Staphyloferrin B, a Citrate Siderophore of *Ralstonia eutropha**

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The structure and stereochemistry of the siderophore isolated from the culture medium of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) could be elucidated as staphyloferrin B by spectroscopic methods and chemical degradation. The relationship of the three species of the new genus *Ralstonia* is reflected in the observation that all three form citrate siderophores.

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. Siderophores may belong to a wide variety of structural classes (e.g., Budzikiewicz, 1997), but related bacterial species usually show a common pattern. The genus *Ralstonia* was established recently (Yabuuchi *et al.*, 1995) by combination of the species *Pseudomonas solanacearum* and *pickettii* with *Alcaligenes eutrophus*. For the two *Pseudomonas* spp. this was the second classificational transfer, for a short time they were attributed to the genus *Burkholderia*. The two former *Pseudomonas* spp. had been found to produce citrate siderophores: schizokinen, citric acid substituted at both terminal carboxyl groups with 1-N-hydroxy-1-N-acetyl-1,3-diaminopropane (Budzikiewicz *et al.*, 1997) and *enantio*-rhizoferrin, 2 citric acids connected at one of their terminal carboxyl groups with 1,4-diaminobutane (Münzinger *et al.*, 1999). It seemed, therefore, to be of interest whether the *Alcaligenes* added to the *Ralstonia* genus conformed also concerning its siderophore. The structure elucidation of the latter (**1**) and of its iron complex will be reported here.

Abbreviations: CAS test, Chrome azurol S test; DAP, 2,3-diaminopropanoic acid; TAP-derivatives, N/O-trifluoroacetyl isopropyl esters; GC, gas chromatography; MS, mass spectrometry; ESI, electrospray ionization; FAB, fast atom bombardment; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane.
Part LXXXIV of the series "Bacterial Constituents". For Part LXXXIII see Schneider *et al.* (1999).

Material and Methods

Instruments

Mass spectrometry: HSQ-30 (FAB, matrix glycerol, gas Xe), 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), ^{1}H 500, ^{13}C 125 MHz, chemical shifts relative to TMS with internal standard DSS: δ(TMS) = δ(DSS) for ^{1}H, δ(TMS) = δ(DSS) + 1.61 for ^{13}C. The samples (15 millimolar in D_2O or H_2O/D_2O 9:1 v/v) were degassed for 2 min in an ultrasonic bath. The H_2O signals were suppressed either by presaturation during the relaxation delay or by the WATERGATE puls sequence. The ^{13}C-spectra were recorded with broad-band decoupling. HMBC experiments were optimized for 6 Hz coupling.

UV/Vis: Ultrospec 2000 (Pharmacia, Uppsala, S), 0.15 millimolar solutions in H_2O. CD: Jasco 720 (Japan Spectroscopic Co. Ltd., Tokyo, Japan, 0.5 millimolar solution in H_2O, reference H_2O, 20 °C.

X-ray: Nonius Kappa CCD-Diffraktometer (Nonius, Delft, NL).

Chromatography: HPLC Knauer (Berlin), column Nucleosil 100 C_{18} (5 μm) (Knauer, Berlin);

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low pressure chromatography columns Biogel P-2 (Bio-Rad, Richmond CA, USA), Sephadex G-10 and QAE Sephadex A-25 (Pharmacia, Uppsala, S).

Chemicals

H_2O was desalted and distilled twice in a quartz apparatus. Organic solvents were distilled over a column and if necessary dried. Chemicals came from Fluka (Buchs, CH), Merck (Darmstadt) and Aldrich (Steinheim) and had p.a. quality, solvents for NMR contained ≥ 99.5% D.

