284.2140, obsd 284.2087; $[\alpha]^{19}_D$ +137.51° (c 3.09, $CHCl_3$).

The three-dimensional structure of **3**, determined crystallographically, is shown in Figure 2.

Double-Bond Isomerization in 49. A heptane solution (2.5 mL) of **49** (40 mg, 0.132 mmol) was treated with thiophenol (1 mL) and AIBN (27 mg, 0.197 mmol) and irradiated with a sunlamp for 3 h while being vigorously stirred. The volatiles were removed under reduced pressure, and the residue was purified by MPLC (silica gel, elution with ethyl acetate/petroleum ether, 1:4.5). There were isolated 14 mg of the less polar alcohol and 10 mg of the more polar alcohol (total yield 60%) corresponding to **54**.

For the less polar stereoisomer: IR ($CHCl_3$, cm^{-1}) 3600, 2960, 2870, 1680, 1455, 1385, 1300, 1110; ^1H NMR (300 MHz, $CDCl_3$) δ 2.95 (dt, J = 9.0, 12.2 Hz, 1 H), 2.71 (t, J = 11.4 Hz, 1 H), 2.55 (quin, J = 6.8 Hz, 1 H), 2.26-1.95 (m, 7 H), 1.82-1.69 (m, 3 H), 1.63-1.36 (m, 4 H), 1.31 (s, 3 H), 1.05 (d, J = 6.5 Hz, 3 H), 1.01 (s, 3 H), 0.92 (d, J = 6.2 Hz, 3 H), 0.90 (d, J = 6.5 Hz, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 216.14, 144.12, 137.41, 82.39, 65.01, 52.77, 48.93, 48.78, 41.85, 36.87, 32.89, 28.67, 27.43 (2 C), 27.33, 26.71, 24.66, 23.77, 21.08, 19.66; MS m/z (M^+) calcd 304.2402, obsd 304.2409.

For the more polar isomer: IR ($CHCl_3$, cm^{-1}) 3580, 2960, 2870, 1670, 1455, 1380, 1340, 1300, 1120; ^1H NMR (300 MHz, $CDCl_3$) δ 2.60-2.48 (m, 3 H), 2.37-1.97 (m, 6 H), 1.92-1.76 (m, 4 H), 1.63-1.39 (m, 4 H), 1.16 (s, 3 H), 1.06 (d, J = 6.5 Hz, 3 H), 1.02 (s, 3 H), 0.90 (d, J = 6.0 Hz, 3 H), 0.88 (d, J = 6.4 Hz, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 216.12, 145.05, 136.80, 80.33, 66.19, 53.36, 49.07, 47.37, 41.06, 37.07, 33.22, 28.78, 27.61, 27.44, 26.71, 24.51, 24.21, 23.20, 21.09, 16.69; MS m/z (M^+) calcd 304.2402, obsd 304.2418.

Double-Bond Isomerization in 3. A. Rhodium Catalysis. Epoxy ketone **3** (1.8 mg, 0.0060 mmol) dissolved in ethyl acetate (1 mL) was treated with 5% Rh/C, and the resulting mixture was stirred under 1500 psi of hydrogen at room temperature for 20 h. The reaction mixture was filtered through a Celite pad and evaporated. Purification of the residue by silica gel chromatography (elution with 2% ethyl acetate in petroleum ether) gave 1.3 mg (72%) of **55**: IR ($CHCl_3$, cm^{-1}) 2960, 1710, 1450, 1385, 1115, 990; ^1H NMR (300 MHz, $CDCl_3$) δ 2.73-2.51 (m, 3 H), 2.37-2.23 (m, 2 H), 2.16-2.05 (m, 3 H), 1.94-1.43 (m, 7 H), 1.50 (s, 3 H), 1.07 (d, J = 6.6 Hz, 3 H), 0.97 (d, J = 6.8 Hz, 3 H), 0.88 (s, 3 H), 0.87 (d, J = 5.9 Hz, 3 H); ^{13}C NMR (75 MHz, C_6D_6) δ 205.87, 145.32, 137.61, 74.06; 73.39, 51.53, 48.76, 47.30, 36.50, 33.14, 32.97, 31.11, 27.89, 27.10, 23.97, 23.10, 22.39, 20.86, 19.79, 17.09; MS m/z (M^+) calcd 302.2246, obsd 302.2241.

B. Thiophenol Catalysis. A solution of **3** (10 mg, 0.033 mmol), thiophenol (1 ml), and AIBN (10 mg) in heptane (5 mL) was irradiated as described above for 3 h. The identical workup afforded 5.9 mg (59%) of **55**, identical in all respects with the material produced in part A.

Acknowledgment. We thank the National Institutes of Health (Grants GM-30827 and CA-12115) for generous financial support and Judith Gallucci and Haibin Deng for performing the X-ray structure determinations.

Registry No. 3, 126458-49-9; **4**, 126458-60-4; **6**, 126458-55-7; **7**, 131934-82-2; **10**, 62994-35-8; **11**, 131934-84-4; **11** (carbinol, isomer 1), 131934-81-1; **11** (carbinol, isomer 2), 131935-08-5; **12**, 131934-85-5; **13**, 131934-86-6; **14**, 131934-87-7; **15**, 131934-88-8; **16**, 131934-89-9; α-**17a**, 132072-20-9; β-**17a**, 131934-90-2; α-**17b**, 132072-21-0; β-**17b**, 131934-83-3; α-**17c**, 132072-23-2; β-**17c**, 131934-91-3; β-**17d**, 132072-24-3; **21**, 131934-92-4; **22**, 131973-37-0; **23**, 132072-22-1; **24**, 131934-93-5; **25**, 131934-94-6; **26**, 131973-38-1; **27**, 117152-55-3; *epi*-**27**, 132072-28-7; **28**, 131934-95-7; *epi*-**28**, 132072-25-4; **29**, 126458-50-2; **29** triol, 131935-09-6; (5*R*)-**29** triol, 132072-29-8; **29** aldehyde, 131935-07-4; **30**, 131934-96-8; **31**, 126458-51-3; **32a**, 126458-52-4; **32b**, 131935-00-7; **33**, 131934-97-9; **37**, 126458-53-5; **38**, 131934-98-0; **39**, 126458-54-6; **40**, 131934-99-1; **42**, 126458-56-8; **43**, 126576-00-9; **47**, 131935-01-8; **48**, 126458-57-9; **48** ketal, 131935-05-2; **49**, 126458-58-0; **50**, 126458-59-1; *epi*-**50**, 132072-26-5; Δ^8-**51**, 131935-06-3; $\Delta^{8(19)}$-**51**, 131935-02-9; **52**, 126458-63-7; **53**, 126458-64-8; **54** (isomer 1), 131935-03-0; **54** (isomer 2), 132072-27-6; **55**, 131935-04-1; (*E*)-$Me_3SiCH{=}CHBr$, 41309-43-7; $CH_2{=}C(CH_3)(CH_2)_2Br$, 20038-12-4; $Ph_3P^+CH_3$ I^-, 2065-66-9; 4-bromo-2-butanone ethylene ketal, 37865-96-6; 4-bromo-2-butanone neopentyl glycol ketal, 87842-52-2.

