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DETERMINATION OF THE STRUCTURE OF AN EXTRACELLULAR PEPTIDE PRODUCED BY THE MUSHROOM SAPROTROPH *PSEUDOMONAS REACTANS*

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Abstract: The primary structure of WLIP, a lipodepsipeptide produced by the mushroom saprotroph *P. reactans*, is determined by a combination of chemical and spectroscopic techniques. The conformation of WLIP in DMSO-d_6 is investigated by molecular modelling calculations using constraints obtained from two-dimensional NMR spectroscopy.

Introduction

Members of the genus *Pseudomonas* characteristically produce a wide variety of extracellular compounds including siderophores, antibiotics, toxins and enzymes. A range of fluorescent *Pseudomonas* species are frequently found in association with the sporophore of the edible mushroom, *Agaricus bisporus* (Lange) Imbach.[1,2,3] Of particular interest is the taxonomically diverse group of saprophytic *Pseudomonas* species, collectively referred to as *Pseudomonas reactans*,[4] which are capable of forming a white line in agar when grown in association with *Pseudomonas tolaasii* Paine, the causal organism of the economically significant brown blotch disease of *A. bisporus*.[5] This white line reaction is a rapid and reliable means of identifying *P. tolaasii* isolates[2,4] and is the result of a specific interaction between diffusible compounds produced by *P. reactans* (called the white line inducing principle, WLIP) and the water soluble toxin produced by *P. tolaasii*.[6,7] Neither the mechanism whereby the white line is formed nor the biological significance of the reaction are understood.

This work reports the isolation, purification and three dimensional structure of WLIP, which has been carried out as part of an integrated study of the significance and biological activity of the components of the white line reaction.

Determination of Primary Structure

Preliminary studies. Peng has shown that the molecular weight of WLIP is of the order of 1000 daltons or less, that it contains no carbohydrate units and that it probably contains an ester functionality (IR absorption at 1745 cm^{-1}). WLIP is weakly acidic and is inactivated towards the white line test by autoclaving.[6]

The ^1H NMR spectrum of WLIP in DMSO-d_6 is shown in Figure I. The spectrum exhibits the three clusters of resonances typical of peptides (downfield amide/aromatic, αCH and side-chain protons). A single resonance at 5.15 p.p.m. lies downfield of the αCH region. All resonances above 5.15 p.p.m. were removed by deuterium exchange after a prolonged period in CD_3OD/DMSO-d_6. The positive ion FAB mass spectrum of crude WLIP

contained an abundant molecular ion at *m/z* 1126 (MH^+), giving a molecular weight of 1125. However, no sequence ions could be confidently assigned.

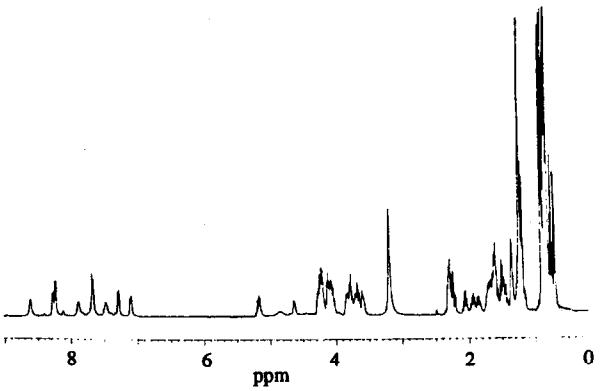


Figure I. ^1H NMR spectrum of WLIP in DMSO-d_6 (400 MHz, 305 K, 5 mM).

Table I. Results of amino acid and chiral GC analysis of WLIP.

Amino acid	Mol / mol peptide	Composition	Chirality
Threonine	0.86	1	D-*allo*
Serine	2.13	2	D
Glutamic acid	1.05	1	D
Valine	0.94	1	D
Isoleucine	0.85	1	L
Leucine	3.17	3	2L, 1D

Amino acid analysis and stereochemistry. Amino acid analysis of WLIP revealed the presence of only six types of amino acid (Table I). The absolute yield of all amino acids increased progressively with time of hydrolysis up to 96 h, suggesting an overall resistance to hydrolytic degradation. The principal molecular ion was still readily observable after this period of hydrolysis but no sequence specific fragments could be detected. WLIP thus has the composition [Glx, Ile, $(Leu)_3$, $(Ser)_2$, Thr, Val]. This represents a mass of 954/955 daltons (dependent on the state of the Glx residue), and leaves *ca.* 170 daltons to be accounted for in the complete molecule.

