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Synthesis and Biological Activity of Pyochelin, a Siderophore of *Pseudomonas aeruginosa*

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Pyochelin, a phenolic siderophore of *Pseudomonas aeruginosa*, was synthesized in three steps from salicylonitrile, L-cysteine, and L-*N*-methylcysteine. The synthetic product was determined to be identical to natural pyochelin by ^{1}H nuclear magnetic resonance spectroscopy, fast atom bombardment mass spectrometry, chromatographic analysis, and chemical reactivity with $FeCl_3$ and ammoniacal silver nitrate reagent. Synthetic and natural pyochelins promoted bacterial growth in iron-depleted medium and were also found to mediate iron transport by *P. aeruginosa* to the same levels. Neopyochelin, a stereoisomeric by-product of the synthesis, showed less biological activity than did pyochelin in iron transport assays.

Many microorganisms possess high-affinity iron uptake systems mediated by the action of low-molecular-weight iron chelators termed siderophores (30). Siderophores are produced by microorganisms in response to iron deprivation and are able to convert insoluble ferric hydroxide polymers into soluble chelates which are substrates for high-affinity transport mechanisms (31). Siderophores are generally classified as either phenolates or hydroxamates, although other siderophores not belonging to these classes have been isolated (37). Siderophores have been implicated in the virulence of several bacteria, i.e., *Escherichia coli* (49), *Vibrio anguillarum* (17), *Salmonella typhimurium* (50), and *Pseudomonas aeruginosa* (2, 12, 40, 44).

P. aeruginosa is a nonfermentative, gram-negative rod considered highly pathogenic for individuals with compromised immunity. Septicemic infections due to *P. aeruginosa* have a very poor prognosis (28); mortality rates in the range of 50 to 80% have been reported (46). *P. aeruginosa* is known to produce two siderophores, pyochelin (14, 15) and pyoverdin (7, 13, 19, 48). Recent reports have suggested that siderophores may be factors in the virulence of this organism. Pyochelin was found to stimulate bacterial growth in murine infections (12). *P. aeruginosa* siderophores were found to be important factors in the interaction of the bacterium with transferrin, an iron-binding glycoprotein which is inhibitory to bacterial growth and is the major serum component responsible for nutritional immunity (10, 16, 47). It was demonstrated that both siderophores promote the removal of iron from transferrin (44) and the growth of mutants defective in siderophore production (2). Recently, it was determined that mutants with defects in ferripyochelin transport are markedly less virulent than wild-type strains of *P. aeruginosa* (40). In addition to directly promoting growth, these siderophores may indirectly affect the production of virulence factors (e.g., toxin A, alkaline protease, and elastase) (4, 5) which are under iron regulation (41). Two other pseudomonads, *Pseudomonas cepacia* and *Pseudomonas fluorescens*, are also able to produce pyochelin (38, 39).

Pyochelin is a structurally unique phenolate siderophore, designated 2-[2-(*o*-hydroxyphenyl)-2-thiazolin-4-yl]-3-methyl-4-thiazolidinecarboxylic acid on the basis of ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (15). The stoichiometry of iron binding appears to be two pyochelin molecules to one Fe(III) ion. When compared with other siderophores, pyochelin has a very low iron-binding coefficient, 5×10^5 (14). The probable reasons for this low coefficient are the iron-binding stoichiometry of two pyochelin molecules per Fe(III) ion and the low molecular weight, 324, of pyochelin. Despite its low iron-binding coefficient, pyochelin is extremely active in iron transport (11) and growth stimulation in media containing transferrin (2) and has been implicated in the pathogenicity of *P. aeruginosa* (2, 12, 40, 44).

These contradictory phenomena have stimulated questions about this unique siderophore and instigated this report concerning the synthesis of pyochelin from salicylonitrile, L-cysteine, and L-*N*-methylcysteine. The analysis of the synthetic pyochelin confirms the previously reported structure for pyochelin. The synthetic product demonstrated chemical and biological activities identical to those of natural pyochelin.