Supplementary Material Available: Figures illustrating the labeling schemes for **39** and **3**, experimental descriptions, and tables of the crystallographic details, final positional and thermal parameters, bond lengths, and bond angles for both compounds (19 pages). Ordering information is given on any current masthead page.

Structure Determination of Tolaasin, an Extracellular Lipodepsipeptide Produced by the Mushroom Pathogen *Pseudomonas tolaasii* Paine

Jennifer C. Nutkins,[†] Russell J. Mortishire-Smith,[†] Leonard C. Packman,[‡] Catherine L. Brodey,[§] Paul B. Rainey,[§] Keith Johnstone,[§] and Dudley H. Williams*[,†]

Contribution from the University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW, U.K., Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW, U.K., and Department of Botany, University of Cambridge, Downing Street, Cambridge, CB2 3EA, U.K. *Received October 1, 1990*

Abstract: The principal active component of tolaasin, the *Pseudomonas tolaasii* toxin, which is responsible for brown blotch disease of mushrooms, is shown to be a lipodepsipeptide of M_r 1985. The structure determined is β-hydroxyoctanoyl-ΔBut-D-Pro-D-Ser-D-Leu-D-Val-D-Ser-D-Leu-D-Val-L-Val-D-Gln-L-Leu-D-Val-ΔBut-D-*allo*-Thr-L-Ile-L-Hse-D-Dab-L-Lys (cyclized via lactone formation between D-*allo*-Thr and the C-terminus) by a combination of fast atom bombardment mass spectrometry (FABMS), ^1H NMR, automated sequencing, and chiral gas chromatography. A minor component of the toxin (M_r 1941) has a related structure in which the homoserine residue is substituted by glycine. In addition, the sequence of a modified form of the toxin, produced by a genetically engineered strain of *P. tolaasii*, is characterized.

Introduction

Pseudomonas tolaasii is the causal organism of the economically significant brown blotch disease of the cultivated mushroom *Agaricus bisporus* (Lange) Imbach.[1] Colonization of mushroom basidiocarps by the bacterium results in unsightly brown lesions that render affected mushrooms unmarketable.

P. tolaasii culture filtrates cause blotch symptoms identical with those caused by the whole organism.[2] Methods for isolation of

[†] University Chemical Laboratory.
[‡] Department of Biochemistry.
[§] Department of Botany.

(1) Tolaas, A. G. *Phytopathology* **1915**, *5*, 51-54.
(2) Nair, N. G.; Fahy, P. C. *Aust. J. Biol. Sci.* **1973**, *26*, 509-512.

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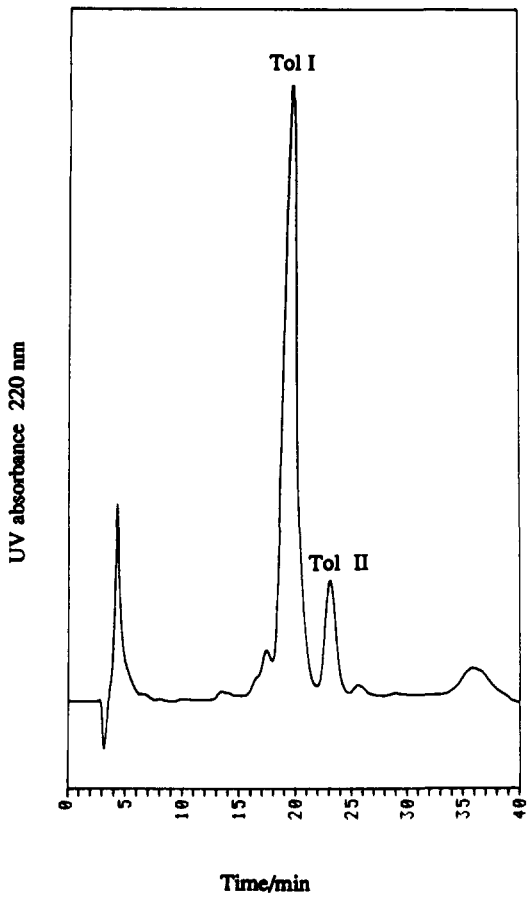

Figure 1. Analytical fractionation of crude tolaasin by reversed-phase HPLC on an Aquapore RP300 (C8) column, 2.1 × 30 mm. A 10-μg sample of material was dissolved in 0.1% TFA and injected onto the column, which was equilibrated with 40% CH_3CN in 0.1% TFA. Elution at 50 μL min^{-1} was by a gradient to 52% CH_3CN (0.1% TFA) over 25 min. Detection was at 220 nm.

the toxin, which we have named tolaasin, were devised by Peng,[3] who reported that it was a polypeptide with molecular weight between 1 and 10 kDa. Addition of partially purified toxin to mushroom basidiocarps reproduces the disease symptoms of the intact organism,[3] and interaction between tolaasin and the "white line inducing principle" (WLIP) from *Pseudomonas reactans*[3] gives rise to the precipitate observed in the diagnostic "white line in agar test".[4]

This work describes the isolation, purification, and primary structure determination of tolaasin, the causative agent of brown blotch disease of the cultivated mushroom.

Results

Tolaasin was isolated from the cell-free culture filtrate of *P. tolaasii* by precipitation with calcium chloride. After partial purification by centrifugation and anion exchange chromatography on a Dowex-1 column, a crude preparation, which retained full biological activity, was obtained. Positive-ion fast atom bombardment mass spectrometry (FABMS) indicated that this material consisted of two principal components with molecular weights of 1985 and 1941, and these were designated Tol I and Tol II. Reversed-phase high performance liquid chromatography (HPLC) on a C8 or phenyl column separated the two compounds and demonstrated that the minor component, Tol II, was marginally more hydrophobic than Tol I (Figure 1). Quantitative amino acid analysis of Tol I identified eight of the common amino acids (Table I) and also showed the presence of two unusual amino acids, X1 and X2. The composition of Tol II was found to be identical

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Table I. Amino Acid Analysis of Tol I and Tol II

amino acid	Tol I	Tol II	chirality
proline	0.94 (1)	1.08 (1)	D
serine	1.89 (2)	1.88 (2)	D
threonine	1.12 (1)	1.03 (1)	D-*allo*
leucine	3.04 (3)	3.13 (3)	1L, 2D
isoleucine	0.89 (1)	1.04 (1)	L
valine	3.81 (4)	3.70 (4)	1L, 3D
glutamic acid	1.08 (1)	1.36 (1)	D
lysine	1.02 (1)	1.04 (1)	L
glycine	absent	1.39 (1)	na[a]

[a] na, not applicable.