The stereochemistries of the constituent amino acids were determined by capillary gas chromatography (GC) using a column in which the stationary phase is L-valine. Intact WLIP was treated with 5 % aqueous triethylamine, and the lactone-opened form (see next section) subjected to hydrolysis in 6 M HCl at 105 °C overnight. The hydrolysate was derivatised with isopropanol/HCl, followed by trifluoroacetic anhydride and the mixture of N, O-trifluoroacetyl *iso*propyl esters analysed by GC. Retention times of peaks from the hydrolysate were compared with those of authentic amino acid standards and the assignment confirmed by co-injection of the hydrolysate with each standard. Results are summarised in Table I and indicate the presence of the D forms of serine, valine and glutamic acid, together with D-*allo*-threonine and L-isoleucine. Leucine was present as a 2:1 mixture of the L and D forms.

Functional group determination. The results of microscale derivatisation experiments were assessed by FABMS (Table II). WLIP was shown to contain a single free carboxyl, a blocked N-terminus and an absence of amino groups or primary amides. These latter data suggest that the Glx residue found by amino acid analysis occurs in the form of glutamic acid in the intact molecule. The presence of three hydroxyl groups in WLIP was confirmed by the assignment of three exchangeable ^1H NMR resonances at 4.54, 4.78 and 4.97 p.p.m.

Table II. Results of microscale functional group determinations on WLIP.

Reaction and conditions	Change in mass of WLIP	Conclusion
Esterification 0.1 M HCl/EtOH	+28	1 CO_2H
Acetylation Ac_2O/H_2O pH 8.4	-	No NH_2
Hofmann rearrangement TIB	-	No $CONH_2$
Trimethylsilylation *Trisil*	+216	3 OH
Base hydrolysis 1 % Et_3N/H_2O	+18	Lactone

HPLC analysis of WLIP showed the presence of a principal component eluting at a relatively high concentration of acetonitrile (60 %). After incubation of WLIP in 1 % (v/v) triethylamine, this hydrophobic material degraded to a major peak eluting at 45 % CH_3CN ($t_{1/2}$ for conversion was approximately 3 h at 55 °C), consistent with a gross change in structure and/or reduction in overall hydrophobicity. The amino acid composition of the base hydrolysis product was unchanged from that of WLIP but FABMS showed that the molecular weight had increased by 18 mass units, suggesting that WLIP might contain a lactone functionality. Derivatisation experiments on this material demonstrated that it contains four hydroxyl groups and a single carboxyl, confirmatory evidence for the presence of a lactone in WLIP.

Sequence determination by Edman degradation and FABMS. The N-terminal amino group of WLIP, both before and after treatment with triethylamine, was unavailable to Edman degradation and the two forms were refractory to digestion with pepsin in 2.5 % (v/v) formic acid. Limited acid hydrolysis of triethylamine treated WLIP gave a number of breakdown products which were purified by reverse phase HPLC and characterised by amino acid composition and sequence analysis (Table III). The untreated form of WLIP gave no significant degradation products under the same hydrolytic conditions. These data combine to reveal the amino acid sequence of WLIP as Φ-Leu-Glu-Thr-Val-Leu-Ser-Leu-Ser-Ile where Φ represents a hydrophobic blocking group. Molecular weight data obtained by FABMS were consistent with these sequence and composition assignments. That Edman sequencing was possible through the glutamic acid residue indicated that it is present in the molecule in the α, α-linked form; this conclusion was supported by the observation of an NOE between Glu2α and Thr3NH, and the absence of NOEs between Glu2γ and Thr3NH.

Table III. Amino acid, Edman and FABMS analyses of hydrolytic fragments of WLIP.

Retention time /min	Composition	Edman generated sequence	Deduced sequence	M_r	Leucine chirality
14.21	$TSVL_2$	TVLSL		531	n.d.
16.00	-	ETVLSL		n.d.	n.d.
18.42	-	TVLSLSI		731	n.d.
22.80	EL	-	ΦLE	430	1L
24.72	L	-	ΦL	301	n.d.
26.05	$TEVL_2$	n.d.	ΦLETVL	743	1L, 1D
27.97	$TSEVL_3$	-	ΦLETVLSL	943	n.d.
29.52	TS_2EVIL_3	-	ΦLETVLSLSI	1143	2L, 1D

n.d. - not determined Φ - as yet unidentified blocking group

Location of the D-leucine residue. The position of the D-leucine residue remained ambiguous and was determined in the following manner. WLIP fragment peptides, generated and purified as described above, were subjected to total hydrolysis; the hydrolysates were then derivatised and analysed by chiral GC. Results from these runs are shown in Table III and demonstrate that the D-leucine residue is located at the centre of the peptide sequence.

Assignment of the ^1H NMR spectrum of WLIP. The assignment of peptide and protein ^1H NMR spectra is now virtually routine for molecular weights up to 20 000 daltons.[8] The main difficulty in assigning spin systems is to establish connectivities past degenerate resonances - a particular problem for molecules such as WLIP which contain a large proportion of leucyl, valyl and isoleucyl residues, the β/γ protons of which generally have a poor dispersion of chemical shift. In the TOCSY experiment,[9] magnetisation is transferred between protons in a coupling network. Proton resonances from amino acid residues can usually be assigned completely if at least one

resonance in the spin system is resolved, and if the mixing time is chosen carefully to transmit magnetisation as far as possible along the side chain.[10] The DQF-COSY experiment[11,12] can then be used to determine the sequence of couplings shown up by the TOCSY spectrum. Assignment of the WLIP spectrum was made in just this manner.