MATERIALS AND METHODS

Bacterial strains and media. *P. aeruginosa* PAO1 and its siderophore-deficient derivative IA1 (2) were used in growth promotion and transport assays. GMM-transferrin medium contained 5 mM K_2HPO_4, 15 mM $(NH_4)_2SO_4$, 20 mM $NaHCO_3$, 1 mM $MgSO_4$, 20 mM morpholinopropane sulfonate (MOPS) hydrochloride (pH 7.3), 20 mM glucose, and 200 μg of transferrin (no. 2252; Sigma Chemical Co., St. Louis, Mo.) per ml. CAA medium contained 0.5% Casamino Acids (Difco Laboratories, Detroit, Mich.), 5 mM K_2HPO_4 (pH 7.0), and 1 mM $MgSO_4$. CAA-MOPS medium contained 1 mM MOPS (pH 7.3) in place of K_2HPO_4.

Spectrometry. The ^{1}H NMR spectra were recorded on a Nicolet NTC-360 spectrometer in chloroform-*d*. Chemical shifts (δ) are reported as parts per million downfield from an internal tetramethylsilane standard. Field desorption and fast atom bombardment (FAB) mass spectra were obtained with Varian MAT 731 and VG Analytical 7070E spectrometers, respectively.

Chromatography. Thin-layer chromatography (TLC) was

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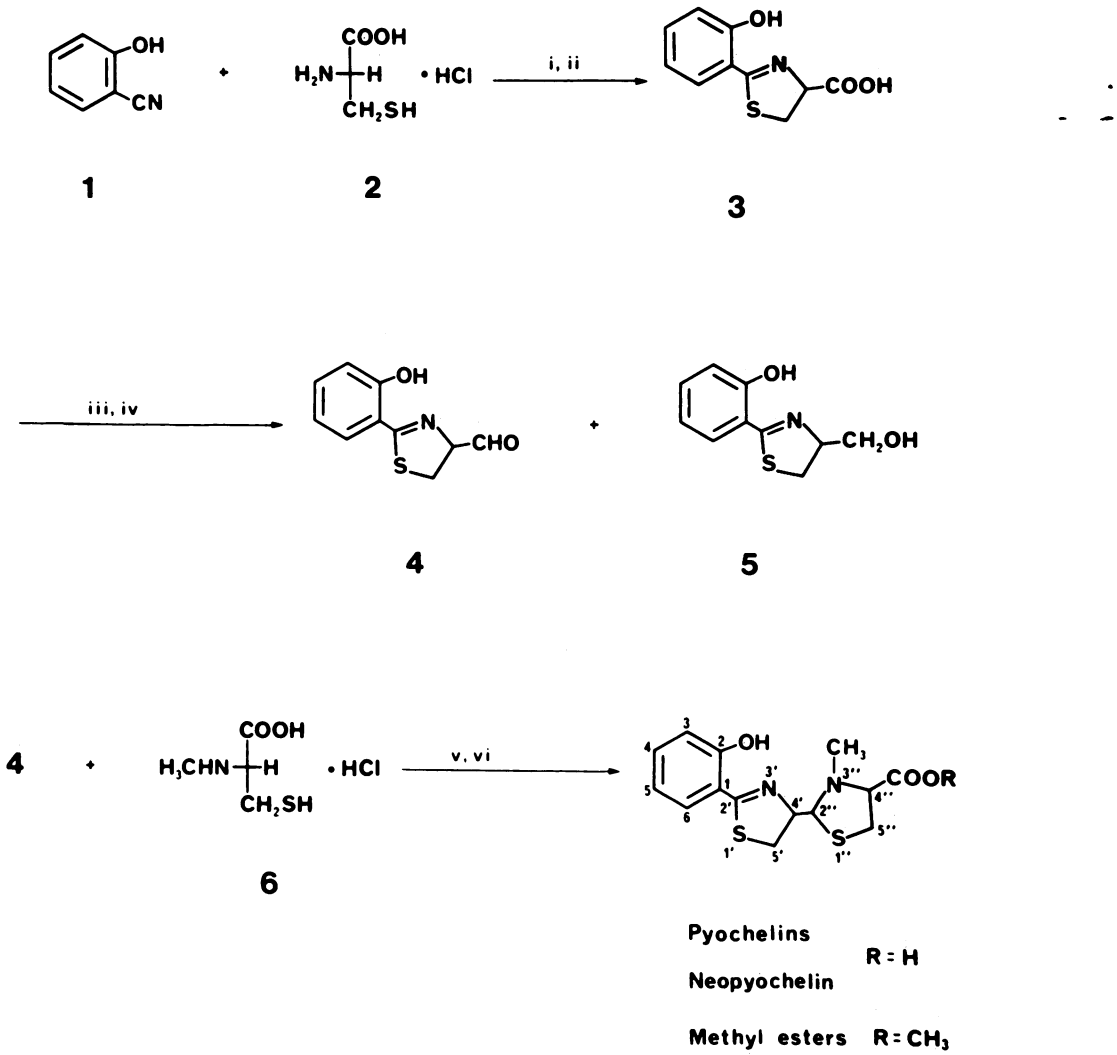

