A Nonhelical, Multiple $\beta$-Turn Conformation in a Glycine-Rich Heptapeptide Fragment of Trichogin A IV Containing a Single Central $\alpha$-Aminoisobutyric Acid Residue

The conformational properties of the protected seven-residue C-terminal fragment of the lipopeptai bol antibiotic Trichogin A IV (Boc-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe) has been examined in CDC$_3$ and (CD$_3$)$_2$SO by $^1$H-nmr. Evidence for a multiple $\beta$-turn conformation [type I' at Gly(1)-Gly(2), type II at Leu(3)-Aib(4), and a type I' at Aib(4)-Gly(5)] suggests that Leu(3) has preferred an extended or semiextended conformation over a helical conformation in CDC$_3$. This structure is thus in contrast to earlier observations of seven-residue peptides containing a single central Aib preferring helical conformations in both solution and crystalline states. A structural transition to a frayed right-handed helix is observed in (CD$_3$)$_2$SO. These results suggest that nonhelical conformations may be important in Gly-rich peptides containing Aib. Further, the presence of amino acids with contradictory influences on backbone conformational freedom can lead to well-defined conformational transitions even in small peptides.

INTRODUCTION

Trichogin A-IV$^1$ a membrane modifying fungal metabolite, isolated from the cultures of Trichoderma longibacterium with the sequence $n$-octyl-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu differs significantly from other channel forming peptaibols.$^{2,3}$ This peptide has a high content of Gly residues and is significantly shorter (11 residues as opposed to 20 residues for alamethicin$^4$ and 16 residues for zervamicin$^5$), in addition to possessing a lipophilic $n$-octanoyl group. While the presence of Aib residues is expected to impose strong stereochemical constraints on backbone folding,$^6,7$ largely stabilizing cylindrical helical structures,$^{8-12}$ the preponderance of Gly residues might be expected to impart appreciable conformational flexibility.$^{13}$ Indeed, the CD spectrum in methanol of a synthetic Trichogin A-IV analogue containing a C-terminal carboxylic acid group was distinctly different from a classical $\alpha$-helical spectrum, having two distinct bands of widely different intensity at 227 and 205 nm (unpublished observations). This intriguing juxtapositioning of
Aib and Gly residues, with contradictory conformational tendencies, prompted us to investigate the structures of Gly-rich, Aib-containing peptides. We describe in this report the multiple conformational states in solution for the protected C-terminal heptapeptide fragment of Trichogin A-IV, Boc-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe (I) and characterize a nonhelical, multiple $\beta$-turn conformation in CDCl$_3$.

**EXPERIMENTAL**

**Peptide Synthesis**

The peptide was synthesized by conventional solution phase methods by using a racemization free fragment condensation strategy. The Boc group was used for N-terminal protection and the C-terminus was protected as a methyl ester. Deprotections were achieved by 98% formic acid or saponification, respectively. All intermediates were characterized by $^1$H-nmr (80 MHz) and thin layer chromatography (tlc) on silica gel and used without further purification. The final peptide was purified by MPLC and high performance liquid chromatography (HPLC) on reverse phase C-18 columns and fully characterized by 400 MHz $^1$H-nmr.

**Boc-Ile-Leu-OMe (II).** 2.3 g (10 mmol) of Boc-Ile-OH was dissolved in 15 mL of dichloromethane (DCM) and cooled in an ice bath. H-Leu-OMe, isolated from 3.64 g (20 mmol) of the hydrochloride by neutralization, subsequent extraction with ethylacetate and concentration (10 mL) was added, followed immediately by 2.36 g (10 mmol) of dicyclohexylcarbodiimide (DCC). The reaction mixture was stirred for 12 h and allowed to come to room temperature. DCM was evaporated, the residue taken in ethyl acetate (60 mL), and the dicyclohexylurea (DCU) filtered. The organic layer was washed with 2N HCl (3 × 50 mL), 1M sodium carbonate (3 × 50 mL), and brine (2 × 50 mL) dried over anhydrous sodium sulphate, and evaporated in vacuo to yield a white solid 2.6 g.

