Azoverdin – an Isopyoverdin*

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Azoverdin, *Azomonas macrocytogenes*, Isopyoverdin, Siderophore

For azoverdin, the siderophore of *Azomonas macrocytogenes* ATCC 12334, a pyoverdin-type structure has been suggested. We now present evidence that it is actually an isopyoverdin. Also the sequence of the peptide chain has to be revised. Azoverdin comprises, therefore, the chromophore (3*S*)-5-amino-1,2-dihydro-8,9-dihydroxy-3H-pyrimido[1,2a]quinoline-3-carboxylic acid whose amino group is bound to a succinamide residue while the carboxyl group is attached to the N-terminus of L-Hse-[2-(*R*-1-amino-3-hydroxypropyl)-3,4,5,6-tetrahydropyrimidine-6*S*-carboxylic acid]-N^5-acetyl-N^5-hydroxy-D-Orn-D-Ser-N^5-acetyl-N^5-hydroxy-L-Orn. In addition to azoverdin congeners with succinic acid (azoverdin A) and with L-Glu (azoverdin G), resp., instead of the succinamide side chain could be isolated.

Introduction

Many microorganisms when grown under iron-limited conditions give off Fe^{3+}-complexing substances, so-called siderophores. A structurally well defined group amongst these are the pyoverdins produced by the fluorescent members of the rRNA homology group I of the bacterial genus *Pseudomonas* (Budzikiewicz, 1993). Pyoverdins consist of three distinct structural parts, viz. the dihydroxyquinoline chromophore **1a**, a peptide chain bound to the carboxyl group of **1a** and a dicarboxylic acid (amide) connected to its NH_2-group. Biogenetically **1a** is derived from the condensation product **2** of D-Tyr and L-Dab (Budzikiewicz, 1994). Ring closure should be possible via either one of the amidine nitrogen atoms of **2**, and in fact recently an isopyoverdin with the chromophore **3a** was isolated from a *Pseudomonas putida* strain (Jacques *et al.*, 1995).

For many years pyoverdins were considered as the typical siderophores of the fluorescent pseudomonads until the isolation of a member of this group ("azoverdin") was reported from *Azomonas macrocytogenes* ATCC 12334 (Linget *et al.*, 1992; Bernhardini *et al.*, 1996) to which the structure **4a** (Fig. 1) was assigned (in the preliminary publication the C-terminal Dab was assumed to form a lactame ring, **4b**). We wish now to demonstrate that (a) azoverdin possesses the isopyoverdin chromophore **3a** and that (b) the tetrahydropyrimidine substructure, i.e. the condensation product of Hse and Dab must not be placed at the C-terminus of the peptide chain but rather between Hse bound to the chromophore **3a** and the

Abbreviations: Common amino acids, 3-letter code; Dab, 2,4-diaminobutyric acid; Hse, homoserine; OHOrn, N^5-hydroxy Orn; AcOHOrn, N^5-acetyl OHOrn; FoOHOrn, N^5-formyl OHOrn; c-OHOrn, cyclo-OHOrn (3-amino-1-hydroxy-piperidone-2); TAB, N/O-trifluoroacetyl (aminoacid) *n*-butyl ester; TAP, the corresponding isopropyl ester; EDTA, ethylenediamine tetraacetic acid; DMSO, dimethyl sulfoxide; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane; GC, gas chromatography; RP-HPLC, reversed phase high perfomance liquid chromatography; MS, mass spectrum or mass spectrometry; EI, electron impact; FAB, fast atom bombardment; NMR-techniques: COSY, correlation spectroscopy; HMBC, ^1H detected multiple bond heteronuclear multiple quantum coherence; NOESY, nuclear Overhauser and exchange spectroscopy; ROESY, rotating frame nuclear Overhauser and exchange spectroscopy; TOCSY, total correlated spectroscopy; NOE, nuclear Overhauser effect.

* Part LXVIII of the series "Bacterial Constituents". For part LXVII see Barelmann *et al.*, 1966.

