Structure Elucidation of Azotobactin 87, Isolated from *Azotobacter vinelandii* ATCC 12837*, **

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Chromopeptide siderophores (azotobactin 87-I and -II) were isolated from an iron deficient culture medium of *Azotobacter vinelandii* ATCC 12837 (=DSM 87). Their structures were elucidated by chemical degradation studies and spectroscopic methods, especially 2D-NMR-techniques. Total assignments of ^{1}H-, ^{13}C-, and ^{15}N-resonances based on 2D-HOHAHA-, ^{1}H/^{13}C-HMQC-, ^{1}H/^{13}C-HMBC-, ^{1}H/^{15}N-HMQC/TOCSY-, and ^{1}H/^{15}N-HMBC-experiments are given as well as sequential information derived from ^{1}H/^{1}H-NOESY-, ^{1}H/^{13}C-HMBC- and ^{1}H/^{15}N-HMBC-experiments. Both Az 87-I and Az 87-II consist of a tetracyclic chromophore – (1*S*)8,9-dihydroxy-4-oxo-2,3,4,5-tetrahydro-1*H*,10c*H*-3a,5,10b-triazaacephenantrylene-1-carboxylic acid – and a decapeptide chain linked with the N-terminus to the carboxy group of the chromophore containing also modified, non-proteinogenic amino acids. The sequence L-Ser-D-Ser-L-Hse-Gly-D-*threo*-OHAsp-Hse-Hse-Hse-D-N^{5}OH-N^{5}-R-Hbu-Orn-L-Hse was determined for Az 87-I, while Az 87-II contains a C-terminal L-Hse-lactone instead. Iron is chelated by the catecholic group of the chromophore, the β-hydroxy aspartic acid, and the hydroxamate function formed by N^{5}-hydroxyornithine and *R*-β-hydroxybutyric acid.

Introduction

Under iron-limited conditions bacteria from the gram-negative genera *Pseudomonas* and *Azotobacter* biosynthesize and secrete Fe^{3+}-complexing siderophores to ensure their iron supply. The chromopeptide siderophores from the "fluorescent group" of *Pseudomonas*, the pyoverdins, and structurally related substances (isopyoverdins, dihydropyoverdins, ferribactins) investigated so far show a great structural diversity in their peptide chains which consist of 6 to 12 amino acids (Budzikiewicz, 1993). In contrast, for the genus *Azotobacter* only one chromopeptide siderophore, viz. azotobactin D (azotobactin δ with a C-terminal Hse-lactone instead of Hse is probably an artifact) from *Azotobacter vinelandii* CCM 289 has been described so far (Demange *et al.*, 1988). (A somewhat spurious azotobactin O (Fukasawa *et al.*, 1972) which has only two complexing sites for Fe^{3+} is possibly an artifact obtained from azotobactin D due to the acidic work up conditions (Page *et al.*, 1991)). From *Azotobacter vinelandii* ATCC 12837 (=DSM 87) a second pair, Az 87-I and Az 87-II, resp., (Fig. 1) could be isolated now. The characteristic of azotobactins is their chromophore, ((1*S*)-8,9-dihydroxy-4-oxo-2,3,4,5-tetrahydro-1*H*-10c*H*-3a,5,10b-triazaacephenantrylene-1-carboxylic acid, Fig. 2). The peptide chain of the

Abbreviations: Common amino acids 3-letter code; Hse, homoserine; OHAsp, *threo*-β-hydroxy Asp; OHOrn, N^{5}-hydroxy Orn; Ac-OHOrn, N^{5}-acetyl OHOrn; Hbu-OHOrn, N^{5}-β-hydroxybutyryl OHOrn; Hbu, β-hydroxybutyric acid; EDTA, ethylenediamine tetraacetic acid; TAB, N/O-trifluoroacetyl (aminoacid) -O-*n*-butyl ester; TAP, the corresponding isopropyl ester; RP-HPLC, reversed phase high pressure liquid chromatography; CD, circular dichroism; FID, free induction decay; FAB-MS, fast atom bombardment mass spectrometry; NMR, nuclear magnetic resonance; DEPT, distortionless enhancement by polarisation transfer; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; TOCSY, total correlated spectroscopy; NOESY, nuclear Overhauser and exchange spectroscopy; HMBC, ^{1}H detected multiple bond heteronuclear multiple quantum coherence; HMQC, ^{1}H detected 2D heteronuclear multiple quantum coherence.

* Part LXV of the series "Bacterial Constituents". For part LXIV see Jacques *et al.*, 1995.

** Preliminary communication see Budzikiewicz *et al.*, 1992.

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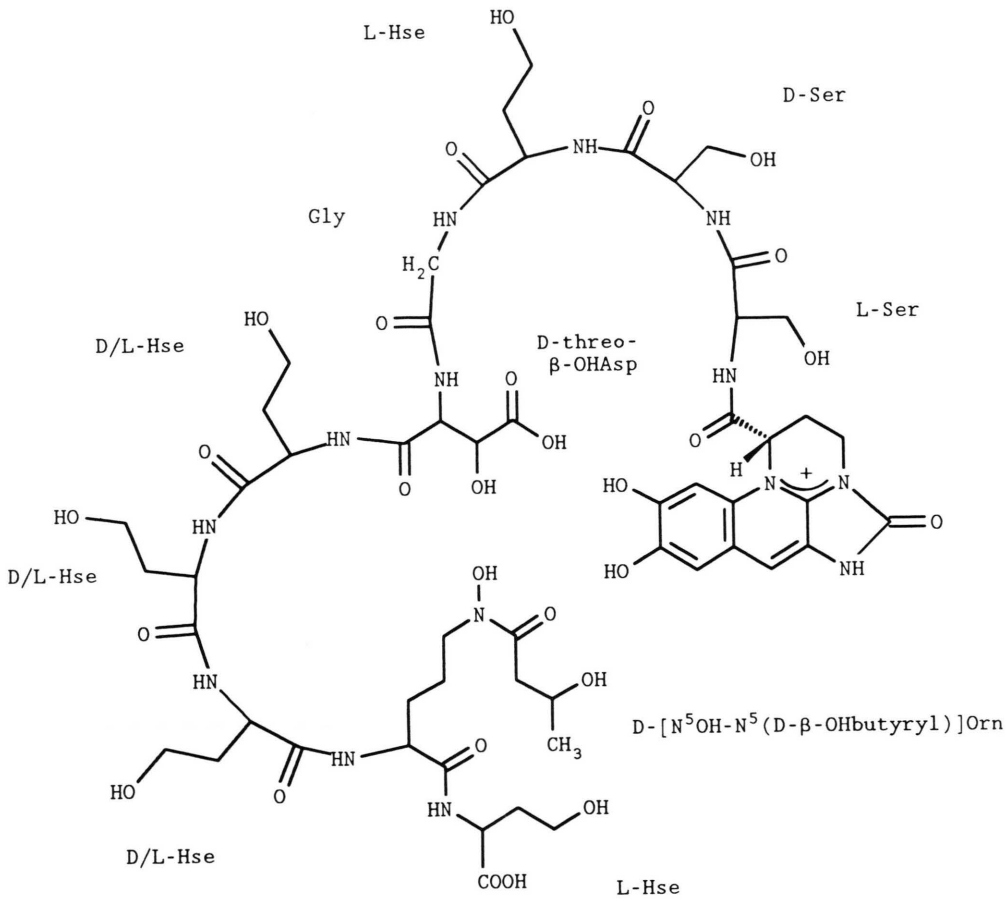

