Classical Synthesis of and Structural Studies on a Biologically Active Heptapeptide and a Nonapeptide of Bovine Elastin

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The synthesis of two elastin sequences incorporating the structural unit X-G-G-X-G (X = A) is described. In particular, the following peptides and polypeptides were synthesized and characterized: $TFA^-H_2^+$LGAGGAG-OH, $TFA^-H_2^+$LGAGGAGVL-OH (both of these inducing stimulation of endogenous elastin production in cultured adult human fibroblast), poly(LGAGGAG), and poly(LGAGGAGVL). The synthesis was accomplished in solution by classical procedures, by using the diphenylphosphoryl azide for the polycondensation step, and the mixed anhydride method for the coupling steps. CD, NMR, and FT-IR measurements gave evidence of quasi-extended (PP II) structures as a peculiar structural feature in the heptapeptide, and a tendency towards flexible β-turns in the nonapeptide. Poly(LGAGGAG) showed greater disorder with respect to the "monomer", the molecular conformation being accountable for by unstructured polypeptide chains together with β-turns. The polymer is able to adopt supramolecular structures reminiscent of those found in elastin. Unlike that of the heptapeptide, polycondensation of the nonapeptide did not afford a linear polymer, but only cyclic derivatives. This could well be due to the tendency of the monomeric nonapeptide to adopt folded conformations.

Introduction

Bovine elastin is widely used in the study of the insoluble vertebrate proteins, because of its ready availability from the neck ligament (ligamentum nuchae) of this ruminant. Most grazing animals have a substantial representation of this ligament, as it serves as a passive elastomeric band in the raising and lowering of the head in grazing. Elastin is easily purified from the ligamentum nuchae, generally by use of hot alkaline extraction or autoclaving procedures.[1] Thermolysin, an endopeptidase produced by thermophilic bacteria, releases a group of small discrete peptides, all of molecular size smaller than 1000 Dalton, from bovine elastin.[2] Our interest lies in a heptapeptide released from the purified elastin by thermolysin digestion. This heptapeptide exhibits biologic activity, as evidenced by its ability to stimulate messenger RNA and cytokine production in fibroblast tissue cultures.[3] A related nonapeptide also possesses this activity. Both have been synthesized by classical procedures in solution, with the mixed anhydride method used for the coupling steps. This synthesis is essential for production of gram amounts of the peptides, so that structure-function relationships and industrial applications − particularly for pharmacological use − can be pursued. Moreover, the polyheptapeptide shows very distinct elastin-like properties and so can be used as a model in the study of this insoluble protein (Figure 1).

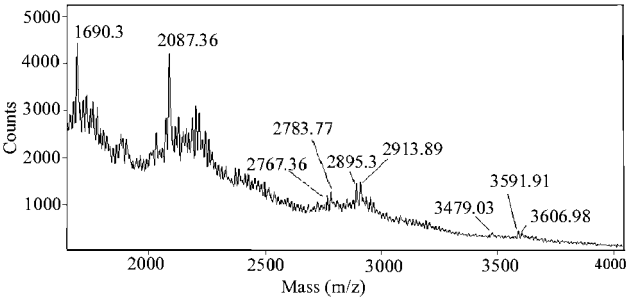

Figure 1. MALDI/MS spectrum of poly(LGAGGAGVL)

Results and Discussion

Synthesis

There are three points of significance regarding this classical synthesis description of these peptides. Most signific-

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antly, all of the yields obtained throughout the classical synthesis scheme are at acceptable levels. However, the experimental conditions for removal of the Boc group to give the fully deprotected heptapeptide could be further explored; the possibility of using different mild acidic conditions that might significantly improve yields appears worth investigation. This crucial step at the end of the synthesis scheme has the lowest yield of any of the steps, and improvement here would greatly enhance total recovery. Secondly, particular attention has been paid to the production of intermediates common to the synthesis both of the heptapeptide and of the nonapeptide: $TFA^-H_2^+$AG-OEt and Boc-LGAGGAG-OH, for example. Finally, and consistently with the chemistry of a classical liquid phase procedure, the isolation of intermediate compounds makes the production of a very pure final product possible. This important matter is not to be overlooked in scaling up of the synthesis for large amounts of both peptides.

Conformational Studies

Boc-LGAGGAG-OEt

Figure 2 shows the CD spectra of the protected heptapeptide in water and TFE at two different temperatures. In both cases a negative band at around 195 nm and a very weak band at around 220−230 nm are observed. The temperature dependence of the intensity of the curves probably indicates a conformational transition from disordered structures (25 °C) to PP II ones. An intense negative band at around 200 nm is generally ascribed either to PP II conformation or disordered structures,[4] but on the basis of the band intensity temperature dependence mentioned above, it is better to assign it to an incipient PP II conformation.[5] In fact, an opposite temperature dependence is expected in the case of essentially unstructured conformations. The CD spectra of the same heptapeptide in TFE (Figure 2) could be interpreted as a mixture of folded conformations, probably a type-II β-turn,[6] and unordered structures, as confirmed by the decrease in the intensity of the band at about 197 nm at 25 °C. The possible β-turn may be generated by a hydrogen bond connecting the C=O function of the Boc group to the NH function of A^3, or one connecting L^1 to G^4. In this context it should be noted that it has previously been demonstrated[7] that β-turns containing XG sequences at the corners are more stable than those possessing GG segments. Figure 3 shows the deconvoluted Amide I band frequencies of Boc-LGAGGAG-OEt in the solid state. The two bands at 1639 cm^{-1} and at 1628 cm^{-1} are both characteristic of antiparallel β-sheet structures.[8,9] The component at 1655 cm^{-1} is normally assigned to disordered structures,[10] while the more intense band at 1665 cm^{-1} is suggestive of a PP II conformation.[11] The band at 1688 cm^{-1} could be assigned to β-sheet or β-turn conformations, or to both.

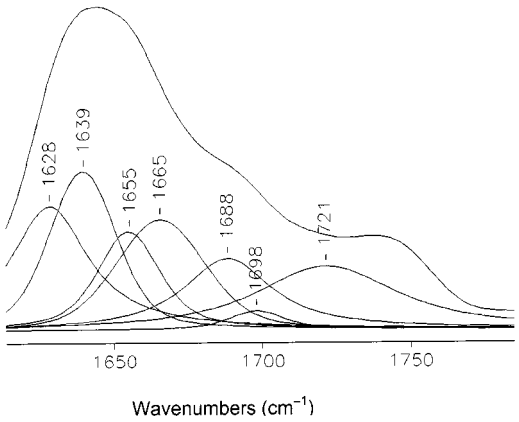


Figure 3. Amide I region of the FT-IR spectrum of Boc-LGAGGAG-OEt in KBr pellets

$TFA^-H_2^+$LGAGGAG-OH

Figure 4 shows the CD spectra of $TFA^-H_2^+$-LGAGGAG-OH in water and in TFE, recorded at two different temperatures. In the first case, as the temperature decreases it is possible to observe a conformational transition from an essentially disordered conformation (25 °C)[12] to the quasi-extended structure of PP II (0 °C).[6,13] As a matter of fact, the appearance of a small positive band at about 215 nm and of a negative band at 197 nm is considered to be diagnostic of the PP II structure. The tendency of the unprotected heptapeptide to adopt the PP II helix is retained even in a less polar solvent such as TFE, albeit to