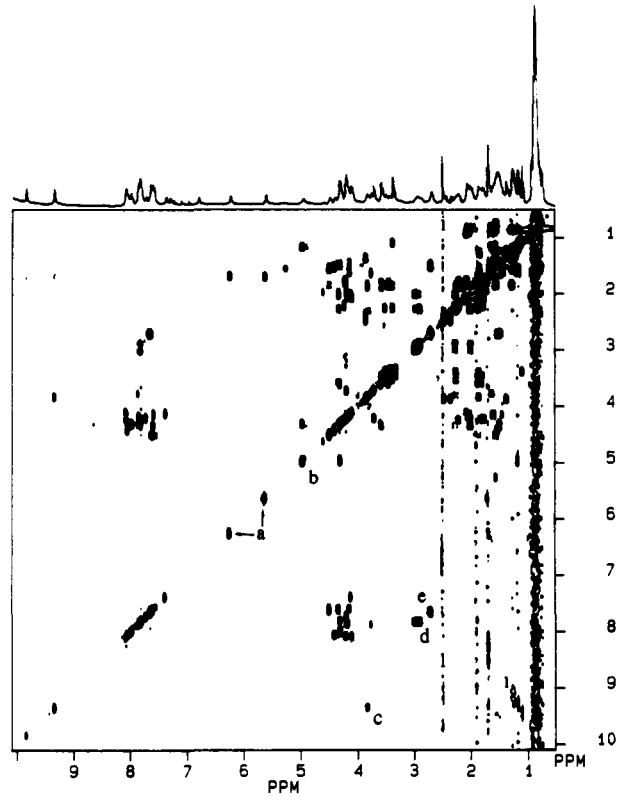

Figure 2. One-dimensional and DQF-COSY spectra of Tol I in DMSO-d^6 at acidic pH. Notable features are (a) diagonal peaks corresponding to alkenic protons of the dehydroaminobutyric acid residues; (b) unusually low-field shift of the threonine β-proton, exhibiting coupling to both α- and γ-protons; (c) Hse NH–αCH coupling, separated from the majority of the NH–αCH correlations; (d) diaminobutyric acid γCH_2–δNH_2 correlation; and (e) lysine ϵCH_2–ϕNH_2 correlation.

with that of Tol I apart from the replacement of the unknown, X1, by glycine.

The chiralities of the known amino acids were determined by capillary gas chromatography (GC) with a Chirasil-Val chiral column. Samples of Tol I and Tol II were hydrolyzed to their constituent amino acids (6 M HCl, 105 °C, 24 h), and the resulting mixtures were analyzed on GC as the *N,O*-trifluoroacetyl (or pentafluoropropionyl) isopropyl esters. Coelution with standard amino acid derivatives gave the results summarized in Table I, demonstrating that tolaasin contains a high proportion of D-amino acids and that both D and L forms of valine and leucine are present.

Analysis of the DQF-COSY[5] and TOCSY[6] spectra of Tol I indicated spin systems consistent with the presence of homoserine (Hse) and 2,4-diaminobutyric acid (Dab) residues in the molecule (Figure 2 and Table II). The identity of these amino acids was

(5) Piantini, U.; Sorenson, O.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 6800–6801.
(6) (a) Braunshweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521–528. (b) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355–360. (c) Weber, P. L.; Sieker, L. C.; Samy, T. S. A.; Reid, B. R.; Drobny, G. P. *J. Am. Chem. Soc.* **1987**, *109*, 5842–5844.

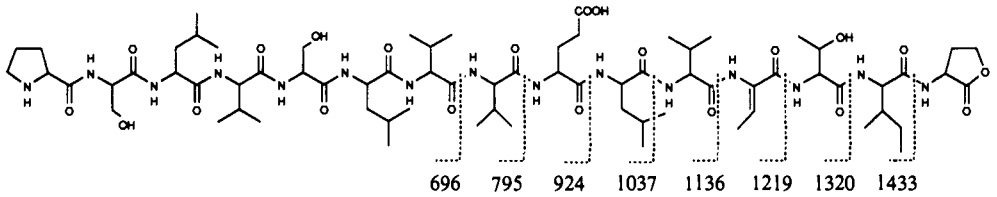

Figure 3. Primary structure of the Tol I fragment, M_r 1533 (MH^+ 1534), showing assignment of acylium fragment ions observed in the positive ion FAB mass spectrum that permitted the partial determination of the C-terminal sequence.

Table II. Experimental Chemical Shifts of Tol I in DMSO-d^6 at 297 K

	NH	αCH	βCH	γCH	δCH	others
Φ		2.25	3.84[a]	1.35	nd[c]	nd[c]
ΔBut1	9.83	5.62	1.69			
Pro2		4.22	2.20[a]	1.79/1.70	3.57/3.44	
Ser3	7.83	4.19	3.72[a]			
Leu4	7.58	4.14	1.58/1.42	nd[c]	0.76/0.73	
Val5	7.37	4.12	2.00	0.84/0.82		
Ser6	7.81	4.12	3.58[a]			
Leu7	7.98	4.32	1.47/1.64	nd[c]	0.87/0.79	
Val8	7.73	4.19	1.99	0.84/0.81		
Val9	7.85	4.18	2.03	0.84/0.81		
Gln10	8.08	4.20	1.83/1.76	2.08[a]		
Leu11	8.06	4.40	1.52[a]	nd[c]	0.90/0.84	
Val12	8.08	4.10	2.07	0.94/0.89		
ΔBut13	9.35	6.24	1.68			
Thr14	8.00	4.30	4.96	1.15		
Ile15	7.86	3.76	1.63	γCH_2 1.55/1.15 γCH_3 0.90	0.84	
Hse16	9.35	3.82	2.46/2.34	3.50/3.37		
Dab17	7.59	4.33	2.27/2.00	2.96/2.90		ϵNH_2 7.82
Lys18	7.61	4.50	1.49[a]	1.26[a]	1.84[a]	ϵCH_2 2.70[a] ϕNH_2 7.65

[a] Degenerate. [b] Φ, β-hydroxyoctanoic acid. [c] nd, not determined.

confirmed by cochromatography of authentic samples of the derivatives of L-homoserine and D-2,4-diaminobutyric acid with previously unidentified peaks in the chiral GC trace of Tol I. In addition, when the amino acid analysis was repeated, homoserine was found to coelute with the peak assigned to X1 and diaminobutyric acid with that assigned to X2. Quantitation with standard samples of the unusual amino acids confirmed that one residue of each type was present.

