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Synthetic Marine Sponge Collagen by Late-Stage Dihydroxylation

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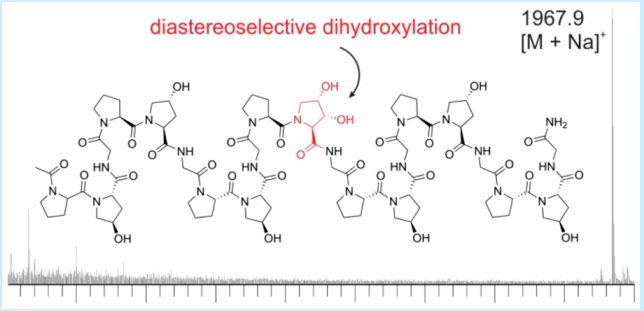
Supporting Information

ABSTRACT: Based on the observation that an increased substrate size is paralleled by an enhanced diastereoselectivity, a late-stage dihydroxylation protocol toward the 21mer CMP (collagen model peptide) Ac-(Pro-Hyp-Gly)$_3$-Pro-Dyp-Gly-(Pro-Hyp-Gly)$_3$-NH_2 is presented. C3 and C4 hydroxylation have a converse effect on the triple-helical stability of collagen. Their combined influence on the melting temperature was studied by NMR spectroscopy.



The mild reaction conditions of osmium-catalyzed olefin dihydroxylation render this reaction well suited for late-stage functionalization of peptides. Nevertheless, dihydroxylations of large substrates with molecular weights significantly above 1 kDa are rare and characterized by moderate yields and mediocre diastereoselectivities even in the presence of chiral ligands.[1] The molecular target investigated here is a peptide natural product with a 3 × 21mer tertiary structure of 3 × 1947 Da (ca. 5.9 kDa). The substrate-controlled diastereoselectivity of complex natural products is difficult to predict. This disadvantage is balanced by the excellent chemoselectivity of dihydroxylation and the reduction of the number of synthetic steps because no protection is needed.

Collagen is composed of all-*trans* configured Xaa-Yaa-Gly tripeptide repeats assembled in a supramolecular right-handed triple helix.[2−4] The Gly in every third position fits into the interior of the helix and forms a hydrogen bond to Xaa of the neighboring strand. Post-translational hydroxylation of the Pro in Yaa position increases the stability of the triple helix, making Pro-Hyp-Gly the dominating repetitive unit of the collagen single strand.[5−7] Synthetic collagen model peptides (CMPs) with different amino acids in the Xaa or Yaa positions avoid the microhetereogeneity of the natural collagens. It was shown that the ring puckering of the Xaa and Yaa residues correlates with the triple helix stability, which is characterized by its melting point.[8−10] In the crystal structure, Pro in the Xaa position shows a C(4)-*endo* puckering of the pyrrolidine ring while 4-Hyp in the Yaa position assumes a C(4)-*exo* puckering (Figure 1).[7] Variation of this puckering pattern using substituents in the 3-position destabilizes the triple helix, as observed for (3*S*)-3-hydroxyproline (3-Hyp in Figure 1). Nevertheless, there are examples known, where an alternative puckering, derived by substituents in 4-position, is tolerated.[11,12] The combined effect of 3- and 4-*cis*-dihydroxylation (Dyp) has not yet been investigated, although this structure plays an important role in the collagen of deep-sea sponges.[13]

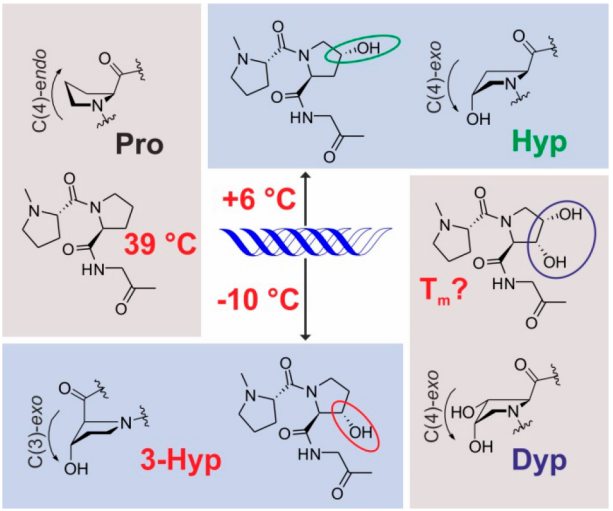


Figure 1. Pro, two Hyp isomers, and Dyp and their puckering in the Yaa position of host−guest collagens of the type (Pro-Hyp-Gly)$_3$-Pro-**Yaa**-Gly-(Pro-Hyp-Gly)$_3$ are shown around a triple helix (blue). Their melting temperatures are indicated relative to the parent amino acid Pro. The influence of Dyp on collagen is investigated in this study.

