

Structure of fuscopeptins, phytotoxic metabolites of *Pseudomonas fuscovaginae*

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Abstract The structure of the fuscopeptins, bioactive lipodepsipeptides produced in culture by the gramineae pathogen *Pseudomonas fuscovaginae*, has been determined. The combined use of FAB mass spectrometry, NMR spectroscopy and chemical and enzymatic procedures allowed one to define a peptide moiety corresponding to ZDhb-DPro-LLeu-DAla-DAla-DAla-DVal-Gly-DAla-DVal-DAla-DVal-ZDhb-DaThr-LAla-LDab-DDab-LPhe with the terminal carboxyl group closing a macrocyclic ring on the hydroxyl group of the *allothreonine* residue. The N-terminus is in turn acylated by 3-hydroxyoctanoate in fuscopeptin A and 3-hydroxydecanoate in fuscopeptin B. Some preliminary data on the biological activity of fuscopeptins are also reported.

Key words: Fuscopeptins; Lipodepsipeptides; Phytotoxins; *Pseudomonas fuscovaginae*

1. Introduction

Pseudomonas fuscovaginae is a phytopathogen identified as the causal agent of bacterial sheath brown rot of rice and other gramineae as *Hordeum vulgare*, *Triticum aestivum*, *Avena sativa* and *Zea mays* [1–5]. This pathogen causes necrotic lesions of the flag leaf sheath and reduction of the peduncle elongation which results in the inhibition of inflorescence emergence and sterility of the panicle. Strains of *P. fuscovaginae* can be distinguished from other fluorescent pseudomonads by several biochemical reactions, as their positive reactions for the oxidase and arginine dihydrolase assays, as well as by serological and pathogenicity tests [2,6].

Many fluorescent pseudomonads are known to produce toxins involved in disease development [7]. The progression of symptoms of sheath brown rot prior to extensive bacterial colonization suggests the involvement of toxins. Recently the presence of bioactive low molecular weight metabolites in *P. fuscovaginae* culture filtrates was reported [8]. One of these metabolites was identified as syringotoxin, a lipodepsinonapep-

tide produced in culture by citrus strains of *P. syringae* pv. *syringae* [9,10], and fully characterized by Ballio et al. [11] and Fukuchi et al. [12].

This paper describes the purification and chemical characterization of two new bioactive metabolites of *P. fuscovaginae*, here called fuscopeptins (FPs). They are lipodepsipeptides structurally related to the syringopeptins (SPs) [13] and the tolaasins [14]. A preliminary description of FPs biological properties is also included.

2. Materials and methods

2.1. Preparation and purification of fuscopeptins

P. fuscovaginae strain UPB 264 [2] was used throughout. Growth of the organism, extraction of cultures, and fractionation of the bioactive metabolites were carried out in Louvain according to [8] and in Rome according to [15].

2.2. Analytical methods

Amino acid analyses of samples hydrolysed with 6 N HCl at 110°C, for 24 h in vacuo were carried out with an Eppendorf-Biotronik LC 3000 analyzer; some analyses were also performed by GC-MS after transformation of the free amino acids into TBDMS derivatives [16]. Configuration of individual amino acids and N-terminal residues was determined as described [17,18].

Peptide sequences were determined by automated Edman degradation using an Applied Biosystems-Perkin Elmer 476A protein sequencer. Dehydroamino acids were determined after modification with mercaptoethanol according to [19].

FAB-MS spectra were recorded on a VG ZAB 2SE (VG Analytical) instrument equipped with a cesium gun operating at 25 kV, 2 mA. Samples dissolved in 5% acetic acid were directly loaded onto the probe tip coated with glycerol/thioglycerol (1:1 v/v). Spectra were recorded on UV-sensitive paper and manually counted.

TBDMS derivatives of amino acids and fatty acid methyl esters were analyzed by a TRIO 2000 (Fisons-VG analytical) GC-MS apparatus equipped with a 5890 Series II Hewlett-Packard gas chromatograph; a silica capillary column crosslinked with methylsilicone and programmed from 100 to 250°C at 3°C/min was used.

The ¹H NMR spectra of samples dissolved in D₂O were run at 27°C on a Bruker AMX600 instrument operating at 600.14 MHz.