Two sections from the DQF-COSY spectrum of WLIP in DMSO-d_6 at 300 MHz and 305 K are presented in Figure II. The two serine and single valine residues were assigned immediately from this spectrum on the basis of their unique coupling patterns within the molecule. The threonine βCH proton has an unusually low chemical shift (5.15 p.p.m.) compared with that expected in the random coil (4.22 p.p.m.)[13] and once this was recognised the complete threonine spin system could be identified. The glutamic acid residue was then assigned; its γCH protons are degenerate and overlap with two protons in a non-amino acid spin system presumed to correspond to the N-terminal blocking group. This left the three leucine and single isoleucine residues to be located. Three intense and one weak unassigned αCH-βCH couplings can be seen in the DQF-COSY spectrum. Of the βCH protons concerned, only one is directly coupled into the methyl region of the spectrum, and this proton must therefore be part of the isoleucine spin system. Despite the degeneracy of βCH and γCH proton shifts in the aliphatic residues, their resonances were completely assigned from a TOCSY experiment run with a spin locking field duration of 100 ms, in which magnetisation transfer directly from resolved αCH protons to δCH protons could be seen. The amide protons of seven of the nine amino acids were assigned from αCH-NH couplings in the DQF-COSY spectrum; the αCH protons of threonine and a serine residue are degenerate in chemical shift and their amide protons were assigned by observation of Hartmann-Hahn transfer directly from resolved βCH to NH protons.

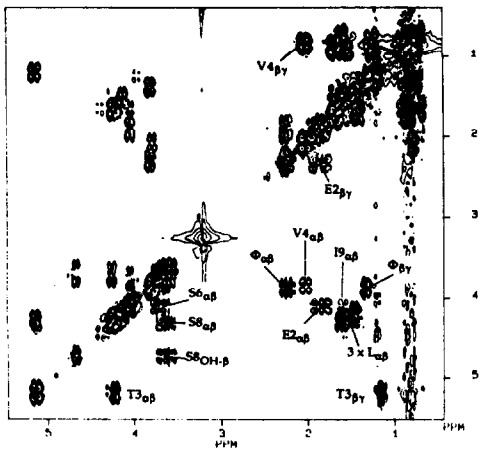


Figure II. DQF-COSY spectrum of WLIP in DMSO-d_6 (300 MHz, 305 K, 5 mM).

Table IV. ^{1}H chemical shift assignments (p.p.m.) for WLIP in DMSO-d_6, 5.0 mM, 305 K.

Residue	NH	αCH	βCH	γCH	δCH
βOHDec		2.25†	3.84	1.35	1.23
Leu1	8.25	4.19	1.48†	1.57	0.87/0.91
Glu2	8.55	4.10	1.82/1.91	2.27/2.30	
Thr3	8.31	4.29	5.15	1.16	
Val4	7.90	3.88	2.05	0.79†	
Leu5	7.38	4.36	1.45/1.66	1.61	0.84/0.90
Ser6	8.19	4.04	3.67/3.76		
Leu7	7.75	4.26	1.60†	1.69	0.81/0.90
Ser8	7.65	4.29	3.61/3.70		
Ile9	7.26	3.94	1.56	γCH_2 1.44† γCH_3 0.68	0.75

† degenerate in chemical shift

Sequential assignment of spin-systems. The assignment of cross peaks in the NOESY and ROESY spectra of WLIP in DMSO-d_6 allowed a sequential assignment of the multiple leucine and serine spin systems to be made. The method has been formalised by Billeter *et al.*[14] and is based on the observation that, irrespective of the secondary fold of a peptide or protein, at least one distance between NH, αCH and βCH protons on adjacent residues is less than 3 Å. The complete set of ^{1}H shift assignments for WLIP in DMSO-d_6 at 305 K is given in Table IV. Two regions of the NOESY spectrum are shown in Figure III and the complete set of NOEs identified in ^{1}H NOESY and ROESY spectra is listed in Table V.

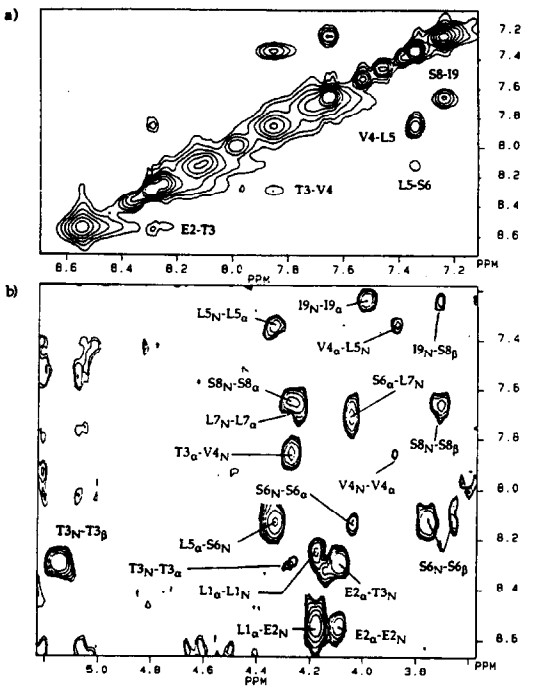


Figure III. Two regions from the 300 ms NOESY spectrum of WLIP (400 MHz, 305 K), a) amide-amide and b) fingerprint NOEs.

