Synthesis and Conformational Analysis of Functionalized Collagen Triple Helices

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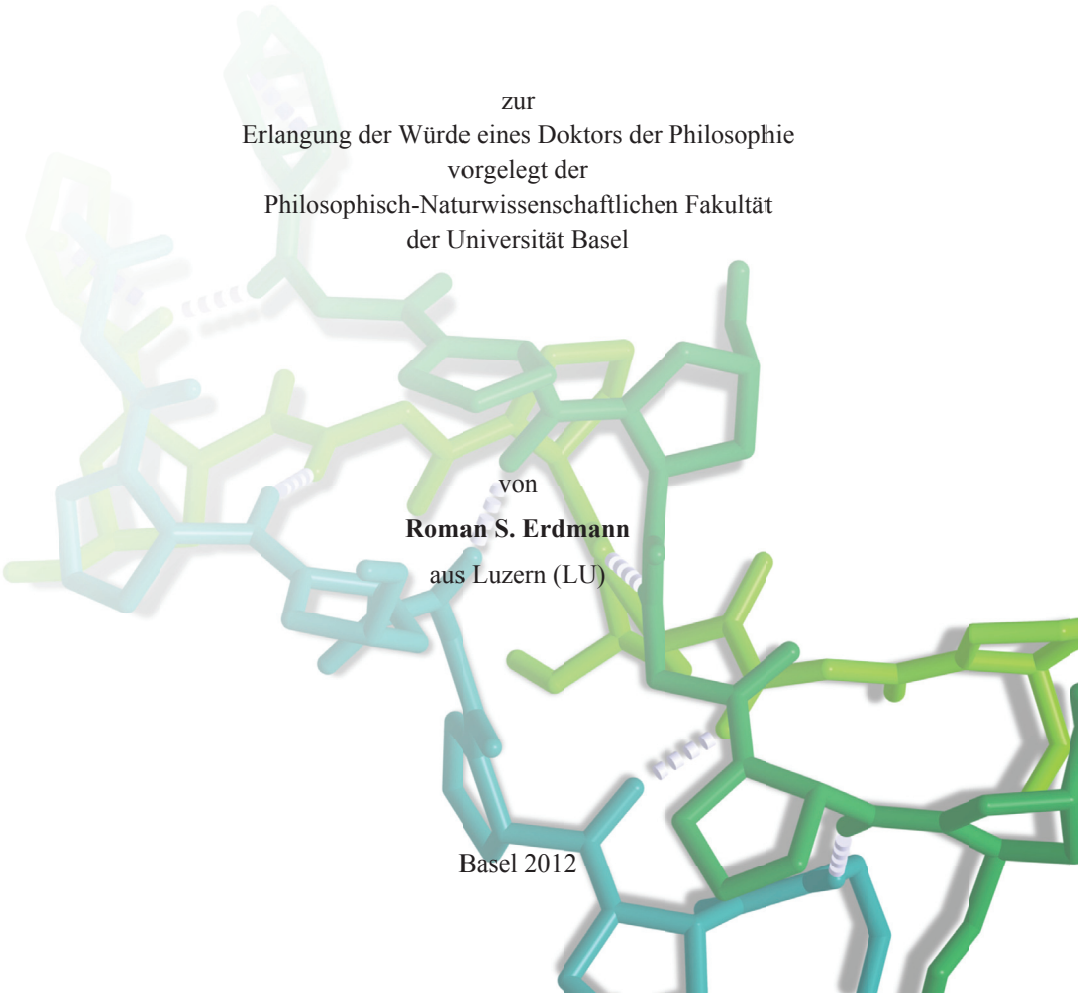

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Basel 2012

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- R. S. Erdmann, H. Wennemers, "*Functionalizable Collagen Model Peptides*"
  J. Am. Chem. Soc. **2010**, *132*, 13957–13959.

- R. S. Erdmann, H. Wennemers "*Synthesis of Fmoc-Pro-Hyp(TBDPS)-Gly-OH and its Application as a Versatile Building Block for the Preparation of Collagen Model Peptides*"
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  Bordeaux, France. January 29 - February 3, 2012.
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In liebevoller Erinnerung an Opa

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Short Summary

In this thesis a “click chemistry” and reduction/acylation approach is presented to functionalize azidoproline containing collagen model peptides. With these approaches moieties ranging from a formamide to triazoles bearing monosaccharide were attached to collagen triple helices. Functionalized triple helices with stabilities up to the extent of triple helices containing only natural amino acids could be achieved. This is important for the development of functional collagen based materials. Furthermore, by varying the position of the incorporated functional group, the stereochemistry at the attachment point of the functional group and the nature of the functional group a deeper insight into the factors that are responsible for the triple helical stability of collagen was gained. The influence of proline ring puckering, the conformation of Xaa-Pro amide bonds, intramolecular H-bonds, steric effects, charges and solvation on the conformational stability of the collagen triple helix was investigated. Additionally, acetylated dimethylamide proline derivatives were investigated. These could be used, instead of acetylated methylester proline derivatives, as models for the conformation of proline residues within biopolymers.

Kurzzusammenfassung

In der vorliegenden Arbeit wird eine “Click Chemie” und Reduktion/Acylierungs Strategie zur Funtionalisierung von azidoprolinhaltigen Collagen Modell Peptiden vorgestellt. Mit diesen Verfahren konnten Einheiten wie Formamide und mit monosaccharidversehene Triazole eingeführt werden. Die so erhaltenen Collagen Triplehelices weisen Stabilitäten auf, die teilweise bis an die Stabilität von Helices welche ausschliesslich aus natürlichen Aminosäuren bestehen, heranreichen. Diese Erkentnisse sind wichtig für die Entwicklung von funktionellen, collagenbasierten Materialien. Desweiteren wurde durch die Variation von der funktionalisierten Stelle, der Stereochemie and der funktionalisierten Stelle, sowie der Art der eingeführten Einheit ein tieferer Einbilck in die Faktoren gewonnen, welche für die triplehelicale Stabilität von Collagen verantwortlich sind. Es wurden die Einflüsse der Prolinringfaltung, der Konformation der Xaa-Pro Amidbindung, intramolekularer Wasserstoffbrücken, sterischer Effekte, Ladungen und Hydratisierung auf die konformationelle Stabilität der Collagen Triplehelix untersucht. Zusätzlich, wurden acetylierte Dimethylamide von Prolinderivaten untersucht. Diese könnten anstatt acetylierten Metylester von Prolinderivaten als Modelle für die Konformation von Prolin Einheiten in Biopolymeren dienen.

Table of contents

I. Introduction

1. Occurrence and Function of Collagen in Nature

Collagen is the most abundant protein in mammals.[1] One third of the human proteome consists of collagen and it accounts for three quarters of the dry weight of skin. It is mainly found in the extracellular matrix where it serves as a structural scaffold in e.g. skin, bones, tendons, and cartilages.[1] Additionally, it is involved in the modulation of cellular activities. Collagen is, for example, crucial for the homeostasis of connective tissue.[2,3] There are 29 different types of collagen within the human proteome.[1,3] These are classified corresponding to their structure in 8 different classes. A big class are the fibrillar collagens which commonly occur in skin, bone, cartilage, and tendon.[1,3] Fibril-associated collagens with interrupted triple helices (FACIT collagens) also form a large class. They occur in cartilage, cornea, dermis, tendon, and some intestines. Membrane associated collagens with interrupted triple helices (MACIT collagens) are the third biggest class and mainly occur in dermis, brain, heart, and eye. Other classes are the basement membrane collagens, short chain collagens, anchoring fibril collagen, multiplexins, and collagen type VI. The triple helices of these classes differ in their amino acid and single strand composition. The triple helices can be formed by three identical polypeptide strands (homotrimers) or two or three different single strands (ABB and ABC heterotrimers, respectively).[1,3]

2. Structure of the Collagen Triple Helix

Collagen consists of three left handed polyproline II-like single strands that are coiled around each other to a triple helix. The triple helices further assemble into fibrils, fibres and bundles (Figure 2.1).[4]

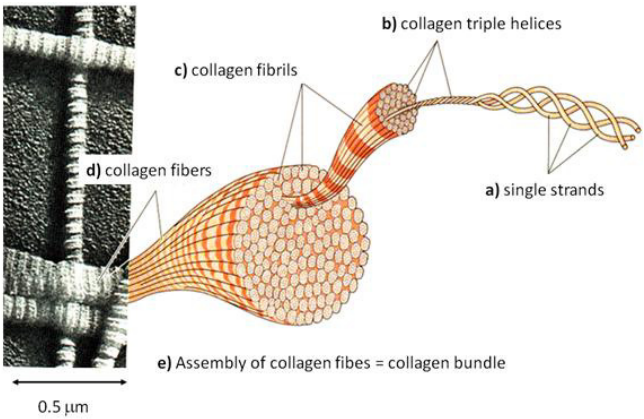


FIGURE 2.1. TEM image of collagen fibers (left); assembly of collagen fibers from collagen single strands (right).[5]

Within the right handed triple helices the single strands are staggered by one residue.[1,3] The single strands exhibit a repetitive Xaa-Yaa-Gly (Gly=glycine) pattern with about 330 of these repeating units. Throughout the whole single strand all amide bonds adopt the *trans* conformation. Furthermore, the peptide chains are held together by hydrogen bonds formed between the amide hydrogen of a glycine residue and the carbonyl oxygen of the amino acid in the Xaa position. Proline (Pro) is with a frequency of 28% the most abundant amino acid in the Xaa position.[6] (4*R*)Hydroxyproline ((4*R*)Hyp) is the most common amino acid in the Yaa position with a abundance of 38%.[6] Additionally Pro-(4*R*)Hyp-Gly is with approximately 11% occurrence the most abundant triplet. X-ray crystal structures of collagen triple helices showed that proline residues in the Xaa position adopt a C(4)-*endo* ring pucker whereas (4*R*)hydroxyproline in the Yaa position adopts a C(4)-*exo* ring pucker (Figure 2.2).[7] Every third residue is glycine since this amino acid is located in the core of the triple helix, close to its axis, where no bulkier amino acids are tolerated because of the very limited space.[1-3,8-13]

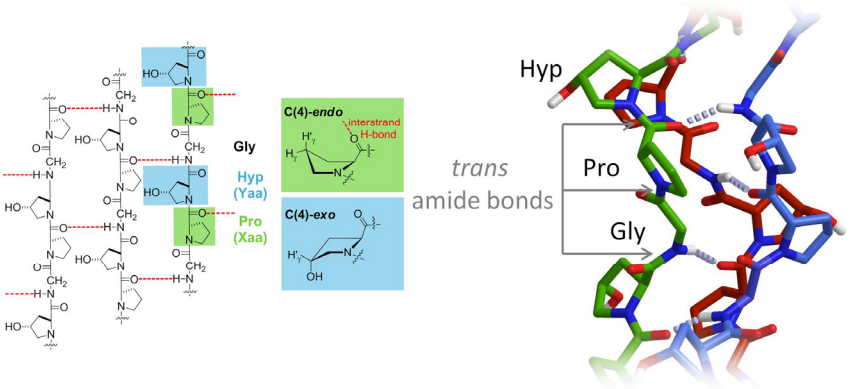


FIGURE 2.2. Structure of the collagen single strands (left). Ring puckering of the imino acids in the Xaa and Yaa position (middle). Triple helical structure of collagen (right).

Ten tripeptidic repeating units form three left-handed turns within the single strands. Thus these helices are called 10/3 helices. A complete turn of the right-handed triple helix contains 3x30 amino acids and spans a length of 8.6 nm (Figure 2.3).[3]

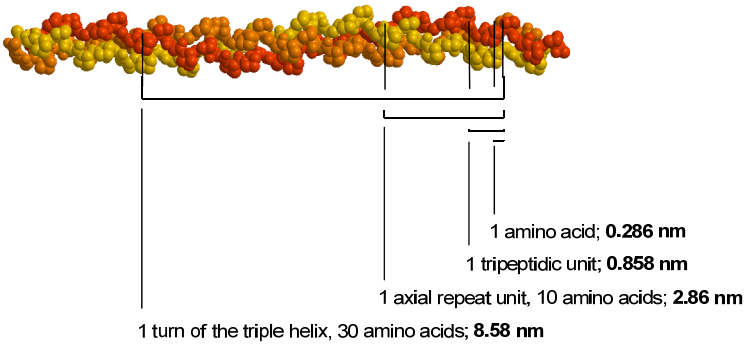


FIGURE 2.3. Dimensions in a 10/3 collagen triple helix.

However, with an increasing imino acid content of the single strands their structure slightly changes into 7/2 helices. Also heterogenic triple helices consisting of different single strands are known, but their structure does not differ significantly from the homo trimers.[3,11]

3. Collagen Biosynthesis

The biosynthesis of collagen starts with the translation of the according mRNA resulting in a precursor called procollagen consisting of N- and C-terminal propeptides (red and blue in Figure 3.1) and a mid section which will later form the triple helix (black in Figure 3.1).[14] Some of the lysine residues and almost all proline residues in the Yaa position of the mid section of procollagen are posttranslationally hydroxylated by prolyl hydroxylases and lysyl hydroxylase. Three procollagen strands nucleate at the C-termini allowing protein disulfide isomerase to link the three chains by disulfide bonds. Subsequently, the peptides fold in a zipper-like fashion with the *cis-trans* isomerisation of Xaa-Hyp amide bonds as the rate limiting step.[15] Finally, procollagen proteinases cleave the C- and N-terminal regions (Figure 3.1).[16]

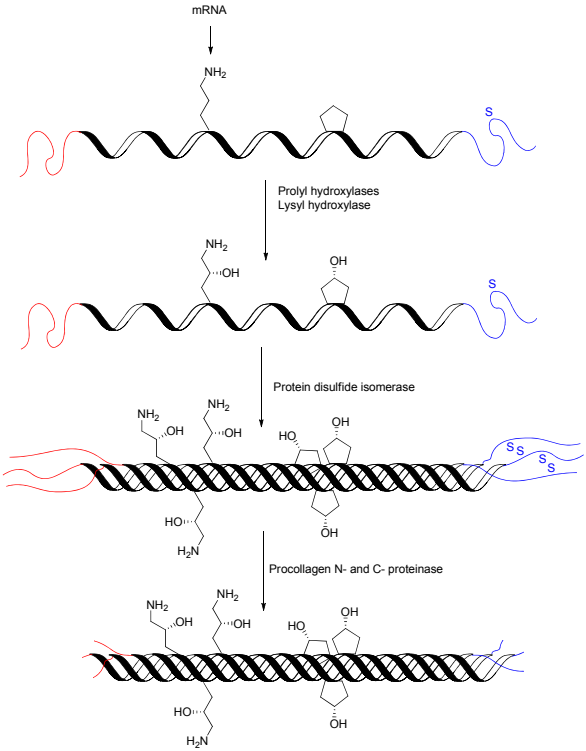


FIGURE 3.1. Biosynthesis of the collagen triple helix. In contrast to the proline residues in the Yaa position only few lysines are hydroxylated.[14-16]

The resulting tropocollagen triple helices further self-assemble into collagen microfibrils, which are then covalently crosslinked to collagen fibers. Already at the stage of the microfibrils the typical D-periodic pattern of alternating darker and brighter bands can be observed in TEM images which arise from the different density of the fibrils due to gap and overlap sections (Figure 3.2).[17]

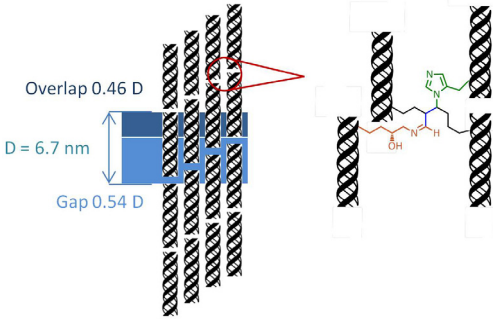


FIGURE 3.2. Alignment of the triple helices within a collagen microfibril.[17]

Lysine and hydroxylysine residues of the tropocollagen are oxidized by lysyl oxidase to allysine, which contains an aldehyde. Two allysine residues of different triple helices can undergo an aldol condensation forming a structure of two crosslinked triple helices. In a 1,4-addition of a histidine residue of a third triple helix the supramolecular structure is further extended. Finally, the resulting aldehyde is able to form an imine with a hydroxylysine residue of a forth triple helix leading to a crosslinked structure of four collagen triple helices (Figure 3.3).[18] This crosslinking is mainly responsible for the tensile strength of collagen.[16]

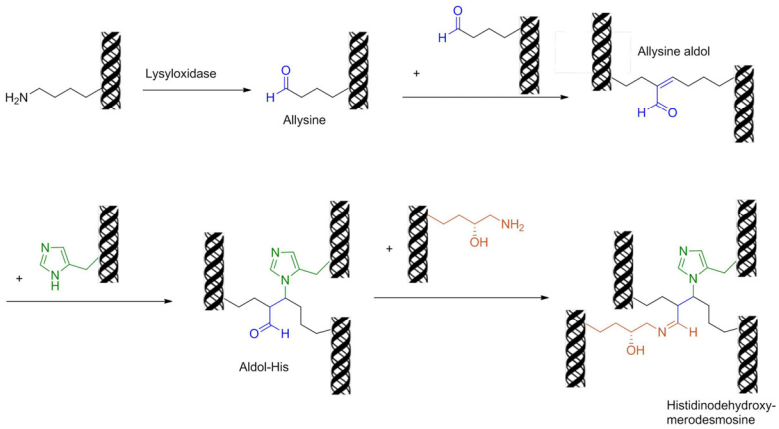


FIGURE 3.3. Crosslinking of up to four triple helices in microfibrils initiated by lysyl oxidase.[18]

3.1 Diseases Related to Defaults in the Collagen Biosynthesis

Errors in the biosynthesis of collagen can lead to severe consequences. Point mutations in the genes encoding collagen often result in the incorporation of an amino acid other than glycine in the third position causing the brittle bone disease (osteogenesis imperfecta, OI).[7,11,19] The mutated amino acid is too bulky to be placed inside the triple helix along its axis. Thus, it causes steric clashes and leads to the destabilization of the triple helix with the consequence of a lower stability of bones. Depending on the location of the mutated residue the brittle bone disease can already be prenatelly lethal.[20]

Other diseases linked to malfunction in the collagen synthesis are lathyrism and the Ehlers-Danlos syndrome.[4,19] In these diseases lysyl oxidase is inhibited or defects in the procollagen proteinases prevent the cleavage of the propeptides.

Also Scurvy, a well know disease among seamen in the time of exploration, is linked to faults in the collagen structure. Diet deficient in vitamin C, which is a cofactor of prolyl hydroxylase, leads to the destabilization of the triple helix and results in skin lesions, blood vessel fragility and poor wound healing. These symptoms were often observed as gum bleeding.[1,4] This disease demonstrates the importance of the hydroxylation of proline residues for the stability of collagen.

4. Stability of the Collagen Triple Helix

4.1 Hydrogen Bond Network or Stereoelectronic Effects?

For a long time the stabilizing effect of hydroxyproline for the collagen triple helix was attributed to a hydrogen bond network between hydroxy groups of Hyp, bridging water molecules of the solvent, and the peptide backbone holding the triple helix together.[7,11] This network was observed in x-ray crystal structures of triple helices formed by collagen model peptides.[7] Raines and co-workers argued that building such a hydrogen bond network would go in line with enormous entropic costs.[21] Additionally, the hydrogen bond theory does not explain the additional stabilization observed in less protic solvents such as methanol.[22] They suggested that the inductive effect of the hydroxy group is involved in the stabilization of the collagen triple helix. To test this hypothesis they compared the stabilizing effect of (4*R*)Hyp and (4*R*)fluoroproline ((4*R*)Flp) within collagen triple helices formed by the peptides H-(Pro-(4*R*)Hyp-Gly)$_{10}$-OH and H-(Pro-(4*R*)Flp-Gly)$_{10}$-OH.[21] In contrast to the hydroxy group the fluoro group is not able to form hydrogen bonds but is more electron withdrawing. The experiments showed that (4*R*)Flp containing collagen model peptides (CMPs) formed conformationally more stable triple helices than the Hyp containing analogues, clearly undermining the hydrogen bond network theory.[21,23-25]

The higher stability of the Flp containing CMPs compared to those containing Hyp was attributed to a stronger gauche effect in Flp than Hyp.[25] This gauche effect has two consequences for the conformational properties of the proline residues and therewith for triple helix stability. On the one hand it strengthens the C(4)-*exo* ring pucker of the proline residue which is desired in the Yaa position and on the other hand it promotes the *trans* conformation of the Pro-Flp amide bond which is necessary for a stable collagen triple helices.[1,10,24-26] The exact interplay of these factors are explained in detail in the next chapters.

4.2 The Gauche Effect

IUPAC defines the gauche effect as the stabilization of the gauche conformer compared to the anti conformer of compounds containing two vicinal electron withdrawing groups.[27]

A typical example is the fluoro gauche effect that determines the conformation of 1,2-difluoroethane in which the gauche conformer is by 3.7 kJ mol^{-1} more stable than the anti

conformer. Consequently, a 3.5 fold excess of the gauche conformer relative to the anti conformer is observed (Figure 4.1).[28,29]

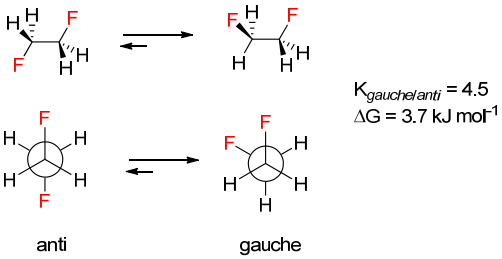


FIGURE 4.1. Anti and gauche conformations of 1,2-difluoroethane with their Newman-projections.

The preference for the gauche conformer can be explained with a σ→σ* interaction. In a gauche conformation of FCH_2-CH_2F the C-H σ-bond orbitals interact with the C-F σ*-antibonding orbital whereas in the anti conformation a C-F σ-bond orbital acts as a weaker donor in this interaction instead of a stronger C-H σ-bond orbital (Figure 4.2 top).[28,29]

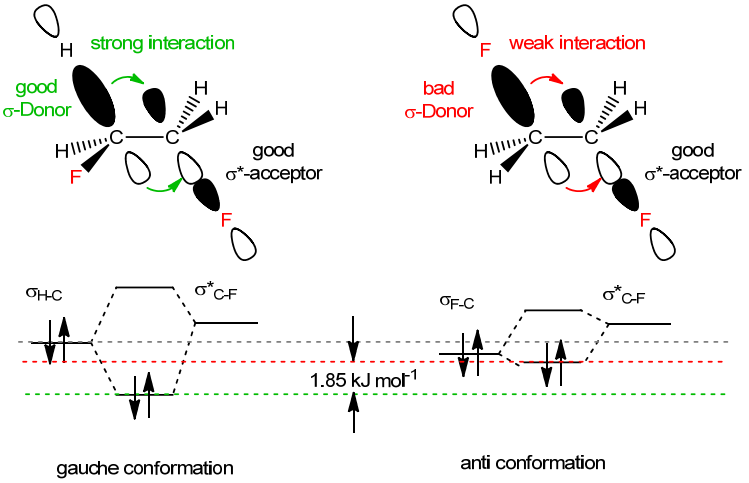


FIGURE 4.2. The fluoro gauche effect arises from a favorable interaction.

Since the energy level of a $\sigma_{H\text{-}C}$ bond orbital is closer to the $\sigma_{C\text{-}F}$* antibond orbital than the $\sigma_{F\text{-}C}$ bond orbital, there is a better orbital overlap resulting in a new molecular orbital which is

lower in energy compared to the molecular orbital formed by a $\sigma_{F\text{-}C} \rightarrow \sigma_{C\text{-}F}^*$ interaction (Figure 4.2 bottom).

The stronger $\sigma_{H\text{-}C} \rightarrow \sigma_{C\text{-}F}^*$ interaction is only possible twice in the gauche conformation and thus this conformation is predominant.

4.3 Impact of the gauche effect on the conformation of proline derivatives

Many proline derivatives substituted with an electron withdrawing group at the 4-position have been investigated over the past years.[24,30-35] The most prominent examples amongst them are probably hydroxy-, fluoro-, and azidoprolines.[30,35,36] In all cases their preferred conformations are dictated by a gauche effect. NMR investigations on acetylated (4*R*)-substituted proline methyl esters revealed that they all adopt a C(4)-*exo* ring pucker.[24,30-35] In contrast the diastereomeric (4*S*)-derivatives adopt a C(4)-*endo* ring pucker. In both diastereoisomers the electron withdrawing group is in a pseudo axial orientation.[24,30-35] This preference for this orientation is due to a gauche effect exerted by the electron withdrawing group. Only in this conformation the $\sigma^*_{C\text{-}EWG}$ and $\sigma^*_{C\text{-}NAc}$ orbitals are aligned in a near 180° angle to the adjacent vicinal $\sigma_{C\text{-}H}$ orbital allowing for an interaction with each other. Figure 4.3 shows how the gauche effect defines the ring pucker using the example of azidoproline (EWG=N_3): The gauche conformation of the azido group and the amide group lead to a C(4)-*exo* ring pucker in Ac-(4*R*)Azp-OMe and a C(4)-*endo* ring pucker in Ac-(4*S*)Azp-OMe.[30]

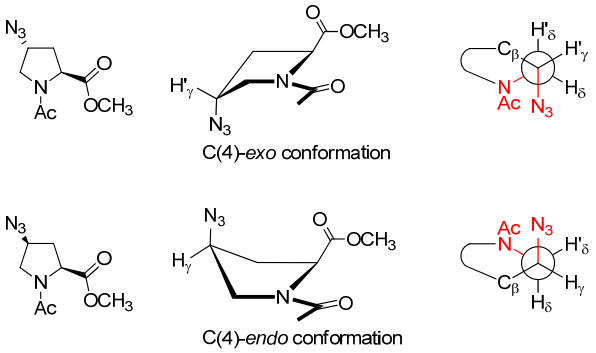


FIGURE 4.3. Preferred conformations of Ac-(4*R*)Azp-OMe and Ac-(4*S*)Azp-OMe with their Newman-projection along the C_γ-C_δ bond.

A second observation is the significant difference in the preference for a *trans* amide bond. The *cis*:*trans* ratio of the (4*R*)-diastereoisomers are considerably higher than those of the (4*S*)-diastereoisomers whereas the ratio of the unsubstituted proline is in between (Table 4.1).

TABLE 4.1. $K_{trans:cis}$ of acetylated methylesters of prolines with electron withdrawing substituents in the 4-position.

	$K_{trans:cis}$	
	(4*R*)	(4*S*)
Ac-Hyp-OMe	6.1	2.4
Ac-Azp-OMe	6.1	2.6
Ac-Flp-OMe	6.7	2.6

All values were measured by ^{1}H-NMR in D_2O at a concentration of 80 mM.

The higher *trans* fraction of the (4*R*)-diastereoisomers is explained with an n→π* interaction between a lone pair of the acetyl oxygen and the π* orbital of the methyl ester.[24,30] X-ray crystal structures of Ac-(4*R*)Azp-OMe and Ac-(4*R*)Flp-OMe support this explanation: In Ac-(4*R*)Azp-OMe the distance between the acetyl oxygen and the carbonyl carbon of the methyl ester is 2.85 Å and the dihedral angle between the acetyl oxygen and the carbonyl of the ester is 98° (Figure 4.4, left).[30] In Ac-(4*R*)Flp-OMe this distance is 2.71 Å and the dihedral angle is the same as in Ac-(4*R*)Azp-OMe.[35] This angle is in the range of the Bürgi-Dunitz trajectory of a nucleophile attacking a carbonyl.[37-40] The optimal angle and the short distance are indicative of the n→π* interaction.[37-40]

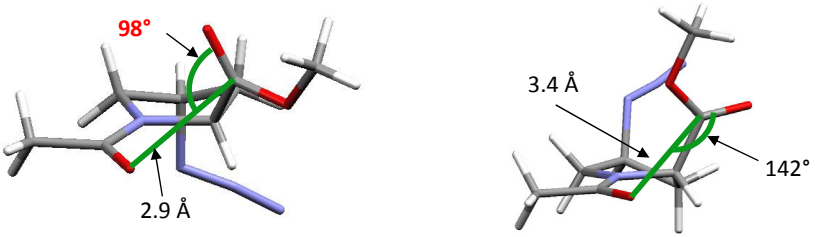


Figure 4.4. Crystal structure of Ac-(4*R*)Azp-OMe and lowest energy structure of Ac-(4*S*)Azp-OMe. Only the conformation of Ac-(4*R*)Azp-OMe allows for an n→π* interaction.

In Ac-(4*S*)Azp-OMe this distance is 3.37 Å and the angle is 142 ° which does not allow for such an interaction (Figure 4.4, right).[30] It was hypothesized that this unfavorable conformation for a n→π* interaction is caused by a repulsion of the pseudo axial azido group and the methyl ester, which leads to an orientation of the methyl ester that is unfavourable for a n→π*-interaction.[31]

4.4 The Stability of the Collagen Triple Helix relies on Stereoelectronic Effects

For investigations of the stability of the collagen triple helix collagen model peptides (CMPs) consisting of 18-30 residues with a high imino acid fraction in the Xaa and Yaa positions have proven useful.[1] Triple helix forming CMPs fold spontaneously in aqueous solutions and the temperature of the midpoint of the thermal transition of their unfolding (T_m value) is used as a measure for the stability of the triple helices.[33,41-54]

As mentioned in chapter 4.1, the Raines group used the 30mers H-(Pro-(4*R*)Hyp-Gly)$_{10}$-OH and H-(Pro-(4*R*)Flp-Gly)$_{10}$-OH to investigated the stabilizing role of hydroxyproline for the conformational stability of the collagen triple.[21] For Hyp containing triple helices a T_m of 69°C was determined whereas for Flp containing triple helices a T_m of 91°C was measured.[21] This demonstrated that the previously described hydrogen bond network[55] could not be responsible for the beneficial effect of hydroxyproline on the conformational stability of the collagen triple helix. Additionally, it demonstrated the higher stability of fluoroproline containing triple helices. This increased stability was assigned to a better preorganization of the single strands for triple helix formation.[1] The stronger gauche effect in fluoroproline compared to hydroxyproline leads to an even stronger preference for a C(4)-*exo* ring pucker which is desired in the Yaa position. Additionally it leads to a higher preference for a *trans* Flp-Gly amide bond which is required for triple helix formation/stability (Figure 4.5).[1,24,25]

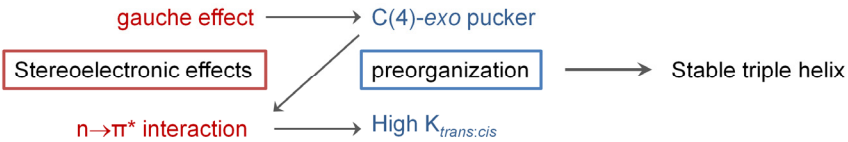


FIGURE 4.3. Interplay of the interactions that lead to a stable collagen triple helix.[1]

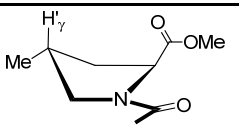
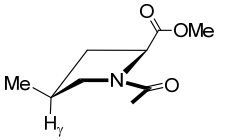
Later, the Raines group also investigated the influence of Flp in the Xaa position on the triple helix stability by comparing the CMPs with the sequences H-(Pro-Pro-Gly)$_7$-OH, H-((4*R*)Flp-Pro-Gly)$_7$-OH and H-((4*S*)Flp-Pro-Gly)$_7$-OH.[47] The peptide containing (4*S*)Flp in the Xaa position was the only one able to form a stable collagen triple helix with a T_m of 33°C. This was explained by comparing the conformational properties of the amino acids in the Xaa position with the requirements for this position.[1,47] In contrast to (4*R*)Flp and Pro only (4*S*)Flp has a strong preference for a C(4)-*endo* ring pucker which is the same that is adopted by the Pro residues in this position in natural collagen. The lower stability of a triple helix built from

H-((4*S*)Flp-Pro-Gly)$_7$-OH compared to H-(Pro-(4*R*)Flp-Gly)$_7$-OH was attributed to the weaker preference of (4*S*)Flp for a *trans* amide bond which is also required in the Xaa position.[10,24,47,56] A study on the CMP H-((4*S*)Flo-(4*R*)Flp-Gly)$_7$-OH which was not able to form stable triple helices due to steric clashes of the fluorine substituents demonstrated that the stabilizing effect of (4*S*)Flp in the Xaa and (4*R*)Flp in the Yaa position are not additive.[56] Related studies using CMPs containing the respective (4*R*)- and (4*S*)-configurated methoxy-,[32] mercapto-,[54] and chloroproline[33,57] derivatives support these conclusions.

