

Succinopyoverdins – a New Variety of the Pyoverdin Chromophore[§]

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Pseudomonas spp. of the fluorescent group produce siderophores (so-called pyoverdins) consisting of a peptide chain attached to a pyrimidoquinoline ring system which is derived from a condensation product of L-Dab and D-Tyr. Commonly several related compounds are found to accompany the pyoverdins having the same peptide chain, but differing in the heterocyclic part. The structure elucidation of a new variety (succinopyoverdin) is described here.

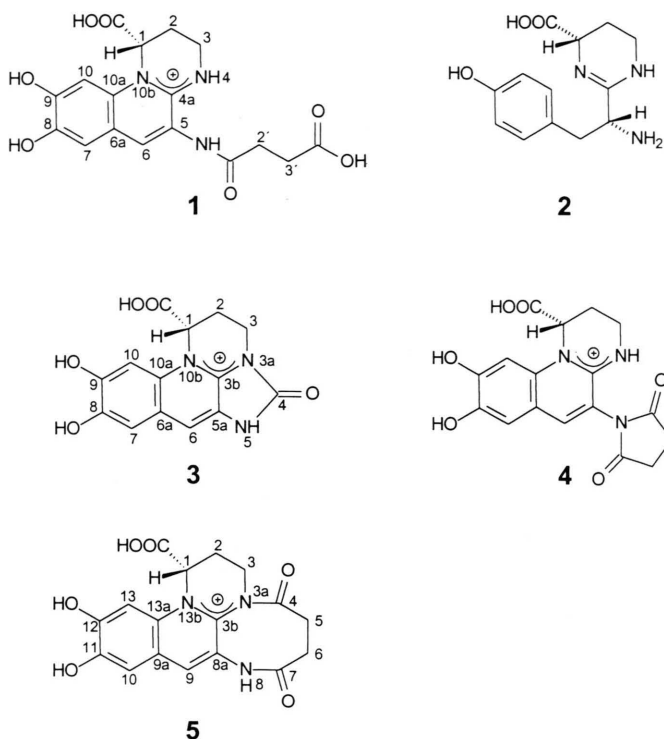
Introduction

Pyoverdins are the characteristic siderophores of the fluorescent species of the bacterial family Pseudomonadaceae in the rRNA homology group I (Budzikiewicz, 1997a and 1997b). They are chromopeptides consisting of the dihydroxyquinoline chromophore, bound amidically to the N-terminus of a peptide chain by its carboxyl group at C-1, and to a small dicarboxylic acid or its amide by the amino group at C-5 (**1**, here with a succinic acid side chain). According to the current biogenetic scheme (Böckmann *et al.*, 1997) the precursor of **1** is the condensation product **2** of L-Dab and D-Tyr (ferribactin system) which cyclizes to 5,6-dihydro-**1**. Occasionally, congeners with the chromophore **3** are also found (Hohlneicher *et al.*, 1994), otherwise typical for the siderophores of *Azotobacter vinelandii*, the azotobactins (Schaff-

ner *et al.*, 1996). Co-occurring pyoverdins, dihydro-pyoverdins, ferribactins and azotobactins always have the same peptide part. They differ only in the nature of the chromophore and the dicarboxylic acids attached to it. The amounts of the various components contained in the fermentation broth depend on the culture conditions such as growth time, oxygen supply etc. In the past, frequently only the main component was isolated for structural work. With the development of a screening system using LC in combination with an UV/Vis detector and an ESI mass spectrometer (Kilz *et al.*, 1999), minor components can be recognized which were overlooked or ignored before. By screening the fermentation extracts of several *Pseudomonas* spp. compounds were detected whose molecular mass amounted to that of a pyoverdin with a succinic acid side chain minus one H₂O. Formal loss of H₂O may have its origin in the peptide chain as an elimination e.g. from Ser or Thr, or by formation of a cyclopeptidic or cyclodepsipeptidic substructure (e.g., Voßen *et al.*, 2000). In the examples discussed below these possibilities can be excluded. Formation of a succinimide cycle (**4**) was observed as an intermediate in the hydrolysis of a succinamide side chain under acidic conditions (Mohn, 1990). The succinimide is reopened to a succinic acid side chain, slowly at acidic and faster at neutral pH values. The compounds to be discussed here are, however, stable under these conditions. It will be shown that cyclization rather occurred by formation of an amide bond to N-4 of **1**,

