Corpeptins, new bioactive lipodepsipeptides from cultures of *Pseudomonas corrugata*

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Abstract The structure of the corpeptins, bioactive lipodepsipeptides produced in culture by *Pseudomonas corrugata*, the causal agent of tomato pith necrosis, has been determined. The combined use of FAB-mass spectrometry, NMR spectroscopy and chemical procedures has allowed us to assign the following primary structure to the peptide moiety: Dhb-Pro-Ala-Ala-Ala-Val-Val-Dhb-Hse-Val-*a*Ile-Dhp-Ala-Ala-Ala-Val-Dhb-*a*Thr-Ala-Dab-Ser-Ile with the terminal carboxy group closing a macrocyclic ring on the hydroxy group of the *allo*-threonine residue. The N-terminus is in turn acylated by 3-hydroxydecanoate in corpeptin A and by *cis*-3-hydroxy-5-dodecenoate in corpeptin B. Some preliminary data on the biological activity of corpeptins are included.

Key words: Phytotoxin; Lipodepsipeptide; *Pseudomonas corrugata*

1. Introduction

In recent years it has been shown that several species of plant pathogenic *Pseudomonas* produce in culture phytotoxic and antimicrobial lipodepsipeptides with membrane disrupting properties. These belong to two groups, one formed by nonapeptides that share many structural features (syringomycins [1–3], syringotoxins [4–6], syringostatins [7,8], pseudomycins [9]), the other by lipodepsipeptides with a longer and more hydrophobic peptide moiety (syringopeptins [10–13], fuscopeptins [14], tolaasins [15]). Both types occurs at the same time in cultures of several ecotypes of *Pseudomonas syringae* pv. *syringae* [10–13], of *P. syringae* pv. *atrofaciens* [16], of saprophytic *P. syringae* [17], of a transposon generated regulatory mutant of a *P. syringae* strain (pathovar not assigned) [9] and of *P. fuscovaginae* [14].

In the present paper we report the isolation and characterization of the structure of two lipodepsipeptides containing 22 amino acid residues, here called corpeptins (CPs), produced by *P. corrugata*, the causal agent of tomato pith necrosis. Leary and Chun found that a strain of this organism isolated from diseased tomato plants produces a heat labile phytotoxin considered a necessary component for pathogenicity [18,19]. The properties reported for that toxin differ from those of the new metabolites described in this paper.

2. Materials and methods

2.1. Microbiological methods

P. corrugata strain NCPPB 2445 (National Collection of Plant Pathogenic Bacteria, Harpenden, UK) was used throughout. It was maintained on nutrient agar containing 2% v/v glycerol. The antagonistic activity of its colony was tested against *Bacillus megaterium* ITM 100 (Collection of the Istituto Tossine e Micotossine da Parassiti Vegetali, Bari, Italy) and *Rhodotula pilimanae* ATCC 26423 (American Type Culture Collection, Rockville, MD, USA) according to the method reported by Lavermicocca et al. [20]. The antimicrobial activity of toxin preparations at each purification step was routinely assayed towards *B. megaterium* [20] and expressed in units per μg. A unit of activity is the amount of toxin in a 10 μl droplet which completely inhibits growth of the *B. megaterium* in the area of application of the droplet [21].

2.2. Preparation of corpeptins

P. corrugata was grown in potato-dextrose broth (Difco) supplemented with 0.4% casamino acids (Difco). 1 ml of bacterial suspension ($A_{600}=0.3$) was added to 500 ml Erlenmeyer flasks containing 200 ml of medium. After 4 days incubation at 26°C in still culture, when the final pH was 7.0, an equal volume of cold acetone was added, the mixture stored overnight at 4°C, cell debris removed by centrifugation at 4°C (9000 $g\times20$ min), and the supernatant flash evaporated to eliminate the acetone. The aqueous residue was assayed for antimicrobial activity against *B. megaterium* and then fractionated on a column of Amberlite XAD-2 according to Bidwai et al. [22]. Fractions active on *B. megaterium* were pooled, freeze-dried and further fractionated by reverse phase-HPLC according to [10].

2.3. Phytotoxic activity

Phytotoxicity was evaluated on leaves of tobacco plants according to [23].