Two previously unassigned 1:3:3:1 quartets (J = 7.2 Hz) in the DQF-COSY spectrum of Tol I were apparent at chemical shifts of 6.24 and 5.62 ppm (Figure 2). Difference decoupling experiments demonstrated that these were coupled to separate 3 H doublets at 1.68 and 1.69 ppm, respectively. No other scalar couplings to these latter resonances were observed in the DQF-COSY spectrum; however, two small cross-peaks corresponding to Hartman–Hahn correlations between these signals and downfield amide resonances at 9.35 and 9.83 ppm were noted in the TOCSY spectrum. These data are consistent with the presence of two dehydroaminobutyric acid residues (ΔBut). The *Z* stereochemistry was assigned to each residue on the basis of the observation of strong nuclear Overhauser effects (NOEs) between their amide NH and γCH_3 protons and the absence of NOEs between their amide NH and alkenic CH protons. Dehydroaminobutyric acid is not stable to acid hydrolysis and consequently cannot be detected by amino acid analysis. The total mass of the amino acids observed left approximately 150 Da to complete the molecular weight of Tol I.

Functional Groups. To determine the functional groups present in tolaasin, small-scale derivatization experiments were carried out on the crude toxin and the products characterized by FABMS. Data were thus simultaneously obtained for both Tol I and Tol II, and the results are shown in Table III.

Automated Edman degradation of the peptides failed to release any amino acids, and therefore, tolaasin was concluded to be N-terminally blocked. Acetylation with pyridine/acetic anhydride resulted in the addition of six acetyl groups to Tol I and five to Tol II. Hence, taking into account the two amino groups of each molecule (Table III), Tol I was deduced to have four hydroxyls whilst Tol II has only three. Dansylation of crude tolaasin, followed by total hydrolysis and comparison of the derivatized amino acids with standard dansylated amino acids on a C8 reversed-phase HPLC column, identified the two primary amino groups of the intact molecules as the side-chain amino groups of lysine and diaminobutyric acid. Thus, the basic amino acids must be linked through their α-amino groups in the peptide backbone. This was corroborated by the observation of NOEs between the amide NH and αCH protons of these residues.

Table III. Functional Groups of Tol I and Tol II

reaction	change in M_r Tol I	Tol II	conclusion
esterification (0.1 M HCl/EtOH)	0	0	no COOH
acetylation (Ac_2O/H_2O (1:1), pH 8.4)	+84	+84	$2NH_2$
Hofmann rearrangement (*I,I*-bis(trifluoroacetoxy)iodobenzene)	−28	−28	$1CONH_2$
base hydrolysis (1% TEA or 1 M NaOH)	+18	+18	lactone
acetylation (Ac_2O/pyridine (1:2))	+252	+210	4OH Tol I 3OH Tol II

Treatment of crude tolaasin with 1–2% aqueous triethylamine (v/v) resulted in the addition of 18 mass units to the molecular weight of Tol I and Tol II, suggesting that both contained a lactone linkage (Table III). Esterification and acetylation of the base hydrolysis products confirmed the presence of an additional carboxyl and hydroxyl group in each. Since no free carboxyl groups were detected in the molecules, the most likely site for the carboxyl group of the lactones was either the C-terminus of the peptide backbone or the Glx residue detected by amino acid analysis.

Identification of the Major Tol I Hydrolytic Fragment Peptide (M_r 1533). Samples of Tol I or Tol II were subjected to partial acid hydrolysis (6 M HCl, 105 °C, 5–25 min) with the aim of generating fragment peptides with free amino termini amenable to sequencing by Edman degradation. The components of the hydrolysis mixture were fractionated by reversed-phase HPLC on a C8 or phenyl column. Recovery of the peptides from HPLC was low, and FAB mass spectra demonstrated that most fractions contained several peptides.

After 10-min acid hydrolyses, a peptide fragment of molecular weight 1533 (protonated molecular ion, MH^+ 1534) was particularly abundant in the hydrolysate of Tol I, and this was characterized in full as an aid to the structure elucidation of Tol I itself. After partial purification by HPLC on a phenyl column, experiments to determine the functional groups in this peptide (cf. Table III) showed that it had one carboxyl, one amino, and three hydroxyl groups. In addition, hydrolysis with 1% triethylamine indicated the presence of a lactone. A pure sample was obtained from the limited acid hydrolysate of Tol I by HPLC on a C8 column, and automated Edman degradation established the sequence of seven residues

ProSerLeuValSerLeuVal

at the N-terminus, but the repetitive yields for each cycle were less than 50%, limiting the length of sequence obtainable by this method. Amino acid analysis gave the following composition: Thr, (0.4); Ser, (0.6); Glu, (1); Pro, (1); Val (4); Ile, (1); Leu, (3); Hse, (1). (On the basis of the earlier sequencing, two serine residues should be found in this fragment; however, it appeared that an unusually large proportion of this amino acid, and of threonine, was destroyed during total hydrolysis.)

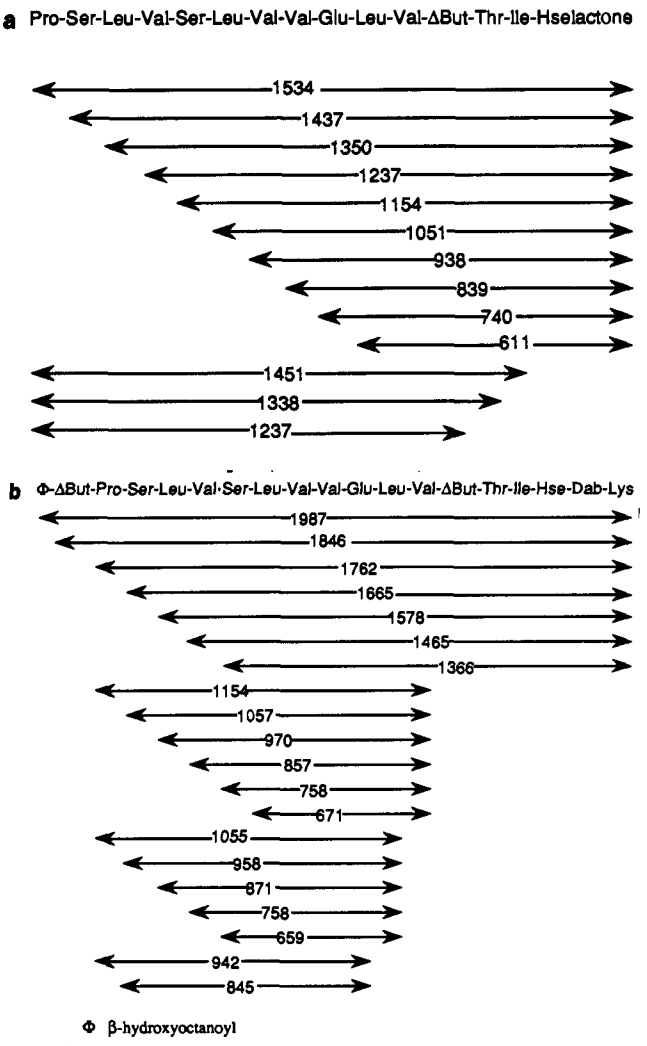

Figure 4. Protonated molecular ions (MH^+) assigned to peptide fragments generated by limited acid hydrolysis of Tol I (6 M HCl, 105 °C, 5–25 min). The masses shown arise from peptides in which the amide has been hydrolyzed to the acid. (a) Peptides related to the major Tol I fragment MH^+ 1534 (M_r 1533), having homoserine lactone at the C-terminus; (b) Peptides related to Tol I having the intact tolaasin lactone (when lysine is the C-terminal amino acid residue) or free C-termini. The molecular ions shown represent only a subset of those observed.

