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Biochimica et Biophysica Acta 1597 (2002) 81–89

BIOCHIMICA ET BIOPHYSICA ACTA
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A new syringopeptin produced by bean strains of *Pseudomonas syringae* pv. *syringae*☆

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Received 16 October 2001; received in revised form 5 February 2002; accepted 5 February 2002

Abstract

Two strains (B728a and Y37) of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae* isolated from bean (*Phaseolus vulgaris*) plants were shown to produce in culture both syringomycin, a lipodepsinonapeptide secreted by the majority of the strains of the bacterium, and a new form of syringopeptin, SP_{22}Phv. The structure of the latter metabolite was elucidated by the combined use of mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy and chemical procedures. Comparative phytotoxic and antimicrobial assays showed that SP_{22}Phv did not differ substantially from the previously characterized syringopeptin 22 (SP_{22}) as far as toxicity to plants was concerned, but was less active in inhibiting the growth of the test fungi *Rhodotorula pilimanae* and *Geotrichum candidum* and of the Gram-positive bacterium *Bacillus megaterium*. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Pseudomonas syringae pv. *syringae*; Lipodepsipeptide; Syringopeptin 22 Phv; Phytotoxin; Structure

1. Introduction

Pseudomonas syringae van Hall pv. *syringae* is a Gram-negative plant-pathogenic bacterium with a very wide host range, which includes economically important crops such as wheat, rice, beans, clover, stone fruits and citrus [1].

The hallmark of the secondary metabolism of this organism, which has been investigated in some of its strains, is the production of two classes of lipodepsipeptides (LDPs), syringopeptins (SPs) and lipodepsinonapeptides [2]. The first class includes compounds whose peptide moiety is composed of 25 or 22 amino acid residues [2]. Syringopeptin 25 (SP_{25}) occurs in two isoforms differing in the structure of the C-terminal amino acid, which is tyrosine in SP_{25} produced by strains isolated from several plants [3], and phenylalanine in [Phe^{25}]-SP_{25} produced by a strain isolated from laurel [4]. Syringopeptin 22 (SP_{22}) also occurs in two isoforms which differ in two amino acid residues: SP_{22} produced by strains from several hosts [3], and SP (SC) produced by sugar cane isolates [5]. In culture, each strain of *P. syringae* pv. *syringae* was found to produce a single type of SP together with one, rarely two [6], members of the class of lipodepsinonapeptides, for example, syringomycins (SRs) [7,8], syringotoxin [9], syringostatin [8] or pseudomycins [10].

SPs contain several non-canonical residues: D- and L-2,4-diaminobutyric acid (Dab), and dehydrobutyrine (Dhb), which are conserved in all the LDPs produced by the bacterium, as well as one *allo*-threonine and several

☆ Paper dedicated to the memory of Prof. Giacomino Randazzo, University of Naples, Italy.

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0167-4838/02/$ - see front matter
PII: S0167-4838(02)00283-2

alanine and valine residues of D-configuration. Both SP_{25} and SP_{22} are secreted as pairs of homologues, designated as A and B (SP_{25}A, SP_{25}B, SP_{22}A, SP_{22}B), which differ in the length of the lipid moiety, a 3-hydroxylated fatty acid chain containing 10 (form A) or 12 (form B) carbon atoms, linked by an amide bond to the N-terminal residue of each peptide.

It was shown that *P. syringae* LDPs are synthesized by a non-ribosomal mechanism of peptide synthesis [11,12] and the origin of four non-canonical amino acids was determined [13]. In this biosynthetic pathway, common to the majority of microbial peptide secondary metabolites, the construction of the peptide molecule is catalyzed by multifunctional enzymes called peptide synthetases, organized in a modular fashion [14]. Each module is responsible for activating and incorporating a single substrate residue of the final peptide product. The modules interact in ordered fashion to generate the peptide product by stepwise incorporation of the thioesterified residues in a series of amino- to carboxy-terminal directed transpeptidations.

The role of *P. syringae* LDPs in the plant–pathogen interaction was investigated mainly by a molecular genetic approach. Evaluation of pathogenicity and virulence of mutant strains impaired in the production of either SR alone or of both SR and SP, showed that LDPs enhance bacterial virulence [15,16] and that their synthesis can substantially increase disease severity. Detection of both SRs and SPs in infected plant tissues provided further evidence of their role in disease development [17–19].

Individual LDPs show various degrees of toxicity to plant and animal cells and display a wide antibiotic spectrum. As far as SPs are concerned, they are potent necrosis-inducing phytotoxins, which in nanomolar concentrations cause cell damage and necrotic lesions on host, and non-host plants [20,21]. The lesions are similar to those observed on plants naturally infected with the producing bacterium. Moreover, SPs also inhibit the growth of many microorganisms, in particular Gram-positive bacteria (e.g. *Bacillus megaterium* de Bary) and plant pathogenic fungi [e.g. *Botrytis cinerea* (de Bary) Whetzel] [21].

