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Circular Dichroism of Designed Peptide Helices and β-Hairpins: Analysis of Trp- and Tyr-Rich Peptides

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Carefully chosen peptide systems can provide valuable insights into the details of protein folding. Construction of the secondary structures most commonly observed in proteins, namely α-helices and β-sheets, from constrained amino acid residues allows detailed investigation of the factors driving the folding and stability of these structural scaffolds.[1] Structure formation in these cases is driven largely by local nonbonded interactions and backbone hydrogen bonding. Helix design by incorporation of constrained amino acids such as Aib (U, α-aminoisobutyric acid) and its analogues has achieved great success.[2] Early studies of β-hairpin design by using sequences derived from proteins did not yield well-folded structures.[3] Subsequently, hairpin design has been effectively achieved with the use of D-Pro-Xxx sequences as turn nucleators in synthetic oligopeptides. D-Pro-Xxx segments favor the formation of type II′/I′ β-turns, which in turn promote hairpin registry.[4] The role of tertiary (side chain–side chain) interactions that are predominant in protein interiors can now be probed by specifically positioning appropriate residues at desired sites in peptides with predefined structures. Specific aromatic–aromatic interactions have been suggested to be important in stabilizing hairpin structures in aqueous solutions.[5] Study of aromatic interactions also gains significance in the light of β-sheet aggregation, which leads to the formation of amyloids and results in protein aggregation and neurodegenerative diseases.[6]

As part of a program to explore aromatic interactions between side chains appropriately positioned on helical and hairpin scaffolds, we have investigated a series of designed peptides rich in aromatic residues. A prerequisite to the unambiguous characterization of the backbone conformation from spectroscopic data rests in the ability of those data to uniquely relate to the conformation. Electronic Circular Dichroism (ECD), which often provides a quick method to assess the conformation of a designed peptide, is usually inapplicable in the case

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of sequences rich in aromatic residues because of the extensive absorption of aromatic chromophores in the far-UV region. NMR spectroscopy is the method of choice for establishing solution conformation, but can prove time consuming if a large number of peptides are to be investigated. Vibrational Circular Dichroism (VCD) is developing into a powerful technique for peptide conformation analysis,[7] providing an attractive alternative to ECD for peptides with aromatic chromophores.[8] VCD is based on the chiral perturbation of a vibrational transition and measures the difference in the absorbance of left and right circularly polarized light in the infrared region.[7,9] VCD studies have recently been applied to the conformation analysis of peptides with the specific intention of identifying secondary structures like helices and sheets. Several groups have developed VCD applications for the characterization of helices.[7,10] Previous studies have described the use of VCD methods in providing a diagnostic of β-hairpin conformations.[8,11] In this paper, we examine model peptide helices and β-hairpins rich in tryptophan and tyrosine residues (Table 1) and demonstrate that VCD spectroscopy provides a clear diagnostic of backbone structure in cases for which ECD spectra are completely anomalous.

Peptides **1–6** were designed to form β-hairpins by placing a D-Pro-Gly segment at the center of the sequence. Peptides **2–5** formed well-defined hairpins with type II′ turns in organic solvents (Table 1), as determined by 2D NMR spectroscopy (see Supporting Information for a summary of unpublished NMR results). Peptide **6** appeared to form a frayed hairpin structure with a type I′ β-turn. This is presumably because of the bulky Trp residues at positions adjacent to the central β-turn; these might result in interactions between the indole ring and flanking amide bonds that, in turn, influence local conformation.[12] The far-UV ECD spectra of peptides **1–6** in methanol are shown in Figure 1 A and B. Peptide hairpins are generally characterized by a broad, negative ECD band at ~218 nm. Inspec-

