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Short communication

Type III collagen mimetic peptides designed with anti- or pro-aggregant activities on human platelets

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Abstract

We report the synthesis of collagen related peptides containing the peptide sequence Lys-Hyp-Gly-Glu-Hyp-Gly-Pro-Lys. The α-triple helix peptides behave as type III collagen analogues supporting platelet aggregation, while the homotrimer which does not exhibit a triple-helical conformation inhibits type III collagen-induced human platelet aggregation. The incorporation of the octapeptide sequence in type III collagen mimetic peptides may lead to the loss of the anti-thrombotic activity for a pro-thrombotic one.

Keywords: Type III collagen analogues; Peptide sequence Lys-Hyp-Gly-Glu-Hyp-Gly-Pro-Lys; TIIICBP; α-Triple helix peptides; Anti-aggregant and pro-aggregant activities

1. Introduction

All multicellular organisms contain collagen, a family of fibrous proteins. Indeed, collagen is the most abundant structural protein in mammals. Fibrillar collagens are composed of three peptide strands (α-chains) of primarily repeating Gly-Xaa-Yaa triplets, which induce each α-chain to adopt a left-handed polyPro II helix. Three left-handed chains then intertwine to form a right-handed triple-helical coiled coil [1]. Twenty-seven distinct types of human collagens have been identified so far. The most abundant of these are found in characteristic collagen fibrils (major types I, II, III and minor types V and XI), which form the structural architecture for skin, cartilage and various tissues [2]. Vascular type I and III collagens exposed to flowing blood upon vessel wall injury, are among the most thrombogenic components of the subendothelial layer [3].

The golden antiplatelet treatment for inhibiting thrombosis during acute coronary intervention is the antagonism of fibrinogen binding to the platelet receptor αIIbβ3. This treatment used as an adjunctive therapy is, however, associated with hemorrhagic risks. In contrast, there is growing evidence that inhibiting platelet adhesion to collagen represents a promising approach to controlling atherothrombotic disorders with only minor bleeding risks [4]. Platelet interactions with collagens involve platelet receptors that bind directly or indirectly to collagen fibers [5]. Among the later receptors, integrin α2β1 and glycoprotein (GP) VI are predominantly responsible for platelet adhesion and activation, respectively. GP VI was shown to recognize the triple-helical motif formed by the repetition of Gly-Pro-Hyp sequences whereas α2β1 preferentially interacts with the Gly-Phe-Hyp-Gly-Glu-Arg and to a lesser extent with the Gly-Leu-Hyp-Gly-Glu-Arg and Gly-Ala-Ser-Gly-Glu-Arg sequences within type I collagen [6]. A new receptor

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for type III collagen named TIIICBP for Type III Collagen Binding Protein has been reported by us [7]. This receptor specifically interacts with the octapeptide Lys-Hyp-Gly-Glu-Hyp-Gly-Pro-Lys (peptide **1**). This sequence **1** is located in the CB4 fragment of the type III collagen α1-chain. The octapeptide specifically inhibits platelet interaction with type III collagen in both static and flow conditions [7,8]. In vivo, the octapeptide **1** prevents photochemically-induced thrombosis in arterioles of mice without affecting bleeding time and may thus lead to novel pharmacological targets in thrombosis [9].

We report here the synthesis, conformational and functional properties of type III collagen related peptides containing the peptide sequence **1**. The rationale for this work was, first, to produce a peptide with enhanced antagonism properties compared to peptide **1**; second, to evaluate the platelet-aggregatory potential of the sequence **1** when incorporated into a α-triple helix construct mimicking the tertiary and quaternary structures of collagen (Graphic 1). For the first purpose, we have synthesized a covalently-linked homotrimer of sequence **1** in order to stabilize the propensity of the latter to form a β-turn secondary structure (peptide **2**) [10]. To test the second issue, the sequence **1** was flanked with Gly-Pro-Pro sequences at both sides to generate covalently-linked (peptide **3**) or spontaneously-formed (peptide **4**) α-triple helix structures.

2. Chemistry

Peptides **2** and **3** were synthesized as homotrimers covalently bonded at the C-terminus, as shown in Scheme 1, and as described by Fields [1a,11]. Employing standard Fmoc chemistry methodology, the corresponding peptides were built from the trimeric frame by first coupling Fmoc-Lys(Dde)-OH followed by Fmoc-Lys(Fmoc)-OH to the Sasrin resin initially coupled to Fmoc-6-aminohexanoic acid-OH (Fmoc-Ahx). The Dde protecting group was manually removed. The remainder of the synthesis was carried out automatically as for peptide **4** using an Applied Biosystem 433A. In the case of peptides **3** and **4**, further Gly-Pro-Pro triplets were added at each end to allow the adoption of a stable triple helix conformation. Gly-Pro-Pro triplets were chosen instead of Gly-Pro-Hyp triplets to avoid any recognition by GP VI [12] and to be sure that these peptides **3** and **4** will specifically interact with TIIICBP. A Gly-Pro-Cys sequence was included at each end to allow cross-linking in order to produce a polymer, since collagen quaternary structure is also essential for the expression of its aggregatory activity (fibrillar network).

