Pyoverdin, Ferribactin, Azotobactin – a New Triade of Siderophores from *Pseudomonas chlororaphis ATCC 9446* and Its Relation to *Pseudomonas fluorescens ATCC 13525**

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Siderophores, Pyoverdin, Ferribactin, Azotobactin, *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*

It is shown that *Pseudomonas fluorescens ATCC 13525* and *Pseudomonas chlororaphis ATCC 9446* produce identical pyoverdins and ferribactins. As the structures of these siderophores are usually species or even strain specific this exception should be kept in mind in view of the reclassification of the genus *Pseudomonas*. From *Pseudomonas chlororaphis* an additional siderophore could be obtained which has the same peptide chain as the co-occurring pyoverdins and ferribactin, but a chromophore which is typical for azotobactins from *Azotobacter vinelandii*.

Introduction

The fluorescent group of the genus *Pseudomonas* produces peptide siderophores when grown in an iron-deficient medium. The most widely described variety are pyoverdins which are characterized by the chromophore (1*S*)-5-amino-2,3-dihydro-8,9-dihydroxy-1*H*-pyrimido-[1,2-a]quinoline-1-carboxylic acid (**1**, Fig. 1), whose carboxyl group is linked to a peptide residue which has been reported as species- (or even strain-) specific (Budzikiewicz, 1993). One exception to this rule seems to be *Pseudomonas fluorescens ATCC 13525* and *Pseudomonas chlororaphis ATCC 9446* (Linget *et al.*, 1992) (see below). Usually several pyoverdins co-occur which differ only in the nature of the small dicarboxylic acid attached to the amino group of **1** (Fig. 1) (Budzikiewicz, 1993). Careful workup of the culture medium frequently allows to isolate in addition dihydro derivatives where the 5,6-double bond of **1** is saturated. The peptide chain is the same as in the corresponding pyoverdins. In some cases in addition a ferribactin was present which again possesses the same peptide chain but contains the chromophore **2**

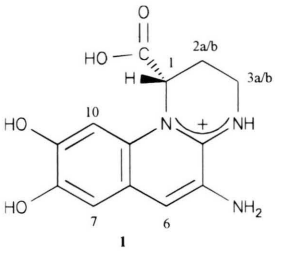

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Fig. 1. Structure of the pyoverdin chromophore.

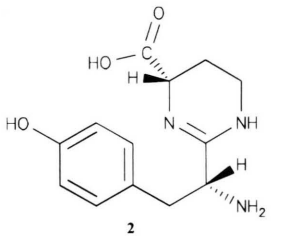

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Fig. 2. Structure of the ferribactin chromophore.

Abbreviations: Chr, chromophore; EDTA, ethylenediamine tetraacetic acid; FAB-MS, fast atom bombardment mass spectrometry; GC, gas chromatography; HMBC, heteronuclear multiple bond coherence; HMQC, heteronuclear multiple quantum coherence; HOHAHA, homonuclear Hartmann-Hahn-experiment; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser effect spectroscopy; (OH)Orn, N^5-hydroxyornithin; Suc, succinic acid; TAP, N/O-trifluoracetyl/*n*-butyl ester; u, mass unit.

* Part LXII of the series "Bacterial constituents". For part LXI see Budzikiewicz (1994).

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(Fig. 2). Dihydropyoverdins and ferribactins are considered to be precursors of the pyoverdins (Budzikiewicz, 1994). From the cultures of *Pseudomonas chlororaphis ATCC 9446* we obtained now a further variety of siderophore which also possesses the same peptide chain now linked to the chromophore **3** (Fig. 3) typical for the azotobactins produced by *Azotobacter vinelandii* (Fukasawa *et al.*, 1972; Demange *et al.*, 1988; Page *et al.*, 1991; Menhart *et al.*, 1991; Budzikiewicz *et al.*, 1992). In the following we will report our investigations regarding *Pseudomonas fluorescens ATCC 13525* and *Pseudomonas chlororaphis ATCC 9446.*

Material and Methods

Cultures: The bacteria were grown in 200 ml cultures in 500 ml Erlenmeyer flasks with passive aeration by shaking for 72 hrs. The culture broth contained 13 g Na-gluconate, 4 g KH_2PO_4, 3 g $(NH_4)_2SO_4$ and 0.5 $MgSO_4 \cdot 7H_2O$ per liter. Isolation and purification were carried out as described earlier (Taraz *et al.*, 1991; Budzikiewicz *et al.*, 1992; Mohn *et al.*, 1990; Briskot *et al.*, 1986; Poppe *et al.*, 1987). Decomplexation was achieved with 8-hydroxyquinoline (Briskot *et al.*, 1986).