FIG. 1. Synthesis of pyochelin. Compound designations: 1, salicylonitrile; 2, L-cysteine hydrochloride; 3, 2-(*o*-hydroxyphenyl)-2-thiazoline-4-carboxylic acid; 4, 2-(*o*-hydroxyphenyl)-2-thiazoline-4-carboxaldehyde; 5, 2-(*o*-hydroxyphenyl)-2-thiazoline-4-methanol; and 6, L-*N*-methylcysteine hydrochloride. Reaction conditions: (i) sodium bicarbonate, absolute ethanol, reflux for 30 min; (ii) piperidine to pH 8.5, reflux for 12 h; (iii) thexylborane in tetrahydrofuran, −20°C, 10 min; (iv) reflux for 40 h; (v) potassium acetate, water–95% ethanol (1:1), 25°C, 1 h; (vi) 0°C, 12 h. Methyl esters of pyochelin and neopyochelin were formed by the reaction of diazomethane with the parent carboxylic acid.

performed by using Adsorbosil (no. 16030; Alltech Associates, Inc., Applied Science Div., State College, Pa.) and Chromagram (no. 13181; Eastment Kodak Co., Rochester, N.Y.) plates. Ammoniacal silver nitrate reagent (24), 0.1 M $FeCl_3$-HCl (14), and UV light were used for visualization of compounds on chromatograms. High-performance liquid chromatography (HPLC) was carried out with a chromatograph (332MP; Beckman Instruments Inc., Fullerton, Calif.) consisting of a monitor for 254-nm light connected to an integrator (model 3390A; Hewlett-Packard Co., Palo Alto, Calif.) which traced elution profiles from a Brownlee Spheri-5 OD-224 (C_{18} reversed-phase) semipreparative column (4.6 by 220 mm) (Brownlee Labs, Santa Clara, Calif.) during a 1.0-ml/min isocratic elution [64% methanol in 0.17 N acetic acid containing 0.5 mM ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid].