tion of the spectra in Figure 1 A and B clearly reveals several features that may be attributed to contributions from the aromatic chromophores, giving rise to anomalous far-UV ECD spectra. The positioning of the aromatic residues influences the interactions between the side chains. For example, in an ideal hairpin, strong cross-strand interactions may be anticipated when aromatic residues are placed at positions 2 and 7 in octapeptide segments.[8,13] Figure 2 A and B illustrates the observed aromatic-proton chemical shifts for the Tyr residue at positions 2 and 7 in peptide **3**. A remarkable upfield shift is observed for the Tyr7 $C\delta H$ proton due to shielding by the ring currents of the facing Tyr2 residue. The temperature dependence means that the effect is substantially more pronounced at low temperatures, at which side-chain conformational exchange is limited. Aromatic interactions are also anticipated for residue pairs 1–3 and 6–8 along the strand segments, as well as 1–6 and 3–8 diagonally across the two strands of the hairpin. Strong aromatic interactions therefore contribute to the far-UV ECD spectra observed for the peptides studied, making unambiguous structural characterization difficult.

Peptides **7–10b** form predominantly helical conformations in chloroform (Table 1), as determined by NMR methods (unpublished NMR data, see Supporting Information). ROESY spectra of the helical peptides carried out in CDCl$_3$ revealed the presence of almost all sequential d_{NN} NOEs. In addition, we obtained medium-to-weak i–i+2 and i–i+3 $d_{\alpha N}$ NOEs; this suggests that the peptides adopted a 3$_{10}$-helical conformation in solution (unpublished). This observation is not surprising, as several Aib-containing short synthetic peptides are shown to adopt a 3$_{10}$-helical conformation in crystals.[14] However, the possibility of facile exchange between the 3$_{10}$- and α-helical conformations must always be considered. Indeed, mixed helical structures are frequently observed in the solid state for hydrophobic helical sequences.[15] Peptide **10a** contains a D-Val residue at position 3, which may be expected to perturb the

Table 1. Peptide sequences, mass spectral data, conformations, and VCD data.

No.	Sequence[a]	Mass [Da] [M+Na]$^+$	[M+K]$^+$	M_{calc}	Solution Conformation[b]	VCD band positions [cm^{-1}][c]
1	Boc-LWV^{D}PGLLV-OMe	1031.8	1047.8	1009	β-hairpin	(−) 1658
2	Boc-WLV^{D}PGWLV-OMe	1105.9	1122.1	1083	β-hairpin	(−) 1649
3	Ac-LYV^{D}PGLYV-OMe	1000.6	1016.8	978	β-hairpin	(−) 1649
4	Boc-YLV^{D}PGWLV-OMe	1081.6	1097.6	1059	β-hairpin	(−) 1651
5	Boc-LLV^{D}PGYLW-OMe	1095.7	1111.8	1073	β-hairpin	(−) 1648
6	Boc-WLW^{D}PGWLW-OMe	1277.8	1293.9	1256	β-hairpin	(−) 1653
7	Boc-WLWUWLW-OMe	1208.7	1224.6	1187	helix	(−) 1678, (+) 1651
8	Boc-UWLWUWLW-OMe	1297.4	1312.9	1273	helix	(−) 1674, (+) 1654
9	Boc-LWVAULWV-OMe	1108.6	1124.5	1084	helix	(−) 1672, (+) 1652
10a	Boc-LW^{D}VUAULWV-OMe	1193.5	1209.6	1169	helix	(−) 1672, (+) 1650
10b	Boc-LWVUAULWV-OMe	1193.7	1209.8	1169	helix	(−) 1670, (+) 1650
11	Boc-WLWUGWLW-OMe	1267.2	1283.9	1244	helix	(−) 1674, (+) 1654