**Boc-Gly-Ile-Leu-OMe (III).** To 2.0 g of Boc-Ile-Leu-OMe, 15 mL of 98% formic acid was added and the removal of the Boc group monitored by tlc. The reaction was worked up after 8 h and removal of the formic acid monitored by tlc. The reaction mixture was stirred for 12 h and allowed to come to room temperature. DCM was evaporated, the residue taken in water (ca. 50 mL) and washed with diethyl ether (2 × 50 mL). The pH of the aqueous layer was then adjusted to 8 with solid sodium bicarbonate and extracted with ethyl acetate (3 × 30 mL). The extracts were pooled, washed with saturated brine, dried over anhydrous sodium sulphate, and concentrated to about 15 mL. This dipeptide-free base was added to an ice-cooled solution of Boc-Gly (1.75 g, 10 mmol) in 15 mL dimethylformamide (DMF) followed by 2.2 g of DCC and 1.35 g 1-hydroxybenzotriazole (HOBT). The reaction was stirred for 2 days, and DCU was filtered off and worked up as described for 2. Yield: 2.5 g.

**Boc-Leu-Aib-Gly-Ile-Leu-OMe (IV).** To 2.0 g of 3, 20 mL of 98% formic acid was added and the reaction monitored by tlc. After 7 h the reaction was worked up as described for 2. This solution (10 mL) was added to an ice-cooled solution of Boc-Leu-Aib-OH$^{16}$ (1.2 g) in 10 mL of DMF followed by 1.08 g DCC and 0.75 g of HOBT and stirred for 3 days. This was worked up by the same procedure as used for compound 2 to yield 1.5 g of white solid.

**Boc-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe (I).** 1.3 g of 4 was deprotected with 15 mL 98% formic acid and worked up as reported for 2. This was coupled to Boc-Gly-Gly-OH (800 mg) in 15 mL DMF using 1.5 g DCC and 700 mg HOBT. After 6 days the reaction was worked up as usual to yield 1.5 g of the crude peptide. The peptide was purified on a reverse phase C-18 MPLC column using methanol-water. The peptide was eluted with 75% methanol. This was subjected to HPLC purification on a Lichrosorb reverse phase C-18 HPLC column (4 × 250 mm, particle size 10 $\mu$m, flow rate 1.5 mL/min) and eluted on a linear gradient of methanol-water (70–95%) with a retention time of 10 min. The peptide was homogeneous on a reverse phase RP C-18 (5 $\mu$m) column and fully characterized by nmr.

**Spectroscopic Studies**

All nmr experiments used undegassed samples and were recorded on a Bruker AMX 400 or WH 270 spectrometers at ambient temperatures unless mentioned otherwise. Peptide concentrations were in the range of 10–12 mM. Structural studies were done on the Bruker AMX 400 in CDCl$_3$/CD$_3$SO solutions. Resonance assignments were made using homonuclear Hartmann–Hahn (HOHAHA) spectra at 293 K (1K points, 512 $t_1$ increments, 48 transients, sweep width 4000 Hz, spin-lock time of 55 ms). Phase sensitive rotating frame nuclear Overhauser effect spectroscopy (ROESY) spectra were recorded using 1024 points, 512 increments, 64 transients, sweep width 3700 Hz, and spin-lock time of 300 ms. All two-dimensional data sets were zero filled to get a 1K × 1K data set with a 90° shifted squared sincbell filter in both dimensions. Temperature dependence, solvent titration, and TEMPO radical broadening experiments were done on a Bruker WH270 spectrometer. Coupling constants were evaluated from one-dimensional experiments. CD spectra were recorded on a JASCO J-500 spectropolarimeter. CD concentrations were in the range of 1–1.5 mM.
A Nonhelical Multiple \( \beta \)-Turn Conformation

RESULTS AND DISCUSSION

CD Studies

Figure 1 shows the CD spectrum of peptide 1 in methanol (MeOH) and in TFE. Three distinct bands at 230 nm (−ve), 212 nm (+ve), and 197 nm (−ve) are observed. The spectrum is distinctly different from those obtained earlier for Aib-containing peptides of similar length adopting right-handed 310/α-helical conformations. The stability of this nonhelical conformation was investigated in a polar solvating medium, trimethyl phosphate, over the temperature range 15–95°C (Figure 2). The positive band at 212 nm shows a sharp fall in intensity upon increasing the temperature suggestive of a transition from a folded structure to a solvated open form. Positive CD bands in the 200–220 nm region have been ascribed to type II \( \beta \)-turns and are also expected to arise from type I/III’ turns.

