Tolaasins A−E, Five New Lipodepsipeptides Produced by *Pseudomonas tolaasii*

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Pseudomonas tolaasii, the causal organism of brown blotch disease of *Agaricus bisporus* and of the yellowing of *Pleurotus ostreatus*, was shown to produce in culture tolaasin I (**1**), tolaasin II (**2**), and five other minor metabolites, tolaasins A, B, C, D, and E (**3**−**7**). These compounds were demonstrated to be important in the development of the disease symptoms. This paper reports on the structural elucidation, based essentially on NMR studies and MS spectra, and biological activity of the above lipodepsipeptides (**3**−**7**). All the above analogues showed differences in the peptide moiety, as observed in other lipodepsipeptides of bacterial origin, and maintained the β-hydroxyoctanoyl ϕ chain at the N-terminus, except tolaasin A, in which the acyl moiety was a γ-carboxybutanoyl ϕ moiety. Among the target microorganisms used (fungi, yeast, and bacteria) the Gram-positive bacteria were the most sensitive, although the antimicrobial activity appeared to be correlated to the structural modification in the different analogues. The structure−activity relationships of these toxins are discussed.

Virulent strains of *P. tolaasii*, the causal organism of brown blotch disease of *A. bisporus* and of the yellowing of *Pleurotus ostreatus*,[1−7] produce in culture tolaasin I (**1**) and the structurally related tolaasin II (**2**).[8] The primary structure of tolaasin I, elucidated by Nutkins et al.,[8] bears a β-hydroxyoctanoic acid moiety linked to the N-terminus, a sequence of seven successive D-amino acids in the N-terminal region of the peptide (Pro2-Val8), with a Ser-Leu-Val repeat, and then alternate L- and D-amino acids. It also contains a 2,3-dehydro-2-aminobutyric acid (ΔBut) residue at positions 1 and 13, a D-homoserine (Hse16), and a D-2,4-diaminobutyric acid (D-Dab17). Finally, a lactone ring is formed between the hydroxyl of D-Thr14 and the C-terminal L-Lys18. Tolaasin II differs from tolaasin I in the replacement of Hse16 with Gly16. Tolaasin I exhibits antimicrobial activity,[9,10] and this feature is apparently correlated to its ability to form transmembrane pores,[3,11−14] as in the case of other antimicrobic lipodepsipeptides produced by phytopathogenic *Pseudomonas* spp.[15]

Herein we report on the purification of five new lipodepsipeptides from *P. tolaasii*, called tolaasins A−E (**3**−**7**), and on their chemical and antimicrobial characterization.

Results and Discussion

Tolaasins A−E (**3**−**7**), together with tolaasins I and II, were isolated from the cell-free culture filtrate of the strain type NCPPB2192 of *P. tolaasii* essentially by precipitation with calcium chloride. After partial purification by fractionated precipitation and desalting by gel-filtration chromatography, a crude preparation, which retained almost the whole antimicrobial activity against *Bacillus megaterium* present in the culture filtrates, was obtained. HPLC analysis of the above preparation showed that besides the main metabolites, tolaasin I (**1**) and tolaaasin II (**2**), also five minor new products, called tolaasins A−E (**3**−**7**), were present.

Through multistep reversed-phase HPLC on a C8 semi-preparative column, these minor substances were purified in very low amounts together with tolaasin I and tolaasin II. The identity of tolaasin I and II was ascertained by comparing their NMR (1D and 2D) and MS data with those reported by Nutkins et al.[8]

A preliminary analysis of ^{1}H NMR and ESIMS data of these metabolites indicated that the new lipodepsipetides are structurally related to tolaasins I (**1**) and II (**2**) as previously suggested by Shirata et al.[16] They isolated eight tolaasins, including tolaasins I and II, from *P. tolaasii* culture filtrates and hypothesized, only on the basis of MS data, that their structural features are similar to **1** and **2**. In our case seven tolaasins, including tolaasins I and II, were isolated. Furthermore the structures of two of them differ from those hypothesized by Shirata et al.[16] A complete NMR-based structural elucidation of tolaasins A−E (**3**−**7**) was not an easy task to accomplish, due to the low amount of substances available. For this reason, while a full characterization of the ^{1}H NMR spin systems was achieved through extensive use of 2D NMR techniques, ^{13}C NMR carbon chemical shifts could not be assigned. Attempts to aquire ^{13}C NMR data by means of suitable 2D NMR experiments (HSQC and HMBC) failed due to very long acquisition time. The assignment of the proton spin systems of each amino acid residue, including the identification of the N-terminal side chain, was mainly obtained from the 2D-TOCSY and the 2Q (double quantum spectroscopy) experiments.[17] In these spectra it is possible to obtain correlation peaks occurring at the sum of the chemical shifts of two coupled spins in ω_1 and at the chemical shift of each spin in ω_2. For this reason, peaks are spread over a much larger dimension with regard to conventional COSY, DQF-COSY, and TOCSY techniques, thus making the 2Q experiment very suitable for assigning crowded homonuclear spectra. The 2Q spectrum was acquired in order to assess the sequence specific assignment of the spin systems preliminarily characterized in the TOCSY spectrum, together with the pattern analysis of sequential dipolar couplings displayed in the 2D NOESY

