The Synthesis and Antibacterial Activity of two Pyoverdin-ampicillin Conjugates, Entering *Pseudomonas aeruginosa via* the Pyoverdin-mediated Iron Uptake Pathway

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Two pyoverdin-ampicillin conjugates were synthesized and their structures were confirmed by mass spectrometry and NMR spectroscopy. In contrast to ampicillin, the conjugates exhibited high antibacterial activity against *Pseudomonas aeruginosa* ATCC 15692 and ATCC 27853, effective only against the strain which is using the parent pyoverdin for iron uptake. This suggests that the conjugates enter the bacterial cell *via* the ferripyoverdin uptake pathway. Growth stimulation studies with conjugates hydrolysed at the β-lactam ring of the ampicillin moiety supported this view.

The low permeability of the bacterial outer membrane is a major factor contributing to the high resistance of *Pseudomonas aeruginosa* against most antimicrobial agents[1,2]. One approach to overcome this barrier is the concept of siderophore-mediated drug delivery. Siderophores are iron-chelating molecules (molecular masses usually lower than 2 kD), excreted by many bacteria under iron deficient conditions. A wide number of the iron complexes enter the cell *via* interactions with specific iron regulated outer membrane proteins (IROMPs)[3,4,12]. Small antibiotic structures, covalently attached to siderophores, so as not to interfere with the siderophore recognition and transport process by the specific IROMPs, may enter the bacterial cell *via* the siderophore pathways[5]. The main siderophores of *P. aeruginosa* are the pyoverdins. Three different *P. aeruginosa* subspecies (siderovars) were found, producing three different pyoverdins, according to different pyoverdin-mediated iron uptake systems (PVD groups I~III, Fig. 1.). The pyoverdins were found to be strictly group-specific, *i.e.* they can be used for iron-uptake only within the same homology group. 84% of 88 strains of different origin tested by MEYER *et al.* belonged to group I and II[6].

PaT II, the pyoverdin isolated from *P. aeruginosa* ATCC 27853 (PVD group II, R=succinyl, Fig. 1.), contains an ornithine residue with a free δ-amino group in its peptide chain. Preliminary studies indicated that this amino group is not necessary for the recognition and transport process by the specific IROMPs[7]. A PaT II-ampicillin conjugate (PaTSebAmp, Fig. 2.) was synthesized, using the free amino group as a point for covalent attachment. An analogous synthesis of a conjugate containing pyoverdin D (PVD group I, R=succinyl, Fig. 1.) was not possible because of the lack of a free primary amino group. Instead, the pyoverdin sucpyoverdin 9446, produced by *P. chlororaphis* ATCC 9446 and *P. fluorescens* ATCC 13525[8] was used to construct a conjugate (Pv9446SebAmp, Fig. 2) effective against *P. aeruginosa* PAO1 (group I). This pyoverdin contains a lysine residue with a free ε-amino group in its peptide chain and can be used for iron uptake by *P. aeruginosa* PAO1[9].

Here we report the synthesis, structural confirmation and preliminary biological evaluation of the two pyoverdin-ampicillin conjugates.