A number of investigations on the mode of action of these metabolites showed that the primary site of their action is the cell membrane [2]. This was demonstrated in studies on natural membranes such as the plasma membrane of maize [22], isolated plant mitochondria [23], tobacco protoplasts and erythrocytes [24]. In particular, detailed investigations on the mechanism of the membrane interaction carried out on artificial model membranes [24–27], showed that LDPs formed pores and ion channels freely permeable by divalent cations. The amphipathic character of these molecules, which is also at the basis of their surfactant properties [24], facilitates their insertion into the lipid bilayers. It is reasonable to assume that this process, leading the disruption of membrane integrity and functions, is at the basis of the observed toxicity of LDPs on different cell types.

Strain B728a of *P. s.* pv. *syringae*, isolated from snap bean (*Phaseolus vulgaris* L.) in USA (Wisconsin) has been used in several investigations on epiphytic competence, pathogenicity and virulence determinants [28–31]. It was assumed to produce SR on the basis of its antifungal activity which is, however, characteristic of all *P. syringae* pv. *syringae* lipodepsinonapeptides. Preliminary chemical investigations on LDPs from the strain B728a showed that it produced SR (Grgurina, unpublished observation) and two previously unrecorded SP homologues containing 22 amino acids, whose structure was studied by chemical degradation and mass spectrometric methods [32]. Two more strains, Y27 and Y37, isolated from bean plants in USA (Wisconsin), were used both in epidemiological and toxicological studies [33]. Their metabolites were recently detected and quantified in cultures of in vitro grown bacteria by mass spectrometry (MS) [6] and also in plants, by immunoassay [19]. In this paper, the structure of the new SP produced by two bean strains (B728 and Y37) of *P. syringae* pv. *syringae*, termed SP_{22}Phv (Phv from *Phaseolus vulgaris*) is elucidated by a combination of MS and nuclear magnetic resonance (NMR) techniques, as well as by chemical degradation studies. Some biological activities of this compound are also reported.

2. Materials and methods

2.1. Bacterial strains and culture conditions

P. syringae pv. *syringae* strains B728a and Y37 were grown in 1-l Roux bottles containing 150 ml of IMM medium [34]. The cultures were incubated at 25 °C for 9 days. Standard methods were used to culture *Geotrichum candidum* Link strain F-260, *Rhodotorula pilimanae* Hedrick et Burke and *B. megaterium* [20] for the antimicrobial assays.

2.2. Purification and quantification of LDPs

The LDPs were isolated from culture filtrates and fractionated by reverse phase high-performance liquid chromatography (RP-HPLC) on an Aquapore RP 300 column (4.6 × 250 mm, 7, Applied Biosystems, Brownlee, USA), using a Beckman System Gold 126 instrument under conditions described in Ref. [35]. The collected chromatographic fractions were lyophilized. SP concentration was assessed spectrophotometrically [13].

2.3. Chemical procedures

Amino acid analyses were performed with an LKB 4151 Alpha plus automatic analyser on samples hydrolysed with 6 N HCl at 110 °C, for 24 h, in vacuo. Derivatization of the hydrolysis mixture with 2,4-dinitrophenyl hydrazine was performed as described in Ref. [36]. A sample obtained from the total hydrolysis of SP_{22}PhvA was treated with 0.2% 2,4-dinitrophenylhydrazine in 2 N HCl at 45 °C for 30 min. After water evaporation, the residue was dissolved in H_2O:CH_3CN (7:3, v/v), centrifuged at 12,000 × *g* for 15 min and analysed by HPLC using the solvent gradient system described in Ref. [35]. The peaks were detected at 360 nm. Hydrolysis of the lactone was obtained by overnight incubation with 6.5% aqueous triethylamine (pH 9) at 25 °C. Partial acid hydrolysis of SP_{22}PhvA was performed with 60 mM HCl at 110 °C. Samples were withdrawn after 1 and 3 h and subsequently analyzed by fast atom bombardment mass spectrometry (FAB-MS).

2.4. Mass spectrometry

LDPs were identified by coupling HPLC with an API-100 single quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Canada) equipped with an atmospheric pressure ionization source. Separation was carried out using an Aquapore RP300 i.d. column (2.1 mm, internal diameter), and a flow rate of 0.2 ml/min to enhance sensitivity and to facilitate the coupling with the mass spectrometer, as described in Ref. [6]. The eluate was monitored at 220 nm and one fourth of it (50 μl) split into the ion source, and also by measuring total ion current (TIC). A probe voltage of 4.8 kV and a declustering potential of 70 V were used. The instrument mass-to-charge ratio scale was calibrated with the ions of the ammonium adducts of polypropylene glycol. Acquisition was made in the positive ion mode with a dwell time of 0.5 ms and a step size of 0.5 amu. Each scan was acquired from 500 to 2500 amu.