Abbreviations: Common amino acids, 3-letter code; FoOHOrn, N⁵-formyl-N⁵-hydroxy Orn; (HP)LC, (high performance) liquid chromatography; ESI, electrospray ionization; FAB, fast atom bombardment; MS, mass spectrometry; CA, collision activation; COSY, correlated spectroscopy; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; NOESY, nuclear Overhauser and exchange spectroscopy; ROESY, rotating frame nuclear Overhauser and exchange spectroscopy; TOCSY, total correlation spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane.

[§] Part LXXXVI of the series "Bacterial Constituents".
For part LXXXV see Budzikiewicz *et al.* (1999).



yielding an (1*S*)-11,12-dihydroxy-4,7-dioxo-2,3,5,6,7,8-hexahydro-1*H*-3*bH*-4*H*-3*a*,8,13*b*-triazacycloocta[*jk*]phenanthrene-1-carboxylic acid system (5).

Materials and Methods

Instruments and chemicals

Mass spectrometry: Finnigan-MAT H-SQ 30 (FAB, matrix thioglycerol/dithiodiethanol), Finnigan-MAT 900 ST (ESI); GC/MS Incos 500 (all Finnigan-MAT, Bremen) with Varian (Sunnyvale CA, USA) GC 3400.

NMR: AM 300 (^1H 300, ^{13}C 75.5 MHz) and DRX 500 (^1H 500, ^{13}C 125 MHz) (both Bruker, Karlsruhe). Chemical shifts relative to TMS with the internal standard DSS; $\delta(\text{TMS}) = \delta(\text{DSS})$ for ^1H , $\delta(\text{TMS}) = \delta(\text{DSS}) - 1.61$ ppm for ^{13}C . Suppression of the H_2O signal by the WATERGATE pulse sequence.

UV/Vis: Lambda 7 (Perkin-Elmer, Überlingen), CD: Jasco 715 (Jasco, Tokyo, Japan).

Chromatography: RP-HPLC columns Nucleosil 100- C_{18} (5 μm) and Eurospher 100- C_{18} (7 μm)

(Knauer, Berlin); low pressure chromatography columns XAD-4 (Serva, Heidelberg), Biogel P-2 (Bio-Rad, Richmond CA, USA), CM-Sephadex C-25, SP-Sephadex C-25, DEAE-Sephadex A-25 and Sephadex G-10 (Pharmacia, Uppsala, S), Sep-Pak RP-18 cartouches (Waters, Milford MA, USA); GC/MS: Chirasil-L-Val (Chrompack, Frankfurt).

Chemicals: Water was desalted and distilled twice in a quartz apparatus; for HPLC it was further purified on XAD-4 resin and filtered through a sterile filter. Organic solvents were distilled over a column. Reagents (Aldrich-Sigma, Deideshofen; Fluka, Neu-Ulm; Merck, Darmstadt; Riedel de Haën, Seelze) were of p. a. quality.

Production and isolation of pyoverdins and succinopyoverdins

Pseudomonas aeruginosa ATCC 15692 (PAO1) was grown, its pyoverdins were isolated and the ferri-pyoverdins were decomplexed with 8-hydroxyquinoline as described earlier (Briskot *et al.*, 1986). In the CM-Sephadex fraction containing

ferri-7 there was a second component (ferri-6) which eluted first upon chromatography on SP-Sephadex with a 0.04 M pyridinium formate buffer (pH 3.1). Ferri-6 was purified by rechromatography under the same conditions and decomplexed subsequently. The ratio of ferri-7 to ferri-6 was found in different cultures as 10:2 to 10:4.

Pseudomonas fluorescens 18.1 (Amann *et al.*, 1999) isolated from fish (Champomier-Verges and Richard, 1994) was grown for 72–96 hrs. in a gluconate medium (Beiderbeck *et al.*, 1998). For the work-up of the culture medium by chromatography on a XAD-4 column see Georgias *et al.* (1999). Ferri-8 and ferri-9 were co-eluted with a 0.2 M pyridinium acetate buffer (pH 5.0) from a CM-sephadex column. They could be separated by preparative HPLC on Eurosphere 100 with 50 mM acetic acid/methanol (gradient 3 to 30% methanol) and were decomplexed subsequently with 8-hydroxyquinoline (Briskot *et al.*, 1986).

