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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Abstract: The efficient synthesis of the tripeptidic building block Fmoc-Pro-Hyp(TBDPS)-Gly-OH and its application for the preparation of collagen model peptides (CMPs) has been achieved. The silyl ether protecting group prevents undesired side reactions during the CMP synthesis thereby facilitating purification and allowing for selective deprotection of the hydroxyproline residue without affecting the solid-supported CMP.

Key words: collagen, peptides, protecting groups, solid-phase synthesis, TBDPS silyl ether

Collagen is the most abundant protein in vertebrates and has applications in medicine and cosmetics.[1] Collagen fibers are assemblies of triple helices that consist of three single strands with the repeat unit Xaa-Yaa-Gly where the amino acids proline (Pro) and (4*R*)-hydroxyproline (Hyp) are most common in the Xaa and Yaa positions, respectively. Defects in the structure of collagen are involved in several diseases such as scurvy and brittle bone disease.[1–3] Thus, understanding the factors that influence the structure of collagen is important. Towards this goal, various collagen model peptides (CMPs) comprising typically 21–30 residues have been prepared in order to investigate their structural properties.[3–6] Since the Pro-Hyp-Gly motif is the most common natural repeat unit within collagen, it is also the most common motif used within CMPs.[3–6] Thus, the effective synthesis of CMPs with at least seven repeat units of Pro-Hyp-Gly is an important goal.

Two different strategies for the synthesis of CMPs are used. Either single Fmoc-protected amino acids are coupled using solid-phase peptide synthesis (SPPS), or trimeric building blocks such as Fmoc-Pro-Hyp-Gly-OH are prepared in solution phase and then utilized in SPPS.[5–7] The first method relies on large excesses of the Fmoc-protected amino acid building blocks and the coupling reagents (5–10 equiv) in every coupling step. This approach can lead to the formation of significant amounts of truncated fragments in which the trimeric repeat unit is not preserved. These impurities can disrupt triple helix formation and are often difficult to separate from the desired CMP. The second method requires the preparation of the trimeric building block (e.g., Fmoc-Pro-Hyp-Gly-OH) in solution phase.[7] This approach is advantageous since fewer coupling steps with less equivalents of the building

block are necessary. As a result, fewer side products form, thereby facilitating the purification of the desired CMP.

Especially the trimer strategy uses Hyp with an unprotected hydroxy function. Thus, in particular with potent coupling reagents such as *O*-(6-chlorobenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HCTU), undesired side reactions involving the hydroxy group can take place.[8] Undesired acylation reactions can also occur during capping with acetic anhydride (Ac₂O) after each coupling step; a protocol typically used to facilitate purification of the final product.[8] An obvious solution to this problem is the protection of the hydroxy group of Hyp. So far, *tert*-butyl ethers in particular have been employed to protect Hyp during the SPPS synthesis of CMPs.[4b,7a] This protecting group is readily removed under the acidic conditions used for the cleavage of CMPs from typically used solid supports such as Rink amide and Wang resin. We sought an alternative protecting group that could be selectively removed without simultaneous cleavage of the CMP from these acid-sensitive solid supports. Here we present the synthesis of the *tert*-butyldiphenylsilyl (TBDPS) protected trimeric building block Fmoc-Pro-Hyp(TBDPS)-Gly-OH and describe its application in the solid-phase synthesis of a CMP consisting of 21 residues.

