Purification, Characterization, and Structure of Pseudobactin 589 A, a Siderophore from a Plant Growth Promoting *Pseudomonas*†

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ABSTRACT: Under conditions of low-iron stress the plant growth promoting bacterium *Pseudomonas putida* 589 (DSM 50202) produced a yellow-green fluorescent iron-binding peptide siderophore, which was designated pseudobactin 589 A and had an affinity constant toward Fe^{3+} of 10^{25} at pH 7. Protonated pseudobactin 589 A had the molecular formula $C_{54}H_{78}O_{26}N_{15}$ and a nominal mass spectral molecular mass of 1353 g/mol. Its structure was determined by a combination of nuclear magnetic resonance, fast atom bombardment mass spectrometry, and Edman degradation. Pseudobactin 589 A consisted of a nonapeptide with the amino acid sequence L-Asp-L-Lys-(D)-β-OH-Asp-D(L)-Ser-L-Thr-D-Ala-D-Glu-L(D)-Ser-L-N^{δ}-OH-Orn, in which lysine was amide bonded via the carboxy and the N^{ϵ}-amino groups. A quinoline-derived chromophore was connectd via an amide bond to the α-amino nitrogen of aspartic acid and an L-malamide residue was attached to the chromophore. The three bidentate Fe^{3+} binding ligands consisted of an *o*-dihydroxy aromatic group from the quinoline derivative, β-hydroxyaspartic acid, and an internally cyclized N^{δ}-hydroxyornithine. The structure of pseudobactin 589 A is unique but strikingly similar to that of other pseudobactin-type siderophores from other plant growth promoting and plant deleterious pseudomonads.

Root-colonizing *Pseudomonas* bacteria can be plant growth promoting (PGP)[1] plant growth deleterious (PGD), or indifferent to plant growth (Schroth & Hancock, 1981). PGP pseudomonads enhance crop yields of a variety of plants (Schroth & Hancock, 1982; Xu & Gross, 1986). This effect is due partly to siderophores that make iron in the rhizosphere less available to deleterious fungi and rhizobacteria (Hemming, 1986; Kloepper et al., 1980; Vandenbergh et al., 1983). Siderophores are virtually Fe^{3+}-specific chelators and are synthesized by a majority of microorganisms under conditions of low-iron stress (Neilands, 1981; Neilands & Leong, 1986). PGD pseudomonads may affect plants in a deleterious fashion by producing phytotoxins, enzymes, and/or hormones (Gross & Cody, 1985) and possibly also by withholding iron from the plant (Becker et al., 1985). The ability of a given *Pseudomonas* strain to colonize plant roots may be affected by its ability to utilize, or be inhibited by, heterologous siderophores. This ability varies and can be classified into three patterns of growth inhibition (Buyer & Leong, 1986; Leong, 1986).

Pseudomonads are metabolically versatile and the yellow-green, fluorescent siderophores from PGP and PGD pseudomonads so far investigated also show extensive structural diversity. Each strain appears to produce a unique siderophore based on a common theme of three iron-binding ligands, one of which is always an *o*-dihydroxy aromatic group derived from quinoline. The other two are acylated N^{δ}-hydroxyornithine (N^{δ}-OH-Orn) residues or N^{δ}-OH-Orn plus a β-hydroxyaspartic acid (β-OH-Asp). The greatest variation occurs in the peptide backbone, which can be linear or partially or fully circular. It consists typically of 6–10 amino acids of various composition and chirality, although alanine, serine, and threonine are commonly found. Microheterogeneity within a given strain is conferred by a dicarboxylic acid, or its amide, attached to the chromophore. This is usually succinic acid, but the presence of malic acid and α-ketoglutaric acid has also been noted. The function of the dicarboxylic acid is unknown (Briskot et al., 1989; Buyer et al., 1986; Poppe et al., 1987; Teintze et al., 1981; Yang & Leong, 1984).

A knowledge of the structure of these siderophores might be of importance to understand mechanisms in microbe–microbe and plant–microbe interactions. *Pseudomonas putida* 589 (DSM 50202) (Molin & Ternström, 1986) has been shown to have plant growth promoting effects on tomatoes. It also displays limited growth inhibition against the plant pathogenic fungus *Verticillium dahliae* (Strenström, 1988). We describe here the purification, characterization, and structure of pseudobactin 589 A, a siderophore from this organism. Pseudobactin 589 A is similar to other siderophores of the pyoverdin/pseudobactin class, but it also possesses unique characteristics, underscoring the great structural diversity among *Pseudomonas* siderophores.

Experimental Procedures

Materials. *threo*-D,L-β-Hydroxyaspartic acid and malic enzyme were obtained from Sigma. Amberlite XAD-4 was

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[1] Abbreviations: BSTFA, bis(trimethylsilyl)trifluoroacetamide; CD, circular dichroism; COSY, correlation spectroscopy; dansyl, 5-(dimethylamino)naphthalene; DQF, double-quantum filtration; EDTA, ethylenediaminetetraacetic acid; FAB, fast atom bombardment; GC, gas chromatography; HAc, acetic acid; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; N^{δ}-OH-Orn, N^{δ}-hydroxyornithine; β-OH-Asp, β-hydroxyaspartic acid; PGD, plant growth deleterious; PGP, plant growth promoting; ppm, parts per million; Py, pyridine; ROE, rotating-frame Overhauser effect; ROESY, rotating-frame Overhauser enhancement spectroscopy; TEA, triethylamine; 2D, two-dimensional.

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from BDH. BSTFA and polyamide sheets (Cheng Chin) were from Pierce. Desferal was a gift from Ciba-Geigy.

Bacteria and Growth Conditions. Pseudomonas putida 589 (DSM 50202) was maintained on King B agar (King et al., 1948). For siderophore production MM9 (Schwyn & Neilands, 1987) without casamino acids was used. *P. putida* was grown with vigorous aeration in 20-L glass flasks at 25 °C for approximately 36 h.

Purification of the Ferric Siderophore. Siderophore-containing supernatant fluid was separated from cells by passage through a Gambro hollow-fiber filter (Gambro Sweden AB). One gram of $FeCl_3$ was added per liter of supernatant to convert all siderophores to their ferric complex. Ferric siderophores were adsorbed overnight to 100 g of XAD-4/L. The XAD was collected on a glass filter and washed with deionized water to remove inorganic salts and debris. Siderophores were eluted with acetone/water (8:2). Following removal of solvent by rotary evaporation, the residue was dissolved in 5–10 mL of 0.02 M pyridine/acetic acid (Py/Hac), pH 6.5. This concentrate was chromatographed at 4 °C on a 1.5 × 20 cm column with DEAE-trisacryl M, equilibrated in the same buffer. Bound ferric siderophores were eluted with a linear gradient (600 mL) from 0.02–1 M Py/Hac, pH 5.3.