2.1. Structural studies

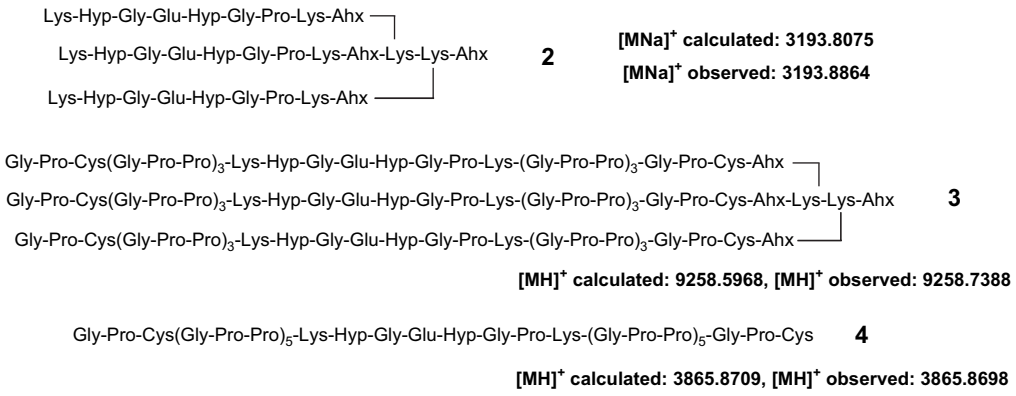
The structuring in triple helix was determined for each peptide by thermal denaturation monitored by optical activity and circular dichroism (CD) measurements (Fig. 1). These spectroscopic studies were carried out in H_2O (polarimetry, 2 mg/ml) or in PBS (CD, 1 mg/ml) and were equilibrated at least 48 h before measurements [13]. The CD data shown in Fig. 1A are indicative of a triple-helical conformation for peptides **3** and **4**, but not for the single-chain molecule **1** and the homotrimer **2**. Interestingly, the homotrimer **2** exhibits a CD spectrum indicative of β-sheet and β-turn secondary structure formations.

As shown in Fig. 1, type III collagen mimetic peptides **3** and **4** prepared from the octapeptidic sequence form thermally stable triple helix. As it has been reported elsewhere the clustering of Gly-Pro-Pro triplets magnifies stabilizing effects, the melting temperature of **3** was determined to be 37 °C, which is 2 °C lower than the melting temperature of **4** (Fig. 1B) [14]. The triple helix stability for **3** and **4** was confirmed by polarimetry studies in comparison with collagen (Table 1) [15]. The peptide **4** seems to be very stable between 20 and 37 °C.

3. Pharmacology

Platelet aggregation assay. Human blood was taken from the antecubital vein of healthy volunteers not taking any medication, and directly added into 3.8% sodium citrate (1:9 v/v blood). Blood was centrifuged for 15 min at 150*g* to obtain Platelet Rich Plasma (PRP). PRP was harvested and centrifugated for 15 min at 1.200*g*. Pelleted platelets were then resuspended in pH 6.5 Patcheke's buffer (36 mM citric acid, 5 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 103 mM NaCl) containing 0.1 μM PGE_1, washed once and resuspended in pH 7.5 Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1.2 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 5.5 mM glucose) and adjusted at 2×108 platelets/ml. Platelet aggregation was monitored by measuring light transmission through the stirred suspension of washed platelets using a Beckman Chrono-Log aggregometer. In the present study, washed platelet conditions were consciously chosen to ascertain that the inhibiting effect of the peptides was on collagen III-induced platelet aggregation solely and not due to a side effect of peptides on other activating, or amplifying, pathways dependent on plasma components such as fibrinogen, von Willebrand factor or thrombin activation. Peptides **3** and **4** were resuspended in phosphate buffer (Na_2HPO_4 100 mM) for three weeks at 4 °C to allow spontaneous triple-helical structure formation.

The peptides **1**–**4** are tested (in triplicates at least), then, in vitro on the human platelets, platelet aggregation being induced by 30 μg/ml of bovine type III collagen. As also described for the peptide **1**, the homotrimer **2** inhibits type III collagen-induced human platelet aggregation with an increased activity compared to peptide **1** [9]. When comparing peptide **2** to peptide **1** on the basis of equal Lys-Hyp-Gly-Glu-Hyp-Gly-Pro-Lys stoichiometry (1 mM for **1** versus 0.33 mM for **2**), the peptide **2** appears to be more potent to inhibit platelet aggregation (Fig. 2). Fig. 2 is a representative figure from experiments done in triplicate, all showing similar and significant differential inhibitory responses between peptide **1** and peptide **2**. The collagen-like triple helix peptides **3** and **4** were stored during three weeks at a temperature of 4 °C to allow the triple helix formation. The compounds **3** and **4** support, in a moderate but significant way, platelet aggregation (20%) (Fig. 3).