Fig. 1. Structure of Az 87-I (Az 87-II with a C-terminal homoserine-lactone).

new siderophores consists of 10 amino acids 5 of which are homoserine. It contains as a hydroxamic acid unit N^5-hydroxy-N^5-*R*-β-hydroxybutyryl-Orn, thus far encountered only once in a pyoverdin (Seinsche *et al.*, 1993). Like azotobactin D and δ the new azotobactins differ only in the C-terminal amino acid Hse in its free (Az 87-I) or lactonized form (Az 87-II).

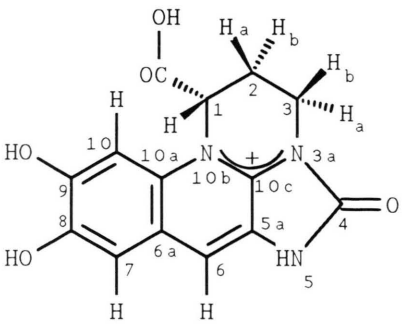

Fig. 2. Chromophore of Az 87-I and Az 87-II.

Material and Methods

Chemicals

Pyridine was treated with chlorosulfonic acid (5 ml/l), distilled and redistilled over KOH. H_2O was deionized and distilled twice. For HPLC it was additionally filtered through a XAD-4 column and a sterile filter (4μ). The other chemicals were of p.a. quality.

High performance liquid chromatography

HPLC was carried out with an equipment supplied by KNAUER (Bad Homburg, Germany)

with stainless steel columns, 210 mm x 4,6 mm, packed with polygosil, 7 µm 60 C-18 (MACHERY & NAGEL, Düren, Germany). Registration and evaluation of the chromatograms were effected with the Chromstar software from Bruker-Franzen Analytik (Karlsruhe, Germany).

Gas chromatography

A CARLO ERBA (Milan, Italy) HRGC 4160 with a flame ionization detector was equipped with a 25 m x 0.25 mm ID fused silica WCOT Chirasil-L-Val-DF-0.12 column (CHROMPACK, Middleburg, Netherlands) for determination of the absolute configurations of the amino acids.

Mass spectrometry

PI- and NI-FAB spectra were obtained with a FINNIGAN MAT (Bremen, Germany) HSQ 30 instrument equipped with an ION TECH LTD. (Teddington, Great Britain) FAB-gun (Xe) or a VARIAN MAT (Bremen, Germany) 731 instrument. Glycerol, thioglycerol and diethyleneamine were used as matrices.

Gas chromatography-mass spectrometry

GC-MS experiments were performed with a KRATOS (Manchester, Great Britain) MS 25 RF and a CARLO ERBA HRGC MFC 500 using a capillary column 25 m x 0.25 mm ID Permabond SE-54-DF-0.25 (MACHERY & NAGEL, Düren, Germany).

Nuclear magnetic resonance

^{1}H-, ^{13}C-, ^{15}N-NMR experiments were performed with a BRUKER (Karlsruhe, Germany) AC 300 and BRUKER AMX 500 using BRUKER DISNMR- and UXNMR-software installed on an ASPECT 3000 and ASPECT X32 computer or SGI Indy 4400 SC-workstation for acquisition and processing.

Az 87-I and Az 87-II were investigated in D_2O (^{1}H, ^{13}C) at 25 °C and in 100 mM CD_3COOH/NaOH-buffer, pH 4.3, 90% H_2O, 10% D_2O for lock (HOHAHA, NOESY, HMBC, HMQC) at 5 °C (necessary to obtain NOESY DATA) and 25 °C. ^{1}H- and ^{13}C-chemical shifts are given relative to TMS with the internal chemical shift standard DSS (2,2-dimethyl-2-silapentan-5-sulfonate) added to the sample using the relations δ(TMS)=δ(DSS) for ^{1}H NMR and δ(TMS)=δ(DSS)-1.61 ppm for ^{13}C NMR. ^{15}N-resonances are given relative to urea. The measurements were carried out with 15 mg of azotobactin in 0.5 ml solvent. The chromophores of the azotobactins were measured in 1 N DCl at 25 °C.

The H_2O resonance was suppressed by presaturation during the relaxation delay or by using the jump and return sequence (Plateau and Guéron, 1982). 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 x 1024 real data points after Fourier transformation.

MLEV17-HOHAHA (Bax *et al.*, 1985) and NOESY (Macura *et al.*,1980) experiments were performed in the phase sensitive mode using the time proportional phase incrementation scheme (TPPI). The spectral width was 5555.56 Hz (11.08 ppm) in both dimensions. After 4 dummy scans, 8 scans (HOHAHA) or 16 scans (NOESY) were collected for each FID. Mixing times were 40 ms for HOHAHA experiments and 200 ms for NOESY. Time domain data were multiplied by a $\pi/2$ or $\pi/3$ shifted squared sine bell to enhance the resolution.

