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Novel bioactive lipodepsipeptides from *Pseudomonas syringae*: the pseudomycins

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Abstract The covalent structure and most of the stereochemistry of the pseudomycins, bioactive metabolites of a transposon-generated mutant of a *Pseudomonas syringae* wild-type strain proposed for the biological control of Dutch elm disease, have been determined. While two pseudomycins are identical to the known syringopeptins 25-A and 25-B, pseudomycins A, B, C, C′ are new lipodepsinonapeptides. For all of these the peptide moiety corresponds to L-Ser-D-Dab-L-Asp-L-Lys-L-Dab-L-aThr-*Z*-Dhb-L-Asp(3-OH) -L-Thr(4-Cl) with the terminal carboxyl group closing a macrocyclic ring on the OH group of the N-terminal Ser. This is in turn *N*-acylated by 3,4-dihydroxytetradecanoate in pseudomycin A, by 3-hydroxytetradecanoate in pseudomycin B, by 3,4-dihydroxyhexadecanoate in pseudomycin C, and by 3-hydroxyhexadecanoate in pseudomycin C′. Some preliminary data on the biological activity of pseudomycin A are reported.

Key words: Phytotoxin; Lipodepsipeptide; Pseudomycin; Syringomycin; *Pseudomonas syringae*

1. Introduction

Pseudomycins are antifungal metabolites produced in elevated amounts by *Pseudomonas syringae* MSU 16H [1] a transposon-generated mutant of a wild-type strain that has attracted interest for its ability to confer a greater protection than the wild-type strain in elms infected with *Ceratocystis ulmi*, the causal agent of Dutch elm disease [2]. Recently the individual pseudomycins have been isolated and their structures have been partially characterized [1]. The aim of our research on pseudomycins was to complete the study of their structure and to investigate their biological properties. Four of them turned out to be new lipodepsinonapeptides related to syringomycins [3,4], syringotoxin [5,6] and syringostatins [7], a group of antimicrobial and phytotoxic compounds produced by different isolates of *P. syringae* pv. *syringae*. This structural relation prompted a comparison of the biological properties of pseudomycins with those of other lipodepsinonapeptides. The preliminary results (details will be published elsewhere) of some biotests carried out with pseudomycin A, the major pseudomycin, in comparison with syringomycin E [3], a lipodepsinonapeptide extensively investigated for its biological activities [8], are included in this paper.

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Abbreviations: TFA, trifluoroacetic acid; FAB-MS, fast atom bombardment mass spectrometry; TBDMS, *t*-butyldimethylsilyl; Asp(3-OH), 3-hydroxyaspartic acid; Thr(4-Cl), 4-chlorothreonine; Dab, 2,4-diaminobutyric acid; Dhb, dehydro-2-aminobutyric acid; aThr, allothreonine; 2D NMR, two-dimensional nuclear magnetic resonance; NOE, nuclear Overhauser effect; TPPI, time proportional phase increment; FID, free induction decay; NOESY, nuclear Overhauser effect correlated spectroscopy; TOCSY, total correlated spectroscopy.

This paper is dedicated to the memory of our much missed colleague and friend Gianpaolo Nitti.

2. Materials and methods

2.1. Microbiological methods

The *P. syringae* MSU 16H strain is an elm tree acclimated transposon (Tn 903) generated mutant of the wild type strain MSU 174. It is equivalent to MSU strain 206 previously described in [2]. It was grown in still culture under conditions reported in [5]. Antifungal activity was assayed with *Rhodotorula pilimanae* [9].

2.2. Preparation of pseudomycins

After 9–10 days growth at 25°C, the bioactive metabolites were extracted and partially purified according to Bidwai et al. [10], and finally fractionated by reverse phase HPLC on an Aquapore RP300 column (4.6 × 250 mm, 7 μm ID, Applied Biosystems) using a Beckman System Gold 126 instrument under conditions described in [11]. Individual peaks were freeze-dried, quantitated by amino acid analysis after HCl hydrolysis, and assayed for activity toward *R. pilimanae*.

2.3. Analytical methods

Amino acids were analyzed as reported in [11], except that an Eppendorf-Biotronik LC 3000 analyzer was used; some analyses were also performed by GC-MS after transformation into TBDMS derivatives [12]. The chirality of amino acid residues was determined by Marfey's method [13]. Peptide sequences were obtained by automated Edman degradation using an Applied Biosystems model 476A sequencer. Samples were spotted on Problott membrane (Applied Biosystems) and sequenced with a Blott Cartridge (Applied Biosystems).