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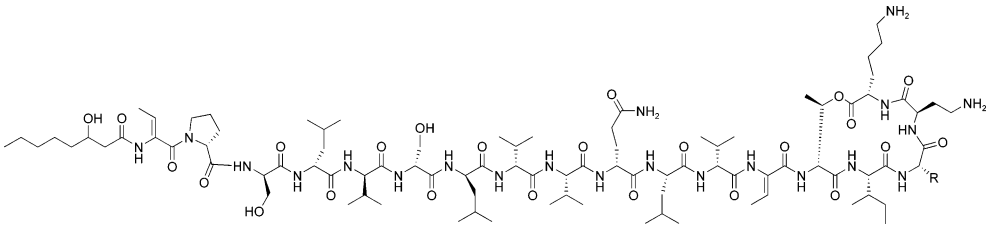
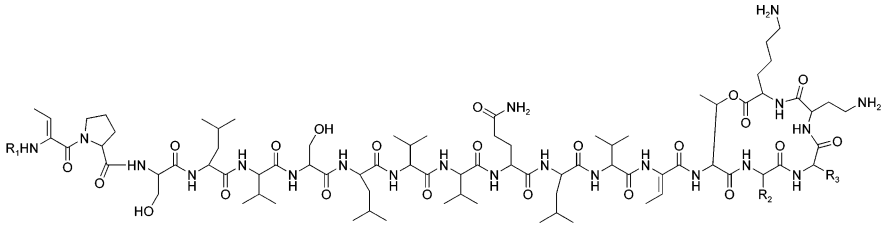
Chart 1



Tolaasin I (**1**): R=CH_2CH_2OH

Tolaasin II (**2**): R=H

Chart 2

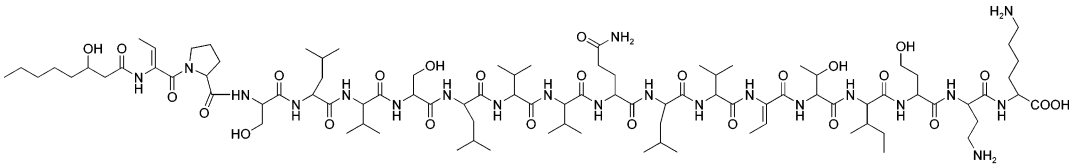


Tolaasin A (**3**): R_1=$HOOC(CH_2)_3CO$; R_2=$CH(CH_3)CH_2CH_3$; R_3=CH_2CH_2OH

Tolaasin B (**4**): R_1=$CH_3(CH_2)_4CH(OH)CH_2CO$; R_2=$CH(CH_3)_2$; R_3=CH_2CH_2OH

Tolaasin D (**6**): R_1=$CH_3(CH_2)_4CH(OH)CH_2CO$; R_2=$CH_2CH(CH_3)_2$; R_3=CH_2CH_2OH

Tolaaasin E (**7**): R_1=$CH_3(CH_2)_4CH(OH)CH_2CO$; R_2=$CH_2CH(CH_3)_2$; R_3=H



Tolaasin C (**5**)

spectrum.[18] Full proton assignments for tolaasins A−E (**3**−**7**), in DMSO-*d* at 300 K, are shown in Tables 1−5.

