Journal of Peptide Science
J. Peptide Sci. **5**: 103–110 (1999)

Heterotrimeric Collagen Peptides Containing Functional Epitopes. Synthesis of Single-Stranded Collagen Type I Peptides Related to the Collagenase Cleavage Site[1]

JOHANNES OTTL, HANS JÜRGEN MUSIOL and LUIS MORODER*

Max-Planck-Institute of Biochemistry, Martinsried, Germany

Received 7 September 1998
Accepted 5 October 1998

Abstract: Synthetic collagen peptides containing larger numbers of Gly-Pro-Hyp repeats are difficult to purify by standard chromatographic procedures. Therefore, efficient strategies are required for the synthesis of higher molecular weight collagen-type peptides. Applying the Fmoc/*t*Bu chemistry, a comparative analysis of the standard stepwise chain elongation procedure on solid support with the procedure based on the use of the synthons Fmoc-Gly-Pro-Hyp(*t*Bu)-OH and Fmoc-Pro-Hyp-Gly-OH was performed. The crude products resulting from the stepwise elongation procedure and from the use of Fmoc-Gly-Pro-Hyp(*t*Bu)-OH clearly revealed large amounts of microheterogeneities that result from incomplete imino acid acylation as well as from diketopiperazine formation with cleavage of Gly-Pro units from the growing peptide chain. Conversely, by the use of the Fmoc-Pro-Hyp-Gly-OH synthon, the quality of the crude products was significantly improved; moreover, protection of the Hyp side chain hydroxyl function is not required using the Fmoc/*t*Bu strategy. With this optimized synthetic procedure, relatively large collagen-type peptides were obtained in satisfactory yields as highly homogeneous compounds. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: collagen peptides; solid-phase synthesis; Fmoc/*t*Bu strategy; tripeptide synthons

INTRODUCTION

The most abundant structural macromolecules present in the extracellular matrix are the collagens, a class of molecules derived from several multigene families. The collagen molecules are composed of three identical or of two or three different α-chains of primarily repeating Gly-Xaa-Yaa triplets that induce each single α-chain to adopt a left-handed poly-Pro-II helix and the three chains to intertwine with a one-residue shift into a right-handed triple-helical coiled coil [1–3].

In collagens like type I and IV, the biological activities, such as cell adhesion mediated by integrins or proteoglycans, cell activation and signaling or matrix metalloproteinase (MMP) binding and catabolism, reside in specific loci of the heterotrimers most probably as structural epitopes [4]. Natural collagens are difficult to work with for studying the effects of the primary, secondary and tertiary structure on the functional properties of such epitopes because of their insolubility and gelating properties. The alternative is to use considerably shorter fragments, but production of such fragments by enzymatic cleavage of natural collagens is difficult and generally their triple-helical structure is thermally rather unstable [5,6]. Corre-

Abbreviations: Hyp, 4-hydroxyproline; DIEA, diisopropylethylamine; DMAP, dimethylaminopyridine; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride; NMP, *N*-methylpyrrolidone; AcOEt, ethyl acetate; HOBt, 1-hydroxybenzotriazole; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; MALDI-TOF, matrix assisted laser desorption time of flight; ESI-MS, electron spray ionization mass spectrometry.

* Correspondence to: Max-Planck-Institut für Biochemie, Am Klopferspitz 18A, D-82152 Martinsried, Germany.
E-mail: moroder@biochem.mpg.de

[1] For preliminary communication see Reference [7].

CCC 1075–2617/99/020103-08$17.50

spondingly, synthetic heterotrimeric fragments folded into sufficiently stable triple-helices could represent ideal model compounds. For the design and production of collagen fragments containing, as a functional epitope, the collagenase binding and cleavage site of collagen type-I, the authors used a simplified mimicry of the cystine knot of collagens for a regioselective assembly of the three chains in the staggered register $\alpha 1/\alpha 2/\alpha 1$, which was proposed for collagen type I as outlined in Figure 1 [7]. Thereby, two main synthetic problems had to be solved: (i) efficient syntheses of α-chain fragments containing increasing numbers of Gly-Pro-Hyp repeats and (ii) the regioselective cross-linking of three α-chain peptides in the correct staggered register.

In the present communication, the authors describe approaches for optimizing the synthesis of single α-chain peptides, since purification of collagen [8] or synthetic collagen peptides [9] by chromatographic procedures is known to be particularly difficult. Deletion by-products differ minimally in their hydrophobic/hydrophilic properties and conformational equilibria are additionally responsible for strong peak broadening. Upon the first successful synthesis of $(Xaa\text{-}Yaa\text{-}Gly)_n$ oligomers on solid support by Sakakibara *et al.* [10] with Xaa-Yaa-Gly as building blocks, this strategy of using tripeptide synthons has been repeatedly applied both with the Boc/Bzl [11–14] and Fmoc/*t*Bu chemistry [15–17]. In the synthesis of the relatively large α-subunits of the heterotrimeric constructs, shown in Figure 1, by the Fmoc/*t*Bu [18] chemistry, serious difficulties were encountered if standard protocols were applied. Because of the resulting low yields of homogeneous products, optimized synthetic procedures had to be elaborated to assure an access to collagen-related peptides of up to 35 amino acid residues in satisfactory yields and at a high degree of homogeneity.

