Solution structures by NMR of a novel antifungal drug: Petriellin A†

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Petriellin A is a novel cyclic depsipeptide antifungal compound consisting of nine L-configured residues, one D-phenyllactic acid (PhLac) and three unknown chiral centres: two *N*-methyl-threonines (MeThr1 & MeThr2) and one *N*-methyl-isoleucine (MeIle). NMR experiments including 2D ROESY, NOESY along with structural and energy calculations predicted that the unknown chiral centres were all L-configured, which was later verified chemically. Simulated annealing, dynamics calculations and minimisation processes showed Petriellin A to have a folded "C-shaped" structure.

Introduction

The *N*-methylated antifungal cyclic tridecadepsipeptide Petriellin A was isolated from the coprophilous fungus *Petriella sordida* (UAMH 7493) by Gloer *et al.*,[1] who used 1D and 2D NMR experiments to identify this compound. Coprophilous fungi are uniquely adapted to herbivore dung, where they play an important role in recycling the nutrients in animal faeces. They are also a good source of antibiotics, enzymes and biological control agents. Petriellin A, produced by antagonistic fungi, is heavily *N*-methylated, containing two *N*-methyl-threonines, two *N*-methyl-valines and one *N*-methyl-isoleucine (Fig. 1). Similar to Cyclosporin A, it has a lactone backbone linkage but contains the relatively rare structures: *N*-methyl-threonine, *N*-methyl-isoleucine and *R* configured phenyllactic acid. In the original report the chirality of three of the amino acid residues were not determined.

This paper reports the determination of the 3D structure of Petriellin A by NMR spectroscopy in order to provide a basis for understanding its biological activity, and to allow a comparison of the 3-dimensional structure of smaller synthetic fragments with the natural product.

Petriellin A has displayed antifungal activity against the early successional coprophilous fungi *Ascobolus furfuraceus* (NRRL 6460) with MIC values of 5 μg mL^{-1} and *Sordaria fimicola* (NRRL 6459), with MIC values of ≤2 μg mL^{-1}. However, no activity was observed in disk assays against *Candida albicans* (ATCC 90029) at 100 μg $disk^{-1}$.[1] Petriellin A also displayed an approximate IC_{50} value of 10 μg mL^{-1} against human fibroblast MRC5 cells, but the diacetate of Petriellin A was found to be significantly more cytotoxic with an IC_{50} of less than 1 μg mL^{-1}. Analogues of Petriellin A have also been isolated including Petriellin B, C and D, which have slight variations in their residues, but remain heavily *N*-methylated in the five amino acids.[2]

Petriellin A represents a potentially promising new class of antifungal compounds unlike three current principal types of antifungal drugs in clinical use for systemic fungal infections, namely azoles, polyenes and pyrimidines.[3–7] *N*-Methylated compounds are important as shown by In *et al.*[8,9] because *N*-methylation causes predictable changes in molecular shape, proteolytic stability, conformational mobility and membrane permeability.[10–13]

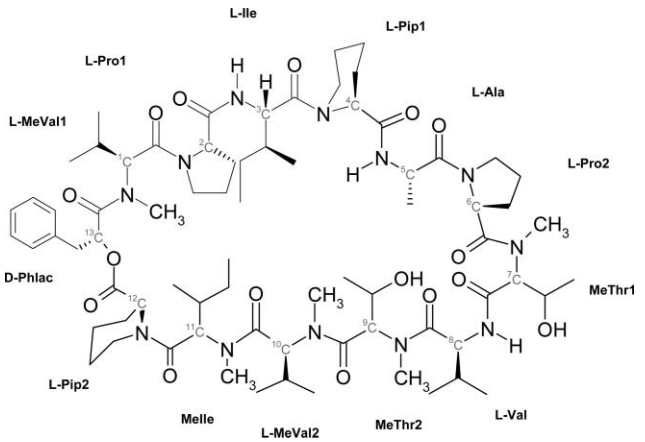

Fig. 1 Petriellin A: L-valinamide, *N*-(2-hydroxy-1-oxo-3-phenylpropyl)-*N*-methyl-L-valyl-L-prolyl-L-isoleucyl-L-2-piperidinecarbonyl-L-alanyl-L-prolyl-*N*-methyl-L-threonyl-L-valyl-*N*-methyl-L-threonyl-*N*-{1-[(2-carboxy-1-piperidinyl)carbonyl]-2-methylbutyl}-*N*,*N*2-dimethyl-μ1-lactone, [1(*R*),10[*S*-(*R**,*R**)].