Chromophore peptides: 40 mg of the respective pyoverdin were hydrolized with 3 ml 9 N HCl for 10 min at 110 °C. After evaporation to dryness the residue was dissolved in H_2O and chromatographed on Bio-Gel P-2 (column 2 cm x 40 cm) with 0.1 N acetic acid. The fractions still containing the chromophore (absorption at 254 nm) were evaporated to dryness and hydrolyzed with 6 N HCl at 110 °C for 21 hrs.

For the *amino acid analysis* see (Briskot *et al.*, 1986) and for the determination of absolute configurations see (Mohn *et al.*, 1990).

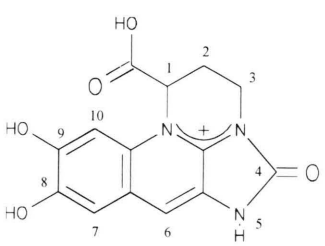

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Fig. 3. Structure of the azotobactin chromophore.

Gas chromatography: A gas-chromatograph CARLO ERBA HRGC 4160 with a FID detector was used. The data were recorded on a Shimadzu Chromatopac C-R 3 A integrator. Quantitative analysis was performed on a Permabond SE-54-DF-0.25 column (Macherey-Nagel, Düren) and the determination of the absolute configurations on a Chirasil-L-Val column ID 0.25 (Chrompack).

UV/VIS-spectra: Perkin-Elmer Hitachi 200.

Mass spectrometry: FAB-MS: Finnigan MAT HSQ 30 equipped with a FAB-gun (IonTech Ltd., Teddington, GB), the FAB-gas was Xenon, the matrix substance thioglycerol.

GC-MS: KRATOS MS 25 RF with a CARLO ERBA HRGC MFC 500 and a Permabond SE-54-DF-0.25 column (Macherey-Nagel, Düren).

Chemicals: Pyridin was treated with chlorosulfonic acid (5 ml/l), distilled and redistilled over KOH. H_2O was deionized and distilled twice.

NMR-spectra: The NMR-experiments were performed with a BRUKER AMX 500 using BRUKER UXNMR software. The siderophores were disolved in 100 mM KH_2PO_4-buffer at pH 4.3 (90% H_2O, 10% D_2O). The acetate of the siderophores was replaced by a Cl^--ion using ion exchange chromatography on DEAE-Sephadex in its chloride form. All spectra were recorded at 5 °C and 25 °C. The H_2O-resonance was suppressed by presaturation during the relaxation delay. 512 experiments with 2048 data points each were acquired for each 2D-spectrum. Zero filling in both dimensions was applied to obtain matrices of 2048×1024 data points.

The MLEV17 HOHAHA (Bax and Davis, 1985) spectra were obtained in the phase sensitive mode using the time proportional phase incrementation scheme. The spectral width was 5263 Hz (10.5 ppm) in both dimensions. After 4 dummy scans 8 scans were recorded for each FID. The spinlock time was 40 ms. For both dimensions a $\pi/3$ shifted squared sine bell was used as window function.

The parameters of the NOESY experiments correspond to those of the HOHAHA experiment, exept that the number of scans was 16 and the delay of the mixing time 200 ms.

For the reverse $^1H-^{13}C$-correlation a HMQC-experiment with GARP-decoupling was performed. The spectral width was 5263 Hz (10.5 ppm) in F_2 and 6240 Hz (49 ppm) in F_1. 512 ex-

periments with 32 scans each were performed in t_1. A $\pi/2$ shifted sine bell was used as window function in both dimensions after zerofilling in t_1. The reverse HC-long-range-correlations were determined by a not decoupled HMBC-experiment (Bax and Summers, 1986). The spectral width was 5263 Hz (10.5 ppm) in F_2 and 23809 Hz (189 ppm) in F_1. 512 experiments with 112 scans each were performed. The delay for the evolution of the long range couplings was 70 ms.