Chemistry

Pyoverdins are poorly soluble in organic solvents and

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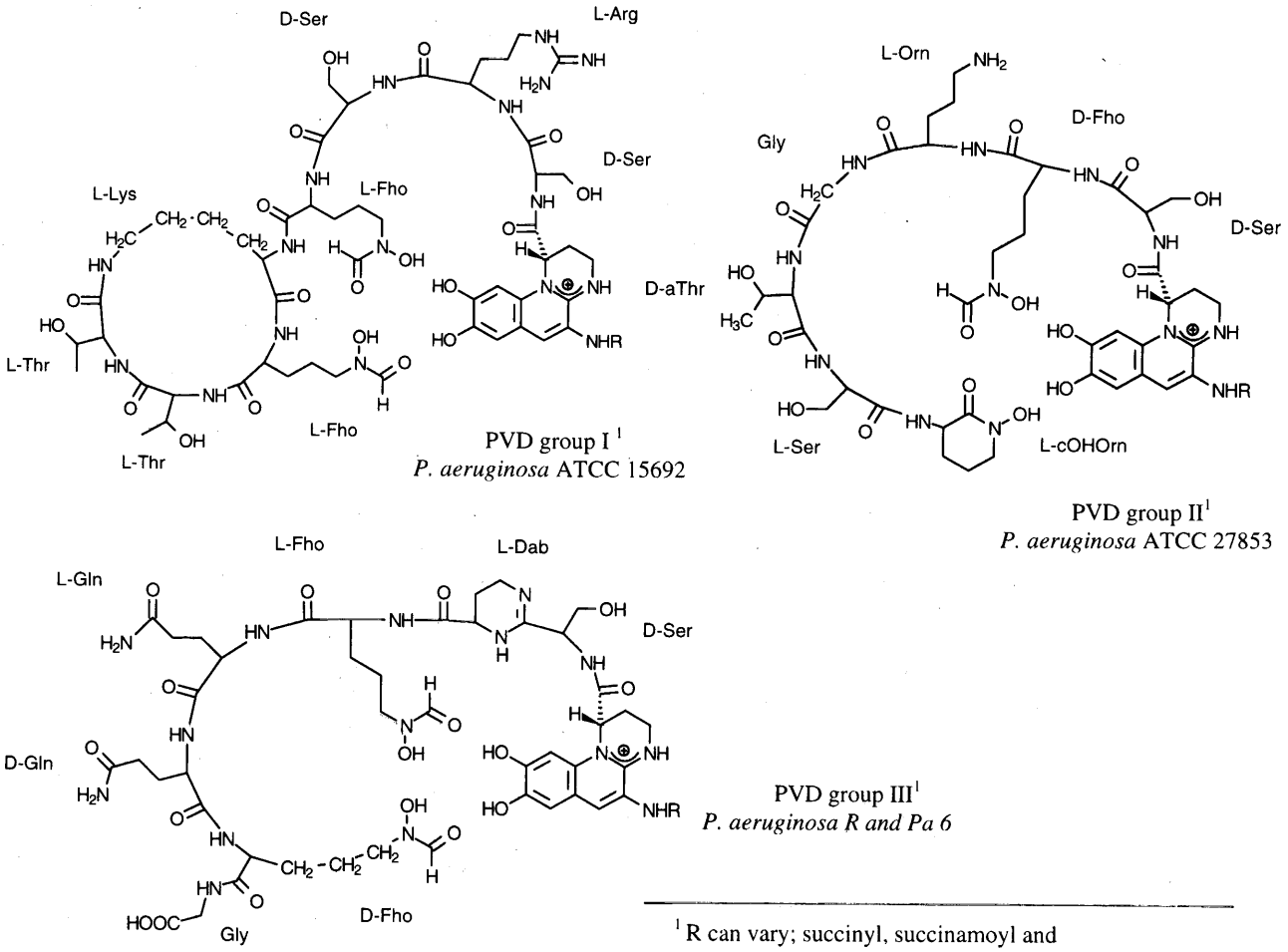
Fig. 1. Pyoverdins produced by *Pseudomonas aeruginosa*.