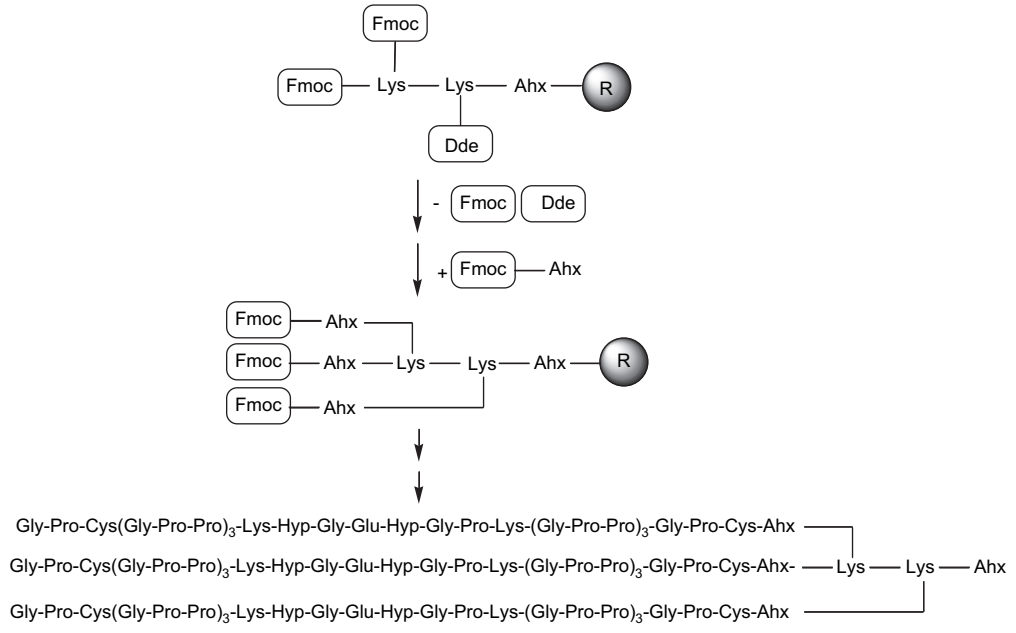
Graphic 1. Structure of peptides **2**, **3**, **4** which incorporate the peptide sequence Lys-Hyp-Gly-Glu-Hyp-Gly-Pro-Lys (peptide **1**).

4. Results and discussion

Type III collagen is a major component of the vessel walls and the most thrombogenic component of the subendothelial layer, together with type I collagen [3]. Type I collagen is classically built of two α1(I) and one α2(I) chains, however in the dermis and in teeth it consists of three α1(I) chains. Type III collagen occurs as a homotrimer of three α1(III) chains. Both collagen types are capable of forming mixed fibers. Moreover, both of them are found in human atherosclerotic plaques [16]. Type III collagen is broadly used as a pro-aggregatory and pro-adhesive agent in many in vitro platelet function assays. The difficulty to obtain pure fibrillar type III collagen makes it less used than type I collagen isolated from horse tendon. However treatment of the type III collagen fragments obtained upon cyanogen bromide digestion allowed the identification of several active sites supporting both platelet adhesion and aggregation. It was found that the collagen's reactive site responsible for the platelet aggregation is localized in α1(III)CB4 fragment and its activity is comparable to the α1(I)CB3 of type I collagen [17]. In addition, there was another sequence identified within the α1(III)CB4 fragment, corresponding to the GP Ia/IIa binding site Gly-Gly-Pro-Hyp-Gly-Pro-Arg (522–528 amino acids).

It was found that a monoclonal antibody against human type III collagen, which is able to inhibit platelet aggregation induced by this collagen, recognizes the epitope of 520–528 amino acids that includes Gly-Gly-Pro-Hyp-Gly-Pro-Arg sequence [18]. Finally, using a range of overlapping synthetic peptides, the vWF binding site was identified within the α1(III)CB4 subunit (positions 541–558) [19].

The goal of our study is to better understand the aggregation role of the sequence **1** (CB4 fragment of the type III collagen α1-chain) in regard with its conformational properties. These experiments demonstrate that the incorporation of peptide **1** into the homotrimer **2** leads to a more stable β-sheet and β-turn secondary structured compound. As a result we show an increased capacity for peptide **1** incorporated in homotrimer **2** to inhibit type III collagen-induced platelet aggregation. In contrast, the sequence **1** introduced in ''collagen-like'' α-triple



Scheme 1. General scheme for the synthesis of branched triple-helical peptide **3**. Ahx stands for 6-aminohexanoic acid.