Isolation of 5

The succinopyoverdin chromophore 5 was obtained by hydrolysis of the corresponding siderophore isolated from *Pseudomonas aeruginosa* ATCC 15692 by treatment with 12 N HCl for 21 days at 37 °C, adsorption of the product mixture on SepPak RP-18, removal of polar material by washing with H₂O, desorption with CH₃OH/0.1 N CH₃COOH (8:2, v/v) and final purification by chromatography on Sephadex G-10 with a 0.1 N pyridinium acetate buffer (pH 5.0).

Results

The succinopyoverdin 6 from *Pseudomonas aeruginosa* ATCC 15692 (PAO1)

From a culture of *Pseudomonas aeruginosa* its ferri-pyoverdins differing in the dicarboxylic acid side chains (Briskot *et al.*, 1989) were obtained by chromatography on CM-Sephadex. From the fraction containing ferri-7 (succinic acid side chain, 1 with the same peptide chain as 6) a second component (ferri-6) could be separated by chromatography on SP-Sephadex. As will be shown, 6 contains the new cationic chromophore 5. Its molecular mass as determined by FAB-MS is 1316 u, 18 u less than that of protonated 7 having a succinic acid side chain. The UV/Vis spectra of 6 (pH 7.0:

414 nm, log ϵ 4.29; pH 3.0: 376 nm, log 4.18, no splitting at lower pH values) and of its Fe³⁺-complex (pH 7.0: 406 nm, log ϵ 4.16; pH 3.0: 404, log ϵ 4.10, charge transfer bands at ca. 460 and 550 nm) resemble those of azotobactins rather than pyoverdins. For the CD-spectra of 6 and 7 see Fig. 1.

The NMR signals recorded for the peptide chain of 6 are in agreement with those reported for 7 (Briskot *et al.*, 1989; Demange *et al.*, 1990): The spin systems of the amino acids in the peptide chain were confirmed by COSY and TOCSY and the sequence of the amino acids by ROESY and NOESY experiments (Fig. 2). The pronounced downfield shift of the Ser¹- α -NH (ca 10 ppm) confirms the linkage to the carboxyl group of the chromophore.

The protons of the chromophore of 6 (Table I) were identified by COSY, ROESY and TOCSY experiments, the H-substituted chromophore C-atoms by HSQC; their multiplicity was confirmed

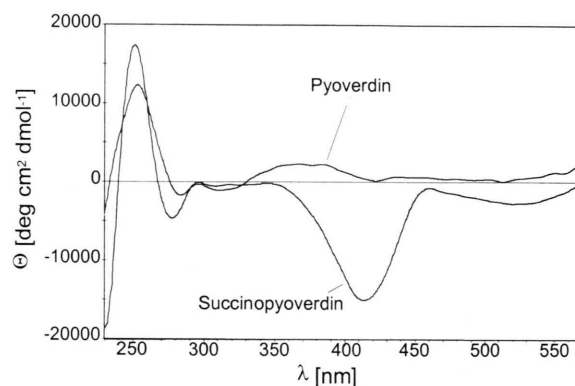
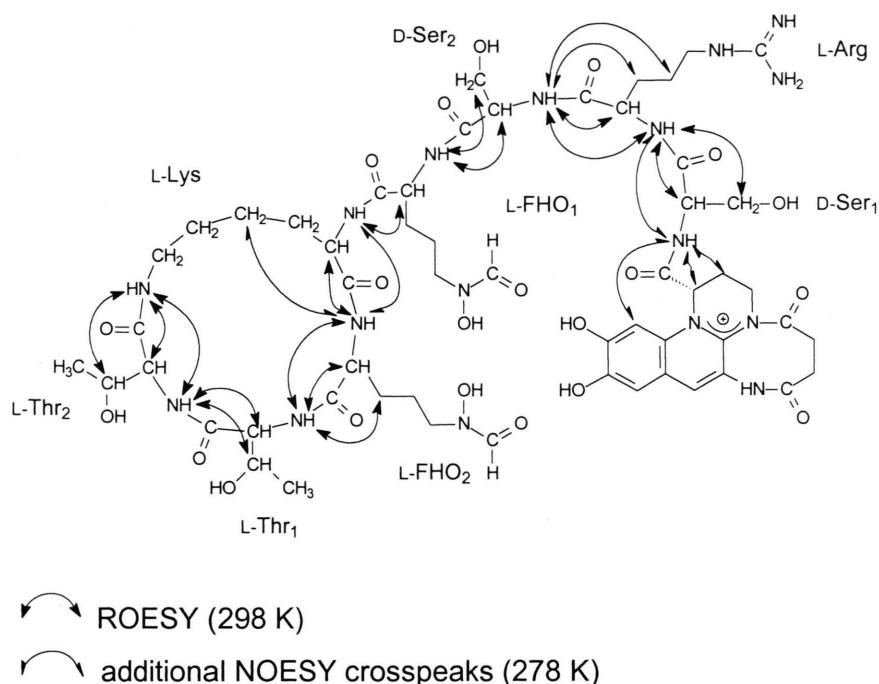