The ^{1}H,^{13}C direct correlation was carried out with a HMQC experiment (Bax *et al.*, 1990) using bird-pulse and GARP decoupling. The spectral width was 5263 Hz (10.5 ppm) in F2 and 8064 Hz (64.5 ppm) in F1. 512 experiments with 32 scans each were performed in t1. A $\pi/2$ shifted sine bell was used as window function in both dimensions after zero filling in t1.

^{1}H,^{13}C long range couplings were determined in a not decoupled HMBC (Bax *et al.*, 1986) with spectral widths of 26315 Hz (209 ppm) in F2, and 5319 Hz (10.6 ppm) in F1, 4 starting dummy scans, 48 scans for each t1, and a 65 ms delay ($J_{C,H}$=7.8 Hz) or an 80 ms delay ($J_{C,H}$=6.3 Hz) for evolution of long range couplings. Processing was done as given for HMQC.

The ^{1}H,^{15}N long range coupling (Schmidt *et al.*, 1991) was determined using a spectral width of 3548 Hz (70 ppm) in F2, and 5814 Hz (11.6 ppm) in F1, 4 starting dummy scans, 64 scans for each t1, and a 60 ms delay ($J_{N,H}$=8.3 Hz) for evolution of the long range couplings.

The gradient-$^{1}H,^{15}N$-HMQC-TOCSY experiment (Schmidt *et al.*, 1991; Williamson, 1993) with GARP decoupling during acquisition and spectral widths of 6024 Hz (12 ppm) in F2, and 1786 Hz (35 ppm) in F1 was carried out using a mixing time of 55 ms for homonuclear Hartman-Hahn transfer, and was optimized for $^{1}J_{N,H}$=90 Hz. Phase sensitive detection of heteronuclear shifts was accomplished by the TPPI method. 256 experiments with 8 scans each (32 dummy scans) were performed in t1. A $\pi/2$ shifted squared sine bell was used as window function in both dimensions after zero filling in t1.

Growth

Azotobacter vinelandii ATCC 12837 (DSM 87) was grown for 120 hrs. in 500 ml Erlenmeyer flasks containing 200 ml of culture medium under rotary shaking, passive aeration, light and a growth temperature of 30 °C. One litre culture medium consisted of 10 g mannitol, 0.2 g NaCl, 1 g K_2HPO_4, 0.2 g $MgSO_4 \cdot H_2O$, 1 g $CaCO_3$ in deionized distilled water. For isolation of the ^{15}N-labelled siderophores 2 g $(^{15}NH_4)_2SO_4$ (96% enriched) was added. The medium was adjusted to pH 7 and autoclaved. At the end of growth Fe(III)citrate was added to the culture to form the ferric complexes from the excreted siderophores.

Isolation

The isolation of the azotobactins followed the steps described for that of pyoverdins (Briskot *et al.*, 1989): The culture fluid was adjusted to pH 6.0 with dilute HCl, to remove bacterial cells the solution was centrifuged, then the ferric siderophores were adsorbed on XAD-4 and eluted with acetone/H_2O 1:1 (v/v). The eluate was brought to dryness i.v. and the residues were stored at -4 °C. Portions of the extracts were chromatographed on Bio-Gel P-2 with a pyridinium acetate buffer (pH 5.0, linear flow rate) and the siderophores were detected at 405 nm. The fraction containing ferric siderophores was then chromatographed with HPLC. The eluent was a mixture of 0.1 N HOAc with acetonitrile (4.5% by volume); the compounds were detected by their absorbance at 405 nm. Two fractions, FeAz 87-I and FeAz 87-II, could be isolated. Decomplexation was achieved with 1% citric acid and 5% 8-hydroxyquinoline in $CHCl_3$ as described (Briskot *et al.*, 1989).

Hydrolysis

For amino acid analysis the pigment material was hydrolyzed with 7.6 N HI for 21 hrs. at 110 °C, the solution was evaporated to dryness i. v., and portions were analyzed for amino acids and other volatile constituents after derivatization to N/O-trifluoracetyl-isopropylesters (Husek and Masek, 1975) by gas chromatography with a flame detector for quantitative results and for the determination of the absolute configurations, or with a mass spectrometer detector for identification of the amino acids by comparison with standard samples (Roach and Gehrke, 1969).

For the sequence analysis azotobactin was hydrolyzed with 0.3 N HCl for 111 hrs. at room temperature, the fraction containing parts of the peptide chain bound N-terminally to the chromophore ("chromophore peptides") was adsorbed on a Sep-Pak RP-18 cartouche (which does not retain free amino acids and oligopeptides) and desorbed with 0.1 N acetic acid/acetonitrile, evaporated to dryness and subjected to FAB-MS analysis.

To determine the position of L- and D-amino acids in the peptide sequence the chromophore peptides were isolated after hydrolysis of the azotobactins with 6 N HCl, 12 min, 90 °C and 3 N HCl, 5 min, 90 °C. After evaporation to dryness the residue was dissolved in H_2O and chromatographed on Bio-Gel P-2 with 0.1 N acetic acid or by HPLC (detection at 254 nm). Fragments still containing the chromophore (6 N HCl: Chr-Ser; 3 N HCl: Chr-Ser-Ser-Hse) were evaporated to dryness, hydrolyzed with 6 N HCl at 110 °C for 21 hrs., and derivatized for GC (see above).

For isolation of the chromophore the azotobactins were hydrolyzed with 3 ml 3 N HCl for 5 days at 120 °C. HCl was removed under reduced pressure, the residue was dissolved in 0.1% trifluoracetic acid, and the compound with white fluorescence was separated from other material by repeated HPLC with 15% MeOH/0.1% trifluoracetic acid. The combined fractions were evaporated to dryness. For CD and ^{1}H NMR measurements the chromophore was dissolved in DCl.

To determine the absolute configuration of Hbu Az 87-I was hydrolyzed in 0.3 N HCl for 10 days

at room temperature. The hydrolysate was brought on a Sep-Pak RP-18 cartouche. The fraction containing chromophore peptides was adsorbed, the free acids and oligopeptides were not retained. After washing with H_2O the eluate was brought to dryness and treated with 1.5 ml 3-pentanol/acetyl chloride (v/v 5/1) for 1 h at 110 °C. After cooling to room temperature the residue was brought to dryness i. v., remaining reagent was removed by twice addition of 0.3 ml CH_2Cl_2 and evaporation to dryness. For the GC analysis the residue was dissolved in CH_2Cl_2.