[a] In the peptide sequence, ^{D}P = D-Pro and U = Aib. [b] The solution conformations of all peptides were inferred from NMR data (Bruker DRX 500 MHz NMR spectrometer; all spectra recorded at 303 K) in CDCl$_3$ for peptides **7–10b**, in CDCl$_3$ + 10% [D$_6$]DMSO for peptides **1–6**, and additionally in CD$_3$OH for peptide **3**. Chemical-shift dispersion of backbone NH and C$^\alpha$H resonances as well as $^3J_{NH-C^\alpha H}$ coupling constants were used as diagnostics. β-Hairpins yielded a large chemical-shift dispersion and high (>7.5) $^3J_{NH-C^\alpha H}$ values, while helices had limited chemical-shift dispersion and small $^3J_{NH-C^\alpha H}$ values (≤6). NOEs were used to confirm conformational assignments. Helices yielded a succession of N$_i$H–N$_{i+1}$H (d$_{NN}$) NOEs, while β-hairpins were characterized by the observation of cross-strand NH–NH (1–8, 3–6) and C$^\alpha$H–C$^\alpha$H NOEs (residues 2–7; unpublished data). Complete NMR analysis was carried out for all peptides except **1** and **11**, for which structural information is based on chemical-shift dispersion and $^3J_{N\alpha}$ coupling constants. [c] VCD spectra were recorded in CDCl$_3$.
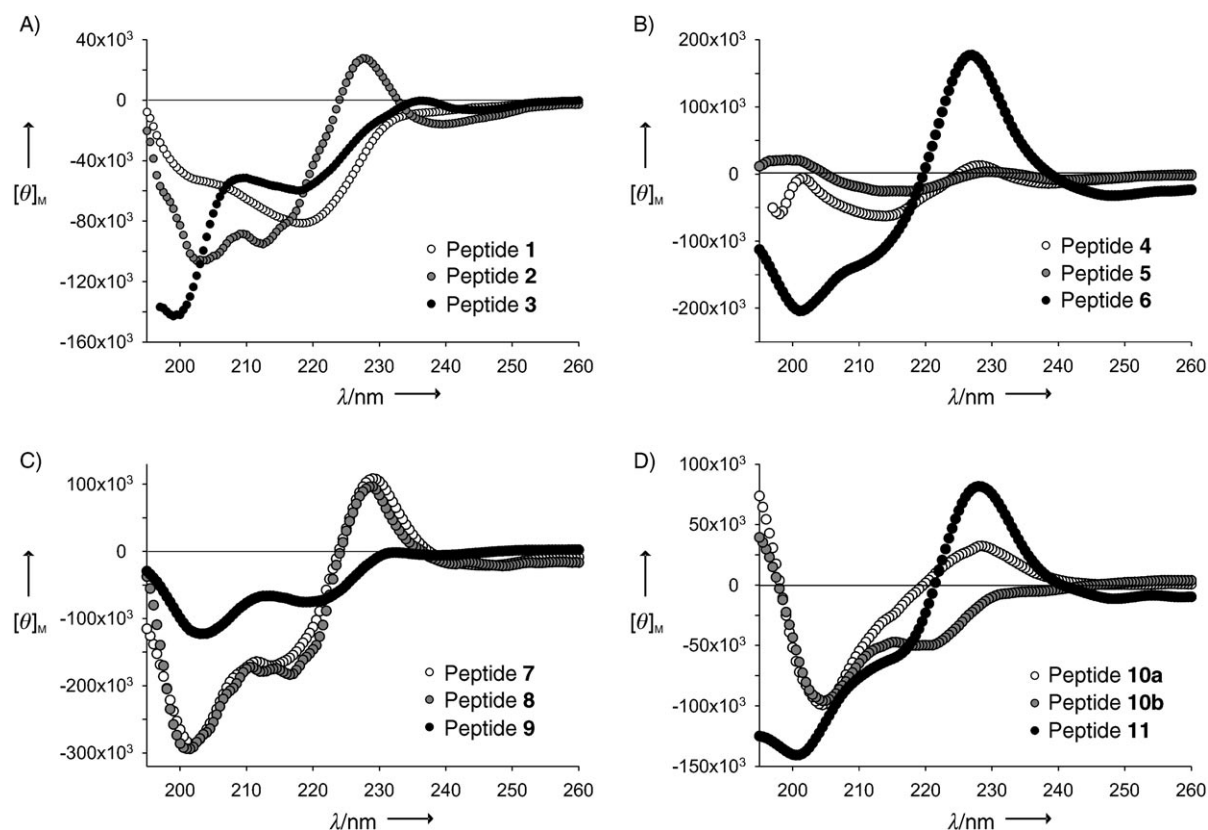
Figure 1. Far-UV ECD spectra of peptide hairpins (A and B) and helices (C and D) recorded in methanol at an ambient temperature of ~298 K. Plot of total molar ellipticities ($[\theta]_M$ /deg cm^2 dmol^{-1}) as a function of wavelength. A) Peptide **1**: Boc-LWV^{D}PGLLV-OMe, peptide **2**: Boc-WLV^{D}PGWLV-OMe, peptide **3**: Ac-LYV^{D}PGLYV-OMe; B) peptide **4**: Boc-YLV^{D}PGWLV-OMe, peptide **5**: Boc-LLV^{D}PGYLW-OMe, peptide **6**: Boc-WLW^{D}PGWLW-OMe; C) peptide **7**: Boc-WLWUWLW-OMe, peptide **8**: Boc-UWLWUWLW-OMe, peptide **9**: Boc-LWVAULWV-OMe; D) peptide **10 a**: Boc-LW^{D}VUAULWV-OMe, peptide **10 b**: Boc-LWVUAULWV-OMe, peptide **11**: Boc-WLWUGWLW-OMe (^{D}P = D-Pro; U = Aib).

