The Pyoverdins of *Pseudomonas syringae* and *Pseudomonas cichorii*

Alain Bultreys[a], Isabelle Gheysen[a], Bernard Wathelet[b], Mathias Schäfer[c], and Herbert Budzikiewicz[c,*]

[a] Département Biotechnologie, Centre Wallon de Recherches Agronomiques, B-5030 Gembloux, Belgium
[b] Unité de Chimie Biologique et Industrielle, Faculté Universitaire des Sciences Agronomiques de Gembloux, B-5030 Gembloux, Belgium
[c] Institut für Organische Chemie, Universität zu Köln, Greinstr. 4, D-50939, Germany. E-mail: aco88@uni-koeln.de

* Author for correspondence and reprint requests

Z. Naturforsch. **59 c,** 613–618 (2004); received June 23, 2004

The structure elucidation of the cyclic (lactonic) forms of the pyoverdins with a succinamide side chain originally produced by the closely related species *Pseudomonas syringae* and *P. cichorii* is reported. Mass spectrometry and nuclear magnetic resonance analyses as well as the determination of the configuration of the amino acids after degradation indicate that these two pyoverdins differ only by the replacement of the first in-chain serine by glycine. The pyoverdins of *P. syringae* and *P. cichorii* and the dihydropyoverdin of *P. syringae* can be used by both species as siderophores.

Key words: Pseudomonas syringae and *cichorii*, Pyoverdin, Siderophore

Introduction

The fluorescent species of the genus *Pseudomonas* are characterized by the production of potent iron chelators, the so-called pyoverdins. The pyoverdins consist of three distinct structural parts, *viz*. a dihydroxyquinoline chromophore responsible for their fluorescence, a peptide chain comprising 6 to 12 amino acids bound to the chromophore carboxyl group, and a small dicarboxylic acid (or its monoamide) connected amidically to its NH_2-group (cf. **1**). The dicarboxylic acids are derived from the citric acid cycle (Schäfer *et al.*, 1991).

The fluorescent *Pseudomonas* species can be divided into two major groups, the arginine dihydrolase-positive saprophytes and opportunistic animal-pathogens *P. aeruginosa, P. fluorescens*, and *P. putida*, and the arginine dihydrolase-negative phythopathogens *P. syringae*, *P. viridiflava*, and *P. cichorii.* Concerning the phythopathogenic group, it was shown in several publications that all pathovars of *P. syringae* and of *P. viridiflava* tested so far (Bultreys and Gheysen, 2000; Bultreys *et al.*, 2001, 2003; Jülich *et al.*, 2001) produce the same pyoverdin. The closely related species *P. cichorii* produces a structurally very similar pyoverdin (Bultreys *et al.*, 2003). In their structures, both pyoverdins differ from the many pyoverdins (Budzikiewicz, 2004) isolated from the saprophytic group in the way that they contain two β-hydroxyaspartic acid units acting as two of the ligand sites for Fe^{3+} (the pyoverdins of the saprophytic group provide either two hydroxamic acid units or one hydroxamic acid and one β-hydroxyaspartic acid unit). The presence of two β-hydroxyaspartic acid units influences *i.a.* the complexing behavior and some spectral properties (Bultreys and Gheysen, 2000; Bultreys *et al.*, 2001, 2003). For the *P. syringae* pyoverdin the structure **1b** had been proposed (Jülich *et al.*, 2001).

In several cases it had been shown (Budzikiewicz, 2004) that pyoverdins with a C-terminal free carboxyl group are actually hydrolysis products of originally produced lactones. These lactonic structures are formed by an ester bond between the C-terminal carboxyl group and an in-chain serine or threonine. A lactonic structure could also be expected for the *P. syringae* pyoverdin, since the molecular mass reported for a succinamide form of this pyoverdin by Bultreys (Bultreys *et al.*, 2001, 2003) was 18 mass units lower than that observed

Abbreviations: Common amino acids, 3-letter code; OHAsp, β-hydroxy-Asp; HPLC, high performance liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; CA, collision activation; NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EDTA, ethylenediaminetetraacetic acid; TMS, tetramethylsilane; pv, pathovar.

0939–5075/2004/0900–0613 $ 06.00

by Jülich (Jülich *et al.*, 2001). The structure elucidation of cyclic forms of the pyoverdin of *Pseudomonas syringae* and of that of *P. cichorii* will be reported here.