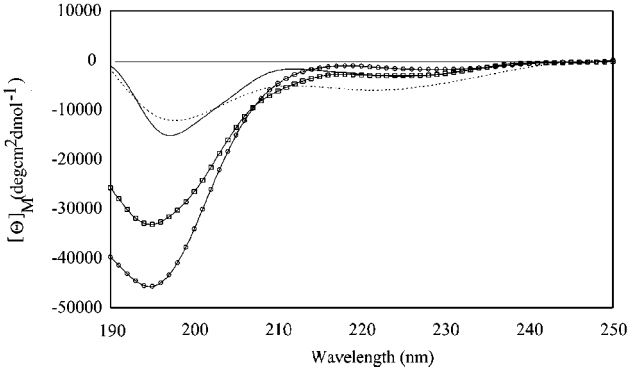


Figure 2. CD spectra of Boc-LGAGGAG-OEt: in water at 25 °C (□) and 0 °C (○), and in TFE at 25 °C (---) and 0 °C (—)

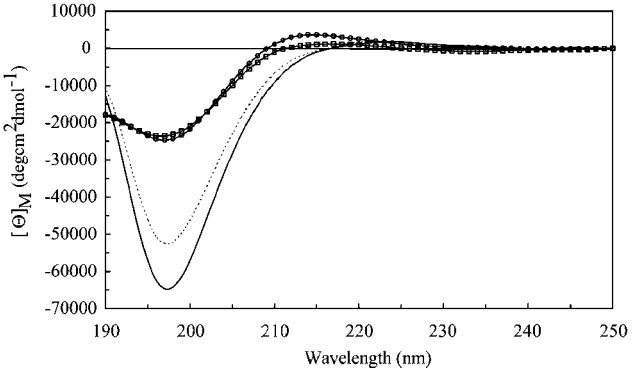


Figure 4. CD spectra of $TFA^-H_2^+$LGAGGAG-OH: in water at 25 °C (□) and 0 °C (○), and in TFE at 25 °C (---) and 0 °C (—)

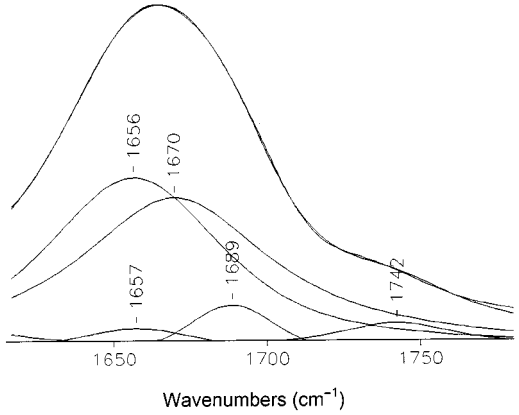


Figure 5. Amide I region of the FT-IR spectrum of $TFA^-H_2^+$LGAGGAG-OH in KBr pellets

lesser extents, as shown by the significant decrease in the negative band. Figure 5 shows the deconvoluted Amide I band frequencies of $TFA^-H_2^+$LGAGGAG-OH in the solid state. The strong band at 1670 cm^{-1} has been viewed as diagnostic of the PP II structure; this component has in fact been found for poly(GPG) in the solid state, for which a compact sheet structure of PP II type helices with two hydrogen bonds per tripeptide has been proposed.[14] The Amide I component at 1656 cm^{-1} is attributable to disordered structures, while the latter band at 1689 cm^{-1} is normally attributed to β-pleated sheets and/or β-turns.

The NMR spectroscopic data in aqueous solution (Figure 6) are compatible with the preceding CD and FT-IR results, revealing a significant amount of extended conformational states. In fact, the only regular and sequential non-intraresidue NOEs found are those between the NH of a residue and the CH_α of the successive one. As a matter of fact, the observation only of the sequential $d_{\alpha N}$ connectivities instead of extensive medium- and long-range interactions is compatible with the PP II structural motif.[15] Furthermore, the ^3J values are also indicative of extended structures.

Boc-LGAGGAGVL-OMe

The CD spectra of Boc-LGAGGAGVL-OMe in H_2O/CH_3CN (1:1, v/v) and in TFE, recorded at 0 °C and 25 °C,

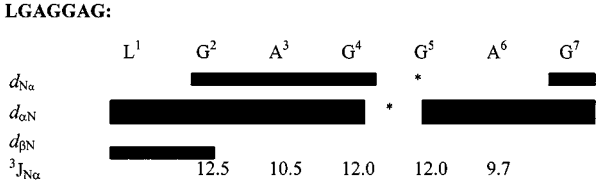


Figure 6. NMR parameters of TFA-LGAGGAG-OH in ^1H_2O/^2H_2O (9:1) at 298 K: semiquantitative estimated NOESY cross-peak intensities and values of the $^3J_{N\alpha}$ coupling constants; the thicknesses of the solid boxes are proportional to the intensity (peak volume) and represent strong and medium intensities for sequential and intraresidue cross-peaks; a star indicates an unresolved overlapping cross-peak; cross-peaks $d_{\alpha N}$ (between protons $C_\alpha H_i - NH_{i+1}$) and $d_{\beta N}$ ($C_\beta H_i - NH_{i+1}$) are shown for two neighbouring amino acids; $d_{N\alpha}$ denotes the cross-peaks between $NH_i - C_\alpha H_i$ protons; the values of the $^3J_{N\alpha}$ coupling constants are expressed in Hz

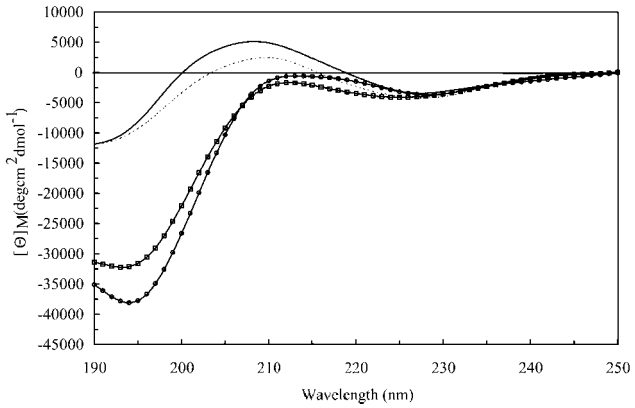


Figure 7. CD spectra of Boc-LGAGGAGVL-OMe: in H_2O/CH_3CN (1:1, v\v) at 25 °C (□) and 0 °C (O), and in TFE at 25 °C (---) and 0 °C (—)

are shown in Figure 7. The curves show prevalent disordered conformations, although at 0 °C there is a trend toward a positive band near 210 nm, suggesting some folding of the molecule. The CD spectra of the same nonapeptide, but recorded in TFE at two different temperatures, are also shown in Figure 7. As is to be expected in a less polar solvent such as TFE, the nonapeptide can adopt a more *folded* conformation as the temperature decreases. In fact, the spectrum is almost characteristic of type-II β-turns together with open (disordered) conformations. Figure 8 shows the deconvoluted Amide I region of the Boc-LGAGGAGVL-OMe in the solid state. The major component at 1660 cm^{-1} is diagnostic of a PP II conformation,[11] whilst the weak band at 1690 cm^{-1} is ascribable to β-turn and/or β-sheet conformations.

