Cepaciachelin, a New Catecholate Siderophore from *Burkholderia (Pseudomonas) cepacia**,**

I. Barelmann[a], J.-M. Meyer[b], K. Taraz[a], H. Budzikiewicz[a]

[a] Institut für Organische Chemie der Universität zu Köln, Greinstr. 4, D-50939 Köln, Deutschland

[b] Laboratoire de Microbiologie et de Génetique, Université Louis-Pasteur, Unité de Recherche, Associée au Centre National de la Recherche Scientifique n° 1481, 28 rue Goethe, F-67000 Strasbourg, France

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In addition to the known hydroxamate siderophores ornibactin C6 and C8 a catecholate siderophore named cepaciachelin, 1-N-[2-N',6-N'-di(2,3-dihydroxybenzoyl)-L-lysyl]-1,4-diaminobutane, was isolated from a *Burkholderia (Pseudomonas) cepacia* PHP7 culture and its structure elucidated by chemical degradation and spectroscopic methods. This is the first case of a member of the *Pseudomonas* group which produces both hydroxamate and catecholate siderophores.

Introduction

To ensure iron supply while growing under iron-limited conditions bacteria from the genus *Pseudomonas* usually synthesize Fe^{3+}-complexing substances (so-called siderophores) (Neilands, 1981). In addition to various small molecules as e. g. pyochelin (Cox *et al.*,1981), cepabactin (Meyer *et al.*, 1989; Winkler *et al.*, 1986) or salicylic acid (Meyer *et al.*, 1992; Visca *et al.*, 1993) more strongly iron binding compounds are produced. They consist of modified oligopeptides with α-hydroxycarboxylate and/or hydroxamate units as complexing sites as, e. g., the pyoverdines from fluorescent pseudomonads (Budzikiewicz, 1993) or the ornibactins from some *Burkholderia* (*Pseudomonas*) species (Meyer *et al.*, 1995). A second group of chelators consists of aliphatic amino compounds substituted with one to three 2,3-dihydroxybenzoic acids (catecholates as binding sites). For *Ps. stutzeri* a member of this class containing Arg was reported (Chakraborty *et. al.*, 1990). So far no *Pseudomonas* sp. has been encountered where the same strain produces both kinds of siderophores. Only for the related *Azotobacter vinelandii* a co-occurrence was reported recently (Cornish and Page, 1995).

We wish now to report a further example. *Pseudomonas cepacia* - reclassified recently (Yabuuchi *et al.*, 1992; Gillis *et al.*, 1995) as *Burkholderia cepacia* - was shown to produce a series of tetrapeptidic siderophores, the ornibactins (**1**) (Meyer *et. al.*, 1995).

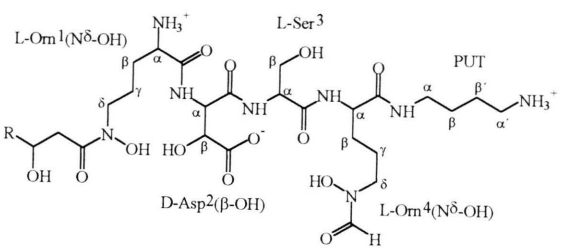

Fig. 1. Structures of ornibactins
a) R = CH_3 ornibactin C4
b) R = C_3H_7 ornibactin C6
c) R = C_5H_{11} ornibactin C8

From a strain (PHP7) isolated from the rhizosphere of *Zea mays* L. in addition to ornibactin C6 (**1b**) and C8 (**1c**) the new catecholate siderophore 1-N-[2-N',6-N'-di(2,3-dihydroxybenzoyl)-L-lysyl]-

Abbreviations: Common amino acids, 3-letter code; TAP, N-trifluoroacetyl (amino acid) O-isopropyl ester; FAB-MS, fast atom bombardement mass spectrometry; GC/MS, gas chromatograph coupled with a mass spectrometer; RP-HPLC, reversed phase high performance liquid chromatography; COSY, (NMR) correlation spectroscopy; HMBC, ^1H detected multiple bond heteronuclear multiple quantum coherence; TOCSY, total correlated (NMR) spectroscopy.

* Part LVII of the series "Bacterial Constituents". For Part LVI see Adolphs *et al.* (1996).

** Reprint requests to Prof. Dr. H. Budzikiewicz. Telefax: 0221-470 5057.

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1,4-diaminobutane (**2**) was obtained for which we propose the name cepaciachelin.