After lyophilization, ferric pseudobactin 589 A was further purified by reversed-phase HPLC on a 10 × 250 mm semipreparative RP C18 column with polygosil, 10-μm particles. A Shimadzu HPLC system equipped with a UV/vis detector was used. Separation was achieved at a flow rate of 5 mL/min, isocratically for 7.5 min, and then by a gradient from 10 to 40% methanol for 22.5 min, in a buffer of 10 mM triethylamine/acetic acid (TEA/HAc). The eluate was monitored at 400 nm, and three major peaks were collected.

Pseudobactin 589 A. The three ferric siderophore peaks were deferrated as described (Briskot et al., 1986). Pure siderophores were obtained after gel filtration on a 2.5 × 90 cm column with Sephadex G-25, equilibrated in 0.02 M Py/Hac, pH 6.0. For NMR, an aqueous solution containing pseudobactin 589 A was passed through a short column of CM-trisacryl M, sodium form, equilibrated in water or two Biogel P-2 columns equilibrated with 5 mM NH_4HCO_3 and distilled H_2O.

Spectroscopy. Ultraviolet and visual spectra from 200 to 600 nm of both the ferric siderophore and the free ligand were taken on a Shimadzu UV-260 spectrophotometer in the following 0.1 M buffers: acetate, pH 5.0; phosphate, pH 6.0, 7.0, and 8.0; Tris-HCl, pH 7.4; and glycine-NaOH, pH 10.0. Fluorescence spectra of the free ligand were measured in 0.1 M acetate, pH 5.6, on a Hitachi F-3000 fluorescence spectrophotometer. CD spectra were obtained on an AVIV Model 60 DS circular dichroism spectrometer.

Formation Constant. In order to determine the formation constant of the Fe^{3+}–siderophore complex, EDTA was used as competing ligand (Anderegg et al., 1963; Meyer & Abdallah, 1978). EDTA in a range from 0.25 to 80 mM was prepared in the same series of buffers as mentioned above, in which the Fe^{3+}–siderophore concentration was 28 μM. Absorbances at 450 nm were measured after equilibration for at least 7 h.

Mass Spectrometry. Pseudobactin 589 A was analyzed with fast atom bombardment mass spectrometry (FAB MS) (Barber et al., 1981; Biemann & Martin, 1987) on a VG ZAB-HF at the Department of Medical and Physiological Chemistry, University of Göteborg, Sweden, and on a Kratos MS-50 at the University of California, Berkeley, Mass Spectrometry Facility. Both mass spectometers were operating at 8 kV and used Xe fast atoms.

Paper Electrophoresis. Horizontal paper electrophoresis was carried out at pH 6.5 in 10% pyridine and 2.5% acetic acid and at pH 1.9 in 12% acetic acid and 2.5% formic acid on Whatman paper No. 1 at 1500 V (25 mA) in a flat-bed apparatus with water cooling. Standards included ferrichrome A (3−) and its methyl esters (0 to 2−) (Emery & Neilands, 1961), pseudobactin (1+) and ferric pseudobactin (0) (Teintze et al., 1981), and ferrioxamine B (1+) (Prelog & Walser, 1962). Spots were identified directly by color, visualized by UV light or by spraying with 0.2% ninhydrin in acetone.

Amino Acid Analysis. Lyophilized material from each of the three siderophore fractions was hydrolyzed in 6 M HCl or 50% HI for 24 h at 110 °C. Samples were analyzed with the amino acid analyzer at the Department of Biochemistry, Uppsala University. The configuration of the amino acids was determined as described (Einarsson et al., 1987), at the Department of Analytical and Marine Chemistry, CTH, University of Götenborg.

NMR Spectroscopy. ^1H NMR spectra were recorded at ambient temperature on a Bruker AM-400 spectrometer, operating at 400.13-MHz proton frequency, equipped with an Aspect 3000 computer and digital phase switcher at the University of California, Berkeley, NMR facility. Some spectra were processed on a Bruker X32 data station equipped with Uxnmr software.

For ^1H spectra, a quantity of about 7 mg of pseudobactin 589 A was dissolved in 0.4 mL of D_2O or 10% D_2O/90% H_2O (referred to as H_2O) at pH 6.5 or 3.5 (for D_2O, pH values refer to pH meter readings before deuterium exchange). Resolution enhancement of regular ^1H spectra was achieved by Gaussian multiplication. Chemical shifts were referred to an internal standard of acetone, equal to 2.225 ppm. For samples in H_2O, a low-power presaturation pulse was employed for solvent suppression during the relaxation delay.

Magnitude mode COSY spectra (Aue et al., 1976; Nagayama et al., 1980) were obtained with a spectral width of 2400 Hz (2.3 Hz/pt) in D_2O at pH 6.5.

The following spectra were acquired in the phase-sensitive mode in H_2O at pH 3.5. DQF COSY was obtained by using the time-proportional phase incrementation (TPPI) phase cycling scheme (Marion & Wüthrich, 1983; Rance et al., 1983) with a spectral width of 3970 Hz (3.9 Hz/pt). 2D rotating-frame nuclear Overhauser effect spectroscopy (ROESY) (Bothner-By et al., 1984; Kessler et al., 1987) was carried out with a spectral width of 3970 Hz (3.9 Hz/pt) and a mixing time of 200 and 300 ms. The spin-locking pulse consisted of a repeating series of 30° pulses separated by delays of 130 μs.

In COSY, 64 scans per t_1 value were accumulated, whereas 32 scans were accumulated in DQF COSY and ROESY. COSY and ROESY had 512 t_1 points and 1024 t_2 data points and DQF COSY was obtained with 504 t_1 points and 2048 t_2 data points. All data were zero filled in the t_1 dimension to yield final 1024 × 1024 real point matrices. Sine bell apodization with a 0° phase shift was used in both dimensions for COSY. Resolution enhancement in both dimensions of DQF COSY and ROESY was obtained by a sine bell function with phase shifts of $\pi/2$ and $\pi/3$, respectively.

^{13}C spectra were recorded in D_2O at pH 2.8 or 6 on a Varian XL-300 spectrometer equipped with software version 6.2 or on a Bruker AM-400 spectrometer. Two-dimensional decoupled ^1H–^{13}C correlation spectra were recorded as described (Bax & Morris, 1981; Bax & Subramanian, 1986). The inverse-mode correlation spectrum was acquired with a proton

spectral width of 9.7 ppm (3.8 Hz/pt) and a carbon spectral width of 216 ppm (21.2 Hz/pt). 64 scans for each of 640 t_1 values and 2048 t_2 data points were accumulated, and a spectrum of 1024 × 1024 data points was obtained with zero filling prior to 2D Fourier transformation. Resolution enhancement in both dimensions was obtained with a $\pi/3$ phase shifted cosine bell function.

