

C^α-Methyl-L-valine: A Preferential Choice over α-Aminoisobutyric Acid for Designing Right-Handed α-Helical Scaffolds

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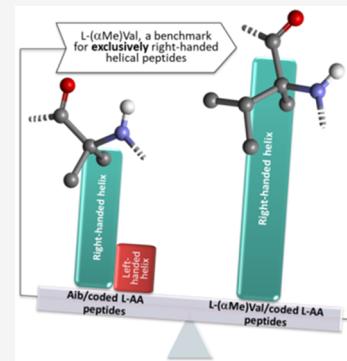
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ABSTRACT: In synthetic peptides containing Gly and coded α -amino acids, one of the most common practices to enhance their helical extent is to incorporate a large number of L-Ala residues along with noncoded, strongly foldameric α -aminoisobutyric acid (Aib) units. Earlier studies have established that Aib-based peptides, with propensity for both the 3_{10} - and α -helices, have a tendency to form ordered three-dimensional structure that is much stronger than that exhibited by their L-Ala rich counterparts. However, the achiral nature of Aib induces an inherent, equal preference for the right- and left-handed helical conformations as found in Aib homopeptide stretches. This property poses challenges in the analysis of a model peptide helical conformation based on chirospectroscopic techniques like electronic circular dichroism (ECD), a very important tool for assigning secondary structures. To overcome such ambiguity, we have synthesized and investigated a thermally stable 14-mer peptide in which each of the Aib residues of our previously designed and reported analogue ABGY (where B stands for Aib) is replaced by C^α-methyl-L-valine (L-AMV). Analysis of the results described here from complementary ECD and ¹H nuclear magnetic resonance spectroscopic techniques in a variety of environments firmly establishes that the L-AMV-containing peptide exhibits a significantly stronger preference compared to that of its Aib parent in terms of conferring α -helical character. Furthermore, being a chiral α -amino acid, L-AMV shows an intrinsic, extremely strong bias for a quite specific (right-handed) screw sense. These findings emphasize the relevance of L-AMV as a more appropriate unit for the design of right-handed α -helical peptide models that may be utilized as conformationally constrained scaffolds.



Although the tertiary and quaternary structures of proteins participate directly in function, their precise arrangements lie on the interactions and assembly of the secondary structures. On the basis of the predominant presence of specific backbone elements, proteins are therefore classified as “all- α ”, “all- β ”, “ α/β ”, “ $\alpha+\beta$ ”, and statistical coil. The 20 coded α -amino acids represent the building blocks of proteins. The legendary comment by C. B. Anfinsen¹ that in proteins the three-dimensional (3D) structural information is embedded within the amino acid sequence, along with statistical and experimental contemporary analyses by Chou and Fasman^{2,3} and Blout and co-workers⁴ indicating that individual amino acids exhibit a preference for characteristic structural elements, has inspired several groups of researchers^{5–10} to investigate the sequence–3D structure relationship in proteins. The aim of those studies has been to design sequences with predefined conformational properties for future applications. Considering the propensities of individual amino acids for a given secondary structure, a chemist can guess the probability of a particular residue to be satisfactorily utilized for the *de novo* planning of a peptide with a well-characterized conformation.

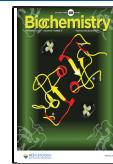
To generate a functionally active peptide with a predetermined 3D structure, one must take care of (i) the type of amino acid units, (ii) the water solubility of the resulting peptides,^{11,12} (iii) the preservation of the L chirality,

(iv) the very limited expectation for alternative 3D structures, and (v) the strong preference for the monomeric state.¹³ In particular, L-Ala as the hydrophobic and L-Lys as the hydrophilic residues stand out as the most utilized, helicogenic, coded amino acids. Accordingly, they have been used to construct relatively short, right-handed helical peptides or even to design small proteins that form helix bundles.^{14–19} The study of such planned secondary structural elements in isolation (in a nonprotein context) would provide an opportunity to measure local versus global interactions that aim to identify what makes them stable when they occur in proteins. Unfortunately, L-Ala-based, synthesized peptides in water show significant helicity only at very low temperatures.^{14,17–20} As often these helical peptides at low temperatures have been used experimentally to derive the helical tendencies of other amino acids,²¹ the conclusions obtained may not properly reflect those physiologically relevant.

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Accordingly, to design synthetic peptides possessing a well-defined helical structure, attempts have been made through incorporation of the strong helix inducer, noncoded, C^{α,a}(*gem*)-dimethylated α -aminoisobutyric acid (Aib),^{22–24} in combination with the coded amino acids.^{25–32} This residue, unlike Ala, can almost exclusively explore regions of the φ and ψ backbone torsion angles of the Ramachandran plot^{23,32} where both 3₁₀- and α -helices are positioned.^{26,32–35} Specifically, to prepare L-Ala rich, relatively short peptides (approximately 10–15 residues long) that would exhibit at least moderate helicity at ambient or higher temperatures, in our previous studies^{30–32} two thermostable model peptides (termed ABK and ABGY, where B stands for Aib) have been designed by insertion of multiple Aib residues. Conformational analyses with chaotropic agents (guanidinium chloride and urea)³⁶ have unambiguously established that the helical stability of the aforementioned L-Ala/Aib mixed peptides at ambient temperature is approximately twice that shown by their related, only L-Ala-based, counterparts at very low temperatures.³¹

However, due to its achiral nature, Aib exhibits an equal preference for the right- and left-handed helices.^{23,32} As a consequence, for example, Aib homo-peptide sequences can easily fold in both enantiomeric helical states (at an equal ratio).^{37–39} This property of Aib not only creates challenges in planning model peptides having helical structures with a specific screw sense but also generates ambiguity in the analysis of the helical conformation in a generic peptide based on chirospectroscopic techniques like the extensively employed electronic circular dichroism (ECD), where the signal intensity, which provides information about the extent of the helicity, might simply arise from the difference in ellipticity of a potential diastereomeric excess.^{40–43} Thus, ECD, a fundamental tool for investigating peptide/protein secondary structures, especially for Ala rich peptides in which, due to resonance overlap, nuclear magnetic resonance (NMR) studies are often quite difficult,^{16,18,19,32} cannot offer the correct global helical content if it originates from admixtures of diastereomeric right- and left-handed folds.⁴⁴

To circumvent this and other types of uncertainties, including that arising from potentially concomitant admixtures of 3₁₀- and α -helices (the relative percentage of which is known to be principally dependent on main-chain length), we have decided that a thermally stable, water-soluble, appropriate model peptide is required to be designed and studied. Moreover, on the basis of our past experience, the most convenient choice is that the three well-separated (at positions 2, 6, and 11) Aib residues occurring in our previously published counterpart ABGY^{31,32,44} should be replaced by the extremely effective helicogenic, C^α-tetrasubstituted chiral C^α-methyl-L-valine (L-AMV), known to be characterized by a remarkable preference for the right-handed helical conformation.⁴⁵ The ECD spectra of the L-AMV peptides are remarkably intense. Available literature results for the L-AMV peptides do not report any evidence for conformations different from α - or 3₁₀-helices.⁴⁶ In particular, neither β -sheets nor any other type of elongated secondary structures has ever been authenticated experimentally. The specific, remarkable conformational rigidity of L-AMV is provided mostly by its isopropyl (in addition to C^α-methylation) side chain with its restricted β -branching character. Peptides from a few additional C^α-methyl-L-amino acids, including in particular C^α-methyl-L-leucine, C^α-methyl-L-phenylalanine, and C^α-methyl-L-

α -aminobutyric acid (also termed isovaline and abbreviated as Iva), have been investigated conformationally, although all of them much less systematically than L-AMV.⁴⁵ In any case, the mixed conclusions from published data point to exclusion of the possibility that peptides rich in these L-AMV congeners might favorably compete with its very strong tendency to fold into the right-handed α -helical structure. It is interesting to note that none of those amino acids is characterized by critical β -branched side-chain restriction.