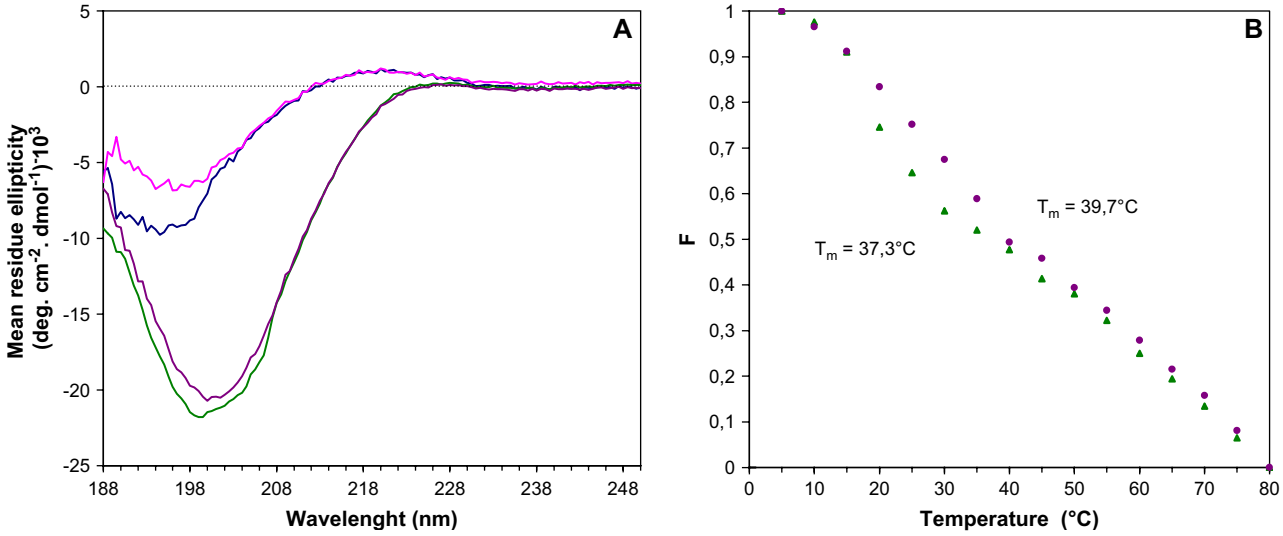


Fig. 1. (A) CD spectra in PBS buffer (10 mM sodium phosphate, 0.15 M NaCl, pH = 7.1) of **1** (magenta, 1 mg/ml), **2** (blue, 1 mg/ml), **3** (green, 1 mg/ml) and **4** (purple, 1 mg/ml) at 5 °C; (B) CD thermal denaturation curves in PBS buffer of **3** (▲, 1 mg/ml) and **4** (●, 1 mg/ml).

helix peptides **3** and **4** is able to trigger platelet aggregation. From the CD spectra and the optical activity studies we show that these type III collagen mimetic peptide derivatives prepared as homotrimer covalently bonded at the C-terminus form a more thermally stable triple helix than the corresponding monomer **1**. These results demonstrate that in vitro, the incorporation of the octapeptide sequence in type III collagen mimetic peptides may lead to the loss of the anti-thrombotic activity for a pro-thrombotic one. These results are in agreement with the new promising alternative which consists in blocking the early phases of the formation of the thrombus, namely the platelet adhesion phase with only minor bleeding risks as a consequence.

5. Experimental section

5.1. Chemistry

High-resolution electrospray mass spectra in the positive ion mode were obtained on a Q-TOF Ultima Global hybrid quadrupole/time-of-flight instrument (Waters-Micromass, Manchester, U.K.), equipped with a pneumatically assisted electrospray (Z-spray) ion source and an additional sprayer (Lock Spray) for the reference compound. The source and desolvation temperatures were kept at 80 and 150 °C, respectively. Nitrogen was used as the drying and nebulizing gas at flow rates of 350 and 50 l/h, respectively. The capillary voltage was 3 kV, the cone voltage 100 V and the RF lens1 energy was optimised for each sample (30–150 V). Lock mass correction, using appropriate cluster ions of an orthophosphoric acid solution (0.1% in 50/50 acetonitrile/water) or of a sodium iodide solution (2 μg/μl in 50/50 propan-2-ol/water + 0.05 μg/μl caesium iodide), was applied for accurate mass measurements. The mass range was typically 50–2050 Da and spectra were recorded at 2 s/scan in the profile mode at a resolution of

Table 1
Triple-helical percentage of collagen mimetic peptide derivatives determined by optical activity[a]

Compound	$T = 20$ °C	$T = 37$ °C
3	43.3%	40.4%
4	63.3%	46.8%
Collagen	100%	88%

[a] Optical rotation was measured on a Jasco polarimeter ($\lambda = 436$ nm) in water, 2 mg/ml.

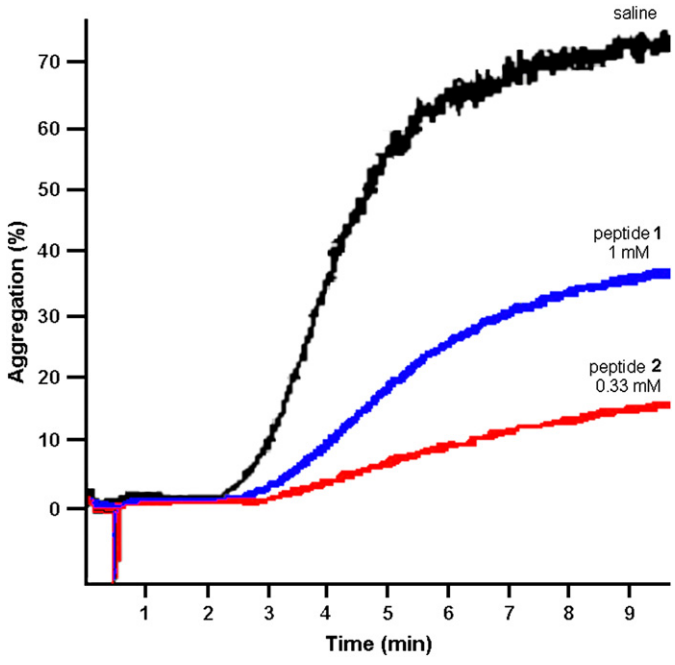


Fig. 2. Inhibitory effects of the peptide **1** (1 mM, blue) and peptide **2** (0.33 mM, red) as compared to saline alone (saline, black) on human platelet aggregation studied for 5 min at 37 °C and induced by type III collagen.