End-Group Analysis. Free amino groups were determined by dansylation of 5 nmol of pseudobactin 589 A, following established procedures (Gray, 1972; Weiner et al., 1972). Dansylated products were separated by ascending chromatography on 7.5 × 7.5 cm polyamide sheets. A mixture of commercial dansylated amino acids was run on the back of each sheet as a control. The sheets were examined under UV light. Automated Edman degradation was performed on an Applied Biosystems 477A liquid-phase protein sequencer at the University of California, Berkeley, Micro Analysis Facility.

Identification of Dicarboxylic Acid. Approximately 3 mg of pseudobactin 589 A was hydrolyzed with HCl and extracted continuously with ether for 3 days. The ether solution was then dried with sodium sulfate and evaporated to dryness at room temperature. The remaining material was silylated with 200 μL of BSTFA at 60 °C for 30 min and analyzed by GC and GC-MS on a capillary column connected to a flame ionization detector. As standards, commercial succinic acid and malic acid were used.

Malic acid was also determined in a NAD^+ coupled assay with malic dehydrogenase. Malic acid was isolated from 5 mg (3.7 mmol) of hydrolyzed pseudobactin 589 A as described (Eldsen et al., 1976). The ether-eluted residue was evaporated to dryness and dissolved in 0.8 mL of 0.1 M glycine buffer, pH 10.0, after which 0.1 mL of 10 mM NAD^+ was added. The reaction was followed spectrophotometrically after the addition of 2 units of malic dehydrogenase. Commercial L-malic acid was used as reference.

Results

Purification and Physicochemical Properties. Growth of *P. putida* 589 in low-iron medium was accompanied by the excretion of yellow-green fluorescent siderophores, the synthesis of which was completely inhibited in rich or iron-repleted minimal medium. The total siderophore concentration in MM9 at harvest was approximately 80 mg/L. DEAE chromatography gave three major red-brown bands, of which the second constituted ferric pseudobactin 589 A and made up approximately 65% of the total siderophore content. The yield of HPLC purified material from this peak was typically 10–15 mg/L.

Red-brown ferric pseudobactin 589 A was very water soluble and had a migration similar that of the monomethyl ester of ferrichrome A (2–) upon electrophoresis at pH 6.5, indicating that it was dianionic. Yellow-green, fluorescent pseudobactin 589 A comigrated with the dimethyl ester of ferrichrome A upon electrophoresis at the same pH, indicating a monoanionic net charge.

The absorption and CD spectra of the free ligand and ferric complex of pseudobactin 589 A are shown in Figure 1. The absorption spectrum of ferric pseudobactin 589 A was insensitive to $[H^+]$ in the pH range from 5 to 10. Absorption maxima were found at 232 nm, with a shoulder at 260 nm (data not shown), and at 398 nm ($\epsilon_{398} = 1.95 \times 10^4$ M^{-1} cm^{-1}). At 450 nm the extinction coefficient was 5.0×10^3 M^{-1} cm^{-1}.

The absorption spectrum of the iron-free siderophore showed considerable variation with pH. As pH was raised from 5 to 8 the absorption maximum shifted from 377 to 403 nm, with a concomitant increase of the extinction coefficient from 1.4

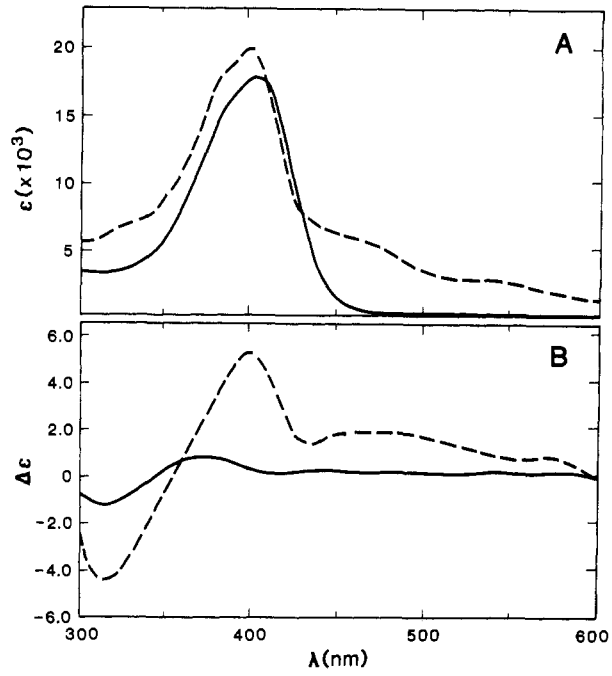

FIGURE 1: UV–vis (A) and CD (B) spectrum of pseudobactin (—) and ferric pseudobactin (---) in aqueous solution at pH 7.4.

× 10^4 to 2.2 × 10^4 M^{-1} cm^{-1} (data not shown). The fluorescence spectrum of pseudobactin 589 A had an excitation maximum at 400 nm and an emission maximum at 460 nm.

When analyzed with FAB in the cationic detection mode, pseudobactin 589 A and ferric pseudobactin 589 A yielded protonated molecular ion peaks at m/z 1352 and 1405, respectively. The presence in the ferric complex of a molecular ion 53 mass units above the free ligand indicated a 1:1 stoichiometry.

Means to indirectly measure complex formation between two molecular species by the addition of an auxiliary, competing species have been described (Anderegg et al., 1963; Rossotti & Rossotti, 1961). Since the absorption at 450 nm for the iron-free siderophore as well as for the Fe^{3+}-EDTA complex was negligible, absorption at this wavelength was directly proportional to the Fe^{3+}–siderophore concentration. As the formation constant of the Fe^{3+}–EDTA complex at various pH values was known, the formation constant of the Fe^{3+}–siderophore complex could be calculated as described (Anderegg et al., 1963; Meyer & Abdallah, 1978). The formation constant for ferric pseudobactin 589 A varied greatly with pH, as shown in Figure 2. The mean K_s at pH 7 was found to be 4.4×10^{25}, which is in the range of previously obtained values (Cody & Gross, 1987; Meyer & Abdallah, 1978; Philson & Llinás 1982a).