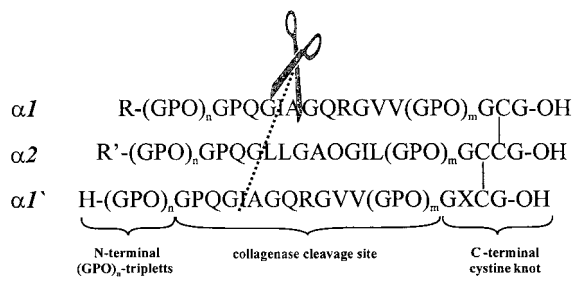


Figure 1 Heterotrimeric collagen peptides containing the collagenase cleavage site 772–784 of collagen type I with a built-in C-terminal cystine-knot; $n = 3$ (heterotrimer A) or 5 (heterotrimers B, C and D); $m = 0$ (heterotrimers A, B and C); R = H (heterotrimers A and B), Ac (heterotrimer C) or Ac-PO (heterotrimer D); R′ = H (heterotrimers A and B); Ac (heterotrimer C) or Ac-O (heterotrimer D); X = L (heterotrimers A, B, and C) or P (heterotrimer D); O = L-4-hydroxyproline.

MATERIALS AND METHODS

Melting points were determined on capillary melting point apparatus (Büchi, Switzerland) and are uncorrected. Optical rotations were measured in a thermostated 1 dm cell on a Perkin-Elmer polarimeter (Model 141). Precoated silica gel 60 TLC plates were from Merck AG (Darmstadt, Germany) and compounds were visualized with the chlorine/tolidine or permanganate reagent. Analytical HPLC was carried out on Nucleosil 300/C8 columns (Macherey and Nagel, Düren, Germany) using a linear gradient of acetonitrile:2% phosphoric acid from 5:95 to 80:20 in 30 min at a flow rate of 1 ml/min and Waters equipment. MALDI-TOF mass spectra were recorded on a Bruker Reflex II instrument and FAB-MS spectra on Finnigan MAT 900. Amino acid analyses of the acid hydrolysates (6 M HCl containing 2.5% thioglycolic acid, 110°C; 72 h) were performed on a Biotronic analyzer (LC 6001).

Resins were purchased from Novabiochem (Läufelfingen, Switzerland) or Rapp Polymere GmbH (Tübingen, Germany). All reagents and solvents used in the synthesis were of the highest quality commercially available. Amino acid derivatives were purchased from Alexis (Grunberg, Germany) or were prepared according to standard protocols. H-Hyp(*t*Bu)-OH was obtained by hydrogenation of Z-Hyp(*t*Bu)-OBzl [19] and it was then converted to Fmoc-Hyp(*t*Bu)-OH by reaction with Fmoc-OSu under standard conditions in 89% overall yield; m.p. 84–87°C; homogeneous on TLC (CH_3CN:H_2O, 4:1, $R_f = 0.3$; $CHCl_3$:MeOH:AcOH, 4:1:0.1, $R_f = 0.1$) and in HPLC ($t_R = 24.20$ min).

Fmoc-Gly-Pro-OH (1)

To a solution of Fmoc-Gly-OSu (20 g; 50.6 mmol) in DMF, H-Pro-OH (6.1 g; 50.6 mmol) was added followed by DIEA (4.2 ml; 25 mmol). After 4 h, the bulk of the solvent was removed and the residue was distributed between 5% $NaHCO_3$ and AcOEt. The aqueous solution was acidified with 5% $KHSO_4$ and extracted with AcOEt. The combined extracts were washed with water and dried over Na_2SO_4. The

solvent was evaporated and the residue recrystallized from petroleum ether; yield: 19.2 g (96%); homogenous on TLC (CH_3CN:H_2O, 8:1, $R_f = 0.5$; $CHCl_3$:MeOH:AcOH, 9:1:0.1, $R_f = 0.4$) and HPLC ($t_R = 20.21$ min).

Fmoc-Gly-Pro-Hyp(*t*Bu)-OH (2)

The dipeptide derivative Fmoc-Gly-Pro-OH (**1**) was converted by standard procedures to the *N*-hydroxysuccinimide ester (yield. 95% upon recrystallization from 2-propanol). To H-Hyp(*t*Bu)-OH (11.4 g; 61 mmol) in DMF, DIEA (4.35 ml; 25.4 mmol) was added followed by Fmoc-Gly-Pro-OSu (25 g; 50.8 mmol). After 3 h the reaction mixture was worked up as described for **1**. The product was recrystallized from ether; yield: 16.7 g (58%); m.p. 139–141°C; homogeneous on TLC (CH_3CN:H_2O, 8:1, $R_f = 0.4$; $CHCl_3$:MeOH:AcOH, 4:1:0.1, $R_f = 0.3$) and HPLC ($t_R = 21.82$ min); FAB-MS: $m/z = 564.3$ [$M + H^+$]; $M_r = 563.24$ calc. for $C_{31}H_{37}N_3O_7$.