Hydrazinolysis

0.1 mg Az 87-I and 0.1 ml of water free N_2H_4 were kept at 80 °C for 19 hrs. The solution was brought to dryness and kept i. v. over conc. H_2SO_4 for 24 hrs. The residue was dissolved in 0.2 ml H_2O and shaken with 0.15 ml benzaldehyde for 1 h at room temperature. The phases were separated by centrifugation. The organic phase was extracted with 0.05 ml H_2O. The combined aqueous solutions were brought to dryness. The residue was derivatized with acetyl chloride/3-pentanol and trifluoracetic acid anhydride and analyzed by GC as described above.

Complex constants

A buffered solution (pH 5.0 and pH 7.0) of FeAz 87-II was treated with increasing amounts of EDTA and kept at room temperature until the equilibrium was reached. The equilibrium concentration of the azotobactin was determined by measuring the absorption at 550 nm.

Results and Discussion

Characterization of Az 87-I and Az 87-II and their Fe^{3+}-complexes

The reddish-brown Fe^{3+}-complexes exhibit at pH 7.0 charge transfer bands at 560 and 470 nm, an absorption maximum at 410 nm and a shoulder at about 260 nm (referring to the phenantrylene system), and a maximum at 230 nm (amide absorption). In the UV spectra of the iron free compounds the position of the absorption maxima and its pH-dependence (a hypsochromic shift with decreasing pH from 410 nm, pH 7.0, to 380 nm with a shoulder at 360 nm, pH 3.0) is comparable

Table I. ^1H NMR data of Az 87-I in 100 mM CD_3COOH/NaOH, 10% D_2O, pH 4.3 at 5 °C and of Az 87-II in 100 mM CD_3COOH/NaOH, 10% D_2O, pH 4.3 at 5 °C and 25 °C.

Proton	Az 87-I 5 °C Chemical shift	Az 87-II 5 °C Chemical shift	Az 87-II 25 °C Chemical shift
Chromophore[a]			
H-1	6.06	6.09	6.11
H-2a	2.70	2.70	2.69
H-2b	3.07	3.07	3.08
H-3a	3.65	3.64	3.66
H-3b	4.38	4.37	4.37
H-6	7.77	7.90	8.07
H-7	7.02	7.19	7.30
H-10	7.19	7.24	7.38
β-Hydroxybutyric acid[b]			
Hα	2.58/2.77	2.56/2.77	2.60/2.77
Hβ	4.23	4.23	4.25
Hγ	1.23	1.23	1.21
Glycine[b]			
NH	8.50	8.50	8.34
Hα	3.95	3.94	3.92/3.97
Homoserine-1[b]			
NH	8.55	8.54	8.34
Hα	4.55	4.48	4.48
Hβ	1.76/2.02	1.76/2.01	1.71/1.98
Hγ	3.58/3.66	3.56	3.56
Homoserine-2[b]			
NH	8.53	8.52	8.38
Hα	4.45	4.45	4.44
Hβ	1.94/2.05	1.94/2.04	1.92/2.04
Hγ	3.56/3.61	3.60	3.62
Homoserine-3[b]			
NH	8.42	8.45	8.32
Hα	4.44	4.43	4.42
Hβ	1.93/2.06	1.93/2.04	1.91/2.04
Hγ	3.58/3.66	3.60/3.64	3.59/3.65
Homoserine-4[b]			
NH	8.42	8.45	8.32
Hα	4.44	4.43	4.42
Hβ	1.93/2.06	1.93/2.04	1.91/2.04
Hγ	3.58/3.66	3.60/3.64	3.59/3.65
Homoserine-5[b]			
NH	8.21	8.63	8.51
Hα	4.37	4.63	4.64
Hβ	1.90/2.10	2.31/2.58	2.29/2.60
Hγ	3.56/3.65	4.34/4.50	4.37/4.53
β-Hydroxyaspartic acid[b]			
NH	8.25	8.21	8.08
Hα	4.85	4.81	4.80
Hβ	4.56	4.51	4.49

Tab. I. (Continued).

Proton	Az 87-I 5 °C Chemical shift	Az 87-II 5 °C Chemical shift	Az 87-II 25 °C Chemical shift
N^5-Hydroxyornithine[b]			
NH	8.50	8.57	8.42
Hα	4.35	4.29	4.32
Hβ	1.84	1.72/1.82	1.68/1.82
Hγ	1.72	1.69	1.64/1.69
Hδ	3.60/3.66	3.59/3.65	3.62/3.64
Serine-1[b]			
NH	9.61	9.60	9.46
Hα	4.61	4.59	4.66
Hβ	4.02	4.00	4.00
Serine-2[b]			
NH	8.80	8.81	8.64
Hα	4.52	4.50	4.52
Hβ	3.90/3.94	3.87/3.94	3.89/3.94

δ (ppm). DSS as internal reference; [a] for the numbering see Fig. 2; [b] for the numbering see Fig. 6.

with the data given in the literature for pyoverdins (Budzikiewicz, 1993).

The complex-formation constants were determined for Az 87-I as described (Anderegg *et al.*, 1963) as $2.61 \cdot 10^{17}$ (pH 5.0) and $2.51 \cdot 10^{24}$ (pH 7.0).

The molecular masses of the two azotobactins were obtained from their FAB-mass spectra: M^+ *m/z* 1385 for Az 87-I and *m/z* 1367 for Az 87-II. The M^--ions of the corresponding Fe-complexes were *m/z* 1436 and *m/z* 1418, resp. As expected for a 1:1-stoichiometry the molecular masses (M) of the ferri compounds are 53 u higher than those of the free azotobactins ($+^{56}Fe^{3+} - 3H^+$). The difference of 18 u between Az 87-I and Az 87-II (as well as for the corresponding iron complexes) suggests a structure of Az 87-II formed by the loss of H_2O.