Structure of Pseudobactin 589 A. The three siderophores isolated by DEAE chromatography had identical amino acid compositions. Hydrochloric acid hydrolysis revealed seven amino acids: Ala, Ser, Thr, Asp, Glu, Lys, and β-HO-Asp, with molar ratios of 1:2:1:1:1:1:1. β-HO-Asp was identified with commercial D,L-*threo*-β-OH-Asp as reference. Hydrolysis with HI identified an additional molar equivalent of ornithine, which suggested the presence of N^{δ}-OH-ornithine (Emery & Neilands, 1961). The amino acids of the compound chosen for study, pseudobactin 589 A, were shown to have the following chirality: D-Ala, L-Ser, D-Ser, L-Thr, L-Asp, D-Glu, L-Orn, L-Lys. Chirally pure β-OH-Asp was not commercially available, but judging from the retention patterns of all other amino acid isomers separated with this system, *threo*-β-OH-Asp possibly had the D configuration.

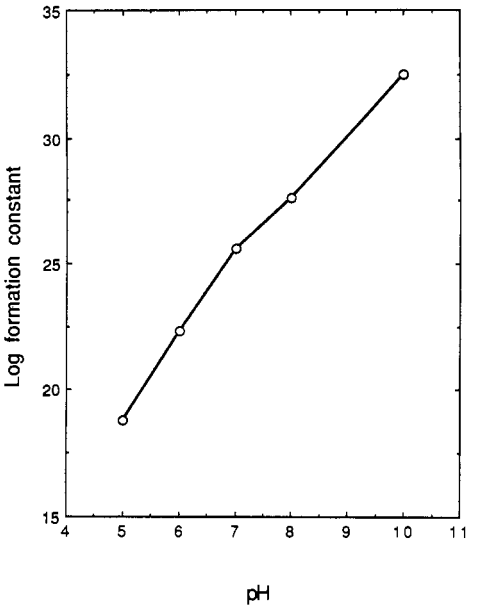

FIGURE 2: Plot of formation constant versus pH for ferric pseudobactin 589 A.

Table I: ^{1}H NMR Chemical Shifts of Pseudobactin 589 A in D_2O at pH 3.5

resonance	δ (ppm)	resonance	δ (ppm)
a	1.22	p	4.35
b	1.28	q	4.35
c	1.33	r	4.35
d	1.40	$S_1\alpha$	4.43
e	1.78	s	4.49
f	1.81	$S_2\alpha$	4.61
g	1.98	t	4.63
h	2.04	u	4.63
	2.16	v	4.67
II	2.43	w	5.00
	2.72	III	5.66
i	2.49	Mal NH	7.00
j	2.75	x	7.03
	2.87	y	7.19
k	2.94	Mal NH	7.74
l	2.98	z	7.95
	3.13	Lys ϵ-NH	8.12
l	3.41	Ser_1 NH	8.27
	3.69	Ala NH	8.27
m	3.64	Thr NH	8.32
$S_1\beta$	3.86	N^δ-OH-Orn NH	8.33
$S_2\beta$	3.88	Glu NH	8.39
	3.96	Ser_2 NH	8.46
n	4.10	β-OH-Asp NH	8.69
o	4.27	Asp NH	9.04

Nature of the Chromophore. The presence in pseudobactin 589 A of a quinoline derivative very similar to that found in other pseudobactin-type siderophores was indicated by virtual identity between its UV–vis and fluorescence spectra to those of other pseudobactin-like siderophores (MacDonald & Bishop, 1984; Philson & Llinás, 1982a,b; Teintze et al., 1981). This was confirmed by comparison of its characteristic ^{1}H and ^{13}C resonances with those of pseudobactin, whose crystal structure is known (Teintze & Leong, 1981; Teintze et al., 1981), as well as other pseudobactin-type siderophores (Briskot et al., 1989; Buyer et al., 1986; Poppe et al., 1987; Yang & Leong, 1984). The ^{1}H NMR spectrum of pseudobactin 589 A in D_2O is shown in Figure 3; the resonances are also summarized in Table I. The proposed structure of pseudobactin 589 A is shown in Figure 4. The ^{13}C chemical shifts are listed in Table II. The ^{1}H–^{13}C correlation spectrum (not shown) allowed all proton-bearing carbons to be assigned. Further assignments were made through the DEPT technique and comparison with

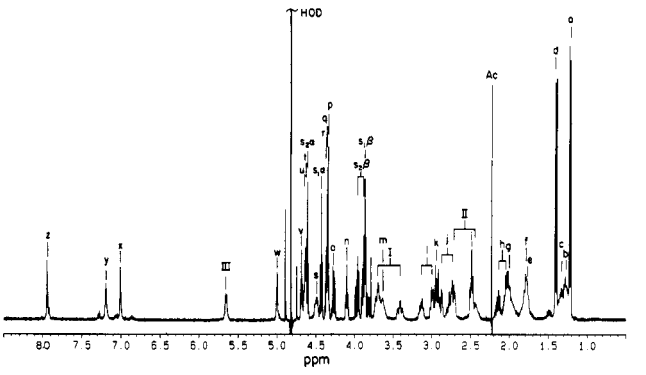

FIGURE 3: Resolution-enhanced ^{1}H NMR spectrum (400 MHz) of pseudobactin 589 A in D_2O at pH 3.5. Chemical shifts are relative to internal acetone (Ac), equal to 2.225 ppm.

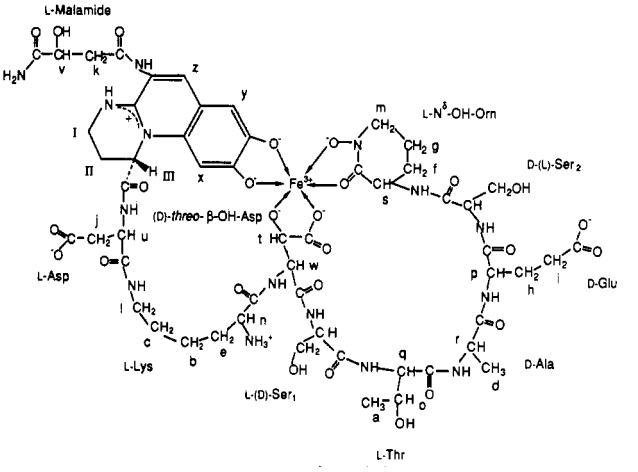

FIGURE 4: Proposed structure of ferric pseudobactin 589 A. The chirality of the two serines was arbitrarily assigned. Letters assigned to hydrogen atoms refer to proton resonances in Table I and Figures 3, 5, 7, and 8.