Synthesis methods. Salicylonitrile (*o*-cyanophenol) and thexylborane preparation kits were obtained from Aldrich Chemical Co., Milwaukee, Wis. Figure 1 displays the reaction sequence for synthesizing pyochelin. Carboxylic acid 3, 2-(*o*-hydroxyphenyl)-2-thiazoline-4-carboxylic acid, was prepared from salicylonitrile (compound 1) and L-cysteine hydrochloride (compound 2) by the method of Mathur et al. (27). L-*N*-Methylcysteine was prepared as its monohydrochloride (compound 6) by the method of Blondeau et al. (6) in 58% yield. Aldehyde 4, 2-(*o*-hydroxyphenyl)-2-thiazoline-4-carboxyaldehyde, was obtained by thexylborane reduction (9) of carboxylic acid 3. Aldehyde 4 was isolated by TLC (Adsorbosil; dichloromethane-ethanol, 100:7) as a fluorescent yellow band at R_f 0.60. A low-resolution field desorption mass spectrum gave the molecular ion at *m/z* 207. Due to the inherent lability of aldehyde 4, a suitable ^1H NMR spectrum could not be obtained. In addition to aldehyde 4, alcohol 5, 2-(*o*-hydroxyphenyl)-2-thiazoline-4-methanol, was also produced in the thexylborane reduction of carboxylic acid 3. Alcohol 5 was identified by ^1H NMR (δ 1.9–2.7 [br s, 1, CH_2OH]; 3.32 [dd, 1, H-5′a, J = 10.8 and 8.3 Hz]; 3.40 [dd, 1, H-5′b, J = 10.8 and 9.0 Hz]; 3.81 [dd, 1, J = 11.3 and

4.7 Hz]; and 3.95 [dd, 1, J = 11.3 and 5.1 Hz] [CH_2OH]; 4.85 [dddd, 1, H-4', J = 9.0, 8.1, 7.6, and 1.4 Hz]; 6.87 [td, 1, H-5, J = 8.0 and 0.7 Hz]; 6.98 [d, 1, H-3, J = 8.1]; 7.34 [td, 1, H-4, J = 8.5 and 1.4]; 7.39 [dd, 1, H-6, J = 7.6 and 1.4 Hz]) and high-resolution FAB mass spectrometry (M + H for $C_{10}H_{12}NO_2S$: calculated, 210.05892; found, 210.05832). Because of its lability, aldehyde 4 was not routinely isolated from the thexylborane reduction mixture, except for qualitative determinations of aldehyde 4. After removal of the solvent from the thexylborane reduction mixture, the residue was suspended in ethanol and subjected to condensation with L-*N*-methylcysteine (compound 6), using the standard method of thiazolidine formation (36). The condensation reaction mixture was added to 200 ml of 10 mM KH_2PO_4-acetic acid (pH 3.0) and was extracted twice with 200-ml volumes of CH_2Cl_2. The aqueous layer was discarded, the organic layers were combined, and the solvent was removed. The residue, containing synthetic pyochelin, was suspended in CH_2Cl_2-ethanol (1:1) and stored at −20°C. The products were purified by preparative TLC (Adsorbosil) with chloroform-acetic acid-ethanol (19:1:1) to give fractions which yielded black bands after being sprayed with ammoniacal silver nitrate reagent (24) and red bands after being sprayed with 0.1 M ferric chloride-HCl (14). Fractions were eluted from the silica with dichloromethane-ethanol (1:1) and yielded pale yellow films when dried. Treatment of these products with diazomethane in methanol (15) gave the corresponding methyl esters, which were subjected to ^1H NMR spectroscopy.

Growth promotion assays. Bacterial strains were prepared for inocula as described previously (2) and were added to GMM-transferrin medium at approximately 5×10^3 CFU/ml. Natural pyochelin and the synthetic products were added to the assays at 3.75 μg/ml. Growth was assayed by measuring the A_{600}.

Iron transport assays. Bacterial strains were grown in CAA medium for 20 h at 37°C and were harvested and washed with water by using centrifugation. The transport abilities of natural and synthetic products were tested by combining 0.019 μCi of $^{55}FeCl_3$ with 10 μg of each of the analyzed compounds per ml and adding this mixture to a suspension of 10^8 bacteria per ml in CAA-MOPS medium. Bacteria were separated from the transport reaction mixtures by filtration through cellulose-acetate filters of 0.45 μm pore size (GA-6; Gelman Sciences, Inc., Ann Arbor, Mich.), which were washed with 10 ml of water to remove unreacted $^{55}FeCl_3$ or ^{55}Fe-binding compounds. Filters were dried and placed in scintillation fluid (Budgetsolve; Research Products, Inc.), and the radioactivity was counted in a scintillation counter. These values were compared with those from reactions lacking ferrisiderophore, bacteria, or ferrisiderophore and bacteria.