helical conformation at the N terminus. Evidence from NMR experiments, however, clearly demonstrates that the overall conformation of the peptide is a helix in solution, with a local distortion in the backbone near the D-amino acid (see Supporting Information). It should be noted that D residues can indeed be comfortably accommodated in right-handed-helix structures, as demonstrated by recent crystal structures of synthetic peptides containing multiple D residues placed in host L-amino acid sequences.[16] Peptide **11** contains a central Aib-Gly segment, which, in principle, can adopt either a type I/III or type I'/III' β-turn conformation. A helical fold of the octapeptide would be supported by type I/III stereochemistry, while a hairpin may be formed with a type I'/III' conformation. The far-UV ECD spectra of peptides **7**–**11** are illustrated in Figure 1 C and D. ECD spectra of helices are generally characterized by negative bands at ~222 and ~208 nm as well as a positive band at ~195 nm. In short helical peptides, which consist of up to 3–4 turns of 3$_{10}$- or α-helices, the relative intensities of the two minima can be significantly different, with the n–π* band at 222 nm exhibiting diminished ellipticity.[15, 17] Inspection of the spectra in Figure 1 C and D reveals anomalous features, specifically, the positive band at ~225–230 nm in peptides **7**, **8**, **10 a**, and **11**. Of the six peptides listed, only two (**9** and **10 b**) yield spectra that resemble those anticipated for short helices.

The far-UV ECD data for the peptides examined in this study suggest that, for both helices and β-hairpins, anomalous far-UV ECD spectra are obtained when the sequence contains one or more Trp/Tyr residues. In the sequences studied, through-space interactions may be expected in a hairpin conformation for peptides **1**–**6**. In the case of the helices, aromatic interactions may be expected when the residues are placed at positions $i/i+3$ or $i/i+4$. This condition is satisfied in sequences **7**, **8** and **11**. In helical structures, variations of the side-chain torsion angles can result in multiple orientations of the projecting aromatic groups, which generally preclude close interactions, such as those observed across antiparallel strands in a hairpin structure. The aromatic chromophores can, however, be sufficiently proximal[18] to contribute to the far-UV ECD spectra, thereby masking transitions of the backbone. Aromatic–amide interactions, observed in several Trp-containing peptides,[12] cause additional absorption effects and complicate the ECD spectrum. The data presented in Figure 1 clearly demonstrate that far-UV ECD does not provide reliable information on peptide conformation when multiple aromatic residues are present. In sharp contrast, VCD spectra provide an unambiguous distinction between the two groups of peptides.