$TFA^-H_2^+$LGAGGAGVL-OH

The CD spectra of the free nonapeptide in water and in TFE are shown in Figure 9. The curve recorded at 25 °C shows an intense negative band at about 195 nm, typical of a disordered conformation. In contrast, the CD spectrum recorded at 0 °C shows a pronounced diminution in the intensity of the band at about 195 nm, which might be in-

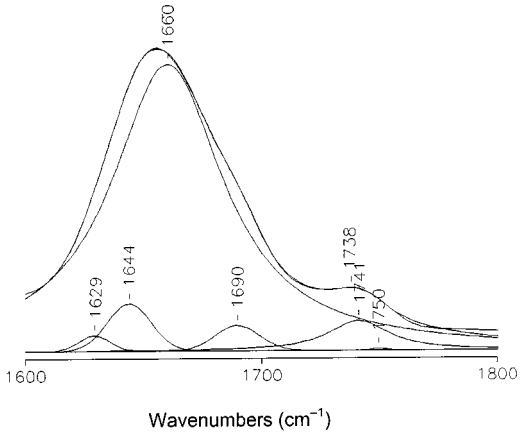


Figure 8. Amide I region of the FT-IR spectrum of Boc-LGAGGAGVL-OMe in KBr pellets

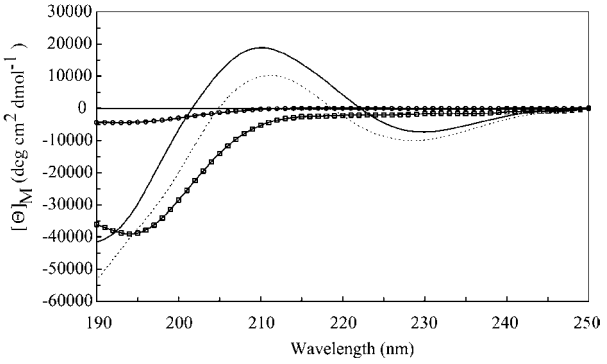


Figure 9. CD spectra of $TFA^{-}H_2^{+}$LGAGGAGVL-OH: in water at 25 °C (□) and 0 °C (O), and in TFE at 25 °C (---) and 0 °C (—)

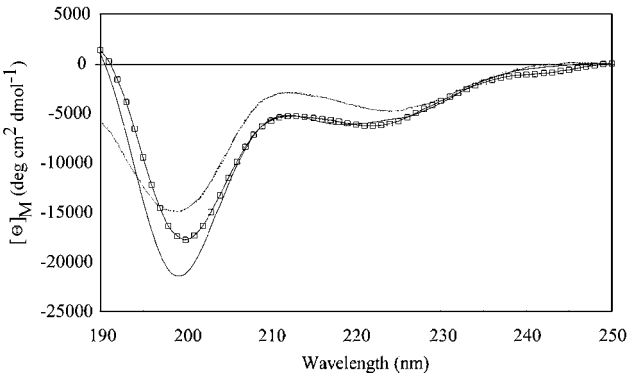


Figure 11. CD spectra of poly(LGAGGAG) in HFIP at: 0 °C (−), 25 °C (---), 60 °C (□)

terpreted as a tendency to a type-II β-turn structure. Figure 9 also shows the CD spectra of $TFA^{-}H_2^{+}$LGAGGAGVL-OH in TFE at two different temperatures. The presence of type-II β-turns is clear as the temperature falls from 25 to 0 °C. This CD interpretation is confirmed by the deconvoluted Amide I band frequencies of the same nonapeptide (Figure 10) in the solid state. The strong band at 1650 cm^{-1} is characteristic of disordered conformations,[10] while the band at 1690 cm^{-1} may be attributable to β-turn structures. Finally the band at 1672 cm^{-1} is attributable to extended structures such as PP II type helixes,[14] in this case more stable in the solid state than in TFE solution. This finding is not unexpected, because the PP II conformation is more stable in aqueous solution,[11,14] while β-turns are more stable in solvents of relatively low dielectric constant, such as TFE.

Poly (LGAGGAG)

CD spectra of poly(LGAGGAG) in hexafluoro-2-propanol (“hexafluoroisopropanol”, HFIP) at three different temperatures are shown in Figure 11. Two main features should be noted: firstly, there is the presence of complex conformational equilibria, which could be interpreted as a mixture of disordered (or extended) components and putative β-turn conformations. Secondly, an increase in the ordered conformation is seen on increasing the temperature from 0 to 25 °C. This finding seems to reflect the inverse temperature conformational change already observed for α-elastin,[16] tropoelastin and also poly(VPGVG).[17] On the other hand, the CD spectrum at 60 °C reveals, as is to be expected, essentially disordered conformers. In passing, the complexity observed in the CD curves is clearly in agreement with the well-documented increase in chain flexibility on chain-lengthening for elastin peptides.[18] For comparison, Figure 12 shows the CD spectra of $TFA^{-}H_2^{+}$LGAGGAG-OH in HFIP recorded at the same three temperatures. In this case, as the temperature decreases it is possible to discern a clear conformational transition from a completely disordered conformation (60 °C) to a more evident tendency to a PP II extended helix (0 °C). It should be noted that the major change is seen on going from 25 to 60 °C, rather than from 0 to 25 °C. Figure 13 shows the deconvoluted Amide I region of poly(LGAGGAG) in the solid state. The two strongest band frequencies, at 1628 cm^{-1} and 1655 cm^{-1}, are characteristic of antiparallel β-sheet structures[8,9] and of disordered conformations, respectively.[10] The weak band at 1677 cm^{-1} is probably attributable to β-turn structures (type I and II).[19]

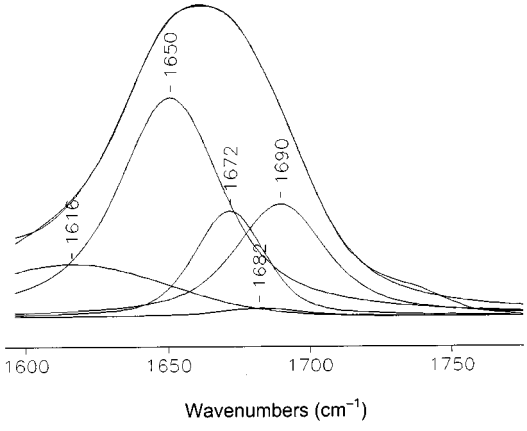


Figure 10. Amide I region of the FT-IR spectrum of $TFA^{-}H_2^{+}$LGAGGAGVL-OH in KBr pellets