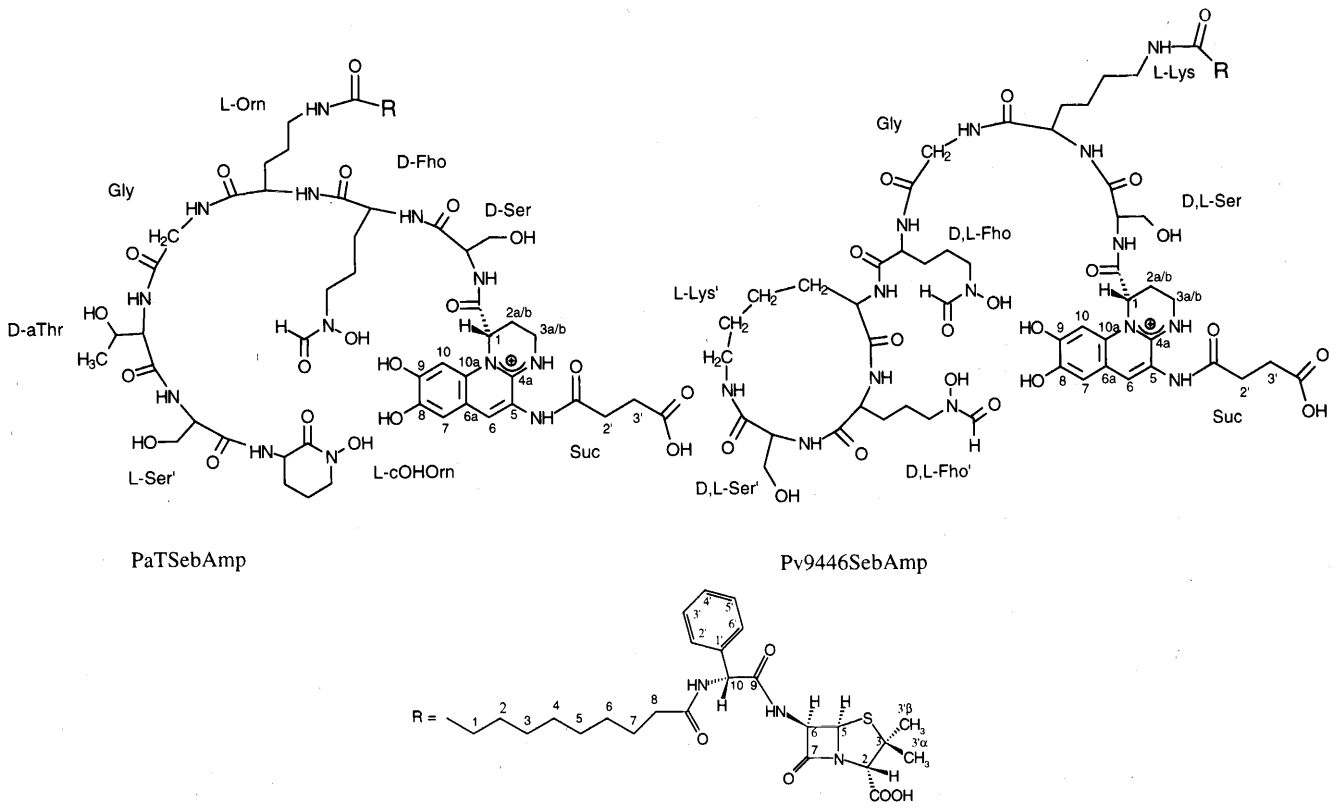
[1] R can vary; succinyl, succinamoyl and α-ketoglutaryl residues were found

their multiple functionalities are sensitive to drastic reaction conditions. We therefore chose as synthetic route a one-step cross-linking reaction in aqueous solution at room temperature. Sebacic acid bis(2-nitro-4-sulphophenyl) ester, developed by GERSHKOVICH *et al.*[10] for protein cross-linking, served as cross-linking reagent. The long, flexible spacer of eight methylene groups was chosen to prevent possible steric hindrance of the penicillin-recognition by the bacterial transpeptidase as well as the pyoverdin-recognition by their specific IROMPs. Although the primary amino groups of the pyoverdins were almost completely acylated during the reaction, the yield of conjugate did not exceed 21%. This was due to the formation of pyoverdin dimers (linked by the sebacic acid spacer) and a beginning decomposition of the conjugates by the hydrolytic cleavage of the β-lactam ring. To reduce the formation of the pyoverdin dimers, a sixfold molar excess of ampicillin was used. The pyoverdin-derivatives were seperated from the reaction mixture by gel filtration on Sephadex G-25. The different pyoverdin-derivatives were subsequently separated by ion-exchange chromatography on DEAE-Sephadex. Positive ion-FAB and -ESI mass spectra of both the iron-complexed and decomplexed conjugates were consistent with the assumed structures and a 1:1 stoichiometry of its Fe(III)-complex typical for pyoverdins.

By one and two dimensional NMR-techniques we were able to confirm the structures of the conjugates (Fig. 2.). A buffered aqueous solvent system (pH 4.3, 278 K) was used to detect the NH-protons. Trideutero-acetonitrile was added to improve the solubility of the conjugates. The complete assignment of the ^{1}H and ^{13}C resonances is given in the Tables 1~4.