Table 1 as well as Figures 3 and 4 summarize the VCD spectra in the region of the amide I band for peptides **1**–**6** and **7**–**11**. It should be mentioned that although the VCD spectra
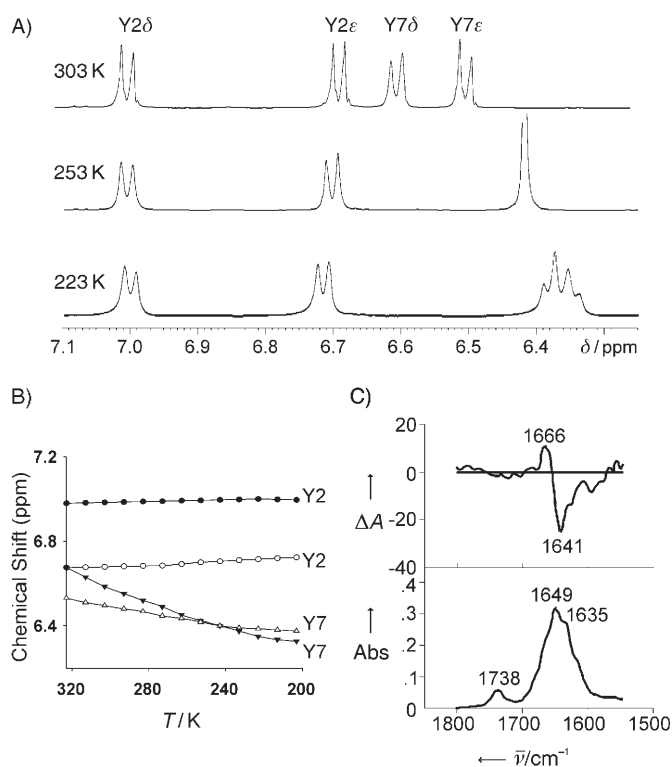
Figure 2. A) Chemical-shift dependence of the aromatic-ring-proton resonances as a function of temperature. Spectra recorded on a Bruker DRX 500 MHz spectrometer with sample concentrations of ~10 mM in CD₃OH. Note the anomalous upfield shift of the Tyr7 CδH protons (*meta* to the phenolic hydroxyl group). B) Plot of the chemical-shift variation of the aromatic resonances in peptide **3** with temperature. C) Vibrational absorption (bottom) and VCD (top; ΔA×10⁶) spectra of peptide **3** in methanol. A negative band at 1641 cm⁻¹, characteristic of β-hairpins, is observed.

were recorded in CDCl₃, the ECD spectra were obtained in methanol, as strong chloroform absorption bands hamper the acquisition of far-UV ECD spectra. However, independent structural information in CDCl₃ was obtained for most of the peptides by using solution NMR methods (some peptide hairpins that showed broad backbone resonances in chloroform at the concentrations used for NMR experiments were recorded in CDCl₃ + 10% [D₆]DMSO; see Supporting Information) and are listed in Table 1. Additionally, 2D NMR experiments were repeated in CD₃OH for one of the peptides (peptide **3**), and the chloroform structure was found to be essentially maintained in this case. For this peptide, VCD spectra were recorded in both chloroform and in CH₃OD.

All the hairpin-forming sequences show a negative VCD band at ~1648–1660 cm⁻¹ (Figure 3), which is associated with a strong IR absorption band, in chloroform. As proposed earlier,[8] this strong absorption band and the associated negative VCD band are thought to originate from β-strands. The spectrum of peptide **3** in methanol (Figure 2C) shows a negative VCD band at 1641 cm⁻¹ accompanied by a broad IR-absorption band centered at 1649 cm⁻¹. The observed VCD bands in CH₃OD are shifted to lower frequencies as compared to chloroform (1649 cm⁻¹). The origin of this shift could be due to solvent polarity and/or H–D exchange, which will influence amide I band positions. The helices, peptides **7–11**, reveal a couplet with a positive component at ~1650–1654 cm⁻¹ and a negative component at ~1670–1678 cm⁻¹ (Figure 4), which is characteristic of a right-handed helical structure. VCD spectra of helical peptides **7** and **8** also showed a positive band at ~1720 cm⁻¹, which might be associated with the carbonyl