Production and isolation of **1**

The mutant AE1153 of *Ralstonia eutropha* CH34 (Khan *et al.*, 1992) was grown in a succinate medium consisting of 4.0 g succinic acid in 400 ml H_2O (pH adjusted to 6.8 with 40% KOH solution), 3.3 g $K_2HPO_4{\cdot}3H_2O$ and 2.6 g KH_2PO_4 in 400 ml H_2O (pH 6.8), 1.5 g $(NH_4)_2SO_4$ in 100 ml H_2O, 0.5 g $MgSO_4{\cdot}7H_2O$ in 100 ml H_2O, 0.5 μmol Fe^{3+} (5% Fe^{3+}-citrate). An amount of ca. 50 μg/l Fe^{3+} is optimal to maintain both bacterial growth and siderophore production. The solutions were mixed and sterilized. The pH was adjusted to 6.5 with 6 M HCl. 125 ml culture medium in a 250 ml Erlenmeyer flask with indentations were inoculated from a stich agar culture and incubated until pH 7.2–7.5 was reached, then the pH was adjusted to 7.0 with 6 M HCl and the culture was stored in the dark at 5 °C. For the main cultures six 3-liter fermenters containing 1.33 l culture medium were inoculated with 1 ml of the storage culture. The pH of 8.5 reached after 48–60 hrs was adjusted to 6.0 with 6 M HCl. To guarantee an iron-free work-up all glass vessels were rinsed with 6 M HCl, the apparatus for tangential filtration and the chromatographic material was freed from Fe^{3+} by treatment with 8% K-oxalate buffer, oxalate was removed by rinsing with 1 M NaCl solution. Cell material was separated from the culture by tangential filtration. To the filtrate 0.2 g/l NaN_3 were added in order to avoid cellular growth and/or infections.

The filtrate was chromatographed on XAD-2 resin with H_2O and the eluate was lyophilized, yield 9–10 g lyophilized material per liter culture medium. Ca. 10 g were dissolved in enough H_2O to give a clear solution (ca. 300 ml) and rechromatographed with H_2O on Bio-Gel P-2. Fractions of 25 ml each were concentrated i.v. to 5 ml and subjected to a CAS test (Schwyn and Neilands, 1987) for Fe^{3+} complexing substances (after concentration to a smaller volume phosphates may interfere with the test). The siderophore was eluted in the first fractions followed by inorganic material and CAS-negative substances. The CAS-positive fractions were combined, brought to dryness i.v. (yield 1–2 g) and redissolved in ca. 20 ml H_2O. The solution was then chromatographed on an anion exchange column (QAE-Sephadex A-25 loaded with Cl^-, detection at 214 nm). A weakly yellow CAS negative fraction eluted with H_2O was discarded. The siderophore was then desorbed with H_2O with a NaCl gradient (increase 0.02 mol/l per hour). 10 ml fractions were collected and CAS tested. Elution of a CAS positive fraction started at 0.1 mol/l NaCl and elution was continued isocratically until all CAS positive material was removed from the column. The siderophore fractions were combined and concentrated i.v. to the point where NaCl just started to precipitate. The clear solution was chromatographed with H_2O on Sephadex G-10 and the CAS positive fraction collected, concentrated i.v. to ca. 5 ml and lyophilized. To remove the last traces of complexed Fe^{3+} (which would interfere with the NMR analysis) a RP-18 Sep-Pak cartouche was rinsed with 5 ml CH_3OH, subsequently twice with 5 ml H_2O and then treated with 5 ml of a saturated solution of 8-hydroxyquinoline, which stayed in the upper portion of the cartouche as a yellow zone. When a saturated solution of the chromophore was passed through the column the upper part of the 8-hydroxyquinoline zone turned black (formation of the Fe-complex). Lyophilization of the eluate gave a white powder, yield ca. 230 mg from 1 l culture medium.

Formation of the Fe^{3+} and the Ga^{3+} complexes of **1**

To a solution of 10 mg **1** in 1 ml H_2O (pH adjusted to 7.0) a 0.01 molar solution of $FeCl_3$ was added drop by drop and the pH continuously readjusted to 7.0, until the brownish color of $Fe(OH)_3$ could be observed. The Fe^{3+}-complex of **1** was chromatographed on Sephadex G-10 (eluent H_2O, detection at 310 nm) and the eluate lyophilized. Yield 10.5 mg of a yellowish powder.

