Achromobactin, a New Citrate Siderophore of *Erwinia chrysanthemi*[+]

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The structure of a citrate siderophore named achromobactin isolated from the culture medium of *Erwinia chrysanthemi* was elucidated by spectroscopic methods and chemical degradation.

Introduction

Many microorganisms when grown under iron deficient conditions produce Fe^{3+}-chelating compounds (so-called siderophores) to make available otherwise insoluble ferric oxide hydrates present in the soil, or organic-bound iron when affecting living organisms. Though to the genus *Erwinia* belong dangerous phytopathogens as *E. amylovora* responsible for the fireblight disease (e.g., Feistner, 1988) of members of the Rosaceae causing severe damages in orchards, rather little is known about its siderophores. They are quite heterogenous: *E. amylovora* produces the hydroxamate proferrioxamines (Feistner *et al.,* 1993; Kachadourian *et al.*, 1996), *E. chrysanthemi* the catecholate chrysobactin (Persmark *et al.,* 1989; Persmark and Neilands, 1992), *E. herbicola* hydroxamate ferrioxamine E (Berner *et al.*, 1989), *E. rhapontici* the pyridyl pyrroline proferrorosamine A (Feistner *et al.*, 1983), and *E. carotovora* chrysobactin (Barnes and Ishimari, 1999) and the citrate siderophore aerobactin (Ishimaru and Loper, 1992). Whether the trihydroxypyridine derivative rubrifacine from *E. rubrifaciens* (Feistner *et al.*, 1984; Feistner and Budzikiewicz, 1985) acts as a siderophore is not known. Systematic studies of *E. chrysanthemi* and of its mutants (Mahé *et al.*, 1995) indicated this species possesses in addition to the chrysobactin mediated one a second iron transport system. The siderophore involved was named achromobactin since it did not give a color reaction upon addition of Fe^{3+}. The structure elucidation of achromobactin will be reported here.

Material and Methods

Instruments

Mass spectrometry: 900 ST (ESI), Incos 50 XL (all Finnigan-MAT, Bremen) with a Varian (Sunnyvale, CA, USA) gas chromatograph 3400 (capillary column CB SE-54, 25 m, 0.25 mm).

NMR: DRX 500 (Bruker, Karlsruhe), ^1H 500, ^{13}C 125 MHz, chemical shifts relative to TMS with internal standard DSS: $\delta(TMS) = \delta(DSS)$ for ^1H, $\delta(DSS) - 1.61$ ppm for ^{13}C. The samples were freed from traces of Fe^{3+} by passing through a Sep-Pak cartouche loaded with 8-hydroxyquinoline. For the measurements 10 mg **1** were dissolved in 0.5 ml H_2O and the pH was brought to 7 by addition of a 0.1 M solution of NaOH. The sample was

[+] Part LXXXIX of the series "Bacterial Constituents". For Part LXXXVIII see Ruangwiryachai *et al.*, 2000.

Abbreviations: CAS test: chrome azurol S test; DAB, 2,4-diaminobutanoic acid; TAP-derivatives, N/O-trifluoroacetyl isopropyl esters; GC, gas chromatography; MS, mass spectrometry; ESI, electrospray ionization; COSY, correlated spectroscopy; DEPT, distortionless enhancement by polarization transfer; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; NOE, nuclear Overhauser effect; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane.

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then lyophilized and redissolved in H_2O/D_2O (9:1, v/v) containing DSS. The H_2O signal was suppressed by presaturation during the relaxation delay. The ^{13}C spctra were recorded with broad-band decoupling. Temperature 25 °C.

UV: Perkin-Elmer (Überlingen) Lambda 7 photometer. Sample concentratiom 150.10^{-6} mol/liter, temperature 25 °C.

Chromatography: HPLC Knauer (Berlin), column Nucleosil 100 C_{18} (5 μm) (Knauer, Berlin); low pressure chromatography columns Sephadex G-10 and G-25 (Pharmacia, Uppsala, S).

