CONFORMATION OF BRANCHED POLYPEPTIDES: THE INFLUENCE OF RELATIVE POSITION OF LEUCINE RESIDUES IN THE SIDE CHAINS

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Dedicated to the memory of Dr Karel Bláha.

Branched polypeptides were synthesized with the general formula poly(Lys-(X_i)) or poly(Lys-(DL-Ala_m-X_i)), where X = Leu or D-Leu, $i < 1$, $1 < m < 2$. Coupling of Leu or D-Leu to poly(Lys) was achieved by the active ester method. The short DL-Ala oligomers were joined to the side chains by polymerisation of N-carboxy-DL-Ala anhydride. The CD measurements performed in water solutions of various pH and ionic strength indicated that joining Leu or D-Leu directly to Lys ε-amino groups increases dramatically the helix forming tendency. However, this ability was somewhat less pronounced with the D-enantiomer of Leu in accordance with our previous observations concerning the role of configuration. The polypeptide with short DL-Ala oligomers as outside determinants was investigated extensively even in trifluoroethanol–water mixtures and SDS solutions. No significant conformational influence could be demonstrated by the elongation of the branches with 1–2 DL-Ala residues.

In the last decade we have developed a new group of branched polypeptides based on a poly(L-lysine) backbone[1,2]. The side chains joining the ε-amino groups of lysine have been composed of a DL-alanine oligomer consisting of about 3 residues (inside area) and terminated by one, two or three residues of another amino acid (the outside determinant). The aim of our studies was to elucidate the correlations between the immunological and carrier properties of the polymers and their constitution and conformation.

The constitution of these polypeptides was characterized extensively[3–5]. Since only very limited information has been available in respect of the conformation of any type of branched polypeptides[6,7] a systematic study was started to determine the factors affecting the conformational trends. From 1977, the beginning of these experiments, Dr Karel Bláha honored us with his interest in the problem. In the frame of a long lasting and fruitful cooperation the conformation of the family of

branched polypeptides has been studied by circular dichroism (CD) spectroscopy in Prague and in Budapest and, as a result, the effect of the branch length and the identity and absolute configuration of the branch terminating amino acids was established[8-11]. We are deeply indebted to Dr Bláha for his contribution, for his ingenious, however careful interpretation of experimental results. Besides our memory the common papers – published mainly in the present Journal – will also preserve his excellent scientific achievements.

Recently we have started to investigate further structural problems related to branched polypeptides: the role of a different sequential order of the side chains[12] and the effect of DL-alanine oligomer spacers. N-benzyloxycarbonyl (Z) protected amino acids were coupled by the active ester method to the ε-amino groups of poly(L-lysine) and after removal of Z groups DL-alanine oligomers were grafted to the free ε- and α-amino groups in the usual way by the aid of DL-alanine N-carboxy anhydride. The average number of DL-alanine residues varied in the end product from 1 to 3.

In the present paper we report the synthesis and conformational analysis of branched polypeptides characterized by the formula poly(Lys-(X_i)) or poly(Lys-(DL-Ala_m-X_i)) (ref.[13]), where X = Leu or D-Leu, $i < 1$, $1 < m < 2$. CD measurements were performed in NaCl solution at various pH and ionic strength, in water–trifluoroethanol mixtures and in sodium dodecyl sulfate (SDS) solution at and above critical micelle concentration (model membrane system)[14,15]. The synthesis and conformational analysis of derivatives containing, in their side chains, DL-alanine oligomers with a higher number of residues has been discussed already in a preliminary paper[16] and will be published in detail later, together with various other similar derivatives.

EXPERIMENTAL

Materials: Amino acids used for these studies were purchased from REANAL, Hungary. Benzyloxycarbonyl chloride, pentafluorophenol, N,N'-dicyclohexylcarbodiimide, 1-hydroxybenzotriazole, 4-methylmorpholine were FLUKA products, while pentachlorophenol was purchased from MERCK.

Intermediates: N-benzyloxycarbonylleucine pentafluorophenyl ester was synthesized according to a procedure described in the literature[17] (amino acids are of L-configuration, unless stated otherwise). Further derivatives used in the synthetic procedures were already listed in the previous papers of this series[1,2]. Amino acid derivatives were checked for purity by elemental analysis, IR spectroscopy and by ascending thin-layer chromatography using Merck precoated plates DC-Alufolien Kieselgel 60, solvent system: ethyl acetate–pyridine–acetic acid–water (240 : 20 : : 6 : 11). Melting points were determined by a VED (GDR) NAGEMA-type apparatus.