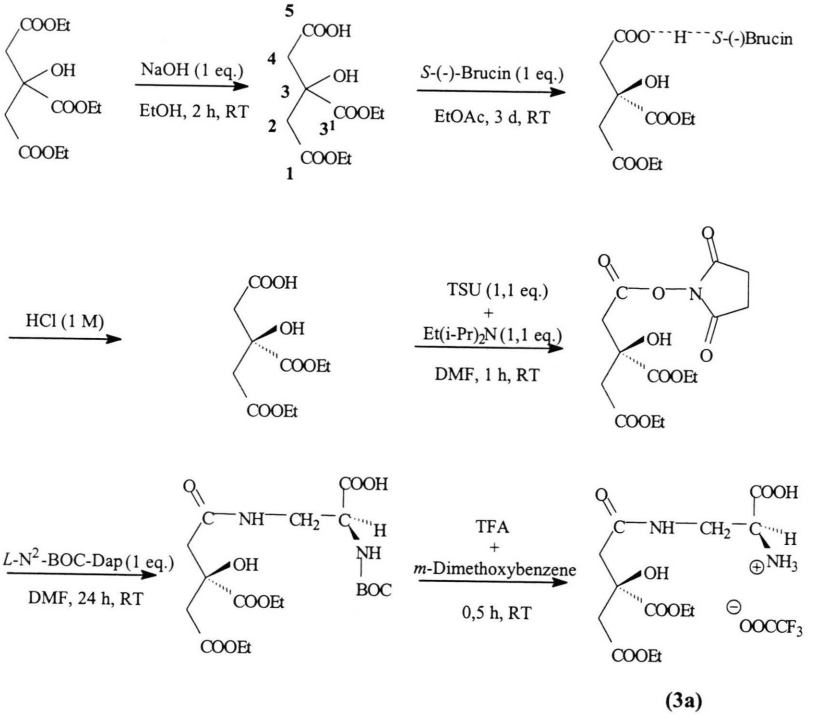
The Ga^{3+} complex of **1** was obtained in the same way by adding a 0.01 molar solution of $Ga(NO_3)_3$ until the solution became turbid from precipitated $Ga(OH)_3$. Detection during the chromatography was effected at 214 nm.

Hydrolysis, TAP-derivatization and analysis of the products

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. Alternatively, 1 mg of **1** dissolved in 2 ml of a 0.1 molar NH_4OCOCH_3 buffer (pH 7.4) was treated with 0.1 mg of the enzyme pronase. After stirring the solution for 24 hrs at 30 °C, the enzyme was removed by centrifugation and the remainder lyophilized. For the TAP derivatization see Voss *et al.* (1999). The products were analyzed by GC/MS on a L-Chirasil-Val column using for comparison standard substances.

Synthesis of stereochemical comparison compounds (Scheme 1)

R-Citric acid 1,3[1]-diethyl ester. The *rac*-diethyl ester was synthesized starting from citric acid triethyl ester in analogy to *rac*-dimethyl ester (Bergeron *et al.*, 1997), yield 45% of a colorless oil. ^{1}H-NMR ($CDCl_3$; δ ppm, mult.) 1.25, t; 1.27, t; 4.13, q; 4.28, q (ethyl groups); 2.80, AB; 2.85, AB (citric acid CH_2-groups). A solution of 3.94 g *S*-(-)-brucin and 2.48 g *rac*-diethyl ester in 150 ml dry ethyl acetate was stirred for 3 days at room temperature. The precipitated brucin salt of the *R*-diethyl ester was collected, washed with a small amount of ethyl acetate and dried i. v.; yield 2.85 g. From the reaction solution after concentration additional 0.11 g could be obtained. Twice recrystallizing from ethyl acetate gave 2.42 g of colorless crystals. The enantiomeric purity and stereochemistry of the brucin salt of *R*-citric acid 1,3[1]-diethyl ester was determined by X-ray crystallography. It forms ortho-



Scheme 1

Scheme 1. Synthesis of *R*-citric acid-5-(*S*-2-amino-2-carboxy-ethyl)-amide 1,3[1]-diethyl ester (**3a**).

rhombic crystals (a = 13.136, b = 14.895, c = 15.835 Å, $\alpha = \beta = \gamma = 90°$). To a solution of 1.93 g of the *R*-ester brucin salt in 50 ml H_2O 1 ml 6 M HCl was added. After stirring at room temp. for 5 min the solution was extracted three times with 50 ml ethyl acetate each, the organic phase was dried with Na_2SO_4 and the solvent removed i.v. It remained 0.63 g of a colorless oil whose ^{1}H-NMR spectrum corresponded to that of the *rac*-diethyl ester.

