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Retention of native-like structure in an acyclic counterpart of a β-sheet antibiotic

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An acyclic derivative of the cyclic peptide antibiotic, ramoplanin, has been prepared. In aqueous solution, two-dimensional NMR spectroscopy indicates that the acyclic form adopts a threshold population of conformers in which at least part of the β-sheet characteristic of the intact ramoplanin persists. Thus, despite losing the entropic benefit which the macrocycle must lend to β-sheet formation, the polypeptide chain of the acyclic ramoplanin appears to display an innate tendency to adopt a native-like conformation.

Loop formation; Ramoplanin; β-Sheet; NMR, 2D; Native-like conformation

1. INTRODUCTION

Loops are a common structural motif in biological systems. For instance, in proteins, a loop in the polypeptide chain may be made by virtue of covalent (e.g. disulphide bridges) or non-covalent (e.g. hydrogen bonds) intramolecular interactions. Loops formed by disulphide bridges are presumed to bestow an entropic benefit on the folded state of the protein by restricting the conformational space available to the unfolded polypeptide chain, thereby reducing the unfavourable entropy change on folding and conferring thermodynamic stability on the native protein [1–5]. To assess further the extent to which loops influence protein integrity, we have chosen to study a system in which a small polypeptide loop (sixteen amino acids) is joined by a lactone linkage rather than a disulphide bond.

Ramoplanin, isolated from an *Actinoplanes* species of microorganism, is a glycolipodepsipeptide antibiotic active against Gram-positive bacteria [6,7]. It comprises a complex of three factors, A1, A2 and A3, which are closely related structurally. All are cyclic depsipeptides with an identical seventeen-residue peptide core. Twelve of these amino acids possess non-standard side chains and seven adopt D-configurations. The macrocycle itself is composed of sixteen amino acids and cyclization occurs from the hydroxyl side chain of (β-hydroxy)asparagine 2 to the C-terminal carboxylate group of (3-chloro-4-hydroxyphenyl)glycine (CHP) 17 via a lactone linkage (Fig. 1a) [6]. The one-dimensional (1D) ^{1}H nuclear magnetic resonance (NMR) spectrum of ramoplanin in aqueous solution is characterized by well-resolved signals, a large chemical shift dispersion and, in D_2O at pD 5.0 and 295 K, seven long-lived ($t_{1/2}$ > 0.5 h) amide proton resonances. This is indicative of ramoplanin adopting a section of stable secondary structure in which the amide NHs are protected from exchange with the solvent by intramolecular hydrogen bonding. The dominant solution conformation of ramoplanose, which differs from ramoplanin only in the presence of a tri- rather than dimannosyl moiety attached to the phenolic side chain of (4-hydroxyphenyl)glycine (HPG) 11 (Fig. 1b), has previously been determined by ^{1}H NMR spectroscopy in conjunction with distance geometry and restrained molecular dynamics calculations [8]. It is characteristized by two antiparallel β-strands interconnected by seven intramolecular hydrogen bonds and two reverse turns (Fig. 2). The virtual coincidence of resonances belonging to the peptide core of both ramoplanose and ramoplanin in ^{1}H NMR spectra of the two compounds recorded under identical conditions indicates that ramoplanin adopts the same fold.

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Abbreviations: NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlated spectroscopy; COSY, ^{1}H–^{1}H correlated spectroscopy; DIPSI, *d*ecoupling *i*n the *p*resence of *s*calar *i*nteractions; NOE, nuclear Overhauser enhancement; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; UHP, ultra-high purity; CHP, (3-chloro-4-hydroxyphenyl)glycine; HPG, (4-hydroxyphenyl)glycine; Orn, ornithine; (βOH)Asn, (D-β-hydroxy)-L-asparagine.