Tolaasin A (**3**) showed a pseudomolecular ion peak in the HRESIMS spectrum at *m*/*z* 980.0912 $(M + 2H)^{2+}$, consistent with the molecular formula $C_{91}H_{155}N_{21}O_{26}$ (exact mass calculated for $C_{91}H_{157}N_{21}O_{26}$/2 980.080435). HRESIMS data were obtained also for the intense fragment ion (M − ϕ chain − ΔBut1 + H), observed as a double charged ion at *m*/*z* 881.5432 (exact mass calculated for $C_{82}H_{146}N_{20}O_{22}$/2 881.54603) and interpreted as originating from the elimination of $[HOOC-(CH_2)_3CONHC{=}CH(CH_2)-C{=}O]^{\bullet}$ with hydrogen transfer to the charged species (see below). Extensive analysis of the ^{1}H NMR data of tolaasin A (**3**), including TOCSY, DQF COSY, and 2Q spectra, permitted confirmation of the same amino acid sequence present in the parent tolaasin I (**1**). Moreover, the Thr14 residue and the C-terminal Lys18 were found to be involved in the lactone formation, as already observed for tolaasin I. On the other hand, a different nature of the N-terminus ϕ chain was suggested by the analysis of the DQF-COSY spectrum, revealing the presence of an aliphatic spin system consisting of three sequential methylene protons at δ 2.07, 1.75, and 1.85 ppm, respectively. In addition, the HMBC spectrum showed the presence of two carbonyl signals at δ 171.8 and 178.2 ppm showing correlations, respectively, with the methylene proton signals at δ 2.07 and 1.85 ppm. To unequivocally identify the N-terminus blocking group, the fragmentation peaks observed in the ESI MS spectrum were interpreted. Upon loss of the N-terminus ϕ chain and the first amino acid ΔBut1, tolaasin A exhibited the same fragment ion at *m*/*z* 1762 $(M + H)^+$ as tolaasin I, a type of fragment found in the ESIMS spectra of all tolaasins. This indicated a $C_5H_7O_3$ formula for the ϕ chain, thus establishing, in accordance with its NMR data, a $HOOC-(CH_2)_3-CO-$ structure for this terminal moiety.

Finally, the ESIMS/MS (collision-induced dissociation) spectrum, executed on the pseudomolecular peaks at *m*/*z* 1959 and 1987 of tolaasin A and tolaasin I, respectively, revealed the same fragmentation pattern, indicating, in agreement with their NMR data, the same amino acid sequence for the two depsipeptides.

Tolaasin B (**4**) showed a pseudomolecular ion peak at *m*/*z* 1973 $(M + H)^+$, 14 mass units less than tolaasin I, suggesting that tolaasin B could differ from the parent toxin by the absence of a methylene group. NMR data along with the above ESIMS data permitted us to deduce for tolaasin B a molecular formula $C_{93}H_{161}N_{21}O_{25}$. Moreover, several peptide fragmentation peaks generated by a stepwise spontaneous loss of amino acid residues from the N-terminus were observed. In particular, five ions at *m*/*z* 1747.8, 1650.8, 1450.7, 1351.7, and 1151.6 could be identified and interpreted as fragments generated by the subsequent loss of the ϕ chain + ΔBut1, Pro2, Ser3 +Leu4, Val5, and Ser6 + Leu7, respectively.

The TOCSY spectrum, almost superimposable to that of tolaasin I, permitted the identification of tolaasin B (**4**), the substitution of the isoleucine spin system present in

Table 1. ^{1}H NMR Chemical Shifts of Tolaasin A (**3**)

	NH	αCH	βCH	γCH	δCH	others
ϕ^a		2.07^b	1.75^b	1.85^b		
ΔBut1	9.81		5.62	1.70		
Pro2		4.24	2.21	1.87	3.59	
			1.79	1.82	3.45	
Ser3	7.85	4.20	3.72^b			
Leu4	7.59	4.14	1.63	1.56	0.78^b	
			1.47			
Val5	7.37	4.13	2.02	0.84^b		
Ser6	7.79	4.32	3.59^b			
Leu7	7.95	4.31	1.64	nd^c	0.86	
			1.49		0.81	
Val8	7.72	4.20	2.00	0.83^b		
Val9	7.76	4.24	2.04	0.84^b		
Gln10	8.09	4.21	1.88	2.08^b	7.26	
			1.76		6.77	
Leu11	8.01	4.41	1.54	nd^c	0.89	
					0.84	
Val12	8.12	4.09	2.08	0.93		
				0.90		
ΔBut13	9.31		6.29	1.67		
Thr14	8.11	4.28	5.00	1.17		
Ile15	7.83	3.84	1.66	1.56	0.85	
				1.17		
				γCH_3 0.90		
Hse16	9.32	3.80	2.25	3.51		
			1.85	3.37		
Dab17	8.02	4.30	2.16	2.84		δNH_2 nd^c
			1.94			
Lys18	7.58	4.48	1.82	1.28	1.49	ϵCH_2 2.69^b
			1.60			ζNH_2 nd^c

^{a} ϕ, pentandioic acid. ^{b} Degenerate. ^{c} Not determined.

