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Relevance of chlorine-substituent for the antifungal activity of syringomycin and syringotoxin, metabolites of the phytopathogenic bacterium *Pseudomonas syringae* pv. *syringae*

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Abstract. Structural analogues of syringomycin and syringotoxin were produced by fermentation, characterized by FAB-MS and amino acid analysis and compared to the parent compounds in the antibiosis test against *Rhodotorula pilimanae*. The C-terminal residue was shown to be important for the activity.
Key words. Pseudomonas syringae pv. *syringae*; syringomycin; syringotoxin; structural analogues; antibiotic activity.

Many strains of *Pseudomonas syringae* pv. *syringae*, a widespread phytopathogenic bacterium, when grown in liquid culture excrete metabolites with phytotoxic and antimicrobial activities[1,2]. The most active antibiotics syringomycins (SRs), syringotoxin (ST) and syringostatins (SSs), isolated from stone fruit and grass hosts, citrus and lilac, are lipodepsipeptides that share many structural features[3-5] (fig. 1). In fact, they all contain 2, 3 - dehydroamino - butyroyl - 3 - hydroxy - aspartyl - 4 - chloro-threonine as C-terminal tripeptide whose carboxyl closes a macrocyclic lactone on the the hydroxyl group of the N-terminal serine residue, which, in turn, is N-acylated by a 3-hydroxy fatty acid. SRE, containing 3-hydroxy dodecanoyl acyl moiety, and a minor 3-hydroxy tetradecanoyl homologue, SRG, were both isolated[6] from mixtures of metabolites produced by the strain B359. Moreover, the strains B359 and B427, which produce SRs and ST respectively, both secrete the same more hydrophobic lipodepsipeptidic metabolite, syringopeptin (SP25A), which does not contain chlorinated residues[7]. Recent work has shown that SRE and SP25A are responsible for most of the phytotoxic and antibiotic activity observed in culture broth extracts[2]. Molecular genetic studies on the role of syringomycin in the development of plant disease indicated that it is not essential for pathogenicity, but contributes significantly to the virulence[8].

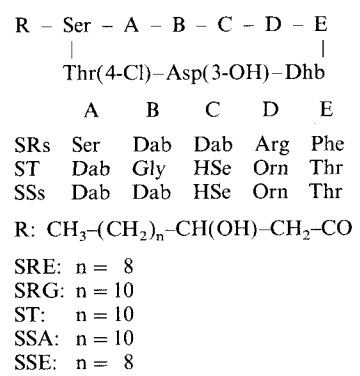

Figure 1. Structure of syringomycins, syringotoxin and syringostatins.

To gain information on molecular features important for the biological activity of syringomycin and syringotoxin, we produced analogues modified at the C-terminal residue, 4-chlorothreonine (Thr(4-Cl)), one of the structural elements common to SRs, ST and SSs. Since a selective modification by chemical methods was hampered by technical difficulties, we tried to replace the chlorine with other substituents by changing the halogenide composition of the medium. This is an approach that has been used successfully with other chlorinated metabolites[9-12].

Materials and methods

Microbiological methods. Strains B359 (SRs producer) and B427 (ST producer), were grown in stationary conditions as described[13], either on IMM medium (medium A), or in IMM medium where histidine monohydrochloride and calcium chloride were replaced by equimolar histidine base (Merck) and calcium nitrate (Sigma) (medium B), or in medium B further supplemented with sodium bromide (Sigma) (medium C) to the same concentration as the chloride in medium A (19.7 mM). The cultures were incubated for 9 d at 25 °C in 1l Roux bottles containing 150 ml of medium. The bottles were inoculated with a suspension of bacteria grown in stirred (150 r.p.m) 250 ml Erlenmeyer flasks containing 100 ml of the same medium for 36–40 h at 25 °C. The antibiotic activity of SR, ST and their derivatives, quantified by amino analysis, was determined on *Rhodotorula pilimanae* as described[2] and expressed in

U/μg. A unit of activity is the amount of toxin in a 10 μl droplet which completely inhibits growth of *R. pilimanae* in the area of application of the droplet.

Purification of lipodepsipeptides. The lipodepsipeptides were extracted and fractionated according to Bidwai[14], with some modifications: an extract obtained from 50 ml of culture broth was applied on an Amberlite XAD-2 (Fluka) column (v/w extract: resin 1:1). The column was washed with HCl acidified H_2O (pH 2.5) (200 ml) and the metabolites were eluted with MeOH:H_2O 9:1 v/v (200 ml). After evaporation of MeOH under reduced pressure, the residue was lyophilized, resuspended in water and further fractionated by reversed phase high performance liquid chromatography (RP-HPLC) on a Gold 126 Beckman System instrument in conditions previously described[2].