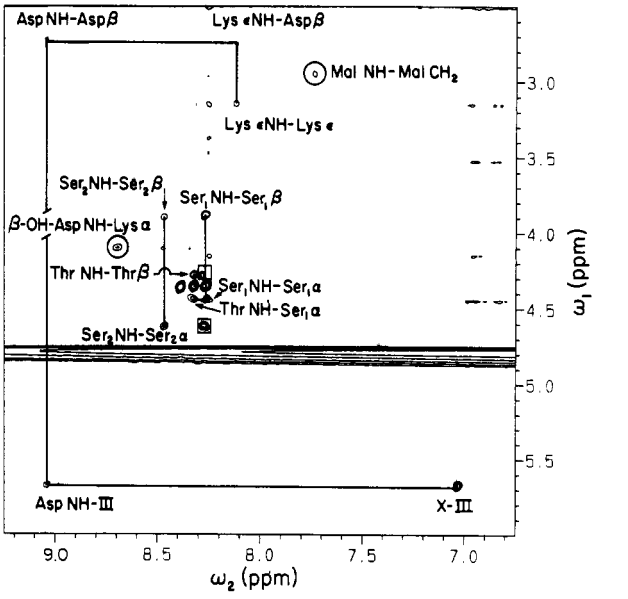

FIGURE 5: Sequential assignments in pseudobactin 589 A. An expansion of the 300-ms ROESY spectrum is shown, corresponding roughly to the COSY spectrum in Figure 8. Some cross peaks also observed in DQF COSY were not labeled. Vertical lines indicate intraresidue ROE's and horizontal lines connect COSY cross peaks and sequential ROE cross peaks. Cross peaks within circles indicate isolated sequential ROE's. The cross peaks in square boxes could not be identified.

standard shifts for amino acids (Wüthrich, 1976). The resonances of the nine aromatic carbons were virtually identical

Table II: ^{13}C Chemical Shifts[a] and Assignments of Pseudobactin 589 A in D_2O at pH 2.8

resonance	symbol[b]	carbon	δ (ppm)
Ala β	d	CH_3	18.3
Thr γ	a	CH_3	20.7
$N^δ$-OH-Orn γ	g	CH_2	21.9
Lys γ	b	CH_2	23.1
Chr[c]	II	CH_2	23.6
Glu β	h	CH_2	27.7
$N^δ$-OH-Orn β[d]	f	CH_2	28.6
Lys δ	c	CH_2	29.6
Glu γ	i	CH_2	31.8
Lys β[d]	e	CH_2	32.3
Chr	I	CH_2	37.0
Asp β	j	CH_2	38.9
Lys ε	l	CH_2	40.8
malamide	k	CH_2	41.0
Ala α	r	CH	51.7
$N^δ$-OH-Orn α	s	CH	52.2
Asp α	u	CH	52.6
$N^δ$-OH-Orn δ	m	CH_2	53.6
Lys α	n	CH	55.1
Glu α	p	CH	55.2
Ser α	$S_1α$	CH	57.5
Ser α	$S_2α$	CH	57.6
β-OH-Asp α	w	CH	57.9
Chr	III	CH	58.9
Thr α	q	CH	61.1
Ser β	$S_2β$	CH_2	62.8
Ser β	$S_1β$	CH_2	62.9
Thr β	o	CH	68.8
β-OH-Asp β	t	CH	70.0
malamide	v	CH	72.7
Chr	x	Arom	102.4
Chr		Arom	116.2
Chr		Arom	116.9
Chr	y	Arom	119.0
Chr		Arom	133.8
Chr	z	Arom	141.4
Chr		Arom	145.5
Chr		Arom	151.2
Chr		Arom	153.3
Chr		C=O[e]	171.6
Ser		C=O	172.2
Ser		C=O	172.8
Thr		C=O	172.9
$N^δ$-OH-Orn		C=O	173.6
Lys		C=O	173.7
Asp		C=O	173.9
Glu		C=O	175.2
Ala		C=O	175.6
malamide		C=O	176.5
β-OH-Asp		C=O	176.9
Asp β-COOH		C=O	177.0
malamide		C=O	178.9
Glu γ-COOH		C=O	179.1
β-OH-Asp β-COOH		C=O	182.5

[a] Chemical shifts are relative to internal 3-(trimethylsilyl)-1-propanesulfonate, equal to −1.10 ppm. [b] Symbols refer to proton–carbon couples in Figure 4. [c] Chr, chromophore; Arom, aromatic carbon. [d] Assignments may be interchanged. [e] All carbonyl carbons were assigned with reference to literature values and in the case of malamide with comparison to shifts of malic acid. Assignments must therefore be regarded as tentative and may be interchanged.

with those previously reported, as was the case for the aromatic and heterocyclic protons. In the ROESY spectrum, which is shown in Figure 5, ROE cross peaks between the aromatic protons y and z and x and III of the heterocycle were identified, which allowed the individual assignments of protons x and y. The CD spectrum of pseudobactin 589 A (Figure 1), in aqueous solution at pH 7.4, showed a maximum at 372 nm ($Δε = +0.8$). In analogy with pseudobactin (Teintze et al., 1981) and pyoverdin C, D, and E (Briskot et al., 1989) the chiral carbon of the quinoline moiety of pseudobactin 589 A was also *S* configurated. Similarly, ferric pseudobactin 589

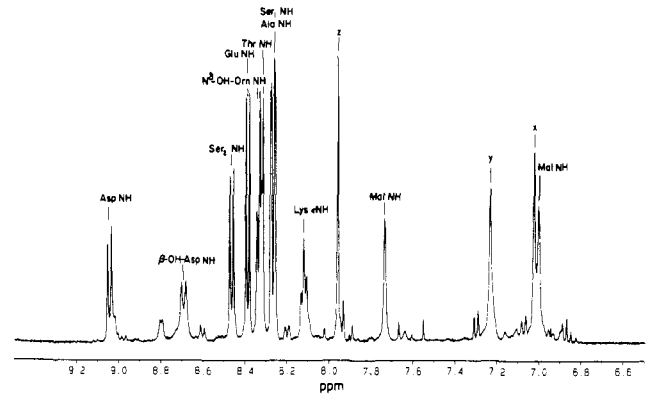

FIGURE 6: Resolution-enhanced ^1H NMR spectrum (400 MHz) of pseudobactin 589 A in H_2O at pH 3.5. Chemical shifts are relative to internal acetone, equal to 2.225 ppm. Only the region corresponding to amide and aromatic protons is shown.

A showed a strong positive Cotton effect at 398 nm ($Δε = +5.3$). Like pseudobactin (Teintze et al., 1981), ferric pseudobactin 589 A therefore appeared to have mainly the Λ configuration in solution.