Aside from the requirement of allowing for selective removal from resin-bound CMPs under mild conditions, a suitable protecting group must also allow for facile introduction and has to be stable during the SPPS and the synthesis of the trimeric Fmoc-protected building block Fmoc-Pro-Hyp(PG)-Gly-OH. In addition, a sterically demanding protecting group is desirable in order to prevent aggregation of the peptide during SPPS. Silyl ethers with aryl and/or sterically demanding alkyl substituents are versatile protecting groups for hydroxy groups that can be easily introduced, and can be removed either with fluoride ions or under acidic conditions but are otherwise robust. We therefore envisioned a silyl ether to be a suitable protecting group for our improved synthesis of CMPs (Figure 1).
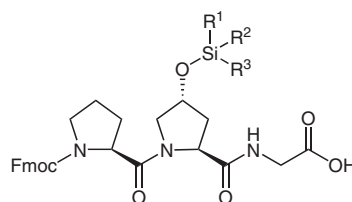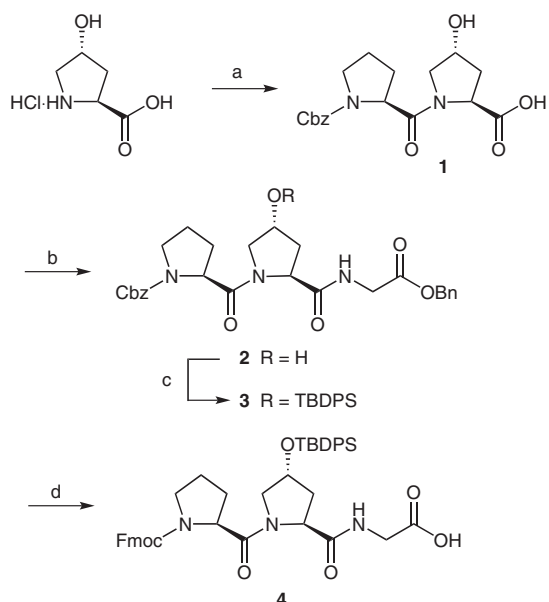

**Figure 1**   Silyl ether protected building block for CMP synthesis

Scheme 1 *Reagents and conditions*: (a) (i) Benzyltrimethylammonium hydroxide, MeOH, r.t.; (ii) Cbz-Pro-OSu, DMF, r.t., 89%; (b) H-Gly-OH·HCl, EDC·HCl, HOBt, *i*-Pr₂NEt, DMF, r.t., 95%; (c) TBDPS-Cl, imidazole, DMF, r.t., 93%; (d) (i) HCO₂NH₄, Pd/C, MeOH, r.t.; (ii) Fmoc-OSu, Et₃N, MeCN–H₂O, r.t., 86%.

Benzyl groups were anticipated to be the most suitable protecting groups for the amino and carboxylic acid functionalities during the synthesis of the trimeric Fmoc-protected building block Fmoc-Pro-Hyp(PG)-Gly-OH. We therefore started our synthesis by condensation of the commercially available Cbz-protected activated succinimide ester of proline (Cbz-Pro-OSu) with Hyp under previously described conditions to isolate the dipeptide Cbz-Pro-Hyp-OH (**1**) in 89% yield (Scheme 1).[7a] Dipeptide **1** was coupled with the glycine benzyl ester H-Gly-OBn using EDC/HOBt as coupling reagents to provide tripeptide Cbz-Pro-Hyp-Gly-OBn (**2**)[7a] in 95% yield. We then investigated the utility of the *tert*-butyldimethylsilyl (TBDMS) ether as a protecting group. Introduction using TBDMS-Cl in the presence of imidazole proceeded smoothly, however, upon hydrogenation of the benzyl protecting groups using Pd/C under a hydrogen atmosphere, cleavage of the TBDMS ether was observed. Several different conditions were tested, however, either cleavage of the TBDMS ether or incomplete removal of the benzyl protecting groups was observed.[9] We therefore turned our attention to the sterically more demanding and stable *tert*-butyldiphenylsilyl (TBDPS) protecting group. Protection of the Hyp residue within tripeptide **2** using TBDPS-Cl and imidazole proceeded smoothly (93% yield) to provide the protected peptide Cbz-Pro-Hyp(TBDPS)-Gly-OBn (**3**). Hydrogenation with Pd/C and H₂ proved sluggish and incomplete cleavage of the Cbz group was typically observed. In contrast, phase-transfer hydrogenation with ammonium formate as the hydrogen source and Pd/C as catalyst led to the C- and N-terminally unprotected tripeptide silyl ether H-Pro-Hyp(TBDPS)-Gly-OH within 45 minutes. Only traces of

the diketopiperazine consisting of Pro-Hyp(TBDPS) were observed as a byproduct. Fmoc-protection of the amino group with Fmoc-OSu under Schotten–Baumann conditions provided the trimeric building block Fmoc-Pro-Hyp(TBDPS)-Gly-OH (**4**) in 86% yield over two steps. Thus, the target building block **4**, ready for SPPS was obtained in an overall yield of 68% (Scheme 1).