Chemicals

H_2O was desalted and distilled twice in a quartz apparatus. Organic solvents were distilled over a column. Reagents were p. a. quality.

Production and isolation of **1**

For the production of **1** four 2-liter flasks containing 500 ml of succinate medium (Meyer and Abdallah, 1978) were inoculated by 5 ml each of an overnight culture of the mutant *cbsE-1 tonB60* of the *Erwinia chrysanthemi* strain 3937 isolated from African violet (*Saintpaulia ionantha* Wendl.) (Enard and Expert, 2000) in a succinate medium. The flasks were incubated on a rotary shaker at 30 °C for 24 hrs, after which the cells were removed by centrifugation. The supernatant was chromatographed on G-25 Sephadex resin with H_2O and the eluate was lyophilized. 73 mg of the lyophilisate were dissolved in 2 ml H_2O. The solution was chromatographed on G-10 Sephadex with H_2O (detection at 214 nm). 10 ml fractions were collected, concentrated and submitted to the CAS-test (Schwyn and Neilands, 1987) for Fe^{3+}-complexing substances. CAS-positive fractions were then tested for the presence of phosphate with $(NH_4)_2MoO_4$ and positive samples were discarded. The phosphate-negative samples were combined and brought to dryness i. v., yield 35 mg. Final purification was achieved by RP-HPLC on Nucleosil 100 C_{18} with an acetronitrile (A) and 0.1% CF_3COOH (B) gradient (3% A, 5 min; 3 to 30% A, 20 min; 30% A, 5 min; 30 to 3% A, 10 min). Only one peak was observed and collected (13–16 min), brought to dryness i. v., redissolved in H_2O and lyophilized; yield 25 mg.

Hydrolysis, TAP-derivatization and analyis of **1**

Hydrolysis was performed in pressure resistant pyrex tubes for 15 hrs with 6 N HCl at 110 °C. The hydrolysate was brought to dryness i. v. For the TAP derivatization see Voss *et al.* (1999). The products were analyzed by GC/MS on a L-Chirasil-Val column using standard substances for comparison.

Results

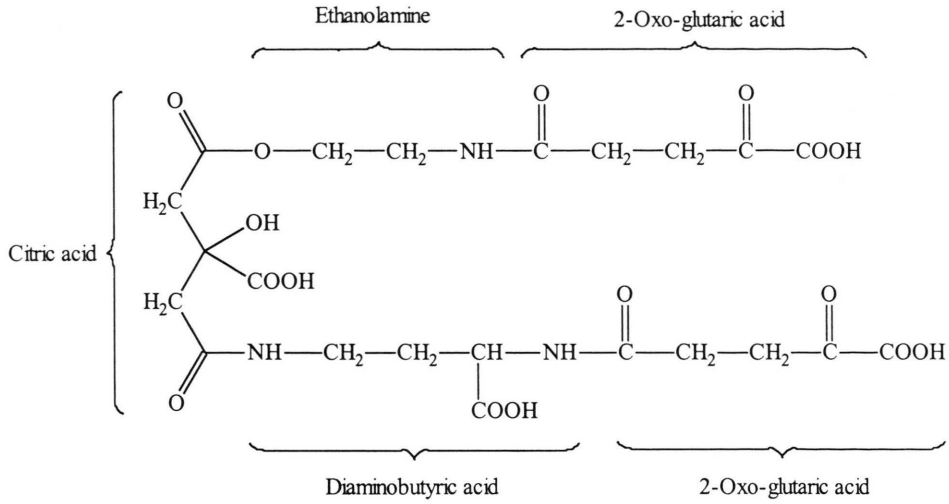
Structure of **1**

Total hydrolysis with 6 N HCl, TAP derivatization and GC-MS-analysis with a chiral column allowed to identify the following compounds: 1-amino-2-hydroxy-ethane (ethanolamine) (Eta), citric acid (Cit) (two peaks are observed which by comparison with a standard could be identified as di- and tri-isopropyl ester), L-2,4-diaminobutanonic acid (Dab) and succinic acid (Suc) (the typical decomposition product of 2-oxo-glutaric acid – Ogl – under hydrolysis conditions). The colorless siderophore shows only one absorption maximum at 195 nm (log ε = 4.18). The molecular mass of **1** was determined by ESI-MS as 591u.