Results and Discussion

Amino acids analysis, mass spectral and NMR data (see Tables I–III for ^{1}H- and ^{13}C-NMR data and Figs. 4 and 5 for 2D-results) confirm that the pyoverdins with a succinic acid side chain from *Pseudomonas fluorescens ATCC 13525* and *Pseudomonas chlororaphis ATCC 9446* are indeed identical (**4**, Fig. 4) as reported by ABDALLAH (Demange *et al.*, 1986; Demange *et al.*, 1987; Demange *et al.*, 1989; Linget *et al.*, 1992). In the same way it could be shown that the ferribactin produced by *Pseudomonas chlororaphis ATCC 9446* is identical with the one isolated from *Pseudomonas fluorescens ATCC 13525* (Hohlneicher *et al.*, 1992). It is the first case that siderophores with identical peptide chains were found in different *Pseudomonas* species. This is the more astonishing as sofar about 30 pyoverdins differing in their peptide chains were described (Budzikiewicz, 1993) and for *Pseudomonas fluorescens* about 10 strains producing different pyoverdins are known. This finding should be kept in mind in view of the current reclassification of the genus *Pseudomonas*.

For the pyoverdins the position in the peptide chain of the D- and L-N^5-formyl-N^5-hydroxy-Orn units had not been determined before. Partial hydrolysis yielded fragments containing the chromophore with D-Ser and L-Orn only. From this it follows that D-Ser is bound directly to the chromophore and that L-Orn occupies position 4 while L-Ser and D-Orn are incorporated into the cyclopeptide moiety that blocks the C-terminal end. The same distribution can also be assumed for the ferribactins.

The complexing constants for the pyoverdin with a succinic acid side chain could be determined as 1.8×10^{19} at pH 5.0 and as 0.8×10^{26} at pH 7 (Anderegg *et al.*, 1963).

Table I. ^{1}H NMR data of Suc-pyoverdin in 100 mM KH_2PO_4, 10% D_2O, pH 4.3 (5 °C).

Amino Acid	α-H	β-H	γ-H	δ-H	ε-H	ε-NH_2	NH	CHO_c/CHO_t
Gly	3.54 3.68	–	–	–	–	–	8.32	–
Ser	4.46	3.97	–	–	–	–	9.63	–
Ser′	4.35	3.88	–	–	–	–	9.02	–
Lys	4.35	1.86 1.66	1.27 1.67	1.67	2.78 2.85	7.58	8.75	–
Lys′	4.16	1.95 1.61	1.01 1.28	1.48 1.58	3.18 3.27	7.41	8.32	–
(OH)Orn	4.21	1.66 1.76	1.67 1.72	3.58	–	–	8.25	7.93/8.29
(OH)Orn′	4.41	1.65 1.77	1.59 1.73	3.58	–	–	8.16	7.93/8.29

Suc	2′	3′
	2.80 2.81	2.72 2.73

Chromophore	H1	H2a/2b	H3a/3b	H6	H7	H10	Chr4 NH^+	Chr NH
	5.73	2.50/2.72	3.40/3.72	7.88	7.06	7.03	8.86	9.97

δ (ppm), DSS as internal standard, c: *cis*, t: *trans*.

Table II. ^{1}H NMR data of Suc-pyoverdin in 100 mM KH_2PO_4, 10% D_2O, pH 4.3 (25 °C).

Amino Acid	α-H	β-H	γ-H	δ-H	ε-H	ε-NH_2	NH	CHO_c/CHO_t
Gly	3.59 3.76	–	–	–	–	–	8.22	–
Ser	4.46	3.96	–	–	–	–	9.45	–
Ser′	4.36	3.87	–	–	–	–	8.80	–
Lys	4.34	1.65 1.84	1.24 1.67	1.50 1.58	2.77 2.85	7.48	8.54	–
Lys′	4.17	1.60 1.93	1.02 1.26	1.48 1.57	3.17 3.26	7.34	8.16	–
(OH)Orn	4.23	1.66 1.77	1.60 1.73	3.56	–	–	8.11	7.92/8.29
(OH)Orn′	4.41	1.72 1.77	1.59 1.73	3.52	–	–	8.00	7.92/8.29

Suc	2′	3′
	2.77 2.79	2.72 2.73

Chromophore	H1	H2a/2b	H3a/3b	H6	H7	H10	Chr NH	Chr $4NH^+$
	5.76	2.49/2.72	3.39/3.73	7.93	7.19	7.07	see text	see text

δ (ppm), DSS as internal standard, c: *cis*, t: *trans*.