In this work, we report the chemical characterization and a detailed analysis of the conformational landscape of L-AMV peptide 1, an analogue of the 14-mer ABGY peptide, using classical spectroscopic techniques (ECD and ¹H NMR). To the best of our knowledge, this is one of the few published cases in which L-AMV residues have been inserted into a sequence for a more appropriate design of a water-soluble, thermostable, right-handed, α -helical peptide. Also, this investigation will permit for the first time an appropriate, direct comparison between the conformational tendencies of the Aib and the L-AMV residues because both have been incorporated into the same model peptide template.

EXPERIMENTAL PROCEDURES

Synthesis, Purification, and Characterization. The crude synthetic peptide containing the three L-AMV residues incorporated at positions 2, 6, and 11 of the original 14-mer model ABGY is a (>95% pure) product of USV Custom Peptide Synthesis Ltd. (Mumbai, India). This compound has been synthesized using a few changes from the well-established 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase standard protocol.^{47,48} In particular, due to the presence of the extremely sterically hindered L-AMV residues,^{45,46} during each step involving them a 5-fold excess of the –COOH component Fmoc amino acid has been employed, along with double coupling and extended time in a semiautomated manner to ensure completeness (checked by the Kaiser test⁴⁹).

Purification of the product from the final step has been performed by using the reverse-phase high-performance liquid chromatography (HPLC) technique [with a gradient from 0% to 60% (v/v) acetonitrile/water and a Waters 2487 instrument having a dual λ absorbance detector at 210 and 275 nm (by taking advantage of the presence in the sequence of the chromophoric C-terminal Tyr residue)]. The molecular weight (expected mass of 1415) of the single HPLC peak obtained was determined with an ESI mass spectrometer (obtained mass of 1415). The HPLC and MS figures are available in the Supporting Information.

Circular Dichroism. Temperature-dependent (5–55 °C) far-ultraviolet (far-UV) ECD spectra of the peptide containing the three L-AMV residues in water were recorded with a J-815 ECD spectropolarimeter equipped with a Peltier temperature controller. In addition, temperature-dependent (5–55 °C) spectra have been also measured at varying concentrations of 2,2,2-trifluoroethanol (TFE) in water [0%, 10%, 20%, and 30% (v/v)] and in CH₃CN and methanol (MeOH), as well. All of the TFE-related spectra have been obtained with a 2 mm path-length cuvette, while in CH₃CN and MeOH, a 1 mm path length has been used. The peptide concentration of the 0%, 20%, and 30% TFE solutions is 65 μ M, while for 10% TFE, it is 55 μ M. For the CH₃CN and MeOH solutions, the peptide concentration is 75 μ M. Our aim has been to investigate the effect of the helix-inducing solvent (TFE), as well as of the less polar aprotic (CH₃CN) and protic (MeOH) organic solvents,

on the peptide conformation. ECD data have been recorded in ellipticity (θ , millidegrees) and reported as mean residue ellipticity ($[\theta]$, degree centimeter square per decimole). Singular-value decomposition (SVD) analysis has been carried out to obtain information about the basis spectra of the original set, which will improve our understanding of the conformational transitions. A custom script is written on the basis of the Python programming language using the Scikit Learn library importing the module called Truncated SVD (<https://scikit-learn.org/stable/modules/generated/sklearn.decomposition.TruncatedSVD.html>) to compute the SVD of each set of ECD signals in different solvents. A downloadable software package called CDtoolX, developed by Miles and Wallace⁵⁰ on the C++ Platform, is also used to generate the SVD analysis of the ECD spectral data. After normalization, the SVD plots obtained from both the tools are compared and reported in the Supporting Information.

Nuclear Magnetic Resonance. One- and two-dimensional (2D) ^1H NMR experiments [TOCSY, ROESY, and NOESY (mixing time of 250 ms)] have been performed at different temperatures: (i) on a Bruker DRX 700 MHz (cryoprobe) spectrometer [for fully aqueous conditions and in 20% TFE with 10% (v/v) D_2O with TSP as the internal standard] and (ii) on a Bruker Avance DRX 600 MHz spectrometer (in CD_3CN and CD_3OH with TMS as the internal standard) using standard protocols.⁵¹ Chemical shifts of the samples (1–2 mM) are expressed in δ (parts per million) relative to the respective internal standards. TOCSY experiments have been used to assign each residue, while ROESY experiments, preferred over NOESY experiments for providing clearer signals, have been useful for sequence and conformational assignments. The programs Sparky 3.113⁵² and TopSpin 4.0.7 (Bruker BioSpin) have been exploited to analyze the 2D NMR data. Chemical shift assignments have been a real challenge due to spectral overlap. In particular, Gly12, Gly13, Lys4, Lys7, and Lys9 show very close NH chemical shifts (especially in organic solvents). Nonetheless, assignments are complete. Indicative long-range ROE signals have been observed. The solvents used (CD_3CN , 99.80% D; CD_3OH , 99.50% D) are Eurisotop products.

RESULTS AND DISCUSSION

Reasons for the Choice of L-AMV. Our extensive study of the conformational preferences of chiral C^α -tetrasubstituted α -amino acids, in particular their preferred helix screw sense in peptides, has revealed that in the Cambridge Structural Database (CSD) 194 AMV residues do occur in published crystal structures.⁴⁵ Of a total of 192 residues in dipeptides or longer linear peptides (two are simple amino acid derivatives), 13 D-configured AMVs are found, and all of them folded in a left-handed helical conformation. The remaining 179 are L-AMVs, 171 of which adopt right-handed and eight left-handed helical conformations. This statistical survey clearly indicates that, overall, all AMV residues are helical and the L-enantiomer has a predisposition to adopt the right-handed helical state to a very large extent. The average values for the backbone φ and ψ torsion angles of right-handed helical L-AMV residues are -54.3° and -35.8° , respectively.

Therefore, on the basis of the aforementioned largely unambiguous 3D structural properties of the L-AMV, we have decided to incorporate it into the three strategically located (2, 6, and 11) positions of our extremely promising, terminally blocked, 14-mer, model peptide ABGY,^{31,32,44} where residues

of the achiral Aib, the prototype of this α -amino acid family, have been originally introduced. The sequences of ABGY and L-AMV peptide **1** are given below. The three Lys residues are essential in imparting an acceptable water solubility to these compounds: amino acid sequence of the Aib-based peptide ABGY, Ac-Ala-Aib²-Ala-Lys-Ala-Aib⁶-Lys-Ala-Lys-Ala-Aib¹¹-Gly-Gly-Tyr-NH₂; amino acid sequence of L-AMV peptide **1**, Ac-Ala-L-AMV²-Ala-Lys-Ala-L-AMV⁶-Lys-Ala-Lys-Ala-L-AMV¹¹-Gly-Gly-Tyr-NH₂ (in both sequences, Ac stands for acetyl, the N^α -blocking group).

ECD Spectra of Helical Peptides and Relevant Parameters.