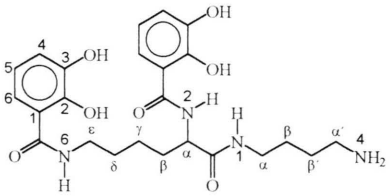

Fig. 2. Structure of cepaciachelin.

Material and Methods

Instruments

Mass spectrometer: Finnigan MAT HSQ-30 (FAB), Finnigan INCOS 50XL (GC/MS)

NMR: Bruker AM 300 (^{1}H: 300 MHz, ^{13}C: 75.5 MHz). ^{1}H- and ^{13}C-chemical shifts are given relative to TMS with the internal standard DSS (2,2-dimethyl-5-silapentan-5- sulfonate) using the relations δ(TMS) = δ(DSS) for ^{1}H-NMR and δ(TMS) = δ(DSS) – 1.61 ppm for ^{13}C-NMR.

UV/Vis: Perkin Elmer Hitachi 200.

HPLC: Knauer, column Nucleosil 100-C-18, 5μ (Macherey & Nagel).

GC: Carlo Erba HRGC 4160, FID detector, capillary column Chirasil-L-Val (Chrompack) 22 m, 0.22 mm i.d.

Column chromatography: Sephadex LH-20 (Pharmacia), Bio-gel P-2 (Bio-Rad).

Production and isolation of the siderophores

Burkholderia cepacia PHP7 (LMG 11351, Gillis *et al.*, 1995) was isolated from the rhizosphere of *Zea mays* L. It was grown in a medium consisting of 6 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$ and 4 g succinic acid in 1 l H_2O (pH adjusted to 7.0; 500 ml medium per 1 l Erlenmeyer flask) for 40 hrs at 30°C with shaking (200 rpm), then ferric citrate was added to the culture medium (1g/l). Cell material was removed by centrifugation. From the supernatant liquid the mixture of ferric siderophores was isolated by $CHCl_3$/phenol extraction (Meyer and Abdallah, 1978) and separated into two fractions by gel chromatography on Sephadex LH-20 with methanol as eluent. Both fractions were purified by column chromatography on Bio-gel P-2 with 0.1 M acetic acid and subsequently by RP-HPLC with a solvent consisting of methanol and a 0.05 M aqueous solution of ammonium acetate (methanol gradient 3

Table I. ^{1}H and ^{13}C NMR data of ornibactin C6 (**1b**) in D_2O at 25 °C.

Structural unit	δ(^{1}H)[a]	δ(^{13}C)[b]	Structural unit	δ(^{1}H)[a]	δ(^{13}C)[b]
HHA–CO	–	174.54	Ser^3–NH	8.34[c]	–
HHA–C-2	2.77	40.55	Ser^3–CO	–	172.99
HHA–C-3	4.12	69.25	Ser^3–α	4.51	57.13
HHA–C-4	1.53	39.73	Ser^3–Cβ	3.93	61.95
HHA–C-5	1.44	19.32	$Orn^4(N^\delta$–OH)–NH	8.31[c]	–
HHA–C-6	0.94	14.32	$Orn^4(N^\delta$–OH)–CO	–	175.17
$Orn^1(N^\delta$–OH)–CO	–	171.20	$Orn^4(N^\delta$–OH)–Cα	4.31	55.04
$Orn^1(N^\delta$–OH)–Cα	4.23	53.98	$Orn^4(N^\delta$–OH)–Cβ	1.79	28.71
$Orn^1(N^\delta$–OH)–Cβ	1.92	29.27	$Orn^4(N^\delta$–OH)–Cγ	1.76	23.89
$Orn^1(N^\delta$–OH)–Cγ	1.70	22.25	$Orn^4(N^\delta$–OH)–Cδ	3.62	51.04
$Orn^1(N^\delta$–OH)–Cδ	3.65	48.35	$Orn^4(N^\delta$–OH)–CHO (cis)	7.98	160.81
Asp^2(β-OH)–NH	8.45[c]	–	$Orn^4(N^\delta$–OH)–CHO (trans)	8.34	165.01
Asp^2(β-OH)–CO	–	173.10	PUT–NH	8.04[c]	–
Asp^2(β-OH)–COOH	–	177.30	PUT–C-α	3.27	39.73
Asp^2(β-OH)–Cα	4.98	57.77	PUT–C-β	1.61	26.54
Asp^2(β-OH)–Cβ	4.57	73.30	PUT–C-β′	1.69	25.24
			PUT–C-α′	3.03	40.55

Abbreviations: PUT, putrescine (1,4-diaminobutane); HHA, 3-hydroxyhexanoic acid.