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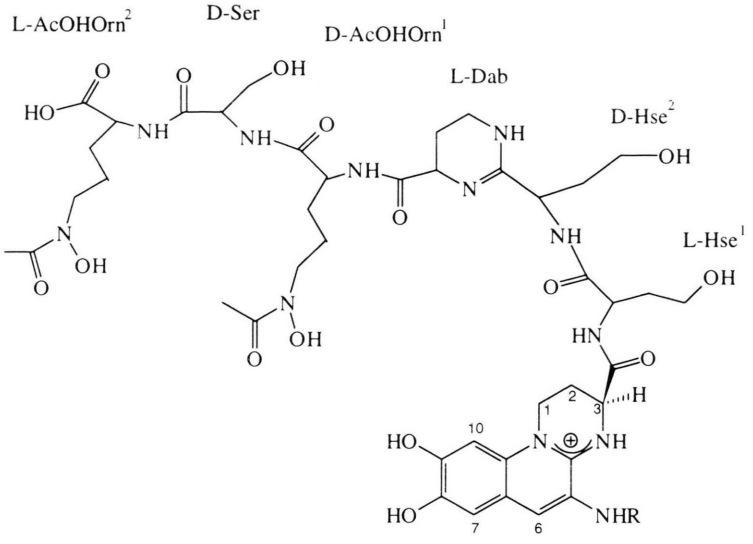
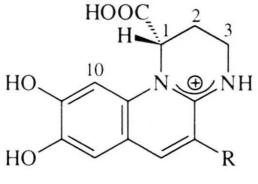
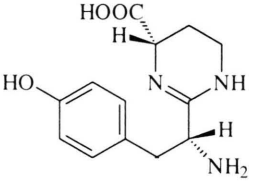


5a: R= $COCH_2CH_2CONH_2$
5b: R= $COCH_2CH_2COOH$
5c: R= $COCH_2CH_2CHNH_2COOH$



1a: R= NH_2
1b: R= OH



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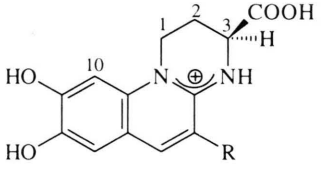


3a: R= NH_2
3b: R= OH

sequence Orn-Ser-Orn (**5a**). Furtheron, in addition to azoverdin and azoverdin A (with a succinic acid instead of a succinamide side chain, **5b**) azoverdin G (with a Glu side chain, **5c**) was found.

