Catecholate Siderophores from *Chryseomonas luteola**

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Catecholate Siderophore, *Chryseomonas luteola*, Chrysobactin, Chryseomonin, Structure Elucidation

Two catecholate siderophores (chrysobactin and chryseomonin) were isolated from an iron-deficient culture medium of *Chryseomonas luteola*. Their structures were elucidated by chemical degradation studies and spectroscopic methods, especially 2D-NMR techniques, and confirmed by synthesis. Chryseomonin constitutes a novel type of catecholate siderophore.

Introduction

Under iron-limited conditions many bacterial species biosynthesize Fe^{3+} complexing compounds, so-called siderophores. Their most common structural features are hydroxamate and/or catecholate units, the latter ones mostly derived from 2,3-dihydroxybenzoic acid attached to amino acids, amino alcohols or aliphatic diamines. We wish now to report the isolation and structure elucidation of two catecholate siderophores from *Chryseomonas luteola*. One of them proved to be chrysobactin (**1**) which had been obtained before from *Erwinia chrysanthemi* (Persmark *et al.,* 1989) and from *Serratia marcescens* (Ehlert *et al.*, 1994). The second one named chryseomonin could be shown to be 6-(1-pyridinio-)chrysobactin, i. e., N^2-[2,3-dihydroxy-6-(1-pyridinio)-benzoyl]-lysylserine (**2**), a novel type of catecholate siderophores.

Material and Methods

Bacterial strain

Chryseomonas luteola was isolated from forest soil in the Rhön mountains. A specimen is deposited at the Institut de Biologie Moléculaire et Cellulaire of the Centre National de la Recherche Scientifique, Strasbourg, France.

Chemicals

Pyridine was treated with chlorosulfonic acid (10 ml/l), distilled, kept over KOH, redistilled and stored at 4 °C over 4 Å molecular sieve. A 56% solution of hydroiodic acid (HI) was heated to 120 °C, decolorized with H_2PO_3, distilled twice, stored at 4 °C under N_2 and diluted to 6 N immediately before use. H_2O was deionized and distilled twice. CH_3OH, ethyl acetate and isopropanol (dried over $CaCl_2$ for 24 hrs) were distilled twice, all other solvents once (CH_2Cl_2 after drying over K_2CO_3). CH_3OH, C_2H_5OH and tetrahydrofuran were freed from H_2O by boiling over Na and distillation. All other chemicals were of p.a. quality.

Gas chromatography

A CARLO ERBA HRGC 4160 with a flame ionization detector was equipped with a 25 m * 0.22 mm ID fused silica WCOT Chirasil-L-Val DF 0.11 μm column (CHROMPACK) for determination of the absolute configuration of the amino acids.

Abbreviations: Common amino acids, 3-letter code; EI-MS and FAB-MS, electron ionisation and fast atom bombardement mass spectrometry; GC, gas chromatography; GC/MS, GC coupled with a mass spectrometer; COSY, correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer; HMBC, ^1H detected multiple-bond heteronuclear multiple quantum coherence; HMQC, ^1H detected 2D heteronuclear multiple quantum coherence; DSS, 2,2-dimethyl-2-silapentan-5-sulfonate; TMS, tetramethylsilane.

* Part LXVI of the series "Bacterial Constituents". For part LXV see Schaffner *et al.* (1996).

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Column chromatography

Adsorbents: DEAE-Sephadex A-25 and Sephadex LH-20 (PHARMACIA), Bio-Gel P-2 (BIO-RAD), RP-18 Sep-Pak(WATERS).

Spectroscopy

UV/VIS: Perkin-Elmer Lambda 7 and Hitachi 200, solvent H_2O; IR: Perkin-Elmer 283, CsI pellets; mass spectrometry: GC/MS Incos 500 XL (Finnigan), FAB-MS HSQ 30 (Finnigan-MAT) and MAT 731 (Varian-MAT) with an IonTech. Ltd FAB gun (Xe), matrix materials thioglycerol, *m*-nitro benzyl alcohol and diethanol amine, EI-MS HSQ 30 and Incos 500 XL; NMR: Bruker AM 300 and AC 300, solvents see text. For exchange of the OH- and NH-protons the samples were twice dissolved in D_2O, evaporated to dryness i.v. and redissolved in D_2O. For samples measured in H_2O/D_2O mixtures (9:1) the H_2O signal was suppressed by presaturation during the relaxation delay (Marion, 1990). Double resonance experiments: $^1J_{C,H}$ and $^3J_{C,H}$ COSY (Aue *et al.*, 1986), HMQC with additional GARP decoupling (Bax *et al.*, 1983) and HMBC experiments (Bax and Summers, 1986); for instrumental parameters see Table I. ^1H- and ^{13}C-chemical shifts are given relative to TMS with the internal standard DSS using the relations δ(TMS) = δ(DSS) for ^1H and δ(TMS) = δ(DSS) - 1.61 ppm for ^{13}C.