[a] Assignments by homonuclear 2D-experiments (H,H-COSY, TOCSY).

[b] Assignments by heteronuclear 2D-experiments (C,H-COSY, HMBC).

[c] In 100 mM KH_2PO_4 in H_2O (pH 4.35)/D_2O 9:1 after presaturation of the H_2O signal by the jump-and-return method.

Table II. ^{1}H data of cepaciachelin (**2**) in D_2O at 25 °C.

Structural unit	δ(^{1}H)	Multiplicity	Structural unit	δ(^{1}H)	Multiplicity
H-4[a]	7.01	dd	α-CH_2-PUT	3.24	m
H-5[a]	6.76	t	α′-CH_2-PUT	2.97	t
H-6[a]	7.17	dd	β-CH_2-PUT	1.50[b]	m
H-4′[a]	6.78	dd	β′-CH_2-PUT	1.49[b]	m
H-5′[a]	6.74	t	2-NH-Lys	8.64[c]	–
H-6′[a]	7.08	dd	6-NH-Lys	8.56[c]	–
α-CH-Lys	4.49	dd	1-NH-PUT	8.33[c]	–
β-CH_2-Lys	1.94	m	4-NH_2-PUT	7.57[c]	–
γ-CH_2-Lys	1.45[b]	m			
δ-CH_2-Lys	1.56[b]	m			
ε-CH_2-Lys	3.43	m			

Abbreviations: DHB, 2,3-dihydroxybenzoyl group; PUT, putrescine (1,4-diaminobutane).
[a] Assignments by TOCSY experiments.
[b] Since these signals could not be identified clearly in the 1D-^{1}H-NMR spectrum there positions were determined by TOCSY experiments at 5 °C.
[c] See Table I, footnote c, at 5 °C.

to 78%, v/v, in 25 min). From the first one two compounds (**1b** and **1c**) were obtained, the second one yielded **2**. All siderophores were adsorbed on Sep-Pak cartridges and decomplexed with oxalic acid (8%, pH 4).

Amino acid analysis

Hydrolysis (6 N HCl, 110 °C, 21 hrs), TAP derivatization, GC/MS and GC analysis on a chiral column was performed as described earlier (Jacques *et al.*, 1995).

Results and Discussion

The siderophores obtained from the first fraction could be identified as ornibactin C6 (**1b**) and C8 (**1c**). FAB-MS of the iron-free ornibactins gave $[M+H]^+$ ions at m/z 709 and 737, resp. As in the literature only NMR data of the Ga^{3+} complex are given (Stephan *et al.*, 1993a, 1993b) those obtained for free **1b** are compiled in Table I (those of **1c** differ only in the integral of the CH_2 region).

The amino acid analysis of **2** demonstrated the presence of L-Lys. Its UV/Vis spectrum shows the typical absorption maxima for 2,3-dihydroxybenzoic acid amides (cf. Taraz *et al.*, 1990): Between 6.74 and 7.17 ppm the proton signals of the superimposed AMX-systems of the two dihydroxybenzoic acid units can be seen which could be identified by TOCSY cross signals. By the same method the positions of the signals of Lys and of 1,4-diaminobutane were established. This was also possible for the NH-signals in a buffered solution by suppressing the water signal by the jump-and-return method.

Besides the fact that the discovery of cepaciachelin (**2**) constitutes the first case of a member of the *Burkholderia* (*Pseudomonas*) group which produces both a peptide hydroxamate and a catecholate siderophore it is of interest also in another way: The most efficient siderophores are those which contain three complexing sites for Fe^{3+}. One of them in the catecholate group is protochelin (**3**) produced by a methanolotrophic bacterium (Taraz *et al.*, 1990) and by *Azotobacter vinelandii* (Cornish and Page, 1995). Two subunits (probably precursors) of it, viz. azotochelin and aminochelin (see brackets in Fig. 3) had also been obtained from this bacterium (Cornish and Page, 1995 and literature cited there). Cepaciachelin (**2**) constitutes a third subunit.

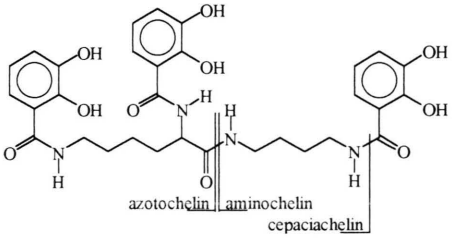

Fig. 3. Structure of protochelin with indication of the subunits azotochelin, aminochelin and cepaciachelin.

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