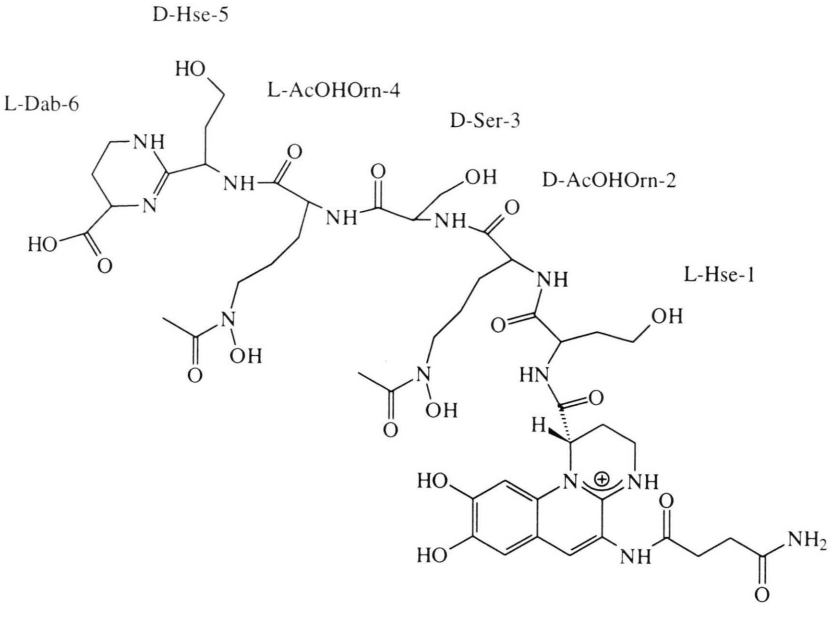
Experimental Procedures

Spectroscopy

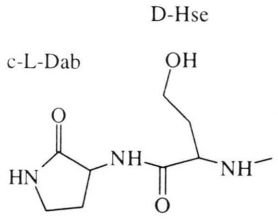
EI-MS: Incos 50 (FINNIGAN-MAT, Bremen); FAB-MS HS-Q 30 (FINNIGAN-MAT, Bremen) with FAB-gun (ION TECH, Teddington, UK), gas Xe; for FAB samples were desalted by adsorption on Sep-Pak RP-18 cartridges, washing with H_2O, desorption with CH_3OH/H_2O 7:3 (v/v) and evaporation of the solvent to dryness. NMR: Bruker AM 300 and Bruker AMX 500 (BRUKER, Karlsruhe). Samples were dissolved in D_2O, brought to dryness (oil pump vacuum) and redissolved in D_2O for measurement; for NOESY, ROESY and TOCSY experiments samples of 20 mg were dissolved in 0.6 ml 0.1 M phosphate buffer (pH 4.3), brought to dryness and redissolved in 0.6 ml D_2O/H_2O 1:9 (v/v). ^1H- and ^{13}C-chemical shifts are given relative to TMS with the internal standard DSS using the correlation $\delta(TMS) = \delta(DSS)$ for ^1H and $\delta(TMS) = \delta(DSS) - 1.61$ ppm for ^{13}C. UV/Vis: Perkin-Elmer Hitachi 200 (PERKIN-ELMER, Überlingen).

Chromatography

Adsorbtion materials for LC: Amberlite XAD-4 0.3–1.0 mm (SERVA, Heidelberg); Biogel P-2 (BIO-RAD, Hercules, USA); CM Sephadex C-25 and DEAE Sephadex A-25 (PHARMACIA, Uppsala, Sweden); Nucleosil 100 C-18, 5 μm and Po-



4a



4b

Fig. 1. Structures proposed for azoverdin.

lygosil 60 C-18, 5 μm (KNAUER, Berlin). GC columns: Chirasil-L-Val (CHROMPACK, Middleburg, Netherlands).

Bacterial Growth

Azomonas macrocytogenes ATCC 12334 was grown at ≈28°C in 250 ml Erlenmeyer flasks containing 100 ml of culture medium under rotary shaking (100 rpm), passive aeration and light. 1 l culture medium consisted of 10 g glucose, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaSO_4 \cdot 2H_2O$, 2 g $(NH_4)_2SO_4$, 4 g KH_2PO_4, 0.2 mg $Na_2MoO_4 \cdot 2H_2O$, pH adjusted to 7.1 with NaOH. After 72 hrs. 10 ml of a 5% Fe(III) citrate solution were added per l culture medium and cell material was removed by tangential filtration.

Isolation

The filtrate was adsorbed on XAD-4 resin, washed with 5 l H_2O and eluted with 3 l CH_3OH/H_2O 7:3 (v/v). The eluate was then brought to dryness. Portions of the extract were chromatographed on Biogel P-2 with a 0.2 N pyridinium acetate buffer (pH 5.0, isocratic), detection at 405 nm. The brown fraction containing the ferric siderophores was then rechromatographed on DEAE Sephadex A-25 (flow rate 1 ml/min) with pyridinium acetate buffer (pH 5.0) 0.02 N (1 hr., 1st brown fraction), 0.02 to 0.2 N (1 hr.), 0.2 N (2 hrs., 2nd brown fraction), detection at 405 nm. The 2nd fraction (containing **5b**) was rechromatographed on the same column material with 0.1 N pyridinium acetate buffer (pH 5.0), the 1st one on CM Sephadex C-25 with 0.02 N pyridinium formiate buffer (pH 4.0). The latter gave 2 fractions (**5a** and **5c**) which were rechromatographed under identical conditions.

Decomplexation

Each of the 3 Fe^{3+}-complexes dissolved in 1% aqueous citric acid was treated with a 5% solution

of 8-hydroxyquinoline in $CHCl_3$. By chromatography on Biogel P-2 with 0.1 N CH_3COOH (detection at 254 nm) **5a** – **5c** could be obtained in pure form as it was shown by RP-HPLC on Polygosil 60-C18 with H_2O containing 0.02 M CH_3COONH_4, 3 ml CH_3COOH and 1 mM EDTA/l, and with CH_3OH.

Total hydrolysis and TAP derivatisation

1 mg of the siderophore was hydrolyzed with 6 N HCl for 15 hrs. at 110°C. The hydrolysate was evaporated to dryness and treated with acetyl chloride and isopropanol (1:5 v/v) for 1 hr. at 110°C. After evaporation to dryness the residue was dissolved in 1 ml CH_2Cl_2 and heated with 0.3 ml trifluoroacetic acid anhydride for 5 min. at 150°C. After removal of the volatile components the residue was dissolved in 0.5 ml CH_2Cl_2 and subjected to GC and GC/MS analysis.