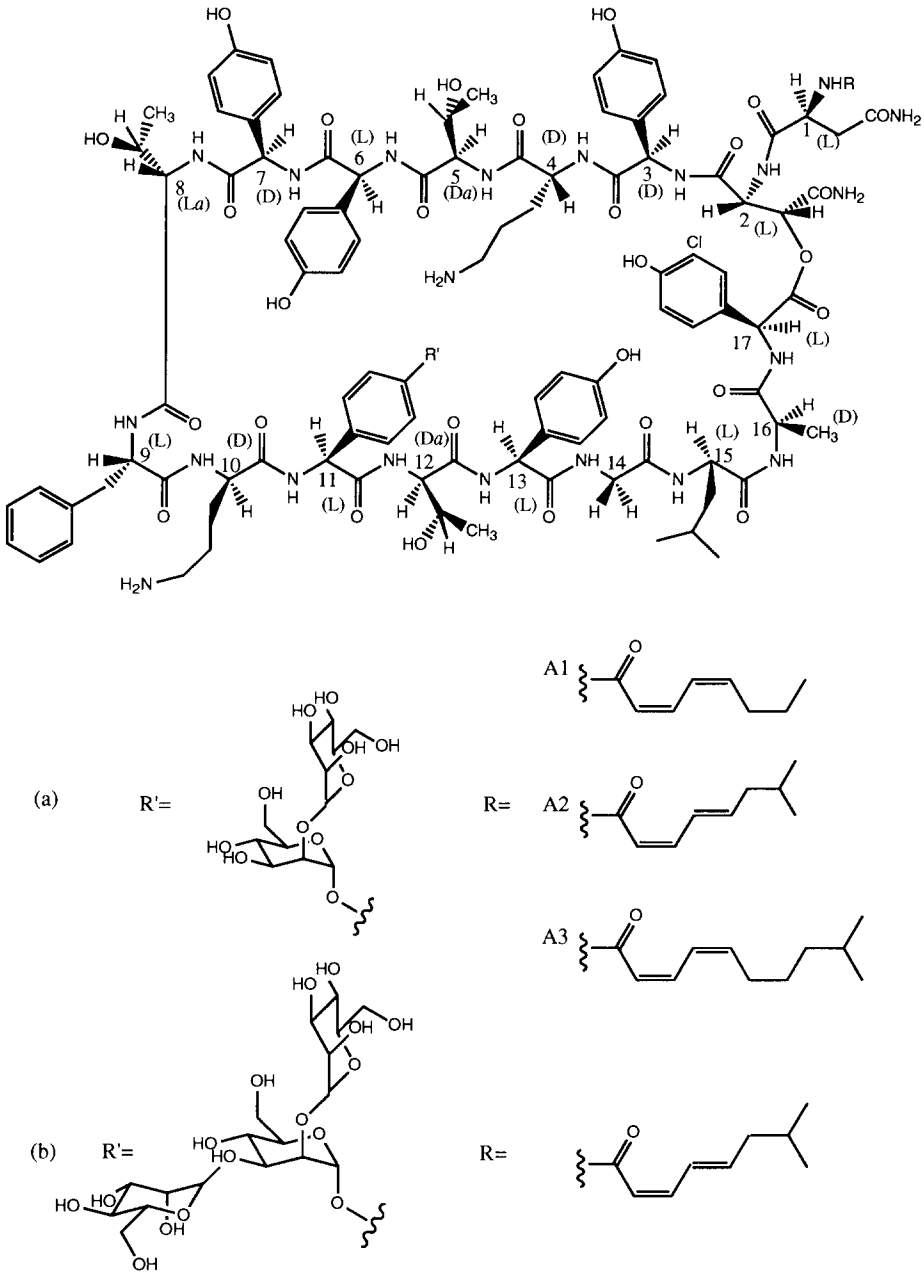

Fig. 1. Molecular structures of (a) ramoplanin and (b) ramoplanose. We find that the diene of the fatty acid derivative of ramoplanin A2 adopts a *cis* transoid *trans* configuration (R.A.M., J.P.L.C., D.H.W., unpublished observations) as opposed to the previously published *cis* transoid *cis* configuration [20].

The small size of ramoplanin (seventeen acids, M_r 2,554 Da) results in much simplified ^{1}H NMR spectra and an uncrowded amide proton region relative to a typical protein spectrum. This, together with the fact that ramoplanin adopts a section of stable secondary structure comparable to that observed in proteins, makes it a convenient system of study.

Presumably the presence of the macrocyclic ring favours β-sheet formation in a predominantly entropic fashion: hydrogen bonding partners are brought into close proximity as a consequence of the ring, thus reducing the entropic penalty in making the subsequent intramolecular hydrogen bonds. The depsipeptide nature of the antibiotic means that, by definition, it contains a lactone link and therefore a unique cleavage point along the peptide backbone. It was intended to selectively open the macrocycle at this point and to compare, principally by ^{1}H NMR spectroscopy, the extent of secondary structure formation, if any, in the cleaved product with that in the intact macrocycle.