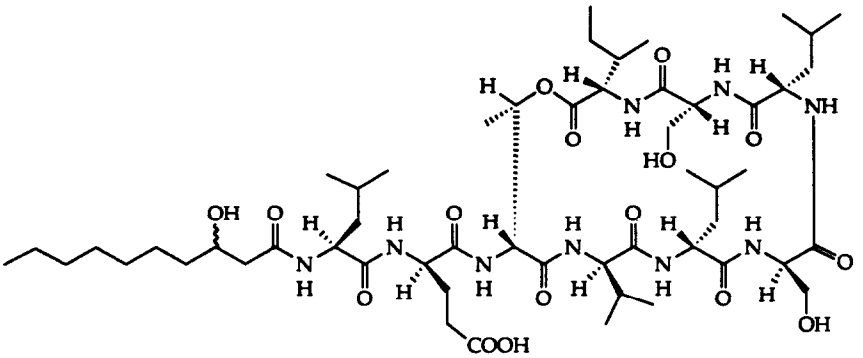
Table V. Inter-residue NOEs identified for WLIP in DMSO-d_6, 5.0 mM, 305 K in NOESY spectra acquired with a range of mixing times from 100 to 400 ms. NOEs were not stereospecifically assigned. NOEs quantitated by relative cross peak intensities at mixing times of 100 ms and 300 ms.

Residue	Proton	Interresidue NOEs
βOHDec	α/α'CH	Leu1NH (m)
Leu1	NH	Glu2NH (m)
Glu2	NH	Thr3NH (m)
	αCH	Thr3NH (l)
	βCH	Thr3NH (s)
Thr3	NH	Val6NH (s)
	αCH	Leu5NH (s), Val4NH (m)
	γCH	Ser8β (s)
Val4	NH	Leu5NH (l)
	αCH	Leu5NH (m)
	βCH	Leu5NH (m), Leu5β (m)
Leu5	NH	Ser6NH (s), Leu7α (m)
	αCH	Ser6NH (l)
Ser6	αCH	Leu7NH (l), Ile9γ (s)
Leu7	αCH	Ser8NH (l)
Ser8	NH	Ile9NH (m)
	βCH	Ile9NH (s)

(s) small (m) medium (l) large

Identification of the N-terminal blocking group. The simplest structure satisfying the criteria a) a mass of 171 daltons, b) a single hydroxyl group and c) a substructure X-$CH_2CH(Y)CH_2CH_2$(...) (the remaining unassigned spin system in the DQF-COSY spectrum) is β-hydroxydecanoic acid. This was synthesised from octanal and ethyl α-bromoacetate using Reformatsky conditions.[15] Derivatised as the O-trifluoroacetyl isopropyl ester, this authentic standard was found to co-elute with an unassigned peak in the chiral GC trace of the similarly derivatised WLIP hydrolysate. That the β-hydroxydecanoic acid was N-terminal to leucine was confirmed by the observation of NOEs between both αCH protons of the fatty acid and a leucine NH.

Lactone connectivities. The hydroxyl group of the lactone is provided by the threonine residue, assigned on the basis of the unusually large chemical shift of Thr3β (5.15 p.p.m.), compared with a random coil value of 4.22 p.p.m. The carboxyl group of the lactone can be provided by either the glutamic acid side chain or the C-terminal isoleucine. Reduction of a lactone results in an overall increase of 4 daltons in molecular mass, and a decrease of 14 daltons in the mass of the amino acid residue providing the carboxyl. Lithium borohydride reduction of WLIP (M_r 1125) gave a molecular ion (MLi^+) at *m/z* 1136 (M_r 1129) as expected. This reduced material was subjected to limited acid hydrolysis and the mixture of fragments analysed by FABMS. A loss of 14 mass units, compared to the equivalent fragment from linear WLIP, was only observed for peptides containing the C-terminal isoleucine, confirming that the lactone is formed between this residue and Thr3. The complete covalent structure of WLIP is shown in Figure IV.



β-hydroxydecanoyl-LLeu-DGlu-D, *allo*-Thr-DVal-DLeu-DSer-LLeu-DSer-LIle

Figure IV. Complete covalent structure of WLIP.