5. Effect of 4-Methylprolines on the Stability of the Collagen Triple Helix

Raines and co-workers also showed that the conformational stability of collagen can be influenced by 4-methylprolines (Mep).[45] In these proline derivatives the ring pucker is not influenced by stereoelectronic effects but by a steric effect which causes the methyl group to be pseudo equatorial. Consequently Ac-(4*R*)Mep-OMe adopts a C(4)-*endo* ring pucker whereas its (4*S*)-diastereoisomer adopts a C(4)-*exo* ring pucker (Table 5.1).[45]

TABLE 5.1. Conformational properties of acetylated (4*R*)- and (4*S*)-methylproline methylester.[45]

	$K_{trans:cis}$	Ring pucker
Ac-(4*R*)Mep-OMe	3.7	 C(4)-*endo*
Ac-(4*S*)Mep-OMe	7.4	 C(4)-*exo*

Incorporation of (4*R*)Mep into the Xaa position (H-((4*R*)Mep-Pro-Gly)$_7$-OH) and (4*S*)Mep into the Yaa position (H-(Pro-(4*S*)Mep-Gly)$_7$-OH) lead to CMPs forming collagen triple helices with T_m values of 13°C and 29°C, respectively.[45] These results were explained with

the matching C(4)-*exo* ring pucker of (4*S*)Mep in the Yaa position and the matching ring pucker of (4*R*)Mep in the Xaa position. The different T_m-values of the triple helices formed of these CMPs were explained by the different preferences of a *trans* amide bond of (4*R*)Mep and (4*S*)Mep. In contrast to fluoroprolines the stabilizing effects of methylprolines are additive when the matching residues are incorporated in the Xaa and Yaa position.[45,56]

The hybrid peptide H-(Mep-Flp-Gly)$_7$-OH forms the most stable triple helix formed by peptides consisting of only 21 residues reported so far (T_m=58°C).[58] The high stability of this peptide further endorses the theory that proline derivatives with a high *trans* amide bond preference and matching ring puckers stabilize the collagen triple helix.[58]

6. Stability of CMPs containing all natural amino acids

The Pro-Hyp-Gly repeating motive is the most abundant but by far not the only one natural collagen. Many other canonical amino acids are found in natural collagen (Table 6.1).[51]

TABLE 6.1. Occurrence of different Xaa-Yaa-Gly tripeptidic units in natural collagen.

Xaa/Yaa	A	D	E	I	K	L	M	N	O	Q	R	S	T	V
A	0.9	0.5			1.2			0.3	3.4	0.4	1.1	0.4		0.4
D	0.4	0.3			1.0				1.5	0.5	1.2	0.3		0.3
E	1.2	0.4		0.4	2.5		0.3	0.3	2.8	0.8	2.7	0.3	0.6	0.4
F									2.5	0.3				
G					0.3				0.3					
H									0.5					
I	0.4				0.3				1.5					
K		0.5							1.4					
L	0.5	0.4			1.0				5.5	0.7	0.6		0.3	
M									0.6					
N									0.7					
P	3.4	0.4	0.4	0.9	2.7	0.5	0.4	0.3	10.5	2.5	2.6	1.4	0.8	1.3
Q	0.3				0.6				1.1	0.3	0.3			
R	0.3	0.5							1.1					
S	0.4				0.4				2.3		0.5			0.3
T									0.8					
V					0.5				1.1	0.3				
Y									0.5					

All numbers are given in percent. Empty cells mean that this triplet was found less often than 0.3 %.[51]

The Brodsky group systematically investigated the effect of all canonical amino acids in the Xaa and Yaa position on the conformational triple helical stability.[6,52,53] For these investigations they used so called "host guest collagen model peptides" comprising of Pro-Hyp-Gly repeating units with a single residue replaced with the amino acid under investigation in the middle of the peptide. For the studies on the Xaa position, peptides with the sequence Ac-(GlyProHyp)$_3$-GlyXaaHyp-(GlyProHyp)$_4$-GlyGly-CONH$_2$ were investigated and for the Yaa position, peptides with the sequence Ac-(GlyProHyp)$_3$-GlyProYaa-(GlyProHyp)$_4$-GlyGly-CONH$_2$ were used. A correlation between amino acid occurrence and their stabilizing properties was found.[6] For the Xaa position it was shown that the stability decreases with the polarity of the amino acid from charged to lipophilic residues and the least stable triple helices were formed by CMPs containing aromatic residues. In the Yaa position aromatic residues seemed to be particularly destabilizing but for all other residues no trend was observed (Table 6.2).[6]

TABLE 6.2. Stability of host guest CMPs containing canonical amino acids.[6]

Gly-Xaa-Hyp	Tm (°C)	Occurrence (%)	Gly-Pro-Yaa	Tm (°C)	Occurrence (%)
Pro	47.3	32.9	Hyp	47.3	34
Glu	42.9	13.0	Arg	47.2	11.4
Ala	41.7	11.1	Met	42.6	9.0
Lys	41.5	3.6	Ile	41.5	2.1
Arg	40.6	2.8	Gln	41.3	6.9
Gln	40.4	2.9	Ala	40.9	10.6
Asp	40.1	4.9	Val	40.0	4.3
Leu	39.0	7.8	Glu	39.7	2.0
Val	38.9	2.6	Thr	39.7	4.2
Met	38.6	0.9	Cys	37.7	0
Ile	38.4	2.0	Lys	36.8	9.0
Asn	38.3	2.1	His	35.7	0.5
Ser	38.0	4.9	Ser	35.0	4.0
His	36.5	1.6	Asp	34.0	4.8
Thr	36.2	1.8	Gly	32.7	0.7
Cys	36.1	0	Leu	31.7	1.7
Tyr	34.3	0.5	Asn	30.3	2.1
Phe	33.5	3.0	Tyr	30.2	0
Gly	33.2	1.6	Phe	28.3	0.2
Trp	31.9	0	Trp	26.1	0

This comprehensive study provided valuable data for the prediction of the stability of all natural amino acid containing CMPs.[59] The data implies that within natural collagen the stability of the triple helix locally differs. Such local variations could lead to different structural properties (e.g. pitch of the helix), which could be used for recognition and binding.[6] Furthermore it is surprising that charged residues not necessarily lead to a destabilization of the triple helix due to the close proximity of three symmetry related charges in close proximity.

7. Investigations on triple helical defects

Research on possible defects in the collagen triple helix is particularly interesting for the understanding of diseases connected with collagen. Among the most prominent example is the brittle bone disease (osteogenesis imperfecta, OI) in which a glycine residue is replaced by another, bulkier amino acid. Brodsky and coworkers used host guest CMPs with the sequence

Ac-(Gly-Pro-Hyp)$_3$-Zaa-Pro-Hyp-(Gly-Pro-Hyp)$_4$-Gly-Gly-NH_2 to quantify the effect of different amino acids which were found to be incorporated in collagen of OI patients.[20] CMPs containing serine and alanine were still able to form triple helices with highly decreased T_m values of about 9°C compared to 45°C of the all-Gly containing peptide. All other mutants failed to form a triple helix. Nevertheless their propensity for triple helix formation was deduced using extrapolation experiments. A correlation of the destabilizing effect of the residues and the severity of osteogenesis imperfecta for mutations in the α1(I) chain of a natural collagen heterotrimer was found. However, this correlation could not be shown for other chains. Following a similar concept the Hartgerink group developed a model for glycine mutations in one, two, or three single strands of a hetero trimer.[60]

Horng and co-workers investigated the thermodynamic and kinetic consequences for triple helix formation by replacing glycine residues. They exchanged glycine in different positions of a Pro-Hyp-Gly containing 24-mer host-guest-peptide to investigate how the stability depends on the location of the mutation. Their study showed that mutations at the C-terminus have less severe consequences on the triple helix stability than mutations at the N-terminus.[61]

The Etzkorn group investigated the effect of fixed *trans* bonds on the stability of the collagen triple helix. They replaced the amide bonds by so called *trans*-alkene isosteres (Figure 7.1).[62,63]

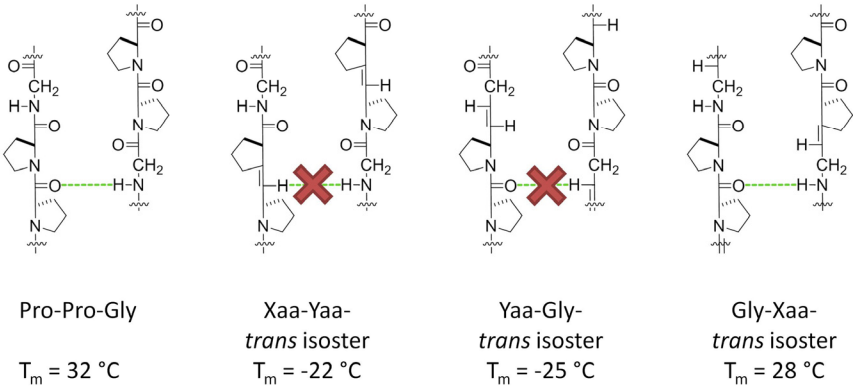


FIGURE 7.1. CMPs containing *trans*-locked isosteres.[62,63]

This replacement of amides by alkenes had dramatic effects on the triple helical stability. The Xaa-Yaa and Yaa-Gly *trans*-locked alkene-isostere containing CMPs were not able to form triple helices anymore. Extrapolation experiments showed that their T_m value is about 55 °C lower than the T_m value of the only amide bond containing triple helix (Figure 7.1).[62] This

highly decreased stability was attributed to the loss of three interstrand hydrogen bonds between the three substituted amides which help to stabilize the triple helix in the native form. Consistent with their explanation, the incorporation of a Gly-Xaa *trans*-locked alkene-isostere did not lead to such a severe destabilization since this amide bond is not involved in hydrogen bonding.[63] However, a slight decrease of 3°C in the T_m value was observed. This result demonstrates that not only the *trans* preference of the amide bond is important for triple helix stability but also other intrinsic properties of the amide bond such as n→π* interactions within the single strands. In a similar study by the Raines group esters as amide isosteres were investigated and similar conclusions were drawn.[64]

An investigation on the role of (4*S*)hydroxyproline in the Xaa position led to the conclusion that this diastereoisomer is disturbing the triple helix formation by the competition of an intramolecular hydrogen bond with an interstrand hydrogen bond (Figure 7.2). The methylation of the (4*S*)Hyp residues prevented the formation of such a competing hydrogen bonds. Consequently, triple helices containing this methylated (4*S*)hydroxyproline ((4*S*)Mop) exhibited a higher conformational stability than their (4*S*)Hyp containing analogues.[41] However, investigations by the Kobayashi group on crystal structures of (4*S*)Hyp containing collagen model peptides did not find these competing H-bonds. Instead unusual C(4)-*exo* ring puckers and quite flat C(4)-*endo* ring puckers were found.[65]

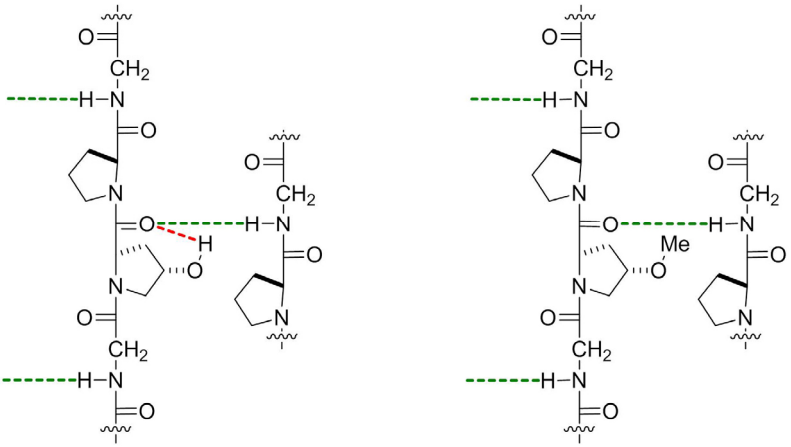


FIGURE 7.2. Destabilizing effect of (4*S*)Hyp by hydrogen bond competition (left). Methylated (4*S*)Hyp cannot form a competing hydrogen bond and is thus not destabilizing.

8. Formation of Synthetic Heterotrimers

In nature some collagens exist as AAB or ABC heterotrimers (see chapter 1). For mimicking such a heteromeric composition the Hartgerink group developed strategies based on ionic interactions.[60,66-68] They synthesized differently charged peptides, namely neutral CMPs containing (4*R*)Hyp in the Yaa position ($(POG)_{10}$, O=Hyp), positively charged CMPs containing arginine and lysine in the Yaa position ($(PRG)_{10}$, $(PKG)_{10}$), and negatively charged CMPs containing glutamic and aspartic acid in the Xaa position ($(EOG)_{10}$, $(DOG)_{10}$). The most stable AAB heterotrimer was found to be formed from a mixture of arginine (R) and aspartate (D) containing CMPs with a T_m of 42°C. The most stable ABC heterotrimer was formed by a mixture of aspartate (D) and lysine (K) containing CMPs and the neutral CMP. The T_m value observed for this triple helix (65°C) was almost as high as the T_m of a $[(POG)_{10}]_3$ triple helix (67.5°C). The surprisingly high stability of the heterotrimers was attributed to the formation of interstrand salt bridges between the charged residues.[68,69] NMR studies on the above mentioned ABC triple helix revealed that the electrostatic interactions not only allow for the formation of heterotrimers but also lead to only one out of the 6 possible alignments of the single strands within the triple helix.[67] By optimizing the charge distribution on the different single strands Hartgerink and co-workers additionally showed that a AAB heterotrimer consisting of two $(EOGPOG)_5$- and one $(PRG)_{10}$-single strand can be highly stable (T_m=56°C).[66]

9. Approaches for more stable triple helices and fibril formation

A long-term goal in chemical collagen research is to stabilize the collagen triple helix and to promote the growth of collagen-like fibrillar structures.[2,70] This is a very challenging topic especially since the factors that govern this process in nature are still under investigation. For feasibility reasons synthetic collagens have to be considerably shorter than their natural analogues that can only be gained from complex organisms such as animals, since a genetically designed expression is very difficult due to the extensive posttranslational modification of natural collagens. Consequently, properties which induce an aggregation have to be distributed on a much smaller area compared to the long natural collagen strands.

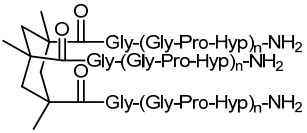
Additionally, the factors driving the aggregation have to be selective to allow not only for aggregation, but also for the formation of well-defined higher order structures. The development of such materials is important since they could not only introduce the excellent gel formation which is highly desirable in applications of natural collagen in cosmetics and medicine,[71-77] but it could also circumvent possible immunogenic responses caused by bovine and fish collagen used today.[78] Additionally the combination of collagenous materials with functional units could provide applications that have not evolved in nature. Functions such as wound healing could be achieved by the attachment of growth and coagulation factors to a synthetic collagen based material. In the following chapters several strategies towards these goals are presented.

9.1 Stabilizing the collagen triple helix using scaffolds

The covalent linkage of collagen model peptides on a tripodal scaffold has three advantages compared to loose single strands. First, all unfolding experiments become concentration independent since the local concentration of CMPs is always very high. Second, the single strands can fold in only one defined triple helix since an alignment of single strands staggered by e.g. 4 residues is impossible. Third, due to the higher stability of these triple helices also intrinsically less stable CMPs can be investigated.

Goodman and co-workers used this approach when they attached short collagen model peptides to Kemps triacid (KTA) (Figure 9.1) in order to investigate the influence of the length of CMPs on triple helix stability.[79] They showed that single strands attached to a scaffold form more stable triple helices. This is mainly caused by the lower entropic cost of bringing the single strands together. They additionally also used a tris(2-aminoethyl)amine scaffold (TREN) (Figure 9.1) to investigate the stability of a rather unusual norleucine (Nle) containing sequence (Gly-Nle-Pro). Identical CMPs attached to a TREN scaffold formed more stable triple helices compared to KTA-bound CMPs.[80] NMR studies showed that this is due to the higher flexibility of the TREN scaffold which facilitates adoption of the staggered alignment of the single strands (Table 9.1).

TABLE 9.1. Comparison of the triple helix stability of CMP attached to a scaffold and untethered CMPs.

		Ac-(Gly-Pro-Hyp)$_n$-NH$_2$
n	**T_m (°C)**	**T_m (°C)**
3	30	< 5
5	72	18
6	82	37

The Raines group attached the CMP Gly-(Pro-Pro-Gly)$_7$-NH$_2$ which does not allow for triple helix formation on its own to a macrocyclic scaffold (Figure 9.1). The scaffold bound CMPs were able to form a triple helix with a T_m of 40°C. This stability was further increased by 4° when the macrocycle chelated a Cs^+ ion.[81]

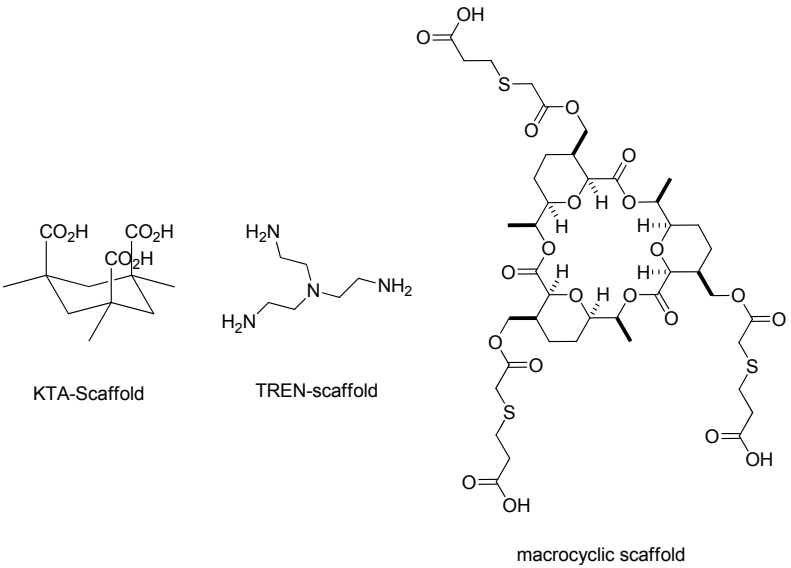


FIGURE 9.1. Scaffolds used to stabilize the collagen triple helix.

9.2 Aggregate formation using covalently crosslinked single strands

Raines and co-workers and the Koide group independently developed collagenous materials from covalently crosslinked single strands.[82,83] Their strategy was to incorporate cysteins into CMPs and then covalently crosslink them in a fashion that the strands would be staggered more than one tripeptidic repeating unit. Such sticky ends are thought to overlap with sticky ends of other single strands and therefore promote fibril growth (Figure 9.2).

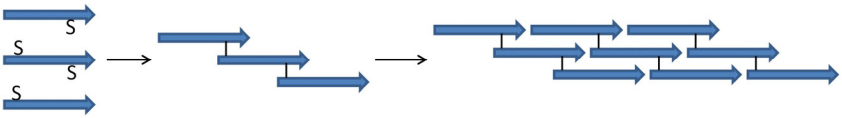


FIGURE 9.2. Crosslinking strategy of single strands for the promotion of fibril formation.[82]

The Raines group demonstrated that their material formed fibrils consisting of up to 8 monomeric units. The Koide group showed that their materials were able to form hydrogels with gelation temperatures higher than that of gelatin.[84] This material was further developed by incorporating an integrin binding sequence into the peptides sequence. This modification allowed for a cell adhesion of this purely synthetic material which is comparable to that of native collagen.[85]

9.3 Aggregate formation using non-covalent interactions

Another approach to promote fibril formation is the use of noncovalent interactions. Chmielewski and coworkers incorporated two histidines at the C-terminus and a residue bearing three carboxylic acids at the N-terminus of a CMP.[86,87] These residues acted as ligands upon addition of different metals and promoted the formation of different microflorette shaped particles. The introduction of a bipyridine-ligand in the central repeating unit of a CMP allowed for the formation of branched fibers upon addition of Fe^{2+}-salts.[88,89] A hybrid of CMPs containing all three ligands formed after addition of metal ions assemblies with fibrous meshes having internal pores approximately 5-20 µm in diameter (Figure 9.3). A further developed CMP was then used for the production of a material capable of encapsulating HeLa cells.[90]

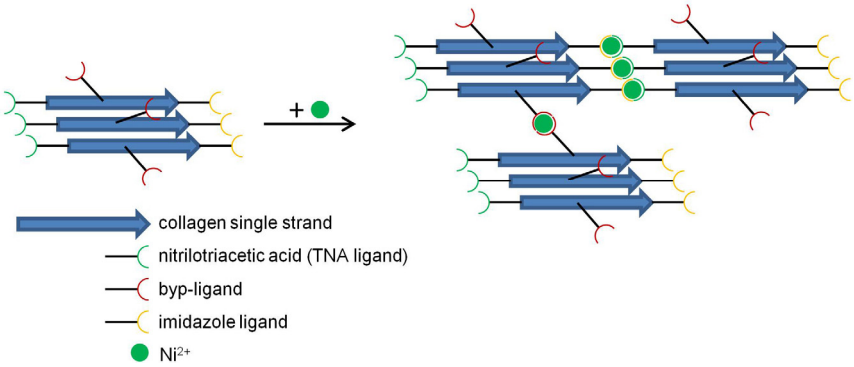


FIGURE 9.3. Summary of the strategies used by the Chmielewski group for the generation of higher order structures.[90]

Maryanoff and coworkers used hydrophobic π-π interactions to promote self assembly of triple helix-based fibrils by attaching phenyl- and pentafluorophenyl units to the termini of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ CMPs (Figure 9.4 top).[91,92] A similar concept was followed by the Horng group by introducing phenylalanin and arginin at the termini. This allowed for the formation of fibrillar structures *via* cation-π interactions (Figure 9.4 bottom).[93]

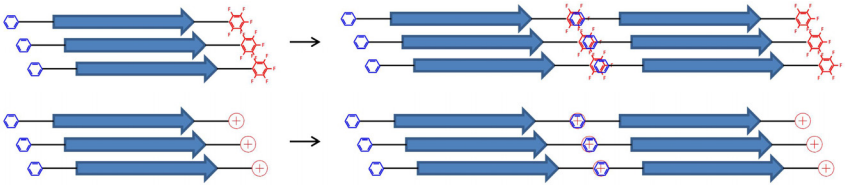


FIGURE 9.4. π-π interactions and cation-π interactions used by Maryanoff and Horng to promote fibril formation.[91-93]

Conticello and coworkers were the first showing that it is possible to produce synthetic collagenous fibres exhibiting the typical D-periodic pattern of collagen fibrils.[94] Surprisingly, this is possible with the 36-mer H-(Pro-Arg-Gly)$_4$-(Pro-Hyp-Gly)$_4$-(Glu-Pro-Gly-)$_4$-OH containing only natural amino acids. The middle region of the CMP serves for the triple helical stability. The positively charged N-terminal block and negatively charged C-terminal block are promoting the fibril formation in a way that always the negatively charged end of a triple helix is aligned next to the positively charged beginning of a triple helix (Figure 9.5).[94]

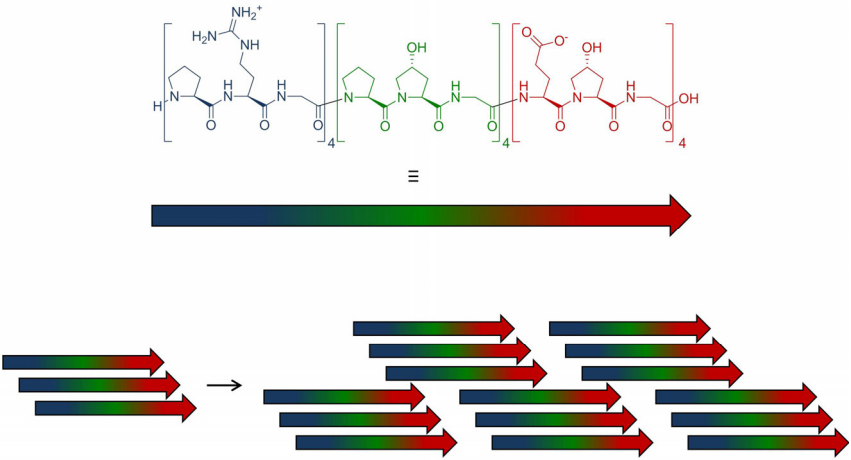


FIGURE 9.5. Strategy using electrostatic interaction for fibril growth promotion.

The Hartgerink group replaced the arginine and glutamate by lysine and aspartate, respectively, since they had observed strong lysine–aspartate salt-bridged hydrogen bonds in a collagen heterotrimer before. Because of these stronger salt bridge interactions the single strands align differently than the peptides of the Conticello group (Figure 9.6). Thus, that peptide is the only one reported so far, able to imitate all steps of the self-assembly of natural

collagen. It forms triple helices which further assemble into nanofibers and finally form a hydrogel.[95]

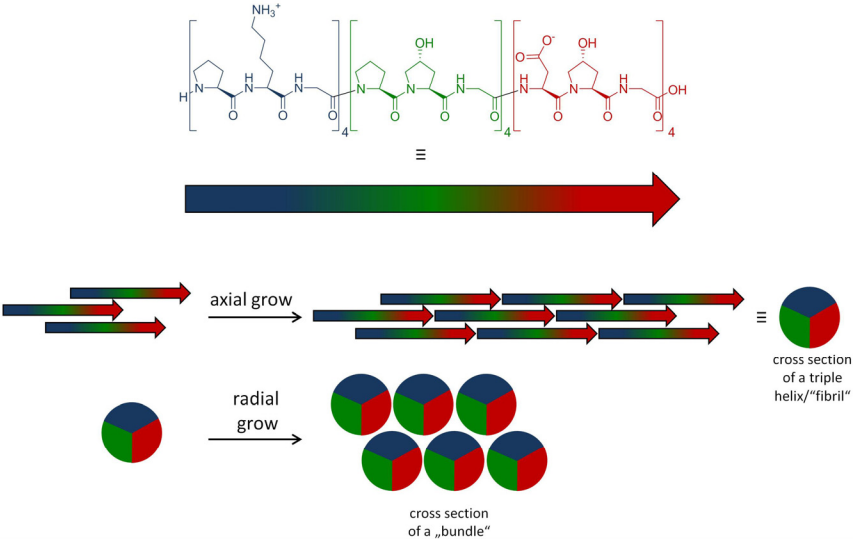


FIGURE 9.6. Design of the CMP self-assembling in a multi-hierarchical way.[95]

10. Kinetic Hysteresis in Collagen folding

The folding and unfolding of CMPs to a triple helix is a rather slow process. This leads to a complication when determining the T_m values of triple helices.[3,96,97] For T_m determinations usually a cooled, equilibrated solution of CMPs is heated and the unfolding process of the triple helix is monitored by CD spectroscopy. However the heating rate in these experiments is much faster than the unfolding process of the triple helices. If the data is fitted to a very simple two state model[98] in which a permanent equilibrium of the sample is assumed the obtained T_m values are higher than the effective ones since the melting process is always lagging behind. This slow folding and refolding behaviour of CMPs results in a hysteresis curve if heating as well as cooling can be easily followed by CD-spectroscopy.[96] Recording the data with a slower heating rate to prevent the hysteresis is not feasible since heating rates as slow as 0.1°C would be necessary[99] which leads to experiments that last over 20 days.

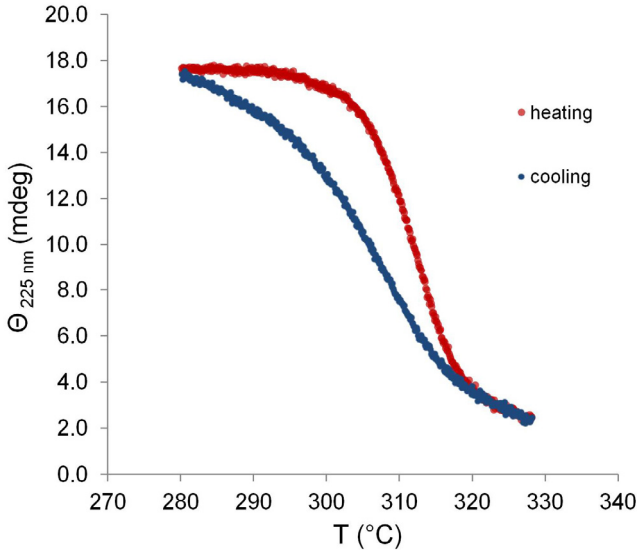
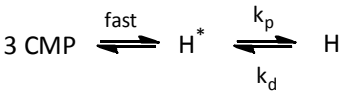


FIGURE 10.1. Hysteresis of unfolding and folding of Ac-(Pro-Hyp-Gly)$_7$-NH$_2$.

The Bächinger and Engel groups studied this hysteresis quantitatively.[99] They proposed a model in which CMPs fold into triple helices with a nucleus as intermediate. Fitting the hysteresis data of a CMP to this model does not only give insight into the thermodynamic parameters of the system, but also provides information on the kinetics of the folding process.[99]



SCHEME 10.1. 3 CMPs form in fast equilibrium a nucleus (H*) and subsequently fold into a triple helix.

Additionally, the obtained thermodynamic parameters allow for the calculation of the effective T_m value.[99] With this method the folding and unfolding behaviour of CMPs can be investigated easier and in more detail than with a combination of CD- and DSC-measurements which were used before.[99]

II. Objectives

The development of functionalized collagen based biomaterials[2,70] is highly desirable. They might not only overcome certain drawbacks of natural collagen, such as the possibility of immunogenic responses, but might also be used to introduce new properties and functions that have not evolved in nature.

A key step in this endeavour is the attachment of functional moieties to the collagen triple helix without losing its triple helical structure. Thus, the goal of this project was the development of a synthetic approach which allows for the attachment of different moieties to the collagen triple helix. Azidoprolines in collagen model peptides were envisioned as sites for the attachment of functional groups. Click chemistry and a reduction/acylation approach were intended for the introduction of different moieties ranging from formamido groups to triazolyl linked monosaccharides. Investigations on the effect of the position (Xaa/Yaa) and the stereochemistry at the functionalized proline residue (4*R*/4*S*) on the conformational stability of the collagen triple helix should provide a guideline for the optimal functionalization of collagen.

Furthermore such functionalizations should be used to gain a deeper insight into the factors that are responsible for the conformational stability of the collagen triple helix. By applying these new functionalization strategies factors such as the conformation of Xaa-Pro amide bonds, proline ring puckering, steric effects, intra- and interstrand H-bonds, charges, and hydration should be investigated. Furthermore the interconnection of these factors should be elucidated.

This project is divided in three parts which are described in the following chapters.

Chapter 11: Design, Synthesis, and Conformational Analysis of Functionalizable Azidoproline Containing Collagen Model Peptides

In this chapter the general design and synthesis of host guest collagen model peptides is described. Furthermore CMPs containing azidoprolines and their tendency for triple helix formation is presented.

This chapter is based on the following publications and manuscript:

- R. S. Erdmann and H. Wennemers "*Synthesis of Fmoc-Pro-Hyp(TBDPS)-Gly-OH and Its Application as a Versatile Building Block for the Preparation of Collagen Model Peptides*" *Synthesis* **2009**, 143-147.
- R. S. Erdmann and H. Wennemers "*Functionalizable Collagen Model Peptides*" *J. Am. Chem. Soc.* **2010**, *132*, 13957-13959.