In order to evaluate building block **4** in the SPPS of CMPs, seven successive coupling and Fmoc-deprotection steps were performed on Rink amide resin with a loading of 0.36 mmol/g. HCTU/*i*-Pr₂NEt served as coupling reagent, and a solution of piperidine in DMF (1:4) was used for Fmoc deprotection (Scheme 2). After each coupling, acetic anhydride was used to cap any remaining free amino groups. The protocol proved efficient enough to be performed in an automated fashion on a peptide synthesizer for the assembly of CMP **5** on Rink amide resin. For the cleavage of the silyl ethers from the Hyp-residues, resin-bound CMP **5** was agitated in a 1 M solution of tetrabutyl ammonium fluoride (TBAF) in tetrahydrofuran overnight. The resin was then washed with tetrahydrofuran and dichloromethane before removing CMP **6** from the solid phase using a solution of trifluoroacetic acid (TFA) in dichloromethane.
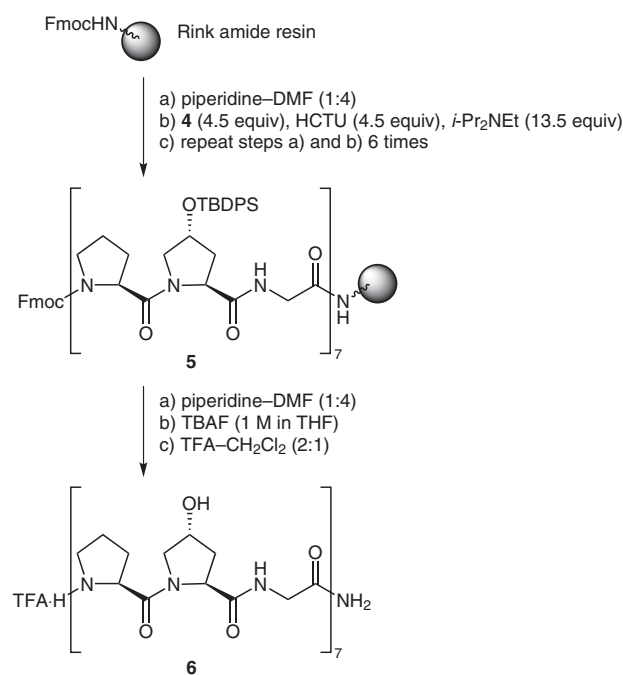
Scheme 2 Solid-phase synthesis of CMP **6**

Analysis of CMP **6** by HPLC demonstrated a purity of ≥60% of the crude material, most of the other signals observed in the chromatogram correspond to fragments derived from incomplete couplings (Figure 2). For comparison, CMP **6** was also prepared under similar conditions (without capping with Ac₂O) using the trimeric building block Fmoc-Pro-Hyp-Gly-OH without a protecting group on the Hyp residue. The chromatogram of the crude material derived from this synthesis indicated a pu-

rity of only ~35%. Purification of CMP **6** prepared by the strategy described above by preparative HPLC proved straightforward and provided **6** in a purity of ≥98% in a yield ranging from 20–40% with respect to the original loading of the resin used. In contrast, yields of the analogous purified CMPs prepared without protecting groups on Hyp ranged from 10 to 20%.
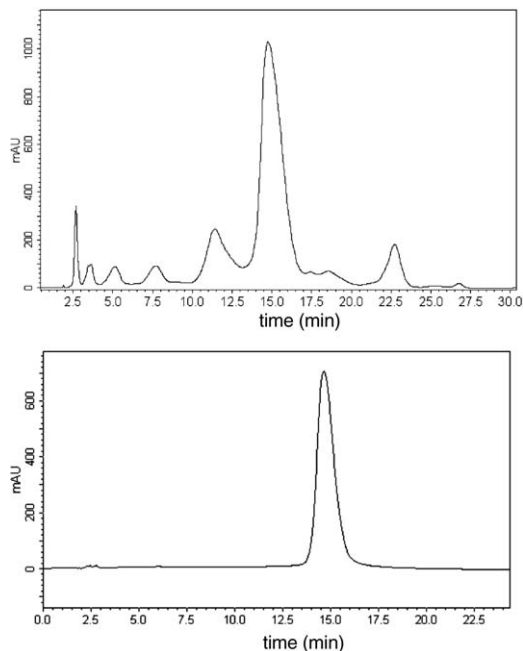