The secondary structures of peptides are responsible for the 3D disposition of the backbone amide moieties. In the solution state, these chromophores are typically characterized by ECD spectroscopy. In the far-UV region, the ECD signature of a right-handed helical structure shows two intense negative bands (at 222 and 208 nm), arising from the $n \rightarrow \pi^*$ and parallel $\pi \rightarrow \pi^*$ excitations of the peptide chromophore, respectively, accompanied by a positive band at ~ 192 nm, which originated from the perpendicular $\pi \rightarrow \pi^*$ excitation. The positive band exhibits an intensity that is significantly higher than those of the negative bands. Thus, for a fully developed, right-handed α -helix, intensity ratio R_2 , $[\theta]_{222}/[\theta]_{208}$ is approximately equal to 1 and intensity ratio R_1 , $[\theta]_{192}/[\theta]_{208}$, is approximately equal to -2 . These two informative parameters can be utilized to distinguish the α -helix from the 3_{10} -helix.^{40,44}

Solvent- and Temperature-Dependent ECD Spectra of L-AMV Peptide **1.** Figure 1 illustrates the temperature-dependent far-UV ECD spectra of L-AMV peptide **1** as a

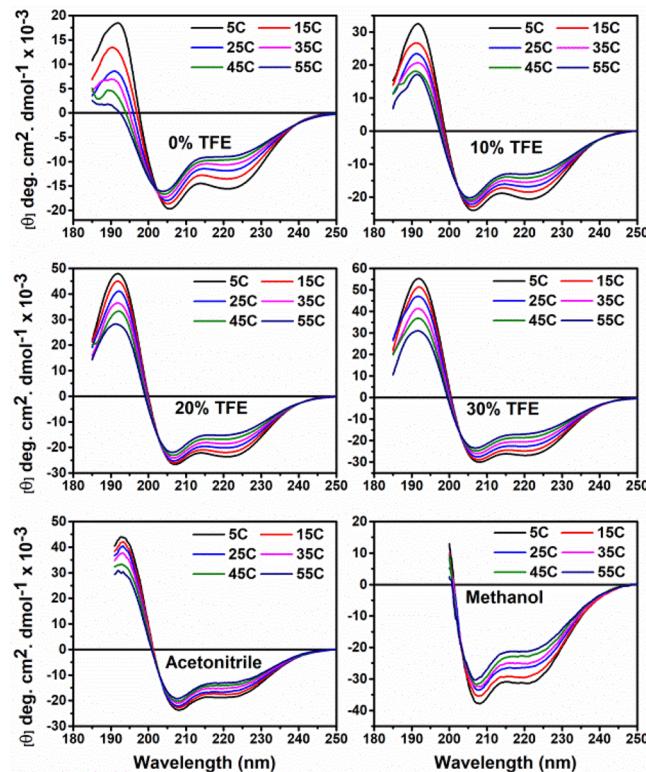


Figure 1. Far-UV ECD spectra of L-AMV peptide **1** in aqueous solution at varying (0%, 10%, 20%, and 30%) concentrations of TFE, in CH_3CN , and in MeOH at different temperatures (5–55 °C). The vertical scales are adjusted to the maximum intensities of the curves.

function of solvent (H_2O , TFE, CH_3CN , and MeOH). The low-noise spectra in the wavelength range of 185–250 nm strongly suggest the remarkable homogeneous nature of the peptide sample under investigation. In all solvents examined, the appearance of two distinct negative ECD maxima (>200 nm) and one positive ECD maximum (<200 nm) (except in MeOH where the curves can be taken to only 200 nm) indicates that under all experimental conditions L-AMV peptide **1** exhibits the typical signature of a right-handed helical screw sense. In TFE/water mixtures, with an increase in TFE concentration (0% → 30%), the ECD curves point toward a significant enhancement of the mean residue ellipticity, $[\theta]$, for all three ECD maxima.

The representative $[\theta]_{222}$ values for L-AMV peptide **1** as a function of increasing TFE concentrations in aqueous solution between 5 and 55 °C are reported in Figure 1. Interestingly, if one takes into consideration the $[\theta]_{222}$ (degree centimeter square per decimole) value of the negative maximum ($-12.5 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$) for its Aib-containing ABGY peptide counterpart (at 30% TFE at 5 °C),⁴⁴ it turns out that it is clearly much lower in absolute magnitude than the corresponding one ($-26.8 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$) seen for L-AMV peptide **1** under the same conditions. Moreover, the magnitude of this negative maximum of ABGY is also lower than that observed for L-AMV peptide **1** ($-15.5 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$) even at 0% TFE and 5 °C. This comparison clearly establishes the conclusion of an enhancement of helicity (negative ellipticity), especially that of the right-handed screw sense, upon incorporation of the L-AMV residues into the peptide. Furthermore, an increase in $[\theta]_{222}$ with the increase in TFE concentration at each particular temperature strongly emphasizes that TFE promotes stabilization of the helical conformation of L-AMV peptide **1**, which is similar to the published observation on the ABGY peptide.⁴⁴ It is worth mentioning that at 30% TFE the ECD signal intensity $[\theta]_{222}$ at 5 °C reaches its maximum for both peptides and that there would not be any significant increase in intensity upon further addition of TFE (data not shown). Such an augmentation of the overall helical structure of the peptide may occur through strengthening of the $\text{C}=\text{O}\cdots\text{H}-\text{N}$ intramolecular H-bonds by desolvation of the backbone upon addition of TFE via participation of nonhelical residues, along with a stabilization of the existing helical stretch^{31,32,44} of the conformation of L-AMV peptide **1** as found in water. With the increase in temperature from 5 to 55 °C at a particular TFE concentration, we have observed a marked decrease in the negative intensity of the bands (at both 222 and 208 nm, especially large at 222 nm) along with a similar decrease in the positive intensity found at 192 nm. In any case, a significant negative intensity at 222 and 208 nm (-9.0×10^3 and $-12.6 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$, respectively) remains even at 0% TFE and 55 °C, which is even higher in magnitude than that obtained for ABGY in 15% TFE at 5 °C. These findings point toward a partial thermal disruption of the helical backbone of the peptide, but because it still retains a substantial helicity even at 55 °C, one is allowed to conclude that the insertion of the L-AMV residues favors a highly thermostable helix for this analogue.

Analogous observations on temperature-dependent ECD spectra and the comparable magnitude of ECD signal intensities at 222 and 208 nm with respect to those obtained in 0–10% TFE are recorded for the L-AMV peptide **1** analogue in both organic solvents examined, namely, CH_3CN

and MeOH (Figure 1). The existence of significant helicity at high temperature (55 °C) in all sorts of solvents examined confirms that the helicity in this peptide is an intrinsic pivotal property of its backbone sequence, as mentioned by Anfinsen,¹ where the nature of the solvent and temperature cannot play the decisive role. This finding can be considered as arising from the conformational rigidity of the L-AMV residue, which forces the sequence deep into the strong helical potential well in the energy landscape. Moreover, during the partial thermal disruption of the helical structure in all solvents, the observation of a single isodichroic point (at approximately 202 nm) (Figure 1) clearly suggests that an equilibrium between two conformational states exists. The appearance of a single isodichroic point in temperature-dependent ECD spectra in peptides based on coded amino acids (e.g., Ala) only³⁰ has been usually interpreted in terms of a two-state helix-coil transition. However, the occurrence of an appreciable helical content (as suggested by the shape of the ECD spectra in Figure 1) even at the highest temperature examined (55 °C) and in 0% TFE (100% aqueous solution) emphasizes that in the observed equilibrium two helical states could be involved (e.g., α - and 3_{10} -helical conformations, or the coexistence of two different types of helical stretches in the same molecule, which cannot be unambiguously determined from the ECD intensities).