Materials and Methods

Pyoverdin production and isolation

The production of pyoverdins was carried out using the liquid-solid technique in Petri dishes with agar blocks (Bultreys and Gheysen, 2000). The unchelated purified pyoverdins of the strains *P. syringae* pv. *syringae* LMG 1247 [identical with the strain ATCC 19310 used by Jülich (Jülich *et al.*, 2001)] and *P. syringae* pv. *morsprunorum* LMG 2222 were obtained from partially purified productions stocked since 1996 and 1997, respectively.

P. syringae pv. *syringae* LMG 1247 was grown either for 5 d at 10 °C in a modified glucose-asparagine medium (GASN) (Bultreys and Gheysen, 2000) containing 5 g/l glucose [ASP medium; this medium was selected in a previous study because – compared with other amino acid-containing agar media – it induced the greatest pyoverdin-related inhibitions of the growth of a yeast by two *P. syringae* strains (Bultreys and Gheysen, 2000)], or for 3 d at 28 °C in the same medium containing 10 g/l glucose. After centrifugation and filtration the culture media containing unchelated pyoverdins were passed through an octadecylsilane column, as previously described for the Fe(III)-chelated pyoverdins (Bultreys and Gheysen, 2000). The water/methanol (1:1, v/v) fractions were brought to dryness and could be stocked for several years. The two conserved fractions were combined and dissolved in water for further purification of the dominant siderophore present in both of them, as ascertained by HPLC. To the solutions of the pyoverdins $FeCl_3$ was added (pH 4.0) and the solvent was evaporated. The residue was then dissolved in a 50 mM NaOH/acetic acid buffer (pH 5.0) and passed through an octadecylsilane column to remove the excess of iron. The column was washed with the buffer and with water, the Fe(III)-chelated pyoverdins were desorbed with water/methanol (1:1, v/v) and the solvent was evaporated.

P. syringae pv. *morsprunorum* LMG 2222 and *P. cichorii* LMG 8401 were grown in a GASN medium for 3 d at 20 °C. The purification steps were as previously described (Bultreys and Gheysen, 2000). The main fractions containing the pyoverdins with a succinamide side chain were selected by HPLC as described earlier (Bultreys and Gheysen, 2000; Bultreys *et al.*, 2001, 2003). After purification, the iron was removed. The ferric complexes were dissolved in 100 mM of a NaOH/phosphoric acid buffer (pH 7.0). Equal volumes of a 500 mM EDTA solution at pH 7.0 were added. The resulting mixtures (ca. pH 5.0) were passed through an octadecylsilane column to remove Fe(III)-EDTA and EDTA. The unchelated pyoverdins were desorbed with water/methanol (1:1, v/v) and brought to dryness.

Mass and NMR spectroscopy

A Finnigan-MAT 900 ST with an ESI source, solvent CH_3OH/H_2O (1:1) was used. Mass selected fragmentation by CA was conducted either in the quadrupole region in front of or in the ion trap. NMR: DRX 500 (^1H 500, ^{13}C 125 MHz) (Bruker, Karlsruhe). Chemical shifts relative to TMS with the internal standard DSS; δ(TMS) = δ(DSS) for ^1H, δ(DSS) = −1.61 ppm for ^{13}C. The MS analyses of the pyoverdin of *P. syringae* were carried out with the pyoverdin of strain LMG 1247 and the NMR analyses with the pyoverdin of strain LMG 2222.

Amino acid analysis

1 mg of each pyoverdin was dissolved in 100 μl of 6 N HCl; nitrogen was flushed into the tube before closure, and the tube was heated to 110 °C for 48 h. 10 μl were brought to dryness, the residue was dissolved in 200 μl of a pH 2.2 citrate buffer and analyzed by ion exchange chromatography (Biochrom 20 Plus).

Amino acid configurations

The amino acids were derivatized to pentafluoropropionyl isopropyl esters with the Alltech derivatisation kit (part. 18093) and separated on an Alltech Chirasil-VAL column (25 m, 0.25 mm ID, 0.16 mm film thickness, part. 13636) injector 250 °C, 4 min at 90 °C, then 4 °C/min until 200 °C. The purified lactonic forms (with a succinamide side chain) of the free pyoverdin of *P. cichorii* LMG 5484 and of the Fe(III)-chelated pyoverdin of *P. syringae* pv. *syringae* LMG 1247 (obtained from six stock samples coming from small volume productions in an ASP medium or in an ASP medium containing 10 g/l glucose) were analyzed in comparison with control amino acids.