2.3. Chemical methods

Partial acid hydrolyses of fuscopeptins were performed with 60 or 120 mM HCl at 110°C for 3 and 4 h, respectively. The resulting peptides were fractionated by reverse-phase HPLC on an Aquapore RP300 column (4.6 × 250 mm, 7 mm, Applied Biosystems) eluted with a linear gradient of acetonitrile/2-propanol (4:1 v/v) in 0.2% TFA, at a flow rate of 0.8 ml/min.

The lactone bond was selectively hydrolysed by 2 h incubation at room temperature with 6.5% aqueous triethylamine buffered at pH 9.0.

Fatty acid methyl esters were prepared by adding ethereal diazomethane to an ethylacetate extract of fuscopeptin hydrolysate or by treatment of the lipodepsipeptide with 14% BF₃ in methanol at room temperature.

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Abbreviations: aThr, *allothreonine*; Dab, 2,4-diaminobutyric acid; Dhb, 2,3-dehydro-2-aminobutyric acid; FAB-MS, fast atom bombardment mass spectrometry; FP, fuscopeptin; GC-MS, gas chromatography mass spectrometry; SP, syringopeptin; SR, syringomycin; TBDMS, *t*-butyldimethylsilyl; TFA, trifluoroacetic acid.

3. Results and discussion

Independently of the different conditions used in the Belgian and Italian laboratories for growth of the microorganism and for extraction of its bioactive metabolites, reverse-phase HPLC of the culture extracts consistently gave an elution pattern very similar to that observed with strains of *P. syringae* pv. *syringae*. As shown in Fig. 1, depicting one typical experiment, the region where lipodepsinonapeptides are eluted, with prevalence of the previously identified syringotoxin (peak 1) [8], is followed by that containing more hydrophobic metabolites (peaks 2 and 3). The complete structure of these compounds was elucidated by the use of FAB-MS, NMR and chemical and enzymatic degradations carried out on microquantities.

FAB-MS and amino acid analyses clearly indicated that these substances were different from previously described *P. syringae* phytotoxins. Thus they were named fuscopeptin A (FP-A) and fuscopeptin B (FP-B). They had the same quantitative amino acid composition: Pro (1), Leu (1), Ala (7), Val (3), Gly (1), Thr (1), Dab (2) and Phe (1). Besides confirming the occurrence of the above amino acid residues, GC-MS analysis of TBDMS derivatives demonstrated the *allo* configuration of the threonine residue. The ^1H NMR spectra were compatible with this composition and furthermore showed the occurrence of two Dhb residues (quartets at 5.87 and 6.76 δ for the β -protons) and a 3-hydroxy fatty acyl moiety. This corresponded to 3-hydroxyoctanoyl for the isoform A (peak 2) and to 3-hydroxydecanoyl for the isoform B (peak 3), as demonstrated by GC-MS analysis of the methyl esters prepared after acid

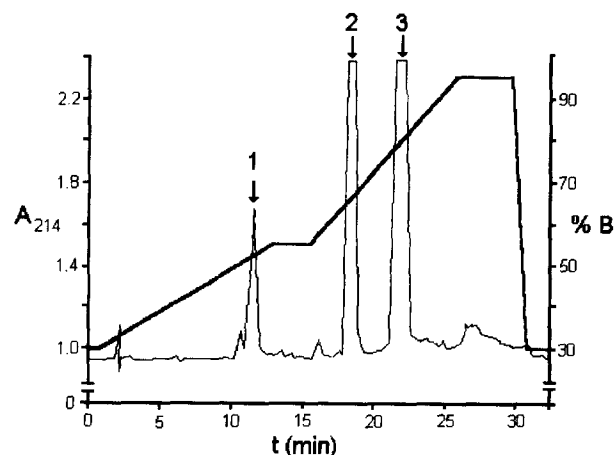


Fig. 1. Reverse-phase HPLC of purified metabolites from *Pseudomonas fuscovaginae* strain UPB 264 culture. The numbers 1, 2 and 3 indicate syringotoxin, fuscopeptin A and B, respectively. A_{214} = UV absorbance (214 nm); %B = percent of solvent B (0.1% TFA in acetonitrile:isopropanol (4:1)) in the eluting solvent. Solvent A = 0.2% TFA in water.

hydrolysis. All amino acids, with the exception of Leu, Phe, one Dab and one Ala, had the D-configuration.