Analytical methods. FAB mass spectra were recorded on a VG ZAB 2SE instrument equipped with the Cesium gun operating at 25 kV, 2A. Samples were dissolved in 5% acetic acid and directly loaded onto the probe tip coated with glycerol-thioglycerol 1:1.

Amino acid analyses were carried out with a Pharmacia Alpha Plus analyzer after hydrolysis in vapour phase 6N HCl at 110°C for 24 h in vacuo, or by gas-chromatography-mass spectrometry (GC-MS) of the N, O(S)*tert*-butyl-dimethyl-silyl derivatives, as described[15]. The chirality of threonine was determined by using a modified Marfey's method[16]. The separation was carried out on a Beckman Ultrasphere octyl column (5 μm, 250 × 2.0 mm) eluted with a linear gradient from 24 to 40% of acetonitrile/2-propanol (4/1, v/v) in 40 mM triethylammonium phosphate buffer, pH 2.2, in 35 min, at a flow rate of 0.2 ml/min, using a Kontron Instruments apparatus.

Chemical methods. Hydrolysis of lactone and simultaneous substitution of bromine by hydroxyl group in Br-SRE and Br-ST (100 nm/ml) was carried out by incubation at 37 °C for 20h with 0.5 M ammonium bicarbonate. The salt was removed by exhaustive lyophilization.

Results and discussion

Mixtures of metabolites produced by growing bacteria of *P. s.* pv. *syringae* on media containing different halogenides were extracted from the culture broths, fractionated in RP-HPLC, and the single components analyzed by FAB-MS. When grown in the medium containing bromide (medium C) instead of chloride, the strain B359 produced bromo-deschloro-syringomycin (Br-SRE), together with smaller amounts of deschloro-syringomycin (H-SRE) and hydroxy-deschloro-syringomycin (OH-SRE). Brominated and hydrogen-substituted SRG analogues were also detected. Similarly, bacteria of the strain B427 in the same medium produced bromo-deschloro-syringotoxin (Br-ST) as the major antimicrobial metabolite, accompanied by deschloro-syringotoxin (H-ST) and hydroxy-deschloro-syringotoxin (OH-ST). The SR and ST deschloro and bromo-deschloro derivatives were structurally characterized by FAB-MS and amino acid analysis; the mass spectral data are summarized in table 1. The structures of 2 bromo-deschloro derivatives Br-SRE and Br-ST were further supported by the following data: the compounds resulting from the incubation of the 2 lipodepsipeptides in 0.5 M ammonium bicarbonate buffer, pH 8.5, at room temperature, overnight, when analyzed by FAB-MS, showed protonated molecular ions MH^+ at m/z 1225 and 1136, respectively. These mass values correspond to bromine free compounds, as demonstrated by the patterns of the molecular ion cluster exhibited in the mass spectra. The recorded mass signals are identical to those observed when SRE and ST were incubated under the same conditions[3,4]. It was suggested that they arise from the hydrolytic cleavage of the lactone ring followed by the replacement of the halogen atom with the hydroxyl group[3]. This result confirms that Br-SRE and Br-ST differ from the corresponding chlorinated metabolites only in the replacement of the chlorine atom with bromine in the structure of the C-terminus threonine derivative.

Table 1. FAB-MS data of syringomycins, syringotoxin and their structural analogues.

Metabolite	MH^+
SRE	1225–27
SRG	1253–55
Br-SRE	1269–71
Br-SRG	1297–99
H-SRE	1191
H-SRG	1219
OH-SRE	1207
OH-SRG	1235
ST	1136–38
Br-ST	1180–82
H-ST	1102
OH-ST	1118

In the absence of halogenide ions, strains B359 and B427 both produced primarily deschloro derivatives H-SRs and H-ST, whereas OH-SR and OH-ST were not detected under these conditions. The structure of H-SRE was further investigated since its molecular weight could, in principle, be accounted for by threonine, *allo*-threonine or homoserine as the C-terminal residue. The metabolite was submitted to 6N HCl hydrolysis and the analysis of the resulting amino acid mixture was carried out both by conventional ion exchange chromatography and by GC-MS analysis of the TBDMS amino acid derivatives[15]. In both cases, threonine was observed, whereas homoserine and *allo*-threonine were not detected. The L-configuration of this amino acid was established by a modified Marfey's procedure[6] as shown in figure 2. The same configuration had been reported for the C-terminal 4-chlorothreonine residues found in