2.4. Analytical methods

Amino acid analyses of acid hydrolyzed CPs, GC-MS analyses of their TBDMS derivatives, automated Edman degradations of peptides, and FAB-MS spectra were carried out as reported in [14]. The ^{1}H-NMR spectra of samples (approximately 0.8 mg/ml) were run at 27°C on a Bruker AMX600 instrument operating at 600.14 MHz. They were performed in water-deuterated trifluoroethanol (2:8, v/v). Some spectra were also performed at a concentration of 0.4 mg/ml to exclude concentration effects due to aggregation. Two-dimensional TOCSY and ROESY experiments were performed by the pulse sequences [24–26]. Fids were collected over 1k for 512 experiments. After a resolution enhancement a zero filling was introduced to obtain

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Abbreviations: FAB-MS, fast atom bombardment-mass spectrometry; GC-MS, gas chromatography-mass spectrometry; SPs, syringopeptins; SP(SC)s, syringopeptins from a sugarcane isolate of *Pseudomonas syringae* pv. *syringae*; FPs, fuscopeptins; CPs, corpeptins; Tol-A, tolaasin A; TBDMS, *tert*-butyldimethylsilyl; Hse, homoserine; Dhp, dehydro-2-aminopropanoic acid; Dhb, 2,3-dehydro-2-aminobutanoic acid; DAB, 2,4-diaminobutanoic acid; FA, 3-hydroxy fatty acyl

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Table 1
Structure of CP-A and of peptides obtained from partial acid hydrolysis

Structure	MH^+
CH_3-$(CH_2)_6$-CHOH-CH_2-CO-Dhb-P-A-A-A-V-V- Dhb-Hse- V-aI-Dhp-A-A-A-V-Dhb-aT-A-Dab-S-I	2094
CH_3-$(CH_2)_6$-CHOH-CH_2-CO-Dhb- P-A-A-A-V-V-Dhb-Hse- V-aI	1176
CH_3-$(CH_2)_6$-CHOH-CH_2-CO-Dhb- P-A-A-A-V-V-Dhb-Hse>	946
P-A-A-A-V-V-Dhb-Hse- V-aI-Dhp-A-A-A-V-Dhb-aT-A-Dab-S-I	1841
P- A-A-A-V-V-Dhb-Hse- V-aI	923
P- A-A-A-V-V-Dhb-Hse>	693
P- A-A-A-V-V-Dhb-Hse	711
A-A-A-V-V-Dhb-Hse>	596
A- A-V-V-Dhb-Hse>	525
A-V-V-Dhb-Hse>	454
V-aI-Dhp-A-A-A-V-Dhb-aT-A-Dab-S-I	1149
Dhp-A-A-A-V-Dhb-aT-A-Dab-S-I	938
A-A-A-V-Dhb-aT-A-Dab-S-I	868

Hse> indicates a homoserine lactone residue.

a 1k×1k real matrix. In the ROESY spectra a spinlock mixing time of 0.080 s was applied. In TOCSY experiments a mixing of 0.1 s was applied to obtain remote scalar connectivities.

2.5. Chemical methods

Hydrolysis of the lactone ring was obtained by overnight incubation at 25°C with 6.5% aqueous triethylamine buffered at pH 9.0. Partial acid hydrolyses of CPs were performed with 120 mM HCl at 110°C. Samples were withdrawn after 1 and 3 h heating, analyzed by FAB-MS and fractionated by reverse phase-HPLC. Individual fractions were characterized by FAB-MS.

3. Results and discussion

P. corrugata NCPPB 2445 inhibited the growth of *B. megaterium*. In the antagonistic assay against *B. megaterium* a *P. corrugata* colony produced an inhibition halo of 10 mm around the colony. The growth of the fungus *R. pilimanae* was unaffected.

Cultures of *P. corrugata*, processed according to Bidway et al. [22], provided a group of fractions active towards *B. mega-*

Table 2
Assignment of the NMR spectrum of corpeptins (PPM from TMS)