Proton resonances were assigned by HOHAHA experiments, carbon resonances by DEPT and HSQC as well as by HMBC experiments for the quarternary carbon atoms. Differences to the previously published NMR-data of the peptide chains of the parent pyoverdins PaT II[11] and suc-pyoverdin 9446[8] are due to the

Fig. 2. Structures of PaTSebAmp and Pv9446SebAmp.



acylation of the ornithine/lysine side chains and are to a certain extent superimposed by the solvent effect due to the addition of acetonitrile to the aqueous buffer (especially a slight high-field shift of the NH-resonances). The linkages of the ampicillin moiety to the amino groups of the ornithine/lysine residues *via* the sebacic acid spacer were proved by cross peaks in ROESY (PaTSebAmp)/NOESY (Pv9446SebAmp) and HMBC experiments. (PaTSebAmp:ROESY: δ-NH(Orn)/1-H(Seb); 10-NH (Amp)/8-H(Seb); HMBC: δ-NH(Orn)/CO-1(Seb), 1-H(Seb)/CO-1(Seb); 10-NH(Amp)/CO-8(Seb), 8-H(Seb)/CO-8(Seb); Pv9446SebAmp: NOESY: ε-NH(Lys)/1-H(Seb); 10-NH(Amp)/8-H(Seb); HMBC: ε-NH(Lys)/CO-1(Seb), 1-H(Seb)/CO-1(Seb); 10-NH(Amp)/CO-8(Seb), 8-H(Seb)/CO-8(Seb)). The amino acid sequences of the pyoverdin moieties and the structural integrity of the ampicillin moieties were likewise confirmed by the ROESY/NOESY and HMBC experiments.

Biological Evaluation

The antibacterial activities of both conjugates against the two strains *Pseudomonas aeruginosa* ATCC 27853 and ATCC 15692 (PAO1) were determined by the agar dilution method. The MICs are given in Table 5.

In contrast to ampicillin, against which *P. aeruginosa* is resistant, PaTSebAmp and Pv9446SebAmp exhibited high antibacterial activities. As expected, they completely lack antibacterial activity against the strain which cannot use the parent pyoverdins for iron uptake. Even at concentrations of 100 μM no effect on the bacterial growth was visible. This suggests that the use of the pyoverdin-uptake pathway by the conjugates is crucial for their antibacterial activity. The lower antibacterial activity of Pv9446SebAmp as compared with that of PaTSebAmp, might be due to the lower iron transport activity of suc-pyoverdin 9446, in comparison with pyoverdin D (PVD group I, R=succinyl, Fig. 1.), produced by *P. aeruginosa* ATCC 15692 (PAO1)[9].

The use of the pyoverdin-uptake pathway by the conjugates was further supported by growth stimulation studies with partially decomposed conjugates (PaTSeb-Ampdec, Pv9446SebAmpdec), isolated as by-products of the synthesis. They contained a hydrolysed β-lactam ring at the ampicillin moiety, excluding the antibacterial effect of penicillins based on the inhibition of the bacterial cell wall synthesis. Their purity was controlled by HPLC and they were characterized by PI-FAB mass spectrometry. Under the severe iron deficiency, created by the

Table 1. ^{1}H NMR data for PaTSebAmp in 100 mM aq KH_2PO_4-CD_3CN, 2:1 (v/v), pH 4.3, 278 K.

Suc	2′	3′						
	2.70	2.68						
Chr	NH	1	2a/2b	3a/3b	4NH$^+$	6	7	10
	9.78	5.62	2.63 2.40	3.67 3.33	8.71	7.90	7.18	7.04
	NH	H_α	H_β	H_γ	H_δ	δNH	CHO	
Ser	9.26	4.46	3.87					
Fho	8.55	4.30	1.73 1.63	1.55 1.49	3.31[a] 3.41[b]		7.78[a] 8.17[b]	
Orn	8.32	4.23	1.75 1.61	1.49	3.10	7.87		
Gly	8.30	3.91						
aThr	8.05	4.31	4.09	1.18				
Ser'	8.38	4.44	3.85					
cOHOrn	8.29	4.43	1.78 1.97	1.95	3.65 3.58			
Seb	1	2	3~6	7	8			
	2.17	1.53	1.23	1.56	2.27			
Amp	2	3′$H_{\alpha/\beta}$	5	6	8NH	10	10NH	2′~6′
	4.14	1.51$_\alpha$ 1.41$_\beta$	5.42	5.47	8.60	5.47	8.33	7.41

[a] *cis*, [b] *trans* conformation.
Suc: succinyl; Chr: chromophore; cOHOrn: cyclo-hydroxyornithine; Fho: formyl-hydroxyornithine; Seb: sebacic acid; Amp: ampicillin.

