Ornicorrugatin, a New Siderophore from *Pseudomonas fluorescens* AF76

Sandra Matthijs[a], Herbert Budzikiewicz[b,*], Mathias Schäfer[b], Bernard Wathelet[c], and Pierre Cornelis[a]

[a] Laboratory of Microbial Interactions, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussel, Belgium
[b] Institut für Organische Chemie der Universität zu Köln, Greinstr. 4, D-50939 Köln, Germany. E-mail: aco88@uni-koeln.de
[c] Chimie Biologique Industrielle, Faculté Universitaire des Sciences agronomiques, Passage des Déportés, 2, B-5030 Gembloux, Belgium

* Author for correspondence and reprint requests

Z. Naturforsch. **63c**, 8–12 (2008); received November 15, 2007

From a pyoverdin-negative mutant of *Pseudomonas fluorescens* AF76 a new lipopeptidic siderophore (ornicorrugatin) could be isolated. It is structurally related to the siderophore of *Pseudomonas corrugata* differing in the replacement of one Dab unit by Orn.

Key words: Pseudomonas fluorescens AF76, Siderophores, Ornicorrugatin

Introduction

The strain *Pseudomonas fluorescens* AF76 was isolated from the rhizosphere of *Arachis hypogaea* L. in India. A pyoverdin-negative mutant of AF76 showed strong ability to decolourize chrome azurol S (CAS) indicating the production of a secondary siderophore. The structure elucidation of this secondary siderophore, ornicorrugatin (corrugatin where one Dab unit is replaced by Orn), will be reported here.

Materials and Methods

Ornicorrugatin was obtained from the supernatant of a 40-h-old culture of the pyoverdin-negative mutant 1G10 of *Pseudomonas fluorescens* AF76 grown in a casamino acid medium. The supernatant was passed on a C-18 column (3×1 cm) and washed twice with distilled water. The siderophore was eluted with H_2O/CH_3CN 4:6. The CAS-positive fraction (Schwyn and Neilands, 1987) was collected and purified by HPLC. Purification was performed on a Gilson system with a 712 HPLC System Controller. A Supelco Discovery® BIO Wide Pore column (C-18, 25×2.12 cm, 10 μm particle size) was used with a flow rate of 20 ml/min and a gradient going from H_2O/CH_3CN 9:1 containing 0.1% CF_3COOH to H_2O/CH_3CN 2:8 containing 0.1% CF_3COOH in 30 min, followed by 10 min isocratic elution with H_2O/CH_3CN 2:8 containing 0.1% CF_3COOH. From the extract CH_3CN was evaporated *in vacuo* and the sample was lyophilized.

Mass spectral data were obtained with a MAT 900 ST instrument providing an electrostatic/magnetic analyzer (EB) geometry connected to an octapole collision cell and a quadrupole ion trap (QIT), and equipped with an ESI II ion source (Finnigan MAT, Bremen, Germany); spray voltage, 3.4–3.6 kV; capillary temperature, 230 °C. Source conditions were set to minimize fragmentation, resolution ca. 5000 (10% valley). The samples were dissolved in water/methanol/trifluoroacetic acid 50:50:0.1 (v/v). Fragmentation induced by low energy collision activation (CA) was effected in the octapole unit and in the QIT ($\sim 2 \cdot 10^{-3}$ Pa He as bath gas diffusing in the collision octapole). Exact ion mass measurements were performed with an LTQ Orbitrap XL (ThermoFisher, Bremen, Germany) instrument with static nano-ESI (needles with 5 μm inner diameter; Mascom, Bremen, Germany). The resolution (full signal width at half hight, FWHH) was 60000 at *m/z* 400 in single stage ESI and 30000 for MS/MS product ion exact mass measurements. The mass accuracy was determined to be < 3 ppm with external calibration.

High resolution ^{1}H NMR (250 MHz) and ^{13}C NMR (62.90 MHz) spectra were recorded in CD_3OD or DMSO-d_6 on a Bruker Avance DRX

Abbreviations: Common amino acids, three letter code; Dab, 2,4-diaminobutanoic acid; OHAsp, *threo*-β-hydroxy Asp; OHHis, *threo*-β-hydroxy His; CAS, chrome azurol S; ESI, electrospray ionization; CA, collision activation.