The incomplete peptide coupling caused by the significantly decreased nucleophilicity of Dyp renders this amino acid ill-suited for the assembly of larger peptides, while its precursor Dhp (3,4-dehydroproline) is acylated in high yield. Here, we synthesize a host−guest CMP containing Dyp by late diastereoselective late-stage dihydroxylation of the Dhp moiety and investigate the influence of Dyp on the melting temperature of the assembled collagen. Instead of the common circular dichroism (CD) spectroscopic approach, we use NMR spectroscopy to determine the melting temperature. The CMPs are

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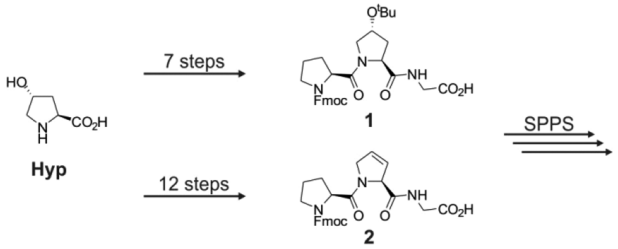
generally obtained by fragment condensation of suitably protected tripeptide building blocks (Fmoc-Xaa^{pg}-Yaa^{pg}-Gly-OH) on a solid support (solid-phase peptide synthesis, SPPS). The coupling of this tripeptide circumvents racemization and the majority of deletion mutants because the number of coupling steps is divided by three.[14,15]

In this work, we describe an improved SPPS protocol for the assembly of CMPs and the protecting-group-free late-stage dihydroxylation to access the peptide natural product. With this collagen derivative in hand, we measured the influence of Dyp on the melting temperature by NMR spectroscopy and ranked the influence of different hydroxylation patterns on the stability of triple-helical collagen. The Fmoc-protected tripeptide building blocks Fmoc-Pro-Hyp(tBu)-Gly-OH (**1**, Figure 2) and Fmoc-Pro-Dhp-Gly-OH (**2**) were synthesized according to established protocols (Supporting Information) and used for the synthesis of the host–guest collagen model peptide CMPs **3**–**5**.[9,12,18,19] With conditions based on our previous work, we obtained purities of 70–79% of the crude peptide.[20] The yields after purification by preparative RP-HPLC based on the 0.05 mM scale were 25–26% for all peptides. With this improved protocol, we could cut down the amount of building blocks to 2 equiv and, simultaneously, doubled the yield of purified CMP compared with standard procedures.[9,14,21]



	sequence	crude[a] purity (%)	purified[b] yield (%)
3	Ac-(PUG)$_3$-Pro-**Hyp**-Gly-(PUG)$_3$-NH$_2$	79	25
4	Ac-(PUG)$_3$-Pro-**Pro**-Gly-(PUG)$_3$-NH$_2$	70	26
5	Ac-(PUG)$_3$-Pro-**Dhp**-Gly-(PUG)$_3$-NH$_2$	70	25

Figure 2. Synthesis of Fmoc-Pro-Hyp(tBu)-Gly-OH (**1**) and Fmoc-Pro-Dhp-Gly-OH (**2**) starting from Hyp. Both tripeptides are suitable for SPPS to obtain CMPs **3**–**5**. (a) Purity of the crude peptide, determined with RP-HPLC. (b) Isolated yield after purification by preparative RP-HPLC based on the 0.05 mM scale.