Poly(Lys).HBr

The synthesis was carried out as reported previously[1] from N^{α}-carboxy-N^{ε}-benzyloxycarbonyllysine anhydride. Conditions for polymerization were chosen to obtain a degree of polymerisation approx. 100 (monomer–diethylamine initiator ratio 1 : 50). Protecting groups were cleaved using

HBr in acetic acid (35%) as confirmed by UV spectroscopy at 254 nm. Ether was used to precipitate poly(Lys).HBr, which was dissolved in water, dialysed extensively against water and isolated by freeze drying. The sample was analysed by sedimentation equilibrium measurements and from data obtained the average degree of polymerization was calculated ($\langle DP \rangle = 92$); the average molar mass, $\langle M_w \rangle$: 19 200 and the relative molar mass distribution: $\langle M_z \rangle / \langle M_w \rangle$: 1·41.

Poly(Lys-(X_i)).HBr X = Leu or D-Leu, $i < 1$

Procedure A. X = Leu: Poly(Lys).HBr (0·5 g, 2·4 mmol) was dissolved in 2 ml distilled water and the HBr content neutralized by the addition of 0·33 ml (2·4 mmol) triethylamine dissolved in 2 ml of dimethylformamide. The stirred mixture was treated with 1·6 g (3·7 mmol) N-benzyloxycarbonylleucine pentafluorophenyl ester (input molar ratio Lys to Z-Leu-OPfp = 1 : 1·5) dissolved in 20 ml dimethylformamide. Stirring was continued at room temperature for 24 h. The solvent was removed in vacuo and the residue triturated several times with ether containing 10% dichloromethane, in order to remove pentafluorophenol and excess active ester. The yield of dried end product was 0·7 g (87%). Cleavage of benzyloxycarbonyl protecting groups was carried out by HBr in acetic acid. The same procedure was applied for all polymers.

Poly(Lys-(Z-Leu_i)) (0·5 g, 1·3 mmol) was left swelling for 3 h with 5 ml glacial acetic acid, followed by the dropwise addition of a 20 times molar excess of HBr in glacial acetic acid (38%) under vigorous stirring at room temperature. Stirring was continued for 3 h. The polypeptide was precipitated with a large excess of ether, filtered and thoroughly washed with ether. The residue, after drying in desiccator (P_2O_5, KOH) was dissolved in water, extensively dialysed against water using Visking casing and finally freeze-dried; yield 0·3 g (75%). The complete removal of protecting groups was checked by UV spectroscopy.

Procedure B. X = Leu or D-Leu: Poly(Lys).HBr (0·5 g, 2·4 mmol) was dissolved in 2 ml distilled water and 0·28 ml (equimolar amount + 5% excess) 4-methylmorpholine dissolved in 2 ml dimethylformamide was added. The stirred solution was treated with 1·85 g (3·7 mmol) N-benzyloxycarbonylleucine pentachlorophenylester and 0·49 g (3·7 mmol) 1-hydroxybenzotriazole dissolved in 20 ml dimethylformamide. Stirring was continued for 12 h. The solvent was removed in vacuo and the residue triturated several times with ether containing 10% dichloromethane. The yield of dried end product was 0·85 g (94%).

After cleavage of protecting groups the yield of purified and freeze-dried polypeptide was 0·59 g (81%). The same synthetic route was applied for the preparation of the D-leucine derivative.

Poly(Lys-(DL-Ala_m-X_i)) X = Leu, $i = 0{\cdot}82$ $1 < m < 2$

Procedure C: Poly(Lys-($Leu_{0.82}$)).HBr (0·25 g, 0·83 mmol) was dissolved in 2·5 ml distilled water and neutralized by equimolar (0·11 ml) triethylamine. To the solution obtained in this way 0·19 g (1·66 mmol) N-carboxy-DL-alanine anhydride dissolved in 0·7 ml dioxane was added. Vigorous stirring was continued for 45 min. The reaction mixture was kept at room temperature for 24 h and afterwards dialysed against distilled water for several days using Visking casing. Finally, the filtered clear solution was freeze-dried. Yield 0·22 g (80%). Characterization of branched polypeptides is presented in Table I.