0939–5075/2008/0100–0008 $ 06.00

250 spectrometer. Chemical shifts are reported in ppm downfield from TMS.

For chiral amino acid analysis, after hydrolysis (6 M HCl, 110 °C, 24 h), the amino acids were derivatized according to the method described by Demange *et al.* (1988), giving *N*-pentafluoropropionyl (PFP) *O*-trimethylsilyl (TMS) esters. 1 μl of the toluene solution of each derivatized amino acid was injected in a Hewlett Packard HP6890 gas chromatograph equipped with an Alltech Chirasil-Val column no. 13636 (25 m × 0.25 mm ID × 0.16 μm) and flame ionization detection. Heating program was 4 min at 90 °C, then 4 °C/min to 200 °C.

Results

The octapole CA spectrum (Fig. 1) of corrugatin (**1**, Fig. 2, n = 2) (Risse *et al.*, 1998) comprises two parts. Starting from $[M+H]^+$ (*m/z* 998) losses of up to three molecules of H_2O are observed (*m/z* 980, 962, 944) as well as of the side chains from the condensation products of β-hydroxy Asp (OHAsp) and β-hydroxy His (OHHis) with Dab, respectively (see Fig 3, loss of 74 Da, *m/z* 924 and of 96 Da, *m/z* 902; the latter ion is even more pronounced in the ion trap CA spectrum). These two ions also lose H_2O (*m/z* 906 and 884, respectively). Loss of both residues results in *m/z* 828 (subsequent loss of water gives *m/z* 810 and 792). Loss

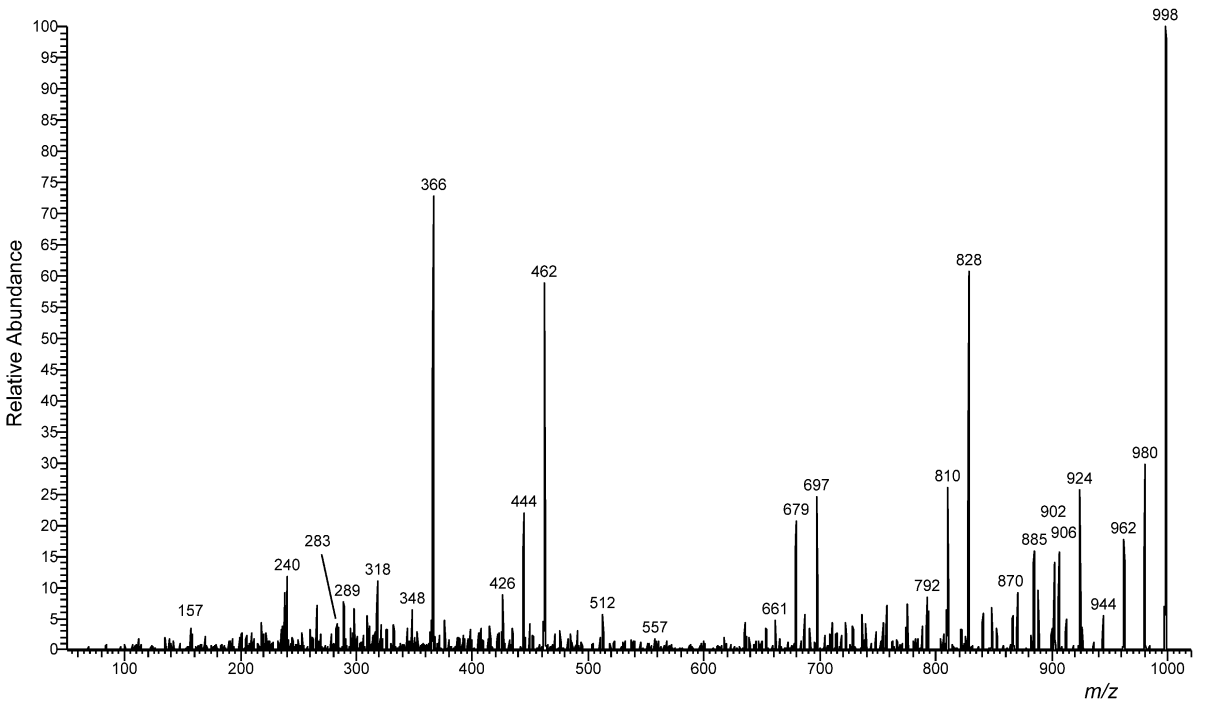

Fig. 1. Octapole CA spectrum of $[M+H]^+$ of corrugatin (**1**).