The double bond in CMP **5** was the substrate for the subsequent late-stage functionalization and solved two synthetic problems at once. Dhp saved two reaction steps because Dyp as a starting point for the assembly of CMP **12** would require a diol protecting group during peptide coupling.[16] Additionally, Dhp is preferred over Dyp in SPPS because of the poor nucleophilicity of the secondary amine of Dyp, resulting in poor acylation yields and long reaction times.[17] The osmate-mediated dihydroxylation of olefins invented by Criegee has undergone significant improvements in the last decades.[22–24] Yet, none of these methods is directly applicable to the synthesis of dihydroxylated collagen. For the dihydroxylation of 21mer peptide **5**, we optimized the reaction conditions and measured the diastereoselectivity of this transformation starting from **6**, **8**, and **10** as model systems of increasing size. In addition to 1 mol % of K_2OsO_4 and 1.33 equiv of NMO co-oxidant, an amount of 0.75 equiv of citric acid was added to accelerate the reaction because slow reaction kinetics were expected for substrates of higher molecular mass.[1,25] Water or a mixture of water/acetonitrile as solvent allowed the direct purification on a preparative RP-HPLC column.[26] A diastereomeric ratio (dr) of 82:18 was determined with NMR spectroscopy for the dihydroxylation of **6**, which was also reported in the literature.[27] Signal overlap made a direct determination of the dr from the ^{1}H NMR spectrum impossible for the dihydroxylation of **8** and **10**. The dr of 92:8 for **9** and 98:2 for **11** was determined by RP-HPLC analysis (Figure 3). For **9** and for **11**, it was possible to separate, isolate, and

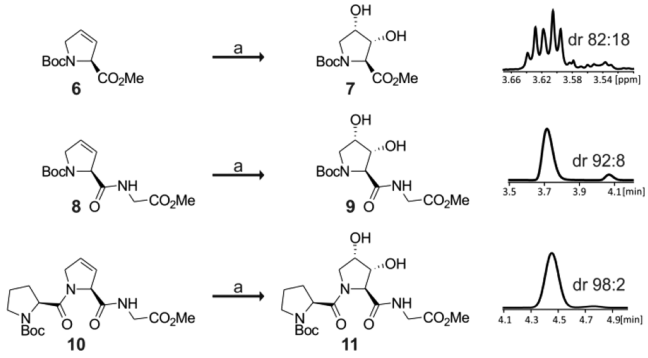


Figure 3. Optimized reaction conditions and dr of the dihydroxylation of derivatives **6** (dr determined with ^{1}H NMR spectrum), **8**, and **10** (dr determined with RP-HPLC): (a) 1 mol % of K_2OsO_4, 1.33 equiv of NMO, 0.75 equiv of citric acid, H_2O/MeCN 4:1, rt, 2.5 h.

characterize both diastereomers. The isolated yields (Supporting Information) confirmed the ratios observed in the HPLC chromatograms. A trend of increasing dr with increasing peptide length is already visible for the tripeptide and follows simple steric reasons.

We expected a high dr for the dihydroxylation of the CMP **5** containing 21 amino acids, but we had to increase the loading of the catalyst in order to shorten the reaction time and to increase the yield.[25] After 6 h at room temperature with a catalyst amount of 20 mol %, only 35% of the starting material **5** was converted. When the reaction temperature was increased to 60 °C, significantly above the melting temperature of the helix, we obtained a complete conversion to **12** in an acceptable reaction time of 6 h with an excellent yield of 98% after purification by preparative RP-HPLC (Figure 4).

Even the reaction mixture, which stagnated at 40% conversion after 24 h at rt, was fully converted to Dyp peptide **12** after heating to 60 °C (Figure 5). Neither the ^{1}H NMR spectrum nor the HPLC chromatogram indicated the formation of detectable amounts of a second diastereomer. The decreased reactivity of **5** can be explained by the limited accessibility of the double bond within the triple helix or by the complexation of the osmate catalyst by substrate **5**, which already bears 6—or 3 × 6 as a triple helix—hydroxy groups. The complexation of metal ions is a known phenomenon.[28] To exclude it in the case of CMPs, we performed the dihydroxylation of the isomer Ac-(Pro-Hyp-Gly)$_6$-Pro-Dhp-Gly-NH$_2$, which, in spite of forming a triple helix, became completely dihydroxylated already at room temperature (Supporting Information).