At least some of the residues must be in a cyclic arrangement since the sum of their masses in a linear structure did not correspond to the molecular weights (1817 for FP-A and 1845 for FP-B) determined by FAB-MS. This consideration,

Table 1
Stereochemical structure of fuscopeptin A

Peptide	D L D D D D D D D D D L L D L										
F-A	FA-Dhb-Pro-Leu-Ala-Ala-Ala-Ala-Val-Gly-Ala-Val-Ala-Val-Dhb-aThr-Ala-Dab-Dab-Phe										
A	Pro-Leu-Ala-Ala-Ala-Ala-Val-Gly										
B	Pro-Leu-Ala-Ala-Ala										
C	Leu-Ala-Ala										
D	Ala-Ala-Ala-Val-Gly										
E	Ala-Ala-Val-Gly										
F	Ala-Val-Gly										
G	Gly-Ala-Val										
H	Ala-Val-Ala-Val-Dhb-aThr-Ala-Dab-Dab-Phe										
I	Val-Ala-Val										
J	Val-Dhb-aThr-Ala-Dab-Dab-Phe										
K	Dab-Dab										

Peptide	Amino acid composition										
	N.t.	DPro	LLeu	DAla	LAla	Gly	DVal	DaThr	LDab	DDab	LPhe
F-A	n.d.	0.8 (1)	0.7 (1)	5.5 (6)	1.0 (1)	0.9 (1)	2.7 (3)	0.7 (1)	0.4 (1)	0.6 (1)	0.8 (1)
A	n.d.	1.3 (1)	1.0 (1)	3.9 (4)		1.0 (1)	0.9 (1)				
B	n.d.	1.0 (1)	1.0 (1)	2.4 (3)							
C	n.d.		1.0 (1)	1.7 (2)							
D	DAla			2.5 (3)		1.0 (1)	1.0 (1)				
E	DAla			1.7 (2)		1.0 (1)	0.8 (1)				
F	DAla			0.8 (1)		1.0 (1)	1.0 (1)				
G	Gly			0.8 (1)		1.0 (1)	0.8 (1)				
H	DAla			1.9 (2)	1.1 (1)		2.1 (2)	1.0 (1)	0.4 (1)	0.3 (1)	1.0 (1)
I	DVal			1.0 (1)			2.0 (2)				
J	DVal				0.7 (1)		1.0 (1)	0.7 (1)	1.0 (1)	0.2 (1)	0.9 (1)
K	LDab								0.7 (1)	1.0 (1)	

The table summarizes the configuration of N-terminal and partial hydrolysate amino acids. In parentheses are given the expected values. FA and N-t are abbreviations for fatty acid moiety and N-terminus, respectively.

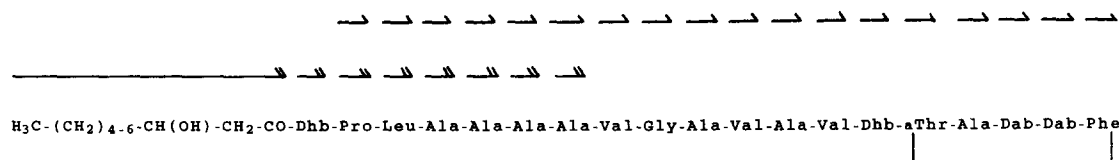


Fig. 2. Chemical structures of FP-A and FP-B. Sequence information was obtained by Edman degradation (—) and FAB-MS (---) for FP-A.

together with (a) the presence of a blocked N-terminus, (b) the occurrence of 3-hydroxyoctanoic acid and 3-hydroxydecanoic in fuscopeptin A and B, respectively, (c) the observed addition of one molecule of water to each substance on treatment with aqueous triethylamine (MH^+ 1836 for FP-A and MH^+ 1864 for FP-B), suggested that these metabolites were lipodepsipeptides. Furthermore the data strongly suggested that the two fuscopeptins are homologues differing only for their fatty acid moieties. A series of fragment ions was observed in the FAB spectra of both FP-A and FP-B following treatment with triethylamine, which eventually led to the partial N-terminal sequence: 3-hydroxyoctanoyl- (for FP-A) or 3-hydroxydecanoyl- (for FP-B) -Dhb-Pro-Ile/Leu-Ala-Ala-Ala-Ala-... and to the C-terminal sequence -Dab-PheCO₂H. The presence of Phe as the

C-terminus in both native FPs was further demonstrated by incubation of the triethylamine-treated lipodepsipeptides with carboxypeptidase A. The MH^+ ion of both FPs was shifted back by 147 Da due to the loss of a phenylalanine residue released by the enzyme.

