THE CONFORMATION OF C-TERMINAL TRIPEPTIDES OF NEUROHYPOPHYSIAL HORMONES. PROTON MAGNETIC RESONANCE AND CIRCULAR DICHROIC STUDIES*

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Proton magnetic resonance (^{1}H-NMR) and circular dichroic (CD) spectra were measured on *a*) two homologous series of vasopressin C-terminal tripeptide-amides containing in position 8 α,γ-diaminobutyric acid, ornithine and lysine in L and D configurations, and *b*) two oxytocin C-terminal tripeptide-amides containing either glycine or alanine in position 9. The ^{1}H-NMR measurements suggested a similar spatial arrangement of the peptide chain in all the studied substances. In solutions of L-Pro-L-Leu-Gly-NH_2 there was no sign of the conformation which the tripeptide takes on in the crystalline state. CD spectra of the tripeptides depended upon the length of the side chains in position 8. This relationship was more marked in all-L-tripeptides, and this is interpreted as a result of interaction of side-chains with the region of the proline amino acid residue.

Considerable attention has been devoted recently to studies of the conformation of both parts of the oxytocin and vasopressin molecules, the twenty-membered heterodetic cycle and the linear tripeptide side chain[1-6]. In the latter case interest has been focused so far on the oxytocin sequence, which has been claimed to be a natural factor inhibiting the release of α-melanotropic hormone[7-9].

Our studies on the structure – activity relations of vasopressins provided two series of vasopressin-(7–9)-tripeptide amides containing in position 8 α,γ-diaminobutyric acid, ornithine and lysine in both L- and D-configurations. ω-Amino functions of the basic amino acids were protected by the benzyloxycarbonyl group. Our preceding communication reported on thermal stability and behaviour in mass spectrometry of these peptides. In the present study we have measured the ^{1}H-NMR and CD spectra in order to obtain information on their conformation in solution and to compare them with the analogous oxytocin peptides.

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EXPERIMENTAL

Material and Methods

Diastereoisomeric pairs of the C-terminal tripeptide-amides of vasopressin were as follows: L-Pro-L-Dab(Z)-Gly-NH_2 (*I*), L-Pro-D-Dab(Z)-Gly-NH_2 (*II*), L-Pro-L-Orn(Z)-Gly-NH_2 (*III*), L-Pro-D-Orn(Z)-Gly-NH_2 (*IV*), L-Pro-L-Lys(Z)-Gly-NH_2 (*V*), L-Pro-D-Lys(Z)-Gly-NH_2 (*VI*), and the oxytocin C-terminal tripeptide-amides: L-Pro-L-Leu-Gly-NH_2 (*VII*) and L-Pro-L-Leu-L--Ala-NH_2 (*VIII*), all of which were prepared as described previously[10].

The proton magnetic resonance spectra of tripeptides *I*—*VIII* were measured on a Varian HA-100 (100 MHz) spectrometer in approx. 0·2M solutions in a mixture of hexadeuteriodimethyl sulphoxide and deuteriochloroform (1 : 1), with tetramethylsilane as an internal reference, at 30°C. Assignment of signals to individual amino acid residues was carried out by means of double resonance experiments in the usual manner and on the basis of characteristic chemical shifts and shapes of proton signals in some structural fragments. Chemical shifts and coupling constants were determined by first order analysis.

Circular dichroic spectra were measured in a Roussel-Jouan Dichrographe CD 185, in cuvettes of optical length 0·5 and 0·1 cm, at 24°C, unless presented otherwise. The tripeptide solutions were about 0·002M. Experimental data are presented in values of molar ellipticity (deg. cm^2 . . $dmol^{-1}$) and are not corrected for the index of refraction of the solvent. The methanol used was of spectroscopic grade (Lachema, Brno), the hexafluoroacetone trihydrate was used without further purification (Hynes Chemical Research Co).