Calculation of the Fractional Helical Content (f_H) from the Experimental $[\theta]_{222}$ ECD Value. Mean residue ellipticity $[\theta]_{222}$, obtained from measurements of the ECD spectra, is the parameter usually taken into consideration for calculation of the fractional helicity (f_H) of each peptide conformational state. In fact, it may provide a quantitative estimation of the helicity of peptides. Although short peptides tend to exist as conformational mixtures, the aforementioned appearance of a single isodichroic point for L-AMV peptide **1** under all experimental conditions investigated highlights a transition between two states, where the limiting value of the 100% helical state, $[\theta_H]_{222}$, can be calculated from $43000(1 - x/n)$, where n is the number of residues in the peptide and x is typically taken as 2.5 at 222 nm.¹⁴ From the N-terminus of this peptide, the contribution of a maximum of 12 residues (n) would be considered as helical, due to the presence of two helix-breaking Gly residues near the C-terminus that force the final $-\text{Tyr-NH}_2$ moiety out of the restricted ordered conformation. A simple calculation makes $[\theta_H]_{222} = -34 \times 10^3 \text{ deg cm}^2 \text{ dmol}^{-1}$. Therefore, eq 1

$$f_H = \frac{[\theta_{\text{obs}}]_{222} - [\theta_C]_{222}}{[\theta_H]_{222} - [\theta_C]_{222}} \quad (1)$$

considering the limiting value for the nonhelical (statistical coil) state $[\theta_C] \approx 0$, yields f_H values at 5 °C [0.46 (0% TFE), 0.61 (10% TFE), 0.70 (20% TFE), and 0.80 (30% TFE)] and f_H values at 55 °C [0.27 (0% TFE), 0.38 (10% TFE), 0.46 (20% TFE), and 0.50 (30% TFE)] that are much higher in magnitude than that obtained for ABGY.^{32,44}

Furthermore, in CH_3CN , the calculated fractional helicity f_H is 0.56 (at 5 °C) and 0.38 (at 55 °C), while in the other organic solvent studied (MeOH) it is remarkably higher: 0.92 (at 5 °C) and 0.62 (at 55 °C).

These f_H values, computed from the experimental $[\theta]_{222}$ data, clearly establish that incorporation of the three L-AMV residues provides exceptionally high helicity in the Ala-based peptide even if compared to that of its analogue ABGY,

reinforced by three Aib residues in the same positions of the sequence, as reported previously.^{32,44}

Utilization of the R_1 and R_2 Ratios for the Measurement of Helicity. Mean residue ellipticity $[\theta]$ and the f_H parameter (obtained from the $[\theta]_{222}$ value) can provide the exact quantitative picture of helicity. However, to establish the nature of the helical structure (whether α - or 3_{10} -helical) of peptide molecules, the best option would be the utilization of the ratiometric ellipticity components R_1 ($[\theta]_{\pi\pi^*\perp}/[\theta]_{\pi\pi^*\parallel} = [\theta]_{192}/[\theta]_{208}$) and R_2 ($[\theta]_{\pi\pi^*}/[\theta]_{\pi\pi^*\parallel} = [\theta]_{221}/[\theta]_{208}$), originally proposed by Manning and Woody⁴⁰ approximately 30 years ago. According to their theoretical calculations,⁴⁰ the limiting values of R_2 for α - and 3_{10} -helices would be >0.8 and ~ 0.4 , respectively, while R_1 for the α -helix would be approximately -2 and for the 3_{10} -helix much less negative than -2 . To date, only a few attempts have been made to characterize these two types of peptide helical structures (using ratio R_2).^{43,53–57} Very recently, on the basis of the experimental evidence, for the first time it has been suggested by Banerjee and Sheet⁴⁴ that ratio R_1 can play a decisive role in the characterization of peptide helices (for the ABGY peptide analogue, the R_1 values for α - and 3_{10} -helices would be approximately -1.8 and approximately -1.2 , respectively).

Figure 2 shows the plots of the R_1 and R_2 ratios for L-AMV peptide 1 under the different environments examined (water, aqueous TFE, CH_3CN , and MeOH) as a function of

temperature (5 – 55 °C). Figure 2 also illustrates a comparison between the histograms of the R_1 and R_2 ratios for L-AMV peptide 1 and the ABGY peptide in aqueous solution with an increase in temperature from 5 to 45 °C.

The detailed scenario (Figure 2) of solvent-dependent (0–30% TFE) and temperature-dependent R_2 values, ranging from 0.93 (5 °C, 20% TFE) to 0.71 (55 °C, 0% TFE), and R_1 values, ranging from -1.88 (5 °C, 20% TFE) to -0.13 (55 °C, 0% TFE), clearly establishes that the L-AMV peptide analogue is biased to adopt a helical conformation, the nature of which is predominantly α -helical even at higher temperatures and lower TFE concentrations. However, under fully aqueous conditions (0% TFE), although R_2 ranges from ≈ 0.90 (5 °C) to 0.71 (55 °C), indicative of highly populated α -helical state, the R_1 value, ranging from ≈ 1.10 (5 °C) to -0.13 (55 °C), is skew, not providing unambiguous support for the aforementioned conformational conclusion. However, this effect may be due to dipole–dipole interactions between the polar water and the π – π^* (both perpendicular and parallel) transitions of the peptide.

The linear dependence of R_1 and R_2 with respect to temperature under each condition examined (Figure 2) also confirms the two-state transition. Furthermore, the linear dependence of $\ln R_2$ with respect to $1/T$ (van't Hoff plot) (Figure 2, middle) clearly indicates that in the range of the experimental temperatures considered the process of helix disruption shows an enthalpy that is independent of temperature.

Not surprisingly, an in-depth comparison of the R_1 and R_2 ratios (Figure 2) between peptide 1 incorporating three L-configured AMV residues (L-AMV) and its counterpart characterized by three achiral Aib residues (ABGY) remarkably emphasizes that insertion of the AMV residues of L chirality into the backbone induces an extremely efficient bias toward the right-handed screw sense. Finally, the relatively lower values of the $[\theta]_{222}$ ellipticity and of the related fractional helicity (f_H) in the organic solvent CH_3CN (see above) are overruled by the magnitude of R_2 , and particularly by that of R_1 (Figure 2), which clearly point to the predominance of the α -helical conformation.⁴⁴

Deconvolution of the ECD Data for Conformational Transitions. Deconvolution of solvent- and temperature-dependent ECD spectra can be employed to study two-state statistical transitions. SVD analysis of the temperature-dependent ECD spectra of L-AMV peptide 1 under all solvent conditions investigated (figure in the Supporting Information) has been performed using two methods, based on the Python programming language via the Scikit Learn library (A) and on the software package CDtoolX (B) to identify the basis spectra that would improve our understanding of the conformational transition pathways. Under all experimental conditions examined, whether aqueous or fully organic, by using both methods, our SVD analysis generates two components: SVD1, which has the spectral signature characteristic of a right-handed α -helix, and SVD2, which resembles a statistical coil curve. Overall, these results are indicative of typical helix-to-statistical coil transitions. Not unexpectedly, depending on the nature of the solvent, the limiting $[\theta]$ value varies. Nevertheless, calculations of R_1 and R_2 for SVD1 and SVD2, respectively, undoubtedly point to a transition between two states involving the α -helix and the statistical coil conformation (for SVD1, R_1 ranges from -0.69 in water to -1.75 in CH_3CN and R_2 ranges from 0.76 in CH_3CN to 0.86 in 20% TFE, while