Table I. Instrumental parameters for NMR double resonance experiments.

		COSY	HMQC	HMBC
TD 1		256	256	512
TD 2		2048	2048	2048
t_1 experiments		256	256	512
NS		32	64	160
SI 1		512	512	1024
SI 2		2048	2048	2048
Spectral width [Hz]	F2	2400	6545	6410
	F1	2400	2451	2404
Delay			4 ms	50–140 ms

Bacterial growth

1 l culture medium contained 13.0 g Na gluconate, 4.0 g $KH_2PO_4 \cdot 3H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 5.0 g $(NH_4)_2SO_4$. The solutions of the organic and the inorganic material were sterilized separately (20 min, 130 °C, $2*10^5$ Pa), poured together and after cooling adjusted to pH 7.4 with 2 N NaOH. 50 ml culture medium in a 250 ml Erlenmeyer flask were inoculated with *Chryseomonas luteola* from an agar plate. After 24 hrs shaking under passive aeration 10 ml each of the culture were transferred to 500 ml Erlenmeyer flasks containing 200 ml culture medium. These main cultures were allowed to grow under rotary shaking (125 rpm), room temp., artificial light and passive aeration. The pH was kept between 7.4 and 8.0 by addition of 6 N HCl. After 3-4 days 4 ml of a 5% aqueous solution of Fe(III) citrate was added to each culture flask, the pH was brought to 5.5 with HCl and the cells were removed by tangential filtration.

Isolation

The solution was brought onto an XAD-4 column, inorganic material was washed out with several liters of H_2O and the ferric siderophores were desorbed with acetone/H_2O 1:1 (v/v), the solution was evaporated to dryness i.v. and the residue (*ca.* 100 mg from a 1 l culture) was stored at −25 °C. The crude extract was dissolved in 0.2 N pyridinium acetate buffer (pH 6.0) and chromatographed on Bio Gel P-2 with the same solvent; detection at 310 nm. The main (violet) fraction was collected and rechromatographed on DEAE-Sephadex with a buffer (pH 6.0) of increasing amounts of pyridinium acetate (0.2 to 1.0 N); detection at 310 and 510 nm. Two fractions were collected, viz. 23 mg of impure **2** and 15 mg of impure **1**. **2** was rechromatographed on DEAE- Sephadex with 0.2 N pyridinium acetate buffer (pH 6.0) (yield 16 mg) and **1** on Sephadex LH-20 with CH_3OH/H_2O 7:3 (v/v) (yield 9 mg). For decomplexation the ferric catecholates were adsorbed on Sep-Pak cartouches which were rinsed with a 8% oxalate solution until complete decolorisation was observed. The siderophores were then desorbed with CH_3OH.

Hydrolysis and amino acid analysis

2 mg samples were hydrolyzed with 2 ml 6 N HI for 22 hrs at 110 °C, the solution was evaporated to dryness i.v. and analyzed after derivatization to their N/O-trifluoroacetyl-isopropyl esters (Husak and Masek, 1975) by GC (for determination of the absolute configuration) and GC/MS (for identification) by comparison with reference samples.

2,3-Dihydroxy-6-(1-pyridinio)-benzoic acid (**3**)

To a solution of 0.5 g 2,3-dihydroxy benzoic acid and 0.52 ml pyridine in 6 ml CH_3OH under vigorous stirring 0.83 g I_2 were added in small portions whereupon the solution turned dark violet. After ~5 min a fine precipitate was formed. After stirring for 2 hrs the mixture was refluxed for 30 min, cooled and the precipitate was removed by centrifugation, washed with a small amount of CH_3OH and dried i.v. Yield 0.81 g (71%). For the ^1H- and ^{13}C-NMR data see Tables II and III.

Chryseomonin (**2**) *and* N^2*-[2,3-dihydroxy-6-(1-pyridinio)-benzoyl]-Lys* (**4**) *from chrysobactin* (**1**)

To 200 mg **1** dissolved in 86 mg pyrdine and 10 ml CH_3OH under vigurous stirring 137 mg I_2 were added whereupon the solution turned dark violet. After additional stirring for 2 hrs and subsquent heating under reflux for 30 min the mixture was cooled to room temperature and stirred for 12 hrs. Then a saturated solution of $Na_2S_2O_3$ was added until the solution was decolorized. The solvent was removed i. v. and the residue was chromatographed on Biogel P2 with 0.1 N CH_3COOH yielding two fractions, *viz.* 25 mg of **2** and 13 mg of **4.**

Results and Discussion

Chrysobactin (**1**)

One of the siderophores obtained from *Chryseomonas luteola* could be shown to be chrysobactin by identification of the degradation products as D-Lys, L-Ser and 2,3-dihydroxybenzoic acid and by comparison of the ^1H- and ^{13}C-NMR and mass spectra with those of authentic material (Persmark *et al.*, 1989; Ehlert *et al.*, 1994).