Compared with standard peptides, relatively little effort has been devoted to the synthesis and function of *N*-methylated peptides. However, recent work on the effects of *N*-methylation on endothelin A/B receptor antagonists has established the utility of *N*-methyl amino acids in a drug design protocol.[11] There are a number of drugs containing *N*-methyl amino acids such as Cyclosporin, Vancomycin and Actinomycin. The success of these drugs has established the importance of *N*-methylation as a peptide modification that can increase proteolytic stability and may be orally delivered.

Results

Petriellin A contains several *N*-methylated amino acid residues and hence the standard NMR method of assigning the H-α protons

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using a combination of COSY and NOESY experiments, using the N–H assignments, then TOCSY experiments to identify the peptide side chains cannot be used. In this case long range HMBC experiments were used to assign the backbone carbons and H-α protons. The complete assignment of Petriellin A extracted from the fungus by us is consistent with the assignment of Gloer *et al.*[1] and is shown in Tables 1 and 2.

It was observed from the NOESY experiment that the cross peaks were small and the opposite sign to the diagonal peaks, indicating that Petriellin A has a relatively fast molecular tumbling rate and falls into the small NOE enhancement region. Thus ROESY data obtained on a 750 MHz spectrometer (Institute for Molecular Bioscience, Brisbane) using a mixing time of 500 ms was used for the structural work.

The assignment process required the use of data from both the 400 MHz and 500 MHz spectra but ROESY data from the 750 MHz spectrometer were used for structural calculations. The analysis of the ROESY spectra yields 147 distance constraints and 3 torsional dihedral angle constraints from NH-coupling constants which were used in the structural minimisation and molecular modelling.

The derived 147 upper and lower distance constraints were calibrated using the standard protocol,[14,15] using the geminal protons of the proline rings in Petriellin A (1.77 Å) for calibrating the ROE intensities. The conformational calculation based on NMR distance constraints was subjected to simulated annealing to generate a group of 200 conformers in DYANA with 6000 steps.[16] The backbone was cyclized throughout the calculations and with peptide bonds defined as all *trans*, except for Pip1 which had a *cis*-conformation. The best 20 conformers with no violations greater than 0.5 Å, and low target function were chosen for further energy minimisation.

Since there are three unknown backbone chiral centres in Petriellin A (MeIle, MeThr1 and MeThr2) hence eight possible configurations, all of these eight structures were derived using the DYANA software package based on the experimental NMR constraints (Fig. 2). Both visual inspection of the resulting structures and comparison of the RMSD data showed that the L-MeIle, L-MeThr1 and L-MeThr2 (LLL) were likely to be the correct configurations having the lowest RMS deviations, with the mean global backbone RMSD of 0.15 ± 0.07 Å. The next two possible structures are D-MeIle, L-MeThr1 and L-MeThr2 (DLL) and L-MeIle, D-MeThr1 and L-MeThr2 (LDL) with a less favoured and greater backbone RMSD of 0.38 Å. These have a mean global backbone RMSD that is twice the value of the (LLL) structure.

Hence from the use of NMR data and modelling, the predictions of the chiral centres for Petriellin A were (LLL). It was later confirmed chemically that the structure was (LLL) using the Marfey analytical method.[17] The chemical chiral analysis also confirmed the side chain chirality in these amino acids.

Table 1 ^{1}H chemical shifts, δ (ppm), for Petriellin A in 1 : 4 $CDCl_3$–d-6 Acetone at 298 K relative to the acetone peak at 2.04 ppm