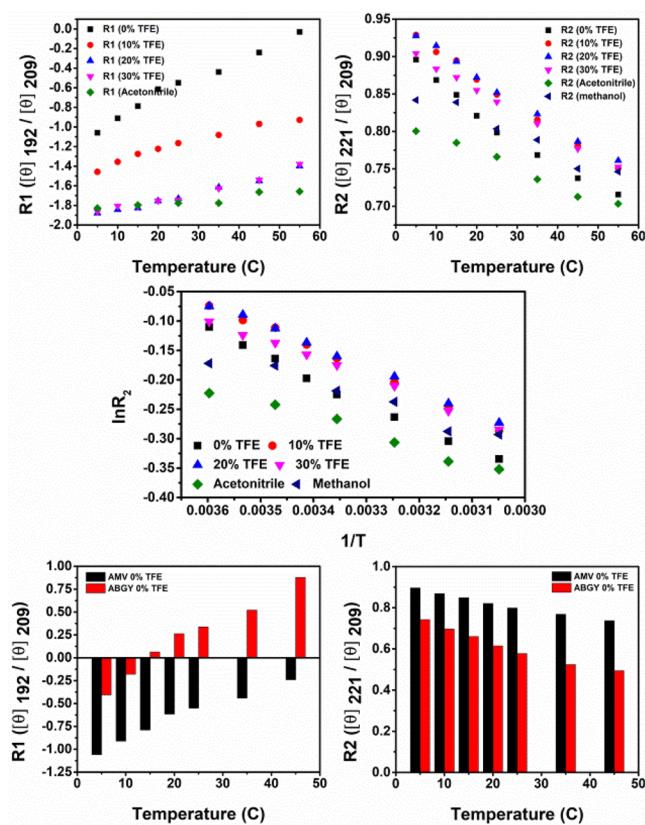


Figure 2. Plots of the R_1 and R_2 ratios (top) for L-AMV peptide 1 in different solutions (0%, 10%, 20%, and 30% TFE, CH_3CN , and MeOH) as a function of temperature in the range of 5 – 55 °C. Linear dependence of $\ln R_2$ (middle) with respect to $1/T$ (van't Hoff plot). Histograms of the R_1 and R_2 ratios (bottom) for L-AMV peptide 1 and the ABGY peptide in aqueous solution (0% TFE) as a function of temperature in the range of 5 – 45 °C.

for SVD2, R_1 ranges from -0.23 in CH_3CN to 1.8 in water and R_2 ranges from 0.29 in water to -1.53 in MeOH). Taken together, these ECD findings well justify our conclusive observation about the largely predominant 3D structural manifestation of the α -helix upon inclusion in this peptide analogue of the three L-AMV residues.

^1H NMR Spectra. Chemical Shifts and $^3J_{\text{N}\alpha}$ Values. L-AMV peptide **1** has been studied by ^1H NMR under different solvent conditions (under fully aqueous conditions and in 20% TFE) and temperatures (from 5 to 50 $^\circ\text{C}$) to investigate its conformational transitions at the residue level, as ECD spectroscopy can indeed describe those changes but only on an average basis. The NH and H^α chemical shift values of each residue have been measured via the respective TOCSY experiments. The chemical shift index (CSI), which measures the directional deviation of the observed H^α chemical shift values from the respective random coil (rc) values, can provide information about the secondary structure of a peptide.^{58,59}

L-AMV peptide **1** under fully aqueous conditions (0% TFE), at both low and high temperatures (5 and 50 $^\circ\text{C}$, respectively), exists predominantly in a helical conformation as judged by the criterion of the observed upfield chemical shift of >0.1 ppm from the respective rc values (negative CSI) of four contiguous H^α protons (Figure 3). Although the negative deviation is

higher) when L-AMV peptide **1** is dissolved in 20% TFE as compared to those in its fully aqueous state (0% TFE) at 5 $^\circ\text{C}$ (Figure 3). From the comparable CSI values at 0% and 20% TFE, one could conclude that, unlike Aib, the inclusion of the L-AMV residues in the sequence of the analogue induces a strong preference for the helical backbone even in the absence of a helicogenic solvent like TFE.

This observation can be further supported by measuring the $^3J_{\text{N}\alpha}$ values (average values of 4.8 Hz for the α -helix, 5.6 Hz for the 3_{10} -helix, and 8.5 Hz for the β -strand) as this comparison offers valuable information about the backbone torsion angle φ for individual residues.⁶¹ Although for short helical peptides an averaging of the $^3J_{\text{N}\alpha}$ values would be expected due to an ensemble of conformational microstates, the temperature-dependent measurements of the $^3J_{\text{N}\alpha}$ values show that at 5 $^\circ\text{C}$ for Ala1–Ala10 they are found in the range of ≤ 5 Hz while at 50 $^\circ\text{C}$ in the range of ≤ 6 Hz. A moderate increase in the $^3J_{\text{N}\alpha}$ values (within the range typical of the helical state) upon warming may reveal helix disruption. However, the observed magnitude clearly justifies the overwhelming existence of the helical conformation for L-AMV peptide **1** over a wide interval of temperatures, as observed from the ECD spectra and the SVD analysis.

Upfield chemical shifts of H^α by >0.1 ppm from the respective rc values (negative CSI) are found throughout the sequence of L-AMV peptide **1** also in different organic solvents (CD_3CN at 5 $^\circ\text{C}$ and MeOH at 25 $^\circ\text{C}$), as observed under aqueous conditions (Figure 3). The magnitude of the absolute deviation (≥ 0.3 ppm) for the H^α chemical shift values is similar, especially in MeOH, when compared to that observed in 20% TFE (5 $^\circ\text{C}$). This result undoubtedly validates the observed ratiometric ellipticities (R_1 and R_2) and calculated fractional helicity f_{H} obtained from the ECD experiments and strongly points to the onset of the helical conformation for L-AMV peptide **1** in all investigated environments.

Further proof of the occurrence of the helical structure could be obtained from the measured $^3J_{\text{N}\alpha}$ values. However, due to an extensive spectral overlap, an unambiguous measurement of the $^3J_{\text{N}\alpha}$ values for all of the residues is not feasible. Nevertheless, in MeOH at 25 $^\circ\text{C}$, it still falls near ~ 4.5 Hz, and in CD_3CN at 5 $^\circ\text{C}$ at ≤ 6 Hz, for the few residues for which the $^3J_{\text{N}\alpha}$ values could be measured.

Solvent Exposure of Amide NH Protons. The nature of a helix, whether α or 3_{10} , can be substantiated by the number of solvent-exposed amide NH protons at the N-terminus of a peptide. To identify these protons, the temperature dependence of amide NH proton chemical shifts ($\Delta\delta/\Delta T$) is typically investigated.^{32,58,60} This parameter provides insight into the type of helix formed from the pattern of H-bonding. Regular α - and 3_{10} -helices exhibit three and two free amide NH protons, respectively, at the N-terminus of a peptide, which are solvent-exposed and found to be H-bonded to solvent molecules. For the intramolecularly non-H-bonded, solvent-exposed state, the reported temperature dependence of the amide NH proton chemical shifts ($\Delta\delta/\Delta T$) is >5.5 ppb/deg, whereas its intramolecularly H-bonded helical states have $\Delta\delta/\Delta T$ values of ≤ 5.0 ppb/deg.³² We found that, in contrast to its Aib (ABGY) analogue under fully aqueous conditions, L-AMV peptide **1** shows that the $\Delta\delta/\Delta T$ values for all of its residues are <4.0 ppb/deg. The exceptions are those of the first three residues (Ala1, AMV2, and Ala3) and of the AMV6 and AMV11 residues, which differ as the Aib residues in the ABGY analogue (Supporting Information). In summary, in the