S-Citric acid-1,3^1-diethyl ester was obtained by HCl cleavage of the brucin salt isolated by bringing to dryness the mother liquid after crystallization of the *R*-ester brucin salt, with an enantiomer excess of about 90% (estimated from the amount of the precipitated *R*-ester salt).

R-citric acid-5-(*S*-2-BOC-amino-2-carboxy-ethyl)-amide 1,3^1-diethyl ester. To a solution of 247 mg *R*-citric acid 1,3^1-diethyl ester and 360 mg N,N,N',N'-tetramethyl(succinimido)uronium tetrafluoroborate (TSU) in 10 ml dry dimethylformamide 0.187 ml ethyl-di-isopropyl-amine were added drop by drop under vigorous stirring continued for 1 hr. The thus formed N-hydroxy-succinimidyl ester was reacted under strict exclusion of H_2O with 204 mg L-N^2-BOC-2,3-diaminopropanoic acid. After stirring for 24 hrs 50 ml H_2O were added and the pH was adjusted to about 2.5 with 0.1 M HCl. The solution was extracted three times with 20 ml ethyl acetate each, the combined organic phases were washed with 10 ml ca. 0.01 M HCl, dried with Na_2SO_4 and freed from the solvent i.v. The remaining yellowish oil was chromatographed on Nucleosil-100 C_{18} (5 μm), solvent 0.1% trifluoroacetic acid – acetonitrile (gradient 3 to 30% acetonitrile), detection at 214 nm, giving 50 mg of a colorless oil. ^{1}H-NMR ($CDCl_3$; δ ppm, mult.) 1.23, t; 1.25, t; 4.10, q; 4.22, q (ethyl groups); 2.72 and 2.85, superimposed multiplets (citric acid CH_2); 3.71, broad; 4.35, broad (CH_2-CH of diaminopropanoic acid); 5.88, broad; 7.90, broad (α- and β-NH); 1.41, s (BOC).

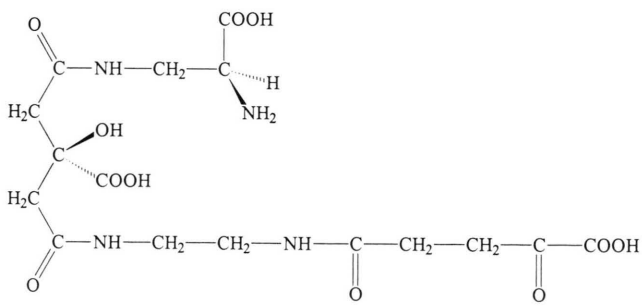
R-citric acid-5-(*S*-2-amino-2-carboxy-ethyl)-amide 1,3^1-diethyl ester (**3a**). A mixture of 50 mg of the BOC derivative, 0.1 ml 1,3-dimethoxybenzene and 5 ml trifluoroacetic acid were stirred for 30 min at room temp. Volatile components were removed i.v., the oily residue was dissolved in a small amount of acetonitrile and chromatographed as described above. To the fraction containing **3a** freed from solvent i.v., 2 ml H_2O were added. Lyophilization gave 40 mg of a crystalline powder. $[M + H]^+$ (by FAB-MS) *m/z* 335. ^{1}H-NMR (D_2O; δ ppm, mult.) 1.18, t; 1.23, t; 4.10, q; 4.20, q (ethyl groups), 2.80, AB; 2.81, AB (citric acid CH_2); 3.72, broad; 4.19, broad (CH_2-CH of diaminopropanoic acid). ^{13}C-NMR (D_2O, δ ppm) 13.1, 13.1, 62.2, 63.1 (ethyl groups); 43.5, 44.0 (citric acid CH_2); 73.5 (citric acid C-3), 171.2, 172.2, 169.4 (CO C-1, C-3^1, C-5), 174.5 (COOH); 116.0 and 162.3, both q (CF_3COO^-).