Table III. ^{13}C NMR data of Suc-pyoverdin 13525 in 100 mM KH_2PO_4, 10% D_2O, pH 4.3 (25 °C).

Amino acid	α-C	β-C	γ-C	δ-C	ε-C	C=O	CHO_c/ CHO_t
Gly	44.0	–	–	–	–	172.7	–
Ser	58.5	62.5	–	–	–	172.7	–
Ser′	58.5	61.4	–	–	–	173.5	–
Lys	55.2	31.8	23.8	27.8	41.0	175.6	–
Lys′	57.3	30.1	20.7	26.7	39.3	176.3	–
(OH)Orn	55.2	29.4	23.5	47.6t 51.5c	–	175.4	160.8c/ 165.2t
(OH)Orn′	54.9	26.5	23.5	47.6t 51.5c	–	174.4	160.8c/ 165.2t

Suc	2′	3′	CO	COOH
	32.8	31.5	178.4	180.6

Chromophore	C-1	C-2	C-3	C-4a	C-5	C-6	CO
	58.5	23.7	36.9	150.4	117.4	140.5	171.9
	C-6a	C-7	C-8	C-9	C-10	C-10a	
	116.4	115.8	145.4	153.2	101.9	135.7	

δ (ppm), DSS as internal reference using δ (CH_3) = −1.61 ppm to conform with reference data using TMS (0 ppm) for calibration. c: *cis*, t: *trans*.

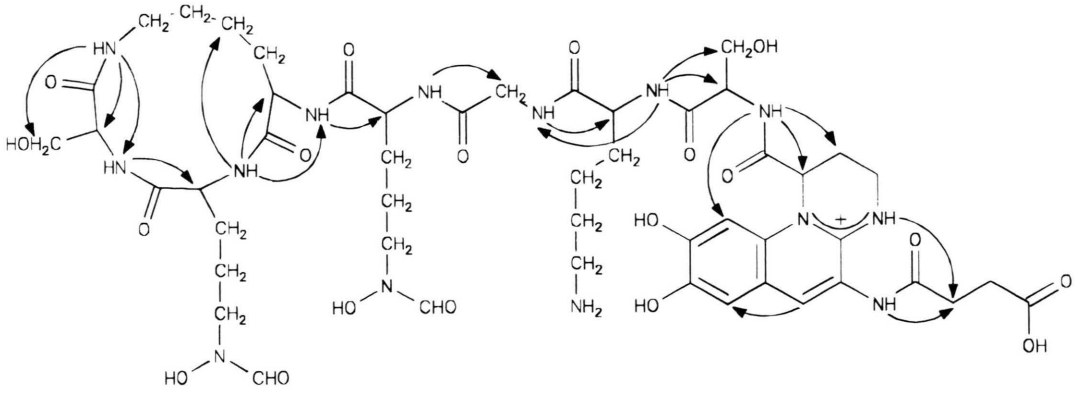

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Fig. 4. Sequential information given by NOESY of Suc-pyoverdin 13525.

ABDALLAH had reported succinic acid (amide), ketoglutaric acid and malic acid (amide) side chains. To this we can add glutamic acid thus completing the common pattern (Budzikiewicz, 1993)

In the culture medium of *Pseudomonas chlororaphis ATCC 9446* in addition to the pyoverdins and the ferribactin (Hohlneicher *et al.*, 1992) a new siderophore could be detected which contains the chromophore **3** (Fig. 3). It can readily be recognized after decomplexation with 8-hydroxyquinoline by its intense green fluorescence (pyoverdins show a more yellowish fluorescence). The amino acid analysis gave the same results as obtained for the pyoverdin and the ferribactin (Gly, 1 D- and 2 L-Lys, 1 L-(OH)Orn, 1 D- and 1 L-Ser), but no dicarboxylic acid could be detected. The mass of the $[M+H]^+$ ion as determined by FAB-MS is *m/z* 1087. The UV/VIS spectra correspond to those of azotobactin D (Demange *et al.*, 1987, Demange *et al.*, 1988) (λ_{max} = 406 nm at pH 7 and λ_{max} = 378 nm at pH 3 without splitting into two maxima as it is typical for pyoverdins).