Partial acid hydrolysis of FP-A with 60 mM HCl followed by reverse-phase HPLC fractionation yielded two main peptide fragments with lower retention times than the starting material. They showed the protonated molecular ion at 1611 and 1188 m/z, respectively. In order to make these peptides suitable for sequencing by automated Edman degradation, they were reacted with mercaptoethanol to form a stable adduct with the Dhb residues [19]. The sequence analysis confirmed previous FAB-MS data and established that the longer, more hydropho-

Table 2

A comparison between some biological activities of 20 μM syringomycin-E (SR), syringopeptin 22-A (SP) and fuscopeptin A (FP)

Test	FP	SP	SR
Toxic activity on:			
<i>Geotrichum candidum</i>	0	0 ^a	++ ^b
<i>Botrytis cinerea</i>	++	+++ ^c	+++ ^{b,c}
<i>Sarocladium oryzae</i>	0	n.d.	0 ^b
<i>Rhodotorula pilimanae</i>	+	+ ^a	+++ ^d
Induction of necrotic lesions:			
on rice leaf sheath	+++	n.d.	+ ^b
on tobacco leaves	+++	+++ ^a	+ ^a
Inhibition of proton extrusion promoted by fusicoccin in maize roots	+	+ ^e	++ ^e
Stomatal closing in <i>Vicia faba</i> epidermal strips	++	++ ^f	+ ^{f,g}
Effect on ATP hydrolysis in right-side-out plasma membrane vesicles (+ for stimulation, – for inhibition):			
maize roots	–	– ^e	+ ^{e,h}
rice leaf sheath	– ⁱ	n.d.	++ ⁱ
Inhibition of ATP hydrolysis in inside-out plasma membrane vesicles:			
maize roots	+++	+++ ^e	+ ^{e,h}
rice leaf sheath	+++ ⁱ	n.d.	+ ⁱ
Inhibition of proton translocation in inside-out plasma membrane vesicles from maize roots	+++	+++ ^e	+ ^e
Dissipation of the pH gradient in inside-out plasma membrane vesicles from maize roots	+++	+++ ^e	+ ^e
Haemolytic activity	+++	+++ ^{c,i}	+ ^{c,i}

0 = no effect; n.d. = not determined.

^a Iacobellis, N.S., Lavermicocca, P., Grgurina, I., Simmaco, M. and Ballio, A. (1992) *Physiol. Mol. Plant Pathol.* 40, 107–116.

^b Flamand, M.C. and Maraite, H., unpublished data.

^c Lavermicocca, P., Iacobellis, N.S. and Simmaco, M. (1995) *Proceedings 5th International Conference on Pseudomonas syringae Pathovars and Related Pathogens* (in press).

^d Grgurina, I., Barca, A., Cervigni, S., Gallo, M., Scaloni, A. and Pucci, P. (1994) *Experientia* 50, 130–133.

^e Di Giorgio, D., Camoni, L. and Ballio, A. [15].

^f Di Giorgio, D., Camoni, L., Mott, K.A., Takemoto, J.Y. and Ballio, A. (1996) *Plant Pathol.* (in press).

^g Mott, K.A. and Takemoto, J.Y. (1989) *Plant Physiol.* 90, 1435–1439.

^h Che, F.S., Kasamo, K., Fukuchi, N., Isogai, A. and Suzuki, A. (1992) *Physiol. Plant.* 86, 518–524.

ⁱ Batoko, H., Flamand, M.C., Boutry, M., Kinet, J.M. and Maraite, H. (1995) *Proceedings 5th International Conference on Pseudomonas syringae Pathovars and Related Pathogens* (in press).

^j Di Giorgio, D., Camoni, L. and Ballio, A., unpublished data.

bic fragment has the primary structure Pro-Leu-Ala-Ala-Ala-Ala-Val-Gly-Ala-Val-Ala-Val-Dhb-Thr-Ala-Dab-Dab-Phe, and the shorter, less hydrophobic, the sequence Ala-Val-Gly-Ala-Val-Ala-Val-Dhb-Thr-Ala-Dab-Dab-Phe (Fig. 2).