S-citric acid-5-(*S*-2-amino-2-carboxy-ethyl)-amide 1,3^1-diethyl ester (**3b**) was synthesized and characterized analogously.

Results

Structure of **1**

Total hydrolysis with 6 M HCl, TAP derivatization and GC-MS-analysis with a chiral column allowed to identify the following compounds: 1,2-diaminoethane, citric acid (two peaks are observed which by comparison with a standard are the di- and tri-isopropyl ester), D- and L-2,3-diaminopropanoic acid 7:10 and a small amount of succinic



1

acid. 2,3-diaminopropanoic acid (Dap) racemizes under acidic conditions (TAP-derivatization of the L-isomer gives D:L 1:10, after treatment with 6 M HCl at 110 °C for 15 hrs 7.5:10, after 21 hrs. 1:1). After TAP derivatization following cleavage of **1** with the enzyme pronase (Garner *et al.*, 1974) a ratio D:L of 0.5:10 was observed indicating that L-Dap is present in **1**.

The colorless siderophore shows only one almost pH independant absorption maximum (pH 3.0: 206 nm, ε = 14800; pH 7.5: 211 nm, ε = 15300 $M^{-1}.cm^{-1}$) that can be attributed to π,π^*-transitions of carbonyl groups. Taking into account the components mentioned above the molecular composition of the $[M + H]^+$-ion could be determined by ESI-MS as $C_{16}H_{25}N_4O_{11}$ (found 449.1505, calc'd 449.1520) and confirmed by the exact mass of the $[M + H - H_2O]^+$-ion (found 431.1409, calc'd for $C_{16}H_{23}N_4O_{10}$ 431.1414). Fragment ions are formed only by the loss of up to 3 H_2O and of CO_2. The molecular composition amounts to one CO-unit more than obtained by the combination of citric acid, Dap, diaminoethane and succinic acid minus 3 H_2O (formation of 3 amide bonds). This suggests the presence of 2-ketoglutaric acid known to decompose under the conditions of hydrolysis giving partially succinic acid.

A siderophore (staphyloferrin B) consisting of these four components was isolated from various *Staphylococcus* spp. (Drechsel *et al.*, 1993; Haag *et al.*, 1994) and structure **1** had been proposed for it. The ^{13}C-NMR data reported agree well with those (Table II) of the *Ralstonia* siderophore. However, several questions regarding the structure had remained open: Thus, amide formation at the carboxyl groups C-1 and C-5 of citric acid had been assumed probably in analogy to other citrate siderophores, and the stereochemistry at C-3 had not been determined. Also, nothing is known about the structure of the Fe^{3+}-complex.

For the discussion of the NMR data (for a detailed description of the various techniques see Evans, 1995) the numbering (^{13}C, Table II) and lettering (^{1}H, Table I) given in Fig. 1 will be used. The chemical shifts observed agree well with those of reference compounds. In the H,H-COSY spectrum (solvent D_2O) four separated spin systems can be recognized correlating geminal (^2J) and vicinal (^3J) protons. Geminal protons can further be identified by their ^1J coupling with the

Table I. ^{1}H-NMR spectroscopic data of **2**, **Ga-2** and differences $\Delta\delta$ between **2** and **Ga-2** H_2O/D_2O, 5 °C, pH 7,0, 500 MHz.