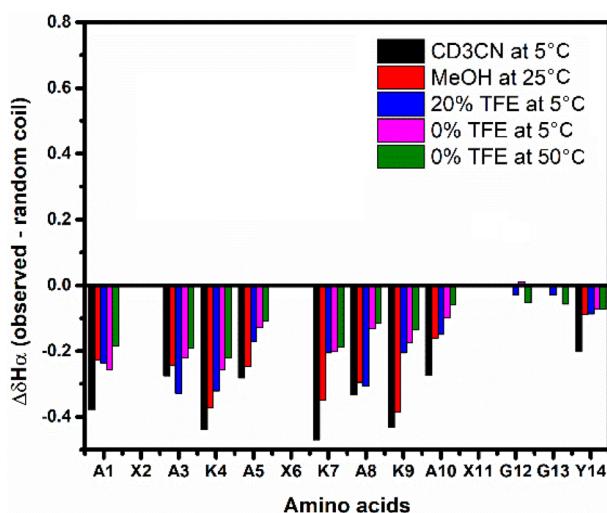


Figure 3. Chemical shift index (CSI) of 14-mer L-AMV peptide **1** under different solvent and temperature conditions.

higher at low temperatures than at high temperatures (a decrease in the negative magnitude at 50 $^\circ\text{C}$ with respect to that at 5 $^\circ\text{C}$), the difference in magnitude is not so significant as that observed for its ABGY analogue. This finding indicates indeed a stronger helical preference at low temperature, but the overall substantial negative CSI even at high temperatures strongly emphasizes the adoption of a thermally stable helical signature by the amino acid sequence. Although the average value of $\Delta\delta \text{ H}^\alpha$ for a helical backbone in proteins is approximately -0.35 ppm,⁵⁸ the comparatively smaller absolute deviations, which are increasingly smaller in its ABGY analogue, could be explained by the presence of a statistical coil state existing in equilibrium (appearance of two states in the SVD analysis of the ECD spectra) with the helical conformer and the solvent-exposed nature of the short monomeric helix.⁶⁰ The absolute deviations for the H^α chemical shift values (negative CSI) are similar (slightly

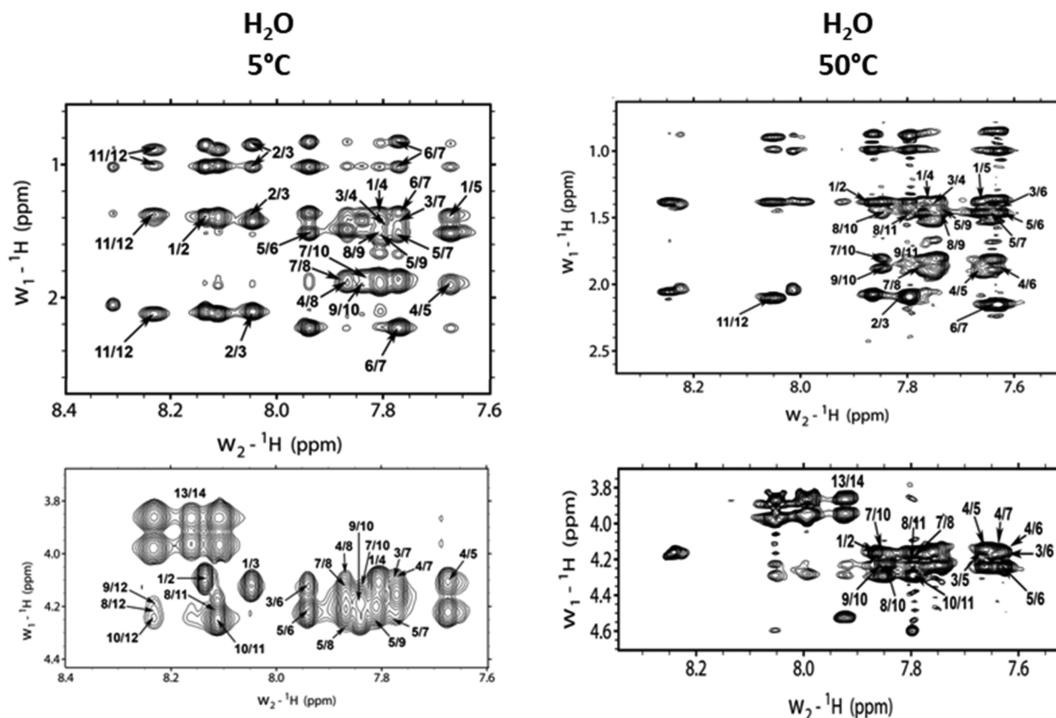


Figure 4. Regions of the ROESY spectrum of 14-mer L-AMV peptide **1** showing the nonsequential NOEs in H₂O at 5 and 50 °C. The long-range $\beta\text{CH}_i \rightarrow \text{NH}_{i+2}$, $\beta\text{CH}_i \rightarrow \text{NH}_{i+3}$, and $\beta\text{CH}_i \rightarrow \text{NH}_{i+4}$ NOEs (top) and $\alpha\text{CH}_i \rightarrow \text{NH}_{i+2}$, $\alpha\text{CH}_i \rightarrow \text{NH}_{i+3}$, and $\alpha\text{CH}_i \rightarrow \text{NH}_{i+4}$ NOEs (bottom) are visible.

sequence from Lys4 to Gly13, the amide NH protons appear to be in a solvent-shielded, intramolecularly H-bonded form. This result represents a clear indication of the attainment of the α -helical structure by L-AMV peptide **1**, unlike the case of its Aib analogue in which only the two N-terminal residues have $\Delta\delta/\Delta T$ values of >5.5 ppb/deg but the third has a temperature-dependent fluctuating behavior.³² These conclusions can be authenticated by a comparison with the reported $\Delta\delta/\Delta T$ values for model rc peptides in water ($\Delta\delta/\Delta T \sim 7$ ppb/deg)⁶² and with those for a helical, 13-residue, lactam-bridged Aib peptide in 0.1 M sodium dodecyl sulfate (average $\Delta\delta/\Delta T$ of ~ 2.5 ppb/deg).²⁹

Nonsequential NOE Cross-Peaks. Nonsequential ROE/NOE cross-peaks provide valuable hints about the conformation of a peptide.⁶³ A considerable number of nonsequential ROE/NOE cross-peaks were observed for L-AMV peptide **1** under different aqueous solvent and temperature conditions (**Supporting Information**). The NH → NH and NH → α/βCH regions of the 700 and 600 MHz ROE cross-peaks (mixing time of 250 ms) are discussed and highlighted in **Figures 4** and **5**. The presence of contiguous NH_i → NH_{i+1} and αCH_i → NH_{i+3} cross-peaks characterizes a regular (α or 3₁₀) helical backbone.⁶³ Furthermore, assignment of βCH → NH ROE/NOE cross-peaks can also be very useful for the conformational analysis of peptides containing α-amino acids that lack the α-hydrogen atom (e.g., AMV). Similar to the αCH_i → NH_{i+3} cross-peak, the helical signature can also be substantiated by the presence of βCH_i → NH_{i+3} cross-peaks along with NH_i → NH_{i+1} cross-peaks.⁶³

Contiguous stretches of $\text{NH}_i \rightarrow \text{NH}_{i+1}$ cross-peaks are observed for almost all residues of L-AMV peptide 1 at low (5 °C) and high (55 °C) temperatures in 0% TFE (Supporting Information). This result demonstrates that even at high temperatures in the absence of a helicogenic agent, due to

presence of the L-AMV residues in the sequence, the corresponding AMV peptide 1 favors a helical conformation. Few $\text{NH}_i \rightarrow \text{NH}_{i+1}$ cross-peaks, which are too close to the diagonal to be resolved, are not identified unambiguously. Several $\text{NH}_i \rightarrow \text{NH}_{i+2}$ cross-peaks are also seen at low temperatures for both 0% and 20% TFE/aqueous conditions. However, the number of $\text{NH}_i \rightarrow \text{NH}_{i+2}$ cross-peaks is decreased when the temperature is increased ([Supporting Information](#)).