The FAB-MS measurements were carried out on a Micromass-ZAB 2SE double-focusing mass spectrometer equipped with a caesium gun operating at 25 kV (2 μA). Mass spectral analyses were carried out by loading 2 μl of sample solution onto a glycerol:thioglycerol coated probe tip. The MS/MS measurements were done on a Micromass Autospec oaTOF (orthogonal acceleration Time Of Flight) equipped with an electrospray source. Molecular ions were measured by introducing the sample into a flowing stream of 50:50 (v/v) methanol:H_2O with 1% acetic acid. The magnet was scanned from 400 to 1800 Da at 5 s per decade. Multiply charged ion series were converted to true molecular masses using the provided OPUS software. Sequence information was obtained by selecting the appropriate molecular ion and using the EBE configuration of the instrument. The molecular ion collided with methane in a collision cell floated at 200 eV below the accelerating voltage. The masses of fragment ions were measured using an orthogonally mounted time-of-flight analyser.

2.5. NMR experiments

Samples for NMR studies were prepared by dissolving approximately 1 mg of lyophilized SP_{22}PhvA either in D_2O (700 μl) without any pH correction, or in a mixed solvent consisting of H_2O (620 μl) acidified with HCl to pH 4.0 and D_2O (80 μl). NMR spectra were run at 300 K on a Bruker AMX 600 instrument operating at 600.13 MHz with *z*-gradient selection. ^{1}H NMR experiments were performed in H_2O:D_2O using a gradient water suppression [37]. To avoid the reduction of the intensity of the signals in the proximity of the water signal, a ^{1}H-NMR spectrum in D_2O was also performed with a soft presaturation of the H–O–D residual signal [38]. Two-dimensional NMR experiments [39] were performed in the phase-sensitive mode with the time proportional phase increment (TPPI) method, using a gradient water suppression. The number of scans was optimized to obtain a satisfactory signal/noise ratio. ^{1}H–^{1}H TOtal Correlation SpectroscopY (TOCSY) was performed under the following conditions: time domain 512 in F1 and 1024 in F2, data matrix size 512 × 512, relaxation delay (r.d.) 2 s, number of scans (n.s.) 80, dummy scans (d.s.) 8, mixing time 80 ms. ^{1}H–^{1}H Rotating frame Overhauser Enhancement SpectroscopY (ROESY) was performed under the following conditions: time domain 512 in F1 and 1024 in F2, data matrix size 512 × 512, r.d. 1 s, n.s. 240, d.s. 8, mixing time 80 ms.

2.6. Biological activity assays

The phytotoxic activity of SP preparations was tested on leaves of 40-day-old tobacco (*Nicotiana tabacum* L.) plants [20] grown in a chamber with an appropriate photoperiod (16 h light by Osram bulb lamps HQ 400 W). Leaves were syringe-injected through their abaxial surface with either 300 μl toxin solutions or distilled water. After injection, the plants were kept at about 23 °C. Appearance of necrotic lesions was checked for 5 days. Antimicrobial assays were performed with *B. megaterium*, *R. pilimanae* and *G. candidum* [21] using 50, 25, 12.5, 6.2, 3.1 and 1.5 μM SP solutions.

3. Results

The HPLC-MS profile of extracts from cultures of the bean strains of *P. syringae* pv. *syringae* (Fig. 1A) showed the typical pattern of *P. syringae* strains characterized by the presence of two groups of substances of different polarity. Mass spectra of the peaks eluted at 14.4 and at 19.7 min correspond to syringomycin E (MH^+ *m/z* 1225) and syringomycin G (MH^+ *m/z* 1253), two homologous forms of SR, N-acylated at the N-terminal amino acid by a 3-hydroxydodecanoyl and 3-hydroxytetradecanoyl residues, respectively. While the occurrence of SR in the Y strains had been reported in Ref. [6], this result provides the first structural evidence on the antifungal metabolite produced by the strain B728a.

In the region of the chromatogram where SPs normally elute, two peaks were observed. The MH^+ values of the corresponding substances, at *m/z* 2129 and 2157, were in the