The quantitative analysis of the amino acids and the determination of their configuration was performed after total hydrolysis with 6 N HI (110 °C, 21 hrs.) by GC of the TAP-derivatives on a chirasil-L-Val column. For both Az 87-I and Az 87-II the same results were obtained: 1 D-*threo*-OHAsp, 1 Gly, 1 D-Hse, 4 L-Hse, 1 D-Orn, 1 D-Ser, 1 L-Ser. OHOrn could be detected in addition to Orn in the HCl hydrolysate which is typical for hydroxamic acids with a N^5-OHOrn unit (Mohn *et al.*, 1990).

Fig. 3 shows the ^1H NMR spectrum of Az 87-I in D_2O. The complete assignment of the ^1H- and ^{13}C-signals of Az 87-I and Az 87-II based on 2D-HOHAHA, $^1H/^{13}C$-HMQC, $^1H/^{13}C$-HMBC-experiments is summarized in Tables I and II.

The azotobactins contain the following polyfunctional amino acids: 2 Ser, 5 Hse, 1 OHAsp, 1 OHOrn. The presence of free β-hydroxyl groups of the two Ser could be demonstrated by comparison of the CH_2-β proton resonances (4.00 and 3.90/3.94, resp.) with literature data for esterified (Yang and Leong, 1984) and non-esterified (Wüthrich, 1976) Ser. In case of esterification they would be shifted by approximately 0.6 ppm to lower fields. The γ-CH_2-group of Hse shows a comparable shift upon esterification or lactonization (-CH_2OH approximately 3.6 ppm, esters and lactones show a downfield shift of 0.8 ppm) (De-

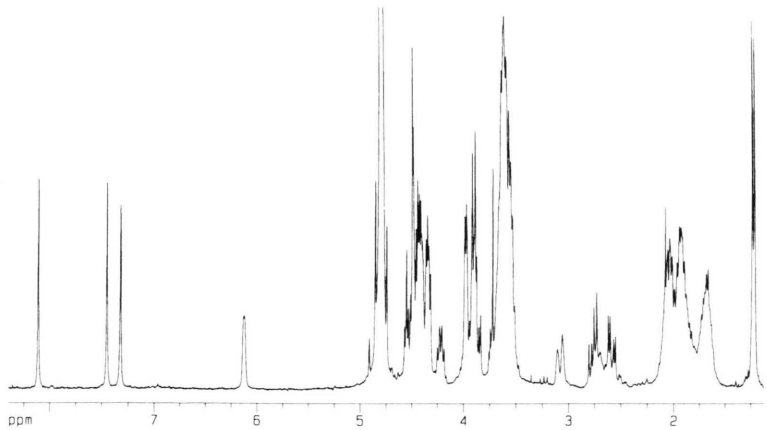

Fig. 3. 300 MHz ^1H NMR spectrum of Az 87-I in D_2O.

Table II. ^{13}C NMR data of Az 87-I in 100 mM CD_3COOH/NaOH, 10% D_2O, pH 4.3, at 5 °C and 25 °C and of Az 87-II in 100 mM CD_3COOH/NaOH, 10% D_2O, pH 4.3 at 25 °C.

Proton	Az 87-I 5 °C Chemical shift	Az 87-I 25 °C Chemical shift	Az 87-II 25 °C Chemical shift
Chromophore[a]			
C-1	57.05	57.69*	57.57*
C-2	24.41	24.83	24.71
C-3	35.53	35.94	35.83
C-4	153.92	154.25	154.21
C-5a	122.15	122.58	122.54
C-6	121.10	121.58	121.84
C-6a	120.19	120.71	120.69
C-7	113.47	114.21	114.26
C-8	146.65	147.02	146.99
C-9	152.26	152.63	152.69
C-10	100.45	100.82	100.52
C-10a	128.75	129.53	129.57
C-10c	140.45	140.94	140.91
CO	169.19	169.48	169.42
β-Hydroxybutyric acid			
CO	174.15	174.48	174.21
Cα	41.78	42.12	42.02
Cβ	65.80	66.09	65.99
C_γ	23.16	23.45	23.34
Glycine			
CO	172.48	172.69	172.61
Cα	43.82	43.88	43.77
Homoserine-1[b]			
CO	175.03	175.22	175.24
Cα	52.10	53.28	52.36
Cβ	33.99	35.03	34.52
C_γ	58.71	59.12	58.10
Homoserine-2[b]			
CO	175.13*	175.25*	175.17*
Cα	52.92	52.98	52.72
Cβ	34.19	34.64	34.43
C_γ	58.94	59.70	59.01
Homoserine-3[b]			
CO	175.17*	175.35*	175.21*
Cα	52.37	52.92	52.79
Cβ	34.21	34.64	34.23
C_γ	58.84	59.35	59.16
Homoserine-4[b]			
CO	175.23*	175.38*	175.28
Cα	52.63	52.79	53.18
Cβ	34.27	34.35	34.06
C_γ	58.84	59.29	59.25
Homoserine-5[b]			
CO	175.51*	175.67*	179.23
Cα	52.56	52.45	50.57
Cβ	34.61	34.54	28.92
C_γ	59.29	59.26	68.63
β-Hydroxyaspartic acid			
CO	172.98	173.21	173.23
Cα	57.80	58.13	53.18
Cβ	72.98	73.21	73.21
CO	177.43	177.57	177.59
N^5-Hydroxyornithine			
CO	174.41	174.52	174.79
Cα	54.68	54.95	54.65
Cβ	29.19	29.57	29.09
C_γ	23.47	23.75	23.56
C_δ	48.18	48.55	48.42
Serine-1[b]			
CO	172.51	172.73	172.58
Cα	57.35	57.45*	57.36*
Cβ	62.19	62.41	62.27
Serine-2[b]			
CO	173.09	173.26	173.09
Cα	56.95	57.16	57.05
Cβ	62.22	62.53	62.41

δ (ppm). DSS as internal reference using $\delta(CH_3) = -1.61$ ppm to conform with reference data using TMS (0 ppm) for calibration; [a] for the numbering see Fig. 2; [b] for the numbering see Fig. 6; * assignments may be interchanged.

mange *et al.*, 1988). Therefore all Hse-hydroxyl groups of Az 87-I are free (3.58/3.66 ppm) whereas one of the five Hse of Az 87-II shows a downfield shift of 0.75/0.92 ppm (4.34/4.50 ppm) which suggests a -CH_2-COR function. The decision between a C-terminal Hse-lactone (cf. azotobactin δ) and a larger ring involving the hydroxy group of another Hse was possible by the observation of a correlation signal in the $^1H/^{13}C$-HMBC experiment between Hse5-Hγ and Hse5-CO due to ^3J-coupling (Fig. 6).