Table 2. ^{1}H NMR Chemical Shifts of Tolaasin B (**4**)

	NH	αCH	βCH	γCH	δCH	others
ϕ^a		2.48	3.86	1.37^b	1.26^b	nd^c OH
		2.35				4.75
ΔBut1	9.82		5.62	1.70		
Pro2		4.23	2.21	1.87	3.59	
			1.79	1.81	3.45	
Ser3	7.84	4.19	3.72^b			OH 4.92
Leu4	7.58	4.15	1.59	1.55	0.78^b	
			1.44			
Val5	7.36	4.13	2.01	0.84^b		
Ser6	7.79	4.32	3.59^b			OH 4.96
Leu7	7.95	4.31	1.63	nd^c	0.83^b	
			1.48			
Val8	7.71	4.20	2.01	0.83^b		
Val9	7.84	4.21	2.05	0.84^b		
Gln10	8.07	4.21	1.87	2.07^b	7.26	
			1.75		6.77	
Leu11	8.04	4.40	1.53	1.53	0.87	
					0.84	
Val12	8.10	4.11	2.08	0.91^b		
ΔBut13	9.31		6.27	1.67		
Thr14	8.15	4.30	5.00	1.16		
Val15	7.93	3.73	1.89	0.95^b		
Hse16	9.33	3.83	2.25	3.52		OH 4.56
			1.89	3.39		
Dab17	7.62	4.33	2.25	2.91		δNH_2 nd^c
			2.00	2.79		
Lys18	7.61	4.49	1.84	1.28^b	1.52	ϵCH_2
			1.57		1.51	2.70^b
						ζNH_2 nd^c

^{a} ϕ, β-hydroxyoctanoic acid. ^{b} Degenerate. ^{c} Not determined.

Table 3. ^{1}H NMR Chemical Shifts of Tolaasin C (**5**)

	NH	αCH	βCH	γCH	δCH	others
ϕ^a		2.48 2.36	3.84	1.39^b	1.27^b	nd^c OH 4.73
ΔBut1	9.81		5.62	1.70		
Pro2		4.24	2.21	1.87	3.58	
			1.79	1.79	3.45	
Ser3	7.84	4.20	3.72^b			OH 4.89
Leu4	7.58	4.16	1.58	1.58	0.78^b	
			1.44			
Val5	7.37	4.13	2.02	0.85^b		
Ser6	7.83	4.34	3.60^b			OH 4.95
Leu7	7.95	4.32	1.63	nd^c	0.86	
			1.49		0.82	
Val8	7.71	4.20	2.02	0.84^b		
Val9	7.82	4.19	2.06	0.85^b		
Gln10	8.08	4.20	1.88	2.08^b	7.26	
			1.78		6.79	
Leu11	8.03	4.40	1.52	nd^c	0.88	
					0.83	
Val12	8.09	4.13	2.09	0.87^b		
ΔBut13	9.55		6.34	1.60		
Thr14	7.88	4.18	3.84	1.14		
Ile15	7.82	3.78	1.65	1.56 1.16	0.89	
				γCH_3 0.89		
Hse16	9.30	3.84	2.27	3.54 3.40		OH 4.54
			1.87			
Dab17	8.03	4.30	2.22	2.90 2.82		δNH_2 nd^c
			1.99			
Lys18	7.59	4.48	1.82	1.25^b	1.54	ϵCH_2 2.72^b
			1.58		1.50	
						ζNH_2 nd^c

^{a} ϕ, β-hydroxyoctanoic acid. ^{b} Degenerate. ^{c} Not determined.

Table 4. ^{1}H NMR Chemical Shifts of Tolaasin D (**6**)

	NH	αCH	bCH	γCH	δCH	others
ϕ^a		2.46 2.35	3.85	1.37^b	1.27^b	nd^c OH 4.73
ΔBut1	9.81		5.62	1.70		
Pro2		4.23	2.21	1.87	3.58	
			1.79	1.81	3.45	
Ser3	7.84	4.19	3.72^b			OH 4.92
Leu4	7.58	4.15	1.59	1.56	0.74^b	
			1.44			
Val5	7.36	4.12	2.01	0.83^b		
Ser6	7.80	4.32	3.59^b			OH 4.96
Leu7	7.95	4.31	1.49	nd^c	0.86	
					0.80	
Val8	7.71	4.20	2.08	0.82^b		
Val9	7.84	4.18	2.05	0.85^b		
Gln10	8.09	4.19	1.87	2.09^b	7.26	
			1.76		6.77	
Leu11	8.02	4.40	1.52	nd^c	0.85	
					0.82	
Val12	8.12	4.07	2.08	0.93		
				0.90		
ΔBut13	9.30		6.29	1.68		
Thr14	7.93	4.27	4.91	1.18		
Leu15	7.91	3.99	1.48	1.56	0.90	
			1.38		0.86	
Hse16	9.37	3.79	2.24	3.48		OH 4.55
			1.87	3.37		
Dab17	7.96	4.31	2.10	2.83		δNH_2 nd^c
			1.92	2.77		
Lys18	7.53	4.47	1.80	1.27	1.49	ϵCH_2 2.68^b
			1.59	1.24	1.45	
						ζNH_2 nd^c