Constitution of Branched Polypeptides

Amino acid analyses were carried out on a Chinoin Model OE 975 analyser. The samples were subjected to hydrolysis with 6M-HCl in sealed tubes at 105°C for 24 h. Complete removal of protecting groups from polypeptides was controlled by the ultraviolet and infrared absorption

spectra using Specord UV VIS and Specord IR spectrophotometers. The N-terminal residues of the side chains were identified from the hydrolysates of the dansylated polypeptides by HPLC (ref.[3]). Similarly HPLC analysis was applied[5] after derivatization of hydrolysates with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) to check whether racemization occurred during coupling of active esters to lysine ε-amino groups.

HPLC separations were performed on a laboratory assembled instrument whose principal components were: a reciprocating piston pump (Type 1515; Orlita, Giessen, FRG), a sample injector (Model 7011 loop injector, Rheodyne, Berkeley, USA) and a variable-wavelength ultraviolet monitor with a flow cell having a pathlength of 10 mm and a volume of 10 μl (Model 212, Cecil, Cambridge, Great Britain). Details of the experimental procedures were reported[3,5]. Sedimentation analysis was carried out in a MOM 3170 ultracentrifuge, as previously described[1].

Conformation of Branched Polypeptides

Circular dichroic spectra were recorded using a Roussel–Jouan Dichrographe No III in cells of optical paths of 1·0, 0·2, 0·1, 0·05 and 0·02 cm or a Jobin–Yvon Dichrographe No V in a cell with an optical length of 0·01 cm at 22–25°C. The samples were dissolved either in 0·0085 to 0·025M-SDS or in 0·02M-NaCl and the pH was adjusted by the addition of 0·1M-NaOH or 0·1M-HCl. Ionic strength was adjusted by the addition of 5·0M-NaCl. The concentration of solutions was about 0·3 mg/ml. Polypeptide solutions in water–trifluoroethanol mixtures were prepared by dissolving the sample in 0·02M-NaCl and adding the appropriate amount of TFE. The pH was adjusted before TFE addition. Spectral data in figures and tables are presented in molar ellipticity values ($[\Theta]$ deg cm^2 dmol^{-1}). The $[\Theta]$ values were calculated for one lysine residue of the main chain carrying a whole side chain[8–11].

RESULTS AND DISCUSSION

Our previous results based on CD measurements, demonstrated that the helix forming capacity of branched polypeptides depends markedly on the structure of the side chains[8–12]. In the case of polypeptides containing branches built up of a sequence of three DL-alanine residues and one L-leucine or D-leucine residue at their N-termini significant differences were found in chiroptical properties. The CD spectrum of poly(Lys-(Leu$_{0.76}$-DL-Ala$_{3.0}$)) (LAK) in 0·2M-NaCl, pH 7·3 has the characteristic features of the spectrum of α-helix[9]. Under identical conditions poly(Lys-(D-Leu$_{0.9}$-DL-Ala$_{3.0}$)) (D-LAK) was found to be unordered[10,11]. To analyse the influence of the side chain structure – specially the role of the DL-alanine oligomer spacer – onto the conformation of branched polypeptides, further model compounds were selected. To this aim polypeptides were synthesized containing no DL-alanine oligomer spacers in the branches, but only one L-leucine or D-leucine residue or 1–2 DL-alanine residues coupled to L-Leu, corresponding to a reversed sequence.

N-Benzyloxycarbonylleucine has been previously coupled to poly(Lys) via its nitrophenyl ester[7]. In our experiments we also applied the active ester method, however, we favoured the use of pentafluorophenyl (Procedure *A*) and pentachlorophenyl esters. Besides a high degree of coupling to lysine ε-amino groups the purification of the resulting branched polypeptides proved to be easier. The highest degree of

coupling could be achieved by adopting the method of König and Geiger[18], i.e. by applying the combination of the active ester derivative and an equimolar amount of 1-hydroxybenzotriazole. This method gave satisfactory results even with the less reactive, but more economical pentachlorophenyl ester derivatives (Procedure *B*). No racemization was observed during the synthesis of these polypeptides. The synthetic polymers were characterized by various methods and their properties are summarized in Table I.