The positive-ion FAB mass spectrum showed N-terminal acylium fragment ions (Figure 3) that partially revealed the C-terminal portion of the sequence and indicated that homoserine formed the lactone of this fragment. The homoserine lactone opened and esterified on treatment with acidified ethanol, and thus, it appeared to be different from the lactone of Tol I that was unaffected by this reagent. The sequence assignment was confirmed by the presence of an analogous set of ions in the mass spectrum of a second Tol I fragment, M_r 1436, which differs from the peptide, M_r 1533, by the absence of the N-terminal proline residue (Figure 4a). As anticipated, the corresponding fragment ions from the smaller peptide were shifted by 97 Da. In addition, the sequence deduced was supported by the masses of some of the minor hydrolytic fragment peptides (Figure 4a). The position of the isoleucine and the connectivity of the glutamic acid residue shown in Figure 3 were initially deduced from the NOESY spectrum of intact Tol I. Subsequent confirmation of these assignments for the fragment (M_r 1533) was obtained by Edman sequence analysis, since after coupling the peptide to aminoethylaminopropyl glass through its C-terminal homoserine lactone, automated Edman degradation generated the sequence PSLVSLVVELV with an improved repetitive yield of 87%.

Structure of Tol I. As Tol I is N-terminally blocked and does not have homoserine lactone at the C-terminus, the abundant fragment peptide (M_r 1533) must represent the central portion of the molecule. Therefore, the terminal groups and the position of the lactone in the intact toxin remained to be established.

Nature of the Glx Residue in Tol I. The residue of mass 129 observed in the FAB mass spectrum of the hydrolytic fragment (M_r 1533) was assigned as glutamic acid. The free carboxyl group must be generated from glutamine in Tol I under acid hydrolysis conditions since fragment ions in the negative-ion mass spectrum of intact Tol I were consistent with the partial sequence

SerLeuValValGlnLeuVal

In this spectrum, the Glx residue had a mass of 128, demonstrating it to be in the amide rather than acid form.

Identification of Terminal Residues. After very short acid hydrolyses of Tol I (6 M HCl, 105 °C, 5 min), the parent molecular ion remained, but a partial shift upwards by 1 mass unit was observed. This was interpreted as incomplete hydrolysis of the glutamine primary amide to the acid. Two large fragment peptides also gave abundant signals that showed a similar distribution of "isotope peak" intensity to the parent ion. In addition, relatively small peaks were observed 18 mass units above each of these signals, presumably arising from acid hydrolysis of the lactone linkage. Minor hydrolysis fragment peptides (Figure 4b) suggested that the peptide MH^+ 1761/2 (M_r 1760/1) had the same N-terminal sequence as the smaller hydrolytic fragment, M_r 1533, characterized previously. Thus, the proline appeared to be N-terminally blocked by residues of mass 141 and 84.

$$MH^+\ 1986/7 \xrightarrow[141]{} 1845/6 \xrightarrow[84]{} 1761/2$$

Since amino acid analysis of the fragment M_r 1761/2 showed that it had the same composition as Tol I, the two basic amino acids must lie at the C-terminus of both the fragment and of Tol I. The mass difference of 84 units was assigned to the acid hydrolysis product of the second dehydroaminobutyric acid residue, and the blocking group therefore has a mass of 143 Da in intact Tol I. This unidentified residue was deduced to have one hydroxyl group on the basis of the functional group data for the whole molecule. The only unassigned spin system in the ^1H NMR spectra was consistent with a β-hydroxy acid residue and, in view of the mass required, it was concluded to be β-hydroxyoctanoic acid. The nature of this acid was confirmed by coelution of a synthetic sample of the methyl ester of its straight-chain isomer with the only major peak in the chiral GC trace of the total hydrolysate of Tol I derivatized by the same method. The two enantiomeric forms of the hydroxy acid were not resolved on the Chirasil-Val column, and thus, the stereochemistry at the β position remains to be determined. The mass separations of 141 and 84 Da, observed in the FAB spectrum on partial acid hydrolysis, were rationalized on the basis of the likely acid hydrolysis pathway of the dehydroaminobutyric acid residue (Figure 5).

Position of the Lactone. The hydroxyl component of the lactone of intact Tol I is provided by the threonine residue. This was deduced from the unusually large downfield shift of the threonine β-proton, 4.96 ppm, compared with an expected random coil value of 4.22 ppm[7] (Figure 2). Since the Glx residue had been identified as glutamine in intact Tol I, the origin of the carboxyl group of the lactone was concluded to be the C-terminal amino acid.

The order of the basic residues at the C-terminus was determined by $NaBH_4$ reduction and limited acid fragmentation of the product peptide. Treatment of Tol I with $NaBH_4$ in methanol resulted in a total mass increase of 8 units, which was assigned to reduction of both the dehydro amino acids and of the lactone, giving a principal product of M_r 1993 (MH^+ 1994). The residue providing the carboxyl group for the lactone had therefore been converted to the alcohol and its mass reduced by 14 units. After purification by HPLC, a short acid hydrolysis of this material (6 M HCl, 105 °C, 5 min) yielded fragment peptides giving molecular ions at MH^+ 1880 and MH^+ 1762. The initial mass

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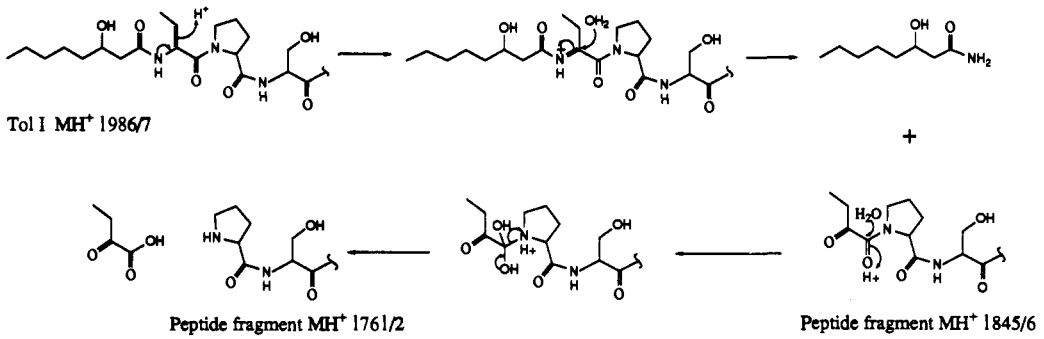

Figure 5. Proposed pathway of acid hydrolysis at the N-terminus of tolaasin, showing how the hydrolytic peptides giving molecular ions MH^+ 1845/6 and MH^+ 1761/2 may arise.