Substructure[a)]	Proton[a)]	δ (**2**) (mult.[b)]; *J* [Hz])	δ (**Ga-2**)	$\Delta\delta$ [δ(**Ga-2**) – δ(**2**)]
***cyc*-Kgl**	*A*	2.63 (m)	2.64	0.01
	B	2.51 (m)	2.57	0.06
	C	2.43 (m)	2.36	−0.07
	D	2.06 (m)	2.03	−0.03
Dae	*A*	3.53 (m)	3.86	0.33
	B	3.38 (m)	3.71	0.33
	C	3.38 (m)	2.86	−0.52
	D	3.07 (m)	2.59	−0.48
	X	7.91 (b)	9.34	1.43
Cit	*A-1*	2.68 (d; *14.6*)	2.78	0.10
	B-1	2.60 (d; *14.6*)	2.59	−0.01
	A-2	2.70 (dd; *1.5*/*14.5*)	2.65	−0.05
	B-2	2.63 (dd; *1.8*/*14.5*)	2.48	−0.15
Dap	*A*	3.89 (dd; *3.5*/*7.4*)	3.79	−0.10
	B	3.88 (m)	4.36	0.48
	C	3.55 (m)	3.39	−0.16
	X	8.14 (b)	7.66	−0.48
	NH_2	6.78 (b)	3.99 / 4.79	−2.79 / −1.99

a) Numbers refer to Fig. 1.
b) Multiplicities are as follows: d doublet, dd doublet of doublets, m multiplet, b broad.

Table II. ^{13}C-NMR-spectroscopic data of **2**, **Ga-2** and the differences Δδ between **2** and **Ga-2**. H_2O/D_2O, 5 °C, pH 7,0, 125 MHz.

Substructure[a)]	C-atom[a)]	δ **2** (Mult.)[b)]	δ **Ga-2**	Δδ [δ(**Ga-2**) – δ(**2**)]
***cyc*-Kgl**	1	177.9 (s)	183.1	5.2
	2	93.8 (s)	93.7	−0.1
	3	33.2 (t)	34.6	1.4
	4	30.5 (t)	31.3	0.8
	5	179.8 (s)	179.1	−0.3
Dae	6	41.1 (t)	41.9	0.8
	7	40.9 (t)	40.4	−0.5
Cit	8	173.8 (s)	174.3	0.5
	9	46.0 (t)	48.2	2.2
	10	76.4 (s)	77.4	1.0
	10^1	180.6 (s)	183.8	3.2
	11	45.8 (t)	50.1	4.3
	12	174.8 (s)	176.8	2.0
Dap	13	41.1 (t)	42.0	0.9
	14	56.5 (d)	56.0	−0.5
	15	173.6 (s)	179.8	6.2

a) Numbers refer to Fig. 1.
b) Multiplicities estimated by the DEPT-135-experiment are as follows: s singlet, d doublet, t triplet.

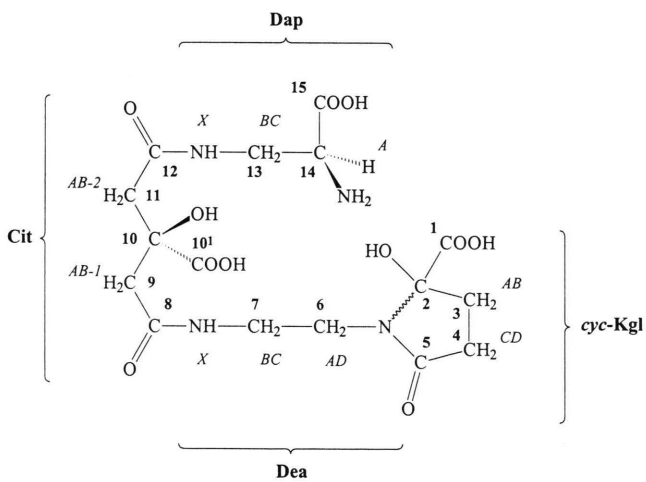


Fig. 1

Fig. 1. Numbering and lettering scheme for the discussion of the NMR data of **2**.