For the discussion of the NMR data (for details of the various techniques see Evans, 1995) the numbering given in Fig. 1 will be used. The chemi-

Table I. ^1H- and ^{13}C-NMR data of **2**.

Substructure	Position	^1H	^{13}C
cyc-Ogl-1	1	–	177.8
	2	–	93.8
	3	2.47/2.07	33.0
	4	2.63/2.50	30.2
	5	–	179.6
Eta	6	3.68/3.19	40.3
	7	4.22/4.19	63.3
Cit	8	–	173.0
	9	2.93/2.76	44.2
	10	–	75.7
	10′	–	180.2
	11	2.64/2.57	46.2
	12	–	173.0
Dab	NH	7.85	–
	13	3.24/3.18	38.5
	14	2.11/2.03	29.0
	15	4.17	56.3
	15′	–	178.2
cyc-Ogl-2	16	–	179.1
	17	2.58/2.57	31.0
	18	2.47/2.04	34.2
	19	–	92.5
	20	–	177.4



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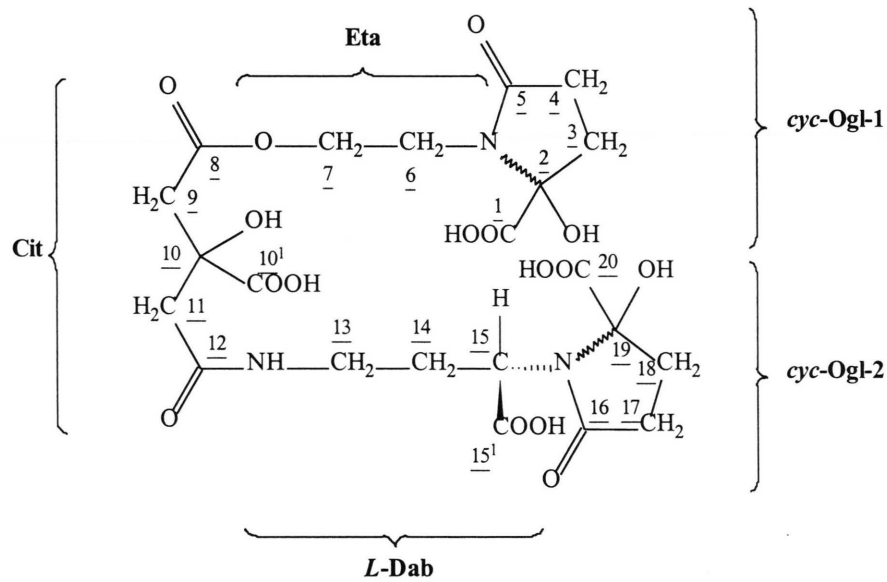

Fig. 1. **2** (cyclized structure of **1**) prevailing in neutral aqueous solution.

cal shifts observed agree well with those of reference compounds. Five separated spin systems can be recognized correlating geminal and vicinal protons (H,H-COSY). Measurement in H_2O/D_2O with H_2O suppression, where the NH-signals can be seen, show in the H,H-COSY spectrum two further cross peaks belonging to the Dab spin system. In the ^{13}C-spectrum 21 signals (2 Cit-CO signals coincide) can be observed whose multiplicity was determined with DEPT-135. C,H-correlations

were effected with the HMQC (^1J-) and HMBC (^2J- and ^3J-coupling). Connectivities between the five subunits were established by HMBC and by 1D-NOE experiments.