addition of the strong synthetic iron-chelator EDDHA to an agar culture medium, bacterial growth is usually much delayed[6]. The addition of the decomplexed homologous pyoverdins, which are able to compete with EDDHA for iron, restored the bacterial growth. In the same way decomplexed PaTSebAmpdec and Pv9446Seb-Ampdec restored the growth of the strains ATCC 27853 and ATCC 15692 respectively. This indicates that they were used for iron uptake *via* the pyoverdin-pathway and it suggests that the intact conjugates likewise enter the cell *via* the pyoverdin-pathway.

For a further investigation of the antibacterial activity, growth curves in broth medium, supplemented with PaTSebAmp and Pv9446SebAmp as Fe(III)-complexes at various concentrations, were monitored (Figs. 3., 4.).

After about 23 hours (delay of about 16 hours as compared with the growth of the control strains without supplementation) growth of both strains was detected. An investigation of the chemical stability of the conjugates in broth medium at 35°C by HPLC proved, that after 24 hours only 24% of the conjugates were decomposed. Moreover, after incubation of fresh conjugate-supplemented broth (concentrations of 10 μM) with the grown bacteria, no delay of bacterial growth was observed.

These findings imply that resistant mutants were selected. The rapid formation of resistant mutants indicates that these mutants were deficiency mutants, probably defective in their pyoverdin-mediated iron uptake systems. BROCHU *et al.*[5] also detected a rapid formation of resistant mutants of *Escherichia coli*, when grown in the presence of synthetic siderophore-penicillin conjugates. They were defective in the expression of some siderophore-specific IROMPs. MEYER *et al.* recently showed that pyoverdins are essential for the virulence of *P. aeruginosa*[13]. Unlike pathogenic *E. coli*, which produces two different high-affinity iron uptake systems[12] (enterobactin, aerobactin), *P. aeruginosa* is dependent on the use of a the pyoverdin-mediated one *in vivo*. Pyochelin, the second siderophore of *P. aeruginosa* seems

Table 2. ^{1}H NMR data for Pv9446SebAmp in 100 mM aq KH_2PO_4-CD_3CN, 2:1 (v/v), pH 4.3, 278 K.

Suc	2′	3′						
	2.68	2.66						
Chr	NH	1	2a/2b	3a/3b	4NH$^+$	6	7	10
	9.74	5.68	2.64 2.38	3.65 3.32	8.76	7.86	7.14	7.14
	NH	H_α	H_β	H_γ	H_δ	H_ε	εNH	CHO
Ser	9.47	4.36	3.90					
Lys	8.33	4.24	1.77 1.53	1.07 1.03	1.28 1.16	2.87 2.81	7.60	
Gly	8.28	3.80 3.64						
Fho	8.05	4.22	1.75 1.63	1.63 1.67	3.51[a] 3.55[b]			7.89[a] 8.25[b]
Lys′	8.09	4.16	1.88 1.57	1.28 0.94	1.42 1.50	3.23 3.09	7.19	
Ser′	8.67	4.27	3.81					
Fho′	7.97	4.32	1.70 1.65	1.54 1.65	3.48[a] 3.53[b]			7.87[a] 8.23[b]
Seb	1	2	3∼5	6	7	8		
	2.10	1.49	1.20	1.22	1.53	2.26		
Amp	2	3′$H_{\alpha/\beta}$	5	6	8NH	10	10NH	2′∼6′
	4.11	1.50$_\alpha$ 1.40$_\beta$	5.41	5.46	8.51	5.47	8.22	7.39

[a] *cis*, [b] *trans* conformation.
Suc: succinyl; Chr: chromophore; Fho: formyl-hydroxyornithine; Seb: sebacic acid; Amp: ampicillin.

not to be essential for the growth *in vivo*. Mutants, defective in their pyoverdin-mediated iron uptake system, should thus be severely impaired in proliferating *in vivo*. Therefore we suggest the pyoverdin-mediated drug delivery as a promising way of drug targeting against multiresistant strains of *P. aeruginosa*. Investigations of the antibacterial activity of PaTSebAmp and Pv9446Seb Amp against clinical isolates *in vivo* are in progress.