Growth stimulation tests

They were carried out as previously described using 0.16 g dipyridyl per liter as iron chelators (Bultreys *et al.*, 2001). The siderophores tested were the Fe(III)-chelated pyoverdins of *P. syringae* pv. *aptata* LMG 5059 and *P. cichorii* LMG 8401, as well as the Fe(III)-chelated dihydropyoverdin of *P. syringae* pv. *aptata* UPB 133; these molecules had been purified as described in previous studies (Bultreys *et al.*, 2001, 2003). The strains tested were *P. syringae* pv. *syringae* B301D, *P. syringae* pv. *tomato* LMG 5093, *P. syringae* pv. *aptata* UPB 110, *P. syringae* pv. *morsprunorum* PmC36, *P. cichorii* LMG 2162 and LMG 8401 and *P. fluorescens* LMG 5822.

Results and Discussion

Some differences in the data published by different groups concerning the pyoverdin of *P. syringae* (Bultreys *et al.*, 2001, 2003; Jülich *et al.*, 2001) prompted a further investigation of the pyoverdin studied by Bultreys (Bultreys and Gheysen, 2000; Bultreys *et al.*, 2001, 2003), which had not yet been fully characterized chemically. Samples purified by chromatography whose purity was carefully assessed by HPLC were investigated by mass spectrometry (electrospray ionization and decomposition of the $[M + 2H]^{2+}$ ion by collision activation). The mass spectra showed that all so-called B-ions obtained by cleavage of the amide bonds (Roepstorff and Fohlman, 1984) – starting from the second threonine – occurred with masses 18 u lower than those obtained for **1b** (Table I). This indicates that a lactone ring is formed with the second threonine (**1a**) (Fig. 1). Under CA conditions a lactone ring is opened by an elimination process transforming threonine into dehydroalanine (Voßen *et al.*, 2000), hence the observed mass shifts (Fuchs and Budzikiewicz, 2001). This conclusion is confirmed by ^{1}H NMR analysis of **1a**. The signal of the CH-group of the second threonine (5.30 ppm) is shifted downfield by 0.9 ppm relative to that of the first threonine (4.39 ppm), the expected effect for esterification of the hydroxyl group (Budzikiewicz, 2004). The MS analyses also indicated the presence of a succinamide residue bound to the chromophore in the dominant pyoverdin. This pyoverdin, with a cyclic peptide chain, has a molecular mass of 1122 u, as shown before (Bultreys *et al.*, 2001). The Fe^{3+} chelate would then have a molecular mass of 1175 u, as previously shown for the pyoverdin of all the pathovars of *P. syringae* investigated so far by Bultreys (Bultreys *et al.*, 2003). The production of pyoverdins in culture media containing asparagine and variable amounts of glucose were suitable to obtain prevailingly this originally produced form of the pyoverdins of *P. syringae* (Bultreys and Gheysen, unpublished). When dried this form was stable during prolonged conservation at –20 °C. However, the ester bond of the lactonic structure can be easily hydrolyzed (Budzikiewicz, 2004) and hydrolysis of the succinamide side chain to give a succinic acid is also a well known process (Schäfer *et al.*, 1991). Consequently, different cul-

Table I. Sequence characteristic ions of **1a** (lactone form) and **1b** (hydrolyzed form) (succinamide side chain) obtained after CA of $[M+2H]^{2+}$ (*m/z* 547).