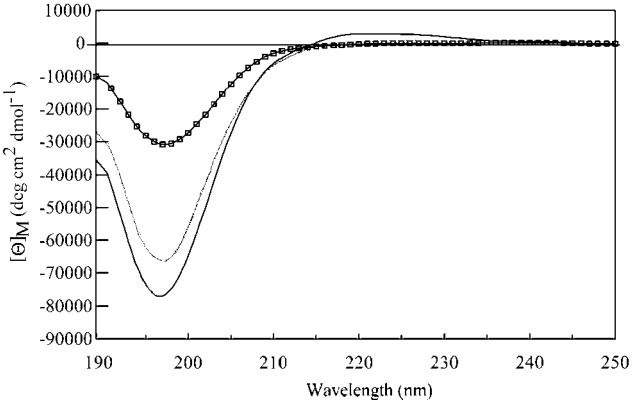


Figure 12. CD spectra of $TFA^{-}H_2^{+}$LGAGGAG-OH in HFIP at: 0 °C (−), 25 °C (---), 60 °C (□)

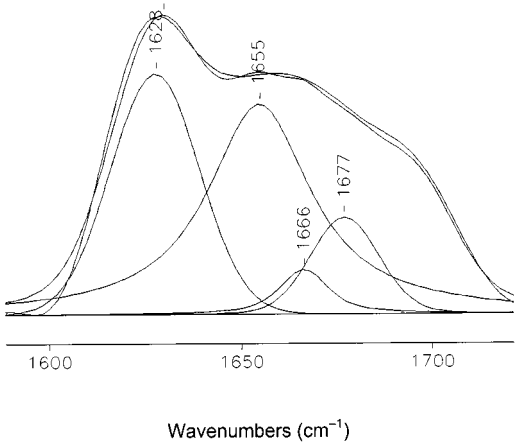


Figure 13. Amide I region of the FT-IR spectrum of poly(LGAGGAG) in KBr pellets

Extensive studies by electron microscopy (ESEM, TEM) were also performed on poly(LGAGGAG), and these revealed different aggregation forms. A very complex pattern emerges from the ESEM micrograph shown in Figure 14, made up of a sort of fragmented and overlapped flake-morphology. In some cases, sheet-shaped aggregates are also seen, as evidenced at higher magnification (Figure 15). The

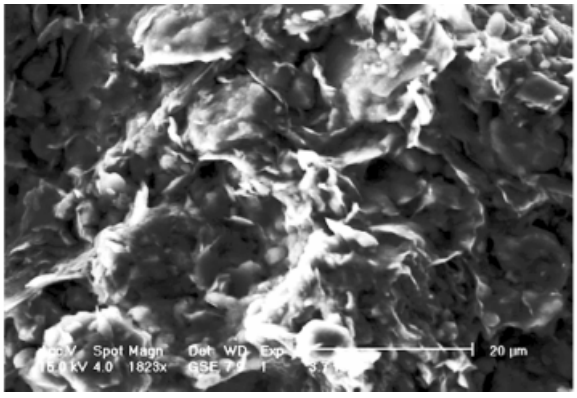


Figure 14. ESEM micrograph of poly(LGAGGAG) showing a sort of flake-morphology; bar represents 20 µm

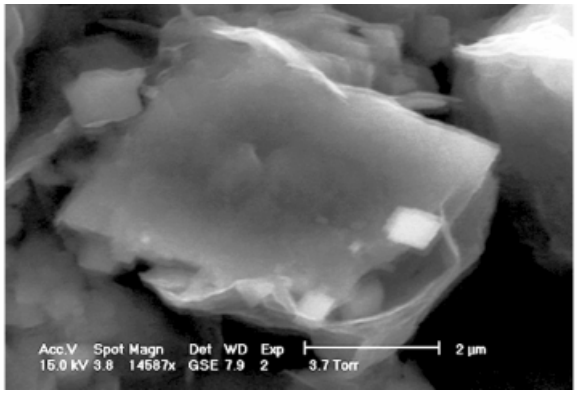


Figure 15. ESEM micrograph of poly(LGAGGAG) showing sheet-shaped aggregates; bar represents 2 µm

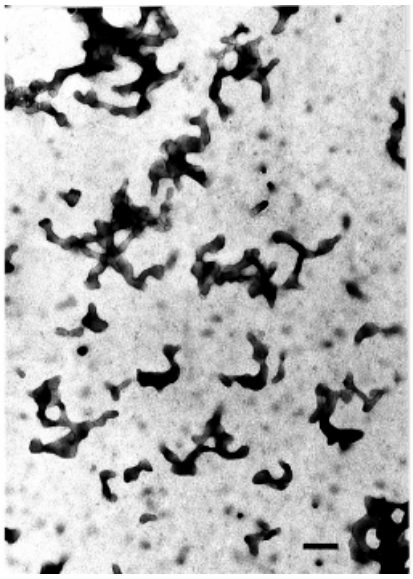


Figure 16. TEM image of poly(LGAGGAG); note the nuclei of diffusion-limited aggregation; bar represents 200 nm

TEM micrograph reported in Figure 16 displays a clear example of nuclei of diffusion-limited aggregation.[20,21] In particular, the higher magnification shown in Figure 17 clearly exhibits intermediate aggregation of the nuclei, which evolves to give more aggregated structures (Figure 18). The determined fractal dimensions are $D = 1.5$ in the first case, and $D = 1.8$ in the second. These results are compatible with a so called cluster-cluster DLA.[22] When the sample was left at room temperature for 10 h, a different organization was found; a fibrous supramolecular structure typical of elastin and its derivatives[23−26] was observed (Figure 19). The diameter of the largest bundle was 90 nm.

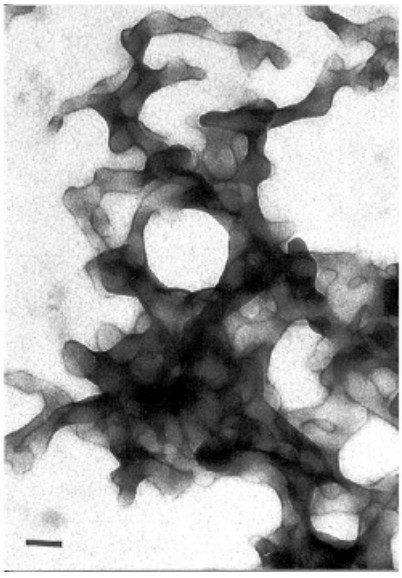


Figure 17. TEM micrograph of poly(LGAGGAG) at a higher magnification that clearly exhibits the aggregation of nuclei; bar represents 100 nm

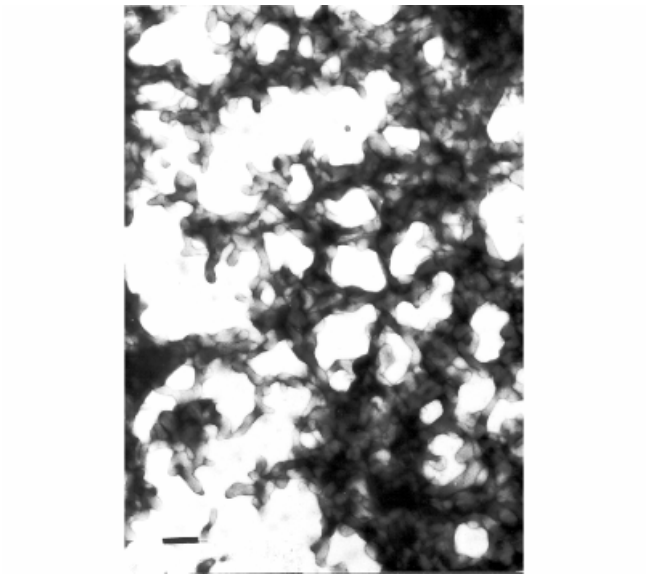


Figure 18. TEM micrograph of poly(LGAGGAG) displaying a more aggregated supramolecular structures; bar represents 200 nm