Dab-system: Cross peaks were observed between CH(15) and C(16) as well as C(19) connecting Dab with *cyc*-Ogl-2, and between NH and CO(12). Since C(12) and C(8) have identical chemical shifts the correlation to C(11) had to be established by an NOE experiment.

Eta-system: Cross peaks were observed between CH_2(6) and C(2) as well as C(5) establishing the connection with *cyc*-Ogl-1, and between CH_2(7) and CO(8). The correlation with CH_2(9) was again established by a NOE experiment.

Cit-system: The 4 quarternary C of Cit could be identified by their connections with the two CH_2-groups. The differences in their chemical shifts demonstrate an asymmetrically substituted Cit.

The two Ogl-systems: The signals of the -CH_2-CH_2-systems could be identified and their connection with the free COOH-groups was established. It had been noted before (Drechsel *et al.*, 1993; Münzinger *et al.*, 1999) that in citrate siderophores at neutral pH the signals expected for the CO-groups of 2-oxoglutaric acid (~200 ppm) are missing and that instead signals at ~93 ppm can be seen because of the formation of lactame structures (cf. Briskot *et al.*, 1986). In neutral aqueous solution the siderophore **1** practically completely exists in the cyclized form **2**.

Discussion

Siderophores produced by microorganisms comprise a wide variety of structural types which seem to reflect the special needs for survival. High complexing constants are necessary when the concentration of available Fe^{3+} is extremely low (as, e.g., enterobactin, the tris-catecholate siderophore of *Escherichia coli*), complex structural types as the peptidic pyoverdins of the fluorescent *Pseudomonas* spp. (Budzikiewicz, 1997) are produced when there are many competitors as in the soil and the ferri-siderophore should be recognized on the cell surface of the producing strain only. Such siderophores request special systems for biosynthesis, transport into the cell and release of the iron. Under less strenuous conditions simpler molecules with lower complexing constants are sufficient to maintain the iron supply. The complexone-type citrate siderophores belong to this group. The chrysobactin and achromobactin dependent iron transport pathways are differentially regulated by iron (Mahé *et al.*, 1995); the de-repression of the chrysobactin system requires more severe conditions of iron deficiency than that of the achromobactin system.

Citrate is readily available from the citric acid cycle. Its central α-hydroxy carboxylic acid part provides one of the binding sites for Fe^{3+}. To the two outer carboxyl groups in most cases rather simple molecules are attached which provide the two other ligands necessary for the formation of an octahedral complex. These ligands determine the complexing constant of the siderophore, its specificity and its physical properties: thus, higher solubility in water is achieved by additional carboxyl groups as in aerobactin (Gibson and Magrath, 1969), enhanced lipophily by longer aliphatic chains as in rhizobactin (Persmark *et al.*, 1993). Citric acid *per se* is achiral, but when the two outer carboxyl groups are substituted differently, the central carbon atom becomes chiral and two enantiomers can be formed (cf. Münzinger *et al.*. 1999). Structure elucidation of citrate siderophores can be complicated by the formation of cyclic products. The pH-dependant cyclization of 2-oxoglutaric acid was discussed before. In addition, acid catalized elimination of H_2O can lead to cycloimidic structures (Persmark *et al.*, 1993; Budzikiewicz *et al.*, 1997).

It deserves mentioning that several siderophores of the genus *Erwinia* are also produced by unrelated microorganisms, such as ferrioxamine E by actinomycetes (v. d. Helm and Poling, 1976), proferrorosamine A by *Bacillus (Pseudomonas) roseus fluorescens* (Pouteau-Thouvenat *et al.*, 1965) or aerobactin from *Aerobacter aerogenes* (Gibson and Magrath, 1969). For relatively simple compounds this may be due to an independently developed biosynthesis, but for more complex systems an exchange of genetic material should be considered.

The genetic variants of the strain 3937 of *Erwinia chrysanthemi* deficient in either chrysobactin or in achromobactin, the implications for pathogenity as well as iron uptake studies will be reported elswhere (Expert *et al.*, 2000).

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