NMR Studies

Figure 3 shows a 400 MHz \( ^1H \) spectrum of peptide 1 in CDCl₃. Assignments of the NH and C\(^{\alpha} \)H resonances were made using a combination of HOHAHA (Figure 4) and ROESY (Figure 5) spectra. Spectral assignments in DMSO were made in a similar fashion. Chemical shifts of backbone protons and \( ^{1}J_{HN-C^\alpha H} \) values in the two solvents are summarized in Table I. The large chemical shift dispersion observed for the C\(^{\alpha} \)H protons of Gly(2) and Gly(5) residues is indicative of a highly structured peptide in CDCl₃. Delineation of solvent-shielded NH groups was carried out in CDCl₃ using free radical (TEMPO) induced line broadening and in (CD₃)₂SO using temperature coefficients of NH chemical shifts. The observed order of TEMPO induced broadening is Aib(4) > Gly(2) > Gly(1) > Leu(7) with Leu(3), Gly(5) and

FIGURE 1 CD spectra of peptide 1 in methanol (MeOH) and in TFE. Peptide concentration is 1.3 mM.

FIGURE 2 Effect of temperature on the CD spectrum of peptide 1 in trimethyl phosphate (TMP). Spectra are shown at 20°C intervals for clarity. Peptide concentration is the same as reported in Figure 1.
FIGURE 3 The 400 MHz $^1$H-nmr spectrum of peptide 1 in CDCl$_3$. One-letter code used for residue assignments is G, Gly; L, Leu; I, Ile; and U, Aib.

Ile(6) being largely unperturbed. The dependence of NH chemical shifts on (CD$_3$)$_2$SO concentration in CDCl$_3$-(CD$_3$)$_2$SO mixtures is illustrated in Figure 7. The free radical perturbation experiment clearly establishes the presence of three solvent-shielded NH groups corresponding to residues Leu(3), Gly(5), and Ile(6). The temperature coefficients in (CD$_3$)$_2$SO are also > 3 ppb/K for all NH groups, with the exception of Ile(6), suggesting that there is only one inaccessible (intramolecularly H-bonded) NH group in this solvent. The solvent titration curves shown in Figure 7 provide clear evidence for a solvent-dependent conformational transitions at about 30% (CD$_3$)$_2$SO-CDCl$_3$. The Leu(3) NH group has a marked discontinuity in its chemical shift dependence on solvent composition, while most other NH resonances show a monotonic downfield shift with increasing (CD$_3$)$_2$SO concentration. The

FIGURE 4 Partial HOHAHA spectrum of peptide 1 in CDCl$_3$. Residue numbers and connected protons are indicated against cross peaks [cf. 7N/$\alpha$, Leu(7)NH-Leu(7)C$^\alpha$H].
A Nonhelical Multiple β-Turn Conformation

FIGURE 5 Partial ROESY spectrum of peptide 1 in CDCl₃. Cross peaks corresponding to $d_{\text{HN}}$ and $d_{\text{HN}}$ connectivities are assigned.

above nmr data suggests that conformations involving three intramolecularly hydrogen-bonded NH groups [Leu(3), Gly(5), and Ile(6)] are populated in CDCl₃. In contrast, in (CD₃)₂SO conformations involving Ile(6) in a single intramolecular hydrogen bond are supported by the evidence.

Nuclear Overhauser Effects

Figures 5 and 6 show partial ROESY spectra in CDCl₃ and (CD₃)₂SO, respectively. A summary of the observed connectivities between backbone protons is provided in Figure 8. In peptides of this size, under the conditions employed, observed NOEs generally correspond to interproton distances $\leq$ 3.0 Å.²² In CDCl₃ $NH_i \leftrightarrow NH_{i-1}$ ($d_{\text{HN}}$) connectivities characteristic of α-helical conformations are not observed between Leu(3) and Aib(4) NH resonances, suggesting the absence of a continuous helical conformation.²³ Indeed, a strong interresidue $d_{\text{HN}}(C^\alpha H \leftrightarrow N_{i-1}H)$ connectivity between Leu(3)