Residue	NH/*N*CH3	αH	β*H*	Others
1-MeVal-1	3.06	4.79	2.16[a]	γ1CH3 0.87[a]; γ2CH3 1.08
2-Pro-1		4.67	1.89, 2.12	γ1CH2 1.90; γ2CH2 2.10; δ1CH2 3.63; δ2CH2 4.36
3-Ile	7.58	4.83*	1.99	γ1CH2 1.18[a]; γ2CH2 1.73; γ3CH3 0.84[a]; δCH3 0.83*
4-Pip-1		4.98	1.48, 2.04	γ1CH2 1.57; γ2CH2 1.72; δ1CH2 1.38; δ2CH2 1.68; ε1CH2 3.42; ε1CH2 4.05
5-Ala	6.96	4.62	1.22[a]	
6-Pro-2		5.00	2.06, 2.13	γ1CH2 1.95[a]; γ2CH2 2.34; δ1CH2 3.66; δ2CH2 3.91
7-MeThr-1	2.85	4.83*	4.18	γ1CH3 1.18[a]
8-Val	8.61	5.09	1.96[a]	γ1CH3 0.85[a]; γ2CH3 0.94
9-MeThr-2	3.24	5.43	4.27	γ1CH3 1.18[a]
10-MeVal-2	3.27	5.26	2.68	γ1CH3 0.77; γ2CH3 0.88[a]
11-MeIle	3.05	4.93	2.16[a]	γ1CH2 1.22[a]; γ2CH2 1.77; γ3CH3 0.90; δCH3 0.86[a]
12-Pip-2		5.37	1.44, 2.17[a]	γ1CH2 1.20; γ2CH2 1.63; δ1CH2 1.19; δ2CH2 1.60; ε1CH2 2.91; ε2CH2 3.90
13-PhLac		5.85	2.92, 3.11	4CH 7.17; 2,6CH 7.24[a]; 3,5CH 7.24

[a] Indicates peaks were overlapping and were resolved using 2D NMR methods.

Table 2 ^{13}C chemical shifts, δ (ppm), for Petriellin A in 1 : 4 $CDCl_3$–d-6 acetone at 298 K relative to acetone at ω2: 2.04 ppm and ω1: 206 ppm

Residue	*N*CH3	C13 chemical shifts δ (ppm)
1-MeVal-1	31.14	γ2 19.03; γ1 20.07; β 28.25; α 61.14; -CO 170.50
2-Pro-1		γ 25.98; β 31.34; δ 48.48; α 60.79; -CO 172.80
3-Ile		δ 11.35; γ-CH3 16.61; γ-CH2 24.90; β 37.32; α 52.70; -CO 173.10
4-Pip-1		γ 20.70; δ 26.38; β 28.25; ε 43.78; α 52.81; -CO 171.50
5-Ala		β 17.92; α 47.24; -CO 171.60
6-Pro-2		γ 26.18; β 30.60; δ 48.21; α 56.39; -CO 174.70
7-MeThr-1	30.47	γ 20.43; β 64.72; α 66.14; -CO 169.80
8-Val		γ1 16.57; γ2 20.87; β 31.50; α 53.91; -CO 172.70
9-MeThr-2	31.09	γ 20.43; α 58.76; β 65.04; -CO 171.80
10-MeVal-2	31.81	γ1 18.65; γ2 19.54; β 27.96; α 58.09; -CO 171.30
11-MeIle	31.14	δ 11.71; γ-CH3 16.82; γ-CH2 24.66; β 34.30; α 58.80; -CO 169.50
12-Pip-2		γ 21.60; δ 26.18; β 26.38; ε 43.99; α 52.81; -CO 169.90
13-PhLac		β 37.50; α 72.13; -4 127.20; -3,5 128.87; -2,6 130.51; -1 137.54; -CO 169.00