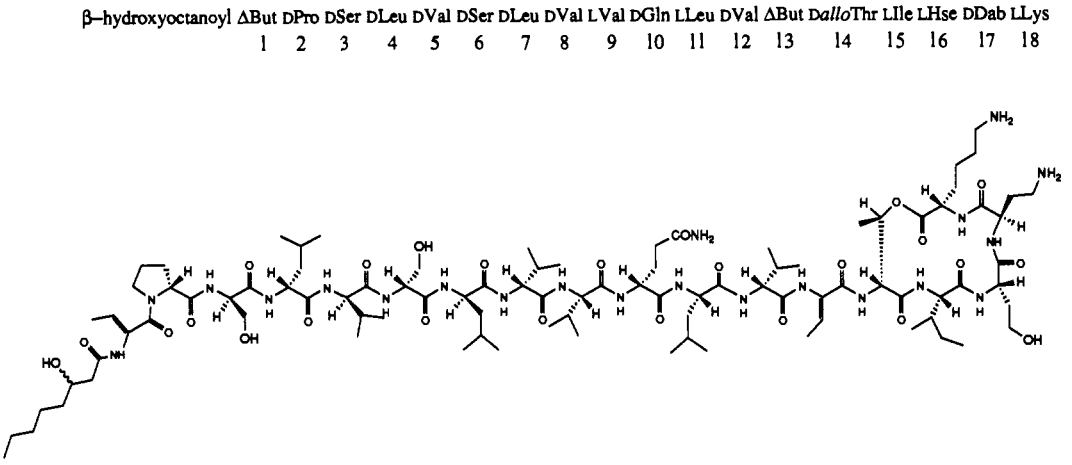

Figure 6. Proposed primary structure of Tol I (M_r 1985).

Table IV. Tol I Fragments Studied by GC

peptide	MH^+	D-Val	L-Val	L-Leu	L-Leu
LVΔButTIHse	611	1			1
VVELVΔButTIHse	938	2	1		1
VELVΔButTIHse	839	1	1		1

difference of 114 corresponds to loss of a reduced lysine residue, consistent with lysine being the C-terminal amino acid of the peptide involved in the lactone linkage. Formation of homoserine lactone with consequent loss of reduced lysine and diaminobutyric acid from the C-terminus gave rise to the second fragment peptide (MH^+ 1762).

Location of the L-Valine and L-Leucine Residues. Fragment peptides containing subsets of the leucine and valine residues of Tol I were generated by acid hydrolysis and manual Edman degradation and isolated by reversed-phase HPLC. Analysis of these peptides by chiral GC permitted the stereochemistries of the amino acids present in each to be determined. The results demonstrated that Val 9 and Leu 11 are of L-configuration (Table IV). The primary structure of Tol I is shown in Figure 6.

Aspects of the ^{1}H NMR Spectra of Tol I. The ^{1}H NMR spectrum of Tol I in DMSO-d^6 exhibits the three spectral windows characteristic of peptides (Figure 2). The assignments summarized in Table II were based on connectivity information obtained from DQF-COSY (Figure 2) and TOCSY experiments. Despite extensive degeneracy of chemical shifts, particularly below 2.0 ppm, all NH, αCH, βCH, and most γCH and δCH resonances could be assigned. With sequence information obtained from FABMS and Edman studies, a sequential assignment of the spectrum from NOESY $d_{NN}(i, i + 1)$ and $d_{\alpha N}(i, i + 1)$ data could be made according to the method of Wüthrich,[8] which supported the proposed structure in all respects.

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Structure of Tol II. The molecular weight, functional group data, and amino acid analysis of Tol II suggested that it is simply an analogue of Tol I in which the homoserine residue has been replaced by glycine. Fragment ions, observed in the positive-, and negative-ion FAB spectra of the intact molecule and the major hydrolytic fragment peptides, confirmed that the sequence is the same as that of Tol I apart from the glycine substitution. The molecular weights of the hydrolytic fragments supported this conclusion. Only the position of the isoleucine was not established due to the mass degeneracy between this residue and leucine. $NaBH_4$ reduction and acid hydrolysis showed that the C-terminal amino acid, lysine, is involved in the lactone. Since the lactone remained in hydrolytic fragments from which the blocking group had been lost and sequence ions in the positive-ion FAB spectrum of the intact molecule showed both serine hydroxyls to be free, the hydroxyl group for the lactone is provided by the threonine residue as in Tol I. The overall chiral analysis of the amino acids of Tol II was found to be the same as that of Tol I, but peptide fragments were not isolated to check the positions of the L-valine and L-leucine residues.

Characterization of a Mutant Tolaasin. Transposon Tn5 was introduced into *P. tolaasii*, and transconjugants were found to occur at a frequency of 1 in 10^{-5} to 10^{-6} per recipient cell. Tolaasin negative mutants arose at a frequency of 1%. Mutants that produced a reduced amount of tolaasin were identified by the formation of a weak white line and were detected at a frequency of 0.2%. A natural product was isolated from one such strain (named PT 144) by use of the same method employed for the preparation of tolaasin. Analysis of this material by positive-ion FABMS showed the presence of two components, M_r 1686 and 1642, corresponding to analogues of Tol I and Tol II but differing in mass from the wild-type peptides by 299 Da. The retention times on reversed-phase HPLC were found to be slightly shorter than those of normal tolaasin. Derivatization experiments demonstrated that the two PT 144 peptides each possess one less hydroxyl group than wild-type Tol I and II, but the other func-

tional groups were found to be unchanged. Amino acid analysis showed that the principal mutant peptide, M_r 1686, contained one less residue of serine, valine, and leucine than wild-type Tol I, consistent with the observed mass difference and loss of a single hydroxyl group. Positive- and negative-ion FABMS of acid hydrolysis fragments and the intact peptide indicated that the sequence of the Tol I analogue was the same (excluding the ambiguities due to mass degeneracy) as that of normal tolaasin, apart from the deletion of one Ser-Leu-Val unit. The chiral GC trace of the derivatized hydrolysate was similar to that of Tol I with the exception of a change in the proportions of the D and L forms of valine and leucine; one residue of D-serine, D-valine, and D-leucine had been lost. Two-dimensional NMR spectra of HPLC purified PT 144 tolaasin (M_r 1686) supported the proposed structure and showed that the isoleucine residue was located in the same position in the sequence as in Tol I.