Isolation of the chromophore **3b**

Three portions of 15 mg each of **5a** were dissolved in 4 ml 3 N HCl each. Through the solutions a stream of N_2 was passed for 10 min. and afterwards they were hydrolyzed at 110°C for 5 days. After cooling the hydrolysates were put together, brought to dryness i.v., dissolved in water, again brought to dryness and redissolved in 1 N HCl. The solution was chromatographed on a Polygosil 60-C18 column with 0.1% aqueous CF_3COOH/CH_3OH 80:20 (v/v), detection at 254 nm. The fraction containing **3b** was brought to dryness, dissolved in 1 N HCl and rechromatographed on Polygosil 60-C18 with the same solvent system 86:14. The UV/Vis spectrum of **3b** in 1 N HCl shows absorption maxima at 352, 365 and 219 nm, log ε 4.11, 4.09 and 4.55, and a shoulder at 236 nm. The FAB-MS shows a $[M+H]^+$-ion at *m/z* 277.

Hydrazinolysis

1 mg bis-desacetyl-bis-desoxy-**5b** (cf. below) was heated with 1 ml waterfree hydrazine for 9 hrs. at 60°C. The mixture was brought to dryness i.v. at 35°C. The residue was dissolved in 0.5 ml H_2O and extracted under shaking with 0.5 ml benzaldehyde for 1 hr. After separation from the organic phase the aqueous solution was extracted 3 times with 1 ml ether and TAP-derivatized (cf. above). By GC L-Orn was detected.

Results and Discussion

General data of azoverdin (**5a**), *azoverdin A* (**5b**) *and azoverdin G* (**5c**)

Molecular masses ($[M+H]^+$-ions in the positive FAB spectra). **5a:** 1090, Fe-**5a:** 1143, **5b:** 1091, **5c:** 1120 u.

Net charges according to the electrophoretic mobility (Offord, 1966). pH 6.9 Fe-**5a** 0, Fe-**5b** -1, Fe-**5c** 0; pH 6.9 **5a** +1, **5b** 0, **5c** +1; pH 1.9 **5a** +2, **5b** +2, **5c** +2.

UV/Vis data. See Table I.

Amino acid analysis. **5a** and **5b:** L-Dab, D- and L-Hse, D- and L-OHOrn, D-Ser, succinic acid, **5c:** same amino acid composition and L-Glu instead of succinic acid.

TAP derivative of the condensation product of Dab and Hse (**6**). EI-MS *m/z* (rel. int.): 513 (1) $M^{+\cdot}$, 494 (1) $[M-F]^+$, 452 (1) $[M-F-C_3H_6]^+$, 426 (3) $[M-COOC_3H_7]^+$, 399 (8) $[M-CF_3COOH]^{+\cdot}$, 357 (2) $[399-C_3H_6]^{+\cdot}$, 330 (12) $[399-CF_3]^+$, 312 (4) $[399-COOC_3H_7]^+$, 288 (12) $[357-CF_3]^+$, 242 (4), 215 (53) $[312-CF_3CO]^{+\cdot}$, 202 (18), 69 (43) CF_3+, 43 (100) $C_3H_7^+$.

Tab. I. UV/Vis data of **5a–5c** and of their Fe^{3+}-complexes.

	λ_{max} [nm]	log ε
Fe-**5a** (pH 6.8)	395	4.13
5a (pH 3.0)	366	4.04
	375	4.05
5a (pH 6.8)	399	4.25
Fe-**5b** (pH 6.8)	394	4.12
5b (pH 3.0)	366	4.05
	375	4.04
5b pH 6.8)	399	4.24
Fe-**5c** (pH 6.8)	395	4.14
5c (pH 3.0)	366	4.04
	375	4.05
5c (pH 6.8)	400	4.23

Presence of the isopyoverdin chromophore **3a**

After hydrolysis **3b** could be isolated (during hydrolysis the C-5 NH_2-group is replaced by OH, cf. Jacques *et al.*, 1995) and compared with the chromophores obtained from isopyoverdin (Jacques *et*

al., 1995) and from pyoverdins (Michels *et al.*, 1991). The ^{1}H- and ^{13}C-data are assembled in Tables II and III. Most important for the structural assignment of **3b** are ROESY cross peaks between H-10 and protons of the CH_2-group at 3.96/4.50 ppm (**1b** shows a cross peak between H-10 and the low-field C**H**CO signal at 5.63 ppm), and the coupling between H-3 and 4-NH (see Fig. 2). The corresponding data of the chromophore **3a** in **5a** and **5c** can be found in Tables IV and V and may be compared with those of isopyoverdin (Jacques *et al.*, 1995) and those for **1a** in e.g., the pyoverdin from *Pseudomonas putida* C (Seinsche *et al.*, 1993). They clearly correspond to those of isopyoverdin. Also here the cross peaks between H-10 and the 1-CH_2-group can be seen in the ROESY

Table II. ^{1}H NMR data of **3b** from **5a** and from isopyoverdin and of **1b** in DMSO-d_6.