The crude product contained 10% diketopiperazine (HPLC, $t_R = 24.63$ min) and 5% Fmoc-Gly-Pro-Gly-Pro-OH (HPLC, $t_R = 20.99$ min) as determined by LC-MS.

Z-Pro-Hyp-OH (3)

H-Hyp-OH (11.5 g; 87.6 mmol) was dissolved in 43 ml (87.6 mmol) of 2 M benzyltrimethylammonium hydroxide in MeOH and the solution was taken to dryness. The residue was redissolved in DMF and reacted for 3 h with Z-Pro-OSu (28.8 g; 83.4 mmol). The bulk of the solvent was removed and the residue distributed between 5% $NaHCO_3$ and ether. The aqueous solution was acidified with 2 M HCl (pH 2.5) and the resulting precipitate was collected by filtration and recrystallized from methyl *tert*-butyl ether; yield: 27.7 g (92%); m.p. 216–217°C (Lit. [20]: 217–218.5°C); homogeneous on TLC (CH_3CN:$CHCl_3$:AcOH, 8:1:1, $R_f = 0.8$; $CHCl_3$:MeOH:AcOH, 4:1:0.1, $R_f = 0.5$) and HPLC ($t_R = 13.52$ min); FAB-MS: $m/z = 363.2$ [$M + H^+$]; $M_r = 362.15$ calc. for $C_{18}H_{22}N_2O_6$.

Z-Pro-Hyp-Gly-OBzl (4)

To an ice-cold solution of Z-Pro-Hyp-OH (**3**) (8.14 g; 50.08 mmol), H-Gly-OBzl × HCl (11.62 g; 57.2 mmol) and HOBt (6.8 g; 50.1 mmol) in DMF, DIEA (20 ml; 55 mmol) was added followed by EDCI (10.5 g; 55 mmol). After 3 h stirring at room temperature, the solvent was removed and the residue was washed in AcOEt with 5% $NaHCO_3$, 5% $KHSO_4$ and water. The organic layer was dried over Na_2SO_4 and concentrated to small volume. Upon addition of ether, the solid was collected by filtration; yield: 25 g (96%); m.p. 55–59°C; homogeneous on TLC (CH_3CN:H_2O, 10:1, $R_f = 0.9$; $CHCl_3$:MeOH:AcOH, 9:1:0.1, $R_f = 0.7$) and HPLC ($t_R = 25.74$ min); FAB-MS: $m/z = 510.5$ [$M + H^+$]; $M_r = 509.56$ calc. for $C_{27}H_{31}N_3O_7$.

H-Pro-Hyp-Gly-OH × Tos-OH (5)

Z-Pro-Hyp-Gly-OBzl (**4**) (25 g; 49 mmol) was hydrogenated over Pd/C in water:MeOH (9:1) containing *p*-toluenesulfonic acid monohydrate (9.32 g; 49 mmol). The catalysator was removed by filtration and the solution was evaporated to dryness. The oily residue was solidified by trituration with ether; yield: 25 g (96%); m.p. 126–129°C; homogeneous on TLC (CH_3CN:H_2O, 4:1, $R_f = 0.2$; $CHCl_3$:MeOH:AcOH:H_2O, 6:4:0.5:1, $R_f = 0.4$) and HPLC ($t_R = 7.64$ min); FAB-MS: $m/z = 286.3$ [$M + H^+$-Tos-OH]; $M_r = 285.3$ calc. for $C_{12}H_{19}N_3O_5$.

Fmoc-Pro-Hyp-Gly-OH (6)

To a solution of H-Pro-Hyp-Gly × Tos-OH (**5**) (20.6 g; 45 mmol) in water:dioxane (2:1), $NaHCO_3$ (9.24 g; 110 mmol) and Fmoc-OSu (16.9 g; 50 mmol) in dioxane were added. After 12 h, the solution was neutralized with 2 M HCl and the bulk of the solvent was evaporated. The residue was distributed between 2% $NaHCO_3$ and AcOEt. The aqueous layer was then acidified to pH 3 with 2 M HCl and the precipitate was filtered off and recrystallized from acetonitrile; yield: 20.1 g (88%); m.p. 177–181°C; homogeneous on TLC ($CHCl_3$:MeOH:AcOH:H_2O, 6:4:0.5:1, $R_f = 0.8$; $CHCl_3$:MeOH:AcOH, 4:1:0.1, $R_f = 0.5$) and HPLC ($t_R = 19.62$ min); FAB-MS: $m/z = 508.2$ [$M + H^+$]); $M_r = 507.20$ calc. for $C_{27}H_{29}N_3O_7$.