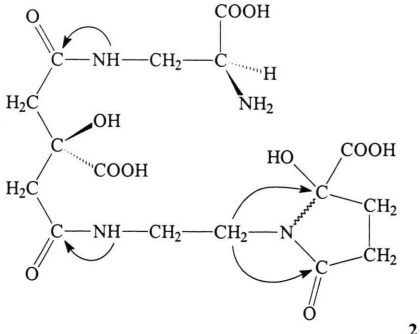
same C-atom in the HMQC spectrum. Measurement in H_2O/D_2O with H_2O suppression, where the NH-signals can be seen, show in the H,H-COSY spectrum three further cross peaks belonging to the Dae and Dap spin systems. Only for the free NH_2-group of Dap the relaxation is too fast for a coupling with other nuclei. In the ^{13}C-spectrum 16 signals 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).

Dap-system: The spin system (in D_2O) A-BC (AB 3.6, AC 7.5 Hz, BC 14.8 Hz) is extended to an A-BC-X system in H_2O/D_2O. A cross peak CH(A)/CO(15) completes the structure. Of importance is the cross peak between the Dap-NH and CO(12) of the citric acid connecting Dap with one of the terminal carboxyl groups.

Cit-system: The 4 quarternary C of Cit can be identified by their connections with the 2 CH_2-groups (showing geminal coupling of 14.4 and 14.5 Hz, respectively). The small differences in chemical shifts of the CH_2-groups demonstrate an unsymmetrically substituted Cit, though with similar residues.

Dae-system: The expected cross peaks allowed the identification of the signals corresponding to the various H- and C-atoms. Of importance is again the cross peak between the NH-signal and CO(8) of the citric acid showing that indeed the two terminal carboxyl groups are derivatized.

Kgl-system: The signals of the -CH_2-CH_2-system can be identified and its connection with the free COOH-group can be established. It had been noted before (Drechsel *et al.*, 1993) that the signal expected for the CO-group of 2-ketoglutaric acid (~ 200 ppm) was missing and that instead one at 96.4 ppm (corresponding to 93.8 ppm in Table II) could be seen. Coupling of the CH_2(AD) protons of Dae with C(2) and C(5) (arrows in Fig. 2) show, that a lactam is formed (cf. Briskot *et al.*, 1986). In aqueous solution the siderophore **1** practically completely exists as **2**.

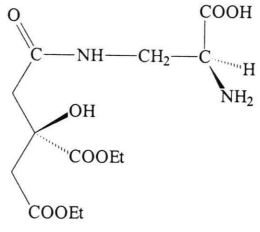


2

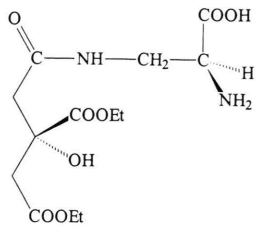
Fig. 2. Cyclized structure **2** of **1** prevailing in aqueous solution (arrows indicate ^1H, ^{13}C-cross peaks in the HMBC spectrum).

Configuration of C-3 of the unsymmetrically substituted citric acid in **1**

1 shows a positive Cotton effect at 217 nm (pH 5.0) and 215 nm (pH 6.0). The further course of the absorption curve indicates a minimum at about 190 nm. In order to correlate the CD-spectrum with the configuration at C-3 of **1** the *R*- and *S*-model compounds **3a** and **3b** had been



3a



3b

synthesized. **3** contains *S*-Dap so that its possible influence on the CD curve is accounted for. The additional chiral center of the amide ring in the cyclic structure **2** is probably irrelevant due to a fast equilibrium or a sterically non preferential ring formation. Comparison of the CD-curve with those of **3a** and **3b** shows a close approximation of the one of **1** with that of **3a** (the *R*-model) suggesting an *S*-configuration at C-3 of the citric acid part of **1** (it should be noted that the *R,S*-nomenclature is a formal one and that the absolute configurations regarding C-3 of the citric acid part of the molecules of *R*-**3** and *S*-**1** are identical).