	3b from azoverdin (**5a**)	**3b** from isopyoverdin	**1b** from pyoverdin
H-1	3.96/4.50	3.96/4.50	5.63
H-2	2.42	2.42	2.29/2.57
H-3	4.39	4.39	3.17/3.61
H-6	7.43	7.45	7.37
H-7	7.11	7.11	7.08
H-10	7.20	7.21	6.99
NH	9.03	9.03	9.26
OH-5	11.76	11.82	11.79
OH-8	10.18	10.19	10.17
OH-9	9.90	9.91	9.79

Table III. ^{13}C NMR data of **3b** from **5a** and from isopyoverdin and of **1b** in DMSO-d_6.

	3b from azoverdin (**5a**)	**3b** from isopyoverdin	**1b** from pyoverdin
C-1	42.2	42.2	55.6
C-2	20.9	20.9	21.1
C-3	49.2	49.2	35.2
C-4a	145.0	144.9	145.6
C-5	137.8	137.8	137.9
C-6	115.9	115.8	115.6
C-6a	115.2	115.2	114.8
C-7	111.2	111.2	111.6
C-8	144.8	144.8	144.6
C-9	148.6	148.5	148.3
C-10	101.0	101.0	100.5
C-10a	126.8	126.7	126.6
CO	171.8	171.7	170.1

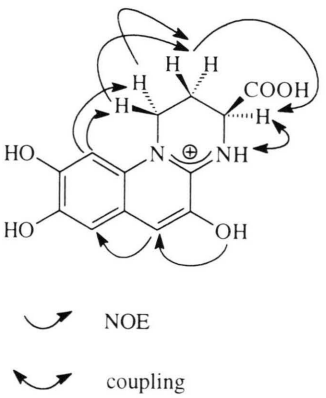


Fig. 2. NOE (ROESY) and couplings observed for **3b.**

Table IV. ^{1}H NMR data of **5a** and **5c** in H_2O (pH 4.3).

	Azoverdin **5a**	Azoverdin G **5c**
Chr-1	3.88/4.47	3.86/4.40
Chr-2	2.51/2.62	2.58
Chr-3	4.60	4.60
Chr-6	7.79	7.81
Chr-7	6.94	6.88
Chr-10	7.00	6.94
NH-Hse[1]	8.87	8.93
α-Hse[1]	4.46	4.46
β-Hse[1]	2.02	2.04
γ-Hse[1]	3.69	3.69
NH-Hse[2]	9.20/9.13	9.17
α-Hse[2]	4.75	4.75
β-Hse[2]	2.12	2.10
γ-Hse[2]	3.68/3.79	3.68/3.78
NH-Dab	–	–
α-Dab	4.43	4.42
β-Dab	1.87	1.87
γ-Dab	3.20/3.11	3.18/3.13
NH-OH-Orn[1]	8.92	8.96
α-OH-Orn[1]	4.20	4.20
β-,γ-OH-Orn[1]	1.6–1.9	1.6–1.9
δ-OH-Orn[1]	3.62	3.62
NH-Ser	8.54	8.57
α-Ser	4.47	4.47
β-Ser	3.89	3.89
NH-OH-Orn[2]	7.90	7.93
α-OH-Orn[2]	4.23	4.23
β-,γ-OH-Orn[2]	1.6–1.9	1.6–1.9
δ-OH-Orn[2]	3.62	3.62
Side chains		
Succinamide-CH_2-2	2.81	
Succinamide-CH_2-3	2.73	
α-Glu		3.87
β-Glu		2.30
γ-Glu		2.76
Acetyl	2.14	2.13

Table V. ^{13}C NMR data of **5a** and **5c** in D_2O (pH 4.3)[a].