Fig. 1. CD-spectra of the pyoverdinin 7 and of the succinopyoverdin 6.

Table I. ¹H-Chemical shifts of the chromophore and succinic acid protons of 5 (CD₃OD, 25 °C), 6 and 7 (H₂O/D₂O 9:1, pH 4.3, 25 °C).

Proton ^a	7	6	5
Chr-1 (1)	5.72	6.01	5.61
Chr-2a/b (2)	2.47/2.69	2.81/3.06	2.60/3.08
Chr-3a/b (3)	3.41/3.73	4.10/4.70	4.12/4.68
Chr-6 (9)	7.90	8.48	8.72
Chr-7 (10)	7.10	7.19	7.51
Chr-10 (13)	7.10	7.13	7.37
Suc-2' (6)	2.73	2.85	2.99
Suc-3' (5)	2.65	3.25	3.28

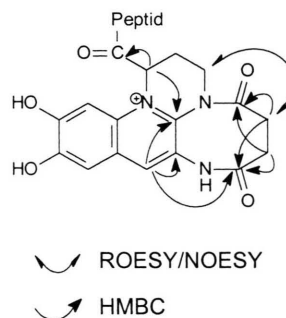
^a Numbering of the pyoverdinin chromophore 1, in parentheses of 5.

Fig. 2. Peptide sequence relevant ROESY and NOESY crosspeaks for the succinopyoverdin **6**.Table II. ¹³C-Chemical shifts of the chromophore and succinic acid carbon atoms of **5** (CD₃OD, 25 °C), **6** and **7** (H₂O/D₂O 9:1, pH 4.3, 25 °C).

Carbon atom ^a	7	6	5
carboxyl-CO	172.1	170.5	172.2
Chr-1 (1)	57.7	56.4	60.7
Chr-2 (2)	23.0	25.3	25.4
Chr-3 (3)	36.3	38.5	39.5
Chr-4a (3b)	150.4	140.3	141.4
Chr-5 (8a)	118.5	24.5	23.6
Chr-6 (9)	139.9	34.5	31.7
Chr-6a (9a)	115.5	159.5	160.2
Chr-7 (10)	114.9	112.5	113.2
Chr-8 (11)	145.1	148.9	148.3
Chr-9 (12)	153.7	162.7	156.7
Chr-10 (13)	101.1	98.9	99.8
Chr-10a (13a)	132.8	133.8	133.4
Suc-CO-1' (7)	178.5	159.5	160.2
Suc-CO-4' (4)	181.7	181.0	175.9
Suc-2' (6)	33.0	34.5	31.7
Suc-3' (5)	33.4	24.5	23.6

^a Numbering of the pyoverdine chromophore **1**, in parentheses of **5**.

by DEPT. The complete assignment of the aromatic carbon atoms was possible by the HMBC technique (see Fig. 3 and Table II). The two CO-carbons of the original succinic acid side chain (C-

Fig. 3. ROESY/NOESY and HMBC correlations for the chromophore part of **6**.