Table 1 NMR Parameters for the Backbone Protons in Peptide 1

<table>
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<tr>
<th>Residue</th>
<th>NH (ppm) (CDCl₃)</th>
<th>NH (ppm) [(CD₃)₂SO]</th>
<th>C^αH (ppm) (CDCl₃)</th>
<th>C^δH (ppm) (CDCl₃)</th>
<th>$J_{HNC^\gamma\delta}$ (Hz) (CDCl₃)</th>
<th>$J_{HNC^\gamma\delta}$ (Hz) [(CD₃)₂SO]</th>
<th>$d_{\delta^H}/dT \times 10^{-3}$ (ppm/K)</th>
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<tr>
<td>Gly(1)</td>
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<td>7.00</td>
<td>3.7, 3.82</td>
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<td>17.3</td>
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<td>8.01</td>
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<td>17.0</td>
<td>7.9</td>
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<tr>
<td>Ile(6)</td>
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<td>6.0</td>
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<td>Leu(7)</td>
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<td>8.34</td>
<td>4.28</td>
<td>4.28</td>
<td>8.3</td>
<td>16.0</td>
<td>4.1</td>
</tr>
</tbody>
</table>
and Aib(4) suggest an extended or a semiextended (\( \phi \sim 120^\circ, \psi \sim 60^\circ \)) at Leu(3). The observation of a semiextended conformation at Leu(3), together with the requirement for the Gly(5)NH to be intramolecularly hydrogen bonded, strongly suggests a type \( \Pi \beta \)-turn centered at the Leu(3)-Aib(4) residues. The involvement of Ile(6) in an intramolecular hydrogen bond is suggestive of a \( \beta \)-turn conformation at the Aib(4)-Gly(5) residues also. A consecutive \( \beta \)-turn structure at residues 3–5 can be generated by a type \( \Pi \)-type \( \Pi' \) \( \beta \)-turn combination with Aib(4) being constrained to a left-handed helical conformation (\( \phi \sim +60^\circ, \psi \sim +30^\circ \)). The Gly(1) and Gly(2) residues exhibit intense \( d_{NN} \) connectivities, i.e., Gly(1)C=H \( \leftrightarrow \) Gly(2)NH and Gly(1)NH \( \leftrightarrow \) Gly(2)NH; Gly(2)C=H \( \leftrightarrow \) Leu(3)NH and Gly(2)NH \( \leftrightarrow \) Leu(3)NH. These nuclear Overhauser effects (NOEs) and the solvent-shielded nature of the Leu(3)NH supports an intramolecularly hydrogen-bonded \( \beta \)-turn conformation at the Gly(1)-Gly(2) segment. The successive \( d_{NN} \) connectivities observed for residues 1–3 is consistent with a type \( 1/1' \) \( \beta \)-turn conformation while a \( \beta \) I structure requires \( \alpha_R \) conformations at residues 1 and 2 and \( \beta \) I structure would accommodate \( \alpha_L \) conformations. Interestingly in the case of the Gly-Gly segment in Leu-enkephalin, both type I' and type I
conformations have been observed in folded conformations in crystals. A recent structure determined of Boc-Gly-Gly-cyclohexylamide reveals a type I/I' conformation in an centric space group. In the case of peptide it is not possible to make a conformational distinction based on the NOE evidence alone. However, an inspection of the anomalous CD spectra presented in Figures 1 and 2 suggest that a type I'@-turn conformation is unlikely in view of the negative CD band observed below 200 nm. Thus a conformational choice consistent with both nmr and CD results involves a structure comprising of three @-turns namely I' at Gly(1)-Gly(2), II at Leu(3)-Aib(4), and I' at Aib(4)-Gly(5). The individual conformational assignments for the first six residues, i.e., Gly-Gly-Leu-Aib-Gly-Ile is @, @, @, @, @, @. The @ stereochemical assignment is chosen at Ile(6) since a strong NOE is observed between Ile(6)NH and Leu(7)NH protons. The solvent-exposed nature of the Leu(7)NH group also precludes continuation of the left-handed helix at Ile(6). The @ stereochemical assignment is also in accordance with the known tendencies of @-branched residues to avoid left-handed helical conformations in proteins. Using idealized backbone dihedral angles (φ, ψ = 60°, ψ = 30°, ψ = 90°, ψ = 0°, ψ = 60°, ψ = 30°, ψ = 60°, ψ = 30°), a computer-generated model of the proposed conformation in CDC13 was generated and refined by energy minimization using the AMBER package. Figure 9 shows a view of the model thus obtained. All interproton distances between backbone protons are consistent with the observed NOEs (weak NOEs ~ 3.4 Å, medium NOEs ~ 2.53.0 Å, strong NOEs ~ 2.5 Å). A comparison of the NOE intensities with the backbone interproton distances in the model is provided in Table II. The hydrogen-bond geometries obtained in the model for are Boc CO...N(3) ~ 3.2 Å, HNO ~ 22.4°, Leu(3)CO...N(6) ~ 3.2 Å, HNO ~ 21.4°, Gly(2)CO...N(5) ~ 3.2 Å, HNO ~ 10.7°. Interestingly, an examination by grid search procedures of a hydrogen-bonded consecutive β-turn structure for residues 3–6 yielded satisfactory hydrogen-bond parameters only for an Aib(4) conformation, which corresponds very closely to those suggested for an ideal 310-helix, i.e., φ ~ 60°, ψ ~ 30°.