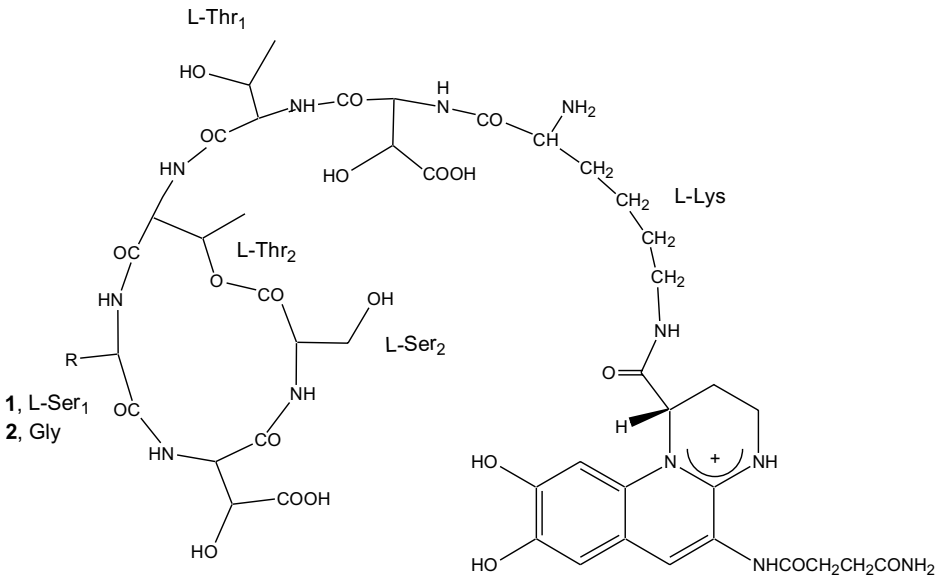
Amino acid		**1a**	**1b**		**1a**	**1b**
	B_0		357[a]			
ε-Lys	B_1	A_1 457[a,b]	457[a,b]			
OHAsp	B_2	616[c]	616[c]			
Thr	B_3	717[c]	717[c]			
Thr–H_2O	B_4	800[c]		Y''_4	407[c]	
Thr			818[c]			
Ser	B_5	887[c,d,e]	905[c]	Y''_3	324[c]	324[c]
OHAsp	B_6	1018[c,d,e]	1036[c,f]	Y''_2	237[c]	237[c]
Ser				Y''_1		
$[M+2H]^{2+}$		562	571			

[a] Observed in the quadrupole spectrum.
[b] A_1 ion is missing (typical for ε-Lys).
[c] Loss of H_2O observed.
[d] A+H_2O ion is observed.
[e] Doubly charged ions of [B_5–H_2O] (*m/z* 435) and [B_6–H_2O] (*m/z* 500.5) are observed.
[f] A doubly charged ion of B_6 (*m/z* 518.5) is observed.

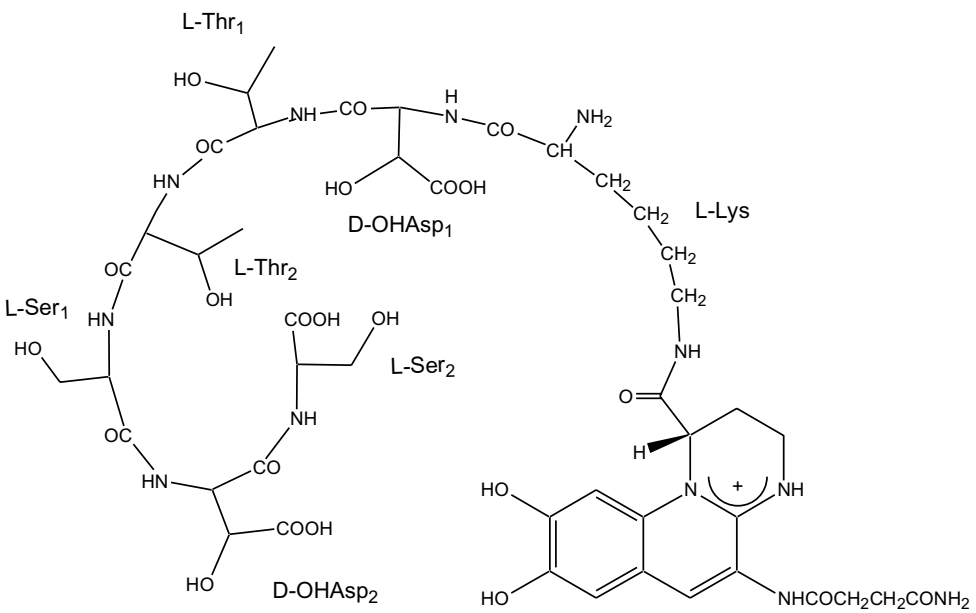
Table II. Sequence characteristic ions of **2** (succinamide side chain) obtained after CA of $[M+2H]^{2+}$ (*m/z* 547).

Amino acids	B-ions		Y″-ions	
	B_0	357[a]		
ε-Lys	B_1	A_1 457[a,b]		
OHAsp	B_2	616[c]		
Thr	B_3	717[c]		
Thr–H_2O	B_4	800[c]	Y''_4	377[c]
Gly	B_5	857[c,d,e]	Y''_3	294[c]
OHAsp	B_6	988[c,d,e]	Y''_2	237[c]
Ser			Y''_1	

[a] Observed in the quadrupole spectrum.
[b] A B_1 ion is missing (typical for ε-Lys).
[c] Loss of H_2O observed.
[d] A+H_2O ion is observed.
[e] Doubly charged ions of [B_5–H_2O) (*m/z* 420) and [B_6–H_2O] (*m/z* 485.5) are observed.