CD spectroscopy is the most common analytical method for the determination of collagen melting temperatures. Typical concentrations are 50–500 μM and temperature gradients of 4–12 °C/h. These variations of experimental conditions lead to deviations of up to several degrees centigrade for similar

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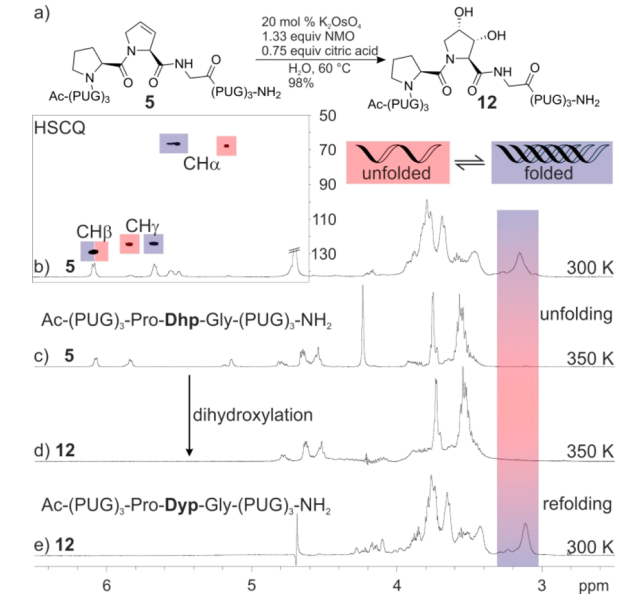

Figure 4. (a) Dihydroxylation of CMP **5**. (b) ^{1}H NMR spectrum of CMP **5** at 300 K (500 MHz in H_2O/D_2O 9:1) and the excerpt from the HSQC. (c) ^{1}H NMR spectrum of monomeric CMP **5** at 350 K. (d) ^{1}H NMR spectrum of CMP **12** at 350 K. (e) ^{1}H NMR spectrum of CMP **12** at 300 K.

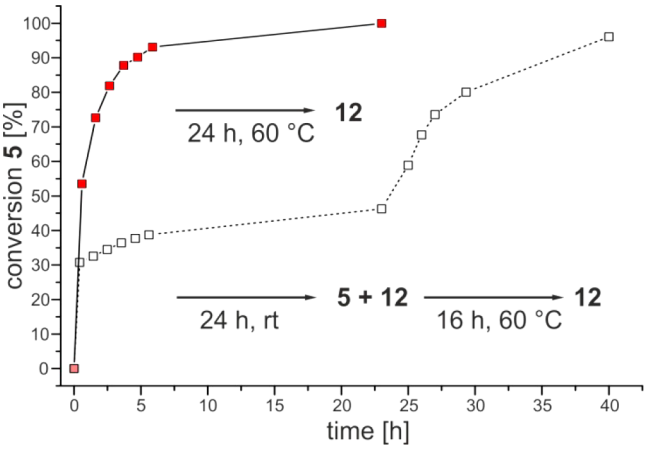

Figure 5. Conversion of the CMP **5** to **12** at different temperatures. A higher reaction temperature enforces full conversion of **5**. The late-stage dihydroxylation shown in red was performed at 60 °C from the beginning, while the dihydroxylation which started at rt (white) was heated to 60 °C after 24 h (points are connected for visualization).

CMPs.[9,18,29] NMR spectroscopy exploits the known phenomenon of the high-field shift of Pro Hδ (Xaa position) in the folded triple-helical form (red in Figure 6).[30,31]

We used the signal of the aliphatic protons, containing a defined number of protons (45 protons in **3**, 47 protons in **4**, 43 protons in **5** and **12**) between 1.80 and 2.40 ppm and plotted the resulting integral of the Pro Hδ (divided by seven for normalization on one Pro-Yaa-Gly-triplet) versus temperature (Figure 7). All normalized integrals were lower than one, indicating that about 80% of the Pro Hδ protons are in a triple-helical environment. This NMR technique not only gives a correct value for the maximal folded fraction but also allows the quantification of the melting temperature at concentrations of 3 mM. The melting temperature at these concentrations is significantly less dependent on the peptide concentration than