Viscosin. A literature search for peptides of similar structure revealed a natural product called viscosin.[16] A structure for viscosin was first proposed by Ohno *et al.* in 1953[16] but this was later shown to be incorrect by synthesis of the putative structure and comparison with authentic material.[17] An alternative structure proposed by Hiramoto *et al.*[18] was confirmed by the total synthesis of viscosic acid (the saponification product of viscosin). Total synthesis of viscosin was finally accomplished by solid phase methods in 1989.[19] Viscosin and WLIP are identical in every respect except for the chirality of Leu5, which has D configuration in WLIP and L configuration in viscosin. We considered it rather surprising that two such natural products should differ solely in the stereochemistry of a single residue and therefore obtained an authentic sample of viscosin for direct comparison with WLIP. The chiral GC traces of WLIP and viscosin are presented in Figure V and confirm that viscosin contains only L-leucine.

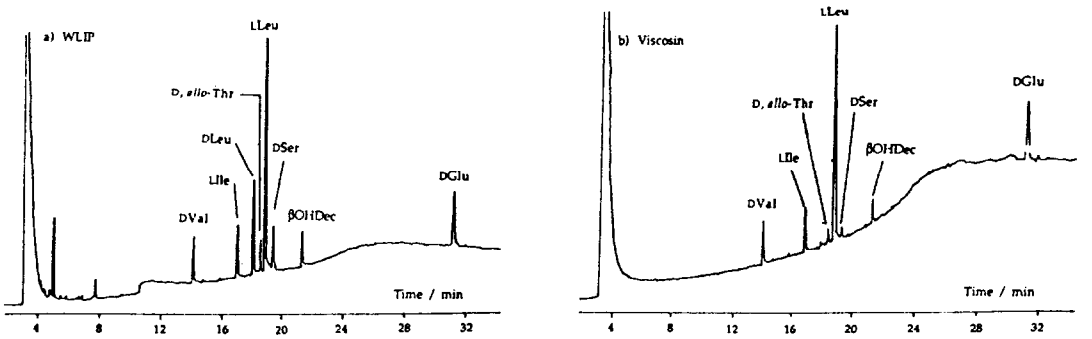


Figure V. Chiral GC analyses of the N, O-trifluoroacetyl isopropyl ester derivatives of the total hydrolysates of a) WLIP and b) viscosin. The low intensities of the D-*allo*-threonine and D-serine peaks in the viscosin trace are due to the longer hydrolysis period employed.

Conformational Studies on WLIP

Amide exchange rates. In a protic solvent, the exchange rate of an amide proton is dependent both upon the extent to which it is hydrogen bonded, since these hydrogen bonds must be broken for exchange to occur, and upon the accessibility of a given proton to solvent. In solvents with exchangeable deuterium atoms, such as D_2O and CD_3OD, proton-deuteron exchange removes the resonances of labile amide protons from the ^1H NMR spectrum. Those protons remaining provide information about the local structure of the molecule. Little overall change in the spectrum of WLIP in DMSO-d_6 was observed on addition of 10 % CD_3OD, suggesting that this did not alter the conformation significantly from that in pure DMSO-d_6. Over a period of twelve hours all amide protons except those of Ile9, Leu5 and Thr3 were completely exchanged. After a period of four weeks, Thr3NH and Leu5NH disappeared and are thus partially protected from exchange, while Ile9NH was reduced in intensity by *ca.* 1/3, and is correspondingly much less accessible to solvent.

Amide temperature coefficients. Several groups of workers have noted that the relationship between amide chemical shift and temperature is indicative of the degree of hydrogen bonding to the proton concerned;[20] a small temperature coefficient ($< 3 \times 10^{-3}$ p.p.b. K^{-1}) suggests the presence of an intramolecular hydrogen bond. Amide chemical shifts were determined for WLIP in DMSO-d_6 over the range 300 to 365 K, and are plotted against temperature in Figure VI. Of particular note are the data for Leu1, Glu2 and Ser8. At first, chemical shifts were measured only up to 330 K, revealing positive temperature coefficients for these protons. This was unexpected, since it suggests that *stronger* hydrogen bonds are formed at higher temperatures. One rationale for this behaviour might be that the local fold of the peptide protects these protons from solvent. At higher temperatures, the greater conformational flexibility of the molecule may expose them to an increased degree of hydrogen bonding either to solvent or an internal acceptor group, shifting them to lower field. Nonetheless, at even higher temperatures these protons should still exhibit negative temperature coefficients and this was indeed found to be the case in spectra acquired between 330 and 365 K. The temperature coefficients for WLIP in DMSO-d_6 range from 2.6×10^{-3} p.p.b. K^{-1} for Thr3 to 11.9×10^{-3} p.p.b. K^{-1} for Ser6. Ile9, which is protected from solvent according to amide exchange experiments, has a $\Delta\delta/T$ coefficient of 6.0×10^{-3} p.p.b. K^{-1}. Only Thr3 and Leu5 (3.7×10^{-3} p.p.b. K^{-1}) have low enough coefficients to suggest that hydrogen bonds are formed to these amide protons.