4 and C-7) could be distinguished by the long-range coupling between C-7 and the proton at C-9. Both the protons of CH₂-5 and CH₂-6 show long-range coupling to CO-4 as well as to CO-7 and can not be distinguished in this way. However, CH₂-5 (2.70 ppm) shows a ROESY cross peak to the signal at 4.10 ppm (CH_a-3) and can thus be identified. The shifts of the benzene ring carbons 9a and 13a as well as 11 and 12 could not be deduced from the HMBC spectrum as they show

coupling with all aromatic protons. Their shift values were assigned by comparison with those of **7**.

Differences in the chemical shifts between **6** and **7** are obvious for the chromophore and the succinic acid signals: All chromophore signals are shifted downfield. The effect is especially pronounced for the protons at C-3 of the chromophore. Due to their neighborhood to the C-4 carbonyl group they are shifted by 0.8 and 1.0 ppm, respectively (see Table II). A downfield shift of 0.5 ppm is also observed for the C-9 proton lying in the influence sphere of the C-7 carbonyl group. There is also a noticeable influence on the C-5 protons of the succinic acid part in **6**.

6 forms a 1:1-Fe³⁺ complex as can be seen from the respective molecular masses as determined by ESI-MS for **6** (1316.7 corresponding to C₅₅H₈₂N₁₇O₂₁) and for ferri-**6** (1369.4 corresponding to C₅₅H₇₉N₁₇O₂₁⁵⁶Fe): 3 H are replaced by 1 Fe³⁺.

The chromophore **5** isolated from **6**

By mild hydrolysis of **6** the cationic chromophore **5** could be isolated. Its mass was determined by ESI-MS as 358.1042 ± 0.0003 (calc'd 358.1039 for C₁₇H₁₆N₃O₆). The absorption maximum in 1 N HCl (377 nm, log ε 4.03) corresponds to that of **6** at low pH values. The CD-spectrum is depicted in Fig. 4. The ¹H- and ¹³C-data of **5** can be found in Tables IV and V. The identification of the various resonances was performed as for **6**. However, since no correlations between C-4 and one of the

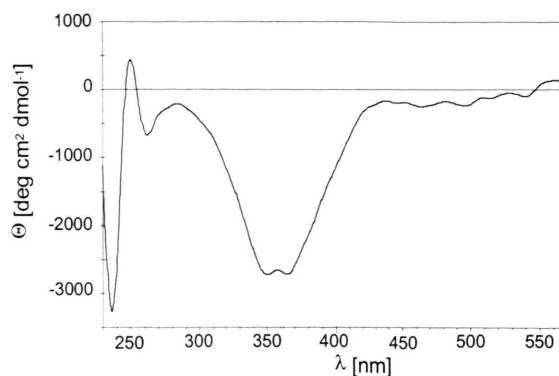


Fig. 4. CD-spectrum of the chromophore **5**.

H of CH₂-3 and between C-7 and CH-9 could be detected, the shift values were assigned by comparison with those of **6**.

The succinopyoverdin **8** from *Pseudomonas fluorescens* 18.1

Also from a culture of *Pseudomonas fluorescens* 18.1 a pyoverdin with a succinic acid side chain (**9** = **1** with the same peptide chain as **8**) (Amann *et al.*, 1999) and a cationic compound (**8**) were obtained. The Biogel fraction containing ferri-**9** and ferri-**8** could be separated by preparative HPLC. After decomplexation the molecular mass of **8** as determined by FAB-MS amounted to 1374, 18 u less than that of the protonated pyoverdin with a succinic acid side chain. The UV/Vis spectra (pH 7.0: 400 nm, pH 3.0: 374 nm, no splitting at

Table III. ¹H-Chemical shifts of the chromophore and the succinic acid protons of **8** and **9** and comparison of the shift differences observed for the pairs **8/9**, azotobactin/pyoverdine from *Pseudomonas fluorescens* ATCC 13525 (chromophores **3** vs. **1**) and the partial hydrolysis products from *Pseudomonas aeruginosa* ATCC 15692 (chromophores **4** vs. **1**) (Briskot, 1999).

$$\Delta_1 = \delta(\mathbf{8}) - \delta(\mathbf{9}) \text{ (5 } ^\circ\text{C, H}_2\text{O/D}_2\text{O 9:1, pH 4.3)}$$

$$\Delta_2 = \delta(\mathbf{3}) - \delta(\mathbf{1}) \text{ (5 } ^\circ\text{C, H}_2\text{O/D}_2\text{O 9:1, pH 4.3)}$$

$$\Delta_3 = \delta(\mathbf{4}) - \delta(\mathbf{1}) \text{ (25 } ^\circ\text{C, D}_2\text{O, pH 3.0)}$$