The substituent attached to carbon 3 of the chromophore was determined to be derived from malamide. An ether extract of hydrolyzed pseudobactin 589 A was trimethylsilylated and malic acid was identified as its tris(trimethylsilyl) derivative by its retention from GC and its mass spectral fragmentation pattern from GC-MS, which yielded an *m/z* peak at 351 for the parent $[MH]^+$ ion. The pattern was identical with that obtained by commercial malic acid treated with BSTFA. The presence of L-malic acid was confirmed by reaction with malic dehydrogenase (data not shown). The net charge and molecular weight of pseudobactin 589 A required that both carboxylic groups of malic acid be amides. Furthermore, DQF COSY in H_2O revealed an isolated spin system consisting of two labile protons in the amide region, the ^1H spectrum of which is shown in Figure 6. These protons were determined to be the malamide NH_2 protons by virtue of their magnetic isolation and spatial proximity to the malamide methylene protons as judged by an ROE cross peak between one amide proton and the methylene protons. This result might suggest that the malamide CHOH group was closest to the chromophore. All three nonlabile malamide protons were, however, within 4 Å of at least one amide proton in this configuration, as determined by computer modeling. Furthermore, as in the case of pseudobactin 7SR1 (Yang & Leong, 1984), the relative downward shifts for the methylene protons were larger than for the CHOH proton, which suggested that the methylene protons are closest to the quinoline moiety.

Nature of the Peptide Chain. The ninhydrin reaction of pseudobactin 589 A was positive, albeit weak, and somewhat obscured by the strong innate color of the siderophore. A spot comigrating with $N^ε$-dansyllysine upon TLC of a dansylated hydrolysate of pseudobactin 589 A indicated a free $N^ε$-lysine amino terminus. Pseudobactin 589 A was not affected by proteolytic enzymes, such as trypsin and chymotrypsin, possibly because of the presence of D-amino acids.

FAB was therefore utilized as the primary tool for the amino acid sequence determination. The fragmentation of pseudobactin 589 A was highly dependent on solvent and matrix. Trifluoroacetic acid with thioglycerol gave a high degree of fragmentation and allowed an almost complete sequence of C ions (Roepstorff & Fohlman, 1984) to be identified, beginning at the peak corresponding to [malamide–chromophore–NH_2]$^+$ at *m/z* 390, as shown in Figure 7. The C ion

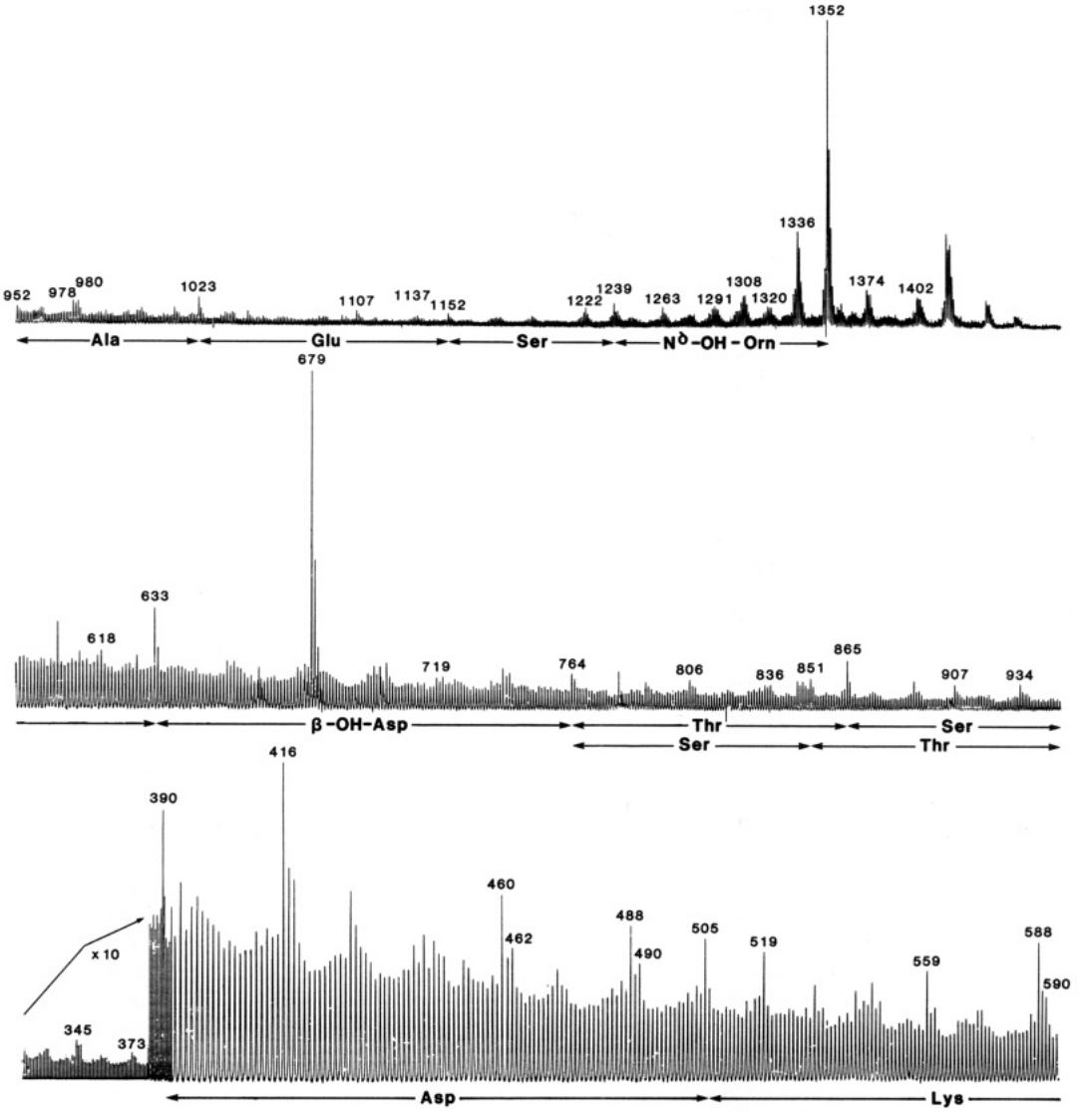

FIGURE 7: FAB spectrum of pseudobactin 589 A. Arrows beneath the spectrum delineate observed N-terminal C ion fragments. The order of one Ser and Thr could not be determined.

was for most peaks accompanied by a cluster of four characteristic sequence ions, 15, 43, 44, and 45 mass units below the amide ion, as in the case of a pseudobactin-like siderophore from *Azotobacter vinelandii* recently sequenced by FAB (Demange et al., 1988). FAB has also previously been observed to yield predominantly C ions in peptides with a positively charged group close to the amino terminus (Williams et al., 1982). The FAB experiment left ambiguous, however, the order of serine$_1$ and threonine, which is indicated in Figure 7.

According to FAB, the carboxy-terminal amino acid was N^{δ}-OH-Orn. It has earlier been shown that N^{δ}-OH-Orn at the carboxy terminus forms an iron-binding hydroxamic acid by either internal cyclization or acylation (Teintze et al., 1981; Yang & Leong, 1984). That the N^{δ}-OH-Orn might have been internally cyclized was indicated by net charge, molecular weight, resistance to carboxypeptidase A, and the absence of methyl or formyl peaks in the ^{1}H NMR spectrum, other than the two attributed to alanine and threonine.