In order to determine the amino acid stereochemistry of these phytotoxins a sample of FP-A was subjected to partial acid hydrolysis with 120 mM HCl and the mixture fractionated by reverse phase HPLC. The purified fragments were identified by Edman degradation and the configuration of their amino acid components was determined according to [17,18] for individual amino acids and N-terminal residues, respectively. The results, summarized in Table 1, allowed to identify the stereochemistry of all amino acids.

Table 2 reports some preliminary results of FPs activities in vitro and in vivo tests in comparison with SR-E and SP-22A. As expected from the high structural similarity of FPs with SPs, the biological properties of the new lipodepsipeptides are much closer to those of SPs than to those of SRs. The contraction of the lactone ring, from eight (SPs) to five (FPs) amino acid residues, is uninfluential for the activity. This contraction leaves untouched the two basic residues, the aromatic nature of C-terminal residue, and the hydroxy-amino acid residue (*allothreonine*). Also other structural features, together with the high content of hydrophobic amino acid residues, and the amphiphilic character, are shared by SPs and FPs, thus allowing the very reasonable assumption that both adopt in solution a similar conformation. This might form the basis for the same mechanism of action at the molecular level, and therefore be the reason for very similar biological activity.

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References

- [1] Miyajima, K. (1983) Report Hokkaido Prefect. Exp. Stn. 43, 57–131.
- [2] Duveiller, E., Miyajima, K., Snacken, F., Autrique, A. and Maraite, H. (1988) J. Phytopathol. 122, 97–107.
- [3] Miyajima, K., Tani, A. and Akita, T. (1983) Int. J. Syst. Bacteriol. 33, 656–657.
- [4] Ziegler, R.S. and Alvarez, E. (1987) Plant Disease 71, 592–597.
- [5] Duveiller, E. and Maraite, H. (1990) Plant Disease 74, 932–935.
- [6] Lelliott, R.A., Billing, E. and Hayward, A.C. (1966) J. Appl. Bacteriol. 29, 470–489.
- [7] Gross, D.C. (1991) Annu. Rev. Phytopathol. 29, 247–278.
- [8] Flamand, M.-C., Pellser, S., Ewbank, E. and Maraite, H. (1995) Physiol. Mol. Plant Pathol. (submitted).
- [9] Gross, D.C., DeVay, J.E. and Stadtman, F.H. (1977) J. Appl. Bacteriol. 43, 453–486.
- [10] Gonzales, C.F., DeVay, J.E. and Wakeman, R.J. (1981) Physiol. Plant Pathol. 18, 41–50.
- [11] Ballio, A., Bossa, F., Collina, A., Gallo, M., Iacobellis, N.S., Paci, M., Pucci, P., Scaloni, A., Segre, A. and Simmaco, M. (1990) FEBS Lett. 269, 377–380.
- [12] Fukuchi, N., Isogai, A., Katayama, J. and Suzuki, A. (1990) Agric. Biol. Chem. 54, 3377–3379.
- [13] Ballio, A., Barra, D., Bossa, F., Collina, A., Grgurina, I., Marino, G., Moneti, G., Paci, M., Pucci, P., Segre, A. and Simmaco, M. (1991) FEBS Lett. 291, 109–112.
- [14] Nutkins, J.C., Mortishire-Smith, R.J., Packman, L.C., Brodey, C.L., Rainey, P.B., Johnstone, K. and Williams, D.H. (1991) J. Am. Chem. Soc. 113, 2621–2627.
- [15] Di Giorgio, D., Camoni, L. and Ballio, A. (1994) Physiol. Plant. 91, 741–746.
- [16] Chaves das Neves, H.J. and Vasconcelos, A.M.P. (1987) J. Chromatogr. 392, 249–258.
- [17] Scaloni, A., Simmaco, M. and Bossa, F. (1995) Amino Acids 8, 305–313.
- [18] Scaloni, A., Simmaco, M. and Bossa, F. (1991) Anal. Biochem. 197, 305–310.
- [19] Scaloni, A., Barra, D. and Bossa, F. (1994) Anal. Biochem. 218, 226–228.