^{a} ϕ, β-hydroxyoctanoic acid. ^{b} Degenerate. ^{c} Not determined.

tolaasin I (**1**) with a proton connectivity consistent with a valine residue. Two intense NOE correlations were observed between Hse16 NH and Val Hα, and Val NH and Thr14 Hα, confirming that the valine residue is at position 15. The presence of the intact lactone moiety was confirmed by CID MS/MS analysis on the fragment ion at *m/z* 1973. This ion generated, by loss of the Lys18-ΔBut13 portion containing the lactone moiety, a peptide fragment ion at *m/z* 1360.4, corresponding to the fragment ϕ chain-Val12. Taken together the above data allowed the establishment of structure **4** for tolaasin B.

Tolaasin C (**5**) showed a pseudomolecular ion peak at *m/z* 2005 $(M + H)^+$ corresponding to a molecular weight of 2003, 18 mass units greater than that of tolaasin I, suggesting that tolaasin C may be the acyclic version of tolaasin I derived by the hydrolysis of its lactone ring

Table 5. ^{1}H NMR Chemical Shifts of Tolaasin E (**7**)

	NH	αCH	βCH	γCH	δCH	others
ϕ[a]		2.49	3.86	1.37[b]	1.26[b]	nd[c] OH 4.76
		2.36				
ΔBut1	9.86		5.63	1.70		
Pro2		4.23	2.20	1.90	3.60	
			1.79	1.81	3.45	
Ser3	7.88	4.19	3.71[b]			OH 4.95
Leu4	7.59	4.14	1.62	1.55	0.76[b]	
			1.42			
Val5	7.38	4.12	2.02	0.83[b]		
Ser6	7.80	4.31	3.59[b]			OH 4.98
Leu7	7.95	4.31	1.64	nd[c]	0.85	
			1.49		0.82	
Val8	7.74	4.21	2.02	0.84[b]		
Val9	7.89	4.19	2.07	0.84[b]		
Gln10	8.12	4.22	1.90	2.09[b]	7.27	
			1.78		6.75	
Leu11	8.07	4.40	1.53	nd[c]	0.89	
					0.83	
Val12	8.18	4.16	2.12	0.91[b]		
ΔBut13	9.31		6.34	1.66		
Thr14	8.31	4.36	5.00	1.17		
Leu15	8.48	3.96	1.42	1.64	0.91	
			1.54		0.84	
Gly16	9.45	3.75				
		3.69				
Dab17	7.60	4.42	2.29	2.96		δNH_2 8.02
			2.03	2.88		
Lys18	7.31	4.51	1.89	1.26	1.53	ϵCH_2 2.72[b]
			1.56			
						ζNH_2 7.84

[a] *ϕ*, *β*-hydroxyoctanoic acid. [b] Degenerate. [c] Not determined.

(molecular formula $C_{94}H_{165}N_{21}O_{26}$). The ESIMS spectrum also contained peptide fragment ions generated by a stepwise spontaneous loss of amino acid residues from the N-terminus. In particular, seven ions at *m/z* 1781.1, 1682.9, 1595.7, 1482.8, 1385.5, 1296.6, and 1183.1 could be identified and interpreted as fragments originated by removal of the *ϕ* chain and ΔBut1, Pro2, Ser3, Leu4, Val5, and Leu7 residues, respectively. Comparison of the fragmentation patterns of tolaasins C (**5**) and I (**1**) showed that tolaasin C fragment ions were found 18 mass units greater than the corresponding ones of tolaasin I, supporting the hypothesis that tolaasin C is a linear peptide generated from the hydrolysis of the lactone ring.

A comparative analysis of the 2D NMR spectra of tolaasin I and tolaasin C provided additional support to the hypothesis of tolaasin C being an acyclic derivative of tolaasin I. Further evidence of the linear nature of the peptide was provided by the analysis of the proton chemical shifts of the dehydroaminobutyric acid residue at position 13 (ΔBut13) and the threonine at position 14. In fact, NMR data of tolaasin C showed a moderate low-field shift for the ΔBut13 NH (*δ* 9.55) and H*β* (*δ* 6.34) protons and a moderate upfield shift for the threonine NH resonance (*δ* 7.88). A large upfield shift was observed for the Hα (*δ* 4.18) and H*β* (*δ* 3.84) of the same residue, in agreement with the expected random coil value of threonine α (4.35 ppm) and *β* protons (4.22 ppm), suggesting that the hydroxyl group of the side chain of this residue is not involved in an ester functionality.