Our approach to complete the structure determination, which involved the point of attachment of the chromophore as well as further information on the peptide sequence, involved 2D NMR techniques developed by Wüthrich and co-workers for the study of biological macromolecules (Billeter et al., 1982; Wider et al., 1984). By this technique, sequence assignments are made through NOE cross peaks between an NH proton and spatially close (<5 Å) NH, Cα, or Cβ protons in the preceding amino acid residue.

Initial 1D spectra and magnitude mode COSY in D_2O at pH 6.5 were utilized for primary assignments due to better resoltuion of some Cα proton resonances. All other spectra were taken at pH 3.5 to minimize exchange of labile protons. Assignment of all peaks in the ^{1}H NMR spectrum in D_2O was afforded by comparison of proton shifts in amino acids (Bundi & Wüthrich, 1979; Gross & Kalbitzer, 1988) and by the identification of individual spin systems (Wüthrich, 1986). Eleven spin systems were identified by COSY, two of which could be assigned to the heterocyclic protons and protons k and v of malamide. The rest belonged to the nine amino acids residues, of which all except Lys and N^{δ}-OH-Orn were readily identified.

A spin system with the number of protons and coupling pattern expected for Orn could be traced. Its chemical shifts were almost exactly identical with those reported for pseudobactin (Teintze & Leong, 1981), another indication that N^{δ}-OH-Orn was internally cyclized. The remaining spin system showed deviations in both the coupling patterns and shifts from those expected for lysine in a linear peptide. The Hδ and Hγ protons were shifted upfield compared to average values and were partially obscured by the Thr Hγ doublet.

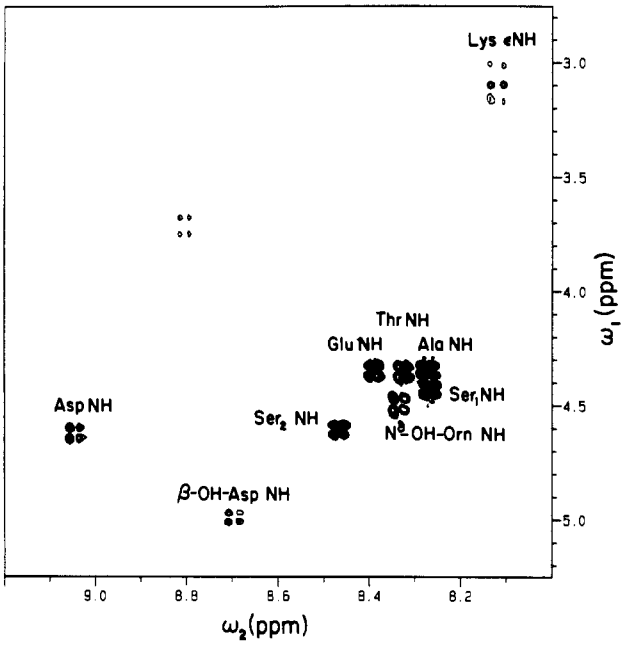

FIGURE 8: Fingerprint region of the DQ COSY spectrum of pseudobactin 589 A in H_2O at pH 3.5. Cross peaks correspond to α-H–NH connectivities except for lysine, where the cross peak is between α-H and ε-NH. The unlabeled peak was not identified.

Furthermore, the ε-methylene protons appeared as two broad multiplets, showing strong scalar coupling, centered at 2.99 and 3.14 ppm. From DQF COSY in H_2O it was obvious that these ε-methylene protons were coupled to an amide proton at 8.1 ppm, which is shown in Figure 8. In the ^1H spectrum of pseudobactin 589 A in H_2O, shown in Figure 6, this amide proton appeared as a triplet and not as a doublet, expected for an NH coupled only to a Cα proton. This led us to reconsider the result obtained by dansylation, see below. Amide protons were assigned by the observation of cross peaks to the corresponding Cα (or Cε) proton(s). Due to degeneracy of Ala, Thr, and Glu Cα protons, the Glu NH and Ala NH could not be unambiguously assigned, thus the assignments of these in Figures 6 and 8 are tentative and based on relative shifts (Bundi & Wüthrich, 1979; Gross & Kalbitzer, 1988). A weak amide signal at 8.80 ppm could not be assigned.

In ROESY, ROE cross peaks indicated that proton III of the heterocycle was spatially close to both the Asp NH and aromatic proton x, which is shown in Figure 5. This suggested the site of attachment of the chromphore to be the amino terminus of Asp via an amide bond. Furthermore, the Asp NH proton was down-shifted more than even that of β-OH-Asp, indicating proximity to the chromophore. This effect has also been pointed out previously (Buyer et al., 1986). In the peptide chain, interresidue ROE's allowed assignment of the sequences chromophore–Asp-Lys-β-OH-Asp and Ser_1-Thr, which is shown in Figure 5. Thus, ROESY established the order of Ser_1 and Thr as Ser_1-Thr.

The proton shifts and couplings for lysine led us to subject a sample of pseudobactin 589 A to automated Edman degradation, which gave the following result; Lys-Xaa-Ser-Thr-Ala-Glu-Ser-Xaa. The yield of lysine after the first cycle was considerably lower than for the following amino acids. Thus, Edman degradation confirmed most of the amino acid sequence. It should be noted that N^α-dansyllysine was not included in the standard mixture of dansyl amino acids for TLC and that it apparently, under the conditions used, could not be resolved from N^ϵ-dansyllysine.

In summary, the proposed structure of pseudobactin 589 A yields a siderophore with a net charge of 1−, which is explained by 1+ from the chromophore and Lys and 1− from Asp, β-OH-Asp, and Glu. Chelation of Fe^{3+}, with the concomitant loss of the two phenolic protons and one proton each from the hydroxyl groups of β-OH-Asp and N^δ-OH-Orn, would give a 2− net charge for ferric pseudobactin 589 A. The proposed structure, with the molecular formula $C_{54}H_{78}O_{26}N_{15}$ ($C_{54}H_{75}O_{26}N_{15}Fe$) and an accompanying mass spectral molecular mass of 1352.5 g/mol (1405.4 g/mol) for the $[MH]^+$ ion, was consistent with its ^1H and ^{13}C NMR spectra and observed mass spectral molecular mass of 1352.4 g/mol (1405.3 g/mol). Figures in brackets refer to the ferric complex.