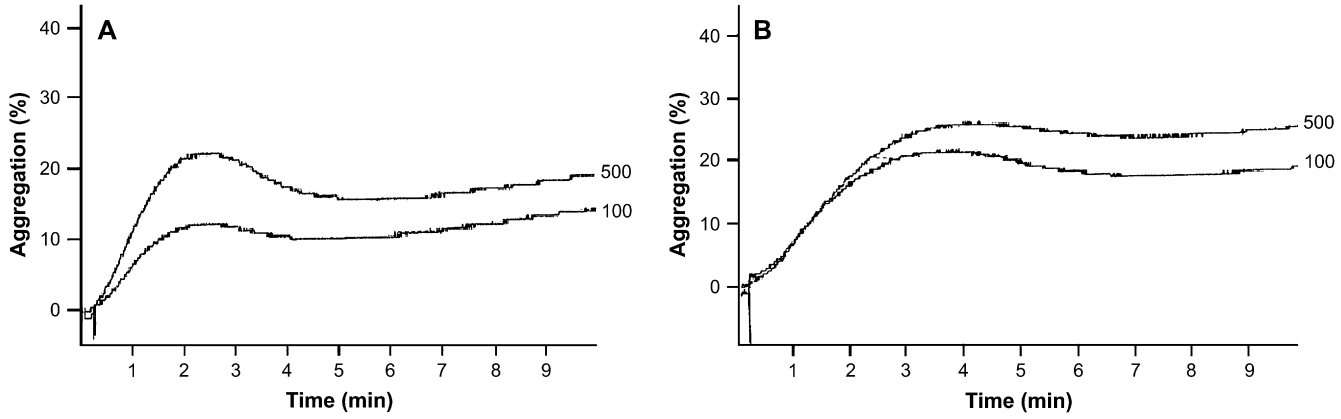


Fig. 3. Biological effects of the soluble peptides **3** and **4** used at 100 and 500 ng/ml on human platelet aggregation studied for 5 min at 37 °C. (A) Pro-aggregatory effects of peptide **3**. (B) Pro-aggregatory effects of peptide **4**.

10 000 (FWMH). Data acquisition and processing were performed with MassLynx 4.0 software. Melting points were determined on a Kofler plate and are given uncorrected. Infrared spectra (IR) were recorded on a NICOLET-210 spectrometer using KBr pellets or a Jasco FT/IR 4200 spectrometer. Nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded on a BRUKER AVANCE 500 spectrometer (500 MHz) and tetramethylsilane (TMS) was used as an internal standard. ^{1}H NMR analyses were obtained at 500 MHz (s: singlet, d: doublet, t: triplet, dd: double doublet, m: multiplet), whereas ^{13}C NMR analyses were obtained at 125 MHz. The chemical shifts (δ) are given in parts per million relative to TMS ($\delta = 0.00$).

5.2. General procedure for preparation of *1–4*

Fmoc(9-fluorophenylmethoxy)-amino acids and Fmoc-amino acids-Sasrin were purchased from Bachem (Germany). The other chemical compounds were purchased from Sigma–Aldrich and used without further purification. The peptides were synthesized on an Applied Biosystems Model 433A Peptide Synthesizer, using standard automated continuous-flow solid-phase peptide synthesis methods. Both manual and automated systems (Applied Biosystems 433A Peptide Synthesizer) were used to prepare the peptides **2** and **3**. Sasrin resin was selected for the synthesis of the protected peptides because protected peptides can be cleaved from the resin with diluted acid solution. Ten-fold molar excess of the above amino acids was used in a typical coupling reaction. Fmoc-deprotection was accomplished by treatment with 20% (v/v) piperidine in *N*-methyl-2-pyrrolidone (NMP). The coupling reaction was achieved by treatment with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*, *N*-diisopropylethylamine (DIEA) in NMP using a standard Fast-Moc protocol.

The primary amine protecting group *N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl] (Dde) was removed with 2% hydrazine (v/v) in DMF at room temperature. The peptides were cleaved and side-chain deprotected by treatment of the peptide resin with water/thioanisole/triisopropylsilane/dichloromethane/trifluoroacetic acid (1:1:1:10:20 v/v) or with dichloromethane/trifluoroacetic acid (1:9 v/v) for 3–4 h at room temperature. The peptides were purified on an RP-HPLC C18 column (Prosphere® C18, 100 Å, 15 μm, 25 × 100 mm) using a mixture of aqueous 0.1% (v/v) TFA (A) and 0.1% (v/v) TFA in acetonitrile/water mixture (80/20, v/v) (B) as the mobile phase (flow rate of 3 ml/min) and employing UV detection at 220 nm. The purity of all peptides was found to be >95%. Electrospray mass spectrometric sequence analysis has been used to confirm the correct sequences (MS–MS, *m/z*).