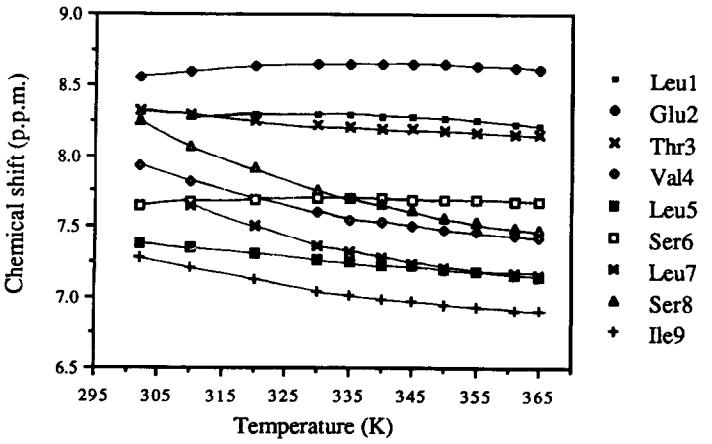


Figure VI. Temperature dependence of amide chemical shifts for WLIP in DMSO-d_6 (400 MHz and 2.5 mM).

Distance geometry, molecular mechanics and dynamics calculations. The NOEs identified in Table V were used as input constraints to the DGEOM package of Blaney.[21] To reduce computational demands and in the light of the lack of NOE data at the N-terminus, the N-terminal Φ-Leu moiety was replaced by an acetyl group. Holonomic constraints were generated from the peptide in a linear β-strand conformation and additional bond length and angular constraints were included to enforce an acceptable geometry on the lactone linkage. Improper constraints were included to fix amino acid stereochemistry and to force amide groups into a *trans* configuration. 30 trial structures were generated and all inter-proton contacts small enough to generate NOEs were calculated. The quality of fit to experimental data was assessed and those 10 structures which best fitted the data were subjected to 200 iterations of restrained minimisation using the AMBER forcefield, during which square well constraints were applied between protons for which an NOE was observed. These minimised structures were put through 4 ps of restrained molecular dynamics using the XPLOR package of Brunger *et al.*[22] and the temperature regulation method of Berendsen *et al.*[23] During the first 1.5 ps the temperature was raised from 20 K to 1000 K, then over the next 1.5 ps the system was allowed to equilibrate at this temperature and was finally cooled to 0 K over 1 ps. Superpositions of five of the ten structures generated are shown in two orientations in Figure VII. The maximum R.M.S. difference between the backbone atoms of any two of the five structures was 1.42 Å, the average pairwise R.M.S. difference for backbone atoms was 1.23 Å and for heavy atoms was 3.56 Å. This latter figure is comparatively large and reflects the lack of NOE data available between the side chains of residues. The structures all fall into a single set, in which the global fold resembles the seam of a tennis ball.

In all conformers, a type II β-turn is apparent between Ser6 and Ile9; the unfavourable 1,3 steric interaction expected between the carbonyl of Leu7 and the sidechain of Ser8 is alleviated by the D stereochemistry of Ser8. The protection of Ile9NH from solvent exchange can then be rationalised in terms of its involvement in the *i, i+3* hydrogen bond of the turn. Note that distance contraints for hydrogen bond formation were not included in structure generation. The unfavourable 1, 3 steric interactions between the carbonyl group of the *i+1* residue and the sidechain of the *i+2* residue in a type II β-turn are typically removed in proteins by the incorporation of glycine at the *i+2* position of the turn.[24] In WLIP, the same effect is produced by placing a residue of D stereochemistry at position *i+2*.

The lactone adopts a *cis* configuration with the γCH_3 group of Thr3 *exo* to the peptide macrocycle. Val4 and Leu5 adopt a bulge-type conformation in which Val4NH and Leu5NH are within hydrogen bonding distance of the sidechain hydroxyl of Ser8. Ser8NH is partially protected from solvent (see above) and is one of the protons to exhibit a downfield shift at increased temperature. One possible rationale for this behaviour might be that a conformational rearrangement of the molecule occurs at higher temperature, in which the position of the turn shifts to residues Leu5 to Ser8; Ser8NH might then be expected to shift to lower field as it forms a stronger hydrogen bond to the Leu5 carbonyl.

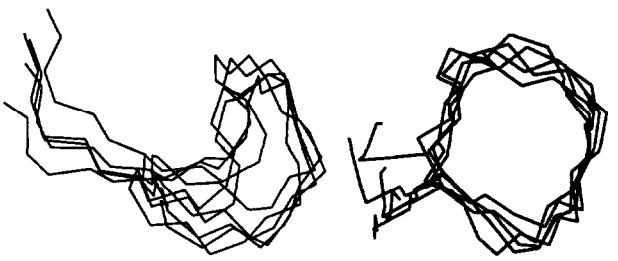


Figure VII. Approximately orthogonal views of five of the ten calculated WLIP structures. The overall fold of the macrocycle resembles the seam of a tennis ball. Backbone atoms only are shown.