Tolaasin D (**6**) exihibited a pseudomolecular ion peak at *m/z* 1987 $(M + H)^+$, suggesting the same molecular formula $C_{94}H_{163}N_{21}O_{25}$ already observed for tolaasin I. Thus tolaasins D (**6**) and I (**1**) are isomers. A careful comparison of the TOCSY spectra of tolaasins D and I indicated that tolaasin D contained a leucine residue in place of the isoleucine present in the parent lipodepsipeptide **1**. The 2Q spectrum allowed the specific assignment of the resonances of the leucine methylene protons. The position 15 of the leucine was supported by two NOE effects between Hse16 NH and Leu15 Hα and between Leu15 NH and Thr14 Hα.

Tolaasin E (**7**) showed a pseudomolecular ion peak at *m/z* 1943 $(M + H)^+$, suggesting the same molecular formula $C_{92}H_{159}N_{21}O_{24}$ as tolaasin II (**2**). Hence, tolaasin E (**7**) is isomeric with tolaasin II (**2**). The latter toxin contains a glycine residue in place of the homoserine residue of tolaasin I (**1**). In fact, in the TOCSY spectrum of tolaasin E it was possible to characterize the spin system of a glycine residue, confirming that **7** is indeed a tolaasin II analogue. The 2Q experiment indicated the presence of four rather than three leucine residues. A careful examination of the NOESY and TOCSY spectra allowed confirmation that the first three leucine residues are at positions 4, 7, and 11, as in tolaasin II (**2**). NOE correlations allowed the placement of the fourth leucine. In fact, dipolar effects between Gly16 NH and Leu15 Hα and between Leu15 NH and Thr14 Hα indicated that the fourth leucine replaces the isoleucine at position 15 in tolaasin II.

Support for the structure of tolaasin E was the presence of the *β*-hydroxyoctanoyl *ϕ* chain at the N-terminus, as deduced by ESIMS data through the spontaneous loss of 225 mass units (the *ϕ* chain + ΔBut1) from the pseudomolecular ion peak at *m/z* 1943.

The antimicrobial activity of HPLC grade tolaasins A−E (**3**−**7**)—assayed in comparison with tolaasin I and II against the yeast *Rhodotorula pilimanae*, the fungus *Rizoctonia solani*, the Gram-positive bacteria *Bacillus megaterium* and *Rodococcus fascians*, respectively, and the Gram-negative bacteria *Escherichia coli* and *Erwinia carotovora* subsp. *carotovora*—is reported in Table 6. *B. megaterium* and *R. fascians* were the most sensitive test microorganisms. In fact, all the analogues, except tolaasin C, inhibited the growth of these bacteria, although differences among their specific activities were observed. The most active analogues appear to be tolaasin D followed by tolaasin I and II with a minimal inhibitory quantity of 0.16, 0.32, and 0.64 *μ*g, respectively. Tolaasins A/B and E were less active, with a minimal inhibitory quantity of 1.28 and 2.56 *μ*g, respectively. A similar sensitivity to the above substances was shown by the fungus *R. solani*. None of the analogues inhibited the growth of the Gram-negative bacteria *E. coli* and *E. carotovora* subsp. c*arotovora* at least at the concentration used in the assays. Finally, the growth of the yeast

Table 6. Antimicrobial Activity of Tolaasins I, II, and A−E (**1**, **2**, and **3**−**7**, respectively)

	tolaasins minimal inhibitory quantity (*μ*g)						
microorganism	I	II	A	B	C	D	E
Rizoctonia solani 1583	0.32	0.64	1.28	2.56	5.12	0.16	5.12
Rhodotorula pilimanae ATCC26423	2.56	5.12	5.12	>5.12	>5.12	2.56	>5.12
Bacillus megaterium ITM100	0.32	0.64	1.28	2.56	>5.12	0.16	2.56
Rodococcus fascians NCPPB3067	0.32	0.64	1.28	1.28	>5.12	0.16	2.56
Escherichia coli K12 ITM103	> 5.12	> 5.12	>5.12	> 5.12	>5.12	>5.12	>5.12
Erwinia carotovora subsp. *carotovora* ICMP5702	> 5.12	>5.12	>5.12	> 5.12	>5.12	>5.12	>5.12