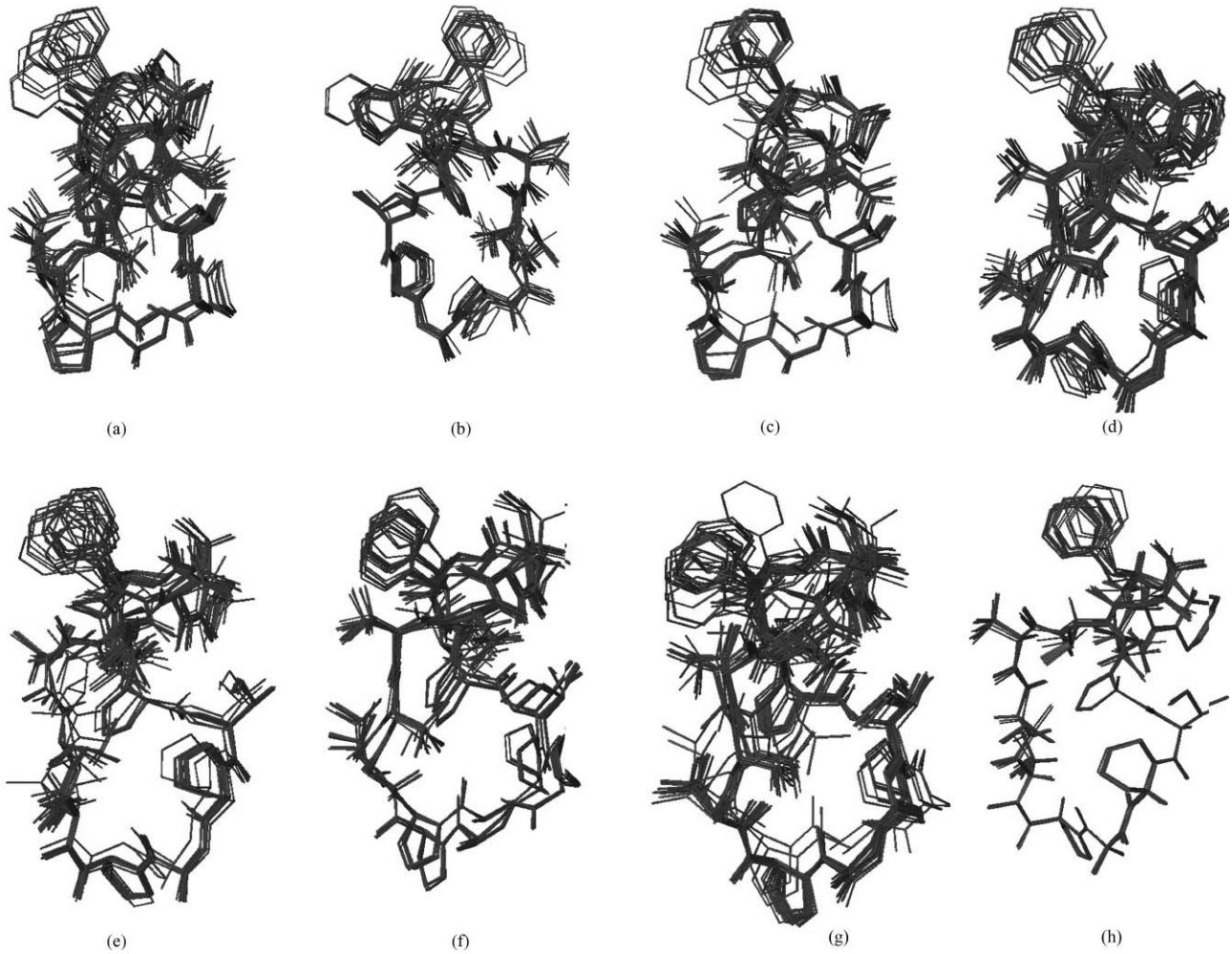

Fig. 2 Petriellin A structures from simulated annealing calculations for the eight possible α-carbon configurations for the three unknown chiral centres (MeIle, MeThr1 and MeThr2). a) DDD, b) DLL, c) DLD, d) DDL, e) LDL, f) LLD, g) LDD, h) LLL.

The backbone structure of Petriellin A is shown in Fig. 3, and is somewhat like the seam on a tennis ball. From an edge-wise view Petriellin A has a "C" shape structure, with bends between residues 3–4, 6–7 and 10–11. When viewed from an angle similar to the schematic diagram in Fig. 1, Petriellin A has a cross sectional diameter of approximately 18.90 Å times 12.30 Å. When viewed from the side it may be described as an oval shape with a slight tail on the right.