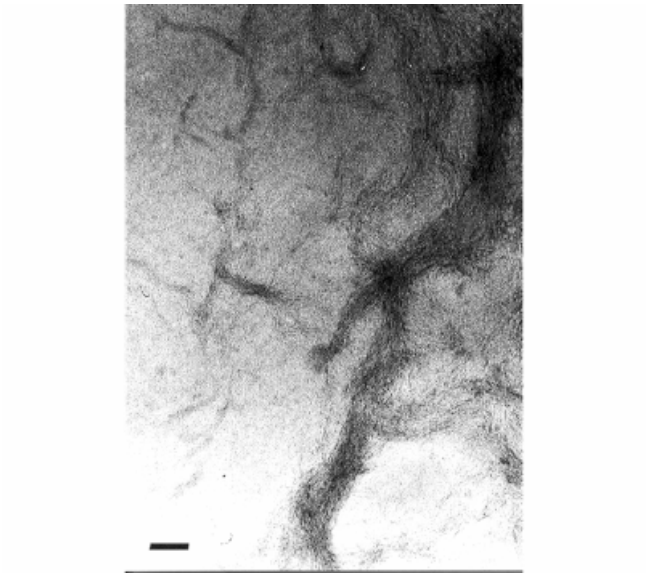


Figure 19. TEM image of poly(LGAGGAG); notice the typical elastin-like fibrous organization; bar represents 100 nm

Conclusions

The results reported in the present paper allow us to draw the following conclusions:

i) A classical synthesis of the hepta- and nonapeptide can be accomplished effectively.

ii) Structure-activity relationships have been indicated. As a matter of fact, judging from the CD spectra, the two peptides, which display essentially the same biological activity, nevertheless appear to be conformationally different. However, it has been previously demonstrated[29] that in elastin different conformations in elastin undergo transconformational equilibria between folded and extended (open) structures. Among these are type-II β-turns and polyproline II helices. Accordingly, the presence of type PPII ⇄ β-turn equilibria, these equilibria being shifted to the right and to the left, respectively, may be proposed for both peptides. (Of course, a significant amount of the so-called "random coil" is always present at room temperature.) Actually, two interpretations are possible: a) Both conformations, β-turn and PPII, interact with the receptor site to give rise to the biological activity. b) Alternatively, only one conformation is able to bind the receptor: Were this the case, it would be tempting to surmise that the conformational equilibria mentioned above should be shifted toward the active conformation (whichever that is) just by binding to the receptor.

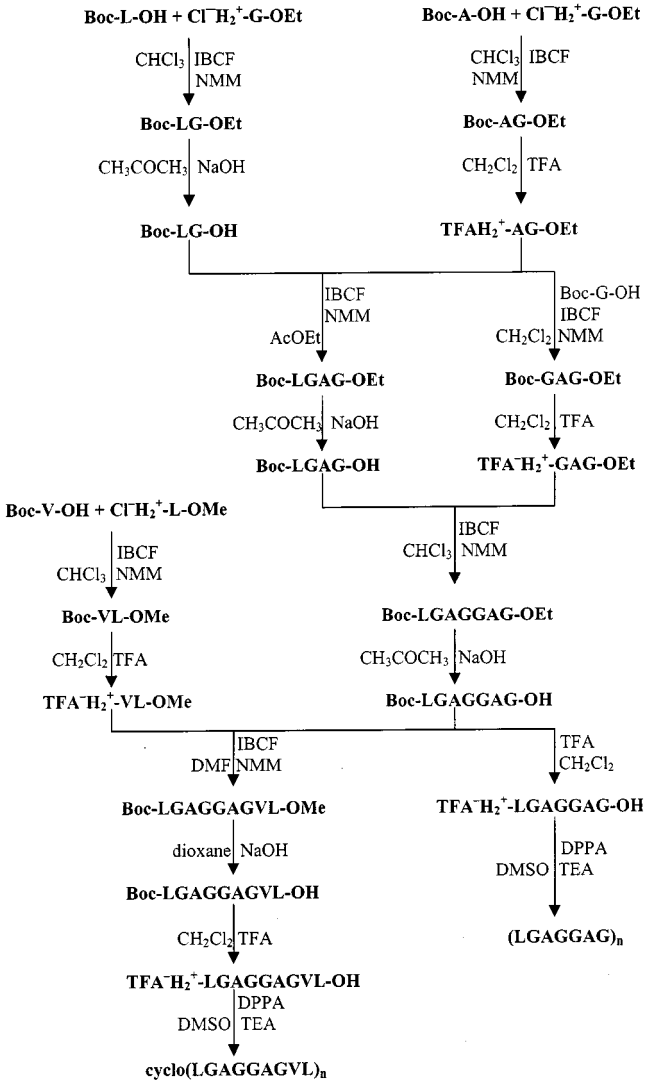
iii) Quite interestingly, the conformational studies carried out on the polyheptapeptide showed the presence of structures currently considered to be typical of elastin;[29] this confirms that the LGAGGAG sequence is indeed almost representative of the conformationally relevant sequences of the protein and suggests that even a simple polyheptapeptide can be a good model for elastin.

iv) Finally, from the standpoint of organic chemistry, we point to the results obtained from the polycondensation reactions: The kind of product obtained is strictly dependent on the conformation of the monomer. As a matter of fact, the extended conformation of heptapeptide monomer favours the obtaining of linear oligomers and polymers, while the folded conformation of the nonapeptide monomer gives rise to macrocyclic products.

Experimental Section

List of Abbreviations: A (or Ala): alanine; G (or Gly): glycine; L (or Leu): leucine; V (or Val): valine; Boc-: *tert*-butyloxycarbonyl; CD: circular dichroism spectroscopy; DMF: *N*,*N*-dimethylformamide; DMSO: dimethyl sulfoxide; DPPA: diphenylphosphoryl azide; DSS: 2,2,3,3-[D_4]-3-(trimethylsilyl)propionic acid sodium salt; ESEM: environmental scanning electron microscopy; FT-IR: Fourier-transformed infrared spectroscopy; HFIP: hexafluoro-2-propanol; HPLC: high-performance liquid chromatography; IBCF: isobutyl chloroformate; NMM: *N*-methylmorpholine; NMR: nuclear magnetic resonance spectroscopy; NOESY: nuclear Overhauser enhancement spectroscopy; PP II: polyproline II; RNA: ribonucleic acid; TEA: triethylamine; TEM: transmission electron microscopy; TFA: trifluoroacetic acid; TFE: trifluoroethanol; TOF-MALDI/MS: time-of-flight, matrix-assisted laser desorption ionization mass spectrometry.