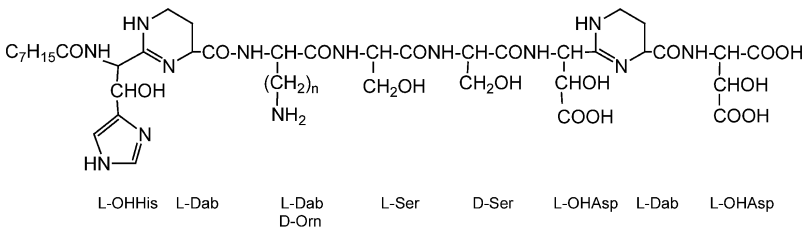

Fig. 2. Corrugatin (**1**, n = 2, Dab) and ornicorrugatin (**2**, n = 3, Orn).

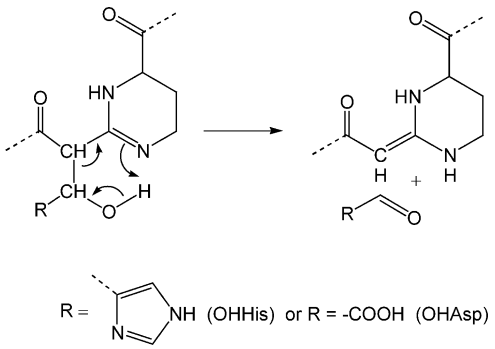

Fig. 3. McLafferty type elimination of amino acid side chains.

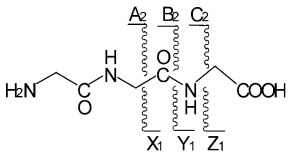

Fig. 4. Designation of peptide fragments. Hyphens (as in Y″) indicate additional H atoms.

of the *C*-terminal OHAsp with back-transfer of the hydroxy group (Fuchs and Budzikiewicz, 2001) yields the ion *m/z* 867 (again more pronounced in the ion trap CA spectrum). Loss of (74 + 96) Da from *m/z* 867 results in *m/z* 697 ($-H_2O$ gives *m/z* 679 and 661). Cleavage after Ser_2 with OH back-transfer yields *m/z* 653 of low abundance (loss of H_2O gives *m/z* 635).

The most pronounced ions in the lower part of Fig. 1 are *m/z* 462 (B_3, cleavage after Dab; for a designation of peptide fragments see Fig. 4, Roepstorff and Fohlman, 1984) which loses twice H_2O (*m/z* 444 and 426) and 96 Da (*m/z* 366, OHHis residue, Fig. 3). Of importance for the subsequent discussion are the ions *m/z* 318 and 240 formed by the loss of $CH_3(CH_2)_5CH{=}CO$ (126 Da, the typical keten elimination from amides) from *m/z* 444 and 366, respectively (Budzikiewicz *et al.*, 1967).

In the octapole CA spectrum of $[M + 2H]^{2+}$ (*m/z* 506.5) Y''_1 (*m/z* 150) and Y''_5 (*m/z* 537) can be seen. Important are the ions *m/z* 444 and 366 (cleavage products of B_3, see above) from which the loss of 126 Da (*m/z* 318 and 240) is much more pronounced than in the singly charged spectrum). A further ion occurs at *m/z* 283 (C''_2 – 96 Da, more pronounced than in Fig. 1) which also loses 126