Experimental

General

ESI mass spectra were obtained on a Finnigan MAT 900 S, positive ion-FAB mass spectra on a Finnigan MAT H-SQ 30 instrument equipped with an IonTech Ltd. FAB gun (Xe), matrix thioglycerol-dithiodiethanol (1:1). NMR experiments were performed on Bruker DPX 300 and DRX 500 instruments. Chemical shifts were measured using DSS as internal reference ($\delta(CH_3)=0.0$ ppm for ^{1}H and -1.61 ppm for ^{13}C). Experiments were performed in 100 mM aqueous phosphate buffer-CD_3CN, 2:1 (v/v), pH 4.3. The H_2O-resonance was suppressed by presaturation during the relaxation delay or by the jump-return method. For reversed phase HPLC investigations a Knauer system (pump type 64.00, UV/VIS filter photometer 97.00) was used, with a Nucleosil 100 C18 column (4 × 22 mm, 0.1 M aq $(NH_4)OAc$-MeOH, 70:30 (v/v), isocratic solvent system).

Chemicals

H_2O was deionized and twice-distilled in a quartz apparatus. For HPLC it was additionally purified at XAD-4 resin. All used buffer salts and MeOH were p.a. grade. Ampicillin trihydrate (>96%) was obtained from Fluka (Buchs, CH).

Table 3. ^{13}C NMR data for PaTSebAmp in 100 mM aq KH_2PO_4-CD_3CN, 2:1 (v/v), pH 4.3, 278 K.

Suc	CO-1′	2′	3′	CO-3′			
	177.3	31.9	31.3	179.4			
Chr	CO	1	2	3	4a	5	6
	170.7	57.9	22.9	36.0	150.4	118.8	139.8
	6a	7	8	9	10	10a	
	115.6	114.8	145.4	152.9	101.2	132.7	
	CO	C_α	C_β	C_γ	C_δ	CHO	
Ser	172.8	56.8	62.3				
Fho	174.6	54.5	28.7	23.5	50.7[a] 46.7[b]	160.1[a] 164.3[b]	
Orn	175.1	54.8	29.0	26.3	39.6		
Gly	172.3	43.5					
aThr	172.8	60.4	68.2	19.6			
Ser′	172.1	56.8	62.3				
cOHOrn	166.9	51.3	27.8	21.0	52.5		
Seb	CO-1	1	2/7	3~6	8	CO-8	
	177.4	37.0	26.5	29.5	36.5	176.9	
Amp	2	CO-2	3	3′C_α	3′C_β	5	6
	74.1	175.0	65.2	31.3	27.5	67.7	58.7
	CO-7	10	CO-9	1′	2′/6′	3′/5′	4′
	175.2	58.3	172.3	137.2	128.5	130.1	129.9

[a] *cis*, [b] *trans* conformation.
Suc: succinyl; Chr: chromophore; cOHOrn: cyclo-hydroxyornithine; Fho: formyl-hydroxyornithine; Seb: sebacic acid; Amp: ampicillin.

Isolation and Purification of Pyoverdins

The pyoverdins PaTII of *Pseudomonas aeruginosa* ATCC 27853 and suc-pyoverdin 9446 of *Pseudomonas chlororaphis* ATCC 9446 were isolated and purified as described earlier[8,14].

PaT II-ampicillin Conjugate (PaTSebAmp)

147.7 mg (227.9 μmol) of sebacic acid bis(2-nitro-4-sulphophenyl) ester sodium salt (prepared according to the procedure of GERSHKOVICH *et al.*[10]) were added to a solution of 73.9 mg (64.6 μmol) of Fe(III)-PaT II and 162.7 mg (403.7 μmol) of ampicillin trihydrate in 4 ml of 0.2 M aq $NaHCO_3/Na_2CO_3$ buffer pH 9.0. The mixture was stirred for 16 hours at room temperature. After adjustment of the pH to 5.0 with 1.0 M HOAc the mixture was applied on a Sephadex G-25 F column (2.6×35.0 cm) and eluted with 0.2 M NaOAc/HOAc buffer pH 5.0 (flux rate 0.5 ml/minute). The first fraction was further chromatographied on a DEAE-Sephadex A-25 column (2.6×30.0 cm, flux rate 2.0 ml/minute), using the same eluent as for the gel filtration. The mixture of pyoverdin-derivatives separated into 8 fractions of which fraction 5 was the conjugate. For seperation from buffer salts the fraction was adsorbed on a Sep-Pak® C-18 cartridge (Waters, Milford, MA), washed with 10 ml of H_2O and desorbed with 5 ml of MeOH-H_2O, 1:1 (v/v). The solvent was removed by evaporation at reduced pressure at 30~35°C. Yield of Fe(III)-PaTSebAmp was 22.5 mg (21%). PI-ESI-MS *m/z* 1660 $(M+Fe\text{-}2H)^+$.