Discussion

Many phytopathogenic fluorescent pseudomonads produce toxins containing peptide moieties that are typically small and are often wholly or partially cyclic. For example, tabtoxin and phaseolotoxin are chlorosis-inducing di- and tripeptides[9] from *Pseudomonas syringae tabaci* and *Pseudomonas syringae phaseolicola*, respectively. Various strains of *Pseudomonas syringae pv. syringae* produce the closely related antimicrobial toxins syringomycin, syringotoxin, and the syringostatins, depending on their host specificity.[10] The structures of these peptides involve fatty acid N-terminal blocking groups, unusual amino acids, and cyclization through a lactone linkage.[11,12] Tolaasin clearly has a number of features in common with this latter group of toxins, including the presence of dehydro and other unusual amino acids. However, its relatively high molecular weight and the small size of its lactone macrocycle, composed of only five residues, appear to be unique.

Electron microscopy supports the plasma membrane as the primary site of action of tolaasin against mushroom tissue.[3,13] Biological activity studies have shown that tolaasin causes lysis of horse erythrocytes and necrosis in higher plant cells, in addition to its activity against fungal protoplasts.[14] Experiments with artificial lipid bilayers demonstrate that tolaasin can form voltage-dependent ion channels[15] with similar properties to those of nisin,[16] Pep 5,[17] and melittin.[18] At present, the nature of the interaction between tolaasin and the membrane can only be speculated upon, but interaction of the two basic amino acids in the C-terminal lactone region of the molecule with the phosphate groups of the lipid bilayer is plausible. Circular dichroism studies indicate that tolaasin contains a region of left-handed α-helix in membrane-like environments, which is likely to be associated with the sequence of seven D-amino acids.[19] This central portion of tolaasin has amphiphilic character when drawn in α-helical projection, in common with many other membrane-active peptides.[20] Such a structure may be involved in spanning the lipid bilayer. However, in terms of size the putative helical region of tolaasin shows greater similarity to the central portions of secretory protein signal sequences than to linear helical peptides, the latter typically consisting of 23 residues.[21] If the length of the region of D-amino acids is important in the action of tolaasin, the shortened peptide from strain PT 144 might be anticipated to have altered activity. Ion channel experiments confirm that this is indeed the case.[15] Pure Tol II retains the ability to pit mushrooms and gives a positive white line test, but it is not clear whether it has a defined role in the toxin or its appearance is a consequence of the biosynthetic pathway.

Knowledge of the primary structure of tolaasin should facilitate further studies toward elucidation of its mode of action on the mushroom cap. It may also permit greater understanding of the nature of the interaction between tolaasin and the natural product from *P. reactans*,[22] which gives rise to the white line precipitate in the diagnostic test for *P. tolaasii*.

Experimental Section

Isolation and Purification of Tolaasin. Tolaasin was isolated and partially purified according to a modification of the method described by Peng.[3] A 3-L flask containing 1-L of *Pseudomonas* agar F (PAF, Difco) was inoculated with 8 mL of an overnight culture of *P. tolaasii* NCPPB 1116 and grown in an orbital inoculator (200 rpm) for 36 h at 25 °C. Cells were removed by centrifugation (10000 g, 10 min) and the supernatant frozen overnight. The thawed filtrate was filtered through Whatman no. 42 paper, acidified to pH 3 with concentrated HCl, and left overnight at room temperature and the precipitate removed by centrifugation (8000 g, 20 min). Tolaasin was precipitated by addition of 150 g of $CaCl_2$, collected by centrifugation (8000 g, 25 min), and dried at 45 °C. The pellet was suspended in 50 mL of methanol and centrifuged (10000 g, 15 min) and the supernatant evaporated to dryness in vacuo at 45 °C. The residue was dissolved in 20 mL of methanol, evaporated to dryness as described previously, and then dissolved in 10 mL of methanol and dried again. Water (10 mL) was added to the residue and the suspension centrifuged (10000 g, 15 min). The precipitate was dissolved in 50 mL of water and lyophilized. The resulting powder was weighed and dissolved in water at a concentration of 50 mg mL^{-1} before passing down a Dowex-1 anion exchange column (15 mm × 25 mm) at a flow rate of 35 mL h^{-1}. The eluate was monitored at 206 nm, and fractions were assayed for activity by use of the rapid pitting test.[4] All fractions displaying increased absorbance at 206 nm were active on mushroom tissue and were pooled and lyophilized to yield a white powder.

Transposon Mutagenesis. Transposon Tn5 mutagenesis of *P. tolaasii* NCPPB 1116 was conducted with use of *E. coli* donor strain S17-1 containing the suicide vector pSUP1011 as described by Simon et al.[23] Equal volumes of donor and recipient were mixed together, spread on the surface of a 0.22-μm filter on LB plates, and incubated for 4–6 h at 28 °C. Transconjugants were selected on PAF containing ampicillin (100 μg/mL) and kanamycin (30 μg/mL).

P. tolaasii isolates defective in their ability to produce tolaasin were identified by the white line in agar test.[4] Transconjugants were streaked on PAF alongside *Pseudomonas* sp. NCPPB 387, which produces a lipodepsipeptide designated white line inducing principle (WLIP).[3,21] WLIP interacts with tolaasin in the agar to give a white line. Cells defective in their ability to produce tolaasin gave either no white line or occasionally a white line of reduced intensity.

Reversed-Phase HPLC. A Varian LC5000 chromatograph with detection at 214 nm and Spherisorb S5P (phenyl) columns were used for all peptide samples prepared by reversed-phase HPLC for FABMS and ^1H NMR. A solvent system of water (0.1% TFA, v/v) and CH_3CN (0.08% TFA, v/v) was employed for elution, with flow rates of 1.5 mL min^{-1} for an analytical column and 2.5 mL min^{-1} for a semipreparative column. Water was obtained from a Millipore Milli-Q water purification system and far UV grade CH_3CN was purchased from Fisons. All solvents were degassed by sonication before use.

Crude tolaasin was purified by injection onto the column at 50% organic phase and elution by a gradient to 100% CH_3CN over 25 min. Tolaasin was fragmented by treatment with 6 M HCl at 105 °C for 5–25 min and the resulting peptides fractionated by use of a gradient of 0–60%

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CH_3CN over 30–60 min; the proportion of organic phase was then raised to 100% over a further 10 min.