The presence of the azotobactin chromophore **3** is confirmed by the molecular mass and the NMR-data (see Tables IV–VI). They correspond to those observed for azotobactin D (Demange *et al.*, 1988) and azotobactin DSM87 (Schaffner, unpublished results), the ^{1}H-signals typically appear at lower field compared with the signals of the pyoverdin chromophore. The AA′BB′-system of the succinic acid side chain of the pyoverdin are miss-

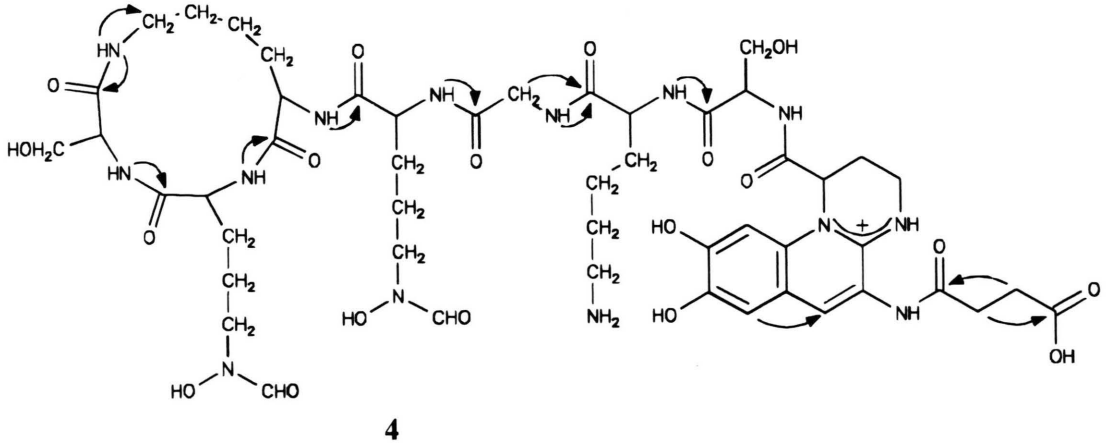

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Fig. 5. Long-range correlations in Suc-pyoverdin 13525.

Table IV. ^{1}H NMR data of azotobactin Pch 9446 in 100 mM KH_2PO_4, 10% D_2O, pH 4.3 (5 °C).

Amino Acid	α-H	β-H	γ-H	δ-H	ε-H	ε-NH_2	NH	CHO_c/CHO_t
Gly	3.43 3.59	–	–	–	–	–	3.36	–
Ser	4.49	3.99	–	–	–	–	9.64	–
Ser′	4.33	3.86	–	–	–	–	9.01	–
Lys	4.34	1.65 1.83	1.27 1.54	1.59	2.79 2.84	7.59	8.81	–
Lys′	4.14	1.59 1.94	0.94 1.26	1.46 1.57	3.16 3.25	7.41	8.30	–
(OH)Orn	4.15	1.68 1.71	1.60 1.72	3.52	–	–	8.17	7.91/8.28
(OH)Orn′	4.39	1.68 1.71	1.60 1.72	3.48	–	–	[a]	7.91/8.28

Chromophore	H1	H2a/2b	H3a/3b	H6	H7	H10	Chr NH
	6.07	2.67/3.01	3.73/4.35	7.94	7.42	7.28	9.90

δ (ppm), DSS as internal standard, c: *cis*, t: *trans*; [a] could not be identified.

Table V. ^{1}H NMR data of azotobactin Pch 9446 in 100 mM KH_2PO_4, 10% D_2O, pH 4.3 (25 °C).

Amino Acid	α-H	β-H	γ-H	δ-H	ε-H	ε-NH_2	NH	CHO_c/CHO_t
Gly	3.52	–	–	–	–	–	8.22	–
Ser	4.47	3.97	–	–	–	–	9.52	–
Ser′	4.35	3.86	–	–	–	–	8.79	–
Lys	4.33	1.63 1.83	1.24 1.57	1.50 1.54	2.77 2.83	[a]	8.58	–
Lys′	4.16	1.76 1.92	1.01 1.25	1.46 1.56	3.16 3.25	7.31	8.13	–
(OH)Orn	4.19	1.63 1.74	1.57	3.53	–	–	8.09	7.93/8.28
(OH)Orn′	4.39	1.53 1.71	1.57	3.49	–	–	7.97	7.93/8.28

Chromophore	H1	H2a/2b	H3a/3b	H6	H7	H10	Chr NH
	6.07	2.69/3.01	3.70/4.35	8.08	7.43	7.28	[a]

δ (ppm), DSS as internal standard, c: *cis*, t: *trans*; [a] could not be identified.

ing. Especially notable is the low field shift of the Ser linked to the chromophore in both cases. Due to the small amount available not all NOE cross peaks could be observed (Fig. 6). However, most of the peptide bonds showed up in the NOESY spectrum and confirmed the same sequence as determined for the pyoverdin and the ferribactin. Thus structure **5** can be proposed for the new compound which will be named azotobactin Pch 9446.