Decomplexation: From aq solution the conjugate was adsorbed on a Sep-Pak® C-18 cartridge and washed with 5 ml of 6% aq potassium oxalate buffer pH 4.0. Remaining oxalate was washed from the cartridge with 5 ml of H_2O and the decomplexed conjugate was desorbed with 5 ml of MeOH-H_2O, 1:1 (v/v). The solvent was removed by evaporation at reduced pressure

Table 4. ^{13}C NMR data for Pv9446SebAmp in 100 mM aq KH_2PO_4-CD_3CN, 2:1 (v/v), pH 4.3, 278 K.

Suc	CO-1′	2′	3′	CO-3′			
	177.1	32.2	31.9	179.8			
Chr	CO	1	2	3	4a	5	6
	170.9	57.7	22.8	36.2	150.4	118.8	139.4
	6a	7	8	9	10	10a	
	115.6	114.6	145.4	153.1	101.3	132.6	
	CO	C_α	C_β	C_γ	C_δ	C_ε	CHO
Ser	172.9	58.0	62.2				
Lys	175.0	54.7	31.5	23.7	28.9	40.0	
Gly	172.1	43.6					
Fho	174.5	54.4	28.7	23.8	51.0[a]		160.0[a]
					47.0[b]		164.2[b]
Lys'	175.8	56.3	29.4	19.8	26.4	38.5	
Ser'	172.7	57.9	60.9				
Fho'	174.2	54.4	26.4	23.6	50.9[a]		160.0[a]
					46.8[b]		164.2[b]
Seb	CO-1	1	2	3~6	7	8	CO-8
	176.9	37.0	26.6	29.5	26.5	36.5	176.7
Amp	2	CO-2	3	3′C_α	3′C_β	5	6
	74.1	174.9	65.4	31.4	27.6	67.7	58.6
	CO-7	10	CO-9	1′	2′/6′	3′/5′	4′
	175.1	58.2	172.0	137.1	128.2	130.0	129.8

[a] *cis*, [b] *trans* conformation.
Suc: succinyl; Chr: chromophore; Fho: formyl-hydroxyornithine; Seb: sebacic acid; Amp: ampicillin.

Table 5. MICs of Fe(III)-PaTSebAmp and Fe(III)-Pv9446SebAmp.

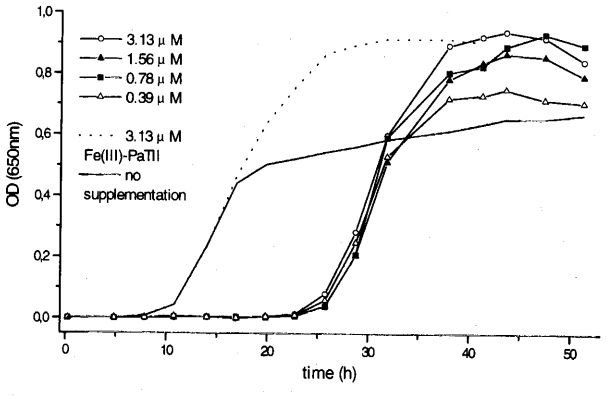
Strain	Fe(III)-PaTSebAmp	Fe(III)-Pv9446SebAmp
ATCC 27853	0.024 μM (0.04 μg/ml)	>100 μM
ATCC 15692 (PAO1)	>100 μM	0.39 μM (0.67 μg/ml)

at 30~35°C. PI-FAB-MS *m/z* 1607 $(M+H)^+$.

Suc-pyoverdin9446-ampicillin Conjugate (Pv9446Seb-Amp)

The synthesis of Pv9446SebAmp followed the same procedure as for PaTSebAmp. Yield of Fe(III)-Pv9446Seb-Amp was 22.3 mg (20%). PI-ESI-MS *m/z* 1729 $(M+Fe-2H)^+$, PI-FAB-MS *m/z* 1676 $(M+H)^+$.