Residue	N	NH	CHα	CHβ	CHγ	Others
Dhb	1	9.132 (9.144)		5.801 (5.796)	1.832 (1.829)	
Pro	2		4.446 (4.435)	2.458 (2.427)	2.060, 2.035 (2.053, 2.012)	3.802, 3.663 (3.797, 3.649)
Ala	3	7.984 (7.958)	4.403 (4.377)	1.550 (1.545)		
Ala	4	7.745 (7.735)	4.121 (4.086)	1.426 (1.416)		
Ala	5	7.965 (7.931)	4.102 (4.086)	1.426 (1.416)		
Val	6	7.640 (7.632)	3.935 (3.916)	2.309 (2.297)	1.044, 1.099 (1.036, 1.094)	
Val	7	7.819 (7.819)	3.864 (3.839)	2.343 (2.330)	1.082, 1.164 (1.082, 1.159)	
Dhb	8	9.438 (9.455)		6.545 (6.535)	1.807 (1.802)	
Hse	9	7.885 (7.868)	4.443 (4.424)	2.187, 2.337 (2.173, 2.318)	3.882, 3.844 (3.872, 3.814)	
Val	10	7.960 (7.932)	3.989 (3.975)	2.406 (2.404)	1.041, 1.138 (1.036, 1.133)	
iIle	11	8.124 (8.130)	4.124 (4.111)	2.006, 0.993 (1.988, 0.988)	1.469, 1.290 (1.475, 1.270)	0.920 (0.906)
Dhp	12	9.500 (9.496)		5.498, 5.690 (5.494, 5.684)		
Ala	13	7.819 (7.830)	4.304 (4.294)	1.582 (1.577)		
Ala	14	8.026 (8.025)	4.205 (4.188)	1.550 (1.545)		
Ala	15	8.124 (8.117)	4.251 (4.231)	1.510 (1.505)		
Val	16	7.984 (7.958)	4.008 (3.988)	2.426 (2.404)	1.112, 1.195 (1.108, 1.191)	
Dhb	17	8.910 (8.906)		6.754 (6.757)	1.853 (1.802)	
Thr	18	8.074 (8.071)	4.343 (4.336)	5.231 (5.227)	1.460 (1.454)	
Ala	19	7.919 (7.891)	4.294 (4.297)	1.533 (1.530)		
Dab	20	8.852 (8.876)	4.251 (4.239)	2.277, 2.544 (2.266, 2.535)	3.143 (3.139)	NH 7.745 (7.779)
Ser	21	8.008 (7.988)	4.147 (4.120)	4.078 (4.065)		
iIle	22	7.566 (7.572)	4.640 (4.621)	2.106 (2.094) 0.981 (0.976)	1.540, 1.176 (1.524, 1.159)	0.920 (0.919)

Values are reported for CP-A and those in parentheses refer to CP-B.
FA SP_{22}-A: CH_3 (10) 0.913; CH_2 (9-6) 1.31–1.34; CH_2 (5) 1.35; CH_2 (4) 1.58–1.61; CH (3) 4.113; CH_2 (2) 2.61–2.58; FA SP_{22}-B: CH_3 (12) 0.899; CH_2 (11) 1.323; CH_2 (10-9) 1.33–1.37; CH_2 (8) 1.378; CH_2 (7) 2.073; CH (6) 5.635; CH (5) 5.425; CH_2 (4) 2.377; CH (3) 4.158; CH_2 (2) 2.615.

Table 3
NOEs observed in CPs ROESY spectra which led to the complete sequential assignment

FA C2-1N	9 s
2α-3N	5.2 m
1N-2β	0.8 w
3N-4N	7.2 s
3α-4N	1.3 w
3β-4N	2.4 m
4N-5N	7.2 s
4α-5N	15.8 s
4β-5N	7.2 s
5N-6N	1.2 w
5α-6N	8.6 s
5β-6N	1.5 w
6N-7N	0.8 w
6α-7N	2.0 m
6γ-7N	1.5 w
N-8N	3.7 m
7α-8N	2.4 m
7β-8N	3.7 m
8N-9N	2.1 m
9α-10N	5.4 m
9β-10N	2.8 m
10N-11N	5.1 m
10α-11N	1.0 w
10β-11N	3.0 m
11N-12N	2.5 m
11α-12N	1.6 w
11β-12N	3.1 m
12N-13N	1.5 w
12β-13N	1.4 w
13N-14N	3.3 m
13β-14N	12.8 s
14N-15N	1.0 w
14α-15N	4.3 m
14β-15N	4.2 m
15N-16N	4.2 m
15α-16N	5.9 m
15β-16N	15 s
16N-17N	2.7 m
16α-17N	3.0 m
16β-17N	2.4 m
17N-18N	2.4 m
18α-19N	7.6 s
18β-19N	2.1 m
19α-20N	16.5 s
19β-20N	1.3 w
20N-21N	1.9 w
20α-21N	5.9 m
21N-22N	5.0 m

Long range NOEs found are omitted. Numbers are integrated intensity in arbitrary units and symbols s, m and w classify the NOEs as strong, medium and weak respectively.

terium. The elution profile resulting from reverse-phase HPLC of this partially purified material (Fig. 1) showed two major peaks in the region where SPs and FPs, respectively from *P. syringae* pv. *syringae* [10] and *P. fuscovaginae* [14], are eluted. Prominent peaks in the region of lipodepsinonapeptides were not observed.