RESULTS AND DISCUSSION

The characteristic parameters of the ^{1}H-NMR spectra of tripeptides *I*—*VIII* are summarized in Table I. Comparison of these values shows a striking similarity in the chemical shifts and coupling constants of protons in different positions in the peptide chain over the entire group of substances. From this it would appear that the peptide chains of the above tripeptides in solution have a very similar spatial arrangement. None of the ^{1}H-NMR spectra contains double signals of protons of the amino acid residues which would suggest the presence of slowly interconverting conformers. For tripeptide *VII* in the crystalline state, a conformation has been found[4] containing a ten-membered ring closed by a hydrogen bond between *trans*--glycine-amide NH and the proline carbonyl. The same conformation has been suggested[1] for this substance also in solution (dimethyl sulphoxide) on the basis of an interpretation of the non-equivalence of glycine-amide protons in the ^{1}H-NMR spectra. An important potential source of stereochemical information contained in the ^{1}H-NMR spectra — the coupling constants (primarily $J_{NH,\,C^\alpha H}$), the temperature coefficients or exchange rates of NH protons with deuterium — were not taken into consideration at the same time. This prompted us to discuss these parameters in the present series of substances, including tripeptide *VII*. The values of $J_{NH,C^\alpha H}$ of the N-terminal residue (proline) are not observable (apparently because of the rapid exchange of proline NH with water[1]). The values of the middle residue and the C-terminal residue are accessible, and in the whole series of tripeptides practically

do not change; for the 8-position residue $J_{NH,C^{\alpha}H} \approx 8{\cdot}0$ Hz; for the 9-position residue $J_{NH,C^{\alpha}H} \approx 5{\cdot}5$ and 5·5 Hz (Gly) and 7·3 Hz (Ala in *VIII*). Assuming sufficiently limited internal motion of tripeptides *I*–*VIII* in the given temperature range, it is possible to characterize the spatial arrangement in solution in terms of a preferred conformation, defined by torsion angles ϕ, ψ, ω, χ. From the relationship $J_{NH,C^{\alpha}H} = f(\phi)$ (ref.[11]) there arise possible approximate values of ϕ: for the 8-position resi-

TABLE I

Characteristic Parameters of ^{1}H-NMR Spectra of Tripeptides

Peptide	1. residue[a]			2. residue					3. residue		
	C_{α}—H	C_{β}—H[b] C_{γ}—H[b]	C_{δ}—H	N—H	C_{α}—H	C_{ω}—H	C_{ω}—NH[c]	$CH_2(Z)$ $C_6H_5(Z)$	N—H[c]	C_{α}—H	$CONH_2$
				J[d]			J[e]			J[d]	
I	3·63	1·45–2·15	2·87	8·20	4·34	3·11	7·04	5·04	8·16	3·72	6·96
				8·0			5·5	7·33	5·5		7·25
II	3·64	1·45–2·15	2·88	8·20	4·36	3·13	6·97	5·03	8·15	3·74	6·87
				7·9			5·5	7·33	5·5		7·23
III	3·63	1·25–2·20	2·89	8·14	4·32	3·07	6·98	5·04	8·11	3·60	6·84
				8·0			5·5	7·32	5·5	3·82	7·14
IV	3·62	1·25–2·15	2·88	8·13	4·29	3·06	7·02	5·04	8·07	3·60	6·88
				~8·0			5·5	7·32	5·5	3·80	7·17
V	3·60	1·05–2·15	2·83	8·19	4·28	2·99	7·22	5·04	8·26	3·66	7·06
				8·4			5·5	7·35	5·6		7·30
VI	3·62	1·05–2·15	2·88	8·12	4·24	3·02	6·94	5·03	8·06	3·60	6·89
				7·8			5·5	7·35	5·5	3·80	7·16
VII	3·62	1·20–2·30	2·86	8·09	4·30	0·87	—	—	8·15	3·55	6·94
				8·0		0·90	—	—	5·5	3·77	7·17
VIII[f]	3·63	1·15–2·20	2·88	8·08	4·33	0·88	—	—	7·93	4·29	6·86
				8·0		0·92	—	—	7·3		7·17

[a] The signal of NH proton of the proline residue was not observed. [b] In the given range of δ-values signals of the protons of the second residue are also present which lie in β- or a more distant position from both nitrogens of this residue. [c] The NH proton occurs as a triplet; separation of lines is given as $J_{NH,CH}$. [d] $J_{NH,C^{\alpha}H}$. [e] $J_{NH,C\omega H}$. [f] The methyl group of the alanine residue gives a doublet at 1·27 p.p.m. and $J_{C^{\alpha}H,C^{\beta}H} = 6{\cdot}9$ Hz.