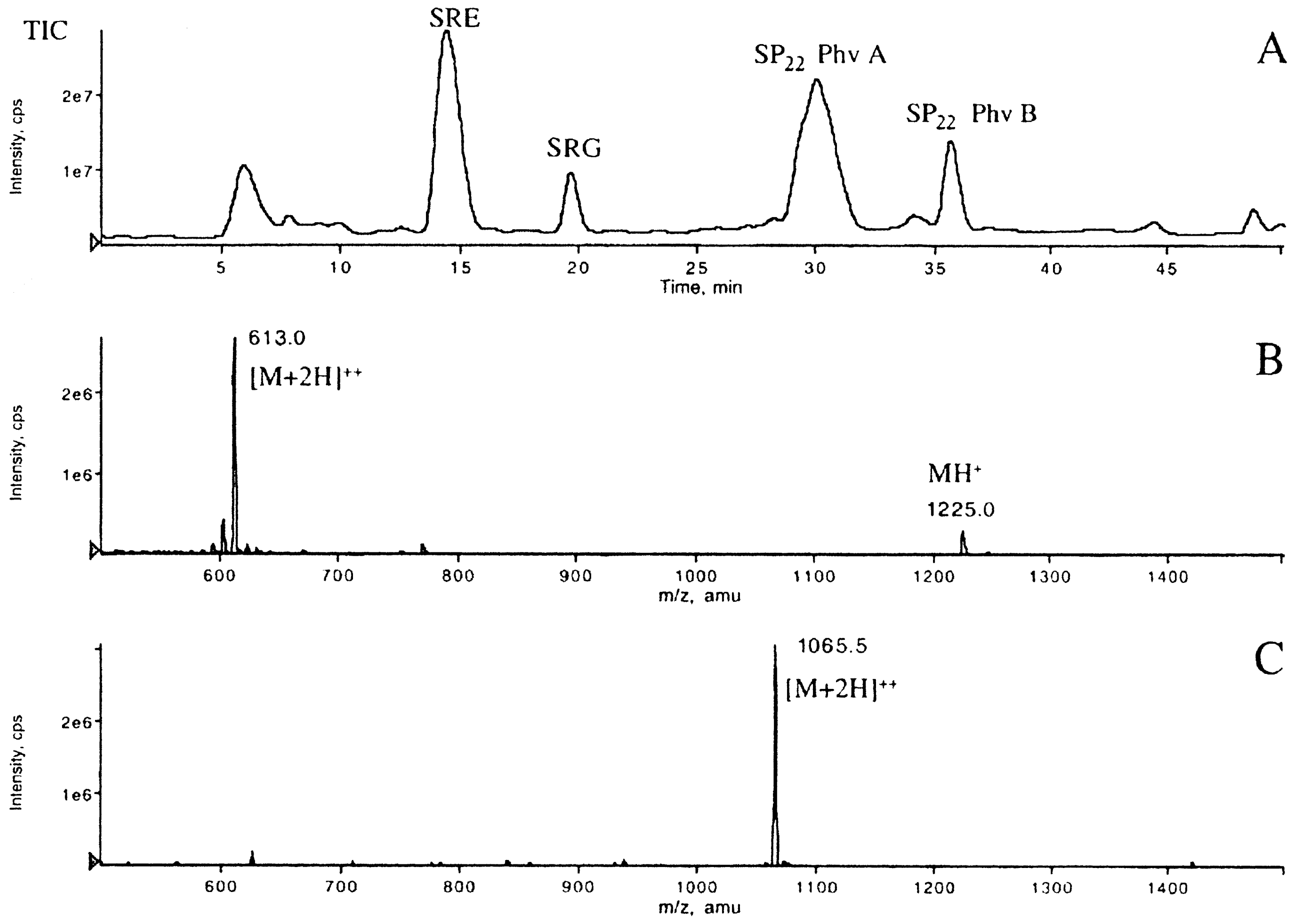

Fig. 1. Representative chromatogram of extracts of the bean strains of *P. syringae* pv. *syringae* (A); mass spectrum of SRE (B); mass spectrum of SP_{22}PhvA (C).

range of those observed for other SPs, but did not match any of the known values. The difference of 28 atomic mass unit (amu), compatible with two methylene units, suggested the presence of a pair of homologous SPs, forms A and B, differing in the length of the acyl moiety. Fig. 1B and C shows the mass spectra of SRE, and of the more abundant form of the new SP (doubly charged ion), respectively. This form was used for structural studies.

The results of the quantitative amino acid analysis: Pro (1), Val (3), Ala (8), Thr (1), Ser (1), Tyr (1), Dab (2) and Leu (1), evidenced the predominance of hydrophobic residues and a close similarity with the known SPs. To show the

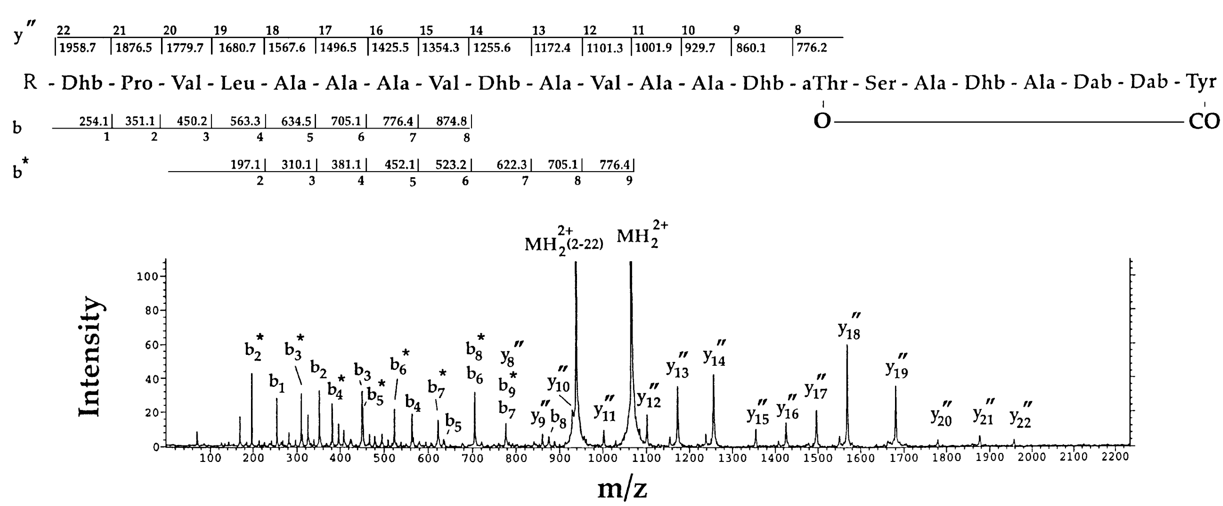

Fig. 2. Tandem MS/MS spectrum of SPA_{22}PhvA.