Azotobactins are the typical siderophores of *Azotobacter vinelandii* (Fukasawa *et al.*, 1972; Demange *et al.*, 1988; Page *et al.*, 1991; Menhart *et al.*, 1991; Budzikiewicz *et al.*, 1992). The occurrence of an azotobactin in the culture broth of a *Pseudomonas* species was mentioned only once in the literature but no details were given (Demange *et al.*, 1990). Ferribactins are most likely the precursors of the pyoverdins (Taraz *et al.*, 1991;

Table VI. ^{13}C NMR data of azotobactin Pch 9446 in 100 mM KH_2PO_4, 10% D_2O, pH 4.3 (25 °C).

Amino acid	α-C	β-C	γ-C	δ-C	ε-C	C=O	CHO_c/ CHO_t
Gly	43.9	–	–	–	–	[a]	–
Ser	58.4	62.5	–	–	–	173.5	–
Ser′	58.4	61.3	–	–	–	173.6	–
Lys	55.1	31.6	23.6	27.6	40.8	[a]	–
Lys′	57.2	30.0	20.5	26.9	39.1	[a]	–
(OH)Orn	55.23	29.1	24.0	51.4c 47.5t	–	[a]	161.0/ 165.2
(OH)Orn′	54.9	27.6	24.0	51.4c 47.5t	–	[a]	161.2/ 165.2

Chromophore	C-1	C-2	C-3	C-4	C-5a	C-6	C-6a
	57.6	24.9	36.1	[a]	122.8	122.2	120.7
	C-7	C-8	C-9	C-10	C-10a	CONHR	
	114.4	147.4 [b]	154.0	101.0	130.1	170.1	

δ (ppm), DSS as internal reference using δ (CH_3) = −1.61 ppm to conform with reference data using TMS (0 ppm) for calibration. c: *cis*, t: *trans*; [a] could not be identified; [b] could not be identified unambiguously.

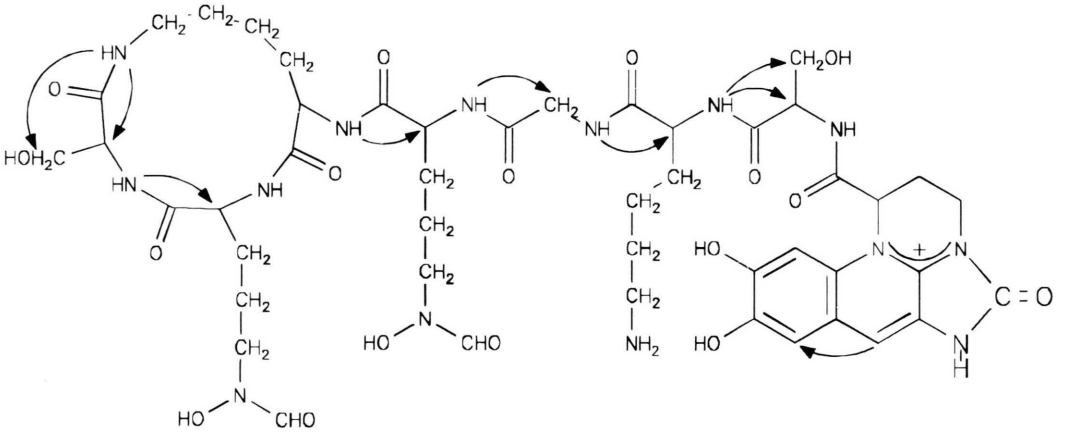

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Fig. 6. Sequential information given by NOESY of azotobactin Pch 9446.

Jacques *et al.*, 1993), ring closure yielding the quinoline system. The various dicarboxylic acids bound to the amino group of the chromophore belong to the citric acid cycle (Schäfer *et al.*, 1991). At which point the formation of the azotobactin chromophore branches off is unknown. A possibility would be a process corresponding to the oxidative decarboxylation of α-ketoglutaric acid giving succinic acid starting from a cyclic form so that the CO_2 remains in the molecule.

Acknowledgement

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