due in L-configuration (−100°, −140°), in D-configuration (+100°, +140°); for the 9-position residue (Gly) (+80°, −80°), (Ala) (−90°, −150°). Values of $\phi \approx -150°$ along with values of $\psi \approx +150°$ (for residues in D-configuration $\phi \approx +150°$, $\psi \approx -150°$) characterize an antiparallel β-chain[12]. For this conformation conformational energy calculations carried out on model N-acetyl-N'-methylamides of amino acids[12] show local energy minima in most amino acids with the exception of Gly. On the other hand, in the region of values of $\phi \approx -100°$ (in the L-configuration) and $\phi \approx +100°$ (in the D-configuration) local minima do not occur. Consequently, of alternative values for residues at position 8 we prefer $\phi \approx -140°$, +140° respectively (for L- or D-configurations) and for angles ψ (on which we have no direct information from ^{1}H-NMR spectra) we assume values close to +150° or −150° respectively, in agreement with the energy calculations. The values determined in this work of $\phi \approx +80°$ or −80° for Gly, with corresponding values of $\psi \approx -40°$ and +40° respectively, characterize two symmetrical, energetically most probable conformational types of Gly as H-7 and H-7' (ref.[12]). The same conformation can also be assumed by the alanine residue in substance *VIII* (with $\phi \approx -90°$).

Comparison of ϕ-values determined here with values from X-ray diffraction analysis of the crystalline hemihydrate *VII* (ref.[4]) (Leu: $\phi = -61{\cdot}2°$, Gly: $\phi = +71{\cdot}8°$) shows a relatively good agreement in the case of Gly, but a markedly different value of ϕ in the central residue. If the same conformation in solution is preferred as that in the solid phase, the central residue should show a value of $J_{NH,C^{\alpha}H} \approx 4$ Hz (instead of the observed 8 Hz). The difference in $J_{NH,C^{\alpha}H}$ is so striking that it excludes an important population of "the crystalline conformation" in solution.

Measurement of the exchange rate of NH protons of tripeptide *VII* with deuterium did not show any significant differences. Since it is known that this method is not always sufficiently sensitive for detection of weak hydrogen bonds in oligopeptides, we also measured the temperature dependence of the ^{1}H-NMR spectra of tripeptides *I*, *V* and *VII* in hexadeuteriodimethyl sulphoxide, over the range of +30° to +75°C. Again we observed approx. the same behaviour of all three studied peptides. With increasing temperature, all the observable NH protons shifted upfield and the widths of signals increased. The other protons showed only minimal changes in chemical shifts and in signal shapes. Repeated measurements gave the same results and excluded the possibility of chemical change of the substances in the temperature range under observation. The signals of the carboxyamide protons of Gly in the temperature range +50° to +60° collapse into a single wide signal, which at the higher temperature narrows. This demonstrates thermally induced exchange of both carboxamide protons, produced by rotation about the C_{α}—C' glycine residue bond. The remaining NH protons shifted approximately linearly upfield with increasing temperature, so that the coefficient $\Delta\delta_{NH}/\Delta T$ of the middle residue in all cases was less than that of the C-terminal residue or C_{ω}—NH (0·1 − 0·3 Hz . deg^{-1} as opposed to 0·4 to

0·6 Hz deg^{-1}). These results are in disagreement with the previously suggested conformation[1] containing a ten-membered ring closed by a hydrogen bond between *trans* glycine-amide NH and the proline carbonyl. Temperature dependence of NH protons showed that only the NH proton of the central residue could be bound by an intramolecular hydrogen bond (either five-membered on the carbonyl group of the same residue or in *I* to *VI* on the carbonyl of the protecting benzyloxycarbonyl group) or was at least markedly shielded from solvent (which can result from steric hindrance by the side-chain of the same residue and the proline ring). The measurement of the temperature dependence therefore did not give any clear indication of the existence of a preferred conformation with intramolecular hydrogen bonding in solution. This could be related either to the fact that the solvation effect results in a stabilisation of a different conformation (less compact) than the energetically preferred conformation in crystals or that there is an equilibrium between several preferred conformations with short life-times (in relation to the ^{1}H-NMR time scale) and therefore to a general flexibility of the studied tripeptides in solution. Measurement of spin-lattice relaxation times T_1 in ^{13}C-NMR spectra led to the conclusion[2]