	5a (azoverdin)	**5c** (azoverdin G)
OH-Orn^2-COOH	179.4	179.4
Succinamide-CO-1	178.8	–
Succinamide-CO-4	177.3	–
Glu-CO	–	176.6
Hse^1-CO	175.7	175.7
Glu-COOH	–	175.2
Ac-CO	175.1	175.0
OH-Orn^1-CO	174.9	174.8
Chr-CO	173.3	173.3
Dab-CO	172.6	172.6
Ser-CO	171.6	171.6
Hse^2-N=C–NH–	165.2	165.2
Chr-9	152.9	152.6
Chr-4a	149.4	149.2
Chr-8	146.9	146.3
Chr-6	139.8	139.6
Chr-10a	134.5	133.9
Chr-5	116.7	117.1
Chr-6a	115.3	115.3
Chr-7	112.9	113.0
Chr-10	101.8	101.7
β-Ser	62.2	62.2
γ-Hse	58.9	58.9
γ-Hse	58.4	58.4
α-Ser	56.8	56.8
α-OH-Orn^1	55.9	55.9
α-OH-Orn^2	55.4	55.3
α-Glu	–	55.1
α-Dab	53.1	53.1
α-Hse^1	52.6	52.5
trans-δ-OH-Orn	52.3	52.3
Chr-3	51.3	51.2
α-Hse^2	50.7	50.7
cis-δ-OH-Orn	48.7	48.6
cis-δ-OH-Orn	48.4	48.3
Chr-1	43.9	43.9
γ-Dab	37.8	37.8
β-Hse	34.7	34.7
β-Hse	34.0	34.0
γ-Glu	–	32.4
Succinamide-C-2	31.9	–
Succinamide-C-3	31.0	–
β-OH-Orn	30.2	30.1
β-OH-Orn	29.1	29.0
β-Glu	–	26.7
γ-OH-Orn	23.7	23.7
γ-OH-Orn	23.6	23.6
Chr-2	23.0	22.9
β-Dab	22.7	22.6
Ac	20.5	20.4

[a] The values given by Bernardini *et al.* (1996) are by 2–3 ppm higher. This may be due to the use of a different standard (*t*-butanol-d_{10}) the correlation of which to TMS is not given.

spectrum. From the NMR data it follows that the chromophore of the azoverdins carries the carboxyl group at C-3 rather than on C-1 and hence the azoverdins belong to the group of isopyoverdins.

The dicarboxylic acid residues bound to the NH_2-group of **3a**

The hydrolysis of **5a** and of **5b** yielded succinic acid. Their molecular masses differ by 1 u and **5b** at neutral pH carries one negative charge more than **5a** (-COO^- vs. -$CONH_2$) in agreement with the presence of a succinamide (**5a**) and a succinic acid (**5b**) substituent. The NMR data (see Tables IV and V) confirm the succinamide grouping in **5a**. The hydrolysate of **5c** lacks succinic acid while as an additional component Glu is obtained. Glu bound with its 5-carboxyl group to the chromophore-NH_2 is one of the substituents commonly encountered. The NMR data (confirmed by TOCSY experiments) correspond to the literature data (Geisen *et al.*, 1992).

The peptide chain

All signals in the ^1H-NMR-spectra of **5a** – **5c** (Table IV) were identified by TOCSY and by comparison with literature data. NOESY correlations are mentioned in the text.

Partial structures of the peptide chain

The presence of 2 N^5-acetyl-N^5-hydroxy-Orn (AcOHOrn) units follows from the NMR signals at 2.13 (^1H) and 175 ppm (^{13}C) (Jacques *et al.*, 1995) which disappear after mild hydrolysis (0.1 M HCl, 6 days at room temperature). The mass of $[M+H]^+$ of the hydrolysis product as determined by FAB-MS *m/z* 1007 corresponds to the loss of 2 x CH_2CO. The hydroxyl groups of the OHOrn units thus formed can be removed subsequently by hydrogenation with H_2/PtO_2 (Briskot *et al.*, 1989) ($[M+H]^+$ *m/z* 975 corresponding to the loss of 2 O-atoms (1007–32); appearance of a signal at 3.02 ppm in the ^1H-NMR spectrum (typical for the δ-CH_2 group of Orn)). When kept for some time at pH 6.8 the bis-desacetyl product loses H_2O ($[M+H]^+$ *m/z* 989) while in the ^{13}C-NMR-spectrum

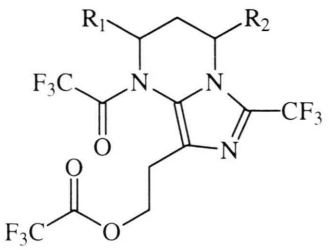


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Fig. 3. TAP derivative of the condensation product of Dab and Hse (R_1 = H, R_2 = $COOC_3H_7$ or R_1 = $COOC_3H_7$, R_2 = H).

a signal at 167 ppm appears. This behavior is typical for the formation of c-OHOrn (cf. Mohn *et al.*, 1990) and suggests that one of the AcOHOrn units is the C-terminus of the peptide chain. This is in agreement with the pH-dependence of the α-CH-signals of *one* AcOHOrn unit (^{1}H: pH 1.0 4.38; 2.1 4.35; 4.3 4.24 ppm) while those of the second one remain unchanged at 4.26 ppm. The final proof was obtained by the hydrazinolytic degradation of bis-desacetyl-bis-desoxy-**5b** which yielded L-Orn.