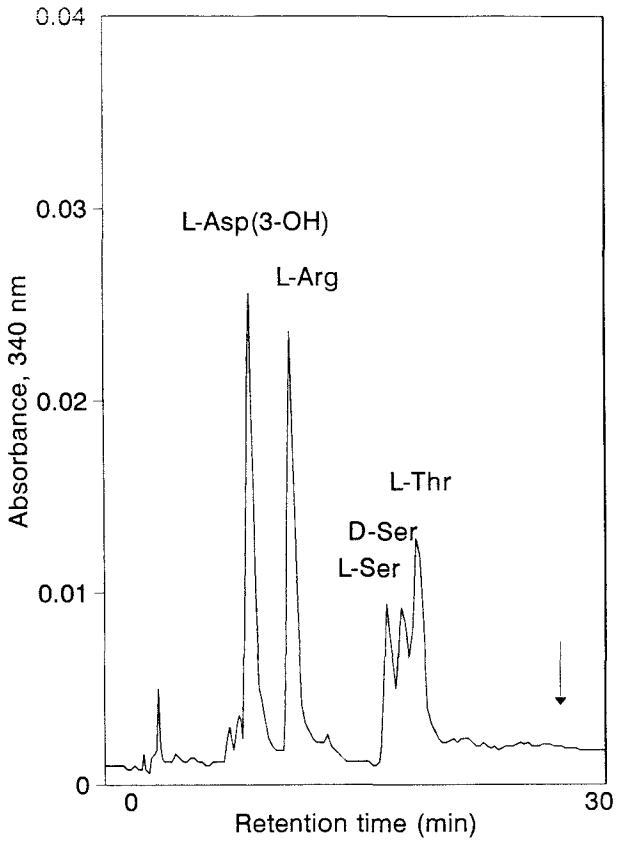

Figure 2. Partial RP-HPLC chromatogram of the H-SRE hydrolysate after derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine. The arrow indicates the expected position for the D-threonine derivative.

SRs, ST and SSs in a study of the stereochemistry of these metabolites[5].

In both media B and C, syringopeptin was produced in amounts comparable to those formed in the chloride-containing medium A.

The antifungal activity of the modified SR and ST compounds was measured on *R. pilimanae*; the results are shown in table 2. The biological activity progressively decreased from the chloride to bromo-deschloro and deschloro derivatives.

The 2 hydroxy-deschloro derivatives, OH-SRE and OH-ST did not show any detectable antifungal activity.

Table 2. Antifungal activities of syringomycin, syringotoxin and their structural analogues.

Metabolite	Specific activity (U/μg)
SRE	42
Br-SRE	21
H-SRE	11
OH-SRE	0
ST	43
Br-ST	20
H-ST	12
OH-ST	0

The results presented in this paper provide the first data on the structure-activity relationship of syringomycin and syringotoxin. The decrease in the antifungal activity observed upon substitution of chlorine by bromine and by hydrogen was expected on the basis of similar findings with other bioactive, chlorine-containing secondary metabolites, for example griseofulvin[9] and chloramphenicol[10], where replacement of chlorine with bromine resulted in a marked decrease of antibiotic activity. The hydroxy-substituted analogues of SR and ST were inactive. These results indicate that the structure of the side chain of the C-terminal amino acid plays an important role in determining the antibiotic activity of SR and ST.

Similar results could reasonably be expected in the case of syringostatins[5] since they have a structural resemblance to the above mentioned toxins, in particular to ST, from which they differ by just one residue (residue B in fig. 1).

Our present knowledge of the mode of action of these lipodepsipeptides is not enough to explain the observed results. However, the activity retained in the threonine-containing analogues shows that the Thr(4-Cl), though important, is not the only structural element responsible for the antibiotic activity.

The pattern of the metabolites detected in broth extracts obtained from growth in different media permits some speculation about how they were formed. The hydroxythreonine which appears as the C-terminal amino acid in OH-SR and OH-ST could either be an artifact due to the hydrolysis of the halogenated amino acid or could be formed by different enzymatic reactions. The origin of this amino acid by hydroxylation of homoserine was shown by Mitchell et al. in studies on the biosynthesis of rhizobitoxine, a phytotoxic metabolite from *Pseudomonas andropogonis*, a widely spread plant pathogen[17]. However, from our knowledge of the biological formation and transformation of halohydrins, other hypotheses can be advanced. Hydroxythreonine could also derive from the corresponding chlorinated metabolites via an epoxidic intermediate whose formation would be catalyzed by a halohydrin epoxidase and the hydrolytic opening by an epoxy hydrolase. Such enzymes, discovered in the course of studies on biodegradation of halogenated compounds by various organisms, were also detected in *Pseudomonas* sp.[18]. This hypothesis might be supported by the presence, among the minor components observed in the HPLC profiles of the extracts from strain B359 of *P. s.* pv. *syringae*, of a compound whose molecular weight, deduced from the protonated molecular ion peak in the FAB-MS spectrum, MH^+ 1189 (ref. 6), matches that calculated for a metabolite containing an epoxidic group at the C-terminal amino acid residue.

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