Along with the number of nonsequential $\alpha\text{CH}_i \rightarrow \text{NH}_{i+3}$ cross-peaks throughout the peptide at 5 °C under fully aqueous conditions, indicative of a helical backbone for L-AMV peptide 1, a variety of $\alpha\text{CH}_i \rightarrow \text{NH}_{i+2}$ and $\alpha\text{CH}_i \rightarrow \text{NH}_{i+4}$ cross-peaks are also observed for L-AMV peptide 1 under different solvent and temperature conditions. The presence of $\alpha\text{CH}_i \rightarrow \text{NH}_{i+4}$ and $\beta\text{CH}_i \rightarrow \text{NH}_{i+4}$ cross-peaks, in combination with the absence of $\alpha\text{CH}_i \rightarrow \text{NH}_{i+2}$ and $\beta\text{CH}_i \rightarrow \text{NH}_{i+2}$ cross-peaks, characterizes an α -helical backbone, while for the 3_{10} -helix, the reverse is true.^{16,63} In a fully aqueous state and at low temperatures, the onset of a larger number of $\alpha\text{CH}_i \rightarrow \text{NH}_{i+4}$ cross-peaks (at the 3–7, 4–8, 5–9, and 8–12 positions), along with $\beta\text{CH}_i \rightarrow \text{NH}_{i+4}$ cross-peaks (at the 1–5, 3–7, 4–8, and 5–9 positions) and a small number of $\alpha\text{CH}_i \rightarrow \text{NH}_{i+2}$ cross-peaks (only at the N- and C-termini, due to end fraying) (Figure 4), clearly confirmed the predominance of an α -helical backbone and validated the corresponding CD spectrum. However, the reverse is seen at high temperatures. Although at high temperatures the $\alpha\text{CH}_i \rightarrow \text{NH}_{i+4}$ cross-peaks cannot be assigned unambiguously (due to spectral overlap), the appearance of $\beta\text{CH}_i \rightarrow \text{NH}_{i+4}$ cross-peaks (at the 1–5 and 5–9 positions) together with a few $\alpha\text{CH}_i \rightarrow \text{NH}_{i+2}$ and $\beta\text{CH}_i \rightarrow \text{NH}_{i+2}$ cross-peaks (at the 4–6 and 8–10 positions) is indicative of a mixed type of helical structure (Figure 4), although the CD signature and the mean

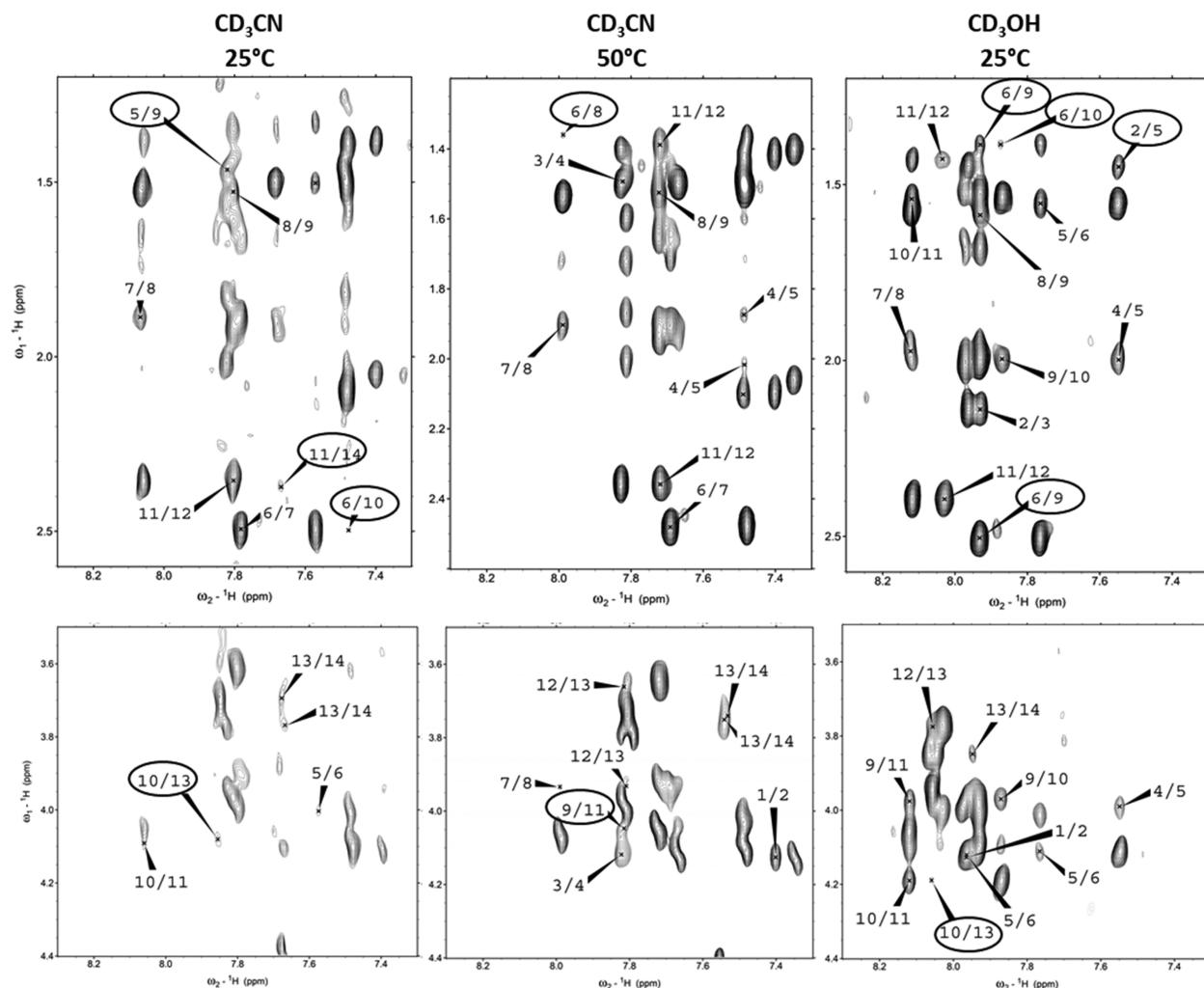


Figure 5. Regions of the ROESY spectrum of the 14-mer L-AMV peptide in CD_3CN at 25°C , in CD_3CN at 50°C , and in CD_3OH at 25°C . The long-range $\beta\text{CH}_i \rightarrow \text{NH}_{i+2}$, $\beta\text{CH}_i \rightarrow \text{NH}_{i+3}$, and $\beta\text{CH}_i \rightarrow \text{NH}_{i+4}$ NOEs (top) and the long-range $\alpha\text{CH}_i \rightarrow \text{NH}_{i+2}$, $\alpha\text{CH}_i \rightarrow \text{NH}_{i+3}$, and $\alpha\text{CH}_i \rightarrow \text{NH}_{i+4}$ NOEs (bottom) are highlighted with ovals.