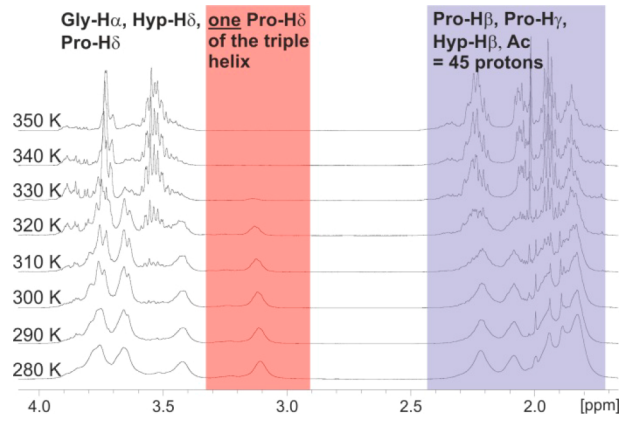

Figure 6. ^{1}H NMR spectra of **3** from 280 to 350 K in 10 K steps, 500 MHz, H_2O/D_2O 9:1. All signals were assigned and highlighted, especially the downfield shifted Pro Hδ (red).

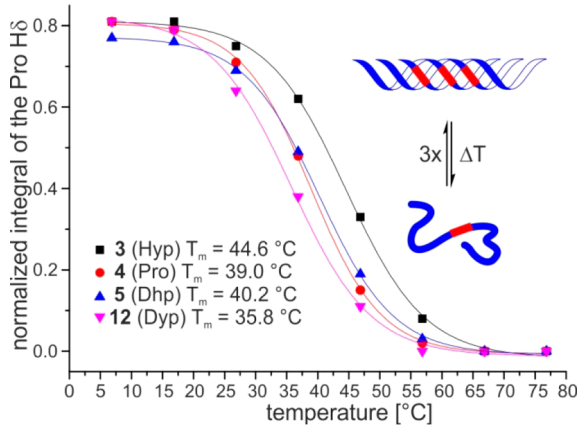

Figure 7. Melting curves of CMPs **3**, **4**, **5**, and **12** calculated from variable-temperature ^{1}H NMR spectroscopy. The melting temperatures of the host–guest CMPs (melting shown schematically on the right) are listed next to the curves. From multiple measurements, we estimate an inaccuracy below 0.1 °C.

at typical CD concentrations between 50 and 500 μM but nevertheless in good agreement with the data available in the literature.[11,32]

The hydroxylation and the C(4)-*exo* puckering significantly stabilized the triple helix formed by CMP **3**[9,33] and increased its melting temperature 5.6 °C above the melting temperature derived from reference CMP **4**.[12] The melting temperature of the CMP **5** was between both values: 4.4 °C below CMP **3** and 1.2 °C above the CMP **4**. The hydroxy groups in both 3- and 4-position in the dihydroxylated CMP **12** resulted in a destabilization of the helix of about 8.8 °C compared to CMP **3**. Based on CD spectroscopy, a destabilization of 15 °C was described for 3-Hyp (C(3)-*exo*) in the Yaa position relative to the 4-Hyp containing CMP.[12] Therefore, the retention of the C(4)-*exo* puckering of Dyp (CMP **12**) compensated steric interactions of the substituent in the 3-position.[34] CMP **12** matches well into the pucker-based explanation models of melting temperatures. The correlation of melting temperatures with the $K_{trans/cis}$ is well documented for 4-substituted pyrrolidine rings but not for 3- or 3,4-disubstituted ones.[10,12] The trans/cis ratio of 4.9 for Ac-3-Hyp-OMe was in the same range as Ac-Pro-OMe (4.6), although the melting temperature of 3-Hyp containing CMP is significantly lower.[10] The $K_{trans/cis}$ value of Ac-Dhp-OMe (5.5)

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was between the values of Ac-Pro-OMe (4.6) and Ac-Hyp-OMe (6.1), although the melting temperature of CMP **5** is slightly higher than that of CMP **4**.[35] CMP **12** completes the set of 3- and 4-hydroxylated collagens. The dihydroxylation approach described here makes polyhydroxylated peptide natural products accessible with less effort.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.7b03525.

Experimental procedures for all reported compounds, ^{1}H and ^{13}C spectra, HPLC chromatograms (PDF)

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Notes

The authors declare no competing financial interest.

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