The CD measurements performed at various conditions were used to classify the polypeptide conformations as either ordered (helical) or unordered. As helical we denote such conformation whose CD spectrum is similar to that of helical poly(L-Lys). In the uncharged state (i.e. alkaline solution) poly(L-Lys) assumes α-helical conformation. The respective CD spectrum is characterized by two negative maxima of about the same intensity at 221 and 208 nm. In neutral and acidic solutions the ε-amino groups of poly(L-Lys) are in the charged state and the backbone of the polymer adopts an essentially unordered structure. The respective CD spectrum is characterized by a weak negative and a weak positive maxima at 218 and 214 nm, respectively, and a strong negative maximum at 199 nm (refs[19–21]). Conformations whose CD spectra were similar to that of the charged poly(L-lysine) was considered unordered. The CD data for branched polypeptides are summarized in Table II and in Figures 1–3.

All new polypeptides studied show a strong tendency to form α-helical structures. The CD spectra of the polypeptide containing L-leucine residue coupled directly to the poly(L-lysine) backbone (poly(Lys-($Leu_{0.97}$))) in a charged state (0·02M-NaCl, pH 3) show α-helical conformation (Table II, Fig. 1). The spectral characteristics of this polypeptide under these conditions are similar to those of poly(Lys(X_i)) (X = Phe or Leu) in distilled water reported by Annand et al.[7]. Increasing the ionic

TABLE I
Characterization of branched polypeptides

Polypeptide	Preparation procedure	Ratio of amino acids			$\langle M_w \rangle^a$ (±5%)
		Lys	*i*	*m*	
poly(Lys-(Leu_i))[b]	*A*	1	0·82	—	27 700
poly(Lys-(Leu_i))[b]	*B*	1	0·97	—	29 600
poly(Lys-(D-Leu_i))[b]	*B*	1	0·98	—	29 600
poly(Lys-(DL-Ala_m-Leu_i))	*A*, *C*	1	0·82	1·5	30 100

[a] Calculated from the average degree of polymerization of poly(Lys) and the side chain composition based on amino acid analysis; [b] isolated as hydrobromide.

strength, the α-helical content slightly increases (Table II). At pH 7·3 the CD spectrum is almost identical to that found in acidic solution (Table II). In 0·2M-NaCl, pH 7·3, no significant conformational change occured (Table II), in 2M-NaCl, pH 7·3 some polypeptide precipitation was observed. The increase of the pH value leads also to a precipitation.

TABLE II
Characteristic values of CD spectra of branched polypeptides

pH	NaCl mol l^{-1}	λ, nm ([Θ] . 10^{-3})				
		max	cross	max	min	max
		poly(L-Lys(D-Leu$_{0.98}$))				
3	0·02			200 (−29·5)	215·5 (−6·34)	224·5 (−7·22)
7·2	0·02		200	208 (−40·6)	215 (−32·2)	222 (−34·6)
3	0·2		200	206·5 (−43·0)	215 (−31·9)	220 (−31·9)
7·2	0·2		200	208 (−41·6)	214 (−31·2)	220 (−33·5)
3	2·0		201·5	206 (−46·2)	213·5 (−35·5)	220·5 (−37·8)
7·2[a]	2·0		206	208·5 (−17·4)	211·5 (−17·3)	224·5 (−30·4)
		poly(L-Lys(L-Leu$_{0.97}$))				
3·1	0·02		201·5	207·5 (−34·0)	213 (−29·8)	221·5 (−34·4)
7·3	0·02		201·5	208·5 (−34·1)	213 (−31·6)	220·5 (−34·0)
3·1	0·2		201·5	207·5 (−36·4)	211 (−33·8)	222 (−38·2)
7·3	0·2		201·5	209 (−33·0)	214 (−29·7)	220·5 (−32·6)
3·1	2·0		201·5	209 (−43·1)	211 (−37·3)	221·5 (−44·6)