5.2.1. Lys-Hyp-Gly-Glu-Hyp-Gly-Pro-Lys (*1*)

The peptide resin H_2N-Lys(Boc)-Hyp(*t*-Bu)-Gly-Glu (*t*-Bu)-Hyp(*t*-Bu)-Gly-Pro-Lys(Boc)-Sasrin®, obtained from commercially available Fmoc-Lys-Sasrin (174.26 mg, 0.104 mmol), was cleaved from the resin and side-chain deprotected by treatment with a mixture of dichloromethane/trifluoroacetic acid (1:9 v/v) for 3 h at room temperature. The solid support was removed by filtration, the filtrate concentrated under reduced pressure, and the peptide **1** precipitated from diethyl ether. The precipitate was washed several times with diethyl ether and dried under reduced pressure. Its composition was verified by electrospray mass spectrometric sequence analysis. Peptide **1** was obtained as a white powder (86.68 mg, 81.2%). MP: 181 °C. IR (cm^{-1}): 3318 (NH, NH_2, OH), 2940 (CH), 1710 (CO), 1659 (CO). ^{1}H NMR (500 MHz, CD_3OD), δ (ppm): 1.55 (m, 2H, $CH_2\gamma$, Pro), 1.57 (m, 2H, $CH_2\beta$, Lys), 1.75 (m, 2H, $CH_2\beta$, Glu), 1.75 (m, 2H, $CH_2\gamma$, Glu), 1.76 (m, 2H, $CH_2\gamma$, Lys), 1.99 (m, 2H, $CH_2\beta$, Lys), 2.00 (m, 2H, $CH_2\delta$, Lys), 2.01 (m, 2H, $CH_2\gamma$, Lys), 2.08 (m, 2H, $CH_2\beta$, Hyp), 2.15 (m, 1H, $CH_2\gamma$, Glu), 2.25 (m, 2H, $CH_2\delta$, Lys), 2.28 (m, 2H, $CH_2\beta$, Hyp), 2.42 (m, 2H, $CH_2\beta$, Pro), 2.97 (m, 2H, $CH_2\varepsilon$, Lys), 3.61 (m, 2H, $CH_2\delta$, Pro), 3.73 (m, 2H, $CH_2\varepsilon$, Lys), 3.78 (m, 2H, $CH_2\delta$, Hyp), 3.83 (m, 2H, $CH_2\delta$, Hyp), 4.11 (m, 4H, CH_2, Gly), 4.34 (t, $J = 5.19$ Hz, 1H, CHα, Lys), 4.45 (m, 2H, CHα, Hyp), 4.48 (m, 1H, CHα, Lys), 4.55 (m, 2H, CHγ, Hyp), 4.60 (m, 1H, CHα, Pro), 4.78 (m, 1H, CHα, Glu), 7.88 (d, $J = 8.06$ Hz, 1H, NH), 8.33 (m, 1H, NH), 8.43 (d, $J = 12.38$ Hz, 1H, NH),

8.91 (t, $J = 5.69$ Hz, 2H, NH_2). ^{13}C NMR (125 MHz, CD_3OD), δ (ppm): 20.6 ($CH_2\gamma$, Pro), 22.1 ($CH_2\beta$, Lys), 24.4 ($CH_2\beta$, Lys), 26.1 ($CH_2\beta$, Glu), 26.7 ($CH_2\gamma$, Glu), 29.2 ($CH_2\delta$, Lys), 29.3 ($CH_2\delta$, Lys), 29.5 ($CH_2\beta$, Pro), 30.3 ($CH_2\gamma$, Lys), 30.4 ($CH_2\gamma$, Lys), 37.3 ($CH_2\beta$, Hyp), 37.6 ($CH_2\beta$, Hyp), 38.8 ($CH_2\varepsilon$, Lys), 39.3 ($CH_2\varepsilon$, Lys), 41.2 (CH_2, Gly), 42.3 (CH_2, Gly), 46.6 ($CH_2\delta$, Pro), 50.4 (CHα, Glu), 51.4 (CHα, Lys), 51.5 (CHα, Lys), 55.3 ($CH_2\delta$, Hyp), 55.6 ($CH_2\delta$, Hyp), 59.2 (CHα, Pro), 59.8 (CHα, Hyp), 60.2 (CHα, Hyp), 69.5 (CHγ, Hyp), 69.7 (CHγ, Hyp), 168.0 (CO, Gly), 168.1 (CO, Lys), 169.8 (CO, Gly), 170.9 (CO, Glu), 172.8 (CO, Pro), 173.0 (CO, Hyp), 173.1 (CO, Hyp), 173.7 (COOH, Glu), 175.5 (COOH, Lys). HRMS (ESI-MS, *m/z*) $(M + H)^+$ calc. for $C_{36}H_{61}N_{10}O_{13}$: 841.4420, found: 841.4403.

*5.2.2. Synthesis of the homotrimer peptide (**2**)*

The trimeric frame was generated by first coupling Fmoc-Lys(Dde), followed by Fmoc-Lys(Fmoc) to the commercially available Fmoc-Ahx-Sasrin (170.73 mg, 0.100 mmol). Removal of both the Fmoc group and the Dde group produced three amino groups to each of which was attached a 6-aminohexanoic acid spacer arm (double coupling), providing three foci for subsequent peptide synthesis. The remainder of the synthesis was carried out automatically (double coupling) using HBTU chemistry and deprotection with 20% piperidine in DMF. The completed peptide was cleaved from the resin and side-chain deprotected by treatment with a mixture of dichloromethane/trifluoroacetic acid (1:9 v/v) for 3 h at room temperature. The solid support was removed by filtration, the filtrate concentrated under reduced pressure, and the peptide **2** precipitated from diethyl ether. The precipitate was washed several times with diethyl ether and dried under reduced pressure. Peptide **2** was purified using reverse-phase chromatography and the composition verified by mass spectrometric sequence analysis. Peptide **2** was obtained as a white powder (143.24 mg, 37.1%). MP: 186 °C. IR (cm^{-1}): 3341 (NH, NH_2, OH), 2949 (CH), 1720 (CO), 1668 (CO).