Table 1
FAB-mapping of the partial acid hydrolysate of syringopeptin 22 Phv produced by the bean isolate of *P. s.* pv. *syringae* (R: 3-hydroxydecanoyl)

Peptide	MH^+	Residues
R-Dhb-Pro-Val-Leu-Ala-Ala-Ala-Val-Dhb-Ala-Val-Ala-Ala-Dhb-Thr-Ser-Ala-Dhb-Ala-Dab-Dab-Tyr-OH	2147	
R-Dhb-Pro-Val-Leu-Ala-Ala-Ala-Val-Dhb-Ala-Val-Ala-Ala-Dhb-Thr-Ser-Ala-Dhb-Ala-Dab-Dab-OH	1984	
Dhb-Pro-Val-Leu-Ala-Ala-Ala-Val-Dhb-Ala-Val-Ala-Ala-Dhb-Thr-Ser-Ala-Dhb-Ala-Dab-Dab-Tyr	1960	
O————————CO		
Pro-Val-Leu-Ala-Ala-Ala-Val-Dhb-Ala-Val-Ala-Ala-Dhb-Thr-Ser-Ala-Dhb-Ala-Dab-Dab-Tyr	1876	2-22
O————————CO		
Pro-Val-Leu-Ala-Ala-Ala-Val-Dhb-Ala-Val-Ala-Ala-Dhb-Thr-Ser-Ala-OH	1377	2-17
Pro-Val-Leu-Ala-Ala-Ala-Val-Dhb-Ala-Val-Ala-Ala-Dhb-Thr-OH	1219	2-15
Pro-Val-Leu-Ala-Ala-Ala-Val-Dhb-Ala-Val-Ala-Ala-OH	1035	2-13
Pro-Val-Leu-Ala-Ala-Ala-Val-Dhb-Ala-Val-Ala-OH	964	2-12
Pro-Val-Leu-Ala-Ala-Ala-Val-Dhb-Ala-Val-OH	893	2-11
Pro-Val-Leu-Ala-Ala-Ala-Val-Dhb-Ala-OH	794	2-10
Pro-Val-Leu-Ala-Ala-Ala-Val-Dhb-OH	723	2-9
Pro-Val-Leu-Ala-Ala-Ala-Val-OH	640	2-8
Pro-Val-Leu-Ala-Ala-Ala-OH	541	2-7
Pro-Val-Leu-Ala-Ala-OH	470	2-6
Pro-Val-Leu-Ala-OH	399	2-5
Pro-Val-Leu-OH	328	2-4
Ala-Dhb-Ala-Dab-Dab-Tyr-OH	607	17-22
Dab-Tyr-OH	282	21-22

Table 2
Assignments of ^1H resonances of syringopeptin SP_{22}PhvA in H_20/D_2O 4:1 by vol., pH 4.0, *T* 300 °K

Fatty acid	Chemical shift (ppm)/signal multiplicity					
	C2	C3	C4	C5	C6–C9	C10
	2.574 (dd, 14.8, 5.2) 2.531 (dd, 14.8, 8.4)	4.084	1.512 (q, 7.4)	1.385	1.27	0.842 (t, 7.6)
Amino acid	**Chemical shift (ppm)/signal multiplicity**					
	NH	Cα	Cβ,β′	Cγ,γ′	Cδ,δ′	
Dhb1	9.598 (s)		5.887 (d, 7.2)	1.762 (d, 7.2)		
Pro2		4.470 (t, 6.0)	2.316	1.889	3.638	
Val3	8.005	4.064 (t, 7.9)	2.133	0.955 (d, 7.1) 0.924 (d, 6.2)		
Leu4	8.386 (d, 6.9)	4.323	1.651	1.593	0.903 (d, 6.4), 0.861 (d, 6.4)	
Ala5	8.161 (d, 5.7)	4.248	1.375 (d, 7.0)			
Ala6	8.065 (d, 5.7)	4.240	1.368 (d, 7.6)			
Ala7	8.142 (d, 6.2)	4.327	1.386 (d, 7.1)			
Val8	8.088 (d, 6.8)	4.103	2.153	1.026 (d, 7.0)		
Dhb9	9.437 (s)	6.646 (q, 7.4)	1.754 (d, 7.4)			
Ala10	7.821 (d, 6.0)	4.366 (d, 8.0)	1.381			
Val11I	8.010 (t, 7.5)	4.052 (d, 6.6)	2.054	0.936		
Ala12	8.456 (d, 6.0)	4.346	1.406 (d, 7.2)			
Ala13	8.321	4.344	1.474 (d, 7.6)			
Dhb14	9.392 (s)	6.747 (q, 7.4)	1.754 (d, 7.4)			
Thr15	7.976	4.540 (d, 9.0)	5.160	5.160	1.209 (d, 6.2)	
Ser16	8.423	4.511 (t, 4.4)	3.990 (dd, 11.6, 4.4), 3.833 (dd, 11.6, 4.4)			
Ala17	8.311	4.125	1.474 (d, 7.6)			
Dhb18	9.405 (s)	6.423 (q, 7.4)	1.738 (d, 7.4)			
Ala19	8.428	4.244	1.456 (d, 7.8)			
Dab20	8.249 (d, 7.1)	4.289	2.133	3.070 (t, 7.6)	(NH_2) 7.70 (bs)	
Dab21	7.980	4.361	2.094, 2.038	2.983, 2.963	(NH_2) 7.62 (bs)	
Tyr22	8.015 (dd, 14.5, 6.0)	4.656	3.040 (d, 8.4), 2.947 (d, 8.4)	(o) 7.037 (d, 8.4)	(m) 6.833	