Proton ^a	9	8	Δ_1	Δ_2	Δ_3
Chr-1 (1)	5.72	6.07	0.35	0.34	0.04
Chr-2a/b (2)	2.55/2.72	2.84/3.08	0.29/0.36	0.17/0.29	0.03/0.02
Chr-3a/b (3)	3.39/3.73	4.13/4.73	0.74/1.00	0.33/0.63	-0.02/-0.04
Chr-6 (9)	7.88	8.55	0.67	0.06	0.08
Chr-7 (10)	7.02	7.25	0.23	0.36	0.07
Chr-10 (13)	6.94	7.20	0.26	0.25	0.05
Suc-2' (6)	2.74	2.90	0.16	—	0.24
Suc-3' (5)	2.65	3.25	0.60	—	0.21

^a Numbering for the pyoverdine chromophore **1**, in parentheses of **5**.

Table IV. ^{13}C -Chemical shifts of the chromophore and the succinic acid carbon atoms of **8** and **9** and comparison of the shift differences observed for the pairs **8/9**, azotobactin/pyoverdins from *Pseudomonas fluorescens* ATCC 13525 (chromophores **3** vs. **1**) and the partial hydrolysis products from *Pseudomonas aeruginosa* ATCC 15692 (chromophores **4** vs. **1**).

$\Delta_1 = \delta(\mathbf{8}) - \delta(\mathbf{9})$ (25 °C, D_2O , pH 4.3)

$\Delta_2 = \delta(\mathbf{3}) - \delta(\mathbf{1})$ (25 °C, D_2O , pH 4.3)

$\Delta_3 = \delta(\mathbf{4}) - \delta(\mathbf{1})$ (25 °C, D_2O , pH 3.0)

Carbon atom ^{a,b}	9	8	Δ_1	Δ_2	Δ_3
carboxyl-CO	170.2	171.7	-1.5	-1.8	-0.2
Chr-1 (1)	56.8	57.9	-1.1	-0.9	-0.3
Chr-2 (2)	25.3	23.0	2.3	1.2	0.1
Chr-3 (3)	38.5	36.1	2.4	-0.8	0.0
Chr-4a (3b)	146.0	150.5	-4.5	-9.2 ^c	-1.0
Chr-5 (8a)	133.4	118.7	14.5	3.8	-2-9
Chr-6 (9)	133.2	139.9	-6.7	-18.3	2.9
Chr-6a (9a)	120.4	114.9	5.5	4.3	-0.3
Chr-7 (10)	112.9	114.5	-1.6	-1.4	-1.2
Chr-8 (11)	148.8	144.8	4.0	2.0	0.4
Chr-9 (12)	150.3	153.1	-2.8	0.8	1.4
Chr-10 (13)	99.2	101.0	-1.8	-0.9	-0.1
Chr-10a (13a)	133.5	132.8	0.7	-5.6	1.2
Suc-CO-1' ^c (7)	^d	178.7	-	-	0.5
Suc-CO-4' ^c (4)	180.8	182.4	-1.6	-	2.8
Suc-2' ^c (6)	34.3	33.1	1.2	-	-1.3
Suc-3' ^c (5)	24.4	33.5	-9.1	-	-1.3

^a Numbering for the pyoverdins chromophore **1**, in parentheses of **5**. ^b For the assignment of the carbon atoms see text. ^c Not reported for azotobactin ATCC 13525; values for azotobactin Az 87 (Schaffner *et al.*, 1996).

^d not reported.

lower pH values) and that of the Fe^{3+} -complex (402 nm, pH independent, charge transfer bands at ca. 460 and 550 nm) correspond to those of **6**. The ^1H - and ^{13}C -NMR data of the peptide chain including two-dimensional correlations and sequence relevant data up to the cross peaks of Ser¹ and Lys¹ to the chromophore correspond to those of pyoverdins Pf 18.1 (Amann *et al.*, 1999), hence an identity of the peptide chain can be assumed (Fig. 5). The ^1H - and ^{13}C -data of the chromophores of **8** and **9** are assembled in Tables III and IV. The assignment for the quaternary C-atoms is tentative, relying on comparison with signals in the spectra of the pyoverdins and azotobactins. The effect on the protons at C-3, C-5 and C-9 is in the same order as reported for **6**.