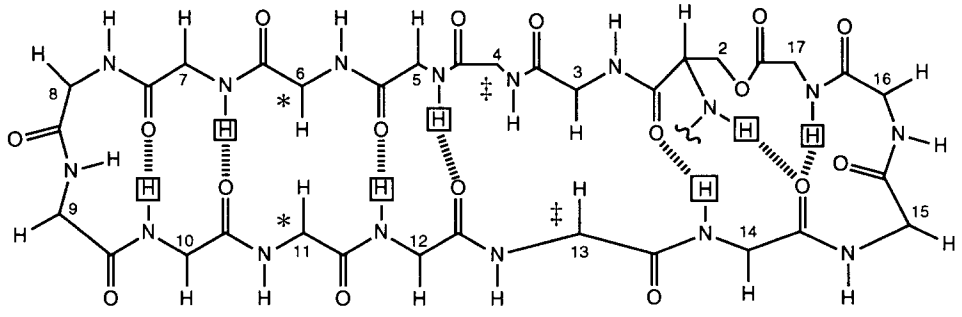

Fig. 2. The β-sheet secondary structure of ramoplanin. Hydrogen bonds are represented by hatched lines. Amide protons exchanging slowly with solvent are boxed. Pairs of residues between which interstand $d_{\alpha\alpha}(i,j)$ NOEs are observed in the acyclic ramoplanin, i.e. HPG6/HPG11 and Orn4/HPG13, are marked by asterisks (*) and double daggers (‡), respectively. Side chains have been omitted for clarity.

2. MATERIALS AND METHODS

Ramoplanin was a gift from Gruppo Lepetit, Milan, and was used without further purification. It was typically present as between 87 and 92% factor A2 ($C_{119}H_{154}ClN_{21}O_{40}.2HCl$, M_r 2,627 Da) with small proportions of related factors, A1 and A3, and contaminating inorganic chlorides also present.

2.1. *Synthesis of the acyclic ramoplanin*

Selective hydrolysis of the depsipeptide linkage was achieved by dissolving ramoplanin (5 mg, 1.9 μmol) in 1% v/v aqueous triethylamine (1 ml) and allowing to stand at ambient temperature (1 h). This solution was then injected directly on to a Waters μBondapak C_{18} reverse-phase preparative HPLC column in 0.1% TFA/ultra-high purity (UHP) water and eluted with a gradient of 0–20% B (acetonitrile/0.1% TFA) over 5 min followed by 20–50% B over 35 min. After lyophilising, the product was redissolved in UHP water (2 ml), dialysed (12 h, 4°C, Spectra/Por 7 membrane (molecular weight cut-off 2 kDa)) to remove any residual triethylamine, and lyophilised once again to give a white solid. Positive ion electrospray mass spectrometry showed this to be consistent with the opened form of the depsipeptide with peaks at 1,287.5 ($[MH_2]^{2+}$), 871.4 ($[MH_2K]^{3+}$), 866.4 ($[MH_2Na]^{3+}$) and 858.7 ($[MH_3]^{3+}$), yield 26% by amino acid analysis.

2.2. *NMR spectra and analysis*

As a result of the low solubility of the acyclic derivative, particularly in buffered systems, NMR samples of this compound were typically prepared by dissolution in D_2O or 90% H_2O/10% D_2O and the pH or pD of the sample adjusted using a solution of DCl. Samples prepared in this manner were 0.8 mM. All pD readings quoted are pH readings uncorrected for isotope effects. Dioxan was used as the internal reference (δ = 3.74 ppm).

^{1}H NMR spectra were recorded at 500.13 MHz using a Bruker 500 AMX spectrometer. Mixing times for NOESY experiments varied from 200–400 ms; TOCSY experiments employed a 60 ms DIPSI-2 isotropic mixing sequence [9,10]. Solvent suppression was achieved either by the Jump and Return method [11], by presaturation during the relaxation delay or by presaturation in conjunction with a NOESY pulse sequence (t_1 = 0) using an irradiation field of the same frequency as and phase coherent with the hard pulse [12]. Two-dimensional (2D) data sets were collected with 2,048 × 512 points in the frequency domain. Up to 128 transients were recorded for each t_1 increment with a relaxation delay of 1.75 s between transients. Spectral widths were 10 or 16 ppm. Prior to Fourier transformation, spectra were zero-filled once in the t_1 dimension and subjected to a phase-shifted sine bell weighting function in both dimensions.