Synthesis of Collagen Peptides on Solid Support

The syntheses on solid support were performed manually or on the automatic synthesizer ABI 431A (Applied Biosystems) with the standard FastMOC strategy using Fmoc-Gly-WANG resin (loading: 0.46 mmol/g) for protocol 1 and Fmoc-Gly-TentaGel-S-PHB-resin (loading: 0.20 mmol/g) for the protocols 2 and 3. The HO-TentagelGel-S-PHB-resin (substitution grade: 0.2 mmol/g) was loaded with Fmoc-Gly-OH by the symmetric anhydride/DMAP procedure in DMF. Cleavage of the Fmoc group with 20% piperidine in DMF was used to determine spectroscopi-

Table 1 Standard Protocol 1 of Peptide Synthesis on Fmoc-Gly-Wang Resin[a]

Synthetic step	Reagents and solvents	Reaction time
Fmoc-cleavage	20% (v/v) piperidine/NMP	3+10 min
Coupling	Stepwise amino-acylation with four equivalents Fmoc-amino acid derivatives Gln(Trt), Arg(Pmc), Hyp(*t*Bu), Ala, Gly, Ile, Leu, Pro/four equivalents HBTU/four equivalents HOBt/eight equivalents DIEA in NMP and four equivalents Fmoc-Gly-Pro-Hyp(*t*Bu)-OH/four equivalents HBTU/four equivalents HOBt/eight equivalents DIEA in NMP and four equivalents Fmoc-Cys(StBu)-OPfp, Fmoc-Cys(Acm)-Opfp/four equivalents HOBt in NMP	2×30 min
Acetylation	20% (v/v) Ac_2O/NMP	15 min
Acidolytic cleavage	TFA/H_2O/triethylsilane 95/2/3 (v/v)	2 h

[a] Loading: 0.46 mmol/g.

cally the loading (0.198 mmol/g) and cleavage with 95% TFA to exclude by HPLC the presence of Fmoc-Gly-Gly-resin contamination. The syntheses were carried out at a 0.25 mmol scale. For all couplings, Fmoc cleavage and washing steps freshly distilled NMP was used as solvent. Coupling efficiency was monitored by the Kaiser test [21] and for N-terminal Pro and Hyp by the chloranil test [22]. Final N-terminal acetylation, when required, was performed with 20% Ac_2O in NMP followed by treatment with 20% piperidine in NMP to hydrolyse esterified Hyp hydroxyl groups when unprotected Fmoc-Pro-Hyp-GlyOH was used as synthon (protocol 3). Peptide cleavage from the resins was carried out with TFA:H_2O:triethylsilane (95:2:3) for 2 h at room temperature. The resin was filtered off, washed with TFA and the filtrate was evaporated. The residues were dissolved in small amounts of TFE and the crude products were precipitated with methyl *tert*-butyl ether or methyl *tert*-butyl ether:hexane (2:1). The precipitates were collected by centrifugation, dissolved in 50 mM AcOH and lyophilized. The crude products were purified by preparative HPLC on Nucleosil 250/C18 (Macherey and Nagel, Düren, Germany) with a linear gradient of 0.08% TFA in acetonitrile:0.1%TFA from 15:85 to 60:40 in 70 min. Fractions containing homogeneous material as monitored by HPLC were combined and lyophilized.

H-(Gly-Pro-Hyp)$_5$-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Cys(S*t*Bu)-Gly-Gly-OH (7)

The title compound was synthesized by the three protocols; yields: 3% by protocol 1; 5.3% by protocol 2 and 8.5% by protocol 3; HPLC: $t_R = 16.82$ min; MALDI-TOF-MS: $m/z = 2836.6$ $[M]^+$; $M_r = 2836.4$ calc. for $C_{121}H_{191}N_{36}O_{39}S_2$; amino acid analysis: Glu 2.00 (2), Pro 5.56 (6), Gly 11.61 (12), Ala 1.02 (1), Cys 0.95 (1), Val 1.78 (2), Ile 0.97 (1), Arg 1.01 (1), Hyp 4.88 (5); peptide content: 77%.

Ac-Pro-Hyp-(Gly-Pro-Hyp)$_5$-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Pro-Hyp-Gly-Cys(S*t*Bu)-Gly-Gly-OH (8)

The 36mer was synthesized with the optimized protocol 3; yield: 17%; HPLC: $t_R = 14.40$ min; ESI-MS: $m/z = 3298.0$ $[M]^+$; $M_r = 3298.56$ calc. for $C_{143}H_{221}N_{40}O_{46}S_2$; amino acid analysis: Glu 2.00 (2), Pro 7.47 (8), Gly 11.56 (12), Ala 1.00 (1), Cys 0.99 (1), Val 1.88 (2), Ile 0.98 (1), Arg 1.06 (1), Hyp 6.87 (7); peptide content: 70%.