Figure 2 RP-HPLC chromatograms of CMP **6** before (top) and after (bottom) preparative HPLC purification

In conclusion, we have developed the silyl ether protected tripeptide Fmoc-Pro-Hyp(TBDPS)-Gly-OH as a versatile building block for the synthesis of CMPs. The TBDPS protecting group prevents undesired acylation reactions during the synthesis, thereby facilitating the purification and increasing the yield of the CMPs. In addition, the bulkiness of the TBDPS might prove helpful in preventing aggregation of longer CMPs on the solid support.

Materials and reagents were of the highest commercially available grade and used without further purification. Reactions were monitored by TLC using Merck silica gel 60 F254 plates. Compounds were visualized by UV, ninhydrin and $KMnO_4$. Flash chromatography was performed using Merck silica gel 60 (40–63 μm). ^{1}H and ^{13}C NMR spectra were recorded on Bruker DPX 500 and DPX 400 spectrometers. The ^{1}H and ^{13}C NMR spectra of compounds **1–4** contained at least two sets of peaks due to the *cis* and *trans* conformers around the tertiary carbamate and amide bonds. Peak assignments are not listed separately for the conformers. Chemical shifts (δ) are reported in ppm using TMS as a reference. Bruker Esquire 3000 Plus was used for electrospray ionization (ESI) mass spectrometry measurements. High-resolution mass spectra were recorded on a Sciex QStar Pulsar electrospray ionization time-of-flight spectrometer (ESI-TOF). Analytical HPLC was performed using a LiChrospher 100 RP-18e (5 μm, 250 mm × 4 mm) column from Merck. Preparative HPLC was carried out on a LiChrospher RP-18e (5 μm, 250 mm × 10 mm) column from Merck. For automated peptide synthesis, a Syro I Peptide Synthesizer (MultiSynTech GmbH, Witten, Germany) was employed.

Cbz-Pro-Hyp-OH (1)
H-Hyp-OH (10.5 g, 79.7 mmol, 1.0 equiv) was suspended in benzyltrimethlyammonium hydroxide in MeOH (40% w/w, 47.1 mL, 103 mmol, 1.3 equiv) and MeOH (10 mL) was added until a yellow solution was obtained. The solution was stirred for 10 min then all volatiles were removed under reduced pressure. The residual yellow oil was dissolved in DMF (60 mL) and Cbz-Pro-OSu (25.0 g, 72.2 mmol, 0.91 equiv) was added. The yellow solution was stirred overnight then all volatiles were removed under reduced pressure. The yellow residue was dissolved in $NaHCO_3$ (5%, 200 mL) and washed with Et_2O (3 × 150 mL). The aqueous layer was slowly added to 3 M HCl (200 mL) and treated in an ultrasonic bath until precipitation of a white solid was observed. The solid was filtered, washed with cold 1 M HCl (3 × 50 mL) and dried under high vacuum. The analytical data of **1** are identical to those reported by Moroder and co-workers.[7a]

Yield: 23.4 g (89%); white solid; R_f = 0.51 (MeCN–CH_2Cl_2–AcOH, 8:1:1; $KMnO_4$).

^{1}H NMR (400 MHz, DMSO-d_6): δ = 12.84 (s, 1 H), 7.40–7.23 (m, 5 H), 5.11–4.93 (m, 2 H), 4.59–4.47 (m, 1 H), 4.40–4.32 (m, 1 H), 4.25 (q, J = 7.9 Hz, 1 H), 3.62–3.32 (m, 4 H), 2.29–1.99 (m, 2 H), 1.95–1.73 (m, 4 H).