Synthesis: Amino acids were purchased from Novabiochem AG (Laufelfinger, Switzerland). The purities of all synthetic products were ascertained by thin layer chromatography with butanol/acetic acid/water (3:1:1) or chloroform/methanol/acetic acid (9:0.8:0.2) and by reverse-phase HPLC on a 250 mm × 4.6 mm micron Jupiter C-18 column. Further characterization was provided by ^{1}H NMR spectra recorded with a Bruker AM 300 spectrometer. The synthesis of the polypeptides was performed as indicated in Scheme 1. Briefly, for *N*-terminal protection, the Boc group was used; this was removed throughout by TFA, while the coupling steps were accomplished by a classical mixed anhydride procedure. Finally, the polycondensation steps were carried out with the aid of DPPA.



Scheme 1. Synthesis of the polypeptides

Boc-AG-OEt: IBCF (17.3 mL, 132.0 mmol) was added at −15 °C to a solution of Boc-A-OH (25 g, 132.0 mmol) and NMM (14.5 mL, 132.0 mmol) in chloroform (85.0 mL). The temperature was kept at −15 °C for 1 min, and $Cl^-H_2^+$G-OEt (18.5 g, 132.0 mmol) and NMM (14.5 mL, 132.0 mmol) were then added. The mixture was stirred at room temperature for 24 h. The organic solvent was removed under reduced pressure and the oily residue was dissolved in ethyl acetate and washed with 5% sodium bicarbonate, water, 5% citric acid and water. The solution, dried with anhydrous sodium sulfate, was concentrated to dryness and an oily residue (30.0 g) that did not crystallize was obtained (83% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.18 (bt, 1 H, NH G), 6.95 (d, 1 H, NH A), 4.07 (q, 2 H, OC*H*$_2$CH$_3$), 3.98 (m, 1 H, H$_\alpha$ A), 3.80 (ABX system, 2 H, H$_\alpha$ G), 1.37 (s, 9 H, Boc), 1.20 (t, 3 H, OCH$_2$C*H*$_3$), 0.88 (d, 3 H, H$_\beta$ A).

$TFA^-H_2^+$AG-OEt: Boc-AG-OEt (25.4 g, 92.8 mmol) was dissolved in dichloromethane (209.5 mL). Trifluoroacetic acid (TFA; 209.5 mL) was then added at 0 °C and the resulting solution was stirred at 0 °C for 1 h and 30 min and at room temperature for 1 h. The solvent was evaporated under reduced pressure and the obtained oil was dissolved in water and lyophilized to remove the excess TFA (95% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.94 (t, 1 H, NH G), 8.26 (d, 1 H, NH A), 4.12 (q, 2 H, OC*H*$_2$CH$_3$), 3.97 (m, 3 H, H$_\alpha$ A and 2 H$_\alpha$ G), 1.37 (d, 3 H, H$_\beta$ A), 1.20 (t, 3 H, OCH$_2$C*H*$_3$).

Boc-GAG-OEt: IBCF (6.6 mL, 50.5 mmol) was added at −15 °C to a solution of Boc-G-OH (8.8 g, 50.5 mmol) and NMM (5.6 mL, 50.5 mmol) in dichloromethane (70 mL). The temperature was kept at −15 °C for 1 min, and the $TFA^-H_2^+$AG-OEt (14.6 g, 50.5 mmol) and NMM (5.6 mL, 50.5 mmol) were then added. The mixture was stirred at room temperature for 24 h. The organic solvent was removed under reduced pressure and the oily residue was dissolved in ethyl acetate and washed with 5% sodium bicarbonate, water, 5% citric acid and water, and then dried with anhydrous sodium sulfate. The solution was concentrated to dryness and the oil obtained was crystallized from diethyl ether/petroleum ether to give a white solid (10.0 g, 60% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.32 (t, 1 H, NH G^3), 7.95 (d, 1 H, NH A), 6.98 (t, 1 H, NH G^1), 4.30 (m, 1 H, H$_\alpha$ A), 4.08 (q, 2 H, OC*H*$_2$CH$_3$), 3.80 (m, 2 H, H$_\alpha$ G), 3.54 (m, 2 H, H$_\alpha$ G), 1.39 (s, 9 H, Boc) 1.19 (m, 6 H, H$_\beta$ A and OCH$_2$C*H*$_3$).

$TFA^-H_2^+$GAG-OEt: Boc-GAG-OEt (6.9 g, 20.8 mmol) was dissolved in dichloromethane (47.0 mL). The resulting solution was cooled in an ice bath, and TFA (47.0 mL) was added. The reaction mixture was stirred at 0 °C for 30 min and at room temperature for 1 h and 30 min. The solvent was evaporated under reduced pressure and the obtained oil was dissolved in water and lyophilized to remove the excess TFA. The oily residue was crystallized from ethyl acetate/diethyl ether to give crystalline tripeptide (6.4 g, 89% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.60 (d, 1 H, NH A), 8.48 (t, 1 H, NH G), 8.00 (br. s, 3 H, $^+$NH$_3$), 4.40 (m, 1 H, H$_\alpha$ A), 4.08 (q, 2 H, OC*H*$_2$CH$_3$), 3.82 (m, 2 H, H$_\alpha$ G), 3.07 (m, 2 H, H$_\alpha$ G), 1.24 (d, 3 H, H$_\beta$ A), 1.19 (t, 3 H, OCH$_2$C*H*$_3$).

Boc-LGAG-OEt: IBCF (4.7 mL, 36.0 mmol) was added at −15 °C to a solution of Boc-LG-OH (10.4 g, 36.0 mmol)[27] and NMM (4.0 mL, 36.0 mmol) in ethyl acetate (35.0 mL). The temperature was kept at −15 °C for 1 min, and $TFA^-H_2^+$AG-OEt (10.4 g, 36.0 mmol) and NMM (4.0 mL, 36.0 mmol) were then added. The mixture was stirred at room temperature for 24 h, and the organic solution was then washed with 5% sodium bicarbonate, water, 5% citric acid and water, and dried with sodium sulfate. The solution was concentrated to dryness and the oil obtained was triturated with diethyl ether to give a white solid (8.6 g, 54% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.32 (t, 1 H, NH G), 8.01 (m, 2 H, NH G and NH A), 6.99 (d, 1 H, NH L), 4.30 (m, 1 H, H$_\alpha$ A), 4.07 (q, 2 H, OC*H*$_2$CH$_3$), 3.93(m, 1 H, H$_\alpha$ L), 3.80 (m, 2 H, H$_\alpha$ G), 3.70 (m, 2 H, H$_\alpha$ G), 1.59 (m, 1 H, H$_\gamma$ L), 1.38 (m, 11 H, Boc and H$_\beta$ L), 1.18 (m, 6 H, OCH$_2$C*H*$_3$ and H$_\beta$ A), 0.83 (m, 6 H, H$_\delta$ L).

Boc-LGAG-OH: NaOH (1 N, 20.1 mL) was added to a solution of Boc-LGAG-OEt (8.5 g, 19.1 mmol) in acetone (95.5 mL). After 3 h and 45 min at room temperature, the organic solvent was evaporated under reduced pressure and water was added. The unchanged reagent was extracted with ethyl acetate, while the aqueous solution was cooled to 0 °C and neutralized by dropwise addition of 1 N HCl (20.1 mL). The crude material was extracted with ethyl acetate and dried with anhydrous sodium sulfate. The solution was then concentrated to dryness and the obtained residue was crystallized from ethyl acetate/petroleum ether to give 7.1 g of crystalline solid (90% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 12.55 (bs, 1H, OH), 8.19 (t, 1 H, NH G), 8.00 (m, 2 H, NH G and NH A), 6.96 (d, 1 H,

NH L), 4.31 (m, 1 H, H_α A), 3.94 (m, 1H H_α L), 3.71 (m, 4 H, H_α G), 1.60 (m, 1 H, H_γ L), 1.36 (s, 9 H, Boc), 1.20 (m, 2 H, H_β L), 0.88 (m, 6 H, H_δ L).