By applying protocol 3, the 12 α-chains of the heterodimers A, B, C and D illustrated in Figure 1 were obtained in yields of 10–20% as well characterized compounds.

RESULTS AND DISCUSSION

With the choice of a cystine network for cross-bridging the three α-chains in the defined $\alpha1/\alpha2/\alpha1$ order, besides selective protections of the cysteine residues, three different chains, i.e. $\alpha1$, $\alpha2$ and $\alpha1'$, were required (see Figure 1). In the first syntheses of the single chains on solid supports by the Fmoc/*t*Bu chemistry [18], the standard protocol 1 outlined in Table 1 was applied. To avoid undesired side reactions at the level of the cysteine residues directly esterified to the resins [23], these were spaced with one or two glycine residues from the resin linker. All coupling steps were performed with HBTU/HOBt [24] except for the cysteines, which were incorporated via the pentafluorophenyl ester

to prevent extensive racemization [25,26]. Despite the use of 4-fold excesses of acylating amino acid derivatives and even double-coupling steps in the Gly-Pro-Hyp(*t*Bu) portion of the sequences, the resulting crude products exhibited a large microheterogeneity in the HPLC elution profile (see Figure 2A). These contaminants derive mainly from incomplete amino-acylation of the imino acids and from diketopiperazine formation with cleavage of Gly-Pro units in the chain elongation steps as well assessed by mass spectrometric analysis of the crude products. Therefore, extensive purification by HPLC was required with concomitant large losses of material and thus low yields of the single α-chains.

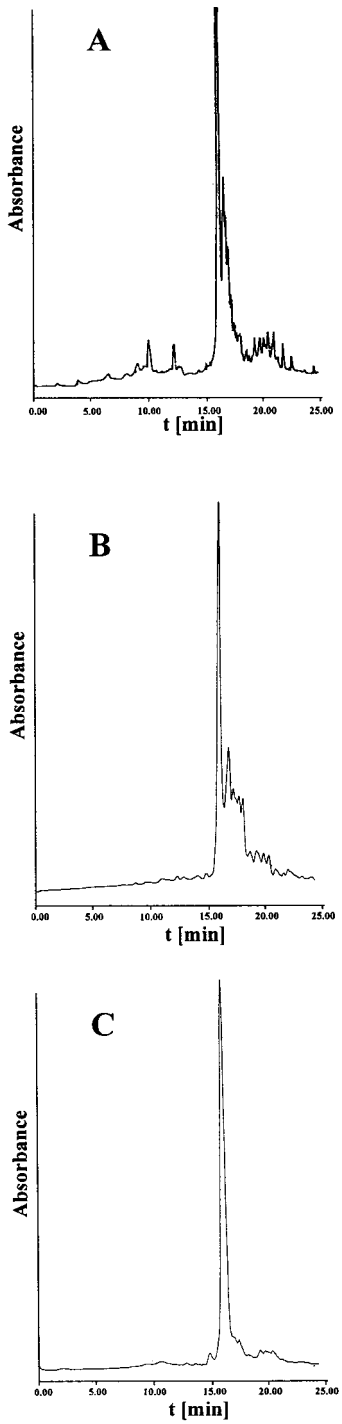
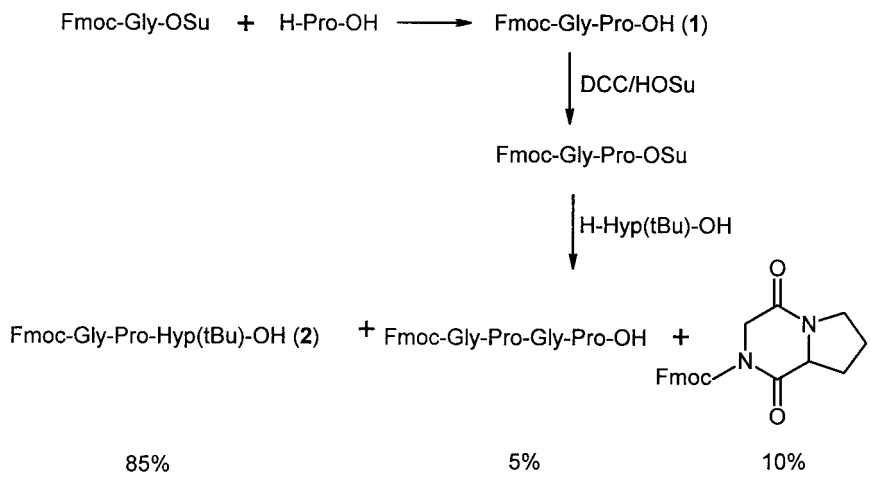