^{13}C NMR (100 MHz, DMSO-d_6): δ = 173.2 (COOH), 170.3, 170.0 (amide), 153.8, 153.5 (carbamate), 137.0, 128.3, 128.2, 127.7, 127.4, 126.7 (Cbz), 69.0, 68.9 (C_γHyp), 65.7, 65.6 (Cbz), 58.0, 57.6, 57.4 (C_αHyp, C_αPro), 54.3, 54.2 (C_δHyp), 46.9, 46.2 (C_δPro), 36.9 (C_βHyp), 29.3, 28.3 (C_βPro), 23.5, 22.8 (C_γPro).

ESI-MS: m/z calcd for $C_{18}H_{22}N_2O_6$: 362.2; found: 385.3 [M + Na]+ (100%), 747.3 [2M + Na]+ (30%).

Cbz-Pro-Hyp-Gly-OBn (2)
Dipeptide **1** (16.4 g, 45.3 mmol, 1.0 equiv) and H-Gly-OBn·HCl (11.0 g, 54.4 mmol, 1.2 equiv) were suspended in DMF (140 mL) and cooled to 0 °C. The suspension was stirred and HOBt (6.94 g, 45.3 mmol, 1.0 equiv) and i-Pr_2NEt (7.31 mL, 47.6 mmol, 1.05 equiv) were added, which gave a clear solution. EDC·HCl (9.56 g, 49.9 mmol, 1.1 equiv) was added and the reaction mixture was stirred overnight. The solvent was evaporated under reduced pressure and the yellow residue was dissolved in CH_2Cl_2 (300 mL) and washed with sat. $NaHCO_3$ (3 × 150 mL). After washing with aq $KHSO_4$ (10%; 3 × 150 mL) the organic layer was dried over $MgSO_4$ and all volatiles were removed under reduced pressure. Drying under high vacuum gave **2**. The analytical data of **2** are identical to those reported by Moroder and co-workers.[7a]

Yield: 22.1 g (96%); white solid; R_f = 0.35 (MeOH–CH_2Cl_2, 5%; $KMnO_4$).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.60 (t, J = 5.6 Hz, 1 H, NHGly), 7.40–7.21 (m, 10 H, Cbz, BnOCO), 5.30–4.94 [m, 4 H, $PhCH_2O(CO)N$, $PhCH_2OCO$], 4.73 (t, J = 7.7 Hz) and 4.66–4.27 (m, 3 H, H_αPro, H_αHyp, H_γHyp), 4.10–3.30 (m, 6 H, H_αGly, H_δPro, H_δHyp), 2.47–1.67 (m, 6 H, H_βPro, H_βHyp, H_γPro).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.0, 171.7, 169.5 (amides, ester), 155.1 (carbamate), 136.4, 135.3, 128.5, 128.4, 128.3, 128.2, 127.9, 127.7 (Cbz, Bn), 70.4 (C_γHyp), 67.1, 66.8 (Cbz, Bn), 58.4, 58.3 (C_αPro, C_αHyp), 55.0 (C_δHyp), 46.8 (C_δPro), 41.3 (C_αGly), 36.4 (C_βHyp), 29.4 (C_βPro), 24.3 (C_γPro).

ESI-MS: m/z calcd for $C_{27}H_{31}N_3O_7$: 509.2; found: 532.4 [M + Na]+ (100%).

Cbz-Pro-Hyp(TBDPS)-Gly-OBn (3)
Tripeptide **2** (22.1 g, 43.3 mmol, 1.0 equiv) and imidazole (13.0 g, 191 mmol, 4.4 equiv) were dissolved in DMF (50 mL) and TBDPS-Cl (31.0 mL, 119 mmol, 2.75 equiv) was added. The reaction mixture was stirred under a nitrogen atmosphere overnight. The reaction was cooled to 0 °C and H_2O (100 mL) and Et_2O (400 mL)

were added. The mixture was washed with sat. NaHCO₃ (3 × 150 mL) and 1 M HCl (3 × 150 mL), then the organic layer was dried over MgSO₄ and all volatiles were removed under reduced pressure. The slightly yellow residue was subjected to column chromatography (SiO₂; EtOAc–pentane, 1:1→2:1) to give **3**.