That the β-OH group of β-OHAsp is free could be shown as describes for Ser. From the pH-dependence of the β-CH-signal in the ^1H NMR spectrum, the shift value of the carbonyl signal and the absence of a ^3J-coupling of another amino acid with the CO of the β-carbonyl group in the HMBC experiment it can be concluded that also the β-carboxyl group of β-OHAsp is free.

As none of the amino acids contains a methyl group the douplet at 1.22 ppm is remarkable in

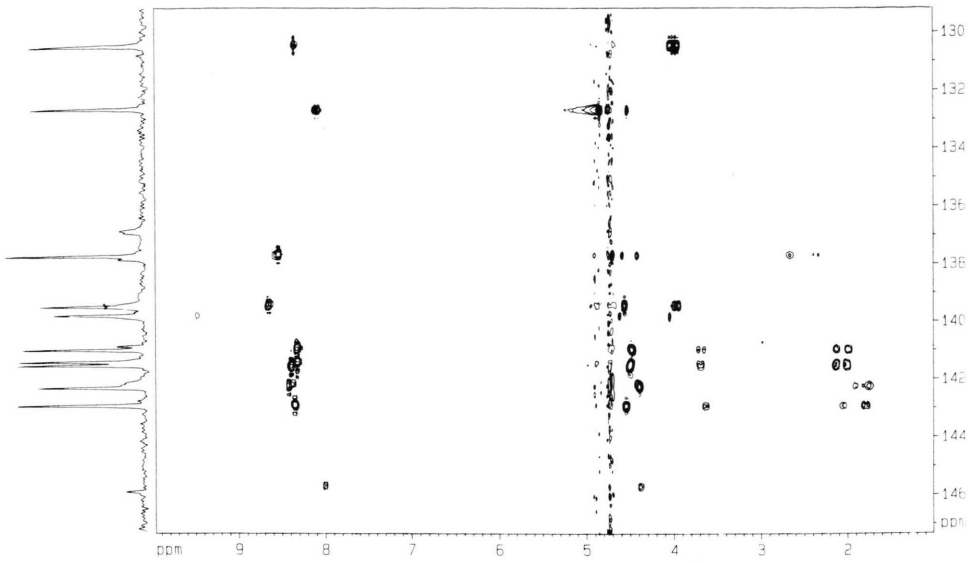

Fig. 4. 500 MHz $^{1}H/^{15}N$-HMQC-TOCSY spectrum of ^{15}NAz 87-II in 100 mM CD_3COOH/NaOH, 10% D_2O, pH 4.3; 25 °C.

the ^{1}H NMR spectra of Az 87-I and of Az 87-II. HOHAHA, DEPT, and $^{1}H/^{13}C$-HMBC demonstrate a CH_3-CHX-CH_2-Y-sequence. Comparison with chemical shifts given in literature and the difference of 86 u between the determined and the calculated molecular mass suggests the presence of a β-hydroxybutyryl group. In the TAB- and TAP-derivatized hydrolysates of Az 87-I/-II no β-OH-butyric acid could be detected because of polymerization and decomposition under the conditions for hydrolysis and derivatization. By treating Az 87-I/-II with dilute HCl (0.3 N) at room temperature for about 10 days it is possible to detect Hbu after derivatization to its 3-pentylester (*m/z* 174, determined by GC/MS). The configuration of the β-OH-butyric acid could be determined as *R* (D) by GC on a Chirasil-L-Val-column.

The typical NMR resonances for OHOrn (Tappe *et al.*, 1993) and the fact that characteristic resonances for formyl, acetyl or cyclic OHOrn usually encountered for hydroxamic acid units in pyoverdins are missing, indicate that the hydroxamic acid unit is formed here by OHOrn and Hbu. NOE-cross peaks between the protons of N^5-OHOrn and Hbu could not be detected in the $^{1}H/^{1}H$-NOESY-experiment, and in the $^{1}H/^{13}C$-HMBC no correlation peak corresponding to a ^{3}J(OHOrn-Hδ/Hbu-CO)-coupling was found. A mild hydrolysis of the hydroxamic acid unit as described for N-formyl OHOrn failed; under these conditions the labile homoseryl peptide bonds were cleaved. The confirmation of the hydroxamate function had therefore to be carried out by a $^{1}H/^{15}N$-HMBC-experiment with the fully ^{15}N-labelled azotobactin ^{15}NAz 87-II: The amide nitrogens of all ten amino acids were identified by a $^{1}H/^{15}N$-HMQC/TOCSY-experiment (Fig. 4), the resonances in the range of 130 to 142 ppm are listed in Table III. In the $^{1}H/^{15}N$-HMBC-experiment the nitrogen with the resonance of 202 ppm shows cross peaks to Hγ and Hδ of OHOrn. Because of these correlation peaks this must be the

Table III. ^{15}N-chemical shifts[a] of amide nitrogens of the amino acids[b] in ^{15}NAz 87-II.

Amino Acid	Gly	OHAsp	Hse-5 (lacton)	Ser-2	Ser-1	Hse-3/4	Hse-3/4	Hse-2	OHOrn	Hse-1
^{15}NH	130.50	132.65	137.75	139.50	139.81	141.00	141.42	141.54	142.31	142.92

[a] δ (ppm) relative to urea; [b] for the numbering see Fig. 6.