Resonances within spin systems were identified using TOCSY and phase-sensitive double quantum-filtered COSY spectra. The COSY spectrum recorded on a sample prepared in 90% H_2O/10% D_2O was extremely weak; measurements of $^3J_{HN\alpha}$ were therefore deemed unreliable. Spin systems were connected in a sequence-specific manner using NOESY and TOCSY spectra [13].

3. RESULTS AND DISCUSSION

The maximum solubility attained for the acyclic analogue (0.8 mM at pH 3.9 and 298 K) was at least a factor of ten lower than for ramoplanin under otherwise identical conditions with a concomitant decrease in the signal-to-noise ratio in ^{1}H NMR spectra. These spectra were of sufficient quality for assignment purposes, however (Table I and Fig. 3).

The chemical shift dispersion in the 1D ^{1}H NMR spectrum of acyclic ramoplanin was markedly reduced compared to the parent antibiotic, particularly within the envelope of the amide protons (Fig. 4), which suggests that any structure the acyclic analogue adopts is more fleeting. This was supported by the comparison of the hydrogen–deuterium exchange behaviour of these amide protons with the equivalent protons in ramoplanin: upon dissolution of the acyclic ramoplanin in D_2O at pD 5.0 and 290 K, ^{1}H NMR studies demonstrated that total isotope exchange occurred within the time taken to dissolve the sample and record the first spectrum (5–10 min). Under similar conditions, ramoplanin exhibits seven amide protons which exchange slowly with solvent.

Any $d_{\alpha N}$, d_{NN} or $d_{\beta N}$ NOEs characteristic of the acyclic ramoplanin adopting secondary structure could not be seen in NOESY spectra recorded on samples prepared in 90% H_2/10% D_2O, possibly because of the poor signal-to-noise ratio of these spectra or possibly because these NOEs were absent anyway. In a NOESY spectrum recorded on a sample of the acyclic ramoplanin in D_2O (pD 3.9, 298 K, τ_m = 400 ms), however, two very clear interstrand $d_{\alpha\alpha}(i,j)$ NOEs were observed between HPG 6 and HPG 11, and between Orn 4 and HPG 13 (Fig. 5). A $d_{\alpha\alpha}(i,j)$ NOE is characteristic of β-sheet secondary structure [13], suggesting that of the ensemble of conformations populated by the acyclic ramoplanin in aqueous solution, at least one native-like β-hairpin

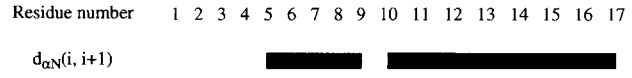

Fig. 3. Observed $d_{N\alpha}(i,i+1)$ NOEs for the acyclic ramoplanin (90% H_2O/10% D_2O, pH 3.9, 298 K).

may be envisaged in which the α-protons of HPG 6 and HPG 11 and those of Orn 4 and HPG 13 are placed close enough to each other (< 5 Å [13] for detactable NOEs to arise between them (Fig. 2). Also from Fig. 2 it is apparent that these NOEs are consistent with the β-sheet structure of ramoplanin itself. Indeed the $d_{\alpha\alpha}(i,j)$ NOE between HPG 6 and HPG 11 was observed in the ^{1}H NMR structural determination of ramoplanose [8]. The $d_{\alpha\alpha}(i,j)$ NOE between Orn 4 and HPG 13 was not observed in that study as a β-bulge in this region orients the α-proton of Orn 4 towards solvent [8]. In the acyclic form we believe the β-bulge is lost, probably reflecting the conformational freedom introduced on severing the lactone linkage. The NOEs observed for acyclic ramoplanin are therefore consistent with the population of a β-hairpin conformation the *position of which is in turn consistent with at least part of the secondary structure of ramoplanin being preserved upon opening the macrocycle.* In the absence of good quality NOESY spectra of the acyclic ramoplanin in 90% H_2O/10% D_2O, it is not possible to comment further on the extent of β-hairpin formation, i.e. whether this conformation is propagated beyond residues 4 and 13, towards the cleavage site (Fig. 2). Also, we would be reluctant, given the quality of our data, to estimate what proportion of the conformations the acyclic ramoplanin adopts is native-like. It is possible to suggest, however, from the reduced chemical shift dispersion in the 1D ^{1}H NMR spectrum of the