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

NMR spectra were run at 27°C on a Bruker AMX600 instrument operating at 600.13 MHz. Samples (1 mg) were dissolved in 0.8 ml of either D_2O, or H_2O/D_2O 90:10, at pH 4.8. 2D NMR experiments were performed in the phase-sensitive mode with TPPI phase cycle [14] typically using 2K of memory for 512 increments. The number of scans were optimized in order to obtain a satisfactory signal-to-noise ratio. Total Correlation experiments (TOCSY) were performed using the MLEV-17 spinlock composite pulse sequence [15,16] with a typical mixing time ranging from 60 to 120 ms (relayed) in order to observe either direct or remote connectivities. NOE dipolar correlated 2D spectra were obtained using the NOESY pulse sequence [17]. The mixing

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time for the magnetization exchange ranged from 60 to 220 ms. Data were processed on a microVax II graphics workstation by the 'TRITON' 2D NMR software of R. Boelsen and G. Vuister, kindly provided by Prof. R. Kaptein of Utrecht University. FIDs were weighted by a sinebell apodization function shifted typically $\pi/3$ in both dimensions. In all homonuclear 2D experiments, a matrix 1,024 × 1,024 in the phase-sensitive mode was thus obtained with a digital resolution of ≅5 Hz/point. A baseline correction was carried out in both dimensions using a polynomial fit. ^{13}C-^{1}H heteronuclear correlations were obtained in the reverse-detection mode on the AMX600 Bruker instrument (1K × 512 w).

2.4. Chemical methods

The lactone ring hydrolysis was obtained by a 3 h incubation at 37°C with 50 mM ammonium bicarbonate.

2.5. Enzymatic hydrolyses

Lipodepsipeptides (250 μg) dissolved in 0.1 M ammonium bicarbonate (150 μl) pH 8.0, were incubated at 37°C for 5 h with TPCK-trypsin (Worthington Biochemicals Co.) using an enzyme/substrate ratio of 1:30. After lyophilization the hydrolysis products were fractionated by HPLC as described under 2.2. Elution was performed with a solvent gradient obtained by mixing 0.2% TFA in water with 70% acetonitrile containing 0.2% TFA, and with a flow rate of 0.8 ml/min. The main peaks were freeze-dried and the samples were analyzed by FAB-MS and Edman degradation.

2.6. Biotests

Tobacco leaf assay was performed as in [18]; the other assays were carried out as in [19].

3. Results and discussion

Reverse phase HPLC of a *P. syringae* MSU 16H extract partially purified according to Bidwai et al. [10] produced an elution pattern of the same type observed with syringomycin- and syringotoxin-producing strains (Fig. 1) [11]. Several peaks appeared in the region where the lipodepsinonapeptides are eluted, followed by two more hydrophobic peaks emerging from the column at higher acetonitrile-isopropanol concentration. FAB-MS and amino acid analyses (see below) of the substances isolated from the six more relevant peaks indicated that four corresponded to the previously described [1] pseudomycin A, B, C and D, another (C′) could be a further pseudomycin, and the sixth (D′) presumably corresponded to the minor component previously found as a contaminant of purified D and labelled D′ [1]. The amino acid composition (see below) clearly indicated that the substances isolated from peaks A, B, C and C′ were different from known *P. syringae* metabolites, while those of compounds in peaks D and D′ compared well with those of the two syringopeptins 25-A and 25-B [11]. The identity of the two compounds from peaks D and D′ with the two syringopeptins was proved by the same MH^+ values, absorbance at 280 nm, HPLC elution times, and detailed ^{1}H-NMR data (not reported); the name pseudomycin should thus be abolished for the two more hydrophobic metabolites.

The complete structure of compounds contained in peaks A, B, C and C′ was elucidated by the use of 2D NMR, FAB-MS and chemical and enzymatic degradations carried out on microquantities. Pseudomycin A, a relatively abundant component, was at first investigated. Amino acid determination, both by conventional ion-exchange chromatography and by GC-MS after derivatization with *N*-methyl-*N*-TBDMS-trifluoroacetamide [12], showed the presence of one mol each of Ser, aThr, Asp, Asp(3-OH), Thr(4-Cl), Lys, and two mol of Dab. The methods commonly used for 2D NMR studies reached the same conclusion and furthermore identified a *Z*-Dhb residue and the 3,4-dihydroxytetradecanoyl moiety; the chemical shifts and assignements of ^{1}H and ^{13}C-NMR spectra are reported in Table 1. All amino acid residues, with the exception of one Dab, had the L configuration.