Discussion

The changes of the NMR data of the chromophore **1** caused by the formation of a succinimide cycle were investigated in detail for partial hydrolysis products of the pyoverdins D and E from *Pseudomonas aeruginosa* comprising Arg-Ser bound to **4** (Briskot, 1988). The influence on the chromophore protons is negligible (<0.1 ppm); only the shifts of the succinimide protons differ from those of a free succinic acid side chain. They form an AA'BB' pattern with a geminal coupling

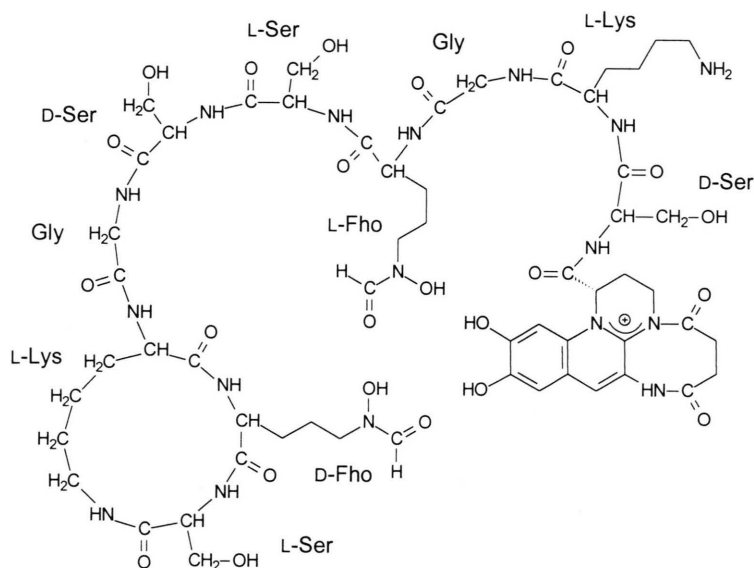


Fig. 5. Structure of the succinopyoverdin **8**.

constant of -19 Hz, and they are not pH-dependent (Briskot, 1988; Mohn, 1990).

The differences between the shifts of the chromophore protons of **6/8** and those of the corresponding pyoverdinin (**7/9**) are comparable to those to be found between the azotobactin and the pyoverdinin Pf ATCC 13525 (Hohlneicher *et al.*, 1994), resulting in downfield shifts especially of the aliphatic protons C-1 – C-3. Remarkable is the pronounced effect on CH-9 of **8** compared with that on the corresponding CH-6 of azotobactin: The chromophore **3** contains a planar five-membered ring while in the case of the non-planar eight-membered ring of **5** the proton of CH-9 gets close to CO-7. The ring-closure to N-3a is confirmed by a NOE in the NOESY spectrum between the H's-3a/b and the H's-5. The downfield shift of the latter is not just the result of the formation of an amide bond: At low pH-values there is no difference in the shifts of -CH₂-COOH and -CH₂CONH₂; only at pH 7 a slight upfield shift (ca. 0.05 ppm) is observed for CH₂COOH (Briskot, 1988). An analogous summary of ¹³C-

data can be found in Table II. The signals of the H-carrying C-atoms could be identified by cross-signals in the HSQC spectrum. Again the correlation with the signals observed for the azotobactin is evident confirming the ring closure to N-3a.

Conclusion

The existence of a new chromophore variety of the siderophores accompanying the pyoverdins could be demonstrated. The formation of **5** shows that a reaction of the carboxyl group of the acid side chain with NH-4 of **1** is possible. This gives some weight to the speculation (Voßen *et al.*, 2000) that the azotobactin chromophore **3** stems from an oxidative decarboxylation of the α-ketoglutaric acid side chain where the CO₂ remains in the molecule, starting from an analogous cyclic form. Congeners of the pyoverdins containing the chromophore **5** may well have been overlooked in the past or even ignored as uninteresting byproducts not worth looking after, as formal loss of H₂O may have many reasons – see the **Introduction**.

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