Figure 2 HPLC elution profiles of the crude products related to the collagen peptide 7 obtained by the different synthetic protocols: A, protocol 1; B, protocol 2 and C, protocol 3

To facilitate the final purification steps and thus improve the yields of the collagen peptides, the use of tripeptide synthons related to the consensus collagen triplets Gly-Pro-Hyp was attempted. In analogy to previous syntheses of collagen peptides based on the use of Boc-Gly-Pro-Hyp(Bzl)-OH [11–13] or Fmoc-Gly-Pro-Hyp-OH [15–17] the related side-chain protected Fmoc-tripeptide was synthesized following Scheme 1. Analogous to previous reports on the synthesis of proline-containing peptides [27–30], acylation of H-Hyp(*t*Bu)-OH as Triton B salt in DMF with Fmoc-Gly-Pro-OSu leads to diketopiperazine formation (10%), but also to partial Fmoc-cleavage and concomitant formation of the tetrapeptide Fmoc-Gly-Pro-Gly-Pro-OH as side product (5%). Attempts to possibly reduce this side reaction by intermediate protection with the benzyloxycarbonyl group failed, since significantly enhanced diketopiperazine formation (30–40%) was observed, a fact that can only by attributed to the reduced sterical hindrance of the Z group if compared with the bulky Fmoc moiety. As the tripeptide synthon Fmoc-Gly-Pro-Hyp(*t*Bu)-OH could be obtained in highly homogeneous form by simple crystallization from ether, it was then applied for the synthesis of the collagen peptides following protocol 2 (Table 2). Again, the crude products were contaminated by large amounts of side products (Figure 2B). Mass spectrometric analysis of the crude products revealed mainly mass losses deriving from diketopiperazine formation and thus, from cleavage of the Gly-Pro dipeptide units at the N-terminus of the growing peptide chains. This side reaction, which was not mentioned in previous analogous syntheses, has to occur both in the Fmoc-cleavage and subsequent acylation step. It is, however, largely prevented in the syntheses of collagen peptides with the Boc-Gly-Pro-Hyp(Bzl)-OH synthon where N^{α}-deprotection is performed with trifluoroacetic acid.

Finally, the strategy of Sakakibara *et al.* [10,31] was applied, which is based on the use of Pro-X-Gly derivatives (X = Pro, Hyp) as synthons for the syn-



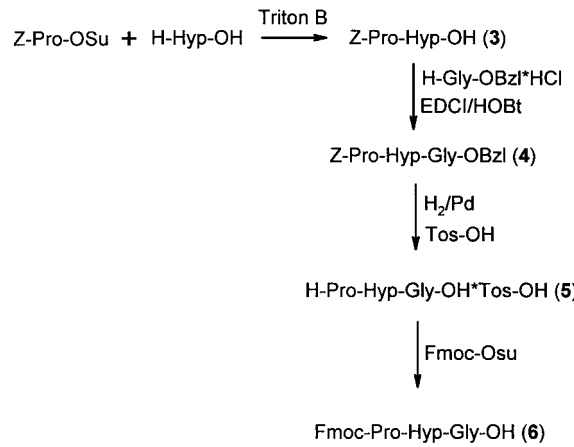
Scheme 1 Synthesis of the tripeptide synthon Fmoc-Gly-Pro-Hyp(*t*Bu)-OH.

Table 2 Standard Protocol 2 of Peptide Synthesis on Fmoc-Gly-Tentagel-S-PHB Resin[a]

Synthetic step	Reagents and solvents	Reaction time
Fmoc-cleavage	20% (v/v) piperidine/NMP	3+10 min
Coupling	Stepwise amino-acylation with four equivalents Fmoc-amino acid derivatives Gln(Trt), Arg(Pmc), Hyp(*t*Bu), Ala, Gly, Ile, Leu, Pro/four equivalents HBTU/four equivalents HOBt/eight equivalents DIEA in NMP and four equivalents Fmoc-Gly-Pro-Hyp(*t*Bu)-OH/four equivalents HBTU/four equivalents HOBt/eight equivalents DIEA in NMP and four equivalents Fmoc-Cys(StBu)-OPfp, Fmoc-Cys(Acm)-Opfp/four equivalents HOBt in NMP	2×30 min
Acetylation	20% (v/v) Ac_2O/NMP	15 min
Acidolytic cleavage	TFA/H_2O/triethylsilane 95/2/3 (v/v)	2 h

[a] Loading: 0.20 mmol/g.

thesis of collagen-type peptides. For this purpose, following Scheme 2, the dipeptide derivative Z-Pro-Hyp-OH was coupled with H-Gly-OBzl by the EDCI/HOBt method and upon hydrogenation in presence of *p*-toluenesulfonic acid to prevent diketopiperazine formation, the resulting tripeptide was acylated with Fmoc-OSu to produce in good overall yield Fmoc-Pro-Hyp-Gly-OH. Due to the low reactivity of the Hyp side-chain hydroxyl function, its acylation was expected to occur only at moderate rates even using strongly acylating reagents such as HBTU/HOBt. Moreover, repeated treatment with piperidine for Fmoc-cleavage should hydrolyse such esters if formed in the chain elongation steps. Conversely, for N-terminal extension with single Hyp residues Fmoc-Hyp(*t*Bu)-OH was used in order to prevent O→N acyl shift in the Fmoc deprotection



Scheme 2 Synthesis of the tripeptide synthon Fmoc-Pro-Hyp-Gly-OH.