Yield: 30.0 g (93%); white solid; R_f = 0.30 (EtOAc–pentane, 3:2; UV and KMnO₄).

^{1}H NMR (400 MHz, DMSO-d_6): δ (major conformer) = 8.37 (t, J = 5.9 Hz, 1 H, NHGly), 7.67–7.18 (m, 20 H, Ph₂Si, Cbz, Bn), 5.14–4.86 [m, 4 H, PhCH₂O(CO)N, PhCH₂OCO], 4.64–4.19 (m, 3 H, H$_a$Pro, H$_a$Hyp, H$_γ$Hyp), 3.96–3.23 (m, 6 H, H$_a$Gly, H$_δ$Pro, H$_δ$-Hyp), 2.35–1.68 (m, 6 H, H$_β$Pro, H$_β$Hyp, H$_γ$Pro), 0.94 (s, 9 H, t-BuSi). Isolated signals of minor conformers: δ = 8.61 (t, J = 5.9 Hz, 1 H, NHGly), 8.55 (t, J = 5.8 Hz, 1 H, NHGly), 1.03, 1.00, 0.99 (s, 9 H, t-BuSi).

^{13}C NMR (100 MHz, DMSO-d_6): δ = 171.7, 171.6, 170.2, 170.0, 169.5 (amides, ester), 153.7, 153.5 (carbamate), 137.0, 135.7, 135.1, 135.0, 133.0, 132.9, 132.8, 129.9, 128.3, 128.1, 128.0, 127.9, 127.8, 127.6, 127.4, 127.0, 126.8 (Ph), 71.6, 71.3 (C$_γ$Hyp), 65.7, 65.6 (Cbz, Bn), 58.0, 57.9, 57.3 (C$_a$Pro, C$_a$Hyp), 54.4, 54.1 (C$_δ$H-yp), 46.8, 46.2 (C$_δ$Pro), 40.5 (C$_a$Gly), 37.7, 37.4 (C$_β$Hyp), 29.3, 28.4 (C$_β$Pro), 26.5 (TBDPS), 23.6, 22.7 (C$_γ$Pro), 18.6, 18.5 (TB-DPS).

HRMS-ESI: m/z calcd for C₄₃H₄₉N₃O₇Si+Na: 770.3237; found: 770.3261 [M + Na]⁺.

Fmoc-Pro-Hyp(TBDPS)-Gly-OH (4)
Under a nitrogen atmosphere, MeOH (15 mL) was added to 10% (w/w) Pd/C (300 mg, 10% w/w) followed by peptide **3** (3.00 g, 4.01 mmol, 1.0 equiv). The suspension was stirred and ammonium formate (2.02 g, 32.1 mmol, 8.0 equiv) was added whereupon the mixture evolved a gas. After 3 h, the mixture was filtered through a glass frit, washed with MeOH (3 × 15 mL) and the solvent was removed under reduced pressure to obtain a white foam. The foam was dissolved in a mixture of MeCN (4 mL), H₂O (4 mL) and Et₃N (1.10 mL, 8.02 mmol, 2.0 equiv). Fmoc-Cl (1.22 g, 3.61 mmol 0.9 equiv) was added and a pH of 10 was secured. The suspension was stirred at r.t. for 30 min, diluted with CH₂Cl₂ (30 mL) and filtered. The filtrate was acidified with 1 M HCl (80 mL) and extracted with CH₂Cl₂ (3 × 30 mL). The combined organic layers were dried over MgSO₄ and all volatiles were removed under reduced pressure. The oily residue was subjected to column chromatography (CH₂Cl₂–MeOH–AcOH, 100:5:2) to yield **4** after co-evaporation with toluene (3×) to remove traces of AcOH.

Yield: 2.31 g (86%); white foam; R_f = 0.29 (CH₂Cl₂–MeOH–AcOH, 100:5:2; UV). HPLC: t_R = 26.1 min [gradient: 50→10% of solvent B (0.1% TFA, 1% MeCN in H₂O) in solvent A (MeCN) over 35 min at a flow rate of 1 mL/min].