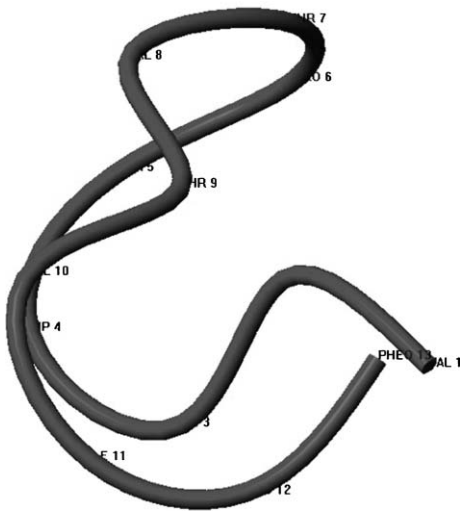

Fig. 3 The backbone structure of Petriellin A. The discontinuity is due to non recognition of the ester linkage.

It can be observed from the structures that H-bonding is seen between residues near the bends of Petriellin A. When hydrogen bonds were calculated based on the criteria of H–H distances of less than 2.40 Å and within the 35 degree angle, there were two hydrogen bonds present in all twenty structures between NH-8-Val and CO-5-Ala, and in fourteen out of the twenty structures between NH-3-Ile and CO-1-MeVal1. The other amide proton of NH-5-Ala was situated near the bend which precluded possible hydrogen bonds.

The Connolly surface[18] model for Petriellin A was generated using the Accelrys[19] package. The hydrophobic part of Petriellin A was coloured green, negatively charged residues were coloured red. Fig. 4 shows the surface as predominantly covered with hydrophobic amino acids. The hydrophilic groups (such as -CO, and the OH of the threonines) are buried in the core of the molecule, and the *N*-methyl groups are facing outwards forcing

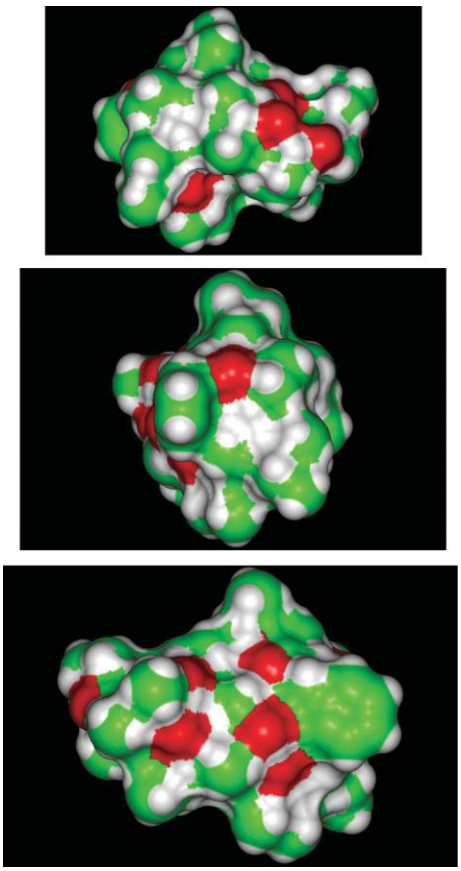

Fig. 4 Connolly surface models of Petriellin A showing distribution of hydrophobic (green) and negatively charged groups (red). The middle and bottom views are the 90° and 180° rotations about the vertical axis relative to the top view. Most of the negatively charged areas are hidden in the grooves.

the hydrophilic groups inwards. The *N*-methyl groups of Petriellin A result in a hydrophobic surface. Four out of the five *N*-methyl groups in Petriellin A faced outwards while N-MeVal2 faced inwards into the centre of the molecule (Fig. 5). Viewing Petriellin A from a similar perspective to the schematic diagram in Fig. 1, the hydrophilic core is formed on the left of the molecule with the surface being mostly hydrophobic (the back view has a centre groove with negative charge).

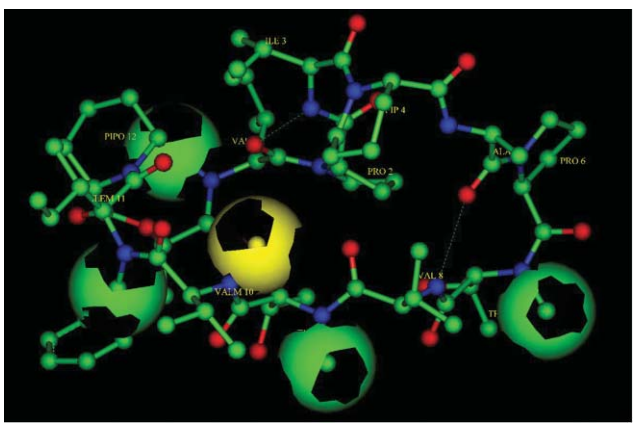

Fig. 5 Four out of the five *N*-methyl groups in Petriellin A face outwards creating a hydrophobic surface shown in green, whereas the *N*-methyl group of residue 10-Val2 (yellow) faces inwards into the core of the molecule. Hydrogen bonds are shown in this structure as green dotted lines.