RESULTS

Synthesis of pyochelin. Synthetic pyochelin was made in a three-step procedure using reactions which have been previously described. The synthetic pathway is shown in Fig. 1. The first intermediate in the pathway, carboxylic acid 3, was obtained in a 92% yield. A variety of reagents were employed in the attempted synthesis of aldehyde 4, but only thexylborane reduction of carboxylic acid 3 yielded aldehyde 4. Unfortunately, alcohol 5 was the main product of the reduction, and the yield of aldehyde 4, 15%, was disappointingly low. Modifications of the thexylborane reduction procedure (9) did not enhance the yield of aldehyde 4. Attempts to convert alcohol 5 to aldehyde 4 were also unsuccessful. Condensation of aldehyde 4 with L-*N*-methylcysteine, compound 6, was carried out as the final step in pyochelin synthesis.

Analytical TLC on Chromagram layers (Eastman) (solvent was $CHCl_3$-acetic acid-ethanol, 19:1:1) of the reaction mixture resulted in the identification of two products similar to pyochelin, one at R_f 0.40 and another at R_f 0.30. At times the spot at R_f 0.40 could be discerned as two very closely spaced spots, but generally the compound chromatographed as one discrete spot on Chromagram layers. Both products behaved similarly to pyochelin in that they (i) had fluorescence and spectral properties similar to those of pyochelin, (ii) yielded red spots on chromatograms after being sprayed with $FeCl_3$ solution, and (iii) yielded black spots after being sprayed with ammoniacal silver nitrate reagent (24). Pyochelin contains an *N*-methylthiazolidine ring which is known to rapidly form silver mercaptide salts during alkaline hydrolysis in the presence of Ag^+ ion (26). Spraying chromatograms with ammoniacal silver nitrate reagent (24) revealed black spots where pyochelin migrated. The product at R_f 0.40 had an R_f identical to that of natural pyochelin.

When preparative TLC was conducted with a different TLC support, Adsorbosil, under the same solvent conditions, three pyochelinlike products with R_fs of 0.40, 0.35, and 0.20 were identified. All three products behaved similarly to pyochelin according to the three properties listed above. When developed on Adsorbosil layers under the same conditions, natural pyochelin yielded two separate spots at R_fs 0.40 and 0.35. The yields of the three synthetic products from aldehyde 4 were as follows: R_f 0.40 product, 16%; R_f 0.35 product, 15%; and R_f 0.20 product, 30%.

Identification of synthetic pyochelin. Natural pyochelin, isolated from dichloromethane extracts of acidified *P. aeruginosa* culture supernatant (14), yielded two spots (R_fs 0.40 and 0.35) on Adsorbosil layers and a single spot (R_f 0.40) on Chromagram layers. Natural pyochelin also demonstrated the occasional separation into two closely spaced spots on Chromagram layers but, like the synthetic product, generally chromatographed as one discrete spot on these layers. The reason for the variability in separation observed with Chromagram layers is unknown but may be due to either atmospheric humidity or solvent saturation of the vapor phase in the TLC chamber. We have made a practice of referring to the species of R_f 0.35 as pyochelin I and to the one at R_f 0.40 as pyochelin II. The two species of natural pyochelin were spontaneously interconvertible when separated on TLC layers and subjected again to chromatography on Adsorbosil layers. Each spot gave rise to a mixture of both species. The two species separated on Adsorbosil chromatograms had the same molecular weight (m/z 325, M + H, FAB mass spectrum). For ^1H NMR spectroscopy, the methyl esters of the two species of natural pyochelin were formed by reaction with diazomethane. Figure 2 shows the ^1H NMR spectra of the methyl esters of synthetic and natural pyochelin I, and Fig. 3 shows the ^1H NMR spectra of the methyl esters of synthetic and natural pyochelin II. The ^1H NMR spectra of the methyl esters of synthetic and natural pyochelin I were virtually identical (Fig. 2). Similarly, the spectra of the methyl esters of synthetic and natural pyochelin II were nearly identical (Fig. 3). The major differences observed between the ^1H NMR spectra of pyochelin I and II methyl esters all appeared to be associated with the 3-methylthiazolidine ring. The peaks which shift between the two forms were due to the H-4', H-2'', H-4'', and N-3'' methyl protons. Comparison of the methyl ester of pyochelin I with that of