Figure 3. Vibrational absorption (bottom) and VCD (top; ΔA×10⁶) spectra of peptide hairpins recorded in deuterated chloroform at 293 K. A) Peptide **1**: Boc-LWVᴰPGLLV-OMe); B) peptide **2**: Boc-WLVᴰPGWLV-OMe); C) peptide **3**: Ac-LYVᴰPGLYV-OMe; D) peptide **4**: Boc-YLVᴰPGWLV-OMe; E) peptide **5**: Boc-LLVᴰPGYLW-OMe; F) peptide **6**: Boc-WLWᴰPGWLW-OMe (ᴰP = D-Pro; U = Aib).
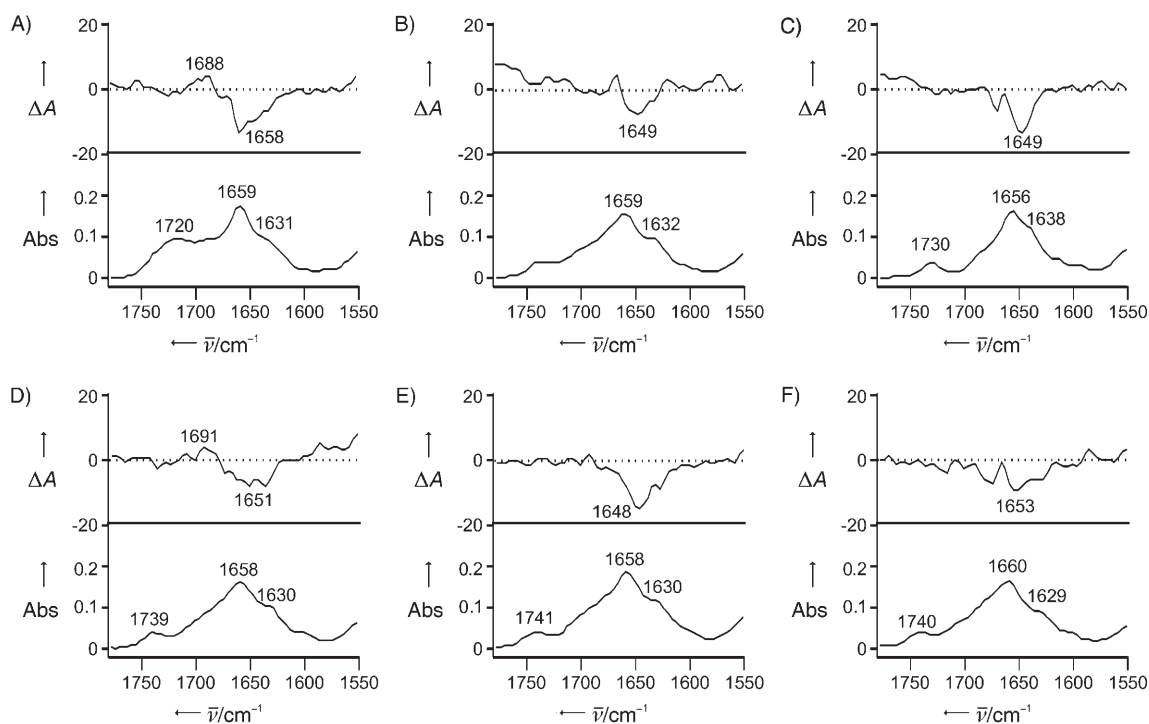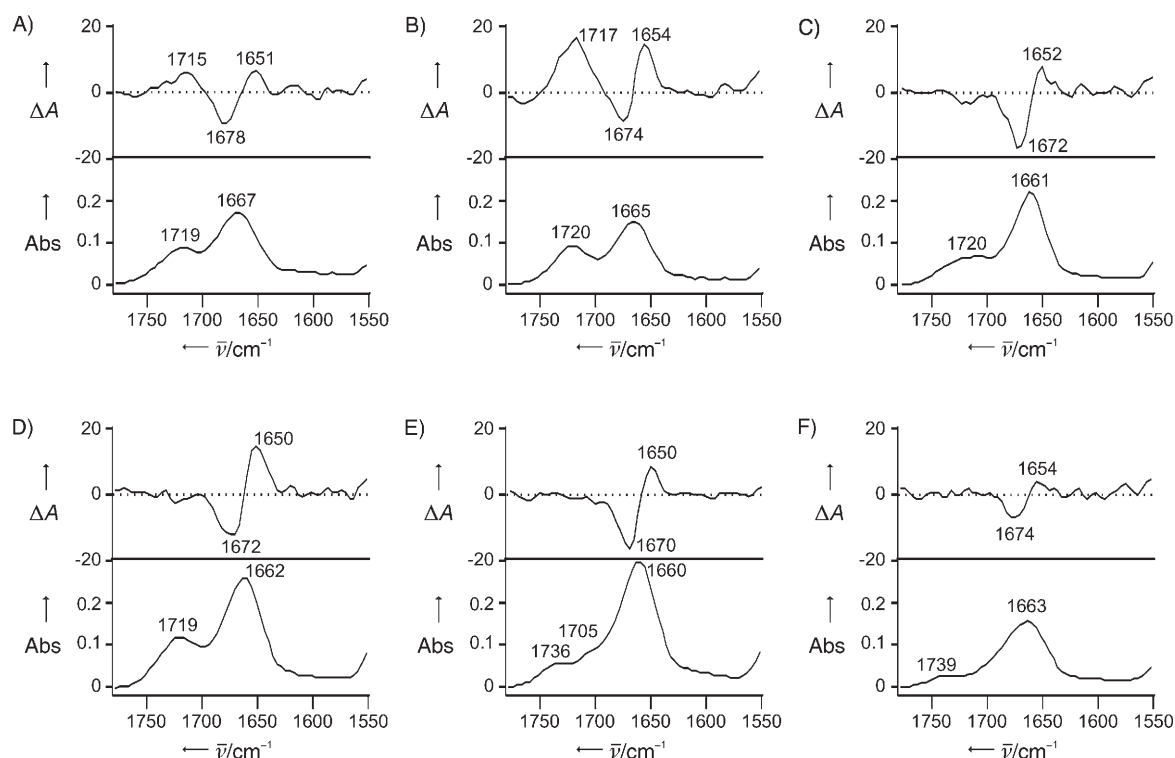
Figure 4. Vibrational absorption (bottom) and VCD (top; $\Delta A \times 10^6$) spectra of peptide helices recorded in deuterated chloroform at 293 K. A) peptide **7**: Boc-WLWUWLW-OMe; B) peptide **8**: Boc-UWLWUWLW-OMe; C) peptide **9**: Boc-LWVAULWV-OMe; D) peptide **10a**: Boc-LW^{D}VUAULWV-OMe; E) peptide **10b**: Boc-LWVUAULWV-OMe; F) peptide **11**: Boc-WLWUGWLW-OMe (^{D}V = D-Val; U = Aib).