- R. S. Erdmann and H. Wennemers "*Conformational stability of collagen triple helices functionalized in the Yaa position by click chemistry*" *Org. Biomol. Chem.* **2012**, *10*, 1982-1986.
- R. S. Erdmann and H. Wennemers "*Conformational Stability of Triazolylproline Containing Collagen Triple Helices*", Manuscript A.

Chapter 12: Collagen Model Peptides Functionalized by Click Chemistry

Copper catalyzed 1,3-dipolar cycloaddition was used to functionalize azidoproline containing CMPs. The impact of the functionalizations on the conformational stability of the collagen triple helix was investigated. Furthermore, the influence of steric effects is discussed in this chapter.

This chapter is based on the following publications and manuscript:

- R. S. Erdmann and H. Wennemers "*Functionalizable Collagen Model Peptides*" *J. Am. Chem. Soc.* **2010**, *132*, 13957-13959.
- R. S. Erdmann and H. Wennemers "*Conformational stability of collagen triple helices functionalized in the Yaa position by click chemistry*" *Org. Biomol. Chem.* **2012**, *10*, 1982-1986.
- R. S. Erdmann and H. Wennemers "*Conformational Atability of Triazolylproline Containing Collagen Triple Helices*", Manuscript A.

Chapter 13: Functionalized CMPs using Reduction/Acylation Chemistry

In this chapter a reduction/acylation approach is presented for the functionalization of azidoproline containing CMPs. The importance of the ring puckering vs. hydrogen bonds within the triple helix for its stability was investigated. Additionally, the effect of steric factors on the triple helical stability are further elaborated. Furthermore, collagen triple helices whose stability changes upon a variation of the pH in its environment is presented. Finally, a hypothesis on the the effect of charges and solvation shells on the conformational stability of the collagen triple helix is presented.

This chapter is based on the following publications and manuscript:

- R. S. Erdmann and H. Wennemers *"Importance of Ring Puckering versus Interstrand Hydrogen Bonds for the Conformational Stability of Collagen"*, Angew. Chem., Int. Ed. **2012**, *50*, 6835-6838.
- R. S. Erdmann and H. Wennemers "*Impact of Steric Effects versus Ring Puckering on the Conformational Stability of the Collagen Triple Helix"*, Manuscript B.

- R. S. Erdmann, H. Gsellinger, D. Häussinger and H. Wennemers "*Effect of Formamidoproline on the Conformational Stability of the Collagen Triple Helix"*, Manuscript C.
- R. S. Erdmann and H. Wennemers "*Protonation Dependent Flipping of the Proline Ring Pucker: Effect on the Conformational Stability of the Collagen Triple Helix"*, Manuscript D.
- R. S. Erdmann and H. Wennemers "*Aminoproline Containing Collagen Model Peptides: Influence of pH, Solvation, Ring Pucker and Hydrogen Bonds on Triple Helix Stability"*, Manuscript G.

The stability of the collagen triple helix but also the stability and function of many other proteins is closely related to the conformational properties of the proline residues which it comprises. Acetylated methyl esters of proline derivatives have proven useful as conformational models of proline residues within peptides and proteins. However an ester bond is not fully representative for the amide bonds within a peptide or protein. Thus, an alternative model compound for proline derivatives within peptides and proteins should be found. In the following chapter a possible alternative is presented.

Chapter 14: Acetylated dimethylamides of proline derivatives as conformational models of proline residues within peptides and proteins

In chapter 14 the conformational properties of acetylated proline dimethyl amides as alternative model compounds are investigated and compared to the corresponding methyl ester analogues. The discussion of both models sheds light on the strengths and limitations of both model compounds.

III. Summary of the Results & Discussions

11. Design, Synthesis, and Conformational Analysis of Functionalizable Azidoproline Containing Collagen Model Peptides

Towards the development of collagen based materials that exhibit more than scaffolding properties an anchor point for the attachment of functional moieties has to be introduced into the collagen triple helix. Azidoproline was envisioned as this attachment point since it has very similar conformational properties as the natural hydroxyproline and can be further functionalized by several different chemical reactions.

11.1 General Design and Synthetic Aspects

The collagen model peptides were designed in a way that they allow for the investigation of the effect of small structural changes in the single strand composition on the triple helix stability. For such investigations so called "host guest CMPs" have proven very useful (see chapter 6).[6,49,51-53] Thus, host guest CMPs with a guest triplet embedded between three host triplets on each terminus were used (Figure 11.1).

- Ac-(Pro-Hyp-Gly)$_3$-**Xaa**-Pro-Gly-(Pro-Hyp-Gly)$_3$-NH_2
- Ac-(Pro-Hyp-Gly)$_3$-Pro-**Yaa**-Gly-(Pro-Hyp-Gly)$_3$-NH_2

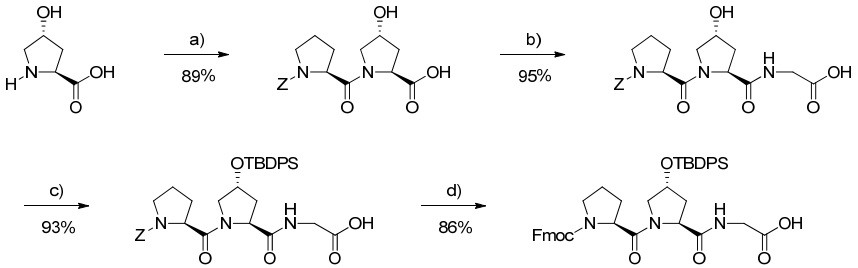
FIGURE 11.1. General sequence of host guest peptides in this thesis.

The host guest peptides were synthesized on solid support using tripeptidic Fmoc-building blocks. This strategy has several advantages compared to the coupling of single amino acid Fmoc-building blocks.[100,101] First, less truncation fragments are possible and thus the purification of the crude peptides is easier. Second, truncation fragments that were not detected in the purification process would have less severe consequences on the triple helical stability since a continuous Pro-Hyp-Gly sequence is conserved. Third, the peptide can be synthesized on a peptide synthesizer over night.

For the Pro-Hyp-Gly containing segment of the CMPs the silyl protected Fmoc-building block Fmoc-Pro-Hyp(TBDPS)-Gly-OH was developed.[101] Hydroxyproline was protected for several reasons. First, possible side reactions such as acylation during the solid phase peptide synthesis can be avoided. Second, side reactions during the functionalization are circumvented. Third, if desired, the fully protected peptide could be cleaved off the solid

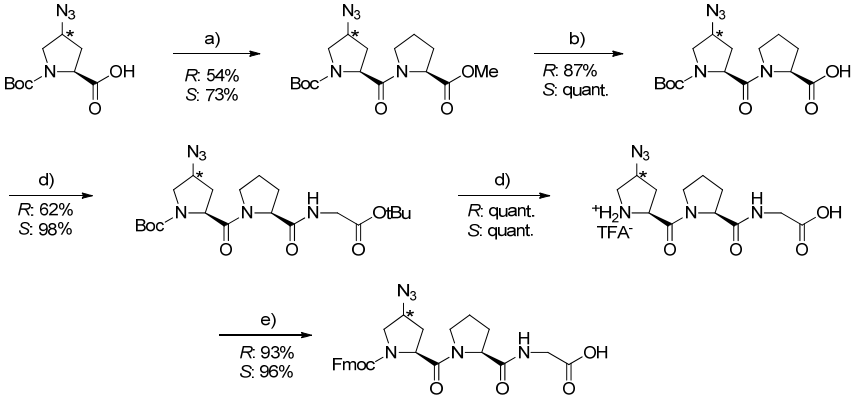
support. Forth, the bulky silyl ether should be able to prevent triple helix formation on the solid support and thus evade negative consequences on the coupling efficiency.[101]

The synthesis of the Fmoc-building block is based on a route developed by Moroder and co-workers.[102] Z-Pro-OSu was coupled with H-Hyp-OH followed by an EDC mediated coupling of H-Gly-OBn.[102] The resulting tripeptide Z-Pro-Hyp-Gly-OBn was protected with TBDPS-Cl and the C- and N-terminus were deprotected in a transfer hydrogenation using ammonium formate as the hydrogen source.[101] Finally the N-terminal amino group was protected using Fmoc-Cl to obtain Fmoc-Pro-Hyp(TBDPS)-Gly-OH in an overall yield of 68% (Scheme 11.1).[101]

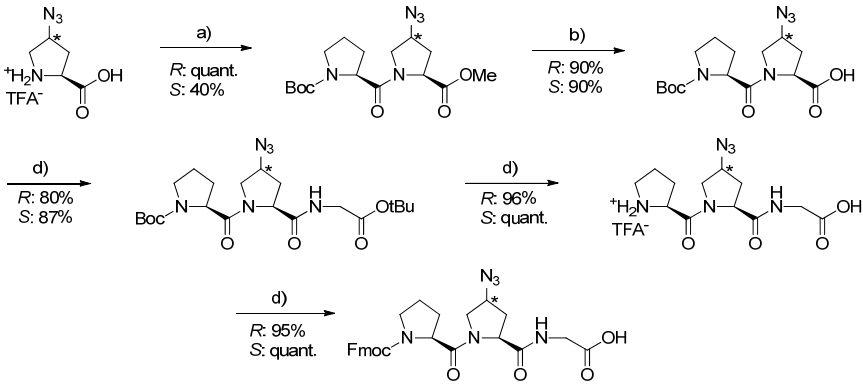


SCHEME 11.1. Synthesis of Fmoc-Pro-Hyp(TBDPS)-Gly-OH. a) 1.3 eq $BnNMe_3^+OH^-$, MeOH, r.t., 30 min; 0.91 Z-Pro-OSu, DMF, r.t., o.n.(over night); b) 1.2 eq H-Gly-OBn·HCl, 1.1 eq EDC·HCl, 1.0 HOBt, 1.05 eq $^iPr_2NEt$, DMF, r.t., o.n.; c) 2.75 eq TBDPS-Cl, 4.4 eq imidazole, DMF, r.t., o.n.; d) 8 eq $HCO_2^-NH_4^+$, 20 % w/w Pd/C, MeOH, r.t., 3 h; 1.2 eq Fmoc-OSu, Et_3N, MeCN:H_2O, r.t., o.n..[101]

For azidoproline containing central tripeptidic units within host guest peptides four different building blocks were synthesized.[103-105] The four building blocks differed in the position (Xaa or Yaa) of the azidoproline residue and in the stereochemistry at C(4) (*R* or S). Fmoc protected tripeptides were synthesized from correspondingly protected prolines, azidoprolines, and glycine building blocks as depicted in schemes 11.2 and 11.3.

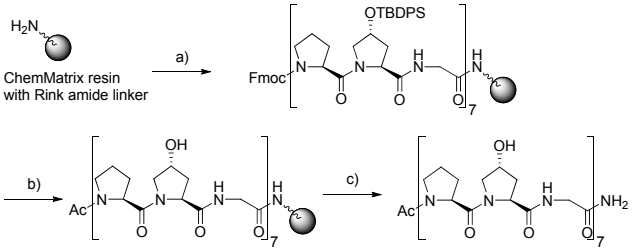


SCHEME 11.2. Synthesis of Fmoc-(4*R*/*S*)-Azp-Pro-Gly-OH. a) 1.5 eq H-Pro-OMe·HCl, 1.5 eq EDC·HCl, 1.1 eq $^{i}Pr_2NEt$, CH_2Cl_2, r.t., o.n.; b) 2 eq NaOH, MeOH:THF, r.t.; c) 1.5 eq H-Gly-O^{t}Bu·HCl, 1.5 eq EDC·HCl, 1.1 eq $^{i}Pr_2NEt$, CH_2Cl_2, r.t., o.n.; (d) TFA:CH_2Cl_2, r.t.; (e) 1.2 eq Fmoc-Cl, 2.5 eq $NaHCO_3$, dioxane:H_2O, r.t. o.n.[104,106]



SCHEME 11.3. Synthesis of Fmoc-Pro-(4*R*/*S*)-Azp-Gly-OH. a) 1.2 eq Boc-Pro-OH, 1.5 eq EDC·HCl, 1.1 eq $^{i}Pr_2NEt$, CH_2Cl_2, r.t., o.n.; b) 2eq NaOH, MeOH:THF, r.t., 3h; c) 1.5 eq H-Gly-O^{t}Bu·HCl, 1.5 eq EDC·HCl, 1.1 eq $^{i}Pr_2NEt$, CH_2Cl_2, r.t., o.n.; (d) TFA:CH_2Cl_2, r.t.; (e) 1.2 eq Fmoc-Cl, 2.5 eq $NaHCO_3$, dioxane:H_2O, r.t., o.n..[103,105]

Finally, the CMPs were synthesized on ChemMatrix resin with a Rink amide linker.[103-106] The trimeric building blocks were coupled with HCTU (2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminiumhexafluorophosphate) on the solid support with $^{i}Pr_2NEt$ as a base. In the following step the N-terminal Fmoc group was removed with piperidine in DMF (2:3 and 1:4). These two steps were repeated six times with the according trimeric Fmoc-building block. Then the silyl protecting groups were removed using TBAF (tetrabutylammonium fluoride) and the peptide was removed from the resin using a mixture of TFA, H_2O, and TIS (triisopropyl silane) and purified by HPLC (Scheme 11.4).



SCHEME 11.4. Solid phase synthesis of CMP **1**. a) (i) Fmoc-Pro-Hyp(TBDPS)-Gly-OH, HCTU, $^{i}Pr_2NEt$, r.t., (ii) piperidine:DMF 1:4, r.t., (iii) repeat (i) and (ii) 6 times; b) (i) Ac_2O, $^{i}Pr_2NEt$, r.t., (ii) TBAF in THF, r.t.; c) TFA:H_2O:TIS 95:2.5:2.5, r.t..

11.2 Stability studies of triple helices formed of Azp containing CMPs

(4*R*)Azp and (4*S*)Azp were incorporated in the Xaa and Yaa position of host guest CMPs to investigate the influence of position and stereochemistry of azidoprolines on the conformational stability of the collagen triple helix. As a reference the peptide Ac-(Pro-Hyp-Gly)$_7$-NH_2 (**1**) was investigated.

- Ac-(Pro-Hyp-Gly)$_3$-Pro-Hyp-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**1**)
- Ac-(Pro-Hyp-Gly)$_3$-**(4*S*)Azp**-Pro-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**2XS**)
- Ac-(Pro-Hyp-Gly)$_3$-**(4*R*)Azp**-Pro-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**2XR**)
- Ac-(Pro-Hyp-Gly)$_3$-Pro-**(4*S*)Azp**-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**2YS**)
- Ac-(Pro-Hyp-Gly)$_3$-Pro-**(4*R*)Azp**-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**2YR**)

The Azp containing CMPs were synthesized in analogy to CMP **1** by coupling the corresponding trimeric building block in the forth coupling step.

Solutions of all 5 peptides showed a maximum at 225 nm in their CD-spectra which is typical for a collagen triple helix.[12] Thermal denaturation experiments using CD-spectroscopy showed for all solutions a sigmoidal thermal transition, confirming that all peptides form triple helices (Figure 11.2).

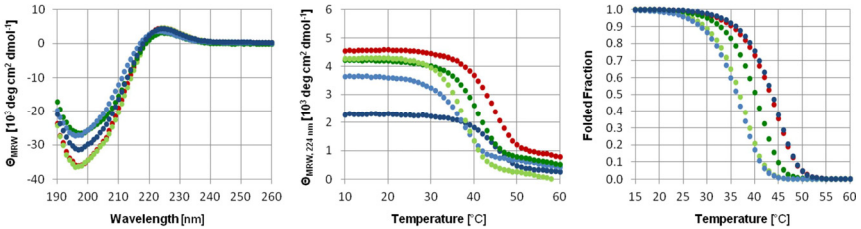


FIGURE 11.2. CD spectra of 0.20 mM solutions of CMPs **1** (●), **2XS** (●), **2XR** (●), **2YS** (●), and **2YR** (●) in 50 mM aqueous AcOH. b) Thermal denaturation curves. c) Folded fractions of the collagen triple helices derived from the CMPs.

11.3 Yaa position

The triple helix formed from **2YR** has a very similar stability as the one derived from the only Hyp containing reference CMP **1**. For both peptides a T_m of 43°C is observed.[105] This similar stability can be explained by the similar conformational properties of Ac-(4*R*)Azp-OMe[30] and Ac-(4*R*)Hyp-OMe[35]: Both adopt a C(4)-*exo* ring pucker which is preferred in the Yaa position. Additionally the they both favor a *trans* over a *cis* amide bond to the same extent ($K_{trans:cis}$=6.1) (see figure 4.3 and table 4.1).

In contrast CMP **2YS** forms triple helices with a lower stability (T_m = 36°C).[103] This value is in agreement with the conformational properties of the corresponding Ac-(4*S*)Azp-OMe[30] model compound. It adopts a C(4)-*endo* ring pucker which is a mismatch in the Yaa position. Furthermore, it only exhibits a low preference for a *trans* amide bond with a *cis*:*trans* ratio of 1:2.6 additionally destabilizing the triple helix (see figure 4.3 and table 4.1).

The stability of the triple helices formed of peptides **2YR** and **2YS** are in agreement with the stability of peptides containing Azp residues in every Yaa position. The peptide containing (4*R*)Azp forms a triple helix with the same stability as triple helices formed of CMPs **1** and **2YR** since (4*R*)Hyp and (4*R*)Azp have very similar conformational properties.[105] The incorporation of (4*S*)Azp in every Yaa position led to a peptide that is unable to form triple helices since the destabilizing effect of (4*S*)Azp occurs in every tripeptidic repeating unit.[105]

11.4 Xaa position

A T_m value of 40 °C was observed for triple helices formed by CMP **2XR**.[104] This destabilization compared to the triple helix formed by CMP **1** can be attributed to the conformational properties of Ac-(4*R*)Azp-OMe. Even though it has a high preference for a *trans* amide bond, its C(4)-*exo* ring pucker is a mismatch in the Xaa position to that of the natural Pro residue and therefore exerts a destabilizing effect. The peptide **2XS** forms a triple helix with a T_m value of 37 °C.[104] Also the destabilizing effect of (4*S*)Azp in the Xaa position of this peptide can be explained by the conformational properties of Ac-(4*S*)Azp-OMe. Even though it adopts a matching C(γ)-*endo* ring pucker, it only slightly prefers the *trans* amide bond conformation over the *cis* conformation.

11.5 Conclusions

The synthesis strategy for Azp-containing collagen model peptides proved to be very useful since the peptides can be assembled in a flexible, modular, and convergent fashion. The stability studies on Azp-containing CMP demonstrated that (4*R*)Azp in the Yaa has very similar stabilizing properties on the conformational stability of the collagen triple helix as the naturally most abundant (4*R*)Hyp residue in this position. This finding further supports that stereoelectronic effects, and not H-bond networks, are responsible for the stability of the collagen triple helix. Incorporation of (4*S*)Azp in the Yaa position and both diastereoisomers in the Xaa position lead to less stable triple helices which can be explained either by a low preference for a *trans* amide bond or a mismatching ring pucker (Figure 11.3).

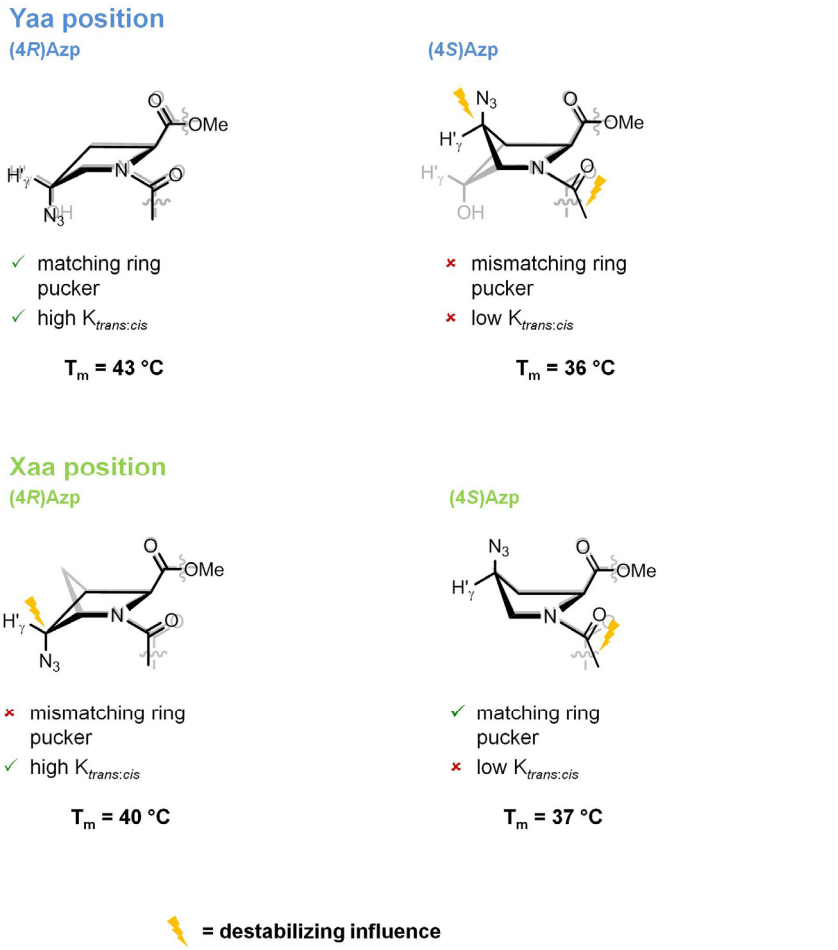


FIGURE 11.3. Influence of (4*R*)Azp and (4*S*)Azp in the Xaa and Yaa position of CMPs.

These findings support the hypothesis that conformationally preorganized single strands form triple helices with a higher stability.[1] The successful formation of Azp containing collagen triple helices allows for the investigation of their functionalizability which will be described in the next chapters.

This chapter is a summary of the following articles or parts thereof:

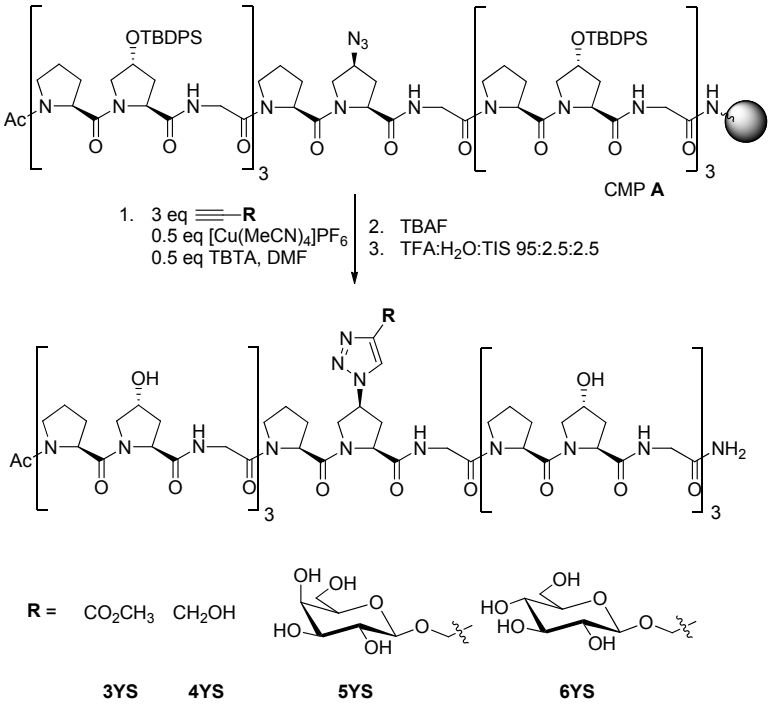
- R. S. Erdmann and H. Wennemers "*Synthesis of Fmoc-Pro-Hyp(TBDPS)-Gly-OH and Its Application as a Versatile Building Block for the Preparation of Collagen Model Peptides*" *Synthesis* **2009**, 143-147.
- R. S. Erdmann and H. Wennemers "*Functionalizable Collagen Model Peptides*" *J. Am. Chem. Soc.* **2010**, *132*, 13957-13959.
- R. S. Erdmann and H. Wennemers "*Conformational stability of collagen triple helices functionalized in the Yaa position by click chemistry*" *Org. Biomol. Chem.* **2012**, *10*, 1982-1986.
- R. S. Erdmann and H. Wennemers "*Conformational Stability of Triazolylproline Containing Collagen Triple Helices*", Manuscript A.

12. Collagen Model Peptides Functionalized by Click Chemistry

The azidoproline containing CMPs described above are very attractive for functionalizations using click chemistry. The high orthogonality of the copper catalyzed alkyne azide cyclo addition[107,108] should allow for the selective attachment of different alkynes to the azidoproline containing CMPs and therewith providing for various functions for different applications. In the following chapter the feasibility of functionalizations using click chemistry on solid support and their effects on the conformational stability of the collagen triple helix are presented. The comprehensive study investigates the effects on the conformational stability of the position of functionalization, the stereochemistry at the functionalized Azp residue and the moieties introduced by the functionalization. The study provides for a guideline for the functionalization of azidoproline containing CMPs maintaining a high propensity to form stable collagen triple helices.

12.1 Functionalization of Azidoproline Containing CMPs on Solid Support

The functionalizations of solid phase bound azidoproline containing CMPs (synthesis see chapter 11) proved to be straightforward.[103-105] Resin bound CMP **A** (scheme 12.1) reacted smoothly with different alkynes in the presence of substoichiometric amounts of $[Cu(MeCN)_4]PF_6$ and TBTA[109] (Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine) under microwave irradiation to the triazolyl proline containing CMPs.[103] After deprotection of the hydroxy functions and removal of the peptide from the resin the crude peptides were purified by RP-HPLC. No fragments with unreacted azido groups could be detected during the purification, confirming the completeness of the click reaction.[105] Under these conditions no difference in the completeness of the reaction between the positions of functionalization, stereochemistry at C(4) of the parent azidoproline and the used alkyne was observed.[103-105] This demonstrated the robustness and general applicability of click chemistry to functionalize azidoproline containing collagen model peptides with different moieties.



SCHEME 12.1. Functionalization of solid phase bound azido proline collagen model peptide **A** using click chemistry.[103]

For this study 16 peptides were synthesized with four different moieties introduced (**3**-**6**) at the Xaa (**X**) or Yaa (**Y**) position and with different stereochemistry at the triazolylproline (**R** or **S**).

- Ac-(Pro-Hyp-Gly)$_3$-**(4*S*)Tzp(CO$_2$Me)**-Pro-Gly-(Pro-Hyp-Gly)$_3$-NH$_2$ (**3XS**)
- Ac-(Pro-Hyp-Gly)$_3$-**(4*R*)Tzp(CO$_2$Me)**-Pro-Gly-(Pro-Hyp-Gly)$_3$-NH$_2$ (**3XR**)
- Ac-(Pro-Hyp-Gly)$_3$-Pro-**(4*S*)Tzp(CO$_2$Me)**-Gly-(Pro-Hyp-Gly)$_3$-NH$_2$ (**3YS**)
- Ac-(Pro-Hyp-Gly)$_3$-Pro-**(4*R*)Tzp(CO$_2$Me)**-Gly-(Pro-Hyp-Gly)$_3$-NH$_2$ (**3YR**)
- Ac-(Pro-Hyp-Gly)$_3$-**(4*S*)Tzp(CH$_2$OH)**-Pro-Gly-(Pro-Hyp-Gly)$_3$-NH$_2$ (**4XS**)
- Ac-(Pro-Hyp-Gly)$_3$-**(4*R*)Tzp(CH$_2$OH)**-Pro-Gly-(Pro-Hyp-Gly)$_3$-NH$_2$ (**4XR**)
- Ac-(Pro-Hyp-Gly)$_3$-Pro-**(4*S*)Tzp(CH$_2$OH)**-Gly-(Pro-Hyp-Gly)$_3$-NH$_2$ (**4YS**)
- Ac-(Pro-Hyp-Gly)$_3$-Pro-**(4*R*)Tzp(CH$_2$OH)**-Gly-(Pro-Hyp-Gly)$_3$-NH$_2$ (**4YR**)
- Ac-(Pro-Hyp-Gly)$_3$-**(4*S*)Tzp(Gal)**-Pro-Gly-(Pro-Hyp-Gly)$_3$-NH$_2$ (**5XS**)

- Ac-(Pro-Hyp-Gly)$_3$-**(4*R*)Tzp(Gal)**-Pro-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**5XR**)
- Ac-(Pro-Hyp-Gly)$_3$-Pro-**(4*S*)Tzp(Gal)**-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**5YS**)
- Ac-(Pro-Hyp-Gly)$_3$-Pro-**(4*R*)Tzp(Gal)**-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**5YR**)
- Ac-(Pro-Hyp-Gly)$_3$-**(4*S*)Tzp(Glc)**-Pro-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**6XS**)
- Ac-(Pro-Hyp-Gly)$_3$-**(4*R*)Tzp(Glc)**-Pro-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**6XR**)
- Ac-(Pro-Hyp-Gly)$_3$-Pro-**(4*S*)Tzp(Glc)**-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**6YS**)
- Ac-(Pro-Hyp-Gly)$_3$-Pro-**(4*R*)Tzp(Glc)**-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**6YR**)

12.2 Conformational Stability of functionalized Collagen triple helices

The relative conformational stability of the 16 peptides (**3-6XS/XR/YS/YR**) was investigated in thermal denaturation studies with CD-spectroscopy as a monitoring tool as described above for the Azp containing CMPs. For all peptides a maximum at 225 nm was observed in their CD spectra and in the denaturation experiments T_m values in the range of 36 to 40 °C were observed (Table 12.1).[103-105]

TABLE 12.1. T_m values of triple helices formed of CMPs functionalized by click chemistry

	T_m (°C)			
	Xaa		Yaa	
	R	*S*	*R*	*S*
3 (CO_2Me)	39	36	36	35
4 (CH_2OH)	38	38	35	36
5 (Gal)	39	40	37	37
6 (Glc)	39	40	37	37

0.20 mM peptide in 50 mM aqueous AcOH. Heating rate 36 °C·h^{-1}

Peptides functionalized in the Xaa position generally form slightly more stable triple helices than peptides with their functionalization in the Yaa position ($\Delta T_m \approx 3$°C).[104] Furthermore the stereochemistry within a certain position does not have an influence on the stability.[103,104] This finding is rather surprising since the stability of many previously investigated triple helices were highly dependent on the stereochemistry at C(4) of the proline derivative within the CMP.[1,33,47]

Since the conformational properties of acetylated methyl esters of proline derivatives have proven to be a valuable tool for monitoring the conformational stability of collagen triple helices containing the respective proline derivatives,[1,24,25,45,46,110] we investigated the conformational properties of the corresponding Ac-Tzp-OMe derivatives.[103,105,111] The result of these investigations can be summarized in the following three observations. The nature of the substituent at the triazolylring has no significant influence on $K_{trans:cis}$ of the Ac-Tzp amide bond and the pucker of the pyrrolidine ring.[103,105,111] Thus the conformational differences are limited to the influence of the stereochemistry at C(4). For Ac-(4*S*)Tzp-OMe a $K_{trans:cis}$ of approximately 2.7 was observed whereas its (4*R*)-diastereoisomer exhibits a $K_{trans:cis}$ of approximately 4.7 (Figure 12.1).[103,105,111] Furthermore the analysis of the vicinal ^{1}H-^{1}H-coupling constants revealed a slight preference of a C(4)-*endo* ring pucker of Ac-(4*S*)Tzp-OMe and a slight preference of a C(4)-*exo* ring pucker of Ac-(4*R*)Tzp-OMe.[103,105,111] The reduced preference for a distinct ring pucker compared to the parent azido prolines is likely due to the higher steric demand of the triazolyl substituents compared to the azido group. The higher steric demand leads to a competition of two ring pucker directing effects: a steric effect which directs the substituent to be in a pseudo equatorial position and a stereoelectronic gauche which directs the substituent into a pseudo axial position. As seen in the preferred ring puckers of Ac-Tzp-OMe, the stereoelectronic gauche effect still prevails since the substituents preferentially are in a pseudo axial position.[103,105,111]

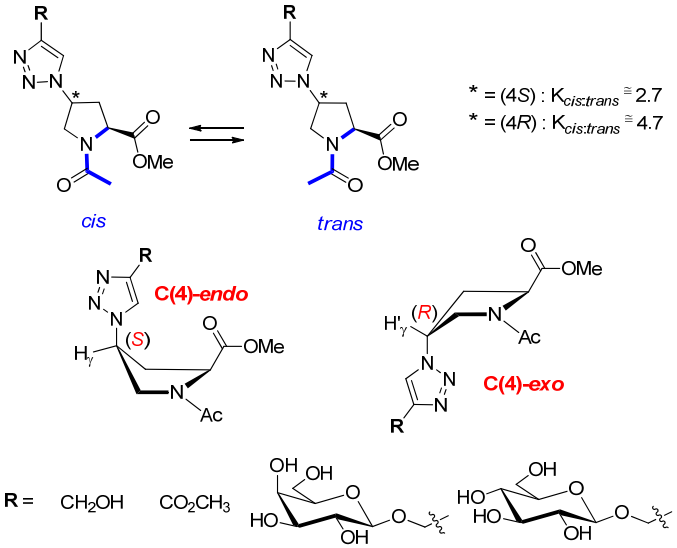


FIGURE 12.1. Structures of acetylated triazolyl proline methyl esters.[104]

Even though the conformational properties of (4*R*)Tzp are a better match in the Yaa position both diastereoisomers of Tzp lead to triple helices of the same stability if they are incorporated in the Yaa position.[103] Even though the C(4)-*endo* ring pucker of (4*S*)Tzp would match to the ring pucker of a proline residue in the Xaa position, both diastereoisomers of Tzp lead to triple helices with the same stability if incorporated in the Xaa position.[104] This clearly demonstrates that there are more factors that influence the conformational stability of the collagen triple helix than ring pucker and preference for a *trans* amide bond. Computer models of triple helices containing both diastereoisomers of Tzp in the Xaa and Yaa positions were generated based on a X-ray crystal structure (PDB ID 1V7H) by the replacement of the residue in the position of investigation by a triazolylproline with the according ring pucker and stereochemistry (Figure 12.2). (4*R*)Tzp residues were generated by the replacement of the hydroxy function of Hyp by a triazolyl moiety and (4*S*)Tzp residues were generated by the replacement of a hydrogen atom by a triazolyl moiety. The analysis of these computer model revealed that steric constraints play an important role.[104] The triazolyl substituent of (4*R*)Tzp in the Xaa position and (4*S*)Tzp in the Yaa position, respectively do not clash with any neighbouring strands since they are directed to the outside of the collagen triple helix and are perfectly accessible to the environment.[104] This accessibility is important for application where the introduced moiety acts as recognition.