Boc-LGAGGAG-OEt: IBCF (2.2 mL, 16.8 mmol) was added at −15 °C to a solution of Boc-LGAG-OH (7.0 g, 16.8 mmol) and NMM (1.8 mL, 16.8 mmol) in chloroform (110.6 mL). The temperature was kept at −15 °C for 1 min, and $TFA^-H_2^+$-GAG-OEt (5.8 g, 16.8 mmol) and NMM (9.11 mmol) were then added. The solution was stirred at room temperature for 27 h. The organic solvent was removed under reduced pressure and the solid residue was suspended in ethyl acetate, filtered, washed with water and finally crystallized from ethanol/diethyl ether to give 7.0 g of crystalline heptapeptide (66% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.31 (t, 1 H, NH G), 8.24 (t, 1 H, NH G), 8.03 (m, 4 H, NH G and NH A), 6.99 (d, 1 H, NH L), 4.30 (m, 2 H, H_α A), 4.09 (q, 2 H, OCH_2CH_3), 3.94 (m, 1 H, H_α L), 3.81 (m, 2 H, H_α G), 3.72 (m, 6 H, H_α G), 1.60 (m, 1 H, H_γ L), 1.38 (m, 11 H, Boc and H_β L), 1.19 (m, 9 H, OCH_2CH_3 and H_β A), 0.85(m, 6 H, H_δ L).

Boc-LGAGGAG-OH: NaOH (1 N, 11.6 mL) was added to a solution of Boc-LGAGGAG-OEt (6.9 g, 11.0 mmol) in acetone (53 mL). After 3 h at room temperature, the organic solvent was evaporated under reduced pressure and water was added. The unchanged reagent was extracted with chloroform, while the aqueous solution was cooled to −4 °C and neutralized by dropwise addition of 1 N HCl (11.6 mL). The crude material was extracted with ethyl acetate and dried with anhydrous sodium sulfate. The solution was then concentrated to dryness and the obtained residue was triturated with diethyl ether to give a white solid (4.0 g, 60% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 12.50 (bs, 1 H, OH), 8.25 (bt, 1 H, NH G), 8.20 (bt, 1 H, NH G), 8.05 (m, 4 H, 2 NH G and 2 NH A), 7.00 (d, 1 H, NH L), 4.29 (m, 2 H, H_α A), 3.92 (m, 1 H, H_α L), 3.71 (m, 8 H, H_α G), 1.59 (m, 1 H, H_γ L), 1.35 (m, 11 H, Boc and H_β L), 1.19 (d, 6 H, H_β A), 0.83 (m, 6 H, H_δ L).

$TFA^-H_2^+$LGAGGAG-OH: Boc-LGAGGAG-OH (0.49 g, 0.818 mmol) was dissolved in dichloromethane (7.00 mL). The resulting solution was cooled to 0 °C, and TFA (1.85 mL) was added. The reaction mixture was stirred at 0 °C for 40 min and at room temperature for 2 h. The solvent was then evaporated under reduced pressure and the oily residue was lyophilized to remove the excess TFA, giving free heptapeptide (0.130 g, 26% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.74 (t, J = 12.5 Hz, 1 H, NH G^2), 8.29 (m, 2 H, NH G^4 and NH A^3), 8.19 (t, J = 12.0 Hz, 1 H, NH G), 8.07 (m, 2 H, NH G and NH A^6), 4.31 (m, 2 H, H_α A), 3.78 (m, 9 H, H_α L and H_α G), 1.65 (m, 1 H, H_γ L), 1.52 (dd, 2 H, H_β L), 1.22 (d, 6 H, H_β A), 0.88 (m, 6 H, H_δ L).

Poly(LGAGGAG): Diphenylphosphoryl azide (DPPA; 0.063 mL, 0.29 mmol) and triethylamine (TEA; 0.068 mL, 0.49 mmol) were added to a stirred solution of $TFA^-H_2^+$LGAGGAG-OH (0.117 g, 0.19 mmol) in dimethyl sulfoxide (DMSO; 0.193 mL).[28] The reaction mixture was kept at room temperature for 47 h, and the resulting polymer was then triturated with cool diethyl ether and repeatedly washed with the same solvent to give a white solid (114 mg, 94% yield). The TOF-MALDI/MS spectrum indicated a distribution of molecular weight in the 1000−4000 Da range.

Boc-VL-OMe: IBCF (0.06 mL, 0.55 mmol) was added at −15 °C to a solution of Boc-V-OH (0.120 g, 0.55 mmol) and NMM (0.06 mL, 0.55 mmol) in chloroform (0.85 mL). The solution was kept at −15 °C for 1 min, and then $Cl^-H_2^+$-L-OMe (0.1 g, 0.55 mmol) and NMM (0.06 mL, 0.55 mmol) were added. The reaction mixture was stirred at room temperature for 24 h and 30 min, the organic solvent was removed under reduced pressure and the oily residue, dissolved in ethyl acetate, was washed with 5% sodium bicarbonate, water, 5% citric acid and water, and dried with anhydrous sodium sulfate. The solution was concentrated to dryness and the crude derivative was crystallized from diethyl ether/petroleum ether to give crystalline dipeptide (102 mg, 54% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.16 (d, 1 H, NH L), 6.68 (d, 1 H, NH V), 4.29 (m, 1 H, H_α L), 3.77 (m, 1 H, H_α V), 3.60 (s, 3 H, CH_3), 1.89 (m, 1 H, H_γ L), 1.53 (m, 3 H, H_β L and H_β V), 1.37 (s, 9 H, Boc), 0.84 (m, 12 H, H_γ V and H_δ L).

$TFA^-H_2^+$VL-OMe: Boc-VL-OMe (79 mg, 0.23 mmol) was dissolved in dichloromethane (0.52 mL). The solution was cooled to 0 °C and TFA (0.52 mL) was added. The reaction mixture was stirred at 0 °C for 50 min and at room temperature for 1 h and 20 min. The organic solvent was then evaporated under reduced pressure and the obtained oil, dissolved in water, was lyophilized twice to remove the excess TFA, giving a white solid (70 mg, 84% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.63 (d, 1 H, NH L), 4.32 (m, 1 H, H_α L), 3.61 (s, 3 H, CH_3), 3.52 (m, 1 H, H_α V), 1.63 (m, 3 H, H_β L and H_β V), 1.54 (m, 1 H, H_γ L), 0.91 (m, 12 H, H_γ V and H_δ L).