The presence of a 3,4,5,6-tetrahydropyrimidine ring formed by condensation of the two amino groups of Dab with the carboxyl group of Hse could be proved as follows: The signal at 165.2 ppm in the ^{13}C-spectra of **5a** – **5c** is typical for the amidine structure of the tetrahydropyrimidine ring (Filsak *et al.*, 1994; Gwose and Taraz, 1992) especially in the absence of c-OHOrn (CO 167.6 ppm, Mohn *et al.*, 1990) and FoOHOrn (*trans*-CHO 164.8 ppm, Briskot *et al.*, 1989); peptide and amide resonances lie below 170 ppm (cf. Table V). In the HMBC-spectrum this peak shows a cross signal with the α-CH of one Hse at 4.75 ppm. The vicinity to the amidine system explains its low-field position as compared with the standard values for Hse (cf. 4.46 ppm for the other Hse in Table IV) in agreement with literature values for analogous systems (4.65 ppm: Gwose and Taraz, 1990; 4.64 ppm: Gipp *et al.*, 1991). Final proof came from the analysis of the products of a partial hydrolysis (3 M HCl, 90°C, 1 hr.): By GC-MS of the TAP derivatives of the amino acids no Dab derivative was found, but instead **6** formed from the condensation product of Dab and Hse by the loss of H_2O (Fig. 3; Filsak *et al.*, 1994).

Sequence of the peptide chain

That a Hse (Hse^1) is bound amidically to the carboxyl group of **3a** follows from a partial hydrolysis (6 M HCl, 90°C, 10 min.) which yielded fragments with masses as determined by FAB-MS *m/z* 477 and 459 corresponding to [succinic acid-**3a**-Hse + H]$^+$ and [*m/z* 477 – H_2O]$^+$ (Hse-lactone). The latter could be isolated and yielded upon further hydrolysis L-Hse. This conclusion is confirmed by cross peaks in the NOESY spectrum of Hse^1 between its NH- and the H-3 as well as the H-2 signals of **3a**. Hse^2 (whose carboxyl group is condensed with the two amino groups of Dab to a tetrahydropyrimidine ring, *v. supra*) is directly connected to Hse^1 as evidenced by the cross peaks between NH-Hse^2 and NH-Hse^1 as well as the α-CH of Hse^1. The C-terminal sequence AcOH-Orn^2-Ser-AcOHOrn^1 can be deduced from the NOESY cross peaks as indicated in **5a** (Fig. 4). The NH of Orn^1 in turn shows cross peaks to the α-CH and to the γ-CH_2 of Dab. These correlations were confirmed by ROESY data from **5c**. The C-terminal position of Orn^2 was unambiguously demonstrated by the hydrazinolysis experiment described above. Thus the complete sequence is **3a**-Hse-[Hse-Dab–H_2O]-AcOHOrn-Ser-AcOHOrn.

Stereochemistry

Hse and Orn are both present in their L- and D-forms. That L-Orn forms the C-terminus follows from the hydrazinolysis experiment described above, and that L-Hse is bound directly to the chromophore from the isolation of a corresponding hydrolysis product (*v. supra*). By partial hydrolysis (Bernardini *et al.*, 1996) fragments were obtained which contained the chromophore and upon further hydrolysis gave only L-Hse and D-OHOrn. D-Hse which in **5a** lies between L-Hse and D-Orn was apparently not detected because it forms part of the tetrahydropyrimidine ring which resists mild hydrolysis (*v. supra*). Regarding the chromophore **3a** it had only been speculated for biogenetic reasons that in the case of isopyoverdin (Jacques *et al.*, 1995) it is 3*S*-configurated. The CD spectra of **3b** from isopyoverdin and from azoverdin are identical.