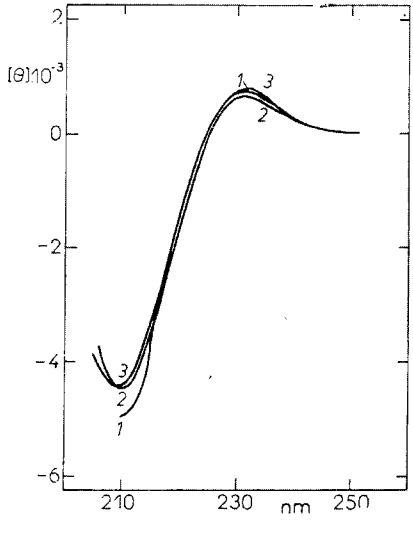

FIG. 1

Circular Dichroism of Tripeptides in Hexafluoroacetone Trihydrate

1 L-Pro-D-Dab(Z)-Gly-NH_2, *2* L-Pro-D--Orn(Z)-Gly-NH_2, *3* L-Pro-D-Lys(Z)-Gly--NH_2.

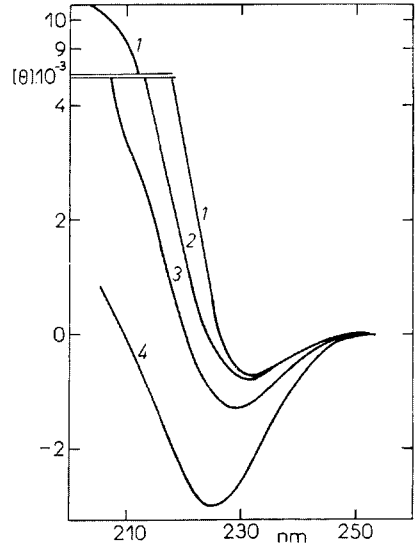

FIG. 2

Circular Dichroism of Tripeptides in Hexafluoroacetone Trihydrate

1 L-Pro-L-Leu-Gly-NH_2, *2* L-Pro-L--Dab(Z)-Gly-NH_2, *3* L-Pro-L-Orn(Z)-Gly--NH_2, *4* L-Pro-L-Lys(Z)-Gly-NH_2.

that tripeptide *VII* was relatively compact in D_2O. Conformational energy calculations for the same peptide have shown[3], however, that there can exist at least three compact conformations with an energy difference of less than 1 kcal mol^{-1}. None of these conformations, nor any combination of populations of such conformations, can explain the observed values of $J_{NH,C^{\alpha}H}$. More detailed studies of the effect of solvent on the conformation of tripeptides *I* – *VIII* are made difficult by the relative insolubility of the substances in non-polar solvents.

The CD spectra of tripeptides *I* – *VII* in hexafluoroacetone trihydrate and in methanol are shown in Figs 1 – 4. The measurements cover only the region of the $n-\pi^*$ transitions of the amide groups. At lower wavelengths it was not possible to obtain reliable data because of the unfavourable ratio of the high absorbance of the aromatic protecting groups to the low intensity of dichroic bands. On the other hand, the long wavelength CD bands of the aromatic protecting groups (250 – 260 nm) are very weak (they are manifested only in derivatives containing α,γ-diaminobutyric acid) and are therefore not presented.

