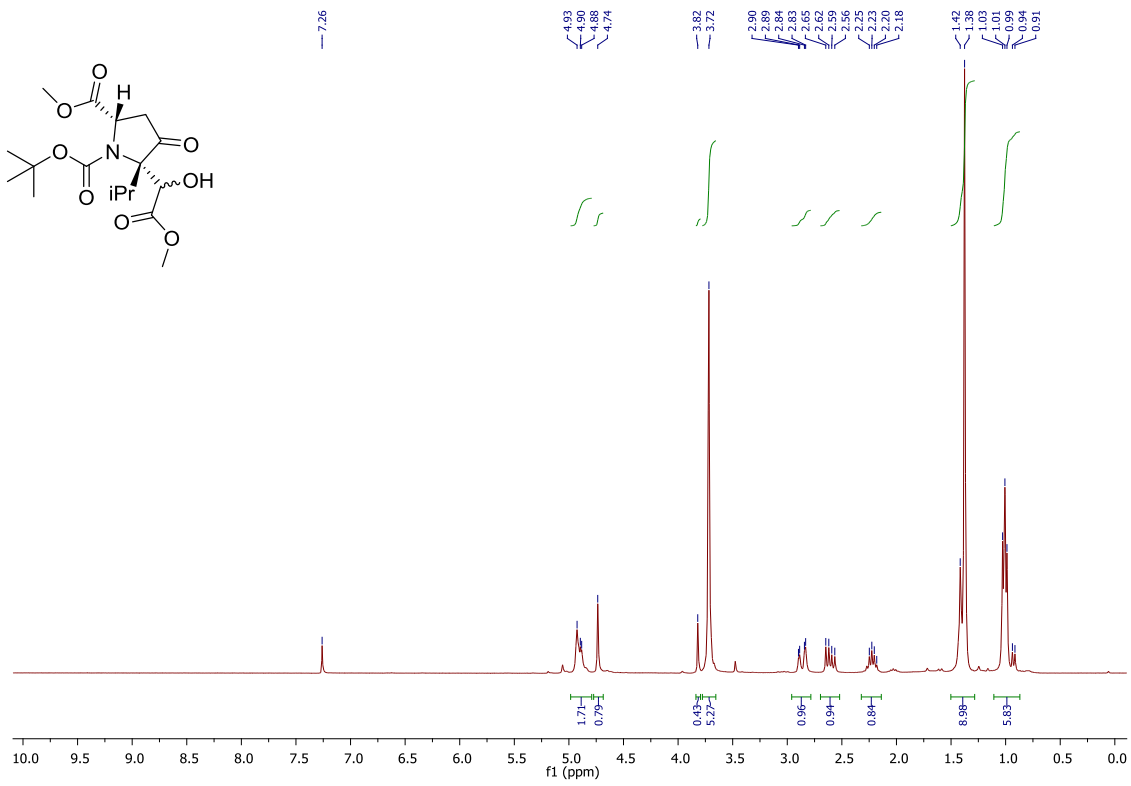


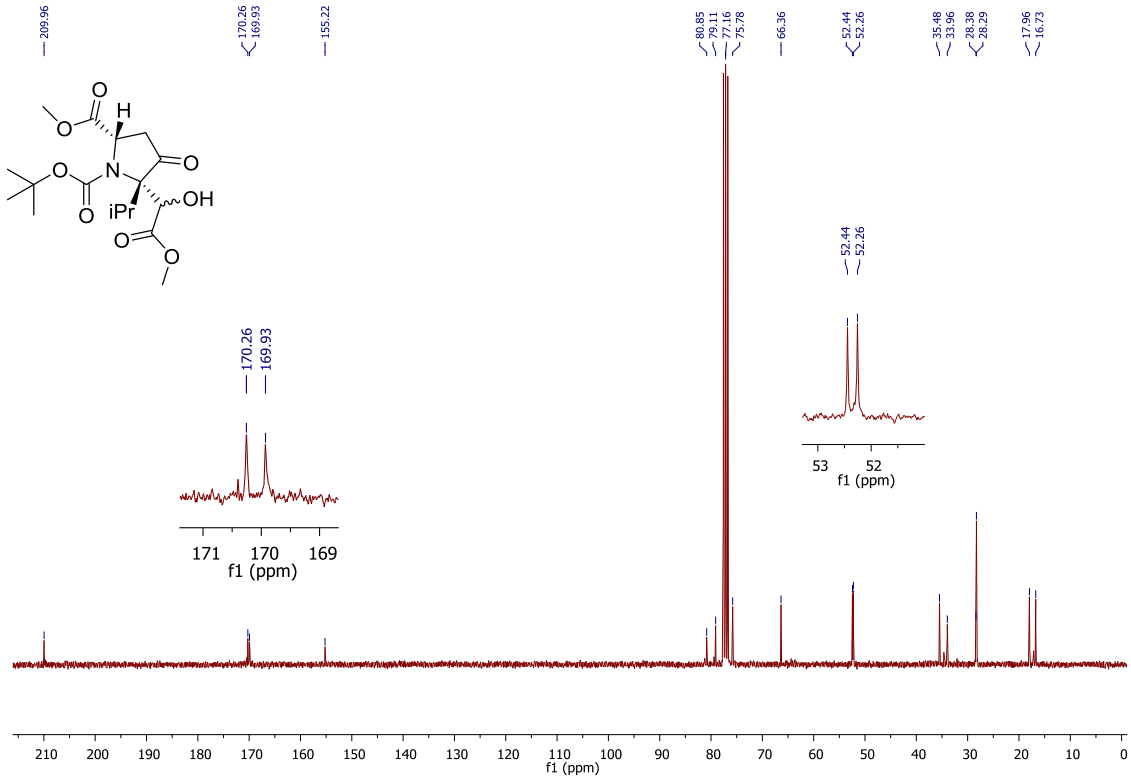
^{1}H and ^{13}C NMR spectra of ((2*R*,4*R*,5*S*)-5-((*S*)-2-Ammonio-3-methylbutan-amido)-2-benzyl-4-hydroxy-6-methylheptanoyl)-L-prolyl-L-tryptophanate (*HER*) (30)





^{1}H and ^{13}C NMR spectra of 1-(*tert*-Butyl) 2-methyl (2*S*,5*S*)-5-(1-hydroxy-2-methoxy-2-oxoethyl)-5-isopropyl-4-oxopyrrolidine-1,2-dicarboxylate (31)





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Danke!

AFFIDAVIT

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Graz, 14.6.2016

Date

Signature