Chryseomonin (**2**)

Catecholate siderophores typically show UV absorption maxima at ~250 and 315 nm (e.g., Taraz *et al.*, 1990; Ehlert *et al.*, 1994). **2**, however, shows a maximum at 325 nm while the one at 254 nm appears as shoulder on the strong π,π*-transition of the pyridinium cation (for 1,2-dihydroxy-4-(1-pyridinio)-benzene at 249 nm; Bandopadhyay *et al.* (1976)). The Fe^{3+}-complex of **2** shows in addition to the absorptions of the aromatic systems a broad pH-dependant (e.g., Taraz *et al.*, 1990) charge transfer band of low intensity (pH 13 ... 470-500 nm, red; pH 7 ... 490-530 nm, violet; pH 4

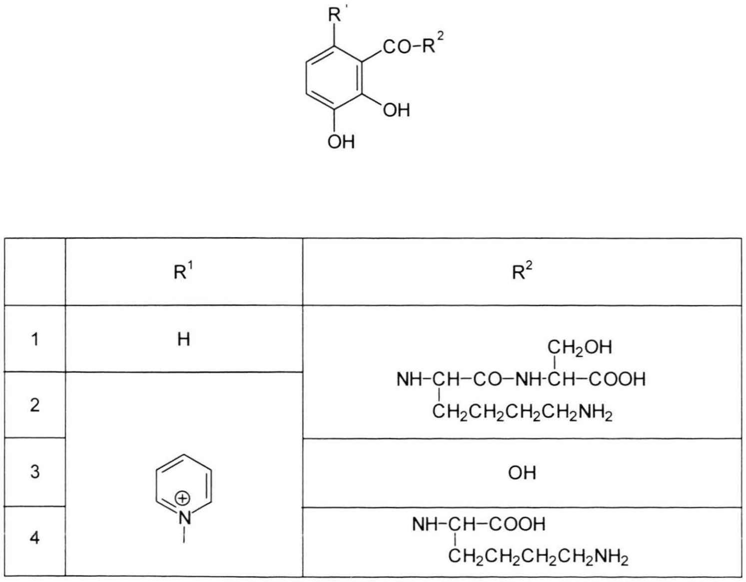

Fig. 1. Structural formulas of the compounds discussed.

Table II. ^{1}H-NMR data of compounds **1–4** (unbuffered D_2O).

Assign-ment	**1**	**2**	**4**	**3**
Pyridine				
H-1, H-5		8.88 d	9.06 d	8.90 d
H-2, H-4		8.15 t	8.15 t	8.15 t
H-3		8.70 t	8.85 t	8.72 t
DHB				
H-4	7.15 dd	7.20 d	7.34 d	7.17 d
H-5	6.94 t	7.13 d	6.94 d	6.97 d
H-6	7.35 dd			
Lys				
α-CH	4.69 dd	4.41 dd	4.30 dd	
β-CH_2	1.93–2.10 m	1.76–1.88 m	1.86–1.93 m	
γ-CH_2	1.50–1.62 m	1.39–1.51 m	1.42–1.55 m	
δ-CH_2	1.77 m	1.68 m	1.81 m	
ε-CH_2	3.05 t	3.00 t	3.13 t	
Ser				
α-CH	4.39 t	4.28 t		
β-CH_2	3.83 d	3.89 d		

DHB, dihydroxybenzoyl unit; d, doublet; dd double doublet; t, triplet; m, multiplet.

Table III. ^{13}C-data of compounds **1–4** (unbuffered D_2O). For C,H-correlations see Fig. 1.

Assign-ment	**1**	**2**	**4**	**3**
Pyridine				
C-1, C-5		146.7	146.8	146.9
C-2, C-4		129.0	129.0	128.6
C-3		148.1	148.1	148.4
DHB				
C-1	118.5	117.8	122.0	115.1
C-2	147.6	145.6	144.4	142.3
C-3	145.7	149.1	148.9	147.3
C-4	120.8	119.1	117.9	118.0
C-5	121.1	119.9	119.3	118.4
C-6	120.7	133.7	133.4	136.1
CO	171.2	166.5	166.2	177.2
Lys				
α-CH	54.9	54.8	54.0	
β-CH_2	31.9	31.6	39.7	
γ-CH_2	23.2	23.1	23.1	
δ-CH_2	27.4	27.3	27.1	
ε-CH_2	40.4	40.3	40.3	
CO	174.6	173.4	175.9	
Ser				
α-CH	58.2	58.2		
β-CH_2	63.1	63.1		
CO	176.9	176.7		

DHB, dihydroxybenzoyl unit.