Chemical shift values (ppm) are referred to a small trace of acetone (δ_{CH_3} 2.220 ppm) present in the sample. Signal multiplicity and J_{H-H} (Hz) are reported in parentheses.

presence and the nature of the unsaturated amino acid residues, which undergo deamination to 2-oxoacids upon heating under acidic conditions [40], the hydrolysis mixture was treated with 2,4-dinitrophenylhydrazine and analysed by RP-HPLC. The retention time of the 2,4-dinitrophenylhydrazone derivative matched that of an authentic sample of 2,4-dinitrophenylhydrazone of 2-oxobutyric acid, indicating that the unsaturated residue was Dhb (data not shown). Mild basic hydrolysis yielded a compound whose MH^+ 2147 was compatible with that of the product of a lactone ring opening. The above data are consistent with SP containing 22 amino acids, differing from SP_{22} in that the two Val residues are replaced by Leu and Ala. The new compound was termed SP_{22}Phv.

The amino acid sequence of SP_{22}PhvA was determined by tandem mass spectrometry (MS/MS) and the FAB mapping of the partial acid hydrolysate and confirmed by two-dimensional nuclear magnetic resonance spectroscopy (2D-NMR). Fragmentation of MH^+ did not produce ions adequate for structural analysis. However, the degradation of the doubly charged ion, the strongest signal in the spectrum, allowed to obtain partial information on the sequence of the peptide. As shown in Fig. 2, the higher part of the spectrum ($m/z > 750$), contained y″-type fragments, whereas in the lower part, b- and b*-type ions were observed. The b*-type ions are generated after the initial cleavage of the peptide bond between Dhb and Pro, as deduced from the value of the doubly charged parent ion (2–22) observed at *m/z* 938.8. Analogous fragmentation was also observed in the spectrum of SP(SC)-2 by Isogai et al. [5] The presence of the 3-hydroxydecanoyl moiety, the N-terminal blocking group in all SPs, could be inferred from the values of the b-type fragments. Comparison between the MS/MS data presented in this study and those reported in the literature [5] relative to SP_{22}A and SP(SC)-2, evidenced the high similarity between the three spectra and also allowed to localize the variant residues. The C-terminal y″-type ions up to *m/z* 1172.4 (fragment y″13 corresponding to residue 10) were common to all the three spectra, suggesting the structural identity of the C-terminal portion of the three SPs 22. The *m/z* value of fragment y″14, 1255.6, matched the corresponding fragment in SP_{22}, showing that the residue 9 in both molecules was Dhb. This fragment differed in 14 amu from y″14 in SP_{22}(SC)-2, which accounted for the difference between Dhb and dehydroaminopropionic acid (Dhp). Position 7 was variable in all the three SPs: Ala in SP_{22}PhvA, Val in SP_{22}A and Leu in SP_{22}(SC)-2. Here the fragment y″16 at *m/z* 1425.5 in SP_{22}PhvA differed from y″15 by 71 amu, denoting the presence of one Ala residue. The difference of 28 amu