^{1}H NMR (500 MHz, CD₃CN): δ = 7.95–7.75, 7.70–7.53, 7.51–7.28 (m, 18 H, Fmoc, TBDPS), 7.24, 7.04 (t, J = 5.6 Hz, 1 H, NHGly), 4.63–4.35 (m, 2 H, H$_a$Hyp, H$_a$Pro), 4.58–4.48 (m, 1 H, H$_γ$Hyp), 4.50–4.33 (m, 2 H, Fmoc), 4.32–4.18 (m, 1 H, Fmoc), 3.90–3.75 (m, 2 H, H$_a$Gly), 3.54–3.11 (m, 4 H, H$_δ$Hyp, H$_δ$Pro), 2.22–1.91 (m, 2 H, H$_β$Hyp), 2.15–2.05, 1.88–1.64 (m, 2 H, H$_β$Pro), 1.88–1.64 (m, 2 H, H$_γ$Pro), 1.04, 1.02, 0.92 (3 × s, 9 H, t-BuSi).

^{13}C NMR (125 MHz, CD₃CN): δ = 173.0, 172.7, 172.6, 171.2 (amides, carboxylic acid), 155.5, 155.2 (carbamate), 145.3, 145.2, 145.1, 142.2, 142.1, 142.0, 138.9, 136.5, 136.4, 134.5, 134.2, 131.0, 128.9, 128.8, 128.7, 128.6, 128.2, 128.1, 126.1, 126.0, 120.9 (Fmoc, TBDPS), 73.0, 72.6 (C$_γ$Hyp), 67.6, 67.5 (Fmoc), 60.2, 59.6, 58.9 (C$_a$Pro, C$_a$Hyp), 55.6, 54.8 (C$_δ$Hyp), 48.2, 48.1 (Fmoc), 48.0, 47.3 (C$_δ$Pro), 41.7, 41.6 (C$_a$Gly), 38.0, 37.6 (C$_β$Hyp), 30.7, 29.7 (C$_β$Pro), 27.2, 27.1 (TBDPS), 24.9, 23.7 (C$_γ$Pro), 19.6, 19.5 (TBDPS). Signals were assigned by 2-D NMR spectroscopy (COSY, HMBC and HMQC).

HRMS-ESI: m/z calcd for C₄₃H₄₇N₃O₇Si+Na: 768.3080; found: 768.3087 [M + Na]⁺.

Collagen Model Peptide 6
The following operations were automated on a Peptide Synthesizer. Rink amide resin (150 mg, 0.36 mmol/g) was Fmoc deprotected by treatment with 40% piperidine in DMF for 3 min followed by treatment with 20% piperidine in DMF for 10 min and washed (5×) with DMF (Protocol A). Building block **4** (4.5 equiv) was coupled on the resin using HCTU (4.5 equiv) and i-Pr₂NEt (13.5 equiv) for 90 min and washed (5×) with DMF (Protocol B). Protocols A and B were repeated seven times, followed by a further Fmoc deprotection (Protocol A) and acetylation with Ac₂O (30 equiv) and i-Pr₂NEt (30 equiv) in CH₂Cl₂ for 60 min.

The resin-bound CMP **6** was washed with DMF and agitated in 1 M TBAF in THF (5 mL) overnight. After washing with THF (5×), a solution of TFA–CH₂Cl₂ (2:1, 4 mL) was added. The mixture was agitated for 60 min and the filtrate was concentrated under reduced pressure. The residue was dissolved in the smallest possible amount of CH₂Cl₂ then Et₂O was added to precipitate the peptide. The precipitate was dried under reduced pressure after centrifugation and decantation to obtain a slightly yellow material (35 mg, quant). The crude material was purified with RP-HPLC [gradient: 91→85.9% of solvent B (0.1% TFA, 1% MeCN in H₂O) in solvent A (MeCN) over 30 min] to obtain CMP **6** (10 mg, 29% yield with respect to the original resin loading) in a purity of ≥99% as a white solid.

RP-HPLC: t_R = 14.8 min (gradient: 91→85.9% of solvent B in solvent A over 30 min at a flow rate of 1 mL/min).

ESI-MS: m/z calcd for C₈₄H₁₂₂N₂₂O₂₈: 1886.9; found: 1910.0 [M + Na]⁺ (100%).

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