Separation of parent and hydrolytic products for amino acid analysis and sequencing was by reversed-phase HPLC on a Brownlee Aquapore RP300 (C8) column (2.1 mm × 30 mm) with a Hewlett-Packard 1090L liquid chromatograph. Samples were loaded in the equilibration buffer, 0.1% (v/v) TFA, and 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 with monitoring at 220 nm.

Automated Sequencing. Amino acid sequence analysis was performed with on an Applied Biosystems Model 477A pulsed-liquid protein sequencer with an on-line model 120A PTH-amino acid analyzer.

Total Acid Hydrolysis and Amino Acid Analysis. Peptide samples were hydrolyzed at 110 °C by the vapor 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 analyzer using ninhydrin detection.

Base Hydrolysis. Peptide samples were dissolved in 1–2% aqueous triethylamine (v/v) and were incubated for up to 2 h at 55 °C. Alternatively, the reaction mixture was left at room temperature for 6 h.

Oxidation with *I,I*-bis(trifluoroacetoxy)iodobenzene (TIB, a reagent that converts primary amides to amines via Hofmann rearrangement.[24]) A 1-mg portion of crude tolaasin was dissolved in 200 μL of 50% aqueous CH_3CN (v/v), and 50 μL of a 1.2 mg/300 μL solution of TIB in the same solvent was added. The mixture was protected from light and left at room temperature for 5 h. HCl (500 μL, 0.01 M) was added and the sample lyophilized and analyzed directly by FABMS.

Dansylation. Tol I or II (0.2 mg) was dissolved in 200 μL of 40 mM Li_2CO_3 (pH 10), and to this was added 200 μL of a 2 mg/0.7 μL solution of dansyl chloride in CH_3CN. The reaction mixture was left in the dark at room temperature for 1 h and then dried under vacuum. The dansylated product was hydrolyzed (6 M HCl, 105 °C, 24 h) prior to HPLC of the amino acids on a C8 reversed-phase column. The conditions employed were isocratic separation with MeOH, H_2O, AcOH (50:50:1, v/v) for 50 min followed by a gradient to 70% MeOH over 10 min. This proportion of organic phase was held for a further 15 min. UV detection at 254 nm was employed. By use of this system, side-chain amino and α-amino dansyl forms of the basic amino acids were well-resolved. γ-Amino dansyl diaminobutyric acid (elution time 42.5 min) and ε-amino dansyl lysine (65.0 min) were identified in the tolaasin hydrolysates by FABMS and comparison of retention times with those of standard dansyl derivatives of the basic amino acids.

Reduction with $NaBH_4$. Crude tolaasin (1 mg) was dissolved in 400 μL of MeOH, and excess (ca. 1 mg) $NaBH_4$ added. The mixture was left at room temperature for 7 h, and then a further 1 mg of $NaBH_4$ was added. After a total of 20 h, the solution was dried in vacuo and dissolved in 200 μL of 0.1% TFA (v/v) for reversed-phase HPLC on a phenyl column. Elution was achieved by a gradient of 0–65% CH_3CN (0.08% TFA, v/v) in 20 min and then to 85% organic phase over a further 20 min.

Chiral GC Analysis. Derivatization steps were performed in 1-mL Wheaton vials (Aldrich). After each step, the sample was cooled and dried in vacuo except for the final derivatization, after which the trifluoroacetyl derivatives were dried in a gentle stream of dry argon. Peptide samples were hydrolyzed with 6 M HCl at 105 °C for 24 h. Derivatization to the isopropyl esters was achieved by dissolving in 1 M HCl/*i*-PrOH and heating at 105 °C for 30 min. Trifluoroacetylation was performed by addition of a 1:1 mixture of CH_2Cl_2 and trifluoroacetic anhydride followed by heating at 105 °C for 10 min. The derivatized amino acids were then analyzed by use of a Carlo Erba 4130 gas chromatograph fitted with a split/splitless injector (splitter ratio 20:1) and a flame ionization detector. The injector temperature was maintained at 240 °C, and hydrogen was employed as carrier gas. For split injection, the amino acid derivatives were dissolved in dry CH_2Cl_2 to give a concentration of 1–2 nmol/μL, and approximately 0.1 μL of this was injected onto a Chirasil-Val capillary column (0.2 mm × 50 m, Alltech Chromatography Ltd). A temperature program running from 80 to 170 °C at 4 °C min^{-1} 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. Identical methodology was employed to prepare standard amino acid derivatives for identification of sample components. Due to the long retention time of the trifluoroacetyl derivatives of lysine, the stereochemistry of this amino acid was determined by reaction with pentafluoropropionyl anhydride, which gives more volatile derivatives.

Isolation of Fragments for Chiral GC. The Tol I fragment LeuValΔButThrIleHse lactone (MH^+ 611) coeluted on reversed-phase HPLC (phenyl column) with three other peptides that had masses less than 1000. However, treatment with 1% triethylamine showed that the fragment of interest was the only one in this fraction that possessed a lactone. Ring opening increased the hydrophilicity of this peptide whilst leaving that of the other components of the mixture unchanged, and thus separation was readily achieved by a second HPLC run under the original conditions. Due to the low susceptibility of Leu–Val and Val–Val peptide bonds to acid hydrolysis, the other peptides in Table IV were generated by two or three cycles of manual Edman degradation performed on a relatively abundant Tol I fragment peptide of sequence SerLeuValValGluLeuValΔButThrIleHse lactone (MH^+ 1138) followed by further purification by HPLC.

FABMS. Mass spectra were obtained on a Kratos MS-50 fitted either with an Ion Tech FAB gun in operation with xenon at an acclerating voltage of 8 kV or with an Antek Cs^+ ion gun operating with a total accelerating voltage of 14–16 kV. 3-Nitrobenzylalcohol was employed as matrix for samples recorded in positive-ion mode and negative-ion spectra were obtained with glycerol (5% 15-crown-5).

NMR Spectroscopy. ^1H NMR spectra were recorded with Bruker AM-400 and AM-500 spectrometers equipped with Aspect 2000 computers using a 3.2 mM solution of Tol I in DMSO-*d*.[6] For samples run at acidic pH, 0.5 μL of TFA was added. Spectra were recorded over spectral widths of 4000 or 5000 Hz (at 400 and 500 MHz, respectively) with quadrature detection employed throughout. Two-dimensional ^1H NMR spectra were acquired in the phase-sensitive mode by quadrature detection in f_2 and with the time proportional phase incrementation method of Marion and Wüthrich in f_1.[25] Data sets resulting from 450 to 512 increments of t_1 were acquired (and zero filled to 1024 points), with each FID composed of 2048 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 artifacts. TOCSY spectra were obtained with a 3-kHz spin-locking field, a DIPSI-2 sequence[26] was used to provide isotropic mixing, and the spin locking field was applied for 75 or 100 ms. Final data sets were subjected to mild Lorentzian–Gaussian apodization prior to Fourier transformation.

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