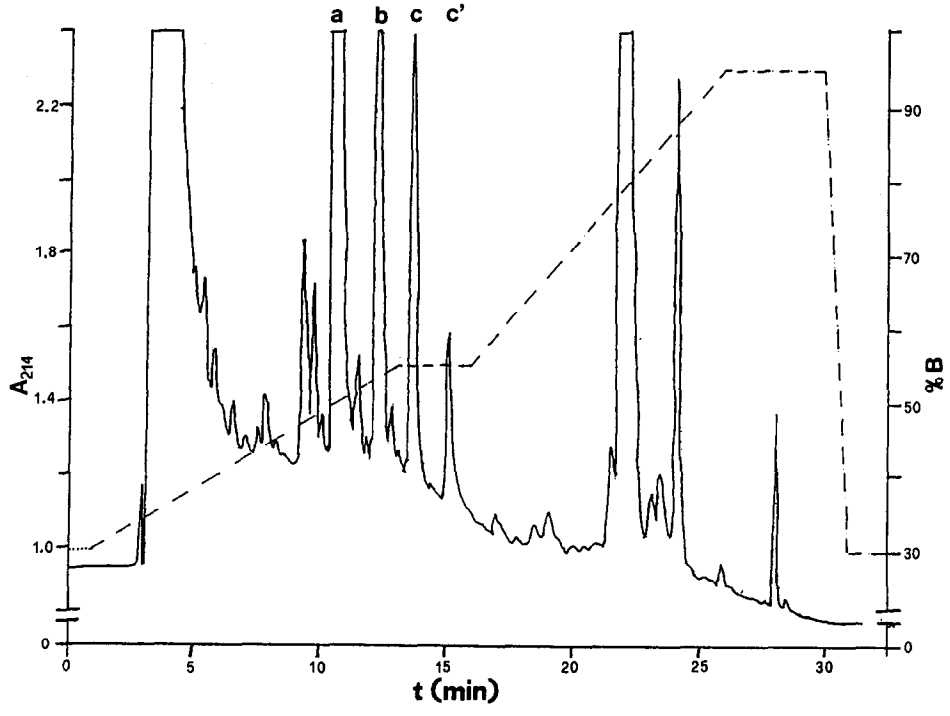

Fig. 1. Reverse-phase HPLC of the metabolites from *Pseudomonas syringae* strain MSU 16H. The letters A, B, C and C′ indicate the pseudomycins; the two main hydrophobic peaks are SP_{25}-A and SP_{25}-B.

The possibility that pseudomycin A is a new lipodepsinonapeptide got support from the occurrence in the molecule of nine amino acid residues and a long chain hydroxyacyl, from the difference of 18 mass units between the calculated sum of the residues and the molecular weight found by FAB-MS (MH^+ 1,223–1,225; the doublet indicates the presence of one chlorine atom), from the observed addition of one mol of water (MH^+ 1,241–1,243) by treatment with ammonium bicarbonate (followed by substitution of chlorine with OH: MH^+ 1,223 singlet), and from the absence of a free N-terminus. FAB-MS of a sample treated with ammonium bicarbonate produced a fragmentation pattern in agreement with the following partial sequence: Asp-Lys-Dab-aThr-Dhb-Asp(3-OH)-Thr(4-OH), where Thr(4-OH) arises from Thr(4-Cl) at basic pH. The occurrence of an L-lysine residue prompted us to cleave pseudomycin A by trypsin. After incubation with the enzyme, the solution was fractionated by reverse phase HPLC. The fragment present in the main hydrophilic peak corresponded to the C-terminal part of the molecule, as ascertained by automated Edman degradation which yielded a Dab residue on the first cycle and a Thr residue on the second; the sequence determination was stopped in correspondence of the dehydro-amino acid [20]. Treatment of the same fragment by a modified Marfey's procedure [13] allowed us to assign the L-configuration to the Dab residue adjacent to Lys and consequently the D-configuration to the other Dab residue. The FAB-MS spectrum of a prominent more hydrophobic peak showed the pseudomolecular ion (MH^+ 691) expected for the rest of the molecule, namely for 3,4-dihydroxytetradecanoyl-(Dab,Ser)-Asp-Lys-OH. The complete sequence of the tetrapeptide moiety and the site of acylation emerged from NMR spectra of pseudomycin A (see Table 1). The chirality of carbons 3 and 4 of the fatty acid moiety has not yet been determined. The otherwise complete structure of pseudomycin A is reported in Fig. 2, where the arrows indicate the short range strong NOE contacts which have allowed us to elucidate, independently from the chemical approach, the amino acid sequence, as well as the position and the type of closure of the macro ring. A number of long range NOE contacts have also been identified; these proximities, together with available information about the chirality of the amino acid residues, are prerequisites for the determination of the solution structure of this molecule.