Table I

Chemical shifts of assigned resonances of the acyclic ramoplanin (90% H_2O: 10% D_2O, pH 3.9, 298 K)

Fatty acid[a] 2 = 5.70 3 = 6.50 4 = 7.05 5 = 6.19 6/6′ = 1.96 7 = 1.61 8/8′ = 0.80
Mannose 1[b]: 5.88(C_1H) Mannose 2[b]: 5.12(C_1H)

Residue	NH	CαH	CβH	CγH	Other protons
Asn-1	7.61	4.32(0.28)[c]	2.67 2.77		
(βOH)Asn 2	8.29[c]	4.99(0.56)[c])	4.72[c]		
HPG-3	8.45	5.37(0.99)		7.26	6.86(CδH)
Orn-4	8.44	4.55(−0.25)	1.63	1.54	2.65(CδH)
Thr-5	8.24	4.48(−0.13)	3.94	1.01	
HPG-6	8.80	6.09(0.70)		6.98	6.62(CδH)
HPG-7	8.50	5.38(0.11)		6.87	6.63(CδH)
Thr-8	8.78	3.94(−0.10)	3.96	1.02	
Phe-9	7.60	4.55(−0.39)	2.98		δ/δ′ = 7.09 ε/ε′ = 7.27 ζ = 7.19
Orn-10	7.61	4.55(0.50)	1.81	1.50	2.91(CδH)
HPG-11	8.91	6.13(0.86)		7.32	7.01(CδH)
Thr-12	9.06	4.64(0.13)	3.97	0.99	
HPG-13	8.80	5.53(0.62)		7.21	6.81(CδH)
Gly-14	8.80	3.60 (−0.78/0.31)			
Leu-15	7.78	4.26(−0.01)	1.50	1.50	0.48
Ala-16	8.37	4.20(0.17)	1.28		
CHP-17	8.06	5.12(0.38)			γ = 7.24 ζ = 6.92 η = 7.12

Figures in brackets indicate the chemical shift difference δ[CαH(ramoplanin)–CαH(acyclic ramoplanin)], with δ[CαH(ramoplanin)] measured at the same temperature and pH as those tabulated for the acyclic ramoplanin.
[a]Numbered from the amide carbonyl of the fatty acid derivative.
[b]Only the anomeric protons of the dimannosyl unit were assigned.
[c]Tentative assignments.

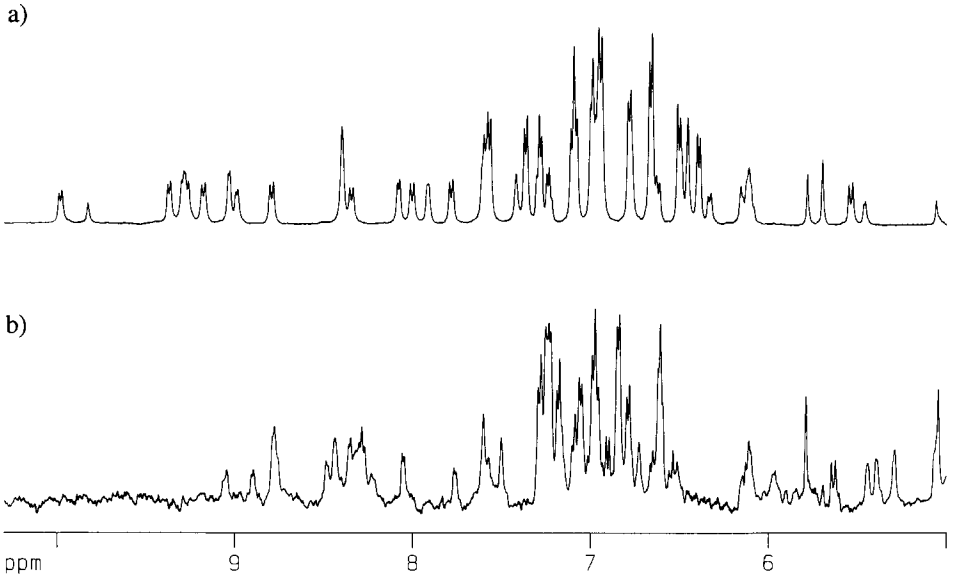

Fig. 4. Regions to high frequency of ID ^{1}H NMR spectra of (a) ramoplanin and (b) the acyclic ramoplanin in 90% H_2O/10% D_2O (pH 3.9, 298 K).