TABLE II
(*Continued*)

pH	NaCl mol l^{-1}	λ, nm ($[\Theta] . 10^{-3}$)				
		max	cross	max	min	max
		poly(L-Lys(DL-$Ala_{1,5}$-L-$Leu_{0,82}$))				
2·8	0·02	191·5 (74·8)	200	207 (−36·3)	213 (−31·4)	220 (−36·2)
7·2	0·02	191·5 (75·0)	200	207·5 (−36·6)	215 (−31·2)	220 (−35·7)
2·8	0·2	192 (75·0)	201	206·5 (−35·0)	212 (−30·4)	220 (−35·1)
7·2[a]	0·2	195·5 (53·9)	201	207·5 (−25·7)	208 (−24·1)	222 (−30·7)
2·9	0·02 +25% TFE[b]	191 (101·9)	201	208 (−54·4)	214 (−45·1)	222 (−47·8)
2·9	0·02 +50% TFE	191 (90·4)	201	208·5 (−47·8)	213·5 (−38·9)	220·5 (−42·4)
2·9	0·02 +75% TFE	192 (73·3)	201	207·5 (−39·3)	214 (−32·9)	221·5 (−36·4)
—[c]			201	208·5 (−31·0)	214 (−21·2)	221 (−25·0)
—[d]			201	206·5 (−32·6)	214 (−24·3)	221 (−26·2)

[a] Slightly turbid solution, [b] TFE — 2,2,2-trifluoroethanol; [c] 8·5 mM-SDS (sodium dodecyl sulphate) in water; [d] 25·0 mM-SDS.

The CD spectrum of poly(Lys(D-$Leu_{0,98}$)) in the charged state (pH 3·1) in 0·02M-NaCl (Table II, Fig. 1) corresponded to the unordered conformation, but at 0·2M-NaCl an α-helical structure was formed (Table II). At high ionic strength (2M-NaCl) no further significant conformational change occured (Table II). The shape of CD spectra in 0·02 or 0·2M-NaCl at pH 7·3 corresponded also to an ordered sterical arrangement (Table II). Similarly to poly(Lys(L-$Leu_{0,97}$)) an increase in ionic strength to 2M-NaCl or an increase in pH resulted in a partial precipitation.

The branched polypeptide containing L-leucine coupled directly to poly(L-lysine) backbone exhibited α-helical conformation even in a charged state at low ionic

strength. The α-helix forming capacity of leucine is probably due to its hydrophobic nature and it is mostly pronounced when the leucine residue is bound directly to the L-lysine which also contains a long hydrophobic chain. The branched peptide which contain D-enantiomer of leucine has a slightly lower tendency to form α-helix than the corresponding peptide containig the L-enantiomer. However, in comparison with poly(L-lysine), its α-helix forming potential is higher confirming the important role of hydrophobic residue in the branches. The CD spectra of α-helical conforma-

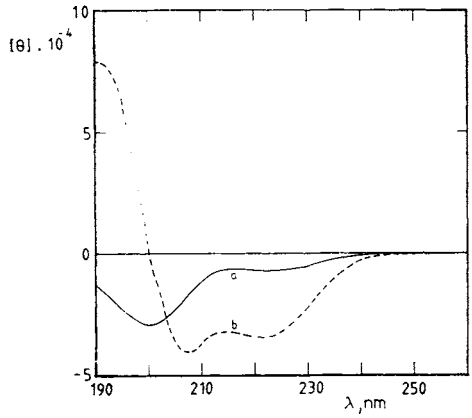

FIG. 1

CD Spectra of poly(L-Lys-(D-Leu$_{0.98}$)); a 0·02M-NaCl, pH 3·0, b 0·02M-NaCl, pH 7·2

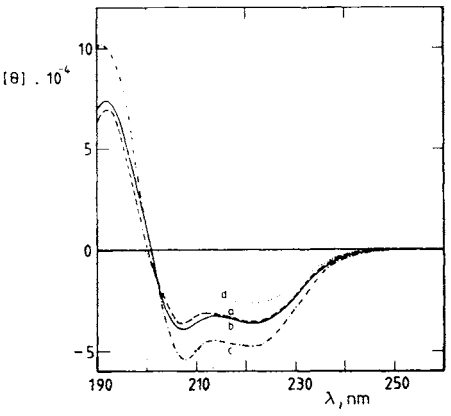

FIG. 2

CD Spectra of poly(L-Lys-(DL-Ala$_{1.5}$-L-Leu$_{0.82}$)); a H_2O, b 0·02M-NaCl, pH 2·9, 75% TFE, c 0·02M-NaCl, pH 2·9, 25% TFE, d 0·025M-SDS