Tripeptides with the central amino-acid residue in D-configuration show in hexa-

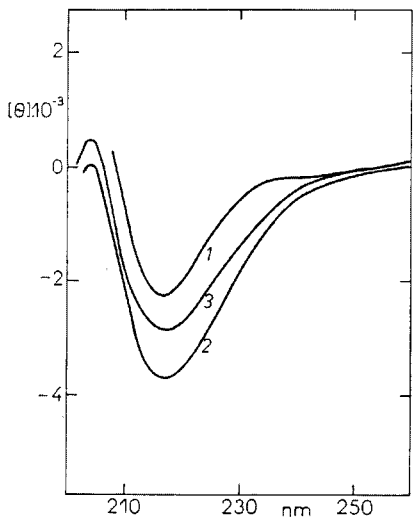

FIG. 3

Circular Dichroism of Tripeptides in Methanol

1 L-Pro-D-Dab(Z)-Gly-NH_2, *2* L-Pro-D--Orn(Z)-Gly-NH_2, *3* L-Pro-D-Lys(Z)-Gly--NH_2.

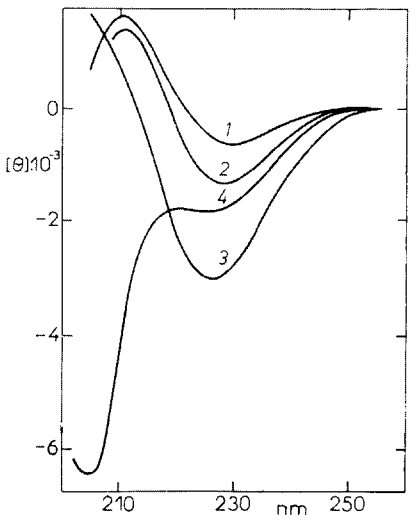

FIG 4.

Circular Dichroism of Tripeptides in Methanol

1 L-Pro-L-Leu-Gly-NH_2, *2* L-Pro-L--Dab(Z)-Gly-NH_2, *3* L-Pro-L-Orn(Z)-Gly--NH_2, *4* L-Pro-L-Lys(Z)-Gly-NH_2.

fluoroacetone trihydrate a CD spectrum such as that shown in Fig. 1. The negative maximum near 210 nm corresponds in position to the $n - \pi^*$ transition of amide groups in a strongly polar solvent[13]. The positive band near 231 nm also lies in the region of the $n-\pi^*$ transition, if we take into consideration that its apparent maximum is strongly red-shifted from the true maximum due to superposition of the more intense negative band. There are, therefore, two $n-\pi^*$ bands differing in energies.

A similar type of CD spectrum is found quite frequently in oligopeptides[14]. It can be explained by the presence of conformers of at least two types. The higher energy negative band can be attributed to solvated amide groups (*e.g.* the hydroxylic solvent is hydrogen-bonded to amide oxygen) and therefore to conformations in which amide bonds are freely accessible to solvent molecules. The low energy band can be attributed to non-solvated amide groups which are found in such a conformation which prevents interaction of amide groups with the solvent.

The spectra of all three substances in Fig. 1 are practically identical and the conformation of the substances is probably very similar in all cases. Steric interaction of the side-chain of residue 8 obviously is not dependent upon its length (we are dealing at most with interaction of β- and γ-methylene groups). In hexafluoroacetone trihydrate, which destabilises intramolecular hydrogen bonds, there is a prevalence in these molecules of an extended, solvated conformation.

A different situation is found in tripeptides with L-amino acids in position 8. Their CD spectra (Fig. 2) in hexafluoroacetone trihydrate show a marked relationship to the length of the side chain of the central residue (including the isobutyl substituent in the oxytoxin C-terminal tripeptide). Just as with the tripeptide of the D-series, here as well there are two $n-\pi^*$ bands. The maximum of the short wavelength positive $n-\pi^*$ band is hidden, since it joins with the more intense positive amide $\pi-\pi^*$ band on the low wavelength side of the spectrum. Only in the case of the ornithine tripeptide the positive $n-\pi^*$ band is observable as a shoulder. (The positive $n-\pi^*$ band can also be seen in the spectra of the same substance in methanol, Fig. 4.) The intensity of the long wavelength negative $n-\pi^*$ band increases and the intensity of the positive band decreases with increasing length of the side-chain of the 8-position residue. This is also manifested by a hypsochromic shift of the negative band in the direction towards the actual wavelength of the transition. The CD curves of all-L tripeptides with short side-chains (curves *1* and *2* in Fig. 2) are nearly mirror images of the curves of the tripeptides of the D series (Fig. 1). This means that the conformations of tripeptides *I* and *VII* resemble the conformations of the D series peptides (*i.e.* primarily extended, unfolded conformations prevail) and steric interactions of short side-chains in the L series are similar to those in the D series, where there is no dependence on the length of the side-chain. In these cases we are dealing apparently only with interaction of the side-chain with the neighbouring amide bonds. The spectra of substances *III* and *V* (Fig. 2) show, however, that in higher

members of the homologous L series other interactions of the side-chains in the 8 position occur with the remnant of the molecule. Since a change in configuration of the central residue does not change quantitatively the spatial relation of its side-chain to the non-chiral glycine-amide part of the molecule, there would appear to be steric interaction with the ring portion of proline. Increasing intensity of the negative long wavelength band indicates an increasing content of non-solvated amide groups. This may be explained by an increasing population of some conformation of the folded type, or a shielding effect of long side-chains.