moiety of the *tert*-butyloxycarbonyl (Boc) group. The urethane band is seen only in the case of the helical peptides containing four Trp residues; however, with the present data, we find it difficult to explain why this observation is restricted to **7** and **8**. In the case of peptide **10a**, it was interesting to note that despite the presence of a D-amino acid in the sequence, which, in principle, should act as a helix breaker, a characteristic VCD spectrum for a helix is obtained. As mentioned earlier, NMR studies in CDCl₃ support the results obtained from the VCD spectrum of this peptide. It is clear from NMR experiments (see Supporting Information) that although there is a local distortion in the helical scaffold, the D-amino acid is largely accommodated in a right-handed helix. Likewise, the VCD spectrum of this peptide also clearly indicates the presence of a negative band at ~1672 cm^{-1} and a positive band at ~1650 cm^{-1}. We therefore conclude that the presence of a D-amino acid does not necessarily disrupt the overall helical scaffold in all cases, as was demonstrated earlier.[16]

There is a clear distinction between the VCD spectra obtained for the groups of peptide hairpins and helices. Peptide **11**, which can, in principle, adopt either a helical or hairpin conformation, shows clear evidence for the formation of a helix in the VCD spectrum. In previous studies from the laboratory, the sequence Boc-LVVUGLVV-OMe was investigated, and NMR studies support a solvent-dependent helix-to-hairpin transition, forming a helix in non-hydrogen-bonding solvents like chloroform and acetonitrile and adopting a β-hairpin conformation in hydrogen-bonding solvents like methanol and di-

methyl sulfoxide.[19] Such solvent-dependent structural transitions can now be easily characterized by using VCD spectroscopy in cases such as peptide **11**, in which aromatic interactions obscure peptide transitions in the far-UV ECD spectrum.