It has been postulated that Petriellin A could be hydrolysed at the ester linkage to generate other active fragments. The structures show that this ester linkage is accessible on the outside of the molecule and hence may be readily hydrolysed.

The determination of the chirality of the α-carbons and the 3-dimensional structure was accomplished in the same solvent (acetone–chloroform) that was used for the original identification of Petriellin A[1] since an unequivocal identification of the structure was required. It is appreciated that a study in a range of solvents that mimic membranes (cyclohexane) and an aqueous environment is desirable. The structure derived here is relatively hydrophobic and is likely consistent with a membrane bound structure. It is known that a number of cyclic peptides including Cyclosporin change conformation between hydrophobic and hydrophilic environments, and thus we envisage a broader solvent structural study in the future.

Materials and methods

Petriellin A was isolated from an ethyl acetate extract of 5 litres of culture broth following the procedures of Gloer *et al.*[1] giving approximately 300 mg crude material. The crude material was semi-purified using 50% methanol–H_2O and 90% hexane–ether solution. Mass spectrometry (Supplementary Information†) showed that Petriellin A was present in the hexane layer. Final purification was performed by HPLC using an analytical VYDAC reverse phase C18 column with a gradient dilution of CH_3CN with 0.1% TFA yielding 9 mg of Petriellin A.

The 1D NMR experiments were recorded at 300 K using standard protocols[20,21] on Bruker DRX 400, and AVANCE 500 and 750 MHz spectrometers. The probe was matched and tuned and the 90° pulse was measured before all experiments.

COSY spectra were acquired with 2048 data points in F2, with 512 increments and 16 scans. They were processed with 2048 × 1024 data points with zero filling applied in F1 direction. Although the magnitude COSY experiment is considered to be inferior to the phase sensitive double quantum COSY experiment, the recent application of gradient pulses has changed this into a simple robust experiment.

For other 2D experiments including TOCSY,[22] ROESY[23] gradient HSQC Echo-Antiecho and NOESY, spectra were acquired using 2 K of data and 512 experiments with 32 scans over 11 ppm spectral width. A range of ROESY mixing times were used with the spin lock power of 20.00 dB. Spectra were processed using QSINE functions with $\pi/2$ shift applied to both dimensions and linear prediction in F1 direction.

Heteronuclear correlation experiments such as ^{13}C and HSQC, the long-range HMBC C–H correlation experiments were recorded using low-pass J-filter to suppress one-bond correlation, and gradient pulses with delay for evolution of 60 ms.

All NOESY and ROESY data were processed with the Bruker XWIN-NMR program and further analysed and assigned with the XEASY software package.[24] All assigned peak intensities were integrated using the elliptical curve method in XEASY, and the calculations were performed on an SGI octane computer.

The distance constraints derived from the integrated cross peaks were used in DYANA[16] to calculate a family of structures. The structures from DYANA that satisfied the distance constraints were then subjected to minimisation and molecular dynamic processes using the "DISCOVER" modules in Accelrys[19] to produce the final NMR solution structures. Chloroform (in the chloroform–acetone mixture) was used as the reference peak calibrated at 7.924 ppm for the temperature variation studies.

Modelling protocol

The long-range NOE data were examined in detail to ensure that the correct assignments had been made and that there were sufficient constraints to determine 3-dimensional structures.[25,26] Initial structural calculations based on the NOE distance constraints for cyclic peptides often result in a number of violations, which were reduced by adding 1.0 Å to the upper limit of methylene or methyl side chain protons, and 0.5 Å to the backbone protons.[27] The ASNO software module in DYANA was used to evaluate the consistency of the NOE data from the proposed structures. These were re-checked by back-calculation with the preliminary set of distance constraints and a bundle of conformers previously calculated[28] using the ASNO module.[16,29] For each cross peak ASNO determined the set of all possible chemical shift-based assignments, checked against the corresponding distance constraints, and discarded them if they were not consistent. The refinement and checking of the distance constraints using the ASNO module was conducted over several iterations until an optimum family of Petriellin A structures was obtained.