R. pilimanae was inhibited only by tolaasins I, II, and D, although the sensitivity of the yeast was lower in comparison to *B. megaterium* (Table 6). The results of the antimicrobial activity of the tolaasins suggest the importance in this respect of the lactone and the N-terminus acyl moiety. Tolaasin A, which has the pentadioic acid instead of the β-hydroxyoctanoic acid, showed a reduced activity with respect to tolaasin I, while tolaasin C, the linear derivative of tolaasin I, due to the lactone opening, lacked activity at least at the concentration tested. The effect of the lactone ring opening on the antimicrobial activity has already been reported for other bacterial lipodepsipeptides.[19] Furthermore, the importance of the position 15 in the peptide moiety of both tolaasin I and I derivatives with respect to the antimicrobial activity was observed. In fact, the replacement of isoleucine in position 15 with a valine or leucine residue in tolaasins B and D, respectively, determines the decrease or the increase of the antimicrobial activity when compared to tolaasin I. The presence of leucine in postiton 15 in tolaasin E determined the reduction of the activity when compared to tolaasin II. Although the latter result is apparently in contrast with the effect of the same replacement in tolaasin D, it is important to consider that tolaasin II differs from tolaasin I by the replacement of the homoserine in position 16 with a glycine residue.

As recently determined from studies on the high-resolution structure performed in sodium dodecyl sulfate, which is a membrane-like enviroment and simulated its catalytic role, by means of 2D NMR spectroscopy and molecular dynamics simulated annealing calculations, tolaasin I forms an amphipatic left-handed α-helix in the region D-Prod2-D-*allo*Thr14 comprising the sequence of seven D-amino acids and the adjacent L-D-L-D-D-region. Furthermore, the lactone macrocycle adopts a "boatlike" conformation and is shifted from the helical axis to form a "golf-club" overall conformation. These structural features appear of importance to its toxic action on cells.[20] Therefore, structural changes, occurring in position 15 in the case of tolaasins B and D, in position 16 in tolaasin II, and in both positions 15 and 16 in tolaasin E, may modify this lactone conformation and consequently the antimicrobial activity.

Experimental Section

General Experimental Procedures. Optical rotations were measured in MeOH solution on a JASCO P-1010 digital polarimeter. IR (neat) and UV (MeOH) spectra were determined on a Perkin-Elmer Spectrum ONE FT-IR spectrometer and a Perkin-Elmer Lambda 25 UV/vis spectrophotometer, respectively. ^{1}NMR measurements were performed at 300 K on Bruker DRX-600 and Bruker Avance-300 spectrometers operating at 600 and 300 MHz, respectively. All spectra were acquired in the phase-sensitive mode, and the TPPI method was used for quadrature detection in the indirect dimension.[21] All 2D NMR were acquired setting the transmitter at the center of the spectral width, presaturating at the residual HOD resonance (3.323 ppm) in DMSO. The tolaasin samples were prepared by dissolving the available amounts in 0.5 mL of DMSO-*d* (Carlo Erba, 99.95% D). Low-resolution mass spectra were recorded on an LCQ-DECA Finnigan and/or a Perkin-Elmer API 100 LC-MS spectrometer equipped with an electrospray source by direct injection. FABMS spectra were recorded on a VG ZAB 2SE in a glycerol/thioglycerol matrix. High-resolution mass spectra were recorded on a Qstar spectrometer (Applied Biosystems). TLC analyses were carried out on silica gel plates (Merck, Kieselgel, 60 F 254, 0.25 mm), and spots visualized by exposing the plates to UV radiation and spraying with 0.5% ninhydrin in Me_2CO, followed by heating at 110 °C for 10 min. Reversed-phase high-performance chromatography (HPLC) of tolaasin preparations was performed on a Shimadzu instrument, equipped with a HPLC pump LC-10 ADVP and a UV−vis detector SPD-10AV. For the analysis and purification of the tolaasin mixture, analytical (25 cm × 4.6 mm) and semipreparative (25 cm × 10 mm) Brownlee Aquapore RP-300 (C8) columns were used, respectively. A solvent system of water (0.1% TFA, v/v) and CH_3CN was used for elution, with flow rates of 1.0 and 4.7 mL min^{-1} for analytical and semipreparative columns, respectively. A sample of material (10 μg or 1 mg for analytical or preparative separations, respectively) was dissolved in 40% CH_3CN in 0.1% TFA, injected onto an analytical column, which was equilibrated with the same solvent, and eluted by a gradient to 52% CH_3CN (0.1% TFA) over 25 min. Detection was at 220 nm. Gel-filtration chromatography was made on Sephadex G-10 (Pharmacia).