Discussion

The structures of the yellow-green fluorescent siderophores from *Pseudomonas* and *Azotobacter* thus far characterized show extensive structural diversity. There appears to be no general difference between the siderophores produced by PGP and PGD pseudomonads. For example, *P. putida* 589, *Pseudomonas* B10 (Teintze et al., 1981), and *P. putida* WCS358 (van der Hofstad et al., 1986) are plant growth promoting, whereas *Pseudomonas* A214 (Buyer et al., 1986) and *Pseudomonas* 7SR1 (Yang & Leong, 1984) are plant deleterious. An important difference among different strains seems to lie in the specificity of the outer membrane ferric siderophore receptor(s), since this confers the ability to utilize heterologous siderophores (Leong, 1986; Magazin et al., 1986).

The quinoline-derived fluorescent chromophore has been found in all pseudobactin-type siderophores and a dicarboxylic acid or amide is attached to the chromophore. Three siderophores isolated from *P. aeruginosa* and *P. fluorescens* (respectively) differed only in the nature of the side chains (Briskot et al., 1986; Poppe et al., 1987). Three ferric siderophores were isolated from *P. putida* 589 and it appears possible that these might also differ only in the nature of the side chains, as their amino acid compositions were identical and all showed fluorescence and iron-binding capacities. The siderophore chosen for study contained malamide, and its name was given the suffix A to denote the possibility of different dicarboxylic acid side chains.

The second iron-binding ligand in pseudobactin 589 A was an N^δ-OH-ornithine residue at the carboxy terminus, as in all other pseudobactin/pyoverdin siderophores. It forms an Fe^{3+}-chelating hydroxamic acid residue either by acylation or by internal cyclization to the carboxyl group. Charge, resistance to carboxypeptidase A, molecular weight, and the absence of formyl or additional methyl peaks in the ^1H spectrum showed that N^δ-OH-ornithine could only have formed a hydroxamic acid by internal cyclization. Furthermore, a fragment below the molecular ion in FAB corresponded to a loss of cyclized HO-Orn as the first amino acid from the carboxy terminus. Pseudobactin 589 A in this respect resembled pseudobactin (Teintze et al., 1981) and pseudobactin 358 (van der Hofstad et al., 1986). Pyoverdin Pa and pyoverdin Pa B and C (pyoverdin C–E), siderophores from *P. aeruginosa*, were also proposed to possess a cyclic terminal N^δ-OH-ornithine (Briskot et al., 1986; Demange et al., 1986; Demange et al., 1987; Wendenbaum et al., 1983). However, it was recently shown that these siderophores had a N^δ-formylated carboxy-terminal ornithine (Briskot et al., 1989). The structure of these siderophores, as of pyoverdine I, II, and III from *P. fluorescens* (Poppe et al., 1987), was furthermore shown to contain an unusual internal tetrapeptide ring, in the former case cyclized via both amino groups of a lysine residue. The possibility of such a ring in pseudobactin 589 A was ruled out on the basis of charge, molecular weight, NMR, FAB

fragmentation pattern, dansylation, and susceptibility to Edman degradation, which covered eight of the nine amino acids. Interestingly, two other lysine-containing pseudobactin siderophores have also been shown to have free α-amino groups (Teintze et al., 1981; van der Hofstad et al., 1986), although in these cases the chromophore was attached directly to the lysine ϵ-amino group.

The third ligand is found between the chromophore and N^{δ}-OH-Orn and can be either a second acylated N^{δ}-OH-Orn (Briskot et al., 1989, Poppe et al., 1987) or β-OH-Asp as in the case of pseudobactin 589 A.

The structure of pseudobactin 589 A was determined by a combination of results from 2D NMR and FAB, each of which provided only partial sequence and structural information. The results taken together, however, were complementary and to some extent mutually corroborating and afforded the complete structure.

Sequential assignments with 2D NMR relies on interresidue connectivities with NOESY. However, at a certain molecular size and spectrometer frequency, $\omega_0\tau_c$, the product of the Larmor frequency and the rotational correlation time, is close to unity, which results in negligible NOE's. With pseudobactin 589 A, no NOE cross peaks could be observed with NOESY (Bodenhausen et al., 1984) at room temperature. A similar observation was made with pseudobactin A214 (Buyer et al., 1986). Possibly the size of pseudobactin siderophores renders them unsuitable for NOESY, at least under conditions used in this study. Instead of acquiring NOESY at low temperature, at which viscosity might cause line broadening, ROESY was used. In the rotating frame ROE's are always positive and increase for increasing values of τ_c. With ROESY a number of ROE's were observed, which allowed several crucial sequential assignments to be made. However, as in the case of pseudobactin A214 (Buyer et al., 1986), ROE cross peaks in addition to those expected with the proposed structure were observed, indicating a folding of the molecule, which would bring sequentially distant protons close together. Furthermore, the crystal conformation of ferric pseudobactin (Teintze et al., 1981) was found to be stabilized by two strong intramolecular hydrogen bonds. It might thus appear that pseudobactin siderophores display a partially ordered structure.

For the sequence determination 2D NMR served as a complement to FAB, which has been instrumental in the recent determinations of a number of pseudobactin-like siderophores (Briskot et al., 1989; Demange et al., 1987, 1988; Poppe et al., 1987). In the case of pseudobactin 589 A, it likewise provided a major part of the structure. However, certain shortcomings with FAB were noted: how Lys was connected could not be determined, neither could the order of Ser and Thr. Furthermore, FAB did not yield any detailed information on the chromophore. Whichever the preferred method, substantiation by a second and perhaps third method does not appear to be superfluous. With pseudobactin 589 A we were fortunate to be able to confirm the major part of the peptide sequence by Edman degradation. The approach of partial hydrolysis and isolation of fragments was thereby made redundant.

Abdallah and co-workers (Demange et al., 1987) gave the peptide sequence for a siderophore from *P. putida* identical with that of pseudobactin 589 A. However, as detailed information was not provided, the identity between this siderophore and pseudobactin 589 A could not be ascertained.

The structure of pseudobactin 589 A underscores the diversity associated with *Pseudomonas* and pseudobactin siderophores. This diversity might be part of an explanation of the varying effects on plants observed with *Pseudomonas*. Furthermore, as more work is focused on iron in plant–microbe interactions and the rhizosphere, the significance of this element is becoming increasingly recognized. It is of importance primarily in the context of siderophores, their outer membrane receptors, and underlying regulatory genetic elements. However, its role also extends beyond this, as exemplified by an iron-antagonized fungistatic agent that is not a siderophore from a rhizosphere pseudomonad (Gill & Warren, 1988), to iron-controlled toxin production in the phytopathogenic fungus *Stemphylium botryosum* (Barash et al., 1983) and the direct relationship between a functional iron assimilation system and pathogenicity in the bacterium *Erwinia chrysanthemi* (Enard et al., 1988).

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