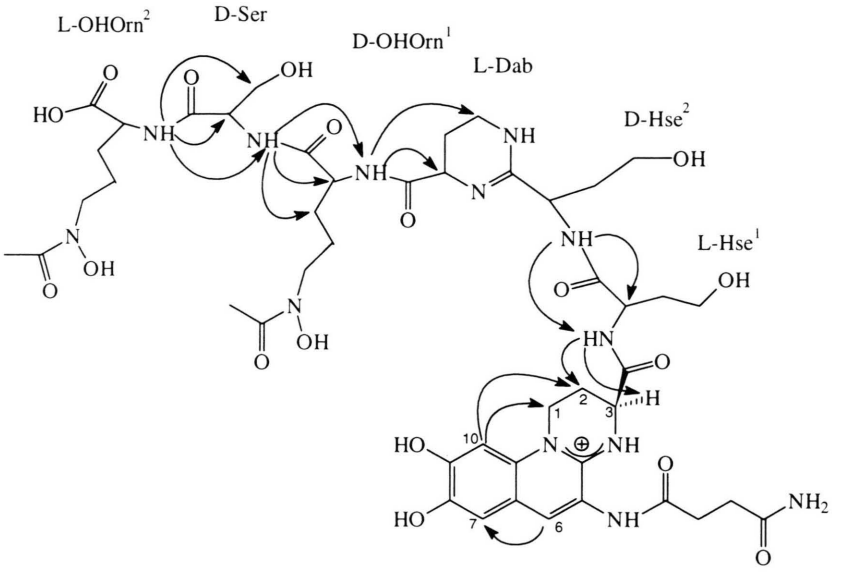

Fig. 4. NOESY cross peaks of **5a** (pH 4.3).

Discussion of the structure proposals by Linget and Bernardini

As mentioned before structures **4b** and **4a** were proposed earlier for azoverdin (Linget *et al.*, 1992; Bernardini *et al.*, 1996). The more recent one (**4a**) differs from **5a** in two ways: (a) It contains the pyoverdin (**1a**) rather than the isopyoverdin chromophore **3a**, and (b) it has the peptide sequence **1a**-Hse-AcOHOrn-Ser-AcOHOrn-[Hse-Dab–H_2O] (i.e. the sequences Orn-Ser-Orn and [Hse-Dab – H_2O] are interchanged).

(a) The chromophore. The authors did not isolate the chromophore after hydrolysis, but rather concluded from the ^{13}C-NMR data that it had the structure **1a**. In the aromatic region the ^{13}C values of **1a** in pyoverdins (P) and **3a** in isopyoverdin (I) do not differ much, but in the aliphatic region (C-1 and C-3) those observed for azoverdin (A) correspond much better to those of I than with those of P (C-1 A 43.9, I 43.8, P 57.9; C-3 A 51.3, I 51.6, P 36.3). For the ^{1}H-resonances the authors give 2 sets for C-1 to C-3 (7 and 50°C) which they attribute to different conformations of the peptide chain, and one of which corresponds to those of I (the C-1 signal at 4.47 ppm is not mentioned), but neither of them to those of P. Without additional information it would be idle to speculate about the origin of the second set of shift values. Probably the authors did not realize the existence of the I-chromophore **3a** and hence attributed their data to **1a**.

(b) The peptide chain. As far as the peptide chain is concerned the proposed structures **4a** and **5a** differ by the placement of the building blocks Orn-Ser-Orn and [Hse-Dab – H_2O]. The masses observed by the authors for their products of partial hydrolysis are compatible with both structures. From the published NMR data it is difficult to show where the sources of misinterpretations lie. So only some obvious points will be mentioned here. (i) Contrary to the literature data the low-field shifted Hse-α-C<u>H</u> is not assigned to the one next to the amidine system but rather to the one bound to the chromophore; (ii) the ROESY cross peaks between AcOHOrn-2-N<u>H</u> and Hse-1-α-C<u>H</u> as well as between Hse-5-N<u>H</u> and AcOHOrn-4-N<u>H</u> (see numbering in **4a**) could not be detected in our spectra of **5a** or **5c**; (iii) the very small signal at 183.3 ppm in their Figure 4 (Bernardini *et al.*, 1996) is attributed to the Dab-<u>C</u>OOH on the basis of an alleged cross peak with Dab-β-<u>C</u>H$_2$ at 2.05 ppm which cannot be be seen in the Figure; (iv) the signal at 181.6 attributed to AcOHOrn-4-CO is a singulet (as for a COOH-group) and not a doublet as required for (CO^{15}NH).

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