Bacterial Growth Conditions. Strain type NCPPB2192 of *P. tolaasii* was grown at 25 °C under shaking (180 rpm) in 500 mL Erlenmeyer flasks filled with 150 mL of liquid King's B medium[22] inoculated with 1.5 mL of a bacterial suspension containing 10^8 cfu/mL. After 48 h incubation cultures were centrifuged (20000*g* for 15 min), and the resulting supernatants were evaluated for the antimicrobial activity against *Bacillus megaterium* following an already established procedure,[23] lyophilized, and stored at −20 °C before further processing.

Production, Extraction, and Purification of Tolaasins. Tolaasins were isolated and purified from cell-free culture (1.350 L) according to a modification of the method described by Peng (1986).[24] The cell-free culture was filtered through Whatman no. 42 paper, acidified to pH 3 with concentrated HCl, and left overnight at room temperature, and the precipitate was removed by centrifugation (10 000 rpm, 30 min). Tolaasins were precipitated by addition of 105 g of $CaCl_2$, collected by centrifugation (10 000 rpm, 45 min), and dried at 50 °C. The pellet was suspended in 50 mL of MeOH and centrifuged (10 000 rpm, 15 min), and the supernatant was evaporated to dryness in vacuo at 45 °C. The residue was dissolved in 20 mL of MeOH, evaporated to dryness as described previously, and then dissolved in 10 mL of MeOH and dried again. Water (35 mL) was added to the residue and the suspension centrifuged (10 000 rpm, 30 min). The precipitate was dissolved in 20 mL of water and lyophilized. The resulting powder (123.2 mg) was dissolved in water and desalted, in three steps, by gel-filtration chromatography on a Sephadex G-10 column (1.5 cm × 21 cm) and eluted with distilled water (18 mL h^{-1}). The collected fractions (1 mL) were monitored by TLC, and the fractions containing tolaasins were combined and lyophilized to give a white solid residue. This procedure yielded a total of 67.6 mg of tolaasins mixture. The tolaasins mixture (67.6 mg) was purified by injection of a 1 mg sample of material onto a semipreparative column, using the same conditions described previously, to give seven tolaasin peaks, named tolaasins A, B, C, I, D, II, and E. The resulting seven fractions were collected, partially dried under reduced pressure, and finally lyophilized. To obtain a suitable purity of the toxins, the HPLC purification for each tolaasin was repeated twice. The purification procedure, applied to five (1.350 L × 5) different culture filtrates of *P. tolaasii*, permitted the isolation of 2.3, 2.7, 1.7, 124.5, 2.0, 19.0, and 3.2 mg of tolaasins A, B, C, I, D, II, and E (**3**, **4**, **5**, **1**, **6**, **2**, and **7**), respectively.

Tolaasin A (3): $[\alpha]^{25}_D$ −7.2° (*c* 0.16); UV λ_{max} (shoulder) 225 nm; IR ν_{max} 3390, 1671, 1204, 1142 cm^{-1}; ^{1}H NMR, see Table 1.

Tolaasin B (4): $[\alpha]^{25}_D$ −3.5° (*c* 0.27); UV λ_{max} (shoulder) 222 nm; IR ν_{max} 3406, 1677, 1205, 1139 cm^{-1}; ^{1}H NMR, see Table 2.

Tolaasin C (5): $[\alpha]^{25}_D$ −18.5° (*c* 0.13); UV λ_{max} (log ϵ) 225 (3.39) nm; IR ν_{max} 3313, 1663, 1205, 1138 cm^{-1}; ^{1}H NMR, see Table 3.

Tolaasin D (6): $[\alpha]^{25}_D$ −27.6° (*c* 0.15); UV λ_{max} (log ϵ) 225 (3.35) nm; IR ν_{max} 3313, 1660, 1205, 1139 cm^{-1}; ^{1}H NMR, see Table 4.

Tolaasin E (7): $[\alpha]^{25}_{D}$ −5.7° (*c* 0.27); UV λ_{max} (log ϵ) 226 (3.13) nm; IR ν_{max} 3397, 1677, 1206, 1144 cm^{-1}; ^1H NMR, see Table 5.

Toxin Bioassays. The antimicrobial activity of 1:1 serial dilutions of stock solutions of tolaasins in distilled water has been evaluated according to the previously reported soft agar procedure.[23,25] After 24−48 h the last dilution determining the growth inhibition of the test microorganisms in the area of drop application was recorded. The amount of substance contained in the above drop was considered the minimal inhibitory quantity. The assays were performed twice with three replicates.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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