Complexing sites of **2**

Fe^{3+}-complexes can not be investigated by NMR spectroscopy as a consequence of the paramagnetism of the metal nucleus. Ga^{3+} has comparable molecular characteristics and hence Ga^{3+}-complexes can serve as reliable models. Ga^{3+} forms a 1:1 complex with **2** (in the same way as Fe^{3+}, see below) as determined by FAB-MS: Ga^{3+} replaces 3 H^+. Chemical shifts in the NMR spectra of the free ligand and the Ga-complex allow to recognize the binding sites. The complex formation is apparently not complete, the ^1H- and ^{13}C-NMR spectra show a superposition of those of the complex and of the free ligands as confirmed by H,H-COSY-, HMQC- and HMBC-measurements. The ^1H- and ^{13}C-data of the Ga-complex can be found in Tables I and II. By the complexation primarily the chemical shifts of the ^{13}C-atoms of the binding sites are influenced. The observed ^1H-shifts stem overwhelmingly from solvatation and/or conformational effects (Tappe, 1995).

Shift differences in the ^1H-spectra (see Table I) in the Kgl and Cit parts of the molecule are negligible, they are evident, however, in the Dea and Dap parts. The increased differences in the chemi-

cal shifts of the geminal CH_2-protons indicate a conformational change and a rigid structure. The rather large shift differences of the NH-protons can be explained by different solvation due to conformational changes. For the ^{13}C-spectrum of the Ga-complex the most significant shift differences are observed for the three carboxyl groups, which obviously are binding sites. For geometrical reasons the three other ligands necessary to form the octahedral complex are the free NH_2- and OH-groups. The almost negligible influence on the shifts of the carbon atoms 2, 10 and 14 (Fig. 1) can be explained by the observation, that in the Ga^{3+} complex the H-atoms are retained (Carrano *et al.* 1996) as indicated by its molecular mass (replacement of 3 H^+ from the carboxyl groups, see above). The protons of the Dap-NH_2 group give two sharp signals in agreement with their fixed position caused by the complexation. The large shift differences especially of the C-atoms 9 and 11 of the citric acid part are due to steric strain: The relatively short Dap chain distorts somewhat the octahedral structure of the complex ($\Delta\delta$ 4.3 ppm for C-11), while the longer Dae-Kgl chain results in a less pronounced effect ($\Delta\delta$ 2.3 ppm for C-9).

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

The Fe^{3+} complex of **2** shows a 1:1 stoichiometry as demonstrated by its molecular mass determined by FAB-MS (replacement of 3 H^+ by Fe^{3+}). The UV/Vis spectrum shows at pH 7.5 a maximum at 200 nm (ε = 14500 $M^{-1}\cdot cm^{-1}$) for the carbonyl π,π^*-transition and a broad charge transfer band with a maximum at 320 nm (ε = 1670). Changes of the pH are of small influence: pH 6.0 and 4.0 198 nm (ε = 14500) and 320 nm (ε = 1650 nm). The positive Cotton effect corresponding to the charge transfer band indicates a Λ-configuration of ferri-**2** (Wong *et al.*, 1983; Teintze *et al.*, 1981).

Discussion

The structure of the siderophore of *Ralstonia eutropha* is that of staphyloferrin B; the configuration at the central carbon atom of the citric acid part could be established as *S* (**1**). It forms 1:1 Ga^{3+}- and Fe^{3+}-complexes. In aqueous solution **1** in free as well as in complexed form cyclizes to **2** at least at neutral pH. The Fe^{3+}-complex has Λ-conformation.

Thus, all three *Ralstonia* spp. produce citrate siderophores. This is a further justification for the grouping together of the former *Pseudomonas* and *Alcaligenes* spp. It is interesting to note that the siderophores are produced also by completely unrelated microorganisms: Schizokinen from *R. solanacearum* by *Bacillus megaterium* and *Anabaena* sp. (Cyanobacteria), rhizoferrin from *R. pickettii* - though with opposite configuration – by *Rhizopus* spp. and other Zygomycetes, and staphyloferrin B from *R. eutropha* by several *Staphylococcus* spp.

To expect a parallel biosynthetic behavior may not be unreasonable for relatively simple compounds as schizokinen or rhizoferrin (that bacteria and fungi produce the enantiomers of rhizoferrin may be an indication for an independant biosynthesis). Staphyloferrin B is a comparatively complex molecule including two chiral centers and incorporating a not very common amino acid (Dap). Exchange of genetic material, a well-known process with bacteria, should be considered for an explanation.

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