... 520-600 nm, blue). The FAB-MS exhibits M^+ at *m/z* 447 corresponding to $[\mathbf{1} - H + C_5H_5N]^+$. Upon hydrolysis as determined by GC and GC/MS D-Lys, L-Ser and 2,3-dihydroxybenzoic acid could be identified.

The structure of **2** was finally established by NMR spectroscopy. In Tables II and III are assembled the ^{1}H- and ^{13}C-data of **1**, **2** and of the comparison compounds 2,3-dihydroxy-6-(1-pyridinio)-benzoic acid (**3**) and of N^2-[2,3-dihydroxy-6-(1-pyridinio)-benzoyl]-Lys (**4**).

^{1}H-NMR experiments

The data for **1** and **2** agree well with the exception that **2** lacks the H-6 signal of the dihydroxybenzoyl unit and it shows the pyridine signals in agreement with **3** and **4.** The coupling pattern of the peptide part was determined by H,H-COSY. Additional measurements in H_2O/D_2O at 5 °C allowed the detection of the NH-signals (Marion, 1990) which could then be identified by decoupling experiments: Lys-NH 9.48 ppm, d; -NH_2 7.69, broad s; Ser-NH 8.31 ppm, d.

^{13}C-NMR experiments

The multiplicity of the carbon atoms was determined by DEPT, direct H,C-connections by a HMQC experiment, while quaternary carbon atoms were identified by HMBC using $^2J_{C,H}$ and $^3J_{C,H}$ coupling. In this way it was possible to determine correlations between a carbonyl C-atom and the α-CH of the same amino acid (^2J) as well as the α-CH of the next amino acid (^3J). This allows *i. a.* to establish the sequence Lys-Ser. The correlations thus determined are assembled in Fig. 2. Again the shift data for **1** and **2** agree well except (cf. **3** and **4**) that the C-6 signal of the dihydroxybenzoyl unit is shifted downfield and the additional pyridine signals are observed.

The ^{1}H-NMR of **2** shows the presence of two hydrogen atoms in *ortho* position in the dihydroxybenzoyl unit. The pyridine ring could, therefore, be either bound to C-4 or to C-6. The former possibility was ruled out by the following observation: A HMBC experiment with **1** optimized for coupling at ~6 Hz shows a cross peak between the signal δ = 171.2 ppm (DHB-CO) and the signal at δ = 7.35 ppm (H-6 of DHB) corresponding to a $^3J_{C,H}$ coupling. An analogous $^3J_{C,H}$ coupling could

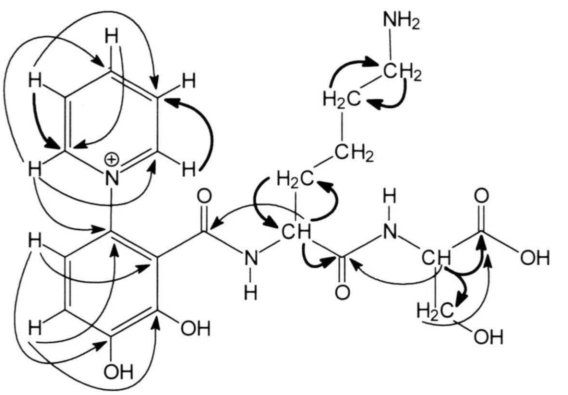

Fig. 2. $^2J_{C,H}$- (dark arrows) and $^3J_{C,H}$-couplings (light arrows) observed for **2**.

not be found for **2.** In the same way the position of the pyridine ring in **3** could be established: Also here no cross signal between the CO- and the H-6 signals was found in HMBC experiments.

2 could be synthesized by treating **1** with pyridine and I_2 in CH_3OH (Bandopadhyay *et al.*, 1976; Saxena *et al.*, 1978). During this reaction the Lys-Ser bond was cleaved in part and **4** was obtained as a side product. Natural and synthetic **2** proved to be identical in every respect (chromatographic behavior, NMR and MS).

The bacterial extract had been chromatographed using a pyridinium acetate buffer. Though chemically rather unlikely there remained the remote chance that **2** was an artefact formed from **1.** To exclude this possibility the following experiments were performed: (a) **1** was kept for several days in contact with the buffer solution and the various column materials used. No trace of **2** could be detected afterwards. (b) The bacterial extract was worked up by using 0.1 N acetic acid instead of the pyridinium acetate buffer. **2** could be identified by ^{1}H-NMR and FAB-MS.

Fe^{3+}-complex of **2**

The FAB-MS of the Fe^{3+}-complex of **2** shows a M^+ at *m/z* 946 which corresponds to $[(\mathbf{2})_2 - 4H + {}^{56}Fe]^+$. M^+ for a ligand-to-Fe ratio of 1:1 or 3:1 were absent. This behavior of **2** corresponds to that of **1** under physiological conditions which also forms a 2:1 complex (Persmark *et al.*, 1989).

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