Pseudomycins B (MH^+ 1,207–1,209), C (MH^+ 1,251–1,253) and C′ (MH^+ 1,235–1,237) are closely related to pseudomycin A. In fact, amino acid composition, and fragmentation observed in the FAB-MS spectra after lactone opening with ammonium bicarbonate gave identical results for all four pseu-

Table 1
^1H and ^{13}C chemical shifts and relative assignments for pseudomycin A.

δ ppm ^1H	Assignment	Residue	δ ppm ^{13}C observed	δ ppm ^{13}C literature
2.58	2 CH	Fatty acid	38.5	43.9 (20)
2.53	2′ CH		38.5	43.9 (20)
3.95	3 CH		72.2	69.7 (20)
3.63	4 CH		74.6	69.7 (20)
1.63	5′ CH		32.5	37.8 (20)
1.57	5 CH		32.5	37.8 (20)
1.46	6 CH		25.5	26.2 (20)
1.43	6′ CH		25.5	26.2 (20)
1.35	7-11 CH		29.3	29.9 (20)
1.35	12 CH		32.2	32.4 (20)
1.33	13 CH		22.7	14.3 (20)
0.9	14 CH		14.3	14.3 (20)
8.90	NH	Ser	/	/
4.76	α CH	Ser	58.1	56.9 (21)
4.39	β CH	Ser	61.8	60.8 (21)
8.83	NH	Dab 1	/	/
4.32	α CH	Dab 1	52.3	52.7 (20)
2.25	β CH	Dab 1	28.2	29.2 (20)
3.22	γ CH	Dab 1	37.5	37.9 (20)
3.15	γ′ CH	Dab 1	37.5	37.9 (20)
8.46	NH	Asp	/	/
4.22	α CH	Asp	55.3	52.7 (21)
2.87	β′ CH	Asp	37.0	37.1 (21)
2.73	β CH	Asp	37.0	37.1 (21)
8.36	NH	Lys	/	/
4.15	α CH	Lys	55.4	54.8 (21)
2.02	β CH	Lys	28.2	30.2 (21)
1.36	γ CH	Lys	27.0	21.9 (21)
1.24	γ′ CH	Lys	27.0	21.9 (21)
1.71	δ CH	Lys	23.0	26.7 (21)
2.99	ε CH	Lys	39.8	39.5 (21)
9.19	NH	Dab 2	/	/
4.59	α CH	Dab 2	51.7	52.7 (20)
2.33	β CH	Dab 2	29.8	29.2 (20)
2.18	β′ CH	Dab 2	29.8	29.2 (20)
3.15	γ CH	Dab 2	37.2	37.9 (20)
8.48	NH	Thr	/	/
4.20	α CH	Thr	61.7	61.0 (21)
4.10	β CH	Thr	66.8	66.6 (21)
1.35	γ CH	Thr	20.3	20.0 (21)
9.65	NH	Dhb	/	/
6.87	β CH	Dhb	129	134 (20)
1.77	γ CH	Dhb	13.6	13.3 (20)
7.85	NH	OHAsp	/	/
5.02	α CH	OHAsp	57.5	57.0 (20)
4.83	β CH	OHAsp	73.5	72.3 (20)
8.72	NH	ClThr	/	/
5.14	α CH	ClThr	55.3	55.8 (20)
4.53	β CH	ClThr	72.1	72.3 (20)
3.62	γ′ CH	ClThr	45.2	45.8 (20)
3.55	γ CH	ClThr	45.2	45.8 (20)