In dimethylsulfoxide [(CD3)2SO] the NOE results yield a succession of δHN connectivities characteristic of a completely helical conformation. However, the temperature coefficients in this solvent are relatively high for all backbone NH protons, with the exception of Ile(6). This is unsurprising in view of the tendency of short peptide helices to fray considerably in strongly solvating media like (CD3)2SO. Despite solvation, the NOE data is supportive of conformations that are largely
helical. It is pertinent to note that the Leu(3)NH ⇔ Aib(4)NH NOE, which was absent in CDC(1)3, is clearly observed in (CD(3))2SO. Furthermore, the Leu(3)C(3)H ⇔ Aib(4)NH connectivity is absent in (CD(3))2SO. A noteworthy feature in (CD(3))2SO is the observation of a strong exchange cross peak involving Gly(1)NH (Figure 6). This presumably arises due to a slow conformational equilibrium, which results in widely differing chemical shifts for Gly(1)NH. It is likely that unfolding of the helix at the amino terminus results in two distinct conformations as noted in crystallographic studies of a helical peptide. These observations strongly suggest a distinct conformational transition on going from an apolar medium to a strongly solvating medium. The results of chemical shift measurements of CDC(1)3-(CD(3))2SO mixtures (Figure 7) provide further evidence for such transitions centered at approximately 30% DMSO, with the NH protons of Leu(3) being the most prominently affected.

CONCLUSIONS

The conformational analysis of the heptapeptide Boc-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe, which constitutes the C-terminal fragment of the fungal metabolite Trichogin A-IV was undertaken with a view to investigate the stereochemical directing role of a central Aib residue in a Gly-rich peptide. The nonhelical CD spectrum of the synthetic fragment is in contrast to a helical CD spectrum obtained for the entire 11 residue natural peptide. The present nmr study establishes a multiple β-turn conformation for the heptapeptide in CDC(1)3 with a consecutive β II'-I' structure centered at Aib(4), which is constrained to a left-handed helical conformation. A conformational transition to a conventional, continuous right-handed helical conformation is observed in (CD(3))2SO on the basis of the nmr data. The absence of strong hydrogen bonds in (CD(3))2SO suggests appreciable fraying of the helix as observed earlier in several short peptide sequences. Several short peptides ranging in length from 6 to 9 residues, containing a single Aib residue, have been shown to adopt helical structures in crystals (Ref. 9 and unpublished results). Ongoing studies in this laboratory have revealed a nonhelical conformation in crystals of the peptide Boc-Gly-Gly-Leu-Aib-Val-Gly-Gly-Leu-Aib-OMe (N. Shamala, personal communication), whereas the octapeptide Boc-Leu-Aib-Val-Gly-Gly-Leu-Aib-OMe folds into a 3₁₀-helix in the crystalline state (I. L. Karle, personal communication). Attempts to crystallize Trichogin A-IV fragments have so far been unsuccessful. The present results emphasize the importance of considering nonhelical conformations in peptides containing a limited number of structure directing Aib residues and incorporating several conformationally flexible Gly residues. The relevance of the observed conformational flexibility of the C-terminal Trichogin A-IV fragment to the membrane modifying activity of the antibiotic also merits further study. The generation of quasi-cyclic conformations in linear peptide sequences may permit metal chelation by nonhydrogen bonded carbonyl groups in membrane environments. Such interactions would be reminiscent of the behavior of acyclic antibiotic ionophores like A23187 and Lasalocid A.

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