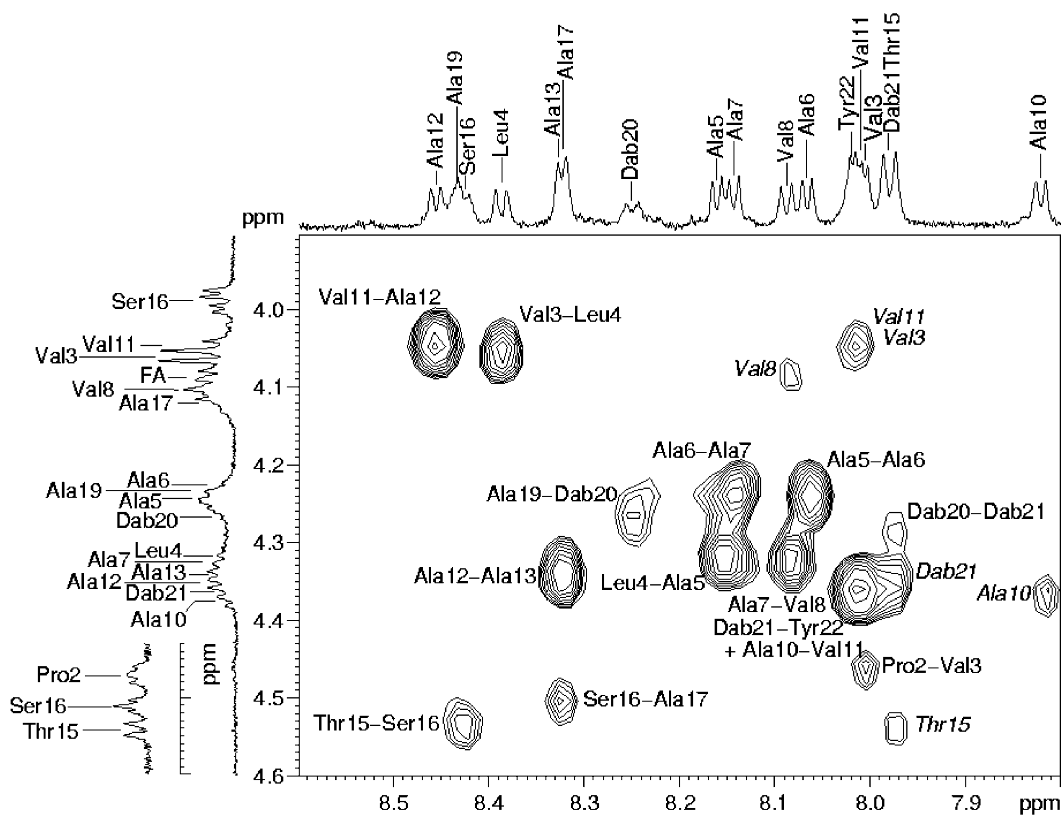

Fig. 3. Slice of $^1H-^1H$ ROESY map of SP_{22}PhvA in H_2O/D_2O by vol., pH 4.0 at 300 K. The major connections leading the amino acid sequence are highlighted. In F1 and F2 dimensions, ^{1}H-NMR spectrum in H_2O/D_2O is reported. In F1 dimension, an expansion of the 4.43- to 4.60-ppm region of the ^{1}H-NMR spectrum in D_2O is also reported.

SYRINGOPEPTIN 22 (SP_{22})

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

R-*z*Dhb-DPro-DVal-LVal-DAla-DAla-DVal-DVal-*z*Dhb-DAla-DVal-LAla-DAla-*z*Dhb-D*a*Thr-DSer-DAla-*z*Dhb-LAla-LDab-DDab-LTyr

O —————————————— CO

R: 3-hydroxydecanoyl (SP_{22}A); 3-hydroxydodecanoyl (SP_{22}B)

SYRINGOPEPTIN SC (SP[SC])

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

R-*z*Dhb-Pro-Val-Leu-Ala-Ala-Leu-Val-Dhp-Ala-Val-Ala-Ala-*z*Dhb-*a*Thr-Ser-Ala-*z*Dhb-Ala-Dab-Dab-Tyr

O —————————————— CO

R: 3-hydroxydecanoyl (SP[SC]-1); 3-hydroxydodecanoyl (SP[SC]-2)

SYRINGOPEPTIN 22 Phv (SP_{22}Phv)

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

R-zDhb-Pro-Val-Leu-Ala-Ala-Ala-Val-*z*Dhb-Ala-Val-Ala-Ala-*z*Dhb-*a*Thr-Ser-Ala-*z*Dhb-Ala-Dab-Dab-Tyr

O —————————————— CO

R: 3-hydroxydecanoyl (SP_{22}PhvA); 3-hydroxydodecanoyl (SP_{22}PhvB)

Fig. 4. Structures of SPs containing 22 amino acid residues.

between the corresponding fragments in SP_{22}A and SP(SC)-2 spectra reflected the difference between Ala (in SP_{22}PhvA) and Val (in SP_{22}A), and between Leu (in SP_{22}PhvA) and Val (in SP_{22}A), taking into account that 14 amu derived from substitution in position 9. Position 4 carried Leu in SP_{22}PhvA, as shown by the difference of 113 amu between y″19 and y″18 at *m*/*z* 1680. The corresponding site in SP_{22}A contained Val. In conclusion, residues of SP_{22}Phv differed from those of SP_{22} in the positions 4 and 7 (Leu and Ala instead of Val and Val), and from those of SP(SC) in positions 7 and 9 (Ala instead of Leu and Dhb instead of Dhp). The structural data obtained by the tandem MS/MS approach were confirmed and complemented by FAB-MS analysis of the peptides obtained by mild acid hydrolysis of SP_{22}PhvA (Table 1). In particular, the data provided information on the sequence within the lactone ring, which appeared to be conserved in the hitherto known 22 amino acids containing SPs.