Comparative studies on viscosin. The ^1H NMR spectrum of viscosin was assigned according to the method described for WLIP. With the exception of the NOEs Ser6NH→Ser8NH and Val4α→Leu7NH all inter-residue NOEs were between adjacent amino acids; this makes the determination of conformation rather difficult. The conformation of the Thr-Val subunit in viscosin can be postulated from the relative intensities of NOEs between Thr3 and Val4, namely Thr3β→Val4NH (s), Thr3β→Thr3NH (m), Thr3α→Val4NH (l) and Thr3α→Thr3NH (s). These place Val4NH on the same side of the macrocycle as Thr3α; both these protons are on the opposite side of the macrocycle to Thr3β, Thr3NH and Thr3CO. However, a $d_{\alpha N}(i, i+3)$ Val4α→Leu7NH NOE suggests that a β-turn may be formed by residues Val4 to Leu7. In viscosin, the stereochemistry of residues 4 to 7 (DLDL) will have the same effect of removing the unfavourable 1, 3 steric interaction noted in WLIP (see above). The anomalous behaviour of amide temperature coefficients observed for WLIP was not reproduced in viscosin. The low temperature coefficient determined for Leu7 (2.3×10^{-3} p.p.b. K^{-1}) is, however, consistent with the placement of Leu7 at the *i+3* position in the turn.

Conclusion. The structure elucidation of WLIP illustrates how the careful use of a combination of FABMS, Edman sequencing, GC and NMR spectroscopy provides solutions to problems which would be intractable to the techniques taken in isolation. The data obtained from NMR were put to good use in the generation of three-dimensional structures compatible with the experimental constraints and it is hoped that this information, together with that available on tolaasin (the second component of the white line interaction)[6,7,25] will allow the nature and significance of the white line reaction to be determined.

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Experimental

Preparation of crude WLIP. The following procedure for preparation and purification of WLIP is based on that of Peng.[6] A 2 l conical flask containing 1 l of *Pseudomonas* F medium (PAF; Difco) at pH 7.4 was inoculated with *Pseudomonas reactans* (National Culture of Plant Pathogenic Bacteria strain 387) and grown for 3 days at 25 °C in a cooled orbital incubator shaking at 200 rpm. The cells were removed by centrifugation (8 000 g; 20 min, 15 °C), the supernatant frozen (-20 °C, overnight), thawed and filtered through Whatman No. 42 filter paper. The filtrate was acidified to pH 5 with 1 M HCl and the resulting precipitate collected by centrifugation (8 000 g; 20 min, 15 °C) and resuspended in 250 ml distilled water. The pH was adjusted to 7.5 with 1 M NaOH, the solution filtered through Whatman No. 1 filter paper and the pH readjusted to 5 with 1 M HCl. The resulting precipitate was collected by centrifugation (8 000 g; 20 min, 15 °C) and dried at 50 °C. The dried residue was resuspended in 100 ml methanol, filtered through Whatman No. 42 filter paper and the filtrate evaporated to dryness *in vacuo* at 45 °C. The residue was washed in 10 ml methanol, recovered by centrifugation (8 000 g; 20 min, 15 °C), dissolved in 100 ml methanol and the resulting solution evaporated to dryness *in vacuo* at 45 °C to yield *ca.* 100 - 200 mg of crude WLIP per litre of culture. Crystalline WLIP of sufficient purity for analysis was prepared by the diffusion of water vapour into a saturated solution of WLIP in methanol.

Total acid hydrolysis. Replicate samples of WLIP were hydrolysed at 110 °C by the vapour of 6 M HCl containing 0.1 % 2-mercaptoethanol and 1 mM phenol under an atmosphere of argon for 24-96 h. The amino acid composition was determined on an LKB 4400 Amino Acid Analyser using ninhydrin detection.

Limited acid hydrolysis. Samples of triethylamine treated WLIP were incubated with 6 M HCl at 110 °C for 10 min in sealed glass tubes. Separation of parent and hydrolytic products was by reverse phase HPLC on a Brownlee Aquapore RP3000 (C8) column (2.1 mm x 30 mm) using a Hewlett Packard 1090L liquid chromatograph. Samples were solubilised in 1% (v/v) triethylamine and loaded as a plug of liquid sandwiched between two plugs of the same solvent to prevent precipitation of the material on contact with the equilibration buffer, 0.1% (v/v) trifluoroacetic acid, in which it was not readily soluble. Once bound to the reverse phase column, the peptides remained soluble. Elution of the parent and fragmentation products was achieved with a linear gradient of 0-80% CH_3CN in 0.1% TFA at 100 μl min^{-1} over 30 min.

Sequencing. Amino acid sequence analysis was performed on an Applied Biosystems model 477A Pulsed-Liquid Protein Sequencer with an on-line model 120A PTH-Amino Acid Analyser.