The preliminary FAB-MS and ^{1}H-NMR data of the substances giving rise to the two peaks showed a close structural relation between them, but a marked difference from previously described peptins. Thus, in order of their appearance in the eluate, they were named corpeptin A (CP-A) and corpeptin B (CP-B). Their molecular weights (MH^+ 2094 and 2120, respectively for CP-A and CP-B) were compatible with those of other previously described peptins, both increased by 18 mass units on standing at pH 9. Quantitative amino acid analyses showed that both compounds contained Pro(1), Ile(2), Ala(7), Val(4), Ser(1), Thr(1), Hse(1), Dab(1). GC-MS analysis of their TBDMS derivatives indicated that the Thr residue and one of the Ile residues have the *allo* configuration. These data coincided with those independently obtained by NMR spectrometry. As for previous work on other lipodepsipeptides from *P. syringae* pv. *syringae*, the complete assignment of the resonances resulted from the spin systems obtained by TOCSY experiments and from the chemical shift values reported for the common amino acids [27]. The spin multiplicity and the characteristic chemical shifts made easy the recognition in both CPs of three *Z*-Dhb, while a single Dhp residue was identified by coupling constants and dipolar connectivity. Thus, 22 amino acids were identified in both CPs, all probably members of a peptide with a cyclic moiety accounting for 1924 mass units.

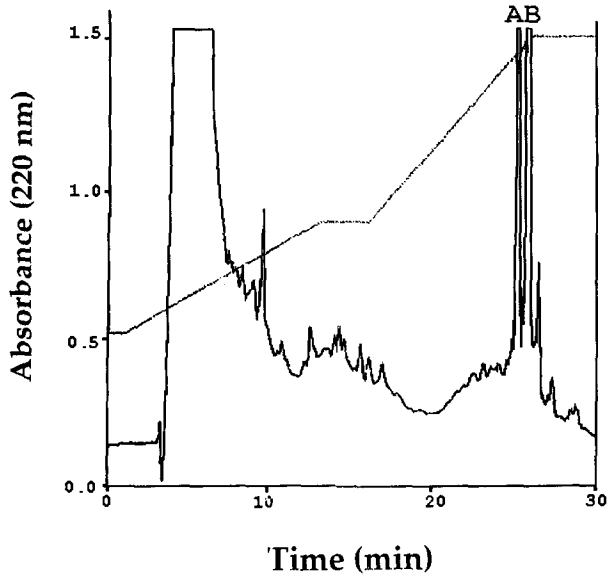

Fig. 1. Reverse-phase HPLC of the metabolites from *P. corrugata* NCPPB 2445. The letters A and B indicate corpeptin A and B respectively.

The amino acid sequence was independently approached by chemical methods and by 2D NMR. Ten peptides isolated from partially hydrolyzed CP-A, examined by FAB-MS, and sequenced by the automated Edman procedure, provided the following partial sequence: FA-Dhb-Pro-Ala-Ala-Ala-Val-

Table 4
Comparison of the amino acid moiety of corpeptins with those of other pseudomonad peptins