1a: R = CH_2OH
2: R = H



1b

Fig. 1. Structures of the pyoverdins from *Pseudomonas syringae* (**1a**, **1b**) and *P. cichorii* (**2**).

ture conditions and experimental procedures probably explain that derived forms of the original pyoverdin **1a** were obtained and studied by Jülich (Jülich *et al.*, 2001), as the hydrolyzed form both with a succinic acid and a succinamide side chain (**1b**). This explains the differences in the published molecular masses and structures for this pyoverdin (Bultreys *et al.*, 2001, 2003; Jülich *et al.*, 2001).

Table III. ^{1}H NMR data of **2** (H_2O/D_2O 9:1, pH 4.5, 25 °C).

Chromophor	1	2	3	6a	7	10
	5.68	2.46 2.70	3.40 3.73	7.95	7.21	7.12
Peptide chain	NH	α	β	γ	δ	ε
Lys	9.14	4.19	1.91	1.36	1.60	3.33
OHAsp[1]	8.80	5.08	4.68			
Thr[1]	8.66	4.71	4.57	1.27		
Thr[2]	9.13	5.10	5.48	1.32		
Gly	8.65	3.93				
OHAsp[2]	8.08	5.01	4.59			
Ser	7.49	4.55	3.90			
Suca	2′	3′				
	2.84	2.75				

P. cichorii belongs to the arginine dihydrolase-negative group, but in contrast to *P. syringae* it is oxidase-positive. From its cultures a pyoverdin was obtained which seemingly was closely related to the pyoverdin of *P. syringae* (Bultreys *et al.*, 2003). Indeed, an amino acid analysis gave 2 OHAsp, Gly, Lys, Ser and 2 Thr, and MS analyses also indicated that the *P. cichorii* pyoverdin probably differed from the one of *P. syringae* only by the replacement of one serine by glycine. In this study, the molecular masses of the two unchelated pyoverdins were confirmed to differ by 30 u. Comparison of the fragmentation pattern (Table II) of the pyoverdin of *P. cichorii* with that of **1a** suggests that the two pyoverdins differ by the replacement of the first (in-chain) serine by glycine. Also here the lactone ring is formed by esterification of the second threonine with the C-terminal serine. The ^{1}H- and ^{13}C-NMR data (Tables III and IV) differ from those published for **1b** (Jülich *et al.*, 2001) only as far as the replacement of serine by glycine is concerned, and by the downfield shift of the CH signal of the second threonine as it had been observed for **1a** above. The observed configurations of the amino acids of the pyoverdin of *P. cichorii* were 2 D-OHAsp, L-Lys, L-Ser and 2 L-Thr. These configurations were identical to those observed in this study for the lactonic succinamide form of the pyoverdin of *P. syringae* pv. *syringae* LMG 1247, and to those previously reported for the open succinic acid form of the pyoverdin of strain ATCC 19310 (Jülich *et al.*, 2001). The unique small modification in the peptide chains of the pyoverdins of *P. syringae* and *P. cichorii* probably explains that both Fe(III)-chelated pyoverdins show the same unusual spectral characteristics between pH 7.0 and pH 3.0. On the other hand, the pyoverdin common to *P. asplenii* and *P. fuscovaginae*, which also contains 2 OHAsp but has a more different peptide chain, behaved differently at pH 3.0 (Bultreys *et al.*, 2003). Therefore, beside the influence of the 2 OHAsp, the rest of the peptide chain also clearly influences the spectral characteristics of these atypical pyoverdins. The pyoverdins of *P. syringae* and *P. cichorii* are the second case where the pyoverdins produced by two different strains differ from each other only by the substitution of a single amino acid. The other case concerns the

Table IV. ^{13}C NMR data of **2** (conditions as for Table III).