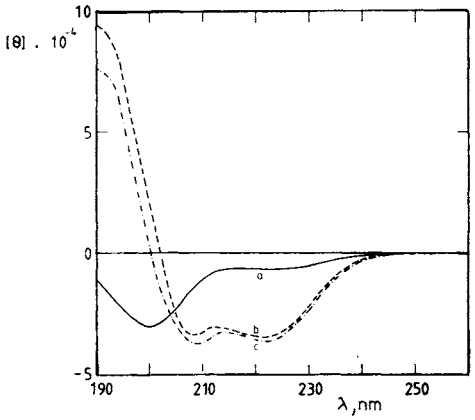

FIG. 3

CD Spectra of a poly(L-Lys-(D-Leu$_{0.98}$)); b poly(L-Lys-(L-Leu$_{0.97}$)) and c poly(L-Lys-(DL-Ala$_{1.5}$-L-Leu$_{0.82}$)) in 0·02M-NaCl, pH 3·0

tion of both branched peptides containing L- or D-leucine are almost identical, which indicates that the L-lysine residues in the backbone contribute to the CD spectrum more than the residues present in the branches. All these observations are in good agreement with our earlier findings[10] concerning leucine containing polypeptides (LAK or D-LAK) in which DL-alanine oligomers were inserted between the poly(L-lysine) backbone and the side chain terminating leucine residues. However, the peptides with DL-alanine inserts exhibit lower tendency to form α-helix[10] than the peptides containing leucine directly coupled to the poly(L-lysine) backbone.

The polypeptide containing 1–2 DL-alanine coupled to L-leucine in the branches, (poly(L-Lys(DL-$Ala_{1.5}$-$Leu_{0.82}$))) was analysed in detail. At pH 2·8 in 0·02M-NaCl, the CD spectrum of this peptide suggests the presence of α-helix (Table II, Fig. 2). The effect of pH on the CD spectrum at low and at medium ionic strength (0·02M- and 0·2M-NaCl) (Table II) is analogous to that observed with the polypeptide containing only L-leucine. An increase of ionic strength to 2·0M-NaCl and an increase of pH causes also precipitation. CD spectrum in 0·0085M-SDS (Table II, Fig. 3) indicates the presence of an α-helical conformation, but the intensity of both bands is lower than in distilled water. The effect of the increase of SDS concentration is negligible (Fig. 2). The shape of the CD spectra in trifluoroethanol–water mixtures corresponds to the α-helical conformation (Table II, Fig. 2). The α-helix content in 25% trifluoroethanol is higher than in distilled water (Fig. 2). An increase in trifluoroethanol concentration from 25% to 50% and from 50% to 75% results in a small but significant decrease of the amplitude of CD bands. Similar behavior was found earlier also for some branched polypeptides containing DL-alanine oligomers inserted between the poly(L-lysine) backbone and side chain terminating residues[9].

The results of CD spectra show that the α-helix forming ability of branched peptides containing leucine is not lowered by the presence of 1–2 DL-alanine residues at the end of the branches in contrast to the case when DL-alanine is inserted between the leucine and the backbone[10]. The charged α-amino group is situated at the end of the branches in both peptides containing DL-alanine while in the case of peptide without DL-alanine the charge is nearer to the backbone. It could be expected that the tendency to form α-helix will be higher in the cases where the distance of positive charges from the backbone is higher. Since experimental results are not in agreement with this suggestion we can conclude that the distance of charged groups from the backbone is not the main factor which influences the conformation i.e. that the repulsive force of charges is compensated by another interaction. The distance of hydrophobic leucine residue from the backbone is identical for the peptide without DL-alanine and for peptide with DL-alanine at the end of the branches. Since these peptides exhibit similar α-helix forming capacity, the distance of hydrophobic groups from the backbone seems to play an important role in conformational behavior of branched polypeptides. The CD spectra of these peptides in ordered α-helical

form are not significantly affected by the presence of DL-alanine residues at the end of the branches which suggests that the contribution of DL-alanine to CD spectrum is negligible probably due to compensation of contributions of D- and L-residues.

The results of the present experiments demonstrated that the helix forming capacity of poly(L-lysine) could be increased dramatically by coupling of leucine directly to the backbone. This effect is probably due to the presence of highly hydrophobic residues in the branches in the vicinity of poly(L-lysine) backbone. The ability to form ordered structure is slightly more pronounced in the case of polypeptide containing L-enantiomer, confirming the importance of the configuration of the amino acid in the side chain. The tendency to form an ordered helical structure is not influenced by the elongation of the branch with 1–2 DL-alanine residues.

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