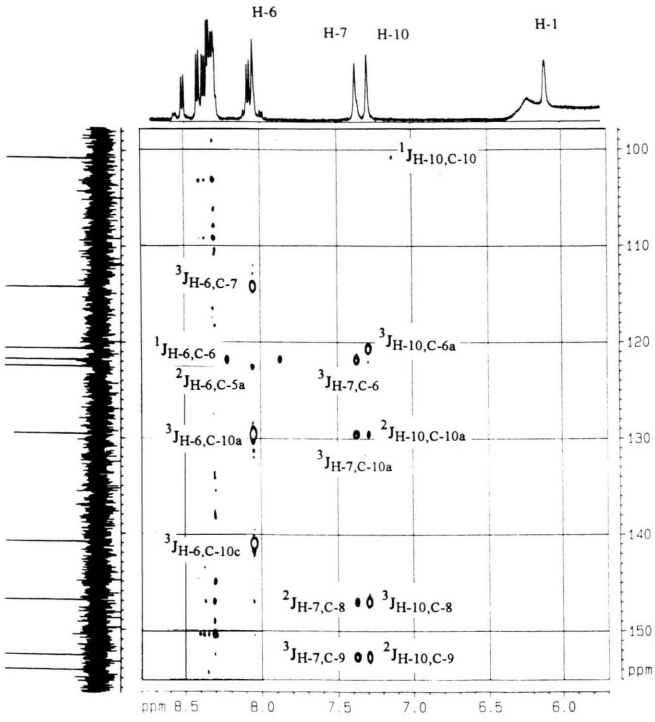

Fig. 5. Expanded region of the $^1H/^{13}C$-HMBC-spectrum (500 MHz) of Az 87-II F1 100–155 ppm; F2 6.0–9.0 ppm in 100 mM $CD_3COOH/NaOH$, 10% D_2O, pH 4.3; 25 °C.

resonance of N^5 of N^5-OHOrn. Additional correlation peaks to Hα of Hbu prove the connection of β-Hbu and N^5-OHOrn.

The NMR-experiments show that the chromophore of Az 87-I and Az 87-II is identical with the chromophore of the pigment of *Azotobacter vinelandii* O, whose structure was determined by X-ray chrystallography of a methylated (Corbin *et al.*, 1970; Karle and Karle, 1971) and a decarboxylated (Sasaki and Hirata, 1973) derivative, and the chromophore of azotobactin D and δ, resp. (*Azotobacter vinelandii* CCM 289). The chemical shifts differ from those of the pyoverdin chromophore (while the azotobactin chromophore contains an urea unit comprising N-4 and C-5-NH, the C-5 amino group in the pyoverdin type chromophore is bound amidically to a dicarboxylic acid). The main differences are observed in the proton resonance of H-3b with a downfield shift of 0.6 ppm and in the resonances of H-2a/H-2b/H-3a (downfield shift of 0.2 ppm). In the ^{13}C-spectra exists an additional CO resonance at 154 ppm. In Fig. 5 the region of the $^1H/^{13}C$-HMBC-experiment relevant for the assignment of the ^{13}C-resonances is shown. The nitrogens N-3a, N-5 and N-10b are identified by ^2J-/^3J-couplings ($^3J_{N\text{-}10b/H10}$, $^2J_{N10b/H1}$, $^2J_{N3a/H3a}$, $^2J_{N3a/H3b}$, $^3J_{N\text{-}5/H\text{-}6}$) in the $^1H/^{15}N$-HMBC. The resonances are listed in Tables I, II, IV. In addition, the chromophore was isolated by HPLC after hydrolysis and its ^1H NMR resonances are similar to those of the intact azotobactins 87-I/-II (1N DCl (ppm): H-6 8.15, H-7 7.50, H-10 7.40, H-1 6.16, H-3b 4.31, H-3a 3.69, H-2b 3.10, H-2a 2.56). The configuration of the asymmetric C-1 of the chromophore of Az 87-I and Az 87-II was determined by comparing the CD spectra of the isolated azotobactin chromophore (OCChr) with the modified

Table IV. ^{15}N-chemical shifts[a] of nitrogen atoms of the chromophore[b] and the N^5 of the hydroxamic acid unit in ^{15}NAz 87-II.

Nitrogen	N-5	N-3a	N-10b	N^5-(Hbu.OHOrn)
δ [ppm]	136.88	151.48	165.43	201.96

[a] δ (ppm) relative to urea; [b] for the numbering see Fig. 2.

hydroxy chromophore (OHChr) isolated from pseudobactin where S-configuration had been demonstrated by X-ray analysis of its iron complex (Taraz *et al.*,1991; Teintze *et al.*, 1981). Since CD spectra are pH-dependent the CD measurements were carried out in 1 N HCl. OCChr as well as the OHChr show similar CD spectra: a positive Cotton effect at the π-π^*-transition of the chromophore in the range of 350–400 nm. Hence, C-1 of the chromophore of Az 87-I/-II is also *S*-configurated.

The amino acid sequence in the peptide chain

The N-terminus of the peptide chain is blocked by the chromophore, the C-terminus of Az 87-II is not free either, C-terminal degradation methods with Az 87-I fail owing to the presence of Hse and OHOrn. Because of the non-proteinogenic amino acids automatic peptide sequencing is not possible, enzymatic cleavages fail because of the large number of D-amino acids. Therefore the amino acid sequence was determined with two different