Table 3 Standard Protocol 3 of Peptide Synthesis on Fmoc-Gly-Tentagel-S-PHB Resin[a]

Synthetic step	Reagents and solvents	Reaction time
Fmoc-cleavage	20% (v/v) piperidine/NMP	3+12 min
Coupling	Stepwise amino-acylation with four equivalents Fmoc-amino acid derivatives Gln(Trt), Arg(Pbf), Hyp(*t*Bu), Ala, Gly, Ile, Leu, Pro/four equivalents HBTU/four equivalents HOBt/eight equivalents DIEA in NMP and 3.15 equivalents Fmoc-Pro-Hyp-Gly-OH/three equivalents HBTU/three equivalents HOBt/eight equivalents DIEA in NMP and four equivalents Fmoc-Cys(StBu)-OPfp, Fmoc Cys(Acm)-Opfp/four equivalents HOBt in NMP	2×30 min
Acetylation	20% (v/v) Ac_2O/NMP	15 min
Acidolytic cleavage	TFA/H_2O/triethylsilane 95/2/3 (v/v)	2 h

[a] Loading: 0.20 mmol/g.

steps. Performing the syntheses of the α-chain peptides according to the protocol 3 outlined in Table 3, the quality of the crude products was significantly improved as well as assessed by the comparative HPLC analysis reported in Figure 2C.

CONCLUSIONS

Collagen peptides of over 35 amino acid residues were obtained by this optimized strategy in satisfactory yields and upon HPLC purification at a high degree of homogeneity as required for their successful assembly by regioselective cysteine-bridging procedures into the four heterotrimers shown in Figure 1.

Acknowledgements

This study was supported by the Sonderforschungsbereich 469 of the Ludwig-Maximilians-Universität (grant A-2 Moroder/Machleidt). The excellent technical assistance of Mrs E. Weyher is gratefully acknowledged. The authors thank Dr Eckerskorn for the mass spectra.

REFERENCES

1. A. Rich and F.C.H. Crick (1961). The molecular structure of collagen. *J. Mol. Biol. 3*, 483–508.
2. G.N. Ramachandran in: *Aspects of Protein Structure*, G.N. Ramachandran, Ed., Academic Press, New York, 1963.
3. R.D.B. Fraser, T.P. Mac Ray and Suzuki, E. (1979). Chain conformation in the collagen molecule. *J. Mol. Biol. 129*, 463–481.
4. Fields, G.B. (1995). The collagen triple helix: correlation of conformation with biological activities. *Connect. Tissue Res. 3*, 235–243.
5. J.H. Highberger, C. Corbett and J. Gross (1979). Isolation and characterization of a peptide containing the site of cleavage of the chick skin collagen α1[I] chain by animal collagenases. *Biochem. Biophys. Res. Commun. 89*, 202–208.
6. S. Netzel-Arnett, A. Salari, U.B. Goli and H. Van Wart (1994). Evidence for a triple helix recognition site in the hemopexin-like domains of human fibroblast and neutrophil interstitial collagenases. *Ann. NY Acad. Sci. 732*, 22–30.
7. J. Ottl, R. Battistutta, M. Pieper, H. Tschesche, W. Bode, K. Kühn and L. Moroder (1996). Design and synthesis of heterotrimeric collagen peptides with a built-in cystine-knot. *FEBS Lett. 398*, 31–36.
8. H.P. Bachinger and N.P. Morris (1996). Analysis of the thermal stability of type II collagen in various solvents used for reversed phase high performance chromatography. *Matrix 10*, 331–338.
9. C.G. Fields, B. Grab, J.L. Lauer and G.B. Fields (1995). Purification and analysis of synthetic, triple-helical 'minicollagens' by reversed phase high performance liquid chromatography. *Anal. Biochem. 231*, 57–64.
10. S. Sakakibara, K. Inouye, K. Shudo, Y. Kishida, Y. Kobayashi and D.J. Prockop (1973). Synthesis of (Pro-Hyp-Gly)$_n$ of defined molecular weights. Evidence for the stabilization of collagen triple helix by hydroxyproline. *Biochim. Biophys. Acta 303*, 198–202.
11. M. Goodman, Y. Feng, G. Melacini and J.P. Taulane (1996). A template-induced incipient collagen-like triple-helical structure. *J. Am. Chem. Soc. 118*, 5156–5157.
12. Y. Feng, G. Melacini, J.P. Taulane and M. Goodman (1996). Acetyl-terminated and template-assembled collagen-based polypeptides composed of Gly-Pro-Hyp sequences. Synthesis and conformational analysis by circular dichroism, ultraviolet absorbance and optical rotation. *J. Am. Chem. Soc. 118*, 10351–10358.