Fast Atom Bombardment Mass Spectrometry (FABMS). Mass spectra were obtained on a Kratos MS-50 spectrometer fitted with an Ion Tech FAB gun in operation with xenon in the positive ion mode at accelerating voltage of 8 kV. 3-Nitrobenzylalcohol was employed as matrix for all samples.

Esterification. 10 nmol samples were treated with 1M HCl in EtOH for 24 h at room temperature and the solvent evaporated under vacuum prior to analysis by mass spectrometry.

Acetylation (to detect free amino groups). WLIP (10 nmol) was dissolved in a 1:1 mixture (v/v) of acetic anhydride and 50 mM ammonium acetate at pH 8.4 and sonicated at room temperature for 2 h.

Oxidation with 1,1-bis(trifluoroacetoxy)iodobenzene (TIB, converts primary amides to amines). WLIP (0.3 mg) was dissolved in 100 µl 50% (v/v) acetonitrile/water and an equimolar amount of TIB added in 25 µl of the same solvent. The mixture was protected from light and left at room temperature for 5 h. 375 µl 0.01M HCl was added and the sample lyophilised and analysed directly by FABMS.

Trimethylsilylation (labels hydroxyls and primary amides).[26] 10 nmol samples were dissolved in 10 µl Trisil reagent (obtained from Pierce, a mixture of trimethylchlorosilane and hexamethyldisilazane in pyridine) and transferred immediately to the probe tip of the mass spectrometer for analysis.

Lithium Borohydride Reduction. WLIP (1 mg) was dissolved in 1 ml ethanol and a small spatula of lithium borohydride added under argon. The mixture was stirred and left at room temperature overnight. The reaction was terminated by the addition of 1 M HCl and the solution lyophilised. The resultant solid was dissolved in 300 µl 0.1 M HCl, extracted three times with chloroform (1.2 ml) and the organic layer dried *in vacuo*. The reduced material was then fragmented by hydrolysis with 6 M HCl for 5 min under the same conditions described for triethylamine treated WLIP.

NMR Spectroscopy. ^{1}H NMR spectra were recorded on a 5.0 mM sample of WLIP in DMSO-d_6 using Bruker AM-400 and AM-500 spectrometers equipped with Aspect 2000 computers. Spectra were recorded over spectral widths of 4 000 or 5 000 Hz (at 400 MHz and 500 MHz respectively) with quadrature detection employed throughout. Two-dimensional proton NMR spectra were acquired in the phase sensitive mode using quadrature detection in F_2, and with the time proportional phase incrementation method of Marion and Wüthrich in F_1.[27] Data sets resulting from 350 to 512 increments of t_1 were acquired (and zero filled to 1 024 points), with each FID composed of 2 048 data points. A relaxation delay of 1 s was used between successive transients. NOESY spectra were recorded with mixing times of 200-400 ms, varied by 20-30 ms between t_1 values to minimize zero quantum artefacts. TOCSY spectra were recorded using a 5 kHz spin locking field; a DIPSI-2 sequence was used to provide the isotropic mixing and the spin locking field was applied for between 75 and 100 ms. Data sets were subjected to mild Lorentzian-Gaussian apodisation prior to Fourier transformation.

Chiral GC analysis. Derivatisation steps were performed in glass vials with close fitting screw caps. 1 mg samples of triethylamine treated WLIP were hydrolysed in 200 µl of 6 M HCl (Fisons, sequanal grade) under nitrogen at 105 °C for 8 h or 24 h. The solvent was then removed *in vacuo*. When dry, the residue was dissolved in 200 µl 1 M HCl in *iso*propanol and heated under nitrogen at 105 °C for 30 min. The sample was again cooled, and dried *in vacuo*. Dichloromethane (250 µl) and trifluoroacetic acid anhydride (250 µl) were added to the mixture of *iso*propyl esters and the mixture heated at 105 °C under nitrogen for 10 min. Solvent was then removed in a stream of dry nitrogen whilst cooling the sample in ice. Amino acid standards were derivatised in the same way. The derivatised mixture was analysed using a Carlo Erba 4130 gas chromatograph fitted with a split/splitless injector (splitter ratio commonly 20:1) and a flame ionization detector. The injector temperature was maintained at 240 °C. The amino acid derivatives were dissolved in 10 µl dry dichloromethane and approximately 0.1 µl of this injected onto a *Chirasil-Val* capillary column (0.2 mm x 50 m, Alltech Chromatography Ltd). A temperature program running from 80 °C to 190 °C at 4 °C min^{-1} generally provided good resolution of enantiomers. HPLC purified fragment peptides were prepared for GC by the same procedure and introduced onto the column with the injector in splitless mode for greater sensitivity.

Molecular modelling. Molecular modelling was performed on a MicroVAX 3600 computer equipped with an Evans and Sutherland PS390 graphics terminal.

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