The twenty best structures of Petriellin A derived from DYANA with no ROESY constraint-violations greater than 0.5 Å were subjected to energetic minimisation using "INSIGHT" and "DISCOVER" modules from Accelrys.[19] Initially the structures were minimised with 2000 iterations using the steepest descent protocol then using the conjugate gradient protocol until a derivative of 0.0001 kcal mol^{-1} $Å^{-1}$ was reached.

Molecular dynamics were performed on Petriellin A at 900 K for 1000 equilibrations with 100 steps at constant pressure and a step time of 1 fs. The structures generated were again energy-minimized. A comparison of the minimized energies calculated for Petriellin A of the twenty structures was used to estimate the relative convergence and the global minimum energy. The energy data for Petriellin A are shown in Table 3. In this study the structures were energy-minimized subject to the NMR constraints without a box of solvent.

Table 3 The 20 energy-minimized NMR structures of Petriellin A in 1 : 4 CD_3Cl–acetone mixture

	Conformational energy/kcal mol^{-1}
E_{total}	566.21 ± 7.73
E_{NOE}	39.91 ± 4.06
E_{bond}	64.74 ± 0.83
E_{phi}	23.25 ± 5.52
$E_{repulsion}$	529.00 ± 6.86
$E_{dispersion}$	−420.43 ± 11.78
Average of the global backbone RMSD[a]	0.98 ± 0.35 Å

[a] RMSD calculated using MOLMOL program.

Table 4 Summarised percentage of dihedral angles of Petriellin A on the Ramachandran plot derived from two different programs

Petriellin A	PROCHECK	DYANA
Residues in most favoured region	50.00%	50.00%
Residues in additional allowed region	37.50%	50.00%
Residues in generously allowed region	12.50%	0%
Residues in disallowed region	0%	0%

The quality of the minimised structures was carefully analysed in detail. Stereochemical quality was examined with the PROCHECK program,[30] which compares the structures with an "ideal" structure. DYANA was also used to check the tolerances, consistencies and distribution of the distance constraints as a function of range. MOLMOL[31] and Accelrys provided a graphical analysis of Petriellin A. The final 20 energy-minimized structures of Petriellin A were examined in detail, structures were superimposed using the MOLMOL[31] program which is used to derive the mean global backbone RMSD of the structures shown in Fig. 2.

The Ramachandran plot was used to indicate stereochemical quality by comparing the (ϕ, ψ) angles in Petriellin A with those allowed. The Ramachandran plot is one of the best guides to check the stereochemistry quality of protein and ideally proteins should have more than 90% of the residues in the allowed regions.[30,32]

It is expected that *N*-methylated residues will influence the plot and therefore the Ramachandran plot may not be completely applicable to these small uncommon cyclic molecules since the *N*-methylated library is unrecognisable by PROCHECK. Both the PROCHECK and DYANA programs showed that all of the dihedral angles in Petriellin A lie within the allowed regions (Table 4).

Temperature variation of N–H shifts

It has been shown[33,34] that the coefficient and magnitude of the temperature dependence of the amide proton chemical shift in water can be used to differentiate protons that are internally H-bonded from those not H-bonded or exposed to the solvent. With internal H-bonds the temperature shift is more positive than −4 ppb K^{-1}.

Even though our data were collected in a chloroform–acetone mixture we have evaluated the temperature coefficients. The data (Supplementary Information†) for the 3 amide protons in Petriellin A show that NH-8-Val and NH-5-Ala have temperature coefficients near 0 ppb K^{-1} and are hence predicted to be (internally) H-bonded. On the other hand the NH-3-Ile proton has a chemical shift coefficient of −4ppb K^{-1} implying that NH-3-Ile is not H-bonded. This is not consistent with the structures derived for Petriellin which show that the NH-5-Ala is exposed on the surface of the molecule, but NH-3-Ile and NH-8-Val are substantially hydrogen bonded. It is probable that these rules are not valid for these solvent systems or for such small molecules.

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