^1H NMR (500 MHz, CD_3OD), δ (ppm): 1.35 (m, 6H, $CH_2\beta$, Lys), 1.35 (m, 6H, $CH_2\beta$, Glu), 1.58 (m, 6H, $CH_2\beta$, Lys), 1.59 (m, 6H, $CH_2\gamma$, Pro), 1.59 (m, 16H, $CH_2\delta$, Lys), 1.70 (m, 16H, CH_2, Ahx), 1.90 (m, 8H, $CH_2\gamma$, Lys), 1.95 (m, 3H, $CH_2\gamma$, Glu), 2.01 (m, 4H, $CH_2\beta$, Lys), 2.01 (m, 8H, $CH_2\gamma$, Lys), 2.09 (m, 6H, $CH_2\beta$, Hyp), 2.15 (m, 3H, $CH_2\gamma$, Glu), 2.19 (m, 8H, CH_2, Ahx), 2.27 (m, 6H, $CH_2\beta$, Hyp), 2.31 (m, 8H, CH_2, Ahx), 2.41 (m, 6H, $CH_2\beta$, Pro), 2.93 (m, 6H, $CH_2\varepsilon$, Lys), 3.21 (m, 8H, $CH_2\varepsilon$, Ahx), 3.57 (m, 6H, $CH_2\varepsilon$, Lys), 3.59 (m, 6H, $CH_2\delta$, Pro), 3.75 (m, 6H, $CH_2\delta$, Hyp), 3.84 (m, 6H, $CH_2\delta$, Hyp), 4.02 (m, 4H, $CH_2\varepsilon$, Lys), 4.13 (m, 12H, CH_2, Gly), 4.26 (m, 2H, CHα, Lys), 4.33 (m, 6H, CHα, Lys), 4.41 (m, 3H, CHα, Hyp), 4.55 (m, 12H, $CH_2\gamma$, Hyp), 4.57 (m, 3H, CHα, Hyp), 4.58 (m, 3H, CHα, Pro), 4.78 (m, 3H, CHα, Glu).

^{13}C NMR (125 MHz, CD_3OD), δ (ppm): 20.6 ($CH_2\gamma$, Pro, 3C), 22.3 ($CH_2\beta$, Lys, 2C), 22.8 ($CH_2\beta$, Lys), 22.9 ($CH_2\beta$, Lys), 24.3 ($CH_2\beta$, Lys, 2C), 24.5 ($CH_2\beta$, Lys, 2C), 25.1 (CH_2, Ahx, 4C), 25.3 (CH_2, Ahx, 4C), 26.3 ($CH_2\beta$, Glu, 3C), 26.6 ($CH_2\gamma$, Glu, 3C), 28.6 ($CH_2\delta$, Lys, 8C), 29.3 ($CH_2\beta$, Pro, 3C), 29.5 ($CH_2\gamma$, Lys, 4C), 30.9 ($CH_2\gamma$, Lys, 4C), 33.4 (CH_2, Ahx, 4C), 35.6 (CH_2, Ahx, 4C), 37.4 ($CH_2\beta$, Hyp, 3C), 37.7 ($CH_2\beta$, Hyp, 3C), 38.8 (CH_2, Ahx, 4C), 38.9 ($CH_2\varepsilon$, Lys, 4C), 39.4 ($CH_2\varepsilon$, Lys, 4C), 41.6 (CH_2, Gly, 3C), 42.3 (CH_2, Gly, 3C), 46.7 ($CH_2\delta$, Pro, 3C), 50.4 (CHα, Glu, 3C), 51.4 (CHα, Lys, 3C), 52.8 (CHα, Lys, 3C), 53.4 (CHα, Lys, 2C), 55.4 ($CH_2\delta$, Hyp, 3C), 55.6 ($CH_2\delta$, Hyp, 3C), 59.0 (CHα, Pro, 3C), 59.8 (CHα, Hyp, 3C), 60.6 (CHα, Hyp, 3C), 69.5 (CHγ, Hyp, 3C), 69.7 (CHγ, Hyp, 3C), 168.0 (CO, Gly, 3C), 168.5 (CO, Lys, 8C), 169.8 (CO, Gly, 3C), 170.9 (CO, Glu, 3C), 172.5 (CO, Pro, 3C), 173.0 (CO, Hyp, 6C), 173.2 (COOH, Glu, 3C), 174.6 (CO, Ahx), 175.4 (CO, Ahx, 2C), 176.1 (COOH, Ahx). HRMS (ESI-MS, *m/z*) $(M + Na)^+$ calc. for $C_{144}H_{244}N_{38}O_{43}Na$: 3193.8075, found: 3193.8864.

*5.2.3. Synthesis of the α-triple helix homotrimer peptide (**3**)*

The peptide **3** was synthesized like the peptide **2** mentioned above from the commercially available Fmoc-Ahx-Sasrin (169.52 mg, 0.100 mmol). The completed peptide was cleaved from the resin and side-chain deprotected by treatment with a mixture of water/thioanisole/triisopropylsilane/dichloromethane/trifluoroacetic acid (1:1:1:10:20 v/v) for 4 h at room temperature. After filtration, the solvent was removed under reduced pressure and the peptide was precipitated in ether and a white powder was obtained. Peptide **3** was purified using reverse-phase chromatography and the composition verified by spectrometric analysis. Peptide **3** was obtained as a white powder (206.21 mg, 42.2%). MP: 186 °C. IR (KBr, cm^{-1}): 3460 (NH, NH_2, OH), 2960 (CH), 2860 (CH_2, CH_3), 1680 (CO), 1675 (CO). HRMS (ESI-MS, *m/z*) $(M + H)^+$ calc. for $C_{420}H_{641}N_{110}O_{115}S_6$: 9258.5968, found: 9258.7388.