Chromophor	CO	1	2	3	4a	5	6	6a	7	8	9	10	10a
	171.3	58.6	23.4	36.7	150.7	118.8	140.3	116.2	115.3	145.2	153.3	101.9	133.3
Peptide chain	CO	α	β	γ	δ	ε	CO′						
Lys	172.0	54.6	31.8	22.7	28.9	40.6							
OHAsp[1]	171.3	58.3	73.7				177.2						
Thr[1]	171.3	60.8	68.3	20.3									
Thr[2]	173.0	57.3	74.1	16.2									
Gly	172.9	45.5											
OHAsp[2]	171.6	58.3	73.7				178.5						
Ser	178.4	55.6	60.7										
Suca	1′	2′	3′	4′									
	179.0	31.9	31.1	177.7									

pyoverdins of the strains *Pseudomonas* sp. A214 and 7SR1 which differ by the replacement of one serine by alanine (Uría Fernández *et al.*, 2003). In both cases, the exchange of one small neutral amino acid does not influence the isoelectrofocusing patterns obtained with each couple of strains in siderotyping analyses (Bultreys *et al.*, 2003; Uría Fernández *et al.*, 2003).

Some non-fluorescent strains of *P. syringae* pv. *aptata* as UPB 110 and UPB 133 were shown to produce only the dihydropyoverdin corresponding to the pyoverdin common in the whole species *P. syringae*. These strains and the non-fluorescent strain *P. syringae* pv. *morsprunorum* PmC36, which is apparently unable to produce these siderophores, were shown to be able to use both siderophores (Bultreys and Gheysen, 2000; Bultreys *et al.*, 2001). In this study, the growth stimulation tests on dipyridyl-containing medium indicated the use of the pyoverdin and dihydropyoverdin of *P. syringae* by *P. cichorii* and the use of the pyoverdin of *P. cichorii* by the fluorescent and non-fluorescent strains of *P. syringae*. On the other hand, *P. fluorescens* LMG 5822 was unable to use the three siderophores. The results indicate that in spite of the small difference in the peptide chain of the pyoverdin of *P. cichorii*, there is still sufficient affinity between this peptide chain and the membrane receptor of the pyoverdin of *P. syringae*, and conversely. Similar reciprocal incorporations were also observed by the strains *Pseudomonas* sp. A214 and 7SR1 of their closely related pyoverdins (Uría Fernández *et al.*, 2003).

Budzikiewicz, H. (2004), Siderophores of Pseudomonadaceae *sensu stricto* (fluorescent and non-fluorescent *Pseudomonas* spp.). In: Progress in the Chemistry of Organic Natural Products (Herz W., Falk H., and Kirby G. W., eds.). Springer, Wien.

Bultreys A. and Gheysen I. (2000), Production and comparison of peptide siderophores from strains of distantly related pathovars of *Pseudomonas syringae* and *Pseudomonas viridiflava* LMG 2352. Appl. Environ. Microbiol. **66**, 325–331.

Bultreys A., Gheysen I., Maraite H., and de Hoffman E. (2001), Characterization of fluorescent and nonfluorescent peptide siderophores produced by *Pseudomonas syringae* strains and their potential use in strain identification. Appl. Environ. Microbiol. **67**, 1718–1727.

Bultreys A., Gheysen I., Wathelet B., Maraite H., and de Hoffman E. (2003), High-performance liquid chromatography analyses of pyoverdin siderophores differentiate among phytopathogenic fluorescent *Pseudomonas* species. Appl. Environ. Microbiol. **69**, 1143–1153.

Fuchs R. and Budzikiewicz H. (2001), Structural studies of pyoverdins by mass spectrometry. Curr. Org. Chem. **5**, 265–288.

Jülich M., Taraz K., Budzikiewicz H., Geoffroy V., Meyer J.-M., and Gardan L. (2001), The structure of the pyoverdin isolated from *Pseudomonas syringae* pathovars. Z. Naturforsch. **56c**, 687–694.

Roepstorff P. and Fohlman J. (1984), Proposal of a common nomenclature for sequence ions in mass spectra of peptides. Biomed. Mass Spectrom. **11**, 601.

Schäfer H., Taraz K., and Budzikiewicz H. (1991), Zur Genese der amidisch an den Chromophor von Pyoverdinen gebundenen Dicarbonsäuren. Z. Naturforsch. **46c**, 398–406.

Uría Fernández D., Geoffroy V., Schäfer M., Meyer J.-M., and Budzikiewicz H. (2003), Structure revision of several pyoverdins produced by plant-growth promoting and plant-deleterious *Pseudomonas* species. Monatsh. Chem. **134**, 1421–1431.

Voßen W., Fuchs R., Taraz K., and Budzikiewicz H. (2000), Can the peptide chain of a pyoverdin be bound by an ester bond to the chromophore? The old problem of pseudobactin 7SR1. Z. Naturforsch. **55c**, 153–164.