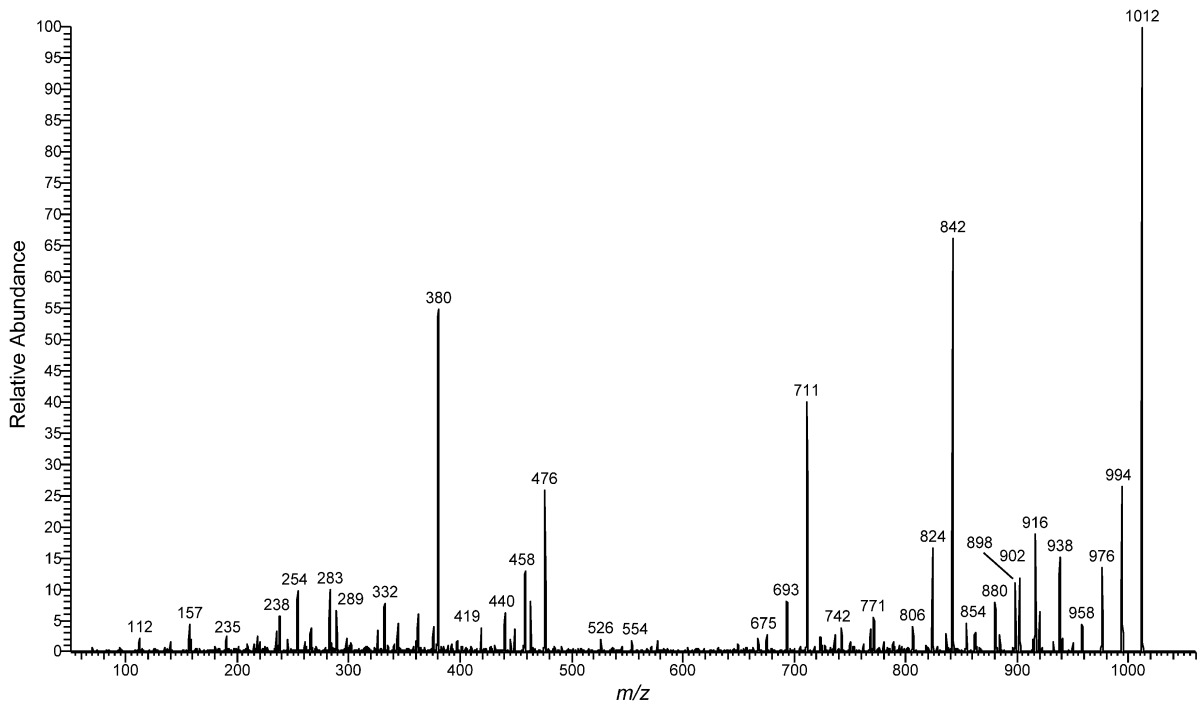

Fig. 5. Octapole CA spectrum of $[M+H]^+$ of ornicorrugatin (**2**).

Da (*m/z* 157). B_2 – 96 Da (*m/z* 266) is of lower abundance. In the corresponding mass spectra of corrugatin labeled with ^{15}N all ions show the expected shift values.

The octapole CA spectrum (Fig. 5) of ornicorrugatin (**2**, Fig. 2, $n = 3$) shows striking similarities with that of corrugatin (Fig. 1), but differs in the following way. The molecular mass is 14 Da higher. This suggests either one additional CH_2 group or a replacement of a CH_2 group by CO. The entire upper group of ions is shifted by 14 Da. It follows that the additional 14 Da can not be located in the last three *C*-terminal amino acids. Since *m/z* 462 and its degradation products characteristic for the lower group of ions are also shifted in mass the additional unit must be located in B_3. The presence of *m/z* 283 and 157 (fragments of C_2'') with identical masses in both spectra demonstrates the absence of the additional 14 Da in these ions. The additional group must therefore be located in the third amino acid. Replacement of Dab by Orn is the most reasonable explanation (Fig. 2, $n = 3$) and is substantiated by the data presented below. The elemental composition of the major ions could be confirmed by exact mass measurements (Table I).

Further evidence is offered by the loss of 126 Da in both spectra from fragments containing the *N*-terminus indicating the presence of the octanoic acid amide structure, and by the presence of the Y_1'' and Y_5'' ions with identical masses for both compounds which indicates that the additional 14 Da can not be located in the five *C*-terminal amino acids.

The ^1H and ^{13}C NMR spectra of ornicorrugatin (**2**) correspond to those of corrugatin (**1**) (Risse *et al.*, 1998) with the exception that one Dab sequence is replaced by the Orn sequence (α: 4.37/53.0, β: 1.97/22.0, γ: 1.77/24.8, δ: 2.97/40.0 ppm as established by H,H-COSY and HMQC).

Table I. Exact mass data of selected ions from Fig. 3.