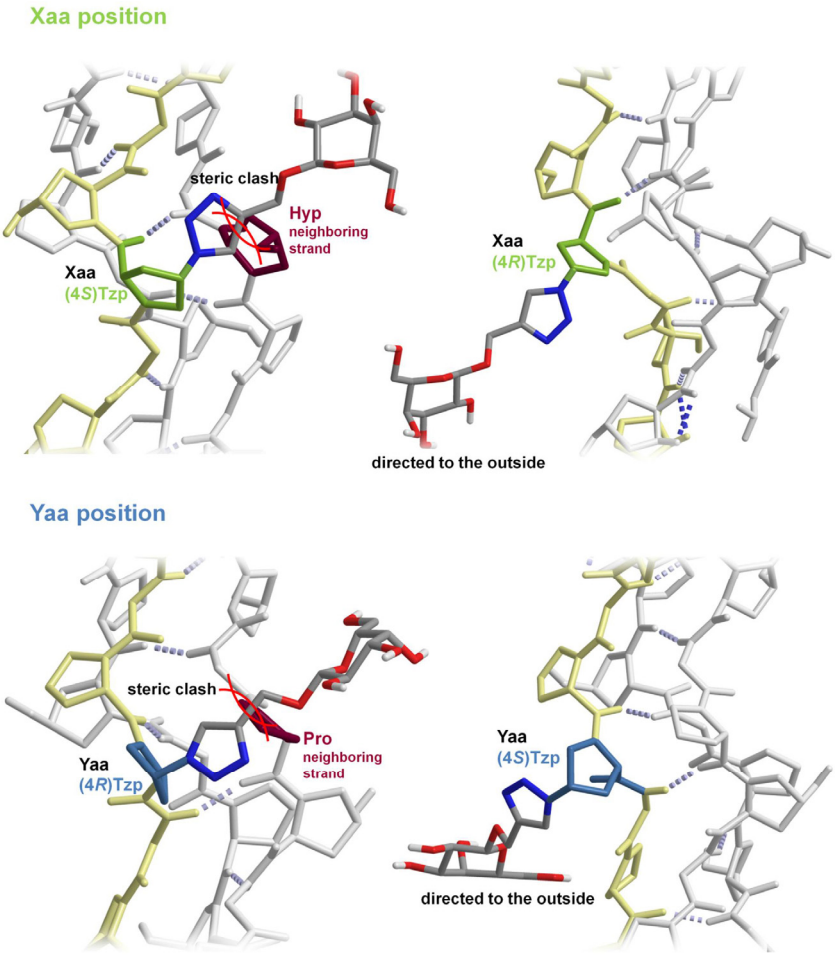


FIGURE 12.2. Computer model of Tzp containing collagen triple helices showing the steric constraints.[104]

On the other hand triazolyl substituents of (4*R*)Tzp in the Yaa position and (4*S*)Tzp in the Xaa position are directed towards a neighbouring strand and thus cause a steric clash, ultimately destabilizing the triple helix (Figure 12.2).[104] These steric constraints explain the independence of the conformational stability of triple helices on the stereochemistry at C(4) of the incorporated triazolyl proline residues. The benefit of the matching ring pucker of (4*S*)Tzp in the Xaa position and (4*R*)Tzp in the Yaa position are nullified by the steric clashes caused

by the triazolyl substituents in these triple helices. The remaining factor is the preference for a *trans* amide bond which explains why (4*R*)Tzp with the higher $K_{trans:cis}$ forms more stable collagen triple helices if incorporated in the Xaa position than (4*S*)Tzp with the lower $K_{trans:cis}$ if incorporated in the Yaa position.[103,104]

The impact of steric effects on the conformational stability of the collagen triple helix is supported by studies on triple helices containing amidoprolines.[112] These studies are presented in chapter 13.

12.3 Conclusions

The synthesis of triazolyl proline containing CMPs is straightforward and is independent of the nature of the herein used alkyne reacted with the azido proline containing precursor. The conformational stability of the collagen triple helices formed by these CMPs is predominantly dependent of the position of the incorporation and the stereochemistry at C(4) of the introduced triazolyl proline. The substituent at the triazolyl ring does not have a significant influence on the stability. It was demonstrated that not only the ring pucker and the preference for a *trans* amide bond are responsible for the conformational stability of the collagen triple helix but that also steric constraints play an important role (Figure 12.3). Incorporation of (4*R*)Tzp in the Xaa position and (4*S*)Tzp in the Yaa position turned out to be the method of choice to attach functional moieties to the collagen triple helix since these residues in the named position are well displayed to the environment of the triple helix and lead to the most stable helices amongst all different triazolyl containing triple helices. This click chemistry approach to functionalized CMPs is particularly intriguing towards the development of functional collagen based materials.

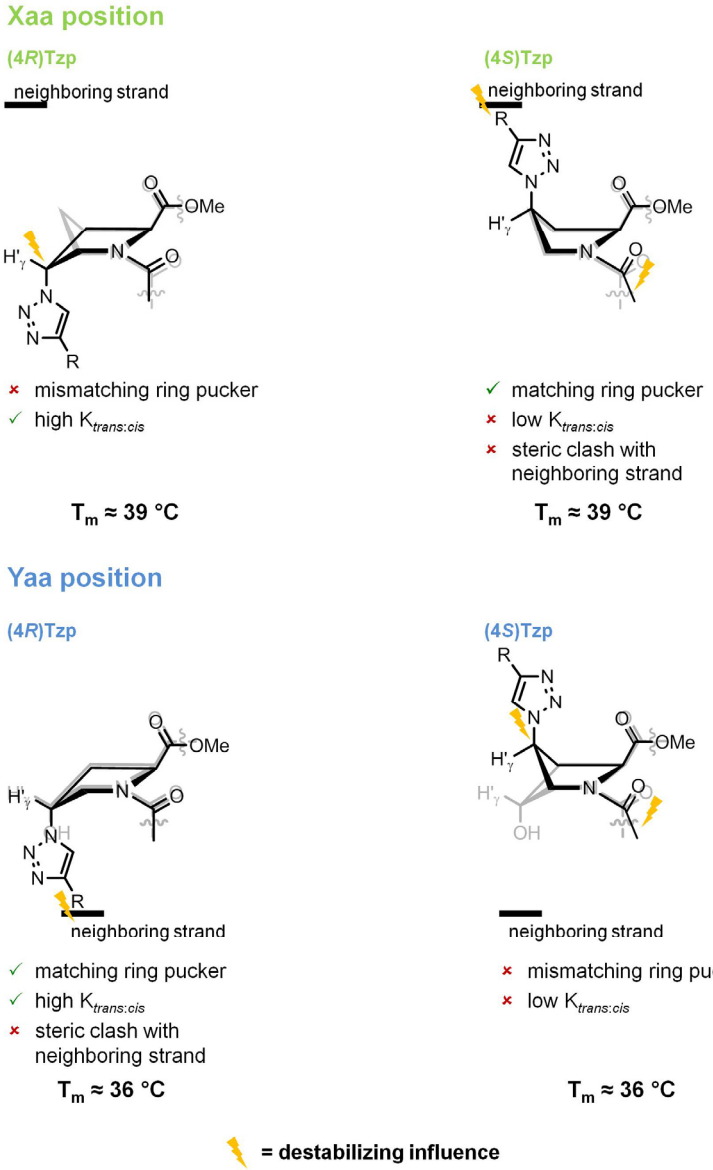


FIGURE 12.3. Influence of (4*R*)Tzp and (4*S*)Tzp in the Xaa and Yaa position of CMPs on the conformational stability of the collagen triple helix.

Chapter 12 is a summary of the following articles or parts thereof:

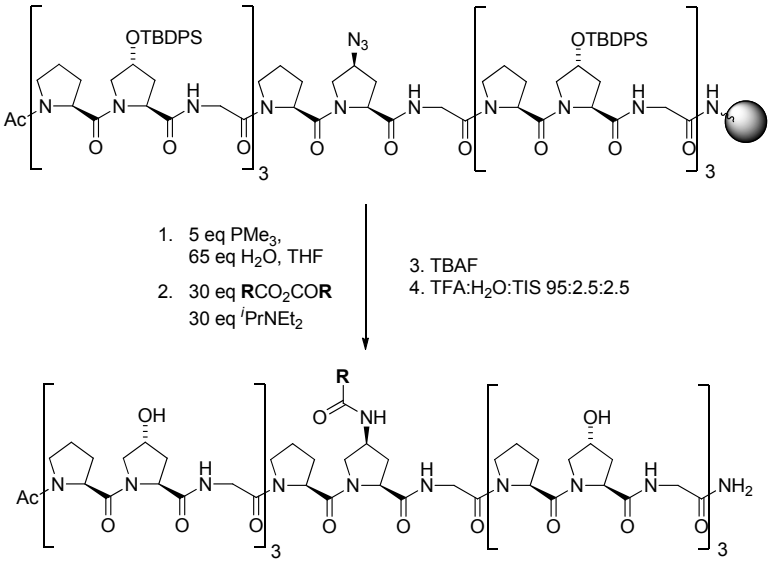
- R. S. Erdmann and H. Wennemers "*Functionalizable Collagen Model Peptides*" *J. Am. Chem. Soc.* **2010**, *132*, 13957-13959.
- R. S. Erdmann and H. Wennemers "*Conformational stability of collagen triple helices functionalized in the Yaa position by click chemistry*" *Org. Biomol. Chem.* **2012**, *10*, 1982-1986.
- R. S. Erdmann and H. Wennemers "*Conformational Stability of Triazolylproline Containing Collagen Triple Helices*", Manuscript A.

13. Collagen Model Peptides Functionalized by Reduction and Acylation

Azidoproline containing collagen model peptides should also be functionalizable by reduction of the azido group and subsequent acylation. This is a valuable alternative to the click chemistry approach even though it is less orthogonal. With an adequate reaction sequence both methods could be combined. In this chapter the feasibility of the reduction/acylation approach is demonstrated and used to elaborate the importance of the interstrand hydrogen bonds compared to ring puckers for the conformational stability of the collagen triple helix. Furthermore, the impact of steric effects on the conformational stability is investigated. These studies provide a useful guideline for the functionalization of the collagen triple helix using reduction and amidation chemistry without losing its conformational stability. In a further study the impact of the conformationally special formamidoprolines on the collagen triple helix is investigated. Finally using only reduction chemistry collagen triple helices whose conformational stability is dependent on the acidity of their environment were investigated. Furthermore, the same approach gave hints on the effect of charges and hydration on the conformational stability of the collagen triple helix.

13.1 Functionalization of Azidoproline Containing CMPs on Solid Support

Many different methods to reduce azides on solid support have been reported so far. [113-121] However they all have drawbacks such as the toxicity of the used reagents,[113,121] the simultaneous cleavage of the resulting amine from the resin,[118,119] or multiple steps have to be carried out.[114-117,120] In the beginning of this project a one step one pot Staudinger reduction that can be carried out on solid support was developed. Solid phase bound azidoproline containing peptides were successfully reduced with trimethyl phosphine (PMe_3) in THF.[106] The addition of small amounts of water allowed for the simultaneous hydrolysis of the resulting imino phosphorane to the amine. The amine can then be further functionalized using different acylating agents such as Ac_2O or carboxylic acids activated with a coupling reagent (Scheme 13.1).[106,112] This method is now not only used for the functionalization of collagen model peptides but also in the synthesis of cell penetrating peptides,[122] peptidic additives for the generation of silver nanoparticles[123] and peptide catalysts for the addition of aldehydes to nitro olefins.[124]



SCHEME 13.1. Transformation of azidoproline containing CMPs to amidoproline containing CMPs.

13.2 Importance of the ring puckering vs. interstrand H-bonds for the conformational stability of the collagen triple helix

The reduction/acylation approach to introduce new moieties into the collagen triple helix ultimately results in the incorporation of amidoprolines such as acetamidoproline (Acp) into collagen. This residue has some special properties amongst proline derivatives substituted at C(4).[31]

13.2.1 (4*S*)Acetamidoproline – A proline derivative with special conformational properties

Acetylated methyl esters of (4*S*)-configurated proline derivatives with a hydrogen bond donating substituent, such as (4*S*)Acp, exhibit an unusually high $K_{trans:cis}$ compared to

derivatives with electron withdrawing substituents lacking a hydrogen bond donating capability (Table 13.1).[31]

TABLE 13.1. $K_{trans:cis}$ of (4*S*)-configurated proline derivatives with an electron withdrawing group attached at C(4).

Ac-(4*S*)Xaa-OMe	$K_{trans:cis}$
Azp[30]	2.6
Flp[24]	2.6
Acp[31]	4.3

Determined by ^{1}H-NMR spectroscopy of 80 mM Ac-(4S)Xaa-OMe in D_2O.

The higher $K_{trans:cis}$ in Ac-(4*S*)Acp-OMe compared to other (4*S*)-substituted proline derivatives was explained by an intramolecular H-bond between the acetamide hydrogen and the methylester oxygen (Figure 13.1, top).[31]

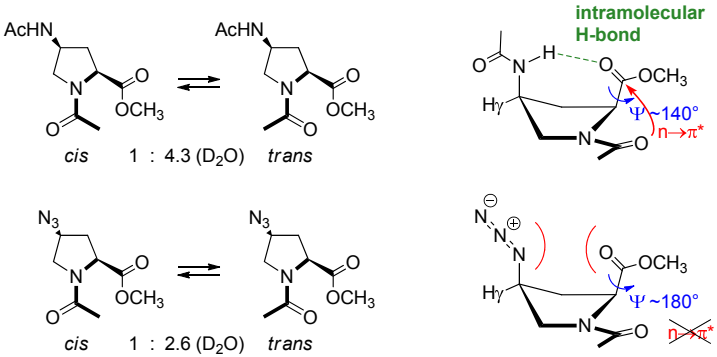


FIGURE 13.1. The *cis*:*trans* ratio of Ac-(4*S*)Acp-OMe is influenced by an intramolecular H-bond which aligns the methyl ester in a favourable position for an n→π* interaction (top) in contrast to Ac-(4*S*)Azp·OMe.[31]

This H-bond orients the methylester in a way which allows for an n→π* interaction between the N-acetyl oxygen lone pair and the methyl ester π* orbital leading to a stabilization of the *trans*-conformer (Figure 13.1, top).[31] In contrast, in (4*S*)-substituted proline derivatives with non H-bond donating substituents there is a repulsion between these substituents and the methylester, leading to an orientation of the methyl ester which is unfavorable for an n→π*-interaction, explaining the lower $K_{trans:cis}$ compared to Ac-(4*S*)Acp-OMe (Figure 13.1 bottom and chapter 4.3).[30,31] *Ab initio* calculations of lowest energy structures revealed

another difference between Ac-(4*S*)Acp-OMe and the corresponding proline derivatives lacking a hydrogen bond donating substituent: The ψ-angle of Ac-(4*S*)Acp-OMe is approximately 140° compared to 176° in Ac-(4*S*)Azp-OMe.[30] 140° is rather in the range of the ψ-angles of (4*R*)-configured proline derivatives with electron withdrawing substituents such as (4*R*)Hyp.[1] Furthermore, the ψ-angle is similar to the ψ-angle found in the proline residues within the collagen triple helix.[7]

13.2.2 Investigation of CMPs Containing (4*S*)Acp

The incorporation of (4*S*)Acp in the Yaa position permits for the first time to investigate whether a C(4)-*endo* ring pucker, which mismatches the ring pucker of the naturally occurring (4*R*)Hyp in this position, is tolerated if the ψ-angle of the incorporated residue is similar compared to the ψ-angle found for (4*R*)Hyp and the $K_{trans:cis}$ is not as low as in other proline derivatives adopting a C(4)-*endo* ring pucker. Furthermore, the incorporation of (4*S*)Acp allows to investigate whether a ring pucker and a ψ-angle that match the one of proline in the Xaa position in combination with a moderate $K_{trans:cis}$ or the interstrand H-bonds are more important for the conformational stability.
For these investigations CMPs **7XS** and **7YS** containing (4*S*)Acp in the Xaa and Yaa position were synthesized. As an additional reference to the (4*R*)Hyp containing CMP **1** a model peptide with Pro-Pro-Gly as guest triplet was synthesized (CMP **8**).

- Ac-(Pro-Hyp-Gly)$_3$-**(4*S*)Acp**-Pro-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**7XS**)
- Ac-(Pro-Hyp-Gly)$_3$-Pro-**(4*S*)Acp**-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**7YS**)
- Ac-(Pro-Hyp-Gly)$_3$-Pro-Pro-Gly-(Pro-Hyp-Gly)$_3$-NH_2 (**8**)

Thermal denaturation experiments using CD-spectroscopy as a monitoring tool showed a T_m-value for triple helices formed by CMPs **7YS** of 40°, which is similar to the stability of the triple helix formed by CMP **8** and comparable to the triple helix formed by CMP **1**.[106]
This demonstrates that the mismatching ring pucker of (4*S*)Acp in the Yaa position does not significantly destabilize the collagen triple helix. An overlay of Ac-(4*S*)Acp-OMe with the Hyp residue in the Yaa position of a crystal structure of a collagen triple helix shows that the ring pucker are a mismatch but that the ψ-angles of both residues are in good agreement (Figure 13.2).[106]

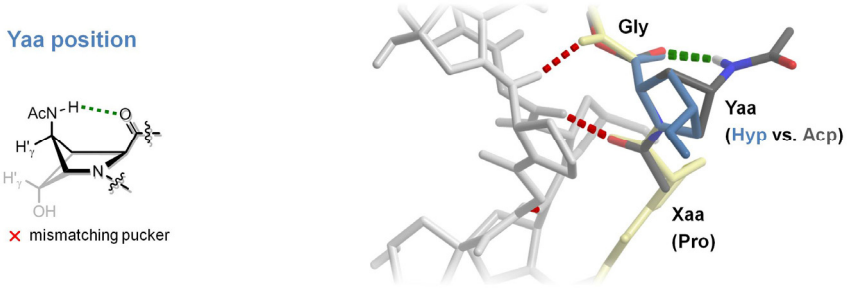


FIGURE 13.2. Conformational difference between (4*S*)Acp and (4*R*)Hyp (left). Overlay of a lowest energy structure of Ac-(4*S*)Acp-OMe[31] (black) and the (4*R*)Hyp residue (blue) in the Yaa position of a crystal structure of a collagen model peptide (PDB 1V7H) (right).[106]

For the triple helices formed by CMP **7XS** a T_m of 32 °C was observed. This is significantly lower than the T_m of the triple helices formed by CMPs **1** and **8** (43°C and 40°C).[106] This suggests a destabilizing effect of the intramolecular H-bond within the (4*S*)Acp residue since this is the main difference between the conformational properties of (4*S*)Acp and the naturally occurring proline residue in the Xaa position. An overlay of a lowest energy structure of Ac-(4*S*)Acp-OMe and a proline residue in the Xaa position within a crystal structure of a collagen model peptide shows that the intramolecular H-bond of (4*S*)Acp is competing with the interstrand H-bond leading from the (4*S*)Acp amide carbonyl to the NH of a neighboring strand (Figure 13.3). Furthermore, the good match of the ring puckers and the ψ-angles can be seen. This demonstrates that the unhindered formation of an interstrand H-bond is more important than a matching ring pucker and a matching ψ-angle.

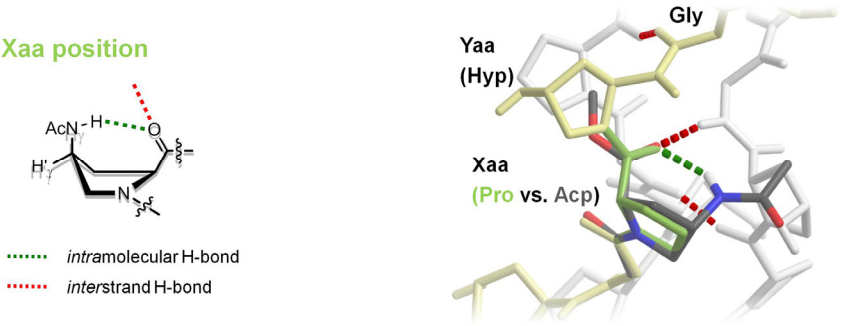


FIGURE 13.3. Conformational similarity of (4*S*)Acp and Pro (left). Overlay of a lowest energy structure of Ac-(4*S*)Acp-OMe[31] (black) and the Pro residue (green) in the Xaa position of a crystal structure of a collagen model peptide (PDB 1V7H) (right).[106]

13.3 Impact of Steric Effects versus Ring Puckering on the Conformational Stability of the Collagen Triple Helix

To provide a guideline for the functionalization of azidoproline containing CMPs using a reduction/acylation approach the previously described study was extended to CMPs containing (4*R*)- and (4*S*)-configured acetamidoprolines in the Xaa and Yaa position and their analogues containing sterically more demanding pivaloylamidoproline residues (Pvp). This investigation was not only envisioned to provide for a functionalization guideline but also to allow to elucidate the effect of sterically demanding groups on the conformational stability of the collagen triple helix. For this investigation the following host guest collagen model peptides containing Acp and Pvp were synthesized.

- Ac-(Pro-Hyp-Gly)$_3$-(**(4*R*)Acp**-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**7XR**)
- Ac-(Pro-Hyp-Gly)$_3$-(**(4*R*)Pvp**-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**8XR**)
- Ac-(Pro-Hyp-Gly)$_3$-(Pro-**(4*R*)Acp**-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**7YR**)
- Ac-(Pro-Hyp-Gly)$_3$-(Pro-**(4*R*)Pvp**-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**8YR**)
- Ac-(Pro-Hyp-Gly)$_3$-(**(4*S*)Pvp**-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**8XS**)
- Ac-(Pro-Hyp-Gly)$_3$-(Pro-**(4*S*)Pvp**-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**8YS**)

13.3.1 Conformational Analysis of Ac-Acp-OMe and Ac-Pvp-OMe

As in chapters 11 and 12, to understand the possible effect of Acp and Pvp on the conformational stability of the collagen triple helix, the conformational properties of the acetylated methylesters of Acp and Pvp were investigated. Ac-(4*S*)Pvp-OMe adopts like Ac-(4*S*)Acp-OMe[31] a C(4)-*endo* ring pucker.[112] The similar $K_{trans:cis}$ (5.0 vs. 4.3) additionally suggests the formation of an intramolecular hydrogen bond also in Ac-(4*S*)Pvp-OMe. This conformational analysis demonstrated that apart from the sterical demand of the substituent at C(4) both proline derivatives have very similar conformational properties (Figure 13.4).[112]
The conformational analysis of Ac-(4*R*)Acp-OMe[31] and Ac-(4*R*)Pvp-OMe revealed that both adopt a C(4)-*exo* ring pucker what is in agreement with other (4*R*)-configured proline derivatives bearing an electron withdrawing substituent.[30,35,112] Also their preference of a *trans*-amide bond over a *cis*-amide bond was similar ($K_{trans:cis}$ of 5.8 and 4.9). This demonstrates as before for the (4*S*)-configured derivatives that the difference of the steric demand of the substituents does not lead to a significant difference of the conformational properties of these proline residues (Figure 13.4).[112]

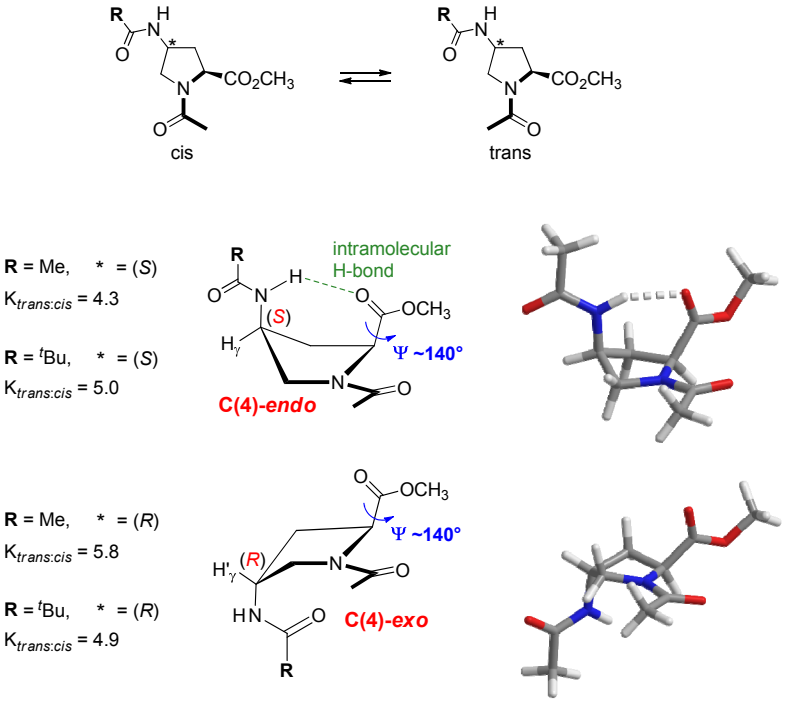


FIGURE 13.4. Conformational properties of Ac-(4*R*/*S*)Acp-OMe and Ac-(4*R*/*S*)Pvp-OMe.[112]

13.3.2 Incorporation of (4*R*)Acp and (4*R*)Pvp in the Xaa Position

The denaturation experiments of triple helices formed by CMPs **7XR** and **8XR** revealed T_m-values of 43°C and 44°C (Table 13.2) which are similar to the triple helices composed of CMP **1** containing the natural Pro-(4*R*)Hyp-Gly guest unit.[112] This results demonstrates that also sterically more demanding groups are tolerated in (4*R*)-configurated amidoprolines in the Xaa position. Additionally it demonstrates that the C(4)-*exo* ring pucker of (4*R*)Acp and (4*R*)Pvp which mismatches the ring pucker of naturally occurring proline residues in this position is well tolerated. An overlay of the lowest energy structure of Ac-(4*R*)Acp-OMe with a proline residue in the Xaa position of a collagen crystal structure[125] shows that the ring pucker is mismatching but the ψ-angles are matching. Furthermore the overlay shows that the substituent is directed to the outside of the collagen triple helix and is not interfering with

neighboring strands (Figure 13.5). Thus, the bulkier pivaloyl substituent compared to the acetyl substituent has no effect on the conformational stability of the collagen triple helix.[112]

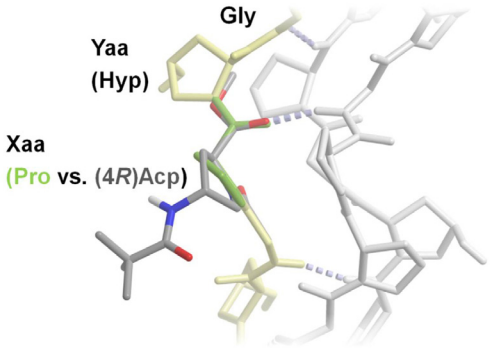


FIGURE 13.5. Overlay of the lowest energy structure of Ac-(4*R*)Acp-OMe (grey) with a Pro residue (green) in the Xaa position of a CMP crystal structure (PDB 1V7H).[112]

TABLE 13.2. T_m-values of triple helices containing (4*R*/*S*)Acp and the sterically more demanding (4*R*/*S*)Pvp in the Xaa and Yaa position.

			T_m (°C)	
entry	Position	Configuration	Acp	Pvp
1	Xaa	(4*R*)	43	44
2	Xaa	(4*S*)	32	26
3	Yaa	(4*R*)	36	32
4	Yaa	(4*S*)	39	40

0.20 mM of CMP in 50 mM AcOH at a heating rate of 36°C·h^{-1}.