The peptides investigated in the present study have been designed in order to populate a specific ordered conformation, either a hairpin or a helix. In the sequences that are rich in aromatic residues, VCD has provided clearly identifiable fingerprints in the amide I region for the two kinds of peptide secondary structures examined. Independent evidence for the nature of the preferred conformations obtained from NMR studies (unpublished) correlates well with the VCD data. The use of well-defined model peptides therefore permits the development of VCD methods as a tool in peptide- and protein-conformation analysis.

Experimental Section

Peptide synthesis: All peptides were synthesized by conventional solution-phase chemistry by using fragment-condensation strategy. The Boc group was used for N-terminal protection and the C terminus was blocked as the methyl ester. For peptide **3**, the acetyl group was employed for N-terminal protection. Deprotection of the Boc- and -OMe groups was carried out by using 98% formic acid and alcoholic NaOH, respectively. Couplings were mediated by using dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt). Some of the intermediates were characterized on a TLC plate (silica gel), by 1D ^{1}H NMR (80 MHz) spectroscopy as well as ESI-MS mass spectrometry and used without further purification.

The final peptides were purified by medium-pressure liquid chromatography (MPLC; C_{18}, 40–60 μm) and, wherever required, by high-performance liquid chromatography (HPLC; C_{18}, 5–10 μm, 10×250 mm) with methanol/water gradients. During the synthesis of peptide **10**, the coupling of the 1–3 fragment (Boc-LWV-OH) to the 4–9 fragment (H$_2$N-UAULWV-OMe; U=Aib, α-aminoisobutyric acid) yielded two peptides (**10a** and **10b**) that had identical mass spectra but were separable by MPLC. Analysis of the 500 MHz NMR spectra (1D ^{1}H, 2D ROESY, DMSO titration experiments) clearly established that the two nonapeptides differed in the configuration of V3, racemization having occurred during the activation of the tripeptide. The Val-Aib coupling is known to be difficult and is a slow reaction often resulting in racemization in couplings involving activation of a Val carboxylic acid group, despite the use of racemization-suppressing additives (unpublished results). Racemization was encountered only in the case of peptide **10**.

MALDI-TOF mass spectrometry: Mass spectra of the purified peptides were recorded on a Kompaq Seq MALDI-TOF mass spectrometer (Kratos Analytical, Manchester, UK). Complete characterizations of all the peptides except peptides **1** and **11** were carried out by 2D ^{1}H NMR (500 MHz) spectroscopy (unpublished results). Table 1 lists peptides studied along with data obtained from mass spectrometry studies.

Electronic circular dichroism: Far-UV ECD spectra were recorded on a JASCO J-715 spectropolarimeter with methanol as the solvent. Calibration of the instrument was done by using d-(+)-10-camphorsulfonic acid. A path length of 1 mm for far-UV was employed. Data were acquired in the wavelength-scan mode by using a 1 nm bandwidth with a step size of 0.2 nm and a scan speed of 20 nm min^{-1}. Typically, five scans were acquired, and the data were averaged. Solvent subtraction was carried out by using methanol as a blank, and the spectra were smoothed.

Vibrational circular dichroism: All VCD spectra were recorded on a commercial Chiralir spectrometer (BomemBiotools, Québec, Canada). The peptide (2 mg) was dissolved in CDCl$_3$ (200 μL), and the spectra were recorded in a fixed-path-length (100 μm) cell at 293 K. All spectra were collected for 1 h at a resolution of 8 cm^{-1}, and solvent contribution was subtracted.

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