Mass found	Mass calcd.	Composition
1012.470	1012.470	$C_{41}H_{66}N_{13}O_{17}$
842.435	842.437	$C_{35}H_{60}N_{11}O_{13}$
476.296	496.299	$C_{23}H_{38}N_7O_4$
458.286	458.288	$C_{23}H_{36}N_7O_3$
380.264	380.266	$C_{19}H_{34}N_5O_3$

Comparison of the free amino acids obtained by acid hydrolysis of authentic (Risse *et al.*, 1998) corrugatin and of ornicorrugatin by gas chromatography on a chiral column after derivatization established an identical composition with the exception that ornicorrugatin contained an additional D-Orn unit. The placement of L- and D-Ser in the peptide chain was established for corrugatin only.

Discussion

So far only twice lipopeptidic siderophores have been described as obtained from fluorescent *Pseudomonas* spp. (Budzikiewicz, 2004), the ferrocins, *cyclo*-depsidekapeptides with slight variations in the peptide chain (Tsubotani *et al.*, 1993) from *Pseudomonas fluorescens* YK-310, and corrugatin (Risse *et al.*, 1998) from *Pseudomonas corrugata*. The taxonomical placement of this species has been controversial, but currently it is placed in close vicinity to the fluorescent *Pseudomonas* spp. (Sutra *et al.*, 1997). The isolation of the structurally closely related ornicorrugatin from a *Pseudomonas fluorescens* strain favours this placement.

The main siderophores of the fluorescent *Pseudomonas* spp. are the pyoverdins, chromopeptides comprising a dihydroxyquinoline chromophore and a peptide chain consisting of six to twelve amino acids, partially modified (Budzikiewicz, 2004). In addition, secondary siderophores with lower ability to bind Fe^{3+} are produced, especially by pyoverdin-negativ strains. A siderophore encountered with several *Pseudomonas* species is pyochelin derived from salicylic acid and two molecules of cysteine (Cobessi *et al.*, 2005; Schlegel *et al.*, 2006; see also Budzikiewicz, 2004). Ornicorrugatin is another secondary siderophore which had been overlooked so far.

Acknowledgement

We wish to thank Dr. E. Darmoc for the exact mass measurements with the LTQ Orbitrap XL (ThermoFisher, Bremen, Germany) instrument.

Budzikiewicz H. (2004), Siderophores of the Pseudomonadaceae *sensu stricto* (fluorescent and non-fluorescent *Pseudomonas* spp.). Prog. Chem. Org. Nat. Prod. **87**, 81–237.

Budzikiewicz H., Djerassi C., and Williams D. H. (1967), Mass Spectrometry of Organic Compounds. Holden-Day, San Francisco, pp. 336 ff.

Cobessi D., Celia H., and Pattus F. (2005), Crystal structurs at high resolution of ferric pyochelin and its membrane receptor FptA from *Pseudomonas aeruginosa.* J. Mol. Biol. **352**, 893–904.

Demange P., Abdallah M., and Frank H. (1988), Assignment of the configurations of the amino acids in peptidic siderophores. J. Chromatogr. **438**, 291–297.

Fuchs R. and Budzikiewicz H. (2001), Rearrangement reactions in the electrospray ionization mass spectra of pyoverdins. Int. J. Mass Spectrom. **210/211**, 603–612.

Risse D., Beiderbeck H., Taraz K., Budzikiewicz H., and Gustine D. (1998), Corrugatin, a lipopeptide from *Pseudomonas corrugata.* Z. Naturforsch. **53c**, 295–304.

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

Schlegel K., Lex J., Taraz K., and Budzikiewicz H. (2006), The X-ray structure of the pyochelin Fe^{3+} complex. Z. Naturforsch. **61c**, 263–266.

Schwyn B. and Neilands J. B. (1987), Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160**, 47–56.

Sutra L., Siverio F., Lopez M. M., Hunault G., Bollet C., and Gardan L. (1997), Taxonomy of *Pseudomonas* strains isolated from tomato pith necrosis: Emended description of *Pseudomonas corrugata* and proposal of three unnamed fluorescent *Pseudomonas* genomospecies. Int. J. Syst. Bacteriol. **47**, 1020–1033.

Tsubotani S., Katayama N., Funabashi Y., Ono H., and Harada S. (1993), Ferrocins, new iron-containing peptide antibiotics produced by bacteria. Isolation, characterization and structure elucidation. J. Antibiot. **46**, 287–293.