13.3.3 Incorporation of (4*S*)Acp and (4*S*)Pvp in the Xaa Position

In comparison to the previously studied Acp containing CMP **7XS** (T_m = 32 °C, chapter 13.2) its analogue containing the sterically more demanding pivaloylamidoproline formed a triple helix with a significantly lower T_m-value of 26 °C (Table 13.2).[112] The difference of 6 °C in the T_m-value arises from steric effects since the conformational analysis of (4*S*)Acp and (4*S*)Pvp (chapter 13.3.1) showed that they only differ significantly in the steric demand of the substituent attached at C(4). This is supported by an overlay of Ac-(4*S*)Acp-OMe with a proline residue of a collagen crystal structure showing a steric clash between the acetyl group

and the residue in the Yaa position of a neighboring strand (Figure 13.6). This clash is destabilizing the collagen triple helix (additional to the competing H-bonds discussed in chapter 13.2.2) and is stronger in case of the bulkier pivaloylamidoproline leading to the lower stability of triple helices formed of CMP **8XS**.[112]

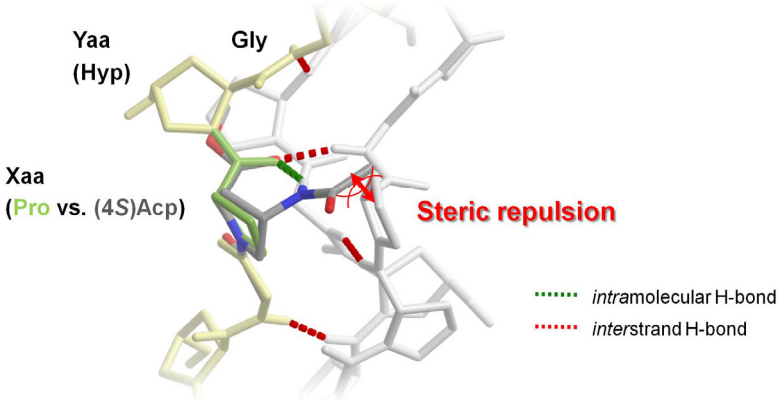


FIGURE 13.6. Overlay of the lowest energy structure of Ac-(4*S*)Acp-OMe (gray) with a Pro residue (green) in the Xaa position of a CMP crystal structure (PDB 1V7H).[112]

13.3.4 Incorporation of (4*R*)Acp and (4*R*)Pvp in the Yaa Position

The incorporation of (4*R*)Acp and (4*R*)Pvp in the Yaa position (CMPs **7YR** and **8YR**) leads to triple helices with T_m-values of 36°C and 32°C (Table 13.2).[112] These values are surprisingly low for the incorporation of proline residues whose ring pucker is matching the C(4)-*exo* ring pucker of the naturally occurring (4*R*)Hyp in the Yaa position and exhibit a reasonably high $K_{trans:cis}$. The significant difference in the conformational stability of these triple helices suggests that the destabilization arises from steric clashes since the properties of (4*R*)Acp and (4*R*)Pvp only differ in their steric demand (see chapter 13.3.1). Indeed, an overlay of a lowest energy structure of Ac-(4*R*)Acp-OMe[31] with the (4*R*)Hyp residue in a collagen crystal structure shows that there is a clash between the acetyl group and a residue in the Xaa position of a neighboring strand (Figure 13.7). This steric clash is stronger in the triple helix formed of CMP **8YR** containing (4*R*)Pvp because it exhibits the sterically more demanding substituent. These results demonstrate that a matching ring pucker and a high preference for a *trans*-amide bond are not sufficient for the formation of a stable collagen triple helix when steric clashes interfere with the triple helix formation.[112]

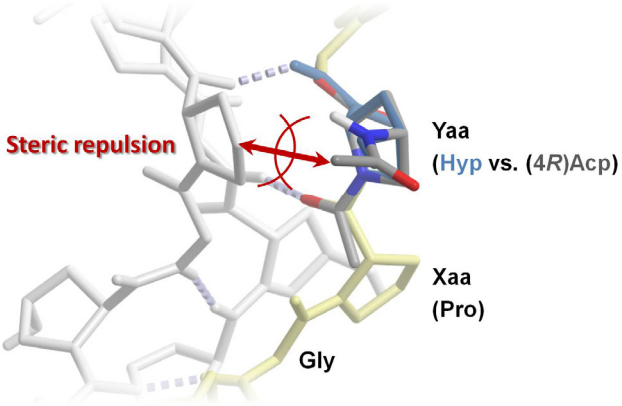


FIGURE 13.7. Overlay of the lowest energy structure of Ac-(4*R*)Acp-OMe with a Hyp residue in the Yaa position of a CMP crystal structure (PDB 1V7H).[112]

13.3.5 Incorporation of (4*S*)Acp and (4*S*)Pvp in the Yaa Position

For triple helices containing a (4*S*)Pvp residue in the Yaa position a T_m-value of 40°C was measured (Table 13.2).[112] This is very similar compared to the previously investicated triple helix containing the sterically less demanding (4*S*)Acp residue in the Yaa position (T_m = 39°C, chapter 13.2.2).[106,112] This suggests that also (4*S*)-configurated aminodoprolines with sterically more demanding substituents are tolerated in the Yaa position. This is supported by the overlay of a lowest energy structure of Ac-(4*S*)Acp-OMe with a (4*R*)Hyp residue in a collagen crystal structure showing that the ring puckers are mismatching but the ψ-angles fit well (Figure 13.8). Additionally it shows that the acetyl substituent is directed to the outside of the triple helix and is perfectly accessible to its environment. Furthermore it supports the previous observations (chapter 13.2.2) made with (4*S*)Acp containing **7YS** that also a mismatching ring pucker C(4)-*endo* ring pucker is tolerated in the Yaa position, even though the destabilization of a mismatching ring pucker is stronger than in the Xaa position (see chapter 13.3.2).[112]

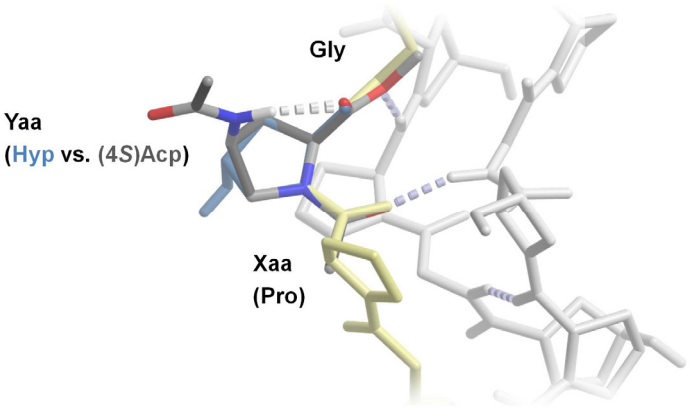


FIGURE 13.8. Overlay of the lowest energy structure of Ac-(4*S*)Acp-OMe with a Hyp residue in the Yaa position of a CMP crystal structure (PDB 1V7H).[112]

13.4 Incorporation of Formamidoproline into the Collagen Triple Helix

Apart from the lower steric demand of the substituent at C(4), formamidoprolines differ from acetamidoproline and pivaloylamidoproline also in the amount observable conformations. In contrast to Ac-Acp-OMe and Ac-Pvp-OMe, Ac-Fmp-OMe adopts at least 4 observable different conformers since there is not only a *cis*:*trans* isomerization around the tertiary Ac-Fmp amide bond but also a *cis*:*trans* isomerization around the formamine bond at C(4) and the possibilty of C(4)-*endo* and C(4)-*exo* ring puckers.[126] Thus, the incorporation of Fmp into collagen triple helices does not only allow to further investigate the impact of steric effects on the conformational stability of the collagen triple helix, but also of the impact of a residue that has more than 2 observable conformations in a monomeric form.

13.4.1 Conformational analysis of Ac-(4*R*)Fmp-OMe and Ac-(4*S*)Fmp-OMe

As in previous studies (chapter 11-13.3) the investigations were started with the conformational analysis of Ac-(4*R*/*S*)Fmp-OMe, since they are a valuable tool to monitor differences in the conformational stability of collagen triple helices. Extensive NMR studies on formamidoprolines revealed the detailed conformations of the three most frequent conformers of Ac-(4*R*)Fmp-OMe and Ac-(4*S*)Fmp-OMe.[126] The studies showed that the

major conformers of Ac-(4*R*/*S*)Fmp-OMe are similar compared to their acetylamidoproline analogues with the exception that Ac-(4*R*)Fmp-OMe adopts only a weakly pronounced C(4)-*exo* ring pucker where C(4) is only slightly above the ring plane.[126] Noteworthy is the fact that the formamidoprolines are the first proline derivatives for which C(4)-*endo* and C(4)-*exo* ring pucker conformations are observable when the Ac-Fmp amide bond adopts a *trans*-conformation (Figure 13.9). Overall, a $K_{trans:cis}$(Ac-Fmp) of 5.4 and a $K_{exo:endo}$ of 2.7 was observed for Ac-(4*R*)Fmp-OMe. For Ac-(4*S*)Fmp-OMe a $K_{trans:cis}$(Ac-Fmp) of 5.3 and a $K_{endo:exo}$ of 8.1 was observed.[126]

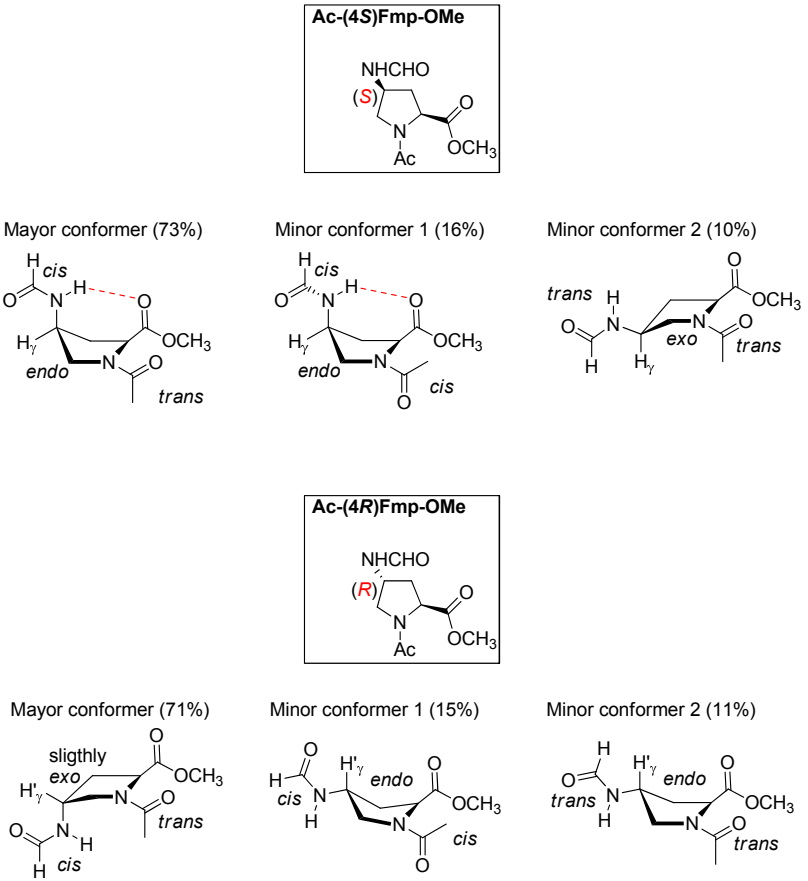


FIGURE 13.9. Conformational properties of Ac-(4*R*)Fmp-OMe and Ac-(4*S*)Fmp-OMe determined by NMR spectroscopy.[126]

13.4.2 Synthesis of Fmp containing CMPs

The synthesis of the Fmp containing CMPs was different compared to the Acp and Pvp containing CMPs. Instead of a functionalization of the corresponding solid phase bound aminoproline containing CMPs, the Fmp residues were introduced by using Fmoc-Fmp-OH in the solid phase synthesis.[126] The following peptides were prepared.

- Ac-(Pro-Hyp-Gly)$_3$-(**(4*S*)Fmp**-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**9XS**)
- Ac-(Pro-Hyp-Gly)$_3$-(**(4*R*)Fmp**-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**9XR**)
- Ac-(Pro-Hyp-Gly)$_3$-(Pro-**(4*S*)Fmp**-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**9YS**)
- Ac-(Pro-Hyp-Gly)$_3$-(Pro-**(4*R*)Fmp**-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**9YR**)

13.4.3 Thermal denaturation studies of Fmp containing collagen triple helices

Based on the previous results obtained for triple helices containing (4*S*)Acp and (4*S*)Pvp in the Xaa position, where bulky substituents destabilized the triple helix (chapter 13.3.3), a slightly more stable triple helix was expected to be formed by CMP **9XS** because the formamido group is sterically less demanding than the acetyl and pivaloyl group in **7XS** and **8XS**. However, the formamido substituent of (4*S*)Fmp is a better H-bond donor compared to the acetamido and pivaloylamido substituents of (4*S*)Acp and (4*S*)Pvp (inductive effects of H vs. Me, ^{t}Bu). This was expected to lead to a destabilization of the (4*S*)Fmp containing triple helix compared to the helices with the bulkier residues since the competing intramolecular H-bond within (4*S*)Fmp is stronger. Finally, the less destabilizing effect of the sterically less demanding substituent and the more destabilizing effect of the stronger intramolecular H-bond were expected to compensate each other. Thus, leading to a stability of the triple helix that is between the stability of the triple helices containing (4*S*)Acp and (4*S*)Pvp in the Xaa position. Thermal denaturation studies confirmed this expectations since a T_m-value of 29°C was measured for triple helices formed by CMP **9XS** (Table 15.3). This is as expected between the T_m-values measured for triple helices formed by **7XS** and **8XS** (32°C and 26°C).[106,112,126]

TABLE 13.3. T_m-values collagen triple helices formed by Fmp containing CMPs.

CMP	T_m (°C)
9XS	29
9YS	39
9YR	36
9XR	40

0.20 mM of CMP in 50 mM AcOH at a heating rate of 36°C·h^{-1}.

For the triple helix containing (4*S*)Fmp in the Yaa position a similar stability compared to the triple helices containing (4*S*)Acp and (4*S*)Pvp was expected since it was demonstrated in chapter 13.3.5 that steric effects do not influence the stability in this position. Furthermore, the similar $K_{trans:cis}$ of Ac-(4*S*)Fmp-OMe compared to Ac-(4*S*)Acp and Ac-(4*S*)Pvp-OMe as well as their similar preference for a C(4)-*endo* ring pucker strengthen the expectation of similar stabilities of triple helices formed by CMPs **7-9YS**. Thermal denaturation experiments revealed a T_m of 39°C for the triple helix containing (4*S*)Fmp in the Yaa position and confirmed the expected similar stability compared to CMPs **8YS** and **9YS** (39 and 40°C).[106,126]

The formamido group of (4*R*)Fmp is sterically less demanding than the acetyl group of (4*R*)Acp, hence, the destabilization caused due to a steric clash (chapter 13.3.4) is reduced in the triple helix containing (4*R*)Fmp compared to (4*R*)Acp and (4*R*)Pvp. However, this relatively stabilizing factor is expected to be compensated by the lower $K_{trans:cis}$ and the less pronounced C(4)-*exo* ring pucker of Ac-(4*R*)Fmp-OMe compared to Ac-(4*R*)Acp-OMe. Thus, the incorporation of (4*R*)Fmp in the Yaa position was expected to lead to triple helices with a similar stability as triple helices containing (4*R*)Acp in the Yaa position. Thermal denaturation experiment confirmed this expectation since a T_m of 36°C was measured for triple helices formed by CMP **9YR** which is comparable to the T_m observed for triple helices consisting of the (4*R*)Acp and (4*R*)Pvp containing CMPs **7YR** and **8YR** (36°C and 32°C).

For the incorporation of (4*R*)Fmp in the Xaa position a triple helix with a similar stability compared to triple helices containing (4*R*)Acp and (4*R*)Pvp were expected. The destabilizing effect of the lower $K_{trans:cis}$ of (4*R*)Fmp compared to (4*R*)Acp and (4*R*)Pvp was expected to be compensated by the stabilizing effect of the less distinct C(4)-*exo* ring pucker of (4*R*)Fmp compared to (4*R*)Acp and (4*R*)Pvp. However, thermal denaturation experiments demonstrated that the incorporation of (4*R*)Fmp in the Xaa position leads to triple helices with a lower stability compared to triple helices containing (4*R*)Acp and (4*R*)Pvp in the same position (T_m = 40°C vs. 43 and 44°C).[126] This result suggests that the effect of the ring pucker is not so

important in this case or that the adoption of a only weakly defined C(4)-*exo* ring pucker has a destabilizing effect on other factors, such as the Φ- and Ψ-angles, that are influencing the conformational stability of the collagen triple helix.[126]

13.5 Conclusions for Amidoproline Containing CMPs

The investigations on CMPs containing Fmp, Acp, and Pvp clearly demonstrated the importance of the interstrand hydrogen bonds for the conformational stability of the collagen triple helix.[106,112,126] Furthermore it has been shown, that the prevention of steric clashes within the triple helices is more important than matching ring puckers, high $K_{trans:cis}$ values and matching ψ-angles for the formation of collagen triple helices (Figure 13.10).[112] The peptides without any steric clashes but with a mismatching ring pucker and a matching Ψ-angle lead to the most stable collagen triple helices. In this case, peptides containing (4*R*)-amidoprolines in the Xaa position are slightly more stable than peptides containing (*4S*)-amidoprolines in the Yaa position.[112] The substituents at C(4) in these triple helices are directed to the outside of the collagen triple helix and are perfectly displayed to the environment of the triple helix, what renders them particularly interesting for the attachment of binding sides such as growth factors, coagulation factors, and integrins for the development of collagen based materials.[112]

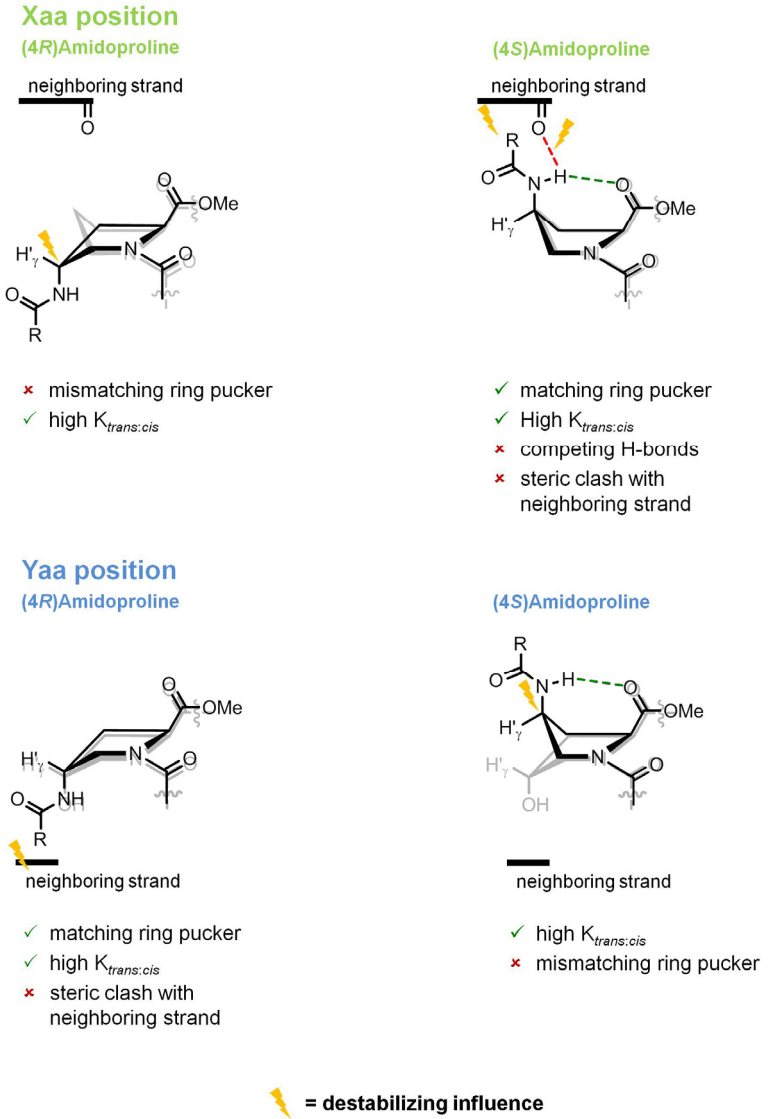


FIGURE 13.10. Influence amidoprolines in the Xaa and Yaa position of CMPs on the conformational stability of the collagen triple helix.[106,112,126] *ns = neighboring strand.

Chapter 13.1-13.5 summarize the following manuscripts:

- R. S. Erdmann and H. Wennemers "*Importance of Ring Puckering versus Interstrand Hydrogen Bonds for the Conformational Stability of Collagen" Angew. Chem., Int. Ed.* **2011**, *50*, 6835-6838.
- R. S. Erdmann and H. Wennemers "*Impact of Steric Effects versus Ring Puckering on the Conformational Stability of the Collagen Triple Helix*" Manuscript B.
- R. S. Erdmann, Heiko Gsellinger, Daniel Häussinger and H. Wennemers "*Effect of Formamidoproline on the Conformational Stability of the Collagen Triple Helix*", Manuscript C.

13.6 Aminoprolines as a pH Dependent Conformational Switch

All previously discussed proline derivatives adopted a distinct ring pucker depending on their substituent at C(4). (4*R*)- and (4*S*)aminoprolines (Amp) have very special properties since their ring pucker is depending on the protonation state of the amino group.[127,128] The protonated ammonium prolines adopt a ring pucker in which the substituent is in the pseudo axial position due to a gauche effect between the highly electron withdawing ammonium group[129] and the amide group within Ac-Amp-OMe·TFA. In contrast, unprotonated aminoprolines adopt the opposite ring pucker in which the amino substituent is in the pseudo equatorial position since it exerts a much weaker gauche effect[129] and thus, steric effects which force the substituent in the pseudo equatorial position predominate (Figure 13.11).[127,128]

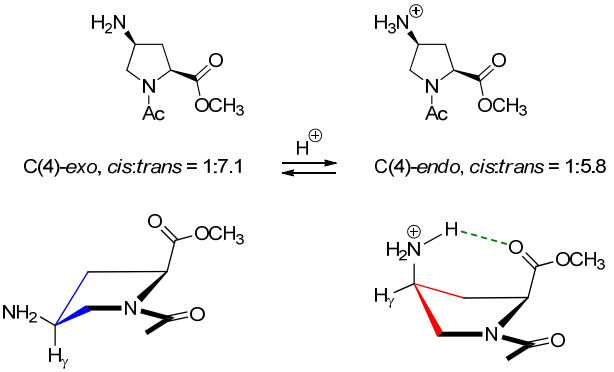


FIGURE 13.11. Effect of the protonation state on aminoproline at the example of Ac-(4*S*)Amp-OMe.

(4*S*)- and (4*R*)aminoprolines were incorporated into collagen model peptides to test whether these switching capability of Amp upon a change of pH can be used to change the conformational stability of collagen triple helices at different pH-values. For this purpose the following aminoproline containing host guest collagen model peptides were synthesized and the stability of the thereof formed triple helices were investigated in thermal denaturation experiments.

- Ac-(Pro-Hyp-Gly)$_3$-((4*S*)Amp-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**10XS**)
- Ac-(Pro-Hyp-Gly)$_3$-((4*R*)Amp-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**10XR**)
- Ac-(Pro-Hyp-Gly)$_3$-(Pro-(4*S*)Amp-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**10YR**)
- Ac-(Pro-Hyp-Gly)$_3$-(Pro-(4*R*)Amp-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**10YR**)

13.6.1 Triple Helices Containing (4*S*)Amp

Proline residues in the Xaa position preferentially adopt a C(4)-*endo* ring pucker, thus the ring pucker of protonated (4*S*)ammonium proline in **11XS·H**$^+$ is matching for this position. However there should also be an intramolecular H-bond formed within (4*S*)AmpH$^+$ which could compete with the interstrand H-bond holding the triple helix together. In analogy to the previous results with (4*S*)Acp in the Xaa position where a competing intramolecular H-bond lead to a destabilization of a collagen triple helix despite a matching ring pucker, a strong destabilization, due to competing H-bonds, of the triple helix containing (4*S*)ammonium-proline was expected (Figure 13.12 top). In contrast, the triple helix containing an non-protonated (4*S*)aminoproline was expected to be more stable since (4*S*)Amp should not be able to form a competing intramolecular H-bond. Furthermore, the mismatching C(4)-*exo* ring pucker of non-protonated (4*S*)Amp should not have a significantly destabilizing effect as demonstrated in chapter 13.3.2 with the incorporation of (4*R*)Acp (CMP **7XR**) which also adopts mismatching ring pucker for this position and does not destabilize the triple helix (Figure 13.12 top).

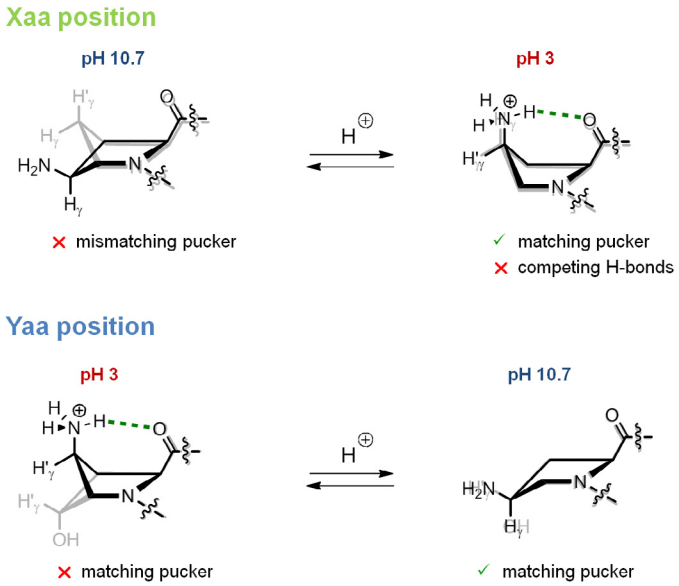


FIGURE 13.12. The ring pucker of amino proline is a mismatch in the Xaa position, whereas the ring pucker of ammonium proline is matching but exhibits an intramolecular H-bond which is competing with an interstrand H-bond responsible for holding the triple helix together. In the Yaa position the ring pucker of ammonium proline is a mismatch and the ring pucker of aminoproline is matching.

Thermal denaturation experiments with the CMP **11XS** under acidic (pH=3; T_m = 18°C) and alkaline (pH=10.7; T_m = 32°C) confirmed these expectations and demonstrated that aminoproline can be used as a pH dependent conformational switch to tune the stability of the collagen triple helix (Table 13.4).[127]

TABLE 13.4. Stability dependence of Amp containing triple helices on the environmental pH.

CMP	T_m (°C)	
	pH 3	pH 10.7
11XS	18	32
11YS	38	44
11YR	32	38
11XR	38	40

0.20 mM solutions of CMP in 50 mM aq. AcOH at a heating rate of 36°C·h^{-1}.

The C(4)-*exo* ring pucker of nonprotonated (4*S*)-aminoproline in the Yaa position matches the ring pucker of the naturally occurring (4*R*)Hyp residue in this position. In contrast, the C(4)-*endo* ring pucker of protonated (4*S*)-ammoniumproline is mismatching in this position (Figure 13.12 bottom). Thus, the CMP **11YS** was expected to form more stable triple helices under basic than under acidic conditions. Thermal denaturation experiments of triple helices formed of CMP **11YS** showed that they are, as expected, more stable under alkaline (T_m = 44°C) than under acidic (T_m = 38°C) conditions.[127]

13.6.2 Triple helices containing (4*R*)Amp

In analogy to the pucker defining factors for (4*S*)Amp, protonated (4*R*)AmpH^+ adopts a C(4)-*exo* ring pucker and non-protonated (4*R*)Amp adopts a C(4)-*endo* ring pucker.[128]

For triple helices formed of CMP **11XR** under alkaline conditions a higher stability was expected than under acidic conditions since under alkaline conditions the non-protonated (4*R*)Amp residue should adopt a C(4)-*endo* ring pucker which matches the ring pucker of the naturally occurring proline residue in the Xaa position whereas protonated (4*R*)ammonium-proline adopts a mismatching C(4)-*exo* ring pucker for this position. Thermal denaturation experiments under alkaline conditions revealed a T_m of 40 °C whereas under acidic condition a lower T_m of 38°C was found.[128] This result is confirms the expectations.

For triple helices formed of CMP **11YR** a higher stability under acidic than under alkaline conditions were expected since protonated (4*R*)AmpH^+ adopts a matching C(4)-*exo* ring

pucker for this position and non-protonated (4*R*)Amp adopts a mismatching C(4)-*endo* ring pucker for this position. However, a T_m of 38 °C was measured under alkaline conditions and under acidic conditions a lower T_m of 32 °C was observed.[128] This result is not in agreement with the expectation. A possible explanation for this surprising reversal of the stability are solvation effects during triple helix formation. This hypothesis is explained in the next chapter.[128]

13.6.3 Possible impact of solvation on the conformational stability of the collagen triple helix

The hypothesis on the impact of solvation on the conformational stability of the collagen triple helix is based on the following assumptions:

- Charged residues exhibit a larger solvation shell than uncharged residues.
- Upon triple helix formation a part of this shell has to be removed depending on the environment of the residue within the collagen triple helix.
- If the residue is directed to the outside of the triple helix only little of the solvent shell is lost upon triple helix formation.
- If the residue is directed to the inside of the triple helix a larger part of the solvation shell has to be removed due to the proximity of the neighboring strands.
- The reduction of the solvation shell upon triple helix formation leads to a loss in enthalpy and consequently results in a destabilization of the collagen triple helix.

In analogy to the study on the steric effects (chapter 13.3), the substituents of (4*S*)-configured ammonium proline in the Xaa position and the substituents of (4*R*)-configured ammonium proline in the Yaa position are directed to the inside of the collagen triple helix. Thus, the solvation shell around the ammonium group in the CMPs **11XS** and **11YR** have to be reduced upon triple helix formation, consequently leading to a destabilization of the triple helix (Figure 13.13). In total, the triple helices formed of **11XS** under acidic conditions should be destabilized by the competition of intramolecular and interstrand H-bonds (in analogy to (4*S*)Acp and (4*S*)Pvp in chapter 13.2.2 and 13.3.3) and the reduction of the solvation shell as well as the charge-charge repulsion within the triple helix. This explains the dramatic decrease in stability (ΔT_m = 14°C) of triple helices formed by **11XS** compared to the triple helices formed by the (4*S*)Acp containing **7XS** and (4*S*)Pvp containing **8XS**.

The unexpected stability of the triple helices formed by (4*R*)Amp containing **11YR** (as described in chapter 13.6.2) can be explained with this solvation shell hypothesis. Under acidic condition the protonated (4*R*)AmpH$^+$ adopts a matching ring pucker for the Yaa position whereas the non-protonated (4*R*)Amp under alkaline adopts a mismatching ring pucker for this position. However, in the formation of the helix containing the protonated (4*R*)AmpH$^+$ there is additionally the strong destabilizing effect of the solvent shell which has to be removed (Figure 13.13). This destabilizing effect of the solvent shell reduction seems to be stronger than the slightly destabilizing effect of a mismatching ring pucker. Thus the triple helix formed by **11YR** under basic conditions (mismatching ring pucker) is more stable than the triple helix formed by **11YR** under acidic conditions (matching ring pucker but reduced solvent shell).

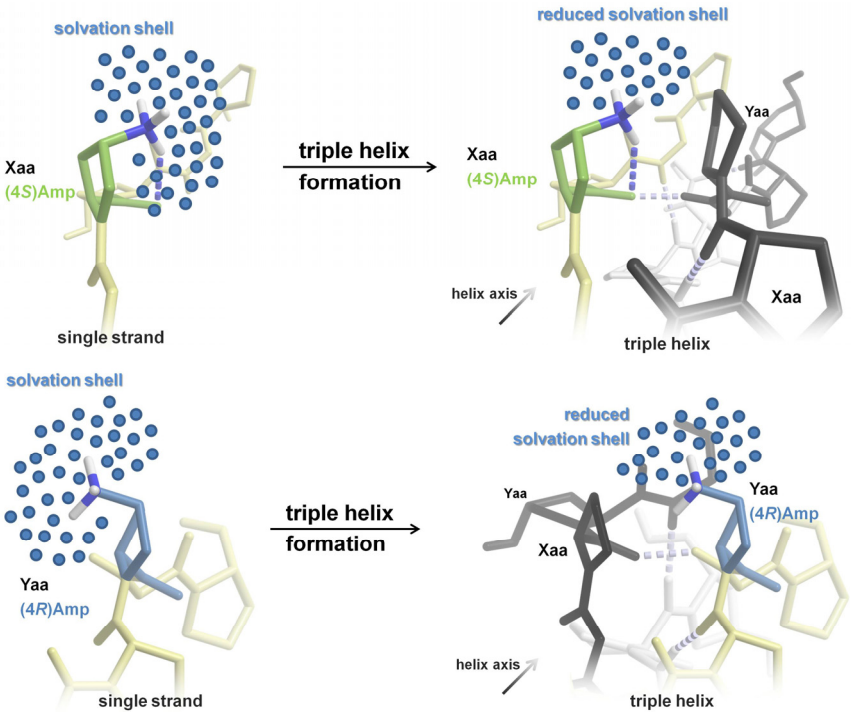


FIGURE 13.13. Destabilizing effect of the solvation shell of (4*S*)Amp in the Xaa position (top) and (4*R*)Amp in the Yaa position (bottom) on the conformational stability of the collagen triple helix.