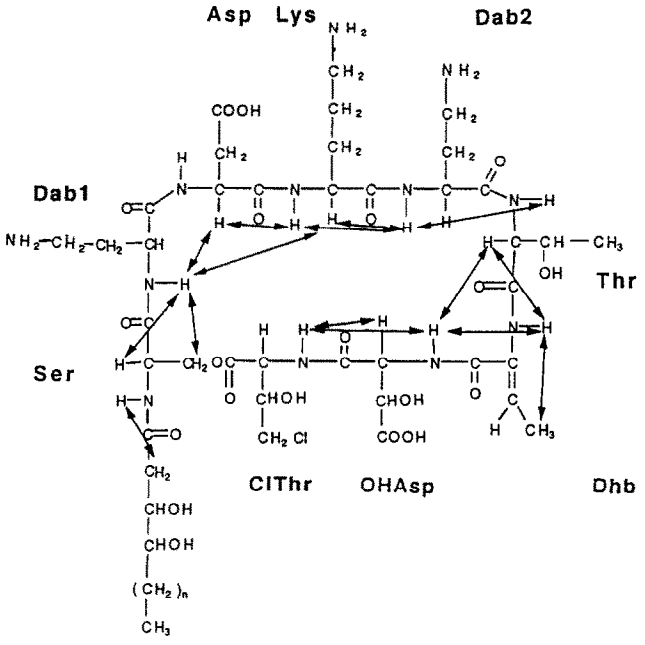

Fig. 2. Chemical structure of Pseudomycins A ($n = 9$) and C ($n = 11$). Only the short range NOE contacts have been reported.

Table 2
Structure of pseudomycins (PSs), syringostatins (SSs), syringotoxin (ST) and syringomycins (SRs).

$Me(CH_2)_n$-CH(X)-CH(OH)-CH_2-CO-L-Ser-aa_2-aa_3-aa_4-aa_5-aa_6-Z-Dhb-L-Asp(3-OH)-L-Thr(4-Cl)

	n	X	aa_2	aa_3	aa_4	aa_5	aa_6
PSs A, C	9.11	OH	D-Dab	L-Asp	L-Lys	L-Dab	L-αThr
PSs B, C	9.11	H					
SSs	7.9	H, OH	D-Dab	L-Dab	D-Hse	L-Orn	L-αThr
ST	9	H	D-Dab	Gly	D-Hse	L-Orn	L-αThr
SRs	5,7,9	H	D-Ser	D-Dab	L-Dab	L-Arg	L-Phe

domycins. Thus, very likely they differ only for the long chain acyl group; their MH^+ values suggest that the nonapeptide moiety is acylated in pseudomycin B by 3-monohydroxytetradecanoate, in pseudomycin C by 3,4-dihydroxyhexadecanoate, and in pseudomycin C′ by 3-monohydroxyhexadecanoate. The position of the four pseudomycins in the reverse phase HPLC elution pattern is consistent with an increased hydrophobicity on passing from A to C′.

As compared to the known lipodepsinonapeptides from *P. syringae* pv. *syringae* (Table 2), the pseudomycins have: (i) the same variety of fatty acids (3-hydroxy and 3,4-dihydroxy) previously found in syringostatins, except that some have a longer aliphatic chain (C_{16}); (ii) an identical N-terminal residue (L-Ser), *N*-acylated by the fatty acid and *O*-acylated by the terminal carboxyl group; (iii) the same C-terminal tripeptide [Dhb-L- Asp(3-OH)-L-Thr(4-Cl)] with the carboxy group closing the lactone ring; (iv) a D-amino acid residue in the second position, similarly to all so far described congeners obtained for isolates of *P. syringae* pv. *syringae* [21]; (v) the third and the fourth residues with the L-configuration, while in their congeners either one or the other has the D-configuration [21]; (vi) the fifth residue correspondent to a basic L-amino acid residue (Dab), as found in syringomycins (Arg) [3,4], syringotoxin (Orn) [5,6], syringostatins (Orn) [7]. The occurrence of L-Asp in the peptide moiety of the pseudomycins is a novel feature of the lipodepsinonapeptides which might affect their conformation and biological properties.

Table 3
A comparison between some biological activities of 20 μM syringomycin-E (SR) and 20 μM pseudomycin A (PS).

	SR	PS
Induction of necrosis in tobacco leaves	+ [18]	+
Inhibition of proton extrusion promoted by fusicoccin in maize roots	++ [19]	+
Stimulation of ATP hydrolysis in right-side-out plasma membrane vesicles from maize roots	+ [19,24]	++
Inhibition of ATP hydrolysis in inside-out plasma membrane vesicles from maize roots	++ [19,24]	±
Inhibition of proton translocation in inside-out plasma membrane vesicles from maize roots	+ [19]	±
Dissipation of the pH gradient in inside-out plasma membrane vesicles from maize roots	++ [19]	±

Preliminary data (Table 3) on the phytotoxicity of pseudomycin A, and on its activity in vitro and in vivo towards some fundamental processes operative at the level of the plant plasma membrane, indicate that the new lipodepsipeptide has a behaviour very similar to that of syringomycin E. At the same molar concentration the pseudomycin is less active than the syringomycin, with the exception of a higher efficiency in stimulating ATP hydrolysis in right-side-out plasma membrane vesicles; this result is compatible with the weaker inhibition of ATP-ase activity.

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