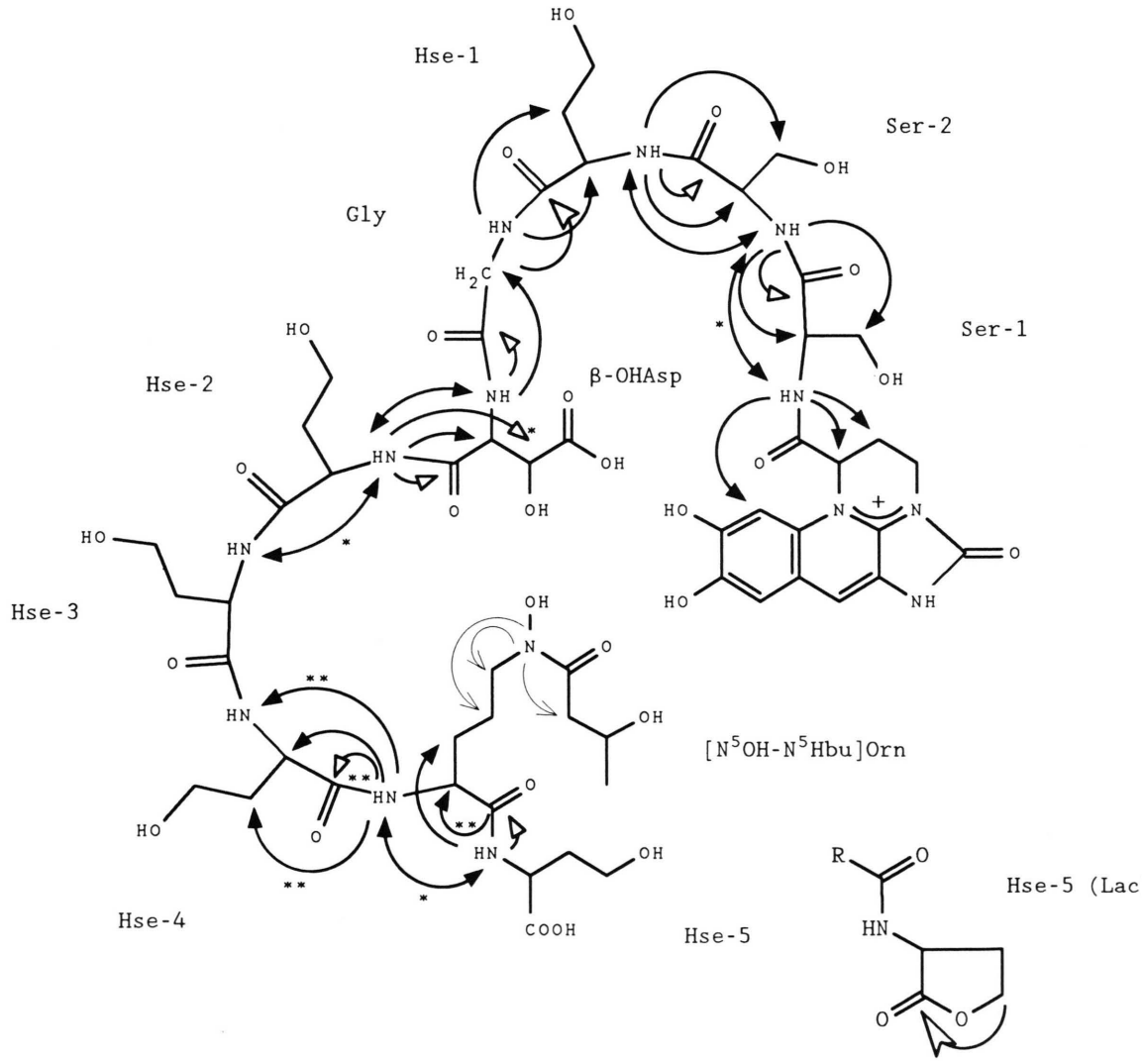

Fig. 6. Sequential information for Az 87-I and Az 87-II given by NOESY: ⟶ (NH-Hα, NH-Hβ); ⟷ (NH–NH) given by HMBC: ⇢ ^2J-(NH,CO); ⟶ ^3J-(Hα,CO); ⟹ ^3J-(Hγ,CO) given by ^{15}N-HMBC with ^{15}NAz 87-II →.

* Sequential information given only for Az 87-I; ** Sequential information given only for Az 87-II.

Table V. Fragments found in the PI-FAB-mass spectrum of the chromopeptide mixture of a partial hydrolysis of Az 87-I (0.3 N HCl, 111 hrs., 20 °C).

m/z	
389	Chr-Ser
476	Chr-Ser-Ser
559	Chr-Ser-Ser-Hse-H_2O
577	Chr-Ser-Ser-Hse
634	Chr-Ser-Ser-Hse-Gly
765	Chr-Ser-Ser-Hse-Gly-OH-Asp
848	Chr-Ser-Ser-Hse-Gly-OH-Asp-Hse-H_2O
949	Chr-Ser-Ser-Hse-Gly-OH-Asp-Hse-Hse-H_2O
1050	Chr-Ser-Ser-Hse-Gly-OH-Asp-Hse-Hse-Hse-H_2O

NMR-sequencing techniques: NOESY-experiments at 5 °C gave sequential information by NOEs between the NH of amino acid (i) and NH, Hα and/or Hβ of the preceding amino acid (i-1). An inverse HMBC experiment at 25 °C gave additional sequential information by showing $^2J(CO_{i-1}\text{-}NH_i)$ cross peaks. The results lead to the primary structures of Az 87-I and Az 87-II which are shown in Fig. 6. The missing correlation between Hse-4 and Hse-3 could be determined by FAB-MS (Table V) of a chromopeptide mixture from a partial hydrolysate (0.3 N HCl, 111 hrs., 20 °C). The molecular masses *m/z* 848, 949, and 1050 indicate the connection of 3 Hse. Also the sequence of the first five amino acids bound N-terminally (*m/z* 389, 476, 559 (577), 634, 765) can be determined in this way and it correlates with the results from NMR. For chromopeptides with C-terminal Hse molecular ions 18 u lower than expected ones are found. The loss of H_2O is the result of a cyclization of Hse during hydrolysis.

The analytical data presented so far do not allow to distinguish between D- and L-Ser and D- and L-Hse in the peptide chain. To get this information a combination of partial hydrolysis, chromatographic isolation, and purification of the hydrolysis products was effected which then were subjected to FAB-MS and amino acid analysis.

The mixtures of chromophore peptides obtained after 90 °C hydrolysis (6 N HCl, 10 min and 3 N HCl, 5 min) was separated partially by chromatography on Bio-Gel P-2 with 0.1 N CH_3COOH. The various fragments could finally be obtained in pure from RP-HPLC (eluent 4,5% CH_3CN in an aqueous acetate buffer, pH 3). FAB-MS, quantitative analysis, and chirality determination of the amino acids after total hydrolysis allowed to identify the partial sequence Chr-L-Ser and Chr-L-Ser-D-Ser-L-Hse.

The stereochemistry of its C-terminal Hse could be determined by hydrazinolysis, separation of free Hse from the hydrazids of the non-terminal amino acids, and its derivatization for chirality analysis as described above. Thus, Hse-5 could be shown to be L-configurated.

Therefore the one D-Hse must belong to the 3 Hse in the positions 6, 7, and 8 of the peptide chain. From hydrolysis experiments carried out with variation in HCl-concentrations, temperature, and time no fragments could be isolated in sufficient amounts that allowed to determine the correct position of D-Hse.

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