The ammonium substituents in the triple helices formed of CMPs **11XR** and **11YS** under acidic conditions are directed to the outside of the triple helix and thus the solvent shells are

not reduced dramatically upon triple helix formation and consequently do not contributing to the destabilization of the triple helix (Figure 13.14).

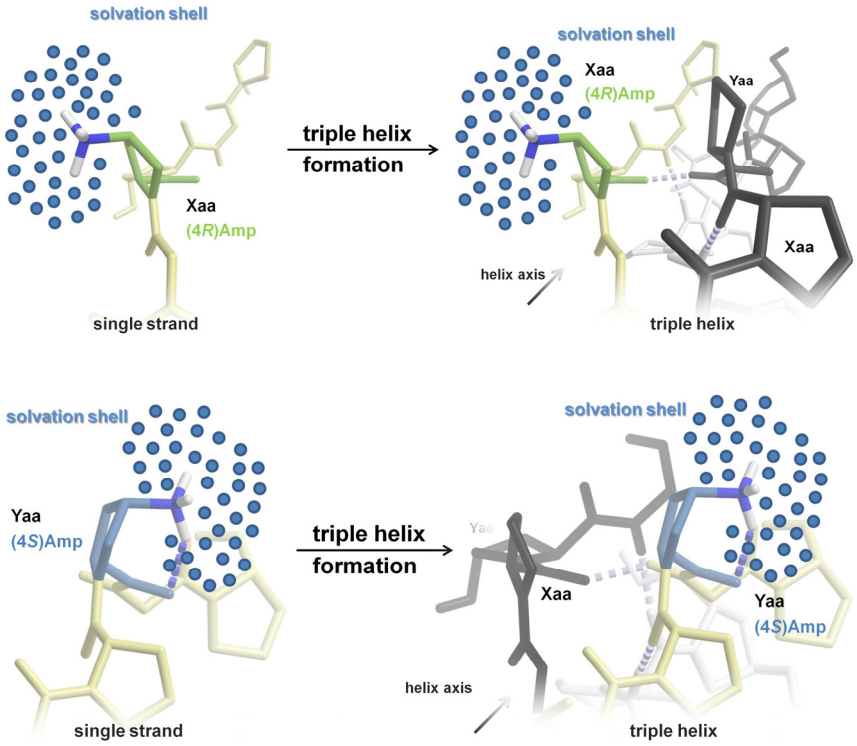


FIGURE 14.14. The solvation shell of (*4R*)Amp in the Xaa position (top) and (4*S*)Amp in the Yaa position (bottom) has no significant effect on the conformational stability of the collagen triple helix since it is not significantly reduced upon triple helix formation.

13.6.4 Conclusions

It has been demonstrated that (4*R*)- and (4*S*)Amp adopt different ring puckers depending on the protonation state of their substituent. This pH-dependent ring flipping can be used to influence the conformational stability of the collagen triple helix. Furthermore the study lead to a hypothesis of the effect of solvation shells around the ammonium substituents on the conformational stability of the collagen triple helix. To endorse this hypothesis the measurement of thermodynamic data with the method introduced by Engel und Bächinger[99] are planned.

Chapter 13.6 is a summary of the following manuscripts:

- R. S. Erdmann and H. Wennemers "*Protonation Dependent Flipping of the Proline Ring Pucker: Effect on the Conformational Stability of the Collagen Triple Helix"*, Manuscript D.
- R. S. Erdmann and H. Wennemers "*Aminoproline Containing Collagen Model Peptides: Influence of pH, Solvation, Ring Pucker and Hydrogen Bonds on Triple Helix Stability"*, Manuscript E.

14. Acetylated dimethylamides of proline derivatives as conformational models of proline residues within peptides and proteins

14.1 Introduction

Proline residues have a unique feature amongst natural amino acids. The linkage of the side chain to the amino function results in a pyrrolidine ring and ultimately leads to tertiary amide bonds within peptides and proteins.[130] In tertiary amides, in contrast to secondary amides, the *cis*-conformation of the amide bond can be populated to a significant extent (Figure 14.1a).[130-134] The *cis*/*trans*-isomerization plays important roles in natural processes such as protein folding[135] and signal transduction.[132,136-142] Proline is often found as residue promoting β-turns and as residue inducing breaks and kinks within α-helices.[130,142,143] Furthermore proline plays an important role in the polyproline structure and the collagen triple helix,[1] where not only the *cis*/*trans*-isomerization[1,99] is important but also the ring puckering of its pyrrolidine ring.[1] Many different proline derivatives have been developed to influence the conformational properties of proline residues in respect to influence the properties of peptides and proteins.[1,30,31,35,42,44,45,54,105,144-149] To study the conformational properties of such proline residues within a biopolymer, the much simpler acetylated proline methyl esters have been established as simple models.[1,30,33,35,54,144] However, these ester containing models are not completely representative of the situation in a biopolymer (Figure 14.1b, left). The ester moiety is more electrophilic than the naturally occurring amide. This leads to an overrepresentation of factors such as n→π*-interactions which are involved in the stabilization of α-helices, oligoprolines and the collagen triple helix.[1,145,150] To overcome this overrepresentation it was suggested to use Ac-Pro-NHMe as model (Figure 14.1b, middle).[151] However it has been shown that a hydrogen bond between the acetyl oxygen and the amide hydrogen is influencing the conformation of such a proline model to a significant extent.[151] Since such a hydrogen bond is not observed in α-helices, oligoprolines or the collagen triple helix these proline monomethyl amides are not representative models for prolyl amide bonds within biopolymers. An alternative to model Xaa-Pro amide bonds are acetylated dimethylamide proline derivatives which are presented in this chapter (Figure 14.1b, right). They exclusively contain amide bonds (compared to the methyl ester models) and cannot form intramolecular hydrogen bonds (compared to the monomethylamides).

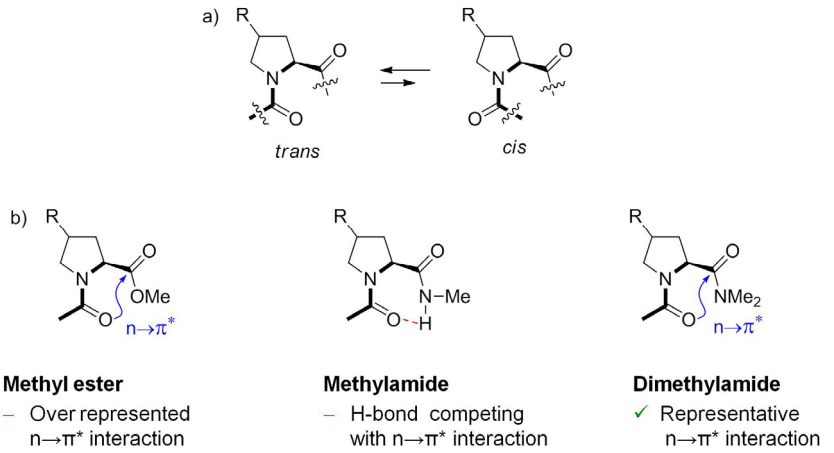
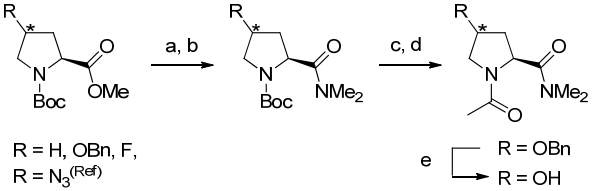


FIGURE 14.1. a) *Cis-trans* equilibrium within a peptide or protein. b) Advantages and disadvantages of methyl esters, methylamides and dimethylamides as model compounds.

14.2 Synthesis of Acetylated Dimethylamide Proline Derivatives

In a first series we prepared and investigated acetylated dimethylamide proline derivatives substituted with a hydroxy, fluoro or azido substituent at C(4). Starting from the corresponding Boc-protected methyl ester the ester was saponified with NaOH in THF/MeOH and the resulting carboxylic acid coupled to $NHMe_2 \cdot HCl$ with EDC·HCl in the presence of $^{i}Pr_2NEt$. Subsequently, the Boc-group was removed under acidic conditions (TFA/CH_2Cl_2 or HCl in dioxane) and the resulting amine was acetylated with Ac_2O in the presence of NEt_3. In case of hydroxy proline, the benzyl protecting group was removed from the hydroxy function under a hydrogen atmosphere in the presence of Pd/C as catalyst (Scheme 14.1).



SCHEME 14.1. Synthesis of acetylated dimethyl proline derivatives. a) 2 eq. NaOH, THF/MeOH, 3h, rt, (4*S*)Hyp: 79%. b) 1.3 eq EDC·HCl, 3.0 eq $^{i}Pr_2NEt$, 1.4 eq $HNMe_2 \cdot HCl$, CH_2Cl_2, rt, o.n. (over night). Pro: 60%, (4*R*)Hyp: 67%, (4*S*)Hyp: 56% , (4*R*)Flp: 62%, (4*S*)Flp: 70%. c) CH_2Cl_2/TFA 1:1 or 4M HCl in dioxane, 3h, rt, Pro: quant., (4*R*)Hyp: quant., (4*S*)Hyp: 63% , (4*R*)Flp: quant., (4*S*)Flp: quant.. d) 2 eq Ac_2O, 2 eq NEt_3, CH_2Cl_2, rt, o.n., Pro: 59%, (4*R*)Hyp: 85%, (4*S*)Hyp: 82% , (4*R*)Flp: 38%, (4*S*)Flp: 37%. e) H_2, Pd/C, MeOH, rt, o.n., (4*R*)Hyp: 47%, (4*S*)Hyp: 98%. Synthesis of Ac-(4*R*/*S*)Azp-NMe_2 was already described in ref. 111.

14.3 Hypothesis

Since the ring pucker of proline derivatives are mainly influenced by steric and stereoelectronic effects of the substituent at C(4) (chapters 4.3 and 5), no difference between the ring puckers adopted in Ac-Xaa-OMe and Ac-Xaa-NMe_2 proline derivatives were expected. For (4*R*)-configured Ac-Xaa-NMe_2 proline derivatives a lower $K_{trans:cis}$ than for Ac-Xaa-OMe was expected since the amides are weaker electrophile than the esters, which leads to a weaker n→π*-interaction within the amides and consequently results in a lower preference of a *trans* amide For (4*S*)-configured proline derivatives bearing no H-bond donor comparable $K_{trans:cis}$ values of the esters and dimethylamides were expected, since it has been demonstrated that there is no n→π*-interaction possible within the (4*S*)-configured

methylester and thus the amide as weaker electrophile should not have an influence on the $K_{trans:cis}$ (chapter 4.3).[30,35]

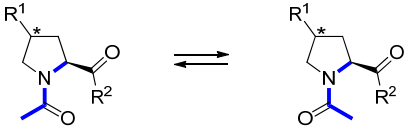
In (4*S*)-configured Ac-Xaa-NMe_2 proline derivatives bearing an H-bond donor an n→π*-interaction should be possible in analogy to the corresponding methyl esters.[31] Due to the lower electrophilicity of the amide compared to the ester a weaker n→π*-interaction is expected which leads to a lower $K_{trans:cis}$ of the dimethylamides compared to the esters. However, the amide is also a stronger nucleophile than the ester and thus a better H-bond acceptor. This could lead to a stronger activation of the amide compared to the ester for n→π*-interactions due to H-bonding. This stronger activation could compensate (partially) the intrinsically lower electrophilicity of the amide compared to the ester. Thus, it is expected, that the relative difference in $K_{trans:cis}$ of (4*S*)-configured, H-bond donor bearing proline methyl esters and dimethylamide is smaller than in (*4R*)-configured proline methyl esters and dimethylamides which do not profit from H-bond activation.

14.4 Conformational Properties of Ac-Xaa-OMe and Ac-Xaa-OMe in D_2O

The conformation of all proline derivatives were analyzed by NMR-spectroscopy in D_2O. For each compound two sets of signals were observed which correspond to the *trans*- and *cis*-isomer of the amide bond. The ring puckering of the proline derivatives were elucidated by the analysis of the vicinal ^1H-^1H-coupling constants. For all (4*R*)-configured proline dimethylamide derivatives a C(4)-*exo* ring pucker was observed and for all (4*S*)-configured proline dimethylamide derivatives a C(4)-*endo* ring pucker was observed which is, as expected, in agreement with the preferred ring pucker of the corresponding methyl esters.

For the dimethylamides of (4*R*)-configured Azp, Hyp, and Flp *cis*:*trans* ratio in the range of 3.7 to 4.5 were observed (Table 14.1). For Ac-Pro-NMe_2 a $K_{trans:cis}$ of 3.8 was observed. As expected, these values are lower than the $K_{trans:cis}$ of the corresponding esters. Surprisingly the $K_{trans:cis}$ of (4*R*)Azp that is comparable to (4*R*)Hyp and (4*R*)Flp in the ester form is on the lower level of Pro within the dimethylamide series. Since the hydroxy-, fluoro- and azido gauche effect are similar, this could arise from the higher steric demand of the azido group compared to the hydroxy and fluorine group. Steric effects influencing other conformational properties of this proline derivate could lead to a less beneficial orientation of the dimethylamide for an n→π*-interaction within Ac-(4*R*)Azp-NMe_2.

TABLE 14.1. $K_{trans:cis}$ of Ac-Xaa-OMe and Ac-Xaa-NMe_2 for Pro, Hyp, Flp and Azp in D_2O.



		$K_{trans:cis}$ (4*R*)		$K_{trans:cis}$ (4*S*)	
entry	R^1	R^2 = OMe	R^2 = NMe_2	R^2 = OMe	R^2 = NMe_2
1	H	4.6	3.8	4.6	3.8
2	OH	6.1	4.4	2.4	3.4
3	F	6.7	4.5	2.6	2.6
4	N_3	6.1	3.7	2.6	2.9

$K_{trans:cis}$ values were determined by NMR-spectroscopy of 80 mM solutions in D_2O.

For the (4*S*)-configured Ac-Xaa-NMe_2 model of Hyp, Flp and Azp $K_{trans:cis}$ values of in the range of 2.6 to 3.4 were observed (Table 14.1). In contrast to their (4*R*)-configured analogues,

as expected, only small differences between the $K_{trans:cis}$ of the methyl esters and the amides are observed. However, the $K_{trans:cis}$ of Ac-(4*S*)Hyp-NMe_2 is significantly higher compared to the corresponding methyl ester. The $K_{trans:cis}$ of Ac-(4*S*)Hyp-OMe is not indicative for a n→π*-interaction mediated by an intramolecular H-bond,[31,41] however, the amide in Ac-(4*S*)Hyp-NMe_2 as a better H-bond donor (see expectations, chapter 14.3) possibly allows for such a H-bond and thus leads to a n→π*-interaction which is responsible for the higher $K_{trans:cis}$ observed in the dimethylamide compared to the methyl ester (Figure 14.2).

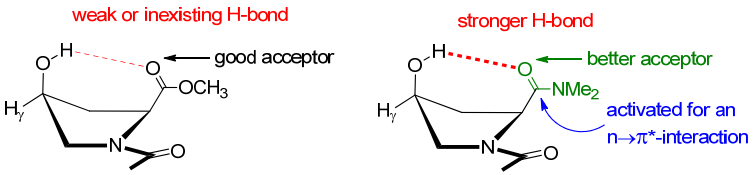
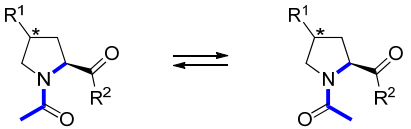


FIGURE 14.2. Formation of H-bonds within (4*S*)Hyp.

In a next series the properties of amino and amidoprolines were investigated. They were synthesized by the hydrogenation of the corresponding Ac-Azp-NMe_2 and in situ acylation of the resulting amine.

Also for the ammonium and amido prolines, as expected, generally a lower $K_{trans:cis}$ of the dimethyl amides compared to the methyl esters was found. (Table 14.2).

TABLE 14.2. $K_{trans:cis}$ of Ac-Xaa-OMe and Ac-Xaa-NMe_2 for Amp, Acp, and Piv D_2O.



			$K_{trans:cis}$			
			(4*R*)		(4*S*)	
entry	R^1	*C(4)	R^2 = OMe	R^2 = NMe_2	R^2 = OMe	R^2 = NMe_2
1	NH_3^+	*R*	3.5	3.1	5.7	5.1
2	NHAc	*R*	5.8	3.9	4.3	4.5
3	NHPiv	*R*	4.9	3.8	5.0	3.9

$K_{trans:cis}$ values were determined by NMR-spectroscopy of 80 mM solutions in D_2O.

As expected, for all (4*S*)-configured derivatives a C(4)-*endo* ring pucker was found and for the (4*R*)-configured derivatives, with the exception of Ac-(4*R*)Pvp-NMe_2, a C(4)-*exo* ring pucker was found. This finding is in agreement with the corresponding methyl esters (except Ac-(4*R*)Pvp-OMe adopts a C(4)-*exo* ring pucker whereas the dimethylamide adopts a C(4)-*endo* ring pucker).

The $K_{trans:cis}$ of (4*S*)-configured ammonium and amidoprolines is, as expected, possibly influenced by an intermolecular H-bond which orientates the dimethylamide in a way that allows for an n→π*-interaction. Furthermore the lower difference in $K_{trans:cis}$ between these (4*S*)-configured dimethylamides and esters compared to the difference in $K_{trans:cis}$ between (4*R*)-configured hydroxy-, azido- and fluoroproline methyl ester and dimethylamides, possibly arises from the H-bond activation which can only occur in Ac-(4*S*)Amp/Acp/Pvp-NMe_2 as hypothesized in chapter 14.2.
As mentioned above, Ac-(4*R*)Pvp-NMe_2 is a special case since it preferentially adopts a C(4)-*endo* ring pucker. A possible explanation for this preference is the high steric demand of the pivaloyl group attached at the amine at C(4). This leads to a competition between the stereoelectronic gauche effect which directs the group into a pseudo axial position (C(4)-*exo* conformation) and the steric effect which directs the substituent in a pseudo equatorial position (C(4)-*endo* conformation). In contrast to the less bulky acetyl group in Ac-(4*R*)Acp-NMe_2, the pivaloyl group seems bulky enough that the steric effect prevails over the stereoelectronic effect and is decisive for the pseudo equatorial alignment of the pivaloylamido group which consequently leads to a C(4)-*endo* ring pucker. However, also the dimethylamide has to play a role in this conformation since the substituent in the corresponding methyl ester is in a pseudo axial position. In Ac-(4*S*)Pvp-NMe_2 the possibility of the formation of a stabilizing intramolecular H-bond is probably decisive for the adoption of a C(4)-*endo* ring pucker with the bulky pivaloylamide in a pseudo axial position (Figure 14.4).

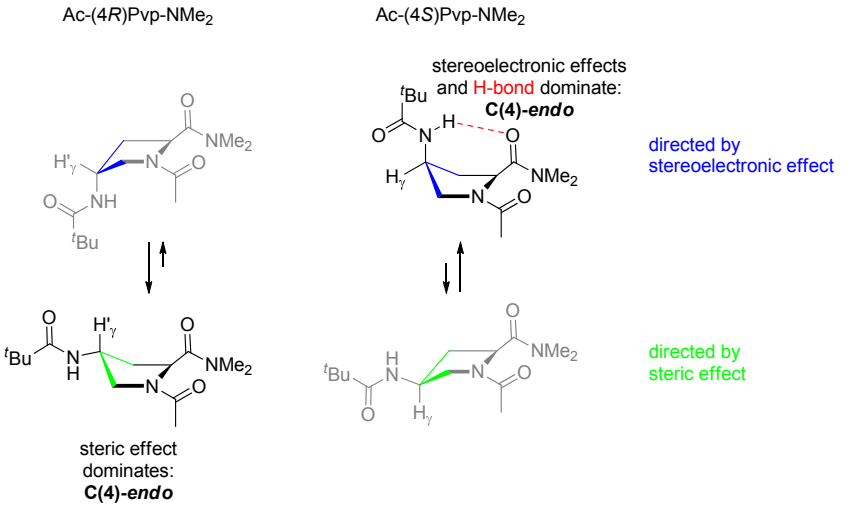


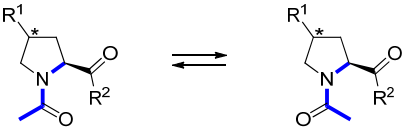
FIGURE 14.4. Competition of effects directing the ring pucker of Ac-(4*R*)Pvp-NMe_2 and Ac-(4*S*)Pvp-NMe_2.

14.5 Conformational properties of Ac-Xaa-OMe and Ac-Xaa-OMe in $CDCl_3$

To probe whether the conformation defining factors in D_2O are the same as in other solvents, the conformational properties of these proline derivatives were investigated in $CDCl_3$.

Whereas the $K_{trans:cis}$ is higher for the methyl esters than for the dimethyl amides in D_2O, in $CDCl_3$ the dimethyl amides surprisingly exhibit a higher $K_{trans:cis}$ than the esters (Table 14.3). (4*R*)-configured acetylated methylester proline derivatives exhibit a $K_{trans:cis}$ in $CDCl_3$ in the range of 3.7 to 5.2. The corresponding dimethylamides preferentially adopt the *trans*-conformer to a significant higher extent with a $K_{trans:cis}$ in the range of 5.7 to 9.8. The $K_{trans:cis}$ of Ac-(*4S*)Xaa-OMe in $CDCl_3$ are in the range of 1.7 to 5.8 and their (4*R*)-configured analogues exhibit $K_{trans:cis}$ values in the range of 3.7 to 13.2.

TABLE 14.3. $K_{trans:cis}$ of Ac-Xaa-OMe and Ac-Xaa-NMe_2 in $CDCl_3$.



		$K_{trans:cis}$ (4*R*)		$K_{trans:cis}$ (4*S*)	
entry	R^1	R^2 = OMe	R^2 = NMe_2	R^2 = OMe	R^2 = NMe_2
1	H	3.8	8.2	3.8	8.2
2	OH	4.4	6.6	4.7	n.d.
3	F	4.5	9.8	1.7	3.7
4	N_3	3.7	9.3	1.9	6.4
5	NHAc	5.2	5.7	5.8	10.8
6	NHPiv	4.4*	7.6*	5.6*	13.2*

Determined by NMR-spectroscopy of 80 mM solutions in $CDCl_3$. * Solutions contained irremovable traces of water.

There are several possible reasons for the higher $K_{trans:cis}$ of the dimethylamides in $CDCl_3$ compared to D_2O. In D_2O the *cis*-conformer of Ac-Xaa-NMe_2 could be stabilized by a hydrophobic interaction of the methyl residues of the acetyl and dimethylamide group leading to a low $K_{trans:cis}$ in D_2O. In $CDCl_3$ this hydrophobic interaction cannot take place and thus the *cis*-conformer is not stabilized. Furthermore the higher $K_{trans:cis}$ of the dimethylamides in $CDCl_3$ compared to D_2O could be explained by a competition between the interactions of the acetyl oxygen and the D_2O oxygen with the π*-orbital of the dimethylamide. This competition weakens the n→π*-interaction in D_2O. In $CDCl_3$ there is no competing D_2O and thus the $K_{trans:cis}$ is higher. Another explanation was found in the *ab initio* calculations of lowest energy structures of Ac-(*4R*/*S*)Azp-NMe.[152] An intramolecular H-bond between the oxygen of the acetyl group and a hydrogen of the dimethylamide methyl groups stabilizes the *trans*-conformer. However, these calculations did not consider solvents.

The exceptionally high $K_{trans:cis}$ of Ac-(4*S*)Acp-NMe_2 and Ac-(4*R*)Pvp-NMe_2 are explainable by the effect of the intermolecular H-bond which is stronger in $CDCl_3$ than in D_2O and further activates the dimethylamide carbonyl for a n→π*-interaction.

However, these arguments cannot explain why the $K_{trans:cis}$ of the methyl esters is higher in D_2O than in $CDCl_3$.

In conclusion the different $K_{trans:cis}$ values of methyl esters and dimethyl amides in different solvents can only be explained partially. Most certainly the $K_{trans:cis}$ is the result of many different factors such as n→π*-interactions, steric interactions, H-bonds, dipole-dipole interactions, hydrophilic and hydrophobic interactions which contribute differently to the stability of each conformer depending on the solvent and the group attached to C(α). Further investigations are necessary to elucidate the weight of each factor and their interplay.

A possible way to eliminate (or reduce) factors such as n→π*-interactions and dipole-dipole interactions would be the investigation of alkene isosteres[153] of the amide and esters, as well as isosteres of their resonance structure (Figure 14.5).

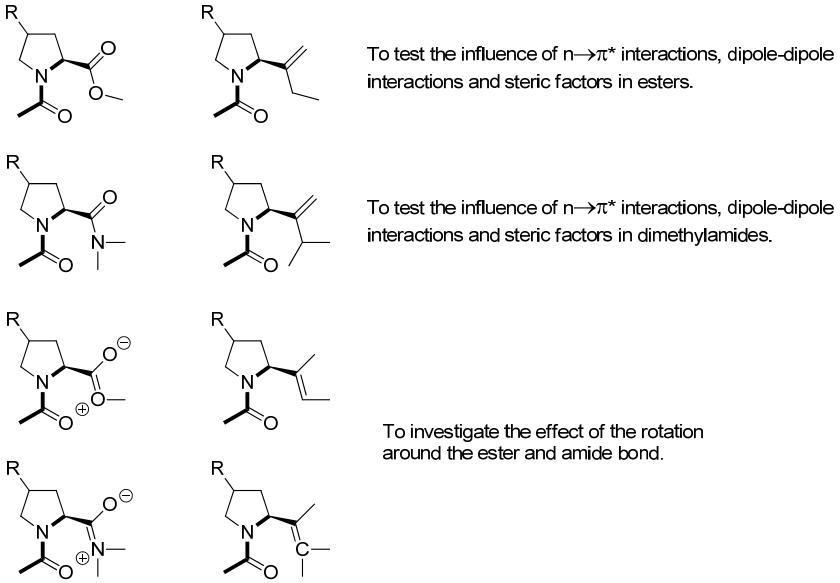


FIGURE 14.5. Alkene isosteres of esters and dimethyl amides as well as of their resonance form.

Also calculations including different solvents could be helpful to gain deeper insight into the fashion how the solvent, n→π*-interactions, steric interactions, H-bonds, dipole-dipole interactions and hydrophilic and hydrophobic effects influence the $K_{trans:cis}$.

However, until a clear understanding of the interconnection of the conformation directing factors is reached, the absolute $K_{trans:cis}$ values of methyl esters and dimethyl amides should be used carefully to predict conformations within biopolymers since it is still not confirmed if all

the factors that are responsible for their conformation also play a role in the conformation of proline containing peptides and proteins. Furthermore, it has to be considered that the second methyl group (compared to the methyl ester) represents an additional steric factor which does not occur in secondary Xaa-Pro bonds. However, this additional steric factor of the two methyl groups in the dimethylamide monomers should very well represent the situation in tertiary Xaa-Pro amide bonds such as in oligoprolines, certain parts of the collagen triple helix, peptoids and N-methylated peptides.

15. Summary

In this thesis it has been demonstrated that azidoproline containing collagen model peptides can be easily functionalized by click chemistry or a reduction/acylation approach. Moieties such as monosaccharides conjugated to a triazol unit or pivaloylamides could be attached to the collagen triple helix. It has been demonstrated that functionalizations of CMPs containing (4*R*)Azp in the Xaa position using a reduction/acylation approach lead to the most stable collagen triple helices (T_m = 44°C). As for functionalizations by click chemistry (4*R*)Azp residues in the Xaa position and (4*S*)Azp residues in the Yaa position are the sites of choice since the resulting peptides lead to the most stable triple helices (T_m up to 40°C). Furthermore a method to design pH-sensitive collagen triple helices was presented. These findings provide a guideline for the design of environment sensitive, stable, and functional collagen based materials.

At the same time a deeper insight into the factors that are responsible for the conformational stability of the collagen triple helix was gained. Whereas previous studies mainly focused on the preference for a *trans*-amide bond of proline residues and its ring pucker within the collagen triple helices, these studies additionally shed light on factors such as ψ-angles, hydrogen bonds and steric effects. It has been demonstrated that a matching ring pucker is not as important as previously thought. The incorporation of residues with a H-bond donating substituent that were able to interfere with the interstrand H-bonds demonstrated their importance for the conformational stability of the collagen triple helix. The incorporation of sterically more demanding substituents demonstrated that an alignment of these substituents towards the outside of the triple helix to prevent a clash with neighboring strands is more important than a matching ring pucker. Studies on triple helices containing charged ammonium substituents lead to the hypothesis of a position and stereochemistry dependent effect of solvation shells on the conformational stability of the collagen triple helix.

The investigation on acetylated dimethylamide proline derivatives as models for proline residues within biopolymers demonstrated that the $K_{trans:cis}$ of proline residues in biopolymers might be lower than previously estimated with acetylated methyl ester proline derivatives due to a the weaker electrophilicity of an amide carbonyl compared to a ester carbonyl. The investigation of methyl esters and dimethylamides in $CDCl_3$ showed in contrast to D_2O higher $K_{trans:cis}$ of the dimethylamides compared to the esters. This phenomenon is not clearly understood yet but it suggests that the $K_{trans:cis}$ is the result of many different factors such as

n→π*-interactions, steric interactions, H-bonds, dipole-dipole interactions, hydrophilic and hydrophobic interactions and the group attached to C(α). Thus, these values should only be used with the knowledge that they might not completely represent the situation within biopolymers.

16. Outlook

The functionalization techniques developed in this thesis can be used for the development of functional collagen based biomaterials. Such materials could be useful in medical applications such as wound healing, cell adhesion, drug delivery and implants.

For such applications a collagen based material that is able to self assemble into fibrils and bundles and to form hydrogels is highly desirable. For this purpose we envision the crosslinking of single strands in a way that overlapping sticky ends allow for self assembly. In contrast to the approach of Koide and coworkers[84] our design should allow for the synthesis of the whole crosslinked triple strand on solid support. The synthetic strategy is to introduce an Azp residue in a single strand and reduce it to the amine, couple a β-alanine linker to the resulting aminoproline and finally start to build up the next single strand on the β-alanine linker (Figure 16.1). The synthesis of two crosslinked single strands was already carried in our lab, the product was identified in the crude mixture and the purification is in progress.

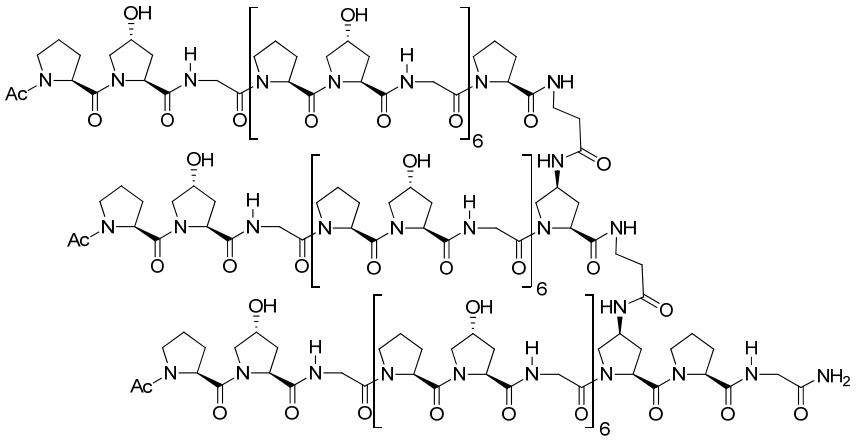


FIGURE 18.1. Example of three cross linked single strands.