Boc-LGAGGAGVL-OMe: IBCF (0.025 mL, 0.19 mmol) was added at −15 °C to a solution of Boc-LGAGGAG-OH (114 mg, 0.19 mmol) and NMM (0.021 mL, 0.19 mmol) in DMF (2.86 mL). The temperature was kept at −15 °C for 1 min, and then $TFA^-H_2^+$ VL-OMe (68 mg, 0.19 mmol) and NMM (0.021 mL, 0.19 mmol) were added. The mixture was kept at room temperature for 24 h, the organic solvent was removed under reduced pressure and the obtained solid − suspended in water, filtered, and repeatedly washed with water − was dried to give the nonapeptide (121 mg, 77% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.33 (d, 1 H, NH L^9), 8.27−8.13 (m, 2 H, NH G and NH V), 8.09−7.98 (m, 4 H, NH A and 3 NH G), 7.66 (d, 1 H, NH A), 6.98 (d, 1 H, NH L^1), 4.31−4.18 (m, 3 H, H_α L^7 and H_α A), 3.91 (m, 1 H, H_α L^1), 3.70 (m, 9 H, H_α V and H_α G), 3.59 (s, 3 H, CH_3), 1.63−1.48 (m, 6 H, H_β and H_γ L), 1.35 (s, 9 H, Boc), 1.20 (d, 6 H, H_β A), 0.90−0.76 (m, 18 H, H_γ V and H_δ L).

Boc-LGAGGAGVL-OH: NaOH (1 N, 0.064 mL) was added to a solution of Boc-LGAGGAGVL-OMe (48 mg, 0.058 mmol) in dioxane (2.00 mL). The solution was stirred at room temperature for 4 h, and the organic solvent was then evaporated under reduced pressure and water was added. The unchanged material was extracted with ethyl acetate, the aqueous solution was cooled to 0 °C, and 1 N HCl (0.064 mL) was added. The obtained crude material was extracted with ethyl acetate and the solvents were evaporated under vacuum to give a pure white solid (31 mg, 66% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.32 (d, 1 H, NH L^9), 8.27−8.13 (m, 2 H, NH G and NH V), 8.09−7.96 (m, 4 H, NH A and 3 NH G), 7.66 (d, 1 H, NH A), 6.98 (d, 1 H, NH L^1), 4.31−4.17 (m, 3 H, H_α L and H_α A), 3.91 (m, 1 H, H_α L), 3.72 (m, 9 H, H_α V and H_α G), 1.64−1.49 (m, 6 H, H_β and H_γ L), 1.36 (s, 9 H, Boc), 1.21 (d, 6 H, H_β A), 0.92−0.77 (m, 18 H, H_γ V and H_δ L).

$TFA^-H_2^+$LGAGGAGVL-OH: Boc-LGAGGAGVL-OH (30 mg, 0.0368 mmol) was dissolved in dichloromethane (1.00 mL). TFA (0.085 mL) was then added at 0 °C and the resulting solution was stirred at 0 °C for 50 min and at room temperature for 3 h. The solvent was evaporated under reduced pressure and the oily residue was dissolved in water and lyophilized to remove the excess TFA, giving 30 mg of product (99% yield). ^{1}H NMR ([D$_6$]DMSO): δ = 8.74 (t, 1 H, NH G), 8.39−8.30 (m, 2 H, NH A and NH G), 8.21 (t, 1 H, NH G), 8.13−8.03 (m, 3 H, NH G, NH V and NH L), 7.70 (d, 1 H, NH A), 4.31−4.09 (m, 4 H, H_α A and H_α L), 3.71

(m, 9 H, H_α V and H_α G), 1.68−1.53 (m, 6 H, H_β and H_γ L), 1.21 (d, 6 H, H_β A), 0.90−0.74 (m, 18 H, H_γ V and H_δ L).

Poly(LGAGGAGVL): Three attempts were made to obtain this polymer. The first two both failed, giving only the monomer and the dimer, as evidenced by MALDI/MS, while the third gave only cyclic derivatives. This could well be due to the tendency exhibited by the monomeric free nonapeptide to adopt folded conformations. In the first attempt we used diphenylphosphoryl azide (DPPA) and triethylamine (TEA) in the same quantity as described for the polycondensation of the heptapeptide (DPPA: 1.5 equiv./mol; TEA: 2.5 equiv./mol). In the second, an additional 0.75 equiv./mol of DPPA and 1.25 equiv./mol of TEA were added after 24 h from the beginning of the reaction. In the third case, 3 equiv./mol (0.093 mmol) of DPPA and 5 equiv./mol (0.155 mmol) of TEA were added to a stirred solution of $TFA^-H_2^+$-LGAGGAGVL-OH (0.031 mmol) in dimethyl sulfoxide (DMSO; 30.6 μL). The reaction mixture was kept at room temperature for 14 d, and water was then added to the obtained oily residue, to give a white solid that was separated by centrifugation and analysed by MALDI/MS (Figure 1), which showed only cyclic derivatives (80% yield).

CD Measurements: Circular dichroism spectra were recorded in a cylindrical cell of path length 0.1 cm with a Jasco J-600 dichrograph. The sample concentration was 0.1 mg/mL. The data are expressed in terms of $[\Theta]_M$, the molar ellipticities (per heptameric or nonameric unit) in units of $^\circ$ cm^2 dmol^{-1}.

FT-IR Measurements: Fourier-transformed infrared spectra were recorded with a Nicolet 5PC spectrophotometer at 4 cm^{-1} resolution using 400 scans. Samples were examined as KBr pellets (1 mg/100 mg). The deconvolution of the Amide I band was performed by using the LabCalc software.

NMR Spectra: NMR measurements were carried out with a Bruker AM 300 spectrometer. Samples (2.0 mg) were dissolved in 0.5 mL of [D_6]DMSO (Aldrich, Milwaukee, WI), with the exception of the TFA-LGAGGAG-OH, which was studied in $^1H_2O/^2H_2O$ (9:1) with DSS as internal standard. The NOESY experiment was recorded by using the standard Bruker microprogramme, with the time-proportional phase incrementation of the first pulse. The mixing time used was 300 ms. Water resonance was suppressed by presaturation for 1.5 s. Values were recorded with 96 acquisitions per spectrum and 2 K points (2048 bytes) were recorded in t_2 dimension. The t_1 domain was zero-filled, thus optimising the digital resolution. Spectra were Fourier-transformed into ω_2 and ω_1 by $\pi/8$ and $\pi/4$ phase-shifted sine-bell window functions, respectively.

Environmental Scanning Eectron Microscopy (ESEM): Poly(LGAGGAG) was put on a stub with a biadhesive film of graphite to ensure sample adhesion and apparatus conductivity. The analysis was performed with a PHILIPS ESEM XL 30 environmental scanning electron microscope, fitted with a lanthanum hexaboride (LaB_6) filament, at different voltages and different pressures of the chamber.

Transmission Electron Microscopy (TEM): The synthetic polyheptapeptide poly(LGAGGAG) was suspended in "HPLC grade" water to a final concentration of 20 mg/mL. The specimen preparations were performed by two different procedures: i) a carbon-coated grid was put onto a drop of sample suspension for 2−3 min; ii) a drop of sample suspension was deposited on a thin carbon film coated grid and it was left for 10 h at room temperature in a humidified Petri dish. In each case the grids were rinsed with 20 drops of water and then stained with 10 drops of a 2% uranyl acetate solution (pH = 3.5). Specimens were examined on a Zeiss EM 10C electron microscope at an accelerating voltage of 60 KV.

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