residue ellipticity ratio point toward a predominant α -helical structure. In 20% TFE, a small number of $\alpha\text{CH}_i \rightarrow \text{NH}_{i+4}$ and $\alpha\text{CH}_i \rightarrow \text{NH}_{i+2}$ cross-peaks is also unambiguously assigned. Moreover, several $\beta\text{CH}_i \rightarrow \text{NH}_{i+2}$, $\beta\text{CH}_i \rightarrow \text{NH}_{i+3}$, and $\beta\text{CH}_i \rightarrow \text{NH}_{i+4}$ cross-peaks are noted for the peptide, although all of them could not be unambiguously identified due to resonance overlap (Supporting Information). At low temperatures, the appearance of $\beta\text{CH}_i \rightarrow \text{NH}_{i+3}$ cross-peaks almost throughout the sequence in 20% TFE suggested that L-AMV peptide **1** adopts a helical conformation. Similar cross-peaks are difficult to identify due to spectral overlap when the peptide is in an aqueous state. Several $\beta\text{CH}_i \rightarrow \text{NH}_{i+4}$ cross-peaks are seen at low temperatures under both full aqueous conditions and in 20% TFE, while only one $\beta\text{CH}_i \rightarrow \text{NH}_{i+2}$ cross-peak is found (between residues 5 and 7). On the contrary, at high temperatures, more $\beta\text{CH}_i \rightarrow \text{NH}_{i+2}$ cross-peaks appear compared to only two $\beta\text{CH}_i \rightarrow \text{NH}_{i+4}$ cross-peaks (between residues 1 and 5 and residues 5 and 9 in 0% TFE; between residues 1 and 5 and residues 3 and 7 in 20% TFE). In a nutshell, from the appearance of characteristic nonsequential ROE cross-peaks, a clear consensus for the α -helical structure could be assigned to the sequence under analysis even in the absence of a helicogenic solvent. This conclusion is

corroborated by considering the R_1 and R_2 ratios obtained from the ECD spectra.

L-AMV peptide **1** has been also studied by ^1H NMR under different organic solvent and temperature conditions. In particular, these experiments have been performed in CD_3CN at 25 and 50°C and in CD_3OH at 25°C . The sequential $\alpha\text{CH}_i \rightarrow \text{NH}_{i+1}$ NOE signals, together with the COSY and TOCSY analyses, allow the full assignment of the spectra. Great support comes from the presence in all spectra of almost all of the sequential $\text{NH}_i \rightarrow \text{NH}_{i+1}$ cross-peaks, which are also indicative of the onset of a helical conformation (Supporting Information).

In addition, in both solvents, a number of sequential $\alpha\text{CH}_i \rightarrow \text{NH}_{i+1}$ NOEs are evident. As illustrative examples, we show the fingerprint region of the peptide in CD_3CN at 25 and 50°C , where several $\alpha\text{CH}_i \rightarrow \text{NH}_{i+1}$ signals are clearly visible. Moreover, at the level of the AMV residues (that lack the αCH proton), $\beta\text{CH}_i \rightarrow \text{NH}_{i+1}$ NOEs are observed in all spectra (Figure 5).

In each CD_3CN spectrum at 25 and 50°C , one long-range NOE suggests the presence of a helical structure. Specifically, the $\alpha\text{CH}_i \rightarrow \text{NH}_{i+3}$ ($10-13$) NOE is seen at 25°C , while the $\alpha\text{CH}_i \rightarrow \text{NH}_{i+2}$ ($9-11$) NOE is observed at 50°C (Figure 5, bottom). In CD_3CN at 25°C , we have evidence of the

presence of the α -helical conformation as we note two $\text{CH}_i \rightarrow \text{NH}_{i+4}$ cross-peaks at the 6–10 and 5–9 positions, and two $\text{CH}_i \rightarrow \text{NH}_{i+3}$ NOEs, compatible with either of the two types of helical structure (10–13 and 11–14). In CD_3CN at 50 °C, we notice a long-range $\alpha\text{CH}_i \rightarrow \text{NH}_{i+2}$ NOE (9–11) and a $\beta\text{CH}_i \rightarrow \text{NH}_{i+2}$ NOE (6–8) that are characteristic of a 3_{10} -helical conformation (Figure 5, central column).

In CD_3OH at 25 °C, we observe a number of $\text{CH}_i \rightarrow \text{NH}_{i+3}$ NOEs (at the 2–5, 6–9, and 10–13 positions) and one $\text{CH}_i \rightarrow \text{NH}_{i+4}$ NOE (6–10), all compatible with the occurrence of the α -helical conformation (Figure 5, right column).

Overall, from our ^1H NMR study, we can reasonably conclude that the 3D structure of L-AMV peptide **1** is helical under all organic solvent and temperature conditions analyzed. The α -helix: 3_{10} -helix ratio varies only a little as a function of the environmental changes. In CD_3CN at 50 °C, our NMR results point to the co-presence of α - and 3_{10} -helices, while in CD_3CN at 25 °C and in CD_3OH at 25 °C, the α -helical conformation seems to largely prevail. The appearance of the 3_{10} -helical state along with the α -helical conformation in a coexisting manner especially at higher temperatures specifically emphasizes the potential role of the 3_{10} -helical state during the onset of the α -helical conformation from the statistical coil state. It should be noted that a more in-depth interpretation of these NMR data is hampered by extensive peak overlapping that will not permit any analysis of molecular dynamics calculations.

CONCLUSIONS

The successful design of a thermostable, short helical peptide formed exclusively by the coded L-amino acids is really a challenging endeavor. However, incorporation of noncoded, helicogenic Aib units into the sequence indeed helps to achieve the desired target, albeit with certain limitations. Although the allowed (ϕ and ψ) main-chain torsion angles of Aib would be fully restricted in the helical region of the Ramachandran plot, due to its achiral nature an inherent, equal preference for both the right- and left-handed helical conformations is induced. In addition, as Aib can adopt both 3_{10} - and α -helices, peptides heavily based on Aib produce an ensemble of canonical microstates, which creates a serious issue to achieve a well-specified helical scaffold, e.g., a right-handed α -helix.

To circumvent the ambiguities arising from the incorporation of Aib, a thermally stable, water-soluble, appropriate right-handed α -helical peptide model was planned by replacing each of the three Aib units of our published 14-mer ABGY by an exceptionally effective, potentially quasi-rigid, helicogenic, L-configured α -amino acid of the C $^\alpha$ -tetrasubstituted Aib class, namely C $^\alpha$ -methyl-L-valine (L-AMV), having a remarkable preference for the right-handed helical conformation. Overall, the findings of our investigation, using complementary spectroscopic tools in aqueous as well as in organic solvents in a temperature-dependent way, clearly emphasize that our hypothesis of multiple L-AMV inclusion is correct in that the designed L-AMV peptide **1** sequence results in an overwhelming α -helical scaffold and the magnitude of its helical contribution is much higher than that exhibited by its ABGY analogue. Moreover, for the adoption of a predominant α -helix even under fully aqueous conditions with a pure two-state transition, our data strongly suggest that the water-soluble, thermostable, right-handed, α -helical model L-AMV peptide **1** template would be much more appropriate to be used as a control for the experimental measurements of the thermody-

namic parameters pertaining to any other amino acid, particularly to those that are physiologically relevant.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.biochem.1c00340>.

MS spectrum and HPLC chromatogram of the 14-mer peptide, additional NMR data, and CD deconvolution spectra (PDF)

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Notes

The authors declare no competing financial interest.

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DEDICATION

This manuscript is dedicated to the memory of our co-author Dr. Raja Banerjee's parents who both passed away during the recent resurgence of the COVID-19 pandemic in Kolkata, India.

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