The attachment of collagen model peptides on the surface of dental implants could lead to a higher bone-implant surface contact, what results in a stronger anchoring of the implant in the jawbone.[154]

Furthermore the possibility to design triple helices which are sensitive to the pH of their environment could be used for the development of collagen based pH-sensitive dendrimers[155]

that encapsulate bioactive molecules. Such a system could be used for the delivery and selective release of drugs to the above-average acidic environment of cancer cells.[156]

IV. Experimental Part for Ac-Xaa-NMe_2

19. General Aspects

Materials and reagents were of the highest commercially available grade and used without further purification. Reactions were monitored by thin layer chromatography using Merck silica gel 60 F254 plates. Compounds were visualized by UV, $KMnO_4$, TDM and ninhydrin. Flash chromatography was performed using Merck silica gel 60, particle size 40 - 63 µm. ^{1}H and ^{13}C NMR spectra were recorded on Bruker DPX 500 and DPX 400 spectrometers at a concentration of 80 mM. Chemical shifts are reported in ppm using TMS (or HOD) as a reference. A Bruker Esquire 3000plus instrument was used for electrospray ionization (ESI) mass spectrometry measurements. High resolution mass spectra were recorded on a LTQ Orbitrap XL spectrometer from Thermo Fischer Scientific with a nanoelectrospray ion source at the University of Bern, Switzerland.

20. Abbreviations of non-canonical amino acids

Acp	4-acetamidoproline
Amp	4-aminoproline
Azp	4-azidoprolin
Flp	4-fluoroproline
Hyp	4-hydroxyproline
Pvp	4-pivaloylproline
Tzp	4-triazolylproline

21. Synthesis of Ac-Xaa-NMe_2 model compounds

21.1 Synthesis of Ac-Pro-NMe_2

Boc-Pro-NMe_2

Boc-Pro-OH (500 mg, 2.32 mmol, 1.0 eq) was dissolved in CH_2Cl_2 (9.3 ml) and EDC·HCl (490 mg, 2.56 mmol, 1.1 eq), $^{i}Pr_2NEt$ (514 µL, 3.02 mmol, 1.3 eq) and $HNMe_2$·HCl (243 mg, 3.02 mmol, 1.3 eq) were added. The solution was stirred over night and diluted with CH_2Cl_2 (50 ml) and washed with 1M HCl (3x 15 ml) and sat. $NaHCO_3$ (3x 15 ml). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was subjected to flash chromatography (5% MeOH in CH_2Cl_2) to obtain 337 mg of the title compound as a colorless oil (60%).

TLC R_f = 0.36 (silica, 5% MeOH in CH_2Cl_2, ninhydrin).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1.2:1).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 4.68 (dd, *J* = 8.3 Hz, 3.2 Hz, 1H; Hα), 3.66-3.54 (m, 1H; Hδ), 3.54-3.37 (m, 1H; Hδ), 3.10 (s, 3H; NMe_2), 2.97 (s, 3H; NMe_2), 2.23-1.98 (m, 2H; Hβ), 1.92-1.79 (m, 2 H; Hβ), 1.47 (s, 9H).

Isolated signals of the minor conformer: 4.55 (dd, *J* = 8.2 Hz, 4.3 Hz, 1H), 3.07 (s, 3H), 2.98 (s, 3H), 1.41 (s, 6H).

^{13}C NMR (101 MHz, $CDCl_3$) δ/ppm = 172.7 (amide), 154.9 (Boc), 79.8 (Boc), 56.7 (Cα), 47.2 (Cδ), 37.3 (NMe_2), 36.3 (NMe_2), 29.9 (Cβ), 28.9 (Boc), 24.6 (Cγ).

Isolated signals of the minor conformer: 174.1 (amide), 154.7 (Boc), 79.8 (Boc), 56.8 (Cα), 47.0 (Cδ), 37.3 (NMe_2), 36.3 (NMe_2), 30.7 (Cβ), 28.7 (Boc), 24.0 (Cγ).

ESI-MS: *m/z* calcd for $C_{12}H_{22}N_2O_3$: 242.2; found: 265.3 $[M+Na]^+$ (100%).

H-Pro-NMe_2·TFA

Boc-Pro-NMe2 (337 mg, 1.39 mmol, 1.0 eq) was stirred in a 1:1 mixture of TFA and CH_2Cl_2 (7 ml) over 3 hours. The solution was concentrated under reduced pressure to obtain 356 mg of the title compound as a slightly yellowish oil (quant.).

TLC R_f = 0.41 (silica, MeCN:H_2O 4:1, ninhydrin).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 10.41 (s, 1H, NH_2^+), 7.66 (s, 1H; NH_2^+), 4.92-4.81 (m, 1H; Hα), 3.62-3.44 (m, 2H; Hδ), 3.09 (s, 3H; NMe_2), 3.05 (s, 3H; NMe_2), 2.63-2.51 (m, 1H; Hβ), 2.25-2.14 (m, 1H; Hβ), 2.13-2.03 (m, 1H; Hγ), 2.02-1.92 (m, 1H; H).
^{13}C NMR (101 MHz, $CDCl_3$) δ/ppm = 168.6 (amide), 58.7 (Cα), 47.5 (Cδ), 37.1 (NMe_2), 36.7 (NMe_2), 29.8 (Cβ), 25.2 (Cγ).
ESI-MS: *m/z* calcd for $C_7H_{14}N_2O$: 142.1; found: 143.1 $[M+H^-]^+$ (100%).

Ac-Pro-NMe$_2$

H-Pro-NMe_2·TFA (356 mg, 1.39 mmol, 1.0 eq) was dissolved in 3.8 ml of CH_2Cl_2 and Ac_2O (263 µL, 2.78 mmol, 2.0 eq) and NEt_3 (391 µL, 2.78 mmol, 2.0 eq) were added. The solution was stirred over night, diluted with CH_2Cl_2 (15 ml), and washed with 1M HCl (3x 5 ml) and sat. $NaHCO_3$ (3x 5 ml). The organic layer was dried under reduced pressure and the residue was subjected to flash chromatography (silica, 5% MeOH in CH_2Cl_2) to obtain 150 mg of the title compound as a colorless oil (59%).
TLC R_f = 0.22 (silica, 5% MeOH in CH_2Cl_2, TDM).
(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:8.2).
^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 4.86 (dd, *J* = 8.2 Hz, 3.6 Hz, 1H; Hα), 3.73 (ddd, *J* = 9.5 Hz, 8.0 Hz, 5.0 Hz, 1H; Hδ), 3.53 (dt, *J* = 9.7 Hz, 7.2 Hz, 1H; Hδ), 3.14 (s, 3H;NMe_2), 2.97 (s, 3H; NMe_2), 2.26-2.10 (m, 2H; Hβ), 2.10 (s, 3H; Ac), 2.02-1.81 (m, 2H; Hγ).
Isolated signals of the minor conformer: 4.63 (dd, *J* = 8.6 Hz, 2.6 Hz, 1H; Hα), 3.10 (s, 3H; NMe_3), 3.01 (s, 3H; NMe_2), 1.91 (s, 3H; Ac).
^{13}C NMR (101 MHz, $CDCl_3$) δ/ppm = 172.4 (amide), 169.5 (amide), 56.4 (Cα), 48.4 (Cδ), 37.5 (NMe_2), 36.3 (NMe_2), 29.5 (Cβ), 25.1 (Cγ), 22.7 (Ac).
ESI-MS: *m/z* calcd for $C_9H_{16}N_2O_2$: 184.1; found: 207.2 $[M+Na]^+$ (100%).

21.2 Synthesis of Ac-(4*R*)Hyp-NMe$_2$

Boc-(4*R*)Hyp(Bn)-NMe$_2$

Boc-(4R)Hyp(Bn)-OH (500 mg, 1.56 mmol, 1.0 eq) was dissolved in CH_2Cl_2 (6.2 ml) and EDC·HCl (388 mg, 2.02 mmol, 1.3 eq), ^{i}Pr$_2$NEt (397 µl, 2.33 mmol, 1.5 eq) and $HNMe_2$·HCl (188 mg, 2.33 mmol, 1.5 eq) were added. The reaction mixture was stirred over night and

diluted with 15 ml of CH_2Cl_2. After washing with 1M HCl (3x 5 ml) and sat. $NaHCO_3$ (3x 5 ml) the organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was subjected to flash chromatography (silica, 5% MeOH) to obtain 362 mg of the title compound as a colorless oil (67%).

TLC R_f = 0.45 (silica, 5% MeOH in CH_2Cl_2, UV, $KMnO_4$).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:1).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 7.40-7.29 (m, 5H; Ph), 4.61-4.45 (m, 2H; Bn), 4.35-4.29 (m, 1H; Hα), 3.78 (dt, *J* = 11.6 Hz, 1.8 Hz, 1H; Hδ), 3.72 (dd, *J* = 11.0 Hz, 4.9 Hz, 1H; Hδ), 3.13 (s, 3H; NMe_2), 2.98 (s, 3H; NMe_2), 2.35-2.20 (m, 2H; Hγ), 2.13-2.03 (m, 2H; Hβ), 1.42 (s, 9H; Boc).

Isolated signals of the other conformer: 4.73 (t, *J* = 7.6 Hz, 1H), 4.27-4.21 (m, 1H; Hα), 3.68 (dd, *J* = 11.6 Hz, 4.4 Hz, 1H; Hδ), 3.60 (dd, *J* = 11.5 Hz, 2.3 Hz, 1H; Hδ), 3.08 (s, 3H; NMe_2), 2.99 (s, 3H; NMe_2), 1.47 (s, 9H; Boc).

ESI-MS: *m/z* calcd for $C_{19}H_{28}N_2O_4$: 348.2; found: 371.3 $[M+Na]^+$ (100%).

H-(4*R*)Hyp(Bn)-NMe_2·TFA

Boc-(4*R*)Hyp(Bn)-NMe_2 (362 mg, 1.04 mmol, 1.0 eq) was dissolved in a 1:1 mixture of TFA and CH_2Cl_2 (5.2 ml) and stirred for 3 hours. The solution was concentrated under reduced pressure to obtain 377 mg of the title compound as a slightly yellowish oil (quant.).

TLC R_f = 0.31 (silica, MeCN:H_2O 4:1, ninhydrin).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 7.83 (s, 2H; NH_2^+), 7.43-7.29 (m, 5H; Ph), 5.04 (dd, *J* = 10.1 Hz, 7.9 Hz, 1H; Bn), 4.64 (d, *J* = 12.0 Hz, 1H; Bn), 4.48 (d, *J* = 12.0 Hz, 1H; Hα), 4.38 (t, *J* = 3.8 Hz, 1H; Hγ), 3.83 (d, *J* = 12.5 Hz, 1H; Hδ), 3.42 (dd, *J* = 12.3 Hz, 3.0 Hz, 1H; Hδ), 3.03 (s, 6H; 2xNMe_2), 2.62 (ddt, *J* = 13.6 Hz, 7.6 Hz, 1.6 Hz, 1H; Hβ), 1.93 (ddd, *J* = 13.8 Hz, 10.4 Hz, 4.3 Hz, 1H; Hβ).

^{13}C NMR (101 MHz, $CDCl_3$) δ/ppm = 161.1 (amide), 137.0, 129.1, 128.7, 128.3 (Ph), 77.4 (Cγ), 71.7 (Bn), 58.0 (Cα), 51.2 (Cδ), 37.0 (NMe_2), 36.7 (NMe_2), 36.0 (Cβ).

ESI-MS: *m/z* calcd for $C_{14}H_{20}N_2O_2$: 248.2; found: 249.3 $[M\text{-}TFA^-]^+$ (100%).

Ac-(4*R*)Hyp(Bn)-NMe_2

H-(4*R*)Hyp(Bn)-NMe_2·TFA (376 mg, 1.04 mmol, 1.0 eq) was dissolved in CH_2Cl_2 (2.1 ml) and Ac_2O (196 µl, 2.08 mmol, 2.0 eq) and NEt_3 (292 µl, 2.08 mmol, 2.0 eq) were added. The solution was stirred over night and diluted with 50 ml CH_2Cl_2. The solution was washed with

1M HCl (3x 15 ml) and sat. $NaHCO_3$ (3x 15 ml) and dried over $MgSO_4$. After concentration under reduced pressure the residue was subjected to flash chromatography (silica, 10 % MeOH in CH_2Cl_2) to obtain 256 mg of the title compound as a colorless oil (85%).

TLC R_f = 0.39 (silica, 10% MeOH in CH_2Cl_2, $KMnO_4$).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:8.8).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 7.40-7.27 (m, 5H; Ph), 4.94 (dd, *J* = 8.1 Hz, 6.2 Hz, 1H; Hα), 4.60-4.48 (m, 2H; Bn), 4.44-4.38 (m, 1H; Hγ), 3.84 (dd, *J* = 10.8 Hz, 5.2 Hz, 1H; Hδ), 3.57 (dd, *J* = 10.7 Hz, 3.0 Hz, 1H; Hδ), 3.15 (s, 3H; NMe_2), 2.96 (s, 3H; NMe_2), 2.24 (ddd, *J* = 12.6 Hz, 8.2 Hz, 4.4 Hz, 1H; Hβ), 2.16-2.08 (m, 1H; Hβ), 2.03 (s, 3H; Ac).

Isolated signals of the minor conformer: 4.81-4.75 (t, *J* = 7.8 Hz, 1H; Hα), 4.23-4.17 (m, 1H; Hγ), 4.09-4.03 (m, 1H; Hδ), 3.62 (dd, *J* = 12.7 Hz, 4.7 Hz, 1H; Hδ), 3.07 (s, 3H; NMe_2), 3.00 (s, 3H; NMe_2), 2.53-2.44 (m, 1H; Hβ), 1.90 (s, 3H; Ac).

^{13}C NMR (101 MHz, $CDCl_3$) δ/ppm = 172.4 (amide), 169.6 (amide), 138.2, 129.0, 128.3, 128.0 (Ph), 78.2 (Cα), 72.0 (Bn), 55.1 (Cγ), 53.7 (Cδ), 37.7 (NMe_2), 36.4 (NMe_2), 35.6 (Cβ), 22.7 (Ac).

ESI-MS: *m/z* calcd for $C_{16}H_{22}N_2O_3$: 290.2; found: 313.3 $[M+Na]^+$ (100%).

Ac-(4*R*)Hyp-NMe_2

Ac-(4*R*)Hyp(Bn)-NMe_2 (256 mg, 882 µmol, 1.0 eq) was added to a suspension of Pd/C (50 mg, 10% Pd, 20 % w/w) in MeOH (3.5 ml) and stirred over night under an H_2-atmosphere. The suspension was filtered over Celite and concentrated under reduced pressure. The residue was purified using MPLC (C18-RP-silica) to obtain 123 mg of the title compound as a colorless oil (70%).

TLC R_f = 0.39 (silica, 10% MeOH in CH_2Cl_2, $KMnO_4$).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:6.6).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 4.95 (t, *J* = 7.5 Hz, 1H; Hα), 4.63 (s, 1H; Hγ), 3.85 (dd, *J* = 10.8 Hz, 4.7 Hz, 1H; Hδ), 3.51 (ddd, *J* = 10.8 Hz, 2.2 Hz, 1.3 Hz, 1H; Hδ), 3.17 (s, 3H; NMe_2), 2.97 (s, 3H; NMe_2), 2.19 (dddd, *J* = 11.7 Hz, 8.1 Hz, 3.5 Hz, 1.2 Hz, 1H; Hβ), 2.13-2.09 (m, 1H; Hβ), 2.08 (s, 3H; Ac).

Isolated signals of the minor conformer: 4.85 (t, *J* = 7.8 Hz, 1H; Hα), 4.48 (s, 1H; Hγ), 3.58 (dd, *J* = 12.6 Hz, 4.0 Hz, 1H; Hδ), 3.12 (s, 3H; NMe_2), 3.01 (s, 3H; NMe_2), 2.50-2.40 (m, 1H; Hβ), 1.90 (s, 3H; Ac).

^{13}C NMR (101 MHz, $CDCl_3$) δ/ppm = 172.5 (amide), 170.0 (amide), 70.8 (Cα), 56.6 (Cδ), 55.2 (Cγ), 38.2 (Cβ), 37.7 (NMe_2), 36.5 (NMe_2), 22.8 (s).

ESI-MS: *m/z* calcd for $C_9H_{16}N_2O_3$: 200.1; found: 223.3 $[M+Na]^+$ (100%).

21.3 Synthesis of Ac-(4*S*)Hyp-NMe_2

Boc-(4*S*)Hyp(Bn)-OMe

Boc-(4*S*)Hyp-OMe (200 mg, 815 µmol, 1.0 eq) was dissolved in DMF (1.0 ml). Benzylbromide (194 µl, 1.63 mmol, 2.0 eq), Ag_2O (378 mg, 1.63 mmol, 2.0 eq), and tetrabutyl ammonium iodide (301 mg, 815 µl, 1.0 eq) were added. The suspension was stirred over night and diluted with CH_2Cl_2 (100 ml) whereupon a yellow solid precipitated. The suspension was washed with 1M HCl (2x 100 ml). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was subjected to flash chromatography (silica, pentane-EtOAc, 10:1→3:1) to obtain 267 mg of the title compound as a colorless oil (98 %).

TLC R_f = 0.77 (silica, pentane:EtOAc 1:1, ninhydrin).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:1.9).

^{1}H NMR (500 MHz, $CDCl_3$): δ/ppm (Hauptkonformer) = 7.25 (m, 5H, Bn), 4.63 (d, *J* = 4.4 Hz, 1H; Hα), 4.39 (dt, *J* = 18.4 Hz, 11.9 Hz, 2H; Bn), 4.25 (t, *J* = 7.8 Hz, 1H; Hγ), 4.04 (dd, *J* = 11.8 Hz, 7.9 Hz, 1H; Hδ), 3.58 (s, 3H; OMe), 3.48 (ddd, *J* = 16.1 Hz, 11.6 Hz, 4.6 Hz, 1H; Hδ), 2.26 (dd, *J* = 14.2 Hz, 8.0 Hz, 1H; Hβ), 2.17 (m, 1H; Hβ), 1.35 (s, 9H, Boc).

Isolated signals of the minor conformer: 7.26 (m, 5H; Ph), 4.64 (d, *J* = 4.4 Hz, 1H; Hα), 4.44 (dt, *J* = 18.4 Hz, 11.9 Hz, 2H; Bn), 4.37 (t, *J* = 7.4 Hz, 1H; Hγ), 4.12 (dd, *J* = 8.0 Hz, 4.1 Hz, 1H; Hα), 3.67 (s, 3H; OMe), 3.55 (ddd, *J* = 16.1 Hz, 11.6 Hz, 4.6 Hz, 1H; Hδ), 2.32 (dd, *J* = 14.2 Hz, 8.0 Hz, 1H; Hβ), 2.00 (m, 1H; Hβ), 1.38 (s, 9H; Boc).

^{13}C NMR (101 MHz, $CDCl_3$): δ/ppm = 173.3 (ester), 154.2 (Boc), 138.1, 128.8, 128.0, 127.4 (Ph), 81.2 (Boc), 76.1 (Cα), 71.3 (Bn), 58.2 (Cγ), 52.4 (OMe), 51.7 (Cδ), 36.5 (Cβ), 28.7 (Boc).

Isolated signals of the minor conformer: 172.9 (ester), 154.7 (Boc), 141.7, 128.9, 128.1, 128.0 (Bn), 81.2 (Boc), 77.1 (Cα), 71.1 (Bn), 57.5 (Cγ), 52.5 (OMe), 52.4 (Cδ), 35.4 (Cβ), 28.8 (Boc).

Boc-(4*S*)Hyp(Bn)-OH

Boc-(4*S*)Hyp(OBn)-OMe (227 mg, 677 µmol, 1.00 eq) was dissolved in THF (7 ml) and MeOH (7 ml) was added. To the solution NaOH (37.9 mg, 948 µmol, 1.4 eq), dissolved in H_2O (632 µL), was added and the mixture was stirred over night. The solution was diluted with CH_2Cl_2 (30 ml) and extracted with sat. $NaHCO_3$ (2x 30 ml). The aqueous layer was acidified with 60 ml of 1M HCl and extracted with CH_2Cl_2 (2x 30 ml) The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The title compound was obtaind as 171 mg of a slightly yellowish oil (79 %).

TLC R_f = 0.17 (silica, 5% MeOH in CH_2Cl_2, ninhydrin).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:1.5).

^{1}H NMR (400 MHz, DMSO-d6): δ/ppm = 7.28 (m, 5H; Ph), 4.41 (d, *J* = 6.7 Hz,2H, Bn), 4.22-4.06 (m, 2H; Hα, Hγ), 3.53 (m, 1H; Hδ), 3.28 (dd, *J* = 16.1 Hz, 4.6 Hz, 1H; Hδ), 2.34 (m, 1H; Hβ), 2.09 (m, 1H; Hβ), 1.33 (s, 9H, Boc).

Isolated signals of the minor conformer: 7.28 (m, 5H; Ph), 4.46 (d, *J* = 7.4 Hz, 2H; Bn), 4.22-4.06 (m, 2H; Hα, Hγ), 3.55 (m, 1H; Hδ), 3.28 (dd, *J* = 16.1 Hz, 4.6 Hz, 1H; Hδ), 2.34 (m, 1H; Hβ), 2.09 (m, 1H; Hβ), 1.38 (s, 9H; Boc).

Boc-(4*S*)Hyp(Bn)-NMe_2

Boc-(4*S*)Hyp(Bn)-OH (171 mg, 532 µmol, 1.0 eq) was dissolved in CH_2Cl_2 (5 ml) and EDC·HCl (133 mg, 691 µmol, 1.3 eq), iPrNEt2 (273 µL, 1.60 mmol, 3.0 eq), and HNMe2·HCl (60.8 mg, 745 µmol, 1.40 eq) were added. The reaction mixture was stirred over night, diluted with CH_2Cl_2 (50 ml) and washed with 1M HCl (3x 30 ml). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was subjected to flash chromatography (silica, 5% MeOH in CH_2Cl_2) to obtain 145 mg of the title compound as a colorless oil (56 %).

TLC R_f = 0.23 (silica, 5% MeOH in CH_2Cl_2, ninhydrin).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:1.2).

^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm = 7.32 (m, 5H; Ph), 4.61 (dd, *J* = 6.7 Hz, 1H; Hα), 4.50 (m, 2H; Bn), 4.10 (m, 1H; Hγ), 3.83 (dd, *J* = 6.7 Hz, 1H; Hδ), 3.43 (m, 1H; Hδ), 3.02 (s, 3H; NMe_2), 2.97 (s, 3H; NMe_2), 2.49 (m, 1H; Hβ), 1.94 (m, 1H; Hβ), 1.45 (s, 9H; Boc).

Isolated signals of the minor conformer: 4.55 (m, *J* = 6.7 Hz, 1H; Hδ), 4.50 (m, 2H; Bn), 3.91 (dd, *J* = 6.8 Hz, 1H; Hδ), 3.43 (m, 1H; Hγ), 3.02 (s, 3H; NMe_2), 2.97 (s, 3H; NMe_2), 2.49 (m, 1H; Hβ), 1.94 (m, 1H; Hβ), 1.39 (s, 9H, Boc).

^{13}C NMR (101 MHz, $CDCl_3$): δ/ppm = 172.2 (amide), 153.9 (Boc), 138.3, 128.8, 128.1 (Ph), 80.9 (Boc), 75.5 (Cα), 72.1 (CH_2Ph), 55.4 (Cγ), 51.8 (Cδ), 37.1 (NMe_2), 36.1 (NMe_2), 36.0 (Cβ), 35.4 (Cβ), 28.7 (Boc).

Isolated signals of the minor conformer: 171.7 (amide), 154.8 (Boc), 138.8, 128.8, 128.2, 128.1 (Ph), 80.9 (Boc), 76.5 (Cα), 71.9 (CH_2Ph), 55.6 (Cγ), 51.0 (Cδ), 37.1 (NMe_2), 36.5 (NMe_2), 36.0 (Cβ), 28.9 (Boc).

H-(4*S*)Hyp(Bn)-NMe_2·TFA

Boc-(4*S*)Hyp(OBn)-NMe_2 (145 mg, 416 µmol, 1.0 eq) was dissolved in 1.5 ml of TFA and diluted with 1.5 ml of CH_2Cl_2. The solution was stirred for 4h and concentrated under reduced pressure to obtain 90 mg of the title compound as a slightly yellowish oil (63%).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:3.2).

^{1}H NMR (400 MHz, D_2O): δ/ppm = 7.26 (m, 5H; Ph), 4.68 (s, 2H; Bn), 4.63 (dd, *J* = 10.6 Hz, 3.9 Hz, 1H; Hα), 4.33 (m, 1H; Hγ), 3.59 (dd, *J* = 14.7 Hz, 4.0 Hz, 1H; Hδ), 3.28 (dd, *J* = 12.7 Hz, 4.1 Hz, 1H; Hδ), 2.86 (s, 3H, NMe_2), 2.81 (s, 3H, NMe_2), 2.54 (dd, *J* = 12.0 Hz, 7.8 Hz, 1H; Hβ), 2.13 (dd, *J* = 14.6 Hz, 3.7 Hz, 1H; Hβ).

Ac-(4*S*)Hyp(Bn)-NMe_2

H-(4*S*)Hyp(Bn)-NMe_2·TFA (90.0 mg, 262 µmol, 1.0 eq) was dissolved in CH_2Cl_2 (2 ml) and NEt_3 (150 µl, 1.12 mmol, 4.0 eq) and Ac_2O (70.0 µl, 746 µmol, 3.00 eq) were added. The suspension was stirred over night and diluted with CH_2Cl_2 until the mixture clarified. A solution of 1M HCl (50 ml) was added and extracted with CH_2Cl_2 (3x 30 ml). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. The residue was subjected to flash chromatography (silica, 5% MeOH in CH_2Cl_2) to obtain 62 mg of the tilte compound as a colorless oil (82 %).

TLC R_f = 0.63 (silica, 10% MeOH in CH_2Cl_2, ninhydrin).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:2.6).

^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm = 7.32 (m, 5H; Ph), 4.75 (t, J = 7.9 Hz, 1H; Hα), 4.54 (s, 2H; Bn), 4.16 (dd, *J* = 7.1 Hz, 1H; Hγ), 3.81 (dd, *J* = 6.7 Hz, 1H; Hδ), 3.62 (dd, *J* = 7.4 Hz, 1H; Hδ), 3.08 (s, 3H; NMe_2), 2.97 (s, 3H; NMe_2), 2.49 (m, 1H; Hβ), 2.07 (s, 3H; Ac), 1.97 (dt, *J* = 7.4 Hz, 1H; Hβ).

Isolated signal of the minor conformer: 4.57 (m, 1H; Hα), 4.54 (s, 2H; Bn), 4.08 (dd, *J* = 8.3 Hz, 1H; Hγ), 3.81 (dd, *J* = 6.7 Hz, 1H; Hδ), 3.62 (dd, *J* = 7.4 Hz, 1H; Hδ), 3.03 (s, 3H, NMe_2), 3.00 (s, 3H; NMe_2), 2.60 (m, 1H; Hβ), 2.07 (s, 3H; Ac), 1.97 (dt, *J* = 7.4 Hz, 1H; Hβ).

^{13}C NMR (101 MHz, $CDCl_3$): δ/ppm = 171.5, 169.6 (amides), 138.1, 128.9, 128.3, 128.1 (Ph), 76.7 (Cα), 72.2 (Bn), 54.9 (Cγ), 52.9 (Cδ), 37.4 (NMe_2), 36.4 (NMe_2), 34.5 (Cβ), 22.7 (Ac).

Isolated signal of the minor conformer: 170.7, 170.3 (amides), 138.2, 128.8, 128.1 (Ph), 75.1 (Cα), 71.5 (Bn), 58.0 (Cγ), 53.9 (Cδ), 37.2 (Cβ), 22.0 (Ac).

MS (ESI): *m/z* = 313.2 $[M+Na]^+$, 603.3 $[2M+Na]^+$, M = 290.3 calcd for $C_{16}H_{22}N_2O_3$.

Ac-(4*S*)Hyp-NMe_2

Pd/C (15.0 mg) was suspended under an inert atmosphere in MeOH (1.5 ml) and Ac-(4*S*)Hyp(Bn)-NMe_2 (62.2 mg, 214 µmol, 1.0 eq) was added. The flask was evacuated and flooded with H_2 three times and stirred under a H_2 atmosphere over night. The suspension was filtered over Celite and the filtrate was concentrated under reduced pressure. The residue was subjected to MPLC (RP-C18 silica, 0%→20% MeOH in H_2O, 15 ml/min) to obtain 20 mg of the title compound as a colorless solid (47%).

TLC R_f = 0.37 (silica, 10% MeOH in CH_2Cl_2, $KMnO_4$).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:3.4).

^{1}H NMR (400 MHz, D_2O): δ/ppm = 4.75 (dd, *J* = 9.0 Hz, 4.9 Hz, 1H; Hα), 4.37 (m, 1H; Hγ), 3.79 (dd, *J* = 11.2 Hz, 5.5 Hz, 1H; Hδ), 3.44 (dd, *J* = 11.2 Hz, 3.9 Hz, 1H; Hδ), 3.01 (s, 3H; NMe_2), 2.82 (s, 3H; NMe_2), 2.48 (m, 1H; Hβ), 1.98 (s, 3H; Ac), 1.75 (dd, *J* = 13.5 Hz, 1H; Hβ).

Isolated signals of the minor conformer: 4.93 (dd, *J* = 6.5 Hz, 4.0 Hz, 1H; Hα), 4.37 (m, 1H; Hγ), 3.68 (dd, *J* = 12.7 Hz, 4.9 Hz, 1H; Hδ), 3.34 (dd, *J* = 12.0 Hz, 1H; Hδ), 3.01 (s, 3H, NMe_2), 2.86 (s, 3H; NMe_2), 2.58 (m, 1H; Hβ), 1.98 (s, 3H; Ac), 1.93 (m, 1H; Hβ).

^{13}C NMR (101 MHz; D_2O): δ/ppm (Hauptkonformer) = 173.3, 172.8 (amides), 69.8 (Cα), 56.4 (Cγ), 55.9 (Cδ), 37.4 (NMe_2), 36.4 (NMe_2), 36.3 (Cβ), 21.3 (Ac).

Isolated signals of the minor conformer: 173.1, 172.6 (amides), 68.5 (Cα), 58.7 (Cγ), 54.9 (Cδ), 37.2 (NMe_2), 36.9 (NMe_2), 36.4 (Cβ), 21.5 (Ac).

ESI-MS: *m/z* calcd for $C_9H_{16}N_2O_3$: 200.1; found: 223.2 $[M+Na]^+$ (100%).

21.4 Synthesis of Ac-(4*R*)Flp-NMe_2

Boc-(4*R*)Flp-NMe_2

Boc-(4*R*)Flp-OH (250 mg, 1.07 mmol, 1.0 eq) was suspended in CH_2Cl_2 (4.3 ml) and $^iPr_2NEt$ (459 µl, 2.68 mmol, 2.5 eq) was added whereupon the solution clarified. Next EDC·HCl (288 mg, 1.50 mmol, 1.4 eq) and $HNMe_2$·HCl (131 mg, 1.61 mmol, 1.5 eq) were added and the solution was stirred over night. The solution was diluted with 50 ml of CH_2Cl_2 and washed with 1M HCl (3x 20 ml), dried over $MgSO_4$ and concentrated under reduced pressure. The oily residue was subjected to column chromatography (7% MeOH in CH_2Cl_2) to obtain 173 mg of the title compound as a colorless oil (62 %).

TLC R_f = 0.38 (silica, 7% MeOH in CH_2Cl_2, ninhydrin).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:1).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 5.35-5.16 (m, 1H; Hγ), 4.88 (t, *J* = 7.8 Hz, 1H; Hα), 3.92 (ddd, *J* = 22.6 Hz, 13.1 Hz, 2.1 Hz, 1H; Hδ), 3.66 (dt, *J* = 12.9 Hz, 4.0 Hz, 1H; Hδ), 3.17 (s, 3H; NMe_2), 3.01 (s, 3H; NMe_2), 2.54-2.39 (m, 1H; Hβ), 2.25-2.05 (m, 1H; Hβ), 1.47 (s, 9H; Boc).

Isolated signal of the other conformer: 4.78 (dd, *J* = 8.4 Hz, 7.7 Hz, 1H; Hα), 3.85 (dd, *J* = 22.4 Hz, 13.0 Hz, 1H; Hδ), 3.75 (ddd, *J* = 12.9 Hz, 6.3 Hz, 3.4 Hz, 1H; Hδ), 3.11 (s, 3H; NMe_2), 2.99 (s, 3H; NMe_2), 1.43 (s, 9H; Boc).