The second solvent used was methanol, where it can be assumed that weaker intermolecular hydrogen bonds with peptide bonds will be formed than in hexafluoroacetone trihydrate, and on the other hand intramolecular hydrogen bonds will not be interfered with. CD spectra of tripeptides of the D series in methanol are shown in Fig. 3. The negative band at 216·5 nm can again be attributed to the amide $n-\pi^*$ transition. The position of the band does not change with length of the side-chain of the central residue. The intensity of the band changes somewhat but does not simply correlate with this structural parameter. The general similarity of these spectra indicates that the conformation of all tripeptides of the D series is similar also in methanol. Transfer of the D series tripeptides from hexafluoroacetone into methanol brings about a quite marked change in spectrum (Figs 1 and 3) which, however, does not necessarily prove a substantial change in conformation. It can also be explained by a bathochromic shift of the negative $n-\pi^*$ band (corresponding to a solvated amide group) in a less polar solvent. Owing to this shift, there appears a positive band of the amide $\pi-\pi^*$ transition at 204 nm and the positive $n-\pi^*$ band (not influenced by change in solvent) is practically hidden by the stronger negative band; a sign of long wavelength positive band can be seen only in curve *1*, Fig. 3. The D series tripeptides are therefore primarily in unfolded conformation also in methanol. This conclusion is in agreement with the effect of increasing temperature. There is only a certain decrease in intensity of the negative band, but the general nature of the spectrum does not change (measured in substances *IV* and *VI* in the temperature range 15 to 40°). The length of the side-chain of the residue at position 8 does not influence the conformation of the D series tripeptides much more than in hexafluoroacetone, but the inverse order of intensities of the negative $n-\pi^*$ band in ornithine and lysine tripeptides indicates that there is a possible intramolecular interaction of the side-chain conditioned by its optimal length.

The CD spectra of the L series tripeptides in methanol are shown in Fig. 4. As in hexafluoroacetone, one can distinguish two $n-\pi^*$ bands of opposite sign. The intensity of the long wavelength negative band increases and its position is hypsochromically shifted with increasing length of the side-chain of the central residue. In comparison with hexafluoroacetone, the relative intensity of the low energy band is higher in methanol. The change in solvent and the increasing length in side-chain therefore shifts in the L series the conformational equilibrium towards a folded conformation con-

taining non-solvated amide groups. The effect of length of the side-chain of the 8 position residue is considerably stronger than in the D series in the same solvent. In the lysine tripeptide we can even observe a CD spectrum which is qualitatively different from the spectra of the other members of the series (curve *4*, Fig. 3). In the short wavelength portion of the spectrum there appears a very intense negative band of the amide $\pi-\pi^*$ transition, which is superimposed to a marked degree on the positive $n-\pi^*$ band, and the intensity of the negative $n-\pi^*$ band is lower than in the ornithine analogue. It is possible to argue that the peptide chain of the lysine analogue takes on a different conformation from those of the other substances in the L series because of intramolecular interactions of the lysine side-chain (*e.g.* formation of the hydrogen bond from the amide group in the side-chain). Increasing temperature (15–40°C) was manifested in the lysine and ornithine analogues by a relative increase in intensity of the negative long wavelength band and a decrease in intensity of the positive $n-\pi^*$ band, which is in agreement with the view of an equilibrium between solvated and non-solvated conformational forms.

Conclusions from studies of these tripeptides using CD and ^{1}H-NMR spectroscopy can be compared only with caution, since the experimental conditions of measurement were quite different. The character of the solvent (hexafluoroacetone trihydrate and methanol are proton donors, dimethyl sulphoxide is a proton acceptor) and the concentration of the dissolved substances (for CD measurement we used concentrations two orders of ten lower than for NMR measurement) are all factors which can significantly influence the studied parameters of these peptides in solution. The results of both methods identify a conformation of the unfolded type as a preferred one. The ^{1}H-NMR spectra can be satisfactorily interpreted by a single conformational type of peptide chain, but the CD spectra suggest a greater conformational variability of the tripeptides in relation to configuration of the central residue and the length of its side-chain.

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