CPs	**FA**	**Dhb**	**P**	**A**	**A**	**A**	**V**	**V**	Dhb	Hse	**V**	aI	**Dhp**	**A**	**A**	**A**	**V**	**Dhb**	**aT**	-	-	-	**A**	**Dab**	S	I
SPs	**FA**	**Dhb**	**P**	**V**	**A**	**A**	**V**	**L**	**A**	**A**	Dhb	**V**	**Dhb**	**A**	**V**	**A**	**A**	**Dhb**	**aT**	S	**A**	V	**A**	**Dab**	**Dab**	**Y**
SPs-25	**FA**	**Dhb**	**P**	**V**	**V**	**A**	**A**	**V**	**V**	-	-	-	**Dhb**	**A**	**V**	**A**	**A**	**Dhb**	**aT**	S	**A**	Dhb	**A**	**Dab**	**Dab**	**Y**
SPs-22	**FA**	**Dhb**	**P**	**V**	**L**	**A**	**A**	**L**	**V**	-	-	-	**Dhp**	**A**	**V**	**A**	**A**	**Dhb**	**aT**	S	**A**	Dhb	**A**	**Dab**	**Dab**	**Y**
SP(SC)s-22	**FA**	**Dhb**	**P**	**L**	**A**	**A**	**A**	**A**	**V**	-	-	-	G	**A**	**V**	**A**	**V**	**Dhb**	**aT**	-	-	-	**A**	**Dab**	**Dab**	**F**
Tol-A	**FA**	**Dhb**	**P**	S	**L**	**V**	S	**L**	**V**	**V**	Q	**L**	V	-	-	-	-	**Dhb**	**aT**	-	-	-	**I**	Hse	**Dab**	K

Identical or closely related residues are in bold.

Val-Dhb-*a*Thr/Hse-Val-Ile/*a*Ile-Dhp-Ala-Ala-Ala-Val-Dhb-X-Ala-Y-Ser-Ile/*a*Ile, with Dab and Hse respectively assigned to X and Y, or to Y and X. The sequential assignment by 2D NMR was performed following the dipolar connectivity along the peptide chain and, particularly, detecting NOEs between amide resonances and CHα (i, i+1) resonances. The above sequence was confirmed by several NH-NH and NH-CHα connectivities and improved with the conclusive assignment of Hse, *a*Thr and Dab to residues 9, 18 and 20, respectively (Table 1). The remaining ambiguity between Ile and *a*Ile was overcome by the results of amino acid analysis of the CP-A-(1–10) peptide lactone (MH^+ 1176) and of the (11–22) peptide (MH^+ 938; the N-terminal Dhp has become a 2-oxo-propanoyl residue); the former contains the *a*Ile and not the Ile residue, the latter contains the Ile and not the *a*Ile residue. The relative positions of *a*Thr and Hse were also defined by the same amino acid analyses.

The lactone ring closure in both CPs was identified by specific NOEs. In particular, that of CP-A was supported by the NOE between CHα Thr and CHα Ile-22, and further corroborated by the unusual chemical shift values of CHα and CHβ of Thr-18. In CP-B a connectivity was found between NH of Ile-22 and CHβ of Thr-18.

Also the structure of the fatty acid chain was elucidated by 2D NMR. TOCSY spectra assigned all the protons of the chains and showed the presence of hydroxy groups. It was established that the acyl moiety is 3-hydroxydecanoyl in CP-A and *cis*-3-hydroxy-5-dodecenoyl in CP-B, a finding that accounts for the difference of 26 mass units between the molecular weights of the two metabolites.

All the NMR results are reported in Tables 2 and 3.

The comparison of the CP peptide moiety with that of other *Pseudomonas* peptins (Table 4) shows a very high homology among CPs, SP_{22}s, SP_{25}s and FPs, somehow smaller but still noticeable in tolaasins (Tol-A). The homology is absent in other antimicrobial and membrane-active peptides which share with the peptins a long sequence of hydrophobic amino acids and a positively charged cyclized C-terminal moiety [28,29].

The fatty acid moiety of CP-A is the same found in SP_{22}-A, SP_{25}-A and FP-B while that of CP-B includes a novelty, namely an unsaturation. As a matter of fact, in all previously described *Pseudomonas* lipodepsipeptides the peptide is acylated by a saturated 3-hydroxy fatty acid.

Preliminary data on the phytotoxic activity of CPs indicate that these new toxins have properties partly different from those of SPs and FPs. In the tobacco leaf assay 4 μM solutions of both CPs induced chlorosis 72 h after tissue infiltration but no apparent necrosis. At the same concentration a high intensity of necrotic symptoms was induced by the two SPs. CP-A displayed detectable necrotic activity only at concentrations higher than 5 μM, while CP-B induced only chlorosis, even at four times higher concentration.

The antimicrobial activities of the CPs are instead very similar to those of the two SPs. In the *B. megaterium* assays 50 μM CP-A and CP-B showed a specific activity of 14.3 and 12.5 units/μg, respectively. Their minimal inhibitory concentrations were 3.75 and 4.20 μM, respectively, comparable to those for SPs [20]. As expected, the activity against *R. pilimanae* was negligible.

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