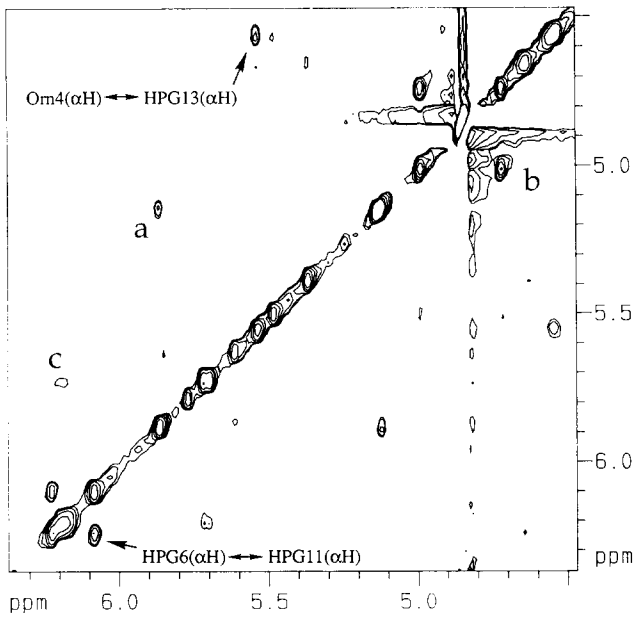

Fig. 5. Two-dimensional NOESY spectrum (τ_m = 400 ms) illustrating the two $d_{\alpha\alpha}(i,j)$ interstrand NOEs observed for the acyclic ramoplanin in D_2O, pD 3.9, 298 K. Additional NOEs are those between the anomeric protons of the dimannosyl unit (a) and the α and β protons of Asn 2 (b). The latter NOE, because the assignments of these two Asn protons are tentative, is also tentative. The cross-peak c, between C_2H and C_5H of the fatty acid derivative, is the result of cross-relaxation via the intervening C_3H. As this cross-peak is very weak compared to those upon which we base our arguments, we believe that its observation does not detract from the latter.

acyclic ramoplanin (Fig. 4) and the absence of protection to deuterium exchange of its amide protons upon dissolution in D_2O at pD 5.0 and 290 K, that the population of this native-like β-hairpin conformation is not as extensive as that of the secondary structure in ramoplanin itself.

Differential scanning calorimetry (DSC) was used to investigate possible thermal unfolding of this β-hairpin conformation between 5–100°C and at pH 4.0. No evidence for a significant cooperative unfolding transition was observed over this temperature range. This does not necessarily mean that the secondary structure observed in the NOESY spectrum recorded on acyclic ramoplanin in D_2O at 298 K, pD 3.9 is present at temperatures up to 100°C. The enthalpy dependence of any unfolding transition may be too low (< 42 $kJ \cdot mol^{-1}$) to detect using DSC as the temperature dependence of the transition would then be too broad to observe above the baseline (A. Cooper, personal communication).

The available evidence suggests that at 298 K and pD 3.9, the acyclic ramoplanin populates a significant β-hairpin conformation, consistent with at least part of the secondary structure in ramoplanin being preserved upon opening the macrocycle. The population of native-like conformers in protein fragments has been linked to the conformation that these same sequences may adopt within the polypeptide chain of the intact protein during the early stages of protein folding, and the possible role such conformations could have as initiation sites in folding reactions [15–19]. It would be unwise to advocate the current study as support for these suggestions as ramoplanin contains amino acids with unusual stereochemistries (D rather than L) and unnatural side chains. Both factors may predispose the conformation of the ramoplanin polypeptide chain towards that of a β-sheet according to the following arguments. Firstly residues 10–13, which form part of one strand of the β-sheet, have alternating stereochemistries D–L–D–L and residues 5–7 on the other strand also show alternating stereochemistries D–L–D. These alternating stereochemistries serve to place the side chains of these residues on one face of the extended polypeptide chain, naturally predisposing it to form a β-sheet. Secondly, the bulky non-standard aromatic side chains of HPG and CHP, lacking the intervening βCH_2 groups of, for instance, tyrosine and phenylalanine, are likely to impose severe limitations on the ϕ and ψ torsion angles of these amino acids, thereby reducing the conformational entropy of the ramoplanin system. Nevertheless, this work does show that this particular polypeptide chain alone and in the absence of ring constraints is able to populate a native-like conformation.

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