13. M. Goodman, G. Melacini and Y. Feng (1996). Collagen-like triple-helices incorporating peptoid structures. *J. Am. Chem. Soc. 118*, 10928–10929.
14. H. Hoyo, Y. Akamatsu, K. Yamauchi and M. Kinoshita (1997). Synthesis and structural characterization of triple-helical peptides which mimic the ligand binding site of the human macrophage scavenger receptor. *Tetrahedron 53*, 14263–14274.
15. C.G. Fields, C.M. Lovdahl, A.K. Miles, V.L. Matthias Hagen and G.B. Fields (1993). Solid-phase synthesis and stability of triple-helical peptides incorporating native collagen sequences. *Biopolymers 33*, 1695–1707.
16. C.G. Fields, D.J. Mickelson, S.L. Drake, J.B. McCarthy and G.B. Fields (1993). Melanoma, cell adhesion and spreading activities of a synthetic 124-residues triple-helical 'minicollagen'. *J. Biol. Chem. 268*, 14153–14160.
17. B. Grab, A.J. Miles, L.T. Furcht and G.B. Fields (1995). Promotion of fibroblast adhesion by triple-helical peptide models of type I collagen-derived sequences. *J. Biol. Chem. 271*, 12234–12240.
18. E. Atherton, C.J. Logan and R.C. Sheppard (1981). Peptide synthesis. Part 2. Procedures for solid-phase synthesis using N^{α}-fluorenylmethoxycarbonylamino-acids on polyamide supports. Synthesis of substance P and of acyl carrier 65–74 decapeptide. *J Chem. Soc. Perkin Trans. 1*, 538–546.
19. C.-D. Chang, M. Waki, M., Ahmad, J. Meienhofer, E.O. Lundell, E.O. and J. Haug (1980). Preparation and properties of N^{α}-9-fluorenylmethyloxycarbonylamino acids bearing *tert*-butyl side-chain protection. *Int. J. Pept. Protein Res. 15*, 59–66.
20. E. Adams (1977). Derivatives and peptides of 4-hydroxyproline. *Int. J. Pept. Protein Res. 9*, 293–309.
21. E. Kaiser, R.C. Colescott, C.D. Bossinger and P.I. Cook (1970). Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem. 34*, 595–598.
22. T. Christensen in: *Peptides, Structure and Biological Functions*, E. Gross and J. Meienhofer, Eds., p. 385–388, Pierce Chemical Co., Rockford, IL, 1979.
23. R. Eritja, J.P. Ziehler-Martin, P.A. Walker, T.D. Lee, K. Legesse, F. Albericio, F. Kalan and B.E. Kaplan (1987). On the use of *S*-*t*-butylsulfenyl groups for protection of cysteine in solid-phase peptide synthesis using Fmoc-amino acids. *Tetrahedron 43*, 2675–2680.
24. R. Knorr, A. Trzeciak, W. Bannwarth and D. Gillessen (1989). New coupling reagents in peptide chemistry. *Tetrahedron Lett. 30*, 1927–1930.
25. H.-J. Musiol, F. Siedler, D. Quarzago and L. Moroder (1994). Redox-active bis-cysteinyl peptides. I. Synthesis of cyclic cystinyl peptides by conventional methods in solution and on solid supports. *Biopolymers 34*, 1553–1562.
26. Y. Han, F. Albericio and G. Barany (1997). Occurrence and minimization of cysteine racemization during stepwise solid-phase peptide synthesis. *J. Org. Chem. 62*, 4307–4312.
27. J.S. Fruton (1949). Peptides of proline and hydroxyproline. *Adv. Protein Chem. 5*, 67–72.
28. S. Sakakibara and Y. Nagai (1960). Synthesis of peptides containing proline. I. Synthesis of glycyl-L-prolyl-L-leucyl-glycyl-L-proline and its related compounds. *Bull. Chem. Soc. Jpn. 33*, 1537–1542.
29. E. Wünsch in: *Peptides 1962*, G.T. Young, Ed., p. 89–91, Pergamon Press, Oxford, 1963.
30. M. Goodman and L. Levine (1964). Peptide synthesis via active esters. IV. Racemization and ring-opening reactions of optically active oxazolones. *J. Am. Chem. Soc. 86*, 2918–2922.
31. S. Sakakibara, Y. Kishida, Y. Kikuchi, R. Sakai and K. Kakiuchi (1968). Synthesis of poly-(L-prolyl-L-propyl-glycyl) of defined molecular weights. *Bull. Chem. Soc. Jpn. 41*, 1273.