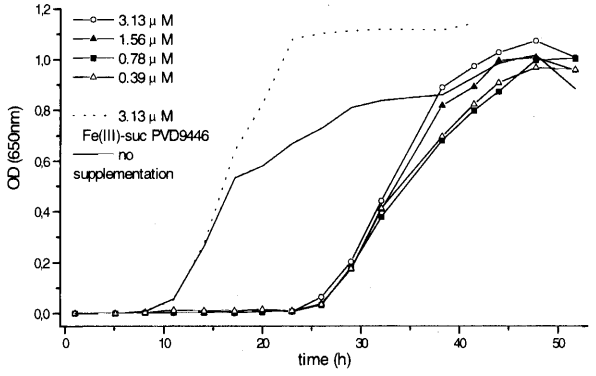
Fig. 3. Growth of *P. aeruginosa* ATCC 27853 in GMM broth containing Fe(III)-PaTSeb-Amp.



Decomposition products of PaTSebAmp and Pv9446SebAmp (PaTSebAmpdec/Pv9446SebAmpdec)

PaTSebAmpdec and Pv9446SebAmpdec were ob-

Fig. 4. Growth of *P. aeruginosa* ATCC 15692 in GMM broth containing Fe(III)-Pv9446Seb-Amp.



tained as by-products in the synthesis of the conjugates. PI-FAB mass spectra of fraction 7 of the chromatography on DEAE-Sephadex A-25 were consistent with the hydrolytic cleavage of the β-lactam ring of the ampicillin moiety and a subsequent decarboxylation. PaTSeb-Ampdec: PI-FAB-MS *m/z* 1581 $(M+H_2O-CO_2)^+$, Pv9446SebAmp-dec: PI-FAB-MS *m/z* 1650 $(M+H_2O-CO_2)^+$.

Determination of MICs

MICs were determined by the agar dilution method using a gluconate minimal medium (GMM), containing Na-gluconate 13.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $(NH_4)_2SO_4$ 5.0 g, KH_2PO_4 2.0 g, K_2HPO_4 4.3 g in 1 liter bidistilled water. The inoculum was adjusted with a UV/VIS spectrophotometer (Perkin-Elmer Lambda 7, optical density at 650 nm), so as to contain approximately 10^4 cfu per inoculation spot. On each agar-plate (i.d. 5 cm) seven to nine inoculation spots (1 μl) were applied. Plates were then incubated at 34°C for 60 hours. The MICs were defined as the lowest concentration of antibiotic that inhibited visible bacterial growth.

Growth Curves

From 50 mM stock solutions of Fe(III)-PaTSebAmp and Fe(III)-Pv9446SebAmp in sterile GMM broth, dilutions were prepared directly in UV-cuvettes by twofold dilution steps to reach twice the desired final concentrations. The inoculum was prepared from GMM broth cultures at the end of the exponential growth phase by dilution with GMM broth, so as to contain approximately 2×10^6 cfu/ml. Aliquots of inoculum and conjugate-containing solutions were mixed and the cuvettes were stirred at 34°C for 50 hours. Every 3 hours growth was monitored by measuring the optical density at 650 nm with a Perkin-Elmer Lambda 7 UV/VIS spectrophotometer.

Growth Stimulation Studies

Growth stimulation studies were performed according to MEYER *et al.*[6]. GMM agar plates (24 hours old), supplemented with 1 mg/ml EDDHA (ethylenediamine-di(*o*-hydroxyphenyl-acetic acid)) (Sigma, St. Louis, MO) were evenly overlaid with 100 μl of a diluted suspension (1×10^7 cfu/ml) of overnight cultures of *Pseudomonas aeruginosa* ATCC 27853 or ATCC 15692 (PAO1), grown in GMM broth. Then two sterile filter discs (i.d. 6 mm, DIFCO Laboratories, Detroit, MI) were laid on each plate and 20 μl of 1 mM solutions of iron free PaTSebAmpdec or Pv9446SebAmpdec as well as the iron free parent pyoverdins in sterile bidistilled water were respectively applied to one filter disc of each plate. The plates were then incubated at 34°C for 18 hours.

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