^{19}F NMR (376 MHz, $CDCl_3$) δ/ppm = -176.2 (s), -177.0 (s) (both conformers).

^{13}C NMR (101 MHz, $CDCl_3$) δ/ppm = 172.6 (amide), 154.7 (Boc), 93.3 (d, *J* = 81.3 Hz; Cγ), 80.5 (Boc), 54.7 (Cα), 53.9 (d, *J* = 22.7 Hz; Cδ), 37.9 (NMe_2), 37.7 (d, *J* = 6.5 Hz; Cβ), 37.5 (NMe_2), 28.8 (Boc).

Isolated signals the other conformer: 172.5 (amide), 91.6 (d, *J* = 80.7 Hz; Cγ), 80.5 (Boc), 54.7 (Cα), 53.6 (d, *J* = 21.8 Hz; Cδ), 37.1 (NMe_2), 36.9 (NMe_2), 36.5 (d, *J* = 5.6 Hz; Cβ), 28.7 (Boc).

ESI-MS: *m/z* calcd for $C_{12}H_{21}FN_2O_3$: 260.2; found: 283.3 $[M+Na]^+$ (100%).

H-(4*R*)Flp-NMe_2·HCl

Boc-(4*R*)Flp-NMe_2 (173 mg, 665 µmol, 1.0 eq) was dissolved in 1.7 ml of a 4M solution of HCl in dioxane and stirred for 3 hours. The solution was concentrated under reduced pressure to obtain 180 mg of the title compound as a slightly yellowish oil (quant.).

TLC R_f = 0.43 (silica, MeCN:H_2O 4:1, ninhydrin).

^{1}H NMR (400 MHz, D_2O) δ/ppm = 5.49 (dt, *J* = 50.8 Hz, 3.3 Hz, 1H; Cγ), 4.93 (dd, *J* = 10.2 Hz, 8.0 Hz, 1H; Cα), 3.72 (ddd, *J* = 19.1 Hz, 13.7 Hz, 2.2 Hz, 1H; Cδ), 3.55 (ddd, *J* = 37.7 Hz, 13.7 Hz, 3.0 Hz, 1H; Cδ), 3.01 (s, 3H; NMe_2), 2.91 (s, 3H; NMe_2), 2.94-2.81 (m, 1H; Hβ), 2.16 (dddd, *J* = 15.0 Hz, 14.4 Hz, 10.3 Hz, 3.8 Hz, 1H).

^{13}C NMR (101 MHz, D_2O) δ/ppm = 168.4 (amide), 93.3 (d, *J* = 175.7 Hz; Cγ), 57.8 (Cα), 52.5 (d, *J* = 23.0 Hz; Cδ), 37.0 (NMe_2), 36.3 (NMe_2), 36.0 (d, *J* = 22.0 Hz; Cβ).

ESI-MS: *m/z* calcd for $C_7H_{14}ClFN_2O$: 196.1; found: 161.1 $[M\text{-}Cl]^+$ (100%).

Ac-(4*R*)Flp-NMe_2

H-(4*R*)Flp-NMe_2·HCl (180 mg, 914 µmol, 1.0 eq) was suspended in 3 ml of CH_2Cl_2 and NEt_3 (380 µl, 2.74 mmol, 3.0 eq) was added. After cooling with an ice bath Ac_2O (242 µl, 2.56 mmol, 2.8 eq) was added and the reaction mixture was stirred over night. After dilution with 20 ml of CH_2Cl_2 the solution was extracted with sat. $NaHCO_3$ (3x 20 ml). The combined aqueous layers were lyophilized and the white foam was extracted with CH_2Cl_2 (3x10 ml). The combined organic layers were concentrated and the colorless oily residue was subjected to MPLC (C18-silica, MeCN-H2O). The title compound was obtained as a colorless oil (68 mg, 38 %).

TLC R_f = 0.40 (10% MeOH in CH_2Cl_2, TDM).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:10).

^{1}H-NMR (500 MHz, $CDCl_3$, 25°C): δ/ppm = 5.34 (dm, *J* = 35 Hz, 1H; Cγ), 4.99 (t, *J* = 7.9 Hz, 1H; Hα), 3.90 (ddd, *J* = 14.5 Hz, *J* = 12.3, *J* = 3.3 Hz, 1H; Hδ), 3.82 (qt, *J* = 12.3 Hz, *J* = 1.5 Hz; Hδ), 3.20 (s, 3H; NMe_2), 2.98 (s, 3H; NMe_2), .2.46 (dddt, *J*= 22.4, *J* = 14.4 Hz, *J* = 8.0 Hz, *J* = 1.8 Hz, 1H; Hβ), 2.21 (dddd, *J* = 37.3 Hz, *J* = 14.4, *J* = 7.9, *J* = 4.4 Hz, 1H; Hβ), 2.09 (s, 3H; Ac).

Isolated signals of the minor conformer: 4.86 (t, *J* = 8.1 Hz, 1H; Hα), 4.24 (ddd, *J* = 21.3 Hz, *J* = 13.7, *J* = 2.2 Hz, 1H; Hδ), 3.61 (ddd, *J* = 37.0 Hz, *J* = 13.9 Hz, *J* = 3.5 Hz, 1H; Hδ), 3.13 (s, 3H; NMe_2), 3.02 (s, 3H; NMe_2), 1.92 (s, 3H; Ac).

^{13}C-NMR (101 MHz, $CDCl_3$, 25°C): δ/ppm = 172.1, 169.6 (amide, ester), 92.8 (d, *J* = 179 Hz; Hγ), 54.9 (d, *J* = 22.6 Hz; Hδ), 54.6 (OMe), δ 37.8 (Me), 36.5 (d, *J* = 21.9 Hz; Hβ), 36.5 (Me).22.7 (Ac).

MS (ESI): *m/z* calcd for $C_9H_{15}FN_2O_2$: 202.1; found: 225.1 [M+Na]+ (100%).

21.5 Synthesis of Ac-(4*S*)Flp-NMe$_2$

Boc-(4*S*)Flp-NMe$_2$

Boc-(4S)Flp-OH (250 mg, 1.07 mmol, 1.0 eq) was converted to the title compound according the protocol for its (4*R*)-configured diastereoisomer. One afforded 195 mg of the title compound as a colorless oil (70 %).

TLC R_f = 0.39 (silica, 7% MeOH in CH_2Cl_2, ninhydrin).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 2:3).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 5.31-5.13 (m, 1H; Hγ), 4.76 (dd, *J* = 9.6 Hz, 2.1 Hz, 1H; hα), 3.96-3.71 (m, 2H; Hδ), 3.05 (s, 3H; NMe_2), 2.99 (s, 3H; NMe_2), 2.60-2.37 (m, 1H; Hβ), 2.31-2.16 (m, 1H; Hβ), 1.48 (s, 9H; Boc).

Isolated signals of the minor conformer: 4.63 (dd, *J* = 9.5 Hz, 2.9 Hz, 1H; Hα), 3.00 (s, 3H; NMe_2), 1.42 (s, 9H; Boc).

^{13}C NMR (101 MHz, $CDCl_3$) δ/ppm = 172.6 (amide), 154.7 (Boc), 93.3 (d, *J* = 81.3 Hz; Cγ), 80.5 (Boc), 54.7 (Cα), 53.9 (d, *J* = 22.7 Hz; Cδ), 37.9 (NMe_2), 37.7 (d, *J* = 6.5 Hz; Cβ), 37.5 (NMe_2),28.8 (Boc).

Isolated signals the other conformer: 172.5 (amide), 91.6 (d, *J* = 80.7 Hz; Cγ), 80.5 (Boc), 54.7 (Cα), 53.6 (d, *J* = 21.8 Hz; Cδ), 37.1 (NMe_2), 36.9 (NMe_2), 36.5 (d, *J* = 5.6 Hz; Cβ), 28.7 (Boc).

ESI-MS: *m/z* calcd for $C_{12}H_{21}FN_2O_3$: 260.2; found: 283.3 $[M+Na]^+$ (100%).

H-(4*S*)Flp-NMe$_2$·HCl

Boc-(4*S*)Flp-NMe2 (195 mg, 749 µmol, 1.0 eq) was dissolved in 4M HCl in dioxane and stirred for 2.5 hours. The mixture was concentrated under reduced pressure and the residue was suspended in little CH_2Cl_2 and dissolved in as little MeOH as possible. Upon addition of

Et_2O a colorless solid precipitated. It was filtered off to obtain 147 mg of the title compound (quant.)

TLC R_f = 0.20 (silica, 10% MeOH in CH_2Cl_2, ninhydrin).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 5.40 (dt, *J* = 50.9 Hz, 3.7 Hz, 1H; Hγ), 4.80 (dd, *J* = 11.1 Hz, 3.9 Hz, 1H; Hα), 3.81 (ddd, *J* = 17.7 Hz, 13.6 Hz, 2.3 Hz, 1H; Hδ), 3.46 (ddd, *J* = 37.6 Hz, 13.6 Hz, 3.2 Hz, 1H; Hδ), 2.96 (s, 1H; NMe_2), 2.89 (s, 1H; NMe_2), 2.78 (dddd, *J* = 40.7 Hz, 15.5 Hz, 11.1 Hz, 4.4 Hz, 1H; Hβ), 2.44-2.30 (m, 1H; Hβ).

^{19}F NMR (376 MHz, D_2O) δ/ppm = -175.0 (ddddd, *J* = 50.9 Hz, 40.9 Hz, 37.7 Hz, 23.3 Hz, 17.8 Hz).

^{13}C NMR (101 MHz, D_2O) δ/ppm = 168.7 (amide), 92.2 (d, *J* = 175.6 Hz; Cγ), 57.8 (Cα), 52.6 (d, *J* = 23.0 Hz; Cδ), 36.9 (NMe_2), 36.2 (NMe_2), 36.0 (d, *J* = 22.1 Hz; Cβ).

ESI-MS: *m/z* calcd for $C_7H_{14}ClFN_2O$: 196.1; found: 161.1 $[M\text{-}Cl]^+$ (100%).

Ac-(4*S*)Flp-NMe$_2$

H-(4*S*)Flp-NMe_2·HCl (120 mg, 610 µmol, 1.0 eq) was acetylated according the protocol for the preparation of its (4*R*)-configured diastereoisomer to obtain 46 mg of the title compound as a colorless solid (37 %).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:3.7).

TLC R_f = 0.42 (10% MeOH in CH_2Cl_2, TDM).

^{19}F NMR (565 MHz, $CDCl_3$) δ/ppm = -170.96 (s), -172.54 (s).

^{1}H{^{19}F} NMR (600 MHz, $CDCl_3$) δ/ppm = 5.29 (dq, *J* = 7.7 Hz, 2.5 Hz, 1H; Hγ), 4.95 (dd, *J* = 9.5 Hz, 2.5 Hz, 1H; Hα), 3.95 (d, *J* = 11.6 Hz, 1H; Hδ), 3.89 (dd, *J* = 11.9 Hz, 5.4 Hz, 1H; Hδ), 3.07 (s, 3H; NMe_2), 2.97 (s, 3H; NMe_2), 2.46 (ddd, *J* = 14.8 Hz, 9.6 Hz, 5.6 Hz, 1H; Hβ), 2.24 (d, *J* = 14.3 Hz, 1H; Hβ), 2.09 (s, 3H; Ac).

^{13}C NMR (151 MHz, $CDCl_3$) δ/ppm = 170.1 (amide), 169.4 (amide), 91.6 (d, *J* = 181.9 Hz; Cγ), 55.6 (Hα), 54.2 (d, *J* = 26.1 Hz; Hδ), 37.1 (NMe_2), 36.2 (NMe_2), 35.8 (d, *J* = 21.7 Hz; Cβ), 22.5 (Ac).

MS (ESI): *m/z* = 225.2 [M+Na]+ (100%), M = 202.1 calcd for $C_9H_{15}FN_2O_2$.

21.6 Synthesis of Ac-(4*R*)Acp-NMe_2

Ac-(4R)Azp-NMe_2[111] (75.0 mg, 333 µmol, 1.0 eq) and Pd/C (10 mg) were suspended in MeOH (5.4 ml) under an inert atmosphere. Ac_2O (63.0 µl, 666 µmol, 2.0 eq) was added and the flask was evacuated and flooded with H_2 three times. After stirring over night under a H_2 atmosphere the suspension was filtered over Celite and concentrated under reduced pressure. The residue was subjected to flash chromatography (silica, CH_2Cl_2 → 10% MeOH in CH_2Cl_2) to obtain 71 mg of the title compound as a colorless oil (89 %).

TLC R_f = 0.32 (silica, 5% MeOH in CH_2Cl_2, TDM).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:3.9).

^{1}H NMR (400 MHz, D_2O) δ/ppm = 4.95 (dd, *J* = 8.1 Hz, 6.9 Hz, 1H; Hα), 4.51-4.44 (m, 1H; Hγ), 3.93 (dd, *J* = 11.2 Hz, 6.0 Hz, 1H; Hδ), 3.62 (dd, *J* = 11.1 Hz, 4.2 Hz, 1H; Hδ), 3.15 (s, 3H; NMe_2), 2.96 (s, 3H; NMe_2), 2.37 (dddd, *J* = 13.7 Hz, 8.5 Hz, 5.4 Hz, 0.5 Hz, 1H; Hβ), 2.16 (dd, *J* = 13.2 Hz, 6.6 Hz, 1H; Hβ), 2.12 (s, 3H; Ac), 2.00 (s, 3H; Ac).

Isolated signals of the minor conformer: 5.14 (dd, *J* = 8.7 Hz, 5.1 Hz, 1H; Hα), 4.43-4.36 (m, 1H; Hγ), 3.74 (dd, *J* = 12.2 Hz, 6.6 Hz, 1H;Hδ), 3.56 (dd, *J* = 12.1 Hz, 5.1 Hz, 1H; Hδ), 3.15 (s, 3H; NMe_2), 3.00 (s, 3H; NMe_2), 2.49 (ddd, *J* = 13.7 Hz, 8.6 Hz, 6.8 Hz, 1H; Hβ), 2.29 (dt, *J* = 13.3 Hz, 5.9 Hz, 1H; Hβ), 1.99 (s, 3H; Ac), 1.93 (s, 3H; Ac).

^{13}C NMR (101 MHz, D_2O) δ/ppm = 174.1 ($CONMe_2$), 172.7 (Ac), 172.5 (Ac), 55.7 (Cα), 53.1 (Cδ), 48.9 (Cγ), 36.9 (NMe2), 35.9 (NMe2), 33.7 (Cβ), 21.8 (Ac), 21.3 (Ac).

Isolated signal of the minor conformer: 174.2 ($CONMe_2$), 173.4 (Ac), 172.3 (Ac), 57.6 (Cα), 51.2 (Cδ), 47.3 (Cγ), 36.9 (NMe_2), 36.1 (NMe_2), 35.3 (Cβ), 21.7 (Ac), 20.6 (Ac).

ESI-MS: *m/z* calcd for $C_{11}H_{19}N_3O_3$: 241.1; found: 264.1 $[M+Na]^+$ (100%).

21.7 Synthesis of Ac-(4*S*)Acp-NMe_2

The title compound was synthesized from Ac-(4*S*)Azp-NMe_2[111] (75.0 mg, 333 µmol, 1.0 eq) in analogy to the protocol for the according (4*R*)-configurated diastereo isomer. The title compound was isolated as 69 mg of a colorless oil (86%).

TLC R_f = 0.37 (silica, 5% MeOH in CH_2Cl_2, TDM).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:4.5).

^{1}H NMR (400 MHz, D_2O) δ/ppm = 4.88 (dd, *J* = 8.5 Hz, 7.0 Hz, 1H; Hα), 4.46 (p, *J* = 6.9 Hz, 1H; Hγ), 4.02 (dd, *J* = 10.9 Hz, 6.8 Hz, 1H; Hδ), 3.49 (dd, *J* = 10.9 Hz, 6.7 Hz, 1H; Hδ),

3.14 (s, 3H; NMe_2), 2.96 (s, 3H; NMe_2), 2.69 (ddd, *J* = 13.2 Hz, 8.7 Hz, 6.9 Hz, 1H; Hδ), 2.10 (s, 3H; Ac), 1.98 (s, 3H; Ac), 1.81 (dt, *J* = 13.2 Hz, 7.1 Hz, 1H; Hβ).

Isolated signal of the minor conformer: 5.08 (dd, *J* = 9.2 Hz, 5.1 Hz, 1H; Hα), 4.41-4.33 (m, 1H; Hγ), 3.98 (dd, *J* = 12.2 Hz, 6.9 Hz, 1H; Hδ), 3.38 (dd, *J* = 12.3 Hz, 5.5 Hz, 1H; Hδ), 3.15 (s, 3H; NMe_2), 2.99 (s, 3H; NMe_2), 2.81 (ddd, *J* = 12.0 Hz, 8.2 Hz, 2.6 Hz, 1H; Hβ), 1.96 (s, 3H; Ac), 1.91 (s, 3H; Ac).

^{13}C NMR (101 MHz, D_2O) δ/ppm = 174.2 ($CONMe_2$), 173.1 (Ac), 173.0 (Ac), 56.2 (Cα), 53.1 (Cδ), 48.8 (Cγ), 37.4 (NMe_2), 36.3 (NMe_2), 33.8 (Cβ), 22.3 (Ac), 21.7 (Ac).

ESI-MS: *m/z* calcd for $C_{11}H_{19}N_3O_3$: 241.1; found: 264.1 $[M+Na]^+$ (100%).

21.8 Synthesis of Ac-(4*R*)Pvp-NMe_2

Ac-(4*R*)Azp-NMe_2[111] (50.0 mg, 222 µmol, 1.0 eq)was reacted with Piv_2O under a H_2 atmosphere in analogy to the protocol for the according methyl ester. The title compound was obtained as 31 mg of a colorless oil (50%).

TLC R_f = 0.33 (silica, 5% MeOH in CH_2Cl_2, TDM).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:3.9).

^{1}H NMR (400 MHz, D_2O) δ/ppm = 4.95 (dd, *J* = 8.1 Hz, 6.9 Hz, 1H; Hα), 4.51-4.44 (m, 1H; Hγ), 3.93 (dd, *J* = 11.2 Hz, 6.0 Hz, 1H; Hδ), 3.62 (dd, *J* = 11.1 Hz, 4.2 Hz, 1H; Hδ), 3.15 (s, 3H; NMe_2), 2.96 (s, 3H; NMe_2), 2.37 (dddd, *J* = 13.7 Hz, 8.5 Hz, 5.4 Hz, 0.5 Hz, 1H; Hβ), 2.16 (dd, *J* = 13.2 Hz, 6.6 Hz, 1H; Hβ), 2.12 (s, 3H; Ac), 2.00 (s, 3H; Ac).

Isolated signals of the minor conformer: 5.14 (dd, *J* = 8.7 Hz, 5.1 Hz, 1H; Hα), 4.43-4.36 (m, 1H; Hγ), 3.74 (dd, *J* = 12.2 Hz, 6.6 Hz, 1H;Hδ), 3.56 (dd, *J* = 12.1 Hz, 5.1 Hz, 1H; Hδ), 3.15 (s, 3H; NMe_2), 3.00 (s, 3H; NMe_2), 2.49 (ddd, *J* = 13.7 Hz, 8.6 Hz, 6.8 Hz, 1H; Hβ), 2.29 (dt, *J* = 13.3 Hz, 5.9 Hz, 1H; Hβ), 1.99 (s, 3H; Ac), 1.93 (s, 3H; Ac).

^{13}C NMR (101 MHz, D_2O) δ/ppm = 174.1 ($CONMe_2$), 172.7 (Ac), 172.5 (Ac), 55.7 (Cα), 53.1 (Cδ), 48.9 (Cγ), 36.9 (NMe_2), 35.9 (NMe_2), 33.7 (Cβ), 21.8 (Ac), 21.3 (Ac).

Isolated signal of the minor conformer: 174.2 ($CONMe_2$), 173.4 (Ac), 172.3 (Ac), 57.6 (Cα), 51.2 (Cδ), 47.3 (Cγ), 36.9 (NMe_2), 36.1 (NMe_2), 35.3 (Cβ), 21.7 (Ac), 20.6 (Ac).

ESI-MS: *m/z* calcd for $C_{14}H_{25}N_3O_3$: 283.2; found: 306.3$[M+Na]^+$ (100%).

21.9 Synthesis of Ac-(4*S*)Pvp-NMe_2

The title compound was prepared in analogy to its (4*R*)-configurated diastereoisomer using Ac-(4*S*)Azp-NMe_2[111] (55.1 mg, 245 µmol, 1.0 eq). The title compound was obtained as 27.1 mg of a colorless oil (39 %).

TLC R_f = 0.32 (silica, 5% MeOH in CH_2Cl_2, TDM).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:13.2).

^{1}H NMR (500 MHz, $CDCl_3$) δ/ppm = 8.16 (d, *J* = 8.9 Hz, 1H; NH), 4.91 (d, *J* = 9.3 Hz, 1H; Hα), 4.85-4.79 (m, 1H; Hγ), 3.80 (dd, *J* = 10.9 Hz, 5.8 Hz, 1H; Hδ), 3.55 (d, *J* = 10.9 Hz, 1H; Hδ), 3.22 (s, 3H; Me), 3.00 (s, 3H; Me), 2.38 (ddd, *J* = 13.8 Hz, 9.7 Hz, 6.6 Hz, 1H; Hβ), 2.06 (s, 3H; Ac), 1.80 (dd, *J* = 13.8 Hz, 0.9 Hz, 1H; Ac), 1.18 (s, 9H; Piv).

Isolated signals of the minor conformer: 6.99 (d, *J* = 9.1 Hz, 1H; NH), 4.73-4.67 (m, 2H; Hα, Hγ), 3.75 (d, *J* = 3.7 Hz, 2H; Hδ), 3.11 (s, 3H; Me), 3.03 (s, 3H; Me), 2.55 (ddd, *J* = 13.4 Hz, 9.6 Hz, 5.8 Hz, 1H; Hβ), 1.92 (s, 3H; Ac), 1.13 (s, 9H; Piv).

^{13}C NMR (126 MHz, $CDCl_3$) δ/ppm = 178.2 (Piv), 173.0 (Ac), 169.5 ($CONMe_2$), 55.8 (Cδ), 55.0 (Cα), 48.9 (Cγ), 38.7 (Piv), 37.6 (Me), 36.3 (Me), 35.3 (Cβ), 27.4 (Piv), 22.4 (Ac).

ESI-MS: *m/z* calcd for $C_{14}H_{25}N_3O_3$: 283.2; found: 306.3$[M+Na]^+$ (100%).

21.10 Synthesis of Ac-(4*S*)Amp-NMe_2·TFA

Synthesis of Ac-(4*S*)Amp(Boc)-NMe_2

Ac-(4*S*)Azp-NMe_2[111] (200 mg, 888 µmol, 1.0 eq) and Pd/C (20 mg) were suspended in MeOH (1.4 ml) under an inert atmosphere. Boc_2O (265 µL, 1.15 µmol, 1.3 eq) was added and the flask was evacuated and flooded with H_2 three times and then the mixture was stirred over night under a H_2-atmosphere. The suspension was filtered over Celite and concentrated under reduced pressure. The residue was redissolved in 15 ml of CH_2Cl_2 and washed with sat. $NaHCO_3$ (3x 5 ml). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was subjected to flash chromatography (silica, 5% MeOH inc CH_2Cl_2) to obtain 150 mg of the title compound as a colorless oil (56%).

TLC R_f = 0.29 (silica, 7% MeOH in CH_2Cl_2, ninhydrin).

^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 6.69 (d, *J* = 9.5 Hz, 1H; NH), 4.88 (dd, *J* = 9.6 Hz, 2.1 Hz, 1H; Hα), 4.55-4.45 (m, 1H; Hγ), 3.81 (dd, *J* = 10.7 Hz, 6.1 Hz, 1H; Hδ), 3.60 (ddd, *J* =

10.8 Hz, 2.1 Hz, 1.1 Hz, 1H; Hδ), 3.23 (s, 3H; NMe_2), 3.01 (s, 3H; NMe_2), 2.41 (ddd, *J* = 13.7 Hz, 9.6 Hz, 7.2 Hz, 1H; Hβ), 2.08 (s, 3H; Ac), 1.85 (ddd, *J* = 13.9 Hz, 3.0 Hz, 2.0 Hz, 1H; Hβ), 1.45 (s, 9H; Boc).

Isolated signals of the minor conformer: 5.69 (d, *J* = 8.2 Hz, 1H; NH), 4.67 (dd, *J* = 9.5 Hz, 2.1 Hz, 1H; Hα), 4.44-4.33 (m, 1H; Hγ), 3.67 (d, *J* = 13.5 Hz, 1H; Hδ), 3.12 (s, 3H; NMe_2), 3.05 (s, 3H; NMe_2), 2.59 (ddd, *J* = 13.4 Hz, 9.5 Hz, 6.3 Hz, 1H; Hδ), 1.92 (s, 3H; Ac), 1.43 (s, 9H; Boc).

^{13}C NMR (101 MHz, $CDCl_3$) δ/ppm = 173.5 (m), 170.6 (m), 156.3 (Boc), 79.9 (Boc), 56.2 (Hα), 55.2 (Hγ), 50.9 (Hδ), 38.1 (NMe_2), 36.8 (NMe_2), 35.5 (Cβ), 28.8 (Boc), 22.8 (Ac).

ESI-MS: *m/z* calcd for $C_{14}H_{25}N_3O_4$: 299.2; found: 300.3 $[M+H]^+$.

Ac-(4*S*)Amp-NMe_2·TFA

Ac-(4*S*)Amp(Boc)-NMe_2 (175 mg, 585 µmol, 1.0 eq) was dissolved in a 1:1 mixture of TFA and CH_2Cl_2 (2.9 ml) and stirred for 90 min. The reaction mixture was concentrated under reduced pressure and treated with cold Et_2O whereupon 175 mg of the title compound precipitated as a colorless solid (100%).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:5.1).

^{1}H NMR (400 MHz, D_2O) δ/ppm = 4.93 (dd, *J* = 9.5 Hz, 3.5 Hz, 1H; Hα), 4.03 (dq, *J* = 9.2 Hz, 3.2 Hz, 1H; Hγ), 3.96 (dd, *J* = 12.0 Hz, 5.8 Hz, 1H; Hδ), 3.80 (ddd, *J* = 12.0 Hz, 3.0 Hz, 1.1 Hz, 1H; Hδ), 3.12 (s, 3H; NMe_2), 2.88 (s, 3H; NMe_2), 2.65 (ddd, *J* = 14.7 Hz, 9.6 Hz, 6.2 Hz, 1H; Hβ), 2.03 (s, 3H; Ac), 2.01(tdd, *J* = 14.7 Hz, 3.4 Hz, 1.3 Hz, 1H; Hβ).

Isolated signal of the minor conformer: 5.13 (dd, *J* = 9.5 Hz, 3.3 Hz, 1H; Hα), 3.89 (dd, *J* = 13.0 Hz, 6.4 Hz, 1H; Hδ), 3.67 (dd, *J* = 13.1 Hz, 3.1 Hz, 1H; Hδ), 3.12 (s, 3H; NMe_2), 2.93 (s, 3H; NMe_2), 2.82 (ddd, *J* = 14.6 Hz, 9.6 Hz, 6.6 Hz, 1H; Hβ), 2.13 (ddd, *J* = 13.4 Hz, 4.5 Hz, 2.2 Hz, 1H; Hβ), 1.86 (s, 3H; Ac).

^{13}C NMR (101 MHz, D_2O) δ/ppm = 173.5, 173.2 (amides), 55.5 (Cα), 52.0 (Cδ), 50.7 (Cγ), 37.8 (NMe_2), 36.6 (NMe_2), 32.4 (Cβ), 21.7 (Ac).

ESI-MS: *m/z* calcd for $C_{14}H_{25}N_3O_4$: 299.2; found: 300.3 $[M+H]^+$.

21.11 Synthesis of Ac-(4*R*)Amp-NMe_2·TFA

Ac-(4*R*)Amp(Boc)-NMe_2

The title compound was synthesized from Ac-(4*R*)Azp-NMe_2[111] (60 mg, 266 µmol, 1.0 eq) in analogy to the protocol for the according (4*S*)-configurated diastereoisomer. The title compound was obtained as 54 mg of a colorless oil (68%).

TLC R_f = 0.20 (silica, 5% MeOH in CH_2Cl_2, ninhydrin).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:7.2).

^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm = 4.90 (dd, *J* = 8.4 Hz, 5.5 Hz, 1H; Hα), 4.63 (m, 1H, N*H*), 4.42 (dd, *J* = 12.6 Hz, 6.1 Hz, 1H; Hγ), 3.95 (dd, *J* = 12.4 Hz, 6.1 Hz, 1H; Hδ), 3.44 (m, 1H; Hδ), 3.13 (s, 3H; NMe_2), 2.95 (s, 3H; NMe_2), 2.19 (dd, *J* = 12.4 Hz, 6.5 Hz, 1H; Hβ), 2.11 (dd, *J* = 12.4 Hz, 6.5 Hz, 1H; Hβ), 2.07 (s, 3H; Ac), 1.45 (s, 9H; Boc.

Isolated signal of the minor conformer: 4.68 (m, 1H; Hα), 4.25 (m, 1H; NH), 4.12 (dd, *J* = 7.1 Hz; Hγ), 3.79 (m, 1H; Hδ), 3.63 (m, 1H; Hδ), 3.07 (s, 3H; NMe_2), 2.99 (s, 3H; NMe_2), 1.90 (s, 3H; Ac), 1.63 (s, 9H; Boc).

ESI-MS: *m/z* calcd for $C_{14}H_{25}N_3O_4$: 299.2; found: 322.1 $[M+Na]^+$.

Ac-(4*R*)Amp-NMe_2·TFA

Ac-(4*R*)Amp(Boc)-NMe_2 (92.9 mg, 310 µmol, 1.0 eq) were dissolved in 3 ml of a 1:1 mixture of TFA and CH_2Cl_2.The solution was stirred for 2h and concentrated under reduced pressure. The residue was covered with ^{t}BuOMe and sonicated whereupon a withe solid precipitated. The title compound was isolated as 68 mg of a colorless solid (74%).

(two isomers are visible in the ^{1}H NMR- and ^{13}C NMR-spectra in a ratio of ≈ 1:3.1).

^{1}H NMR (500 MHz, D_2O): δ/ppm = 4.95 (dd, *J* = 8.6 Hz, 6.1 Hz, 1H; Hα), 4.02 (dd, *J* = 10.3 Hz, 5.4 Hz, 1H; Hγ), 3.96 (dd, *J* = 11.8 Hz, 6.2 Hz, 1H; Hδ), 3.73 (m, 1H; Hδ), 3.04 (s, 3H; NMe_2), 2.84 (s, 3H; NMe_2), 2.43 (m, 1H; Hβ), 2.24 (dd, *J* = 13.4 Hz, 7.0 Hz, 1H; Hβ), 2.03 (s, 3H; Ac).

Isolated signals of the minor conformer: 5.11 (dd, *J* = 8.9 Hz, 4.6 Hz, 1H; Hα), 3.04 (s, 3H, NMe_2), 2.88 (s, 3H, NMe_2), 2.58 (m, 1H; Hβ), 2.37 (dd, *J* = 13.4 Hz, 7.0 Hz, 1H; Hβ), 1.84 (s, 3H; Ac).

^{13}C NMR (101 MHz, D_2O): δ/ppm (mayor conformer) = 174.2, 172.1 (amides), 55.7 (Cα), 51.8 (Cγ), 50.3 (Cδ), 38.2 (NMe_2), 36.4 (NMe_2), 32.4 (Cβ), 22.3 (Ac).

ESI-MS: *m/z* calcd for $C_{11}H_{18}F_3N_3O_4$: 313.1; found: 200.1 $[M-TFA]^+$.

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