*5.2.4. Synthesis of the α-triple helix monomer peptide (**4**)*

Synthesis of octapeptide **4** started from commercially available Fmoc(9-fluorophenylmethoxy)-Cys(Boc)-Sasrin (283.83 mg, 0.111 mmol). The completed peptide was cleaved from the resin and side-chain deprotected by treatment with the scavengers water/thioanisole/triisopropylsilane/dichloromethane/trifluoroacetic acid (1:1:1:10:20 v/v) for 4 h at room temperature. The solid support was removed by filtration, the filtrate concentrated under reduced pressure, and the peptide **4** precipitated from diethyl ether. The precipitate was washed several times with diethyl ether and dried under reduced pressure. Peptide **4** was purified using reverse-phase chromatography and the composition verified by spectrometric analysis. Peptide **4** was obtained as a white powder (188.34 mg, 37.4%). MP: 181 °C. IR (cm^{-1}): 3280 (NH, NH_2, OH), 3070 (CH), 1680 (CO), 1651 (CO). HRMS (ESI-MS, *m/z*) $(M + H)^+$ calc. for $C_{176}H_{260}N_{46}O_{49}S_2$: 3865.8709, found: 3865.8698.

5.3. Structural studies

5.3.1. Circular dichroism

Circular dichroism (CD) measurement was carried out in the far UV (250–190 nm) on a Jobin yvon CD6 (UMR CNRS/USTL 8576-IFR 118, Lille, France) spectropolarimeter. The spectra were recorded with a quartz cell of 0.1–1 cm optical path length, with five replicates at a scan rate of 0.5 nm/min. The CD data were expressed in terms of mean residue ellipticity ($[\theta]$) in deg cm^2 dmol^{-1}. The concentration for CD analysis was 1 mg/ml in PBS buffer (10 mM sodium phosphate and 0.15 M NaCl) at pH 7.0. The CD spectra of all the samples are scanned after 7-day storage at 4 °C. All CD spectra measured were baseline corrected by subtracting the buffer spectrum. The secondary structure elements were determined from the Selcon program. The thermal transition curves were obtained by recording the molar ellipticity ($[\theta]$) in the range of 5–80 °C at $\lambda = 225$ nm at 1 °C/min. Fraction folded was defined as: $U = (\theta_{obs} - \theta_F)/(\theta_U - \theta_F)$ where θ_{obs} was the ellipticity observed, θ_F the mean residue ellipticity of peptide fold and θ_U the ellipticity unfold.

5.3.2. Optical rotation

The optical rotation was carried out on a Jasco polarimeter (EA 1391 ERRMECE, University of Cergy-Pontoise, France), using a 436 nm filter, and a cylindrical cuvette of 0.1 dm optical path length. Temperature control was performed by a Julabo FS18 bath. Cooling and heating ramps of 0.5 °C/min were applied. The acquisition and heating ramps were performed by the Spectra Manager program. The mesures of all the samples are determined after 60 h of storage at 4 °C in water (2 mg/ml).

The triple helix amount χ is derived from:

$$\chi = \frac{[\alpha]_\lambda^{\text{helix}} - [\alpha]_\lambda^{\text{coil}}}{[\alpha]_\lambda^{\text{collagen}} - [\alpha]_\lambda^{\text{coil}}} \quad (1)$$

where λ is the wavelength ($\lambda = 436$ nm), $[\alpha]_\lambda = \alpha/lc$ is the specific optical rotation of the protein in solution, c is the concentration (grams per cubic centimeter), l is the optical path length (0.1 dm), α is the optical rotation angle (degrees) measured experimentally, $[\alpha]_\lambda^{\text{collagen}}$ is the specific optical rotation of native soluble collagen ($\chi = 1$), which contains only triple helices, and $[\alpha]_\lambda^{\text{coil}}$ is the specific optical rotation of the coils ($\chi = 0$). ($[\alpha]_{436\ \text{nm}}^{\text{collagen}} = -800$ deg cm^3 g^{-1} dm^{-1} at 27 °C, $[\alpha]_{436\ \text{nm}}^{\text{collagen}} = -274$ deg cm^3 g^{-1} dm^{-1} at pH = 7, 4 and 40 °C.) The optical rotation angles are indicated in Table 2.

Table 2
The optical rotation angles (degree) measured experimentally

Compound	$T = 20$ °C	$T = 37$ °C
Peptide **3**	-0.0784 ± 0.0007	-0.0766 ± 0.0006
Peptide **4**	-0.1072 ± 0.0007	-0.0885 ± 0.0007

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Appendix. Supporting information

Mass spectrum (MS–MS) of peptides **2** and **3**; ^{1}H and ^{13}C NMR of octapeptide **1**. This material is available free of charge via the internet at http://www.sciencedirect.com. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2006.12.018.

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