The results of structural analysis performed by two-dimensional high-field ^{1}H-NMR spectroscopy were consistent with the MS data. The complete assignment of resonances resulted from the chemical shift values reported for the common amino acids and from TOCSY experiments which allowed to identify all the spin systems. In particular, in the olefinic spectral region, four quartets were assigned to the CH protons of four Dhb residues [39]. The assignment of the tyrosine aromatic protons was also straightforward. The presence of the hydroxyl group in position β of the fatty acid chain was confirmed by the TOCSY experiment. The length of the fatty acid moiety was determined by the integration of the fatty acid chain resonances. The closure of the lactone ring between the carboxyl of the Tyr and the CβHOH of the Thr residue was clearly indicated by the diagnostic downfield shift of CβH-Thr signal (5.160 ppm) in the ^{1}H-NMR spectrum [37]. The assignment of all the resonances present in the ^{1}H-NMR spectrum of SP_{22}PhvA in H_2O/D_2O is shown in Table 2. The ROESY spectrum showed the cross-peaks due to dipolar connectivities. In particular, the CαH*i*/NH($i+1$) cross-peaks (Fig. 3) allowed to obtain the amino acid sequence of the new compound, which was in agreement with the MS data. In addition, the *z*-configuration of all the Dhb residues was assigned on the basis of ROE cross-peaks between CβH-Dhb*i* and NH($i+1$). The structures of the three SPs containing 22 amino acids are shown in Fig. 4.

To evaluate the relevance of the structural differences between SP_{22} and SP_{22}Phv, the antibiotic and phytotoxic activities were compared on test organisms. The more abundant isoforms of both toxins (SP_{22}A and SP_{22}PhvA) were used in these assays. Both metabolites, tested up to 6.2 μM, displayed similar phytotoxic activity, measured as severity of necrotic lesions that formed on tobacco leaves. However, SP_{22}PhvA was less active than SP_{22}A in the antibiotic activity assays. In particular, the growth of *B. megaterium* was inhibited by 6.2 μM SP_{22}A and by 25 mM SP_{22}PhvA. The latter metabolite did not affect the growth of *R. pilimanae* and *G. candidum* up to the maximum concentration used (50 μM), whereas SP_{22}A inhibited the growth of these fungi at 12.5 and 25 μM, respectively.

4. Discussion

The structural characterization of new members of a family of secondary metabolites, not only increases the knowledge of the structural varieties of natural products, but can also be valuable for investigations into the structure–activity relationship (SAR) and the biosynthesis of the compounds under study. Even the replacement of a single amino acid residue may affect the properties of a metabolite. For example, the substitution of Thr(4Cl) by Thr in SR causes a significant decrease in antifungal activity [35]. The replacement of Val by Ala in the lipopeptide surfactin induces modifications in its arrangement at the interface, reflected in a difference in surface tension, although no conformational change is involved [41,42]. The findings of the present study indicate that the two isoforms, $SP_{22}A$ and $SP_{22}PhvA$, differ at least in their antimicrobial activity, the latter compound being less active against the Gram-positive bacterium and the two fungi tested.

The chemical approach for inducing selective structural modifications is limited by the structural complexity of the compounds, hence biological methods are often preferred. For example, the "precursor directed biosynthesis", which relies on the relatively relaxed specificity of peptide synthetases [43], allows the formation of peptide analogues by increasing the concentration of miscognate substrates in the nutrient medium. More recently, modified peptide metabolites were obtained by genetic manipulation of their biosynthetic clusters [44,45]. However, the natural occurrence of isoforms, the "ready-made" structural analogues, is still valuable for SAR investigations.

Another interesting aspect is the relationship between the type of LDPs produced by individual strains of *P. syringae* pv. *syringae* and their host plants. The data so far available show that different isolates of the same pathovar can produce the same metabolite. The question of host specialisation and differentiation between strains has been addressed mainly by genetic studies, based on hybridization with gene probes derived from genes involved in lipopeptide production. For example, an analysis of genetic fingerprint patterns of *P. syringae* pv. *syringae* strains from various hosts and geographical provenances has shown that almost all the strains isolated from stone fruit trees form a cluster distinct from most strains isolated from other hosts [46]. These findings show that at least some strains of the pathovar *syringae* have adapted to a particular host and provide further indication of host specificity within the pathovar.

It is possible to speculate that the host plant influences the biosynthetic machinery of the pathogen and, in particular, the specificity-conferring domains of the synthetase modules. Our finding that two strains of *P. syringae* pv. *syringae* isolated from bean plants, although in the same country, produced the same isoform of SP 22, supports such a hypothesis.

Acknowledgements

This work was supported by a contribution from the "Istituto Pasteur Fondazione Cenci Bolognetti" of the University of Rome "La Sapienza" (grant to I.G.) and other University "La Sapienza" grants. Financial supports of the Italian Ministry of University and Scientific and Technological Research (MURST PRIN 1997 to N.S.I) and the Italian National Research Council (CNR Target project on Biotechnology to A.G. and V.F.), by the EC project "Fondi strutturali" (104131 to A.S.) are acknowledged. Thanks are extended to Dr. K. Willis, University of Wisconsin, USA and Prof. G. Surico, University of Florence, Italy for providing the strains of *P. syringae* pv. *syringae* used in this work, to Prof. D. Barra (University of Rome "La Sapienza") for the amino acid analyses, and to Prof. A. Burlingame (University of California, San Francisco, USA) for the use of oaTOF mass spectrometer.

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