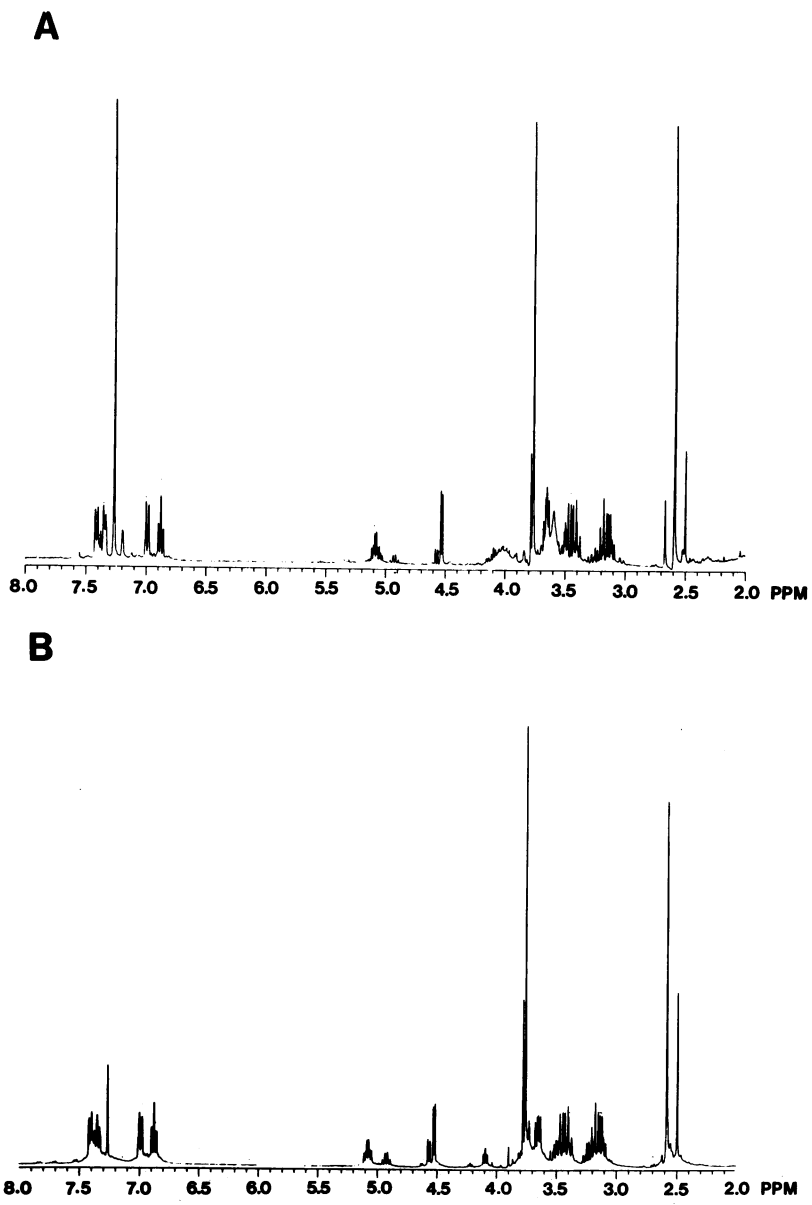

FIG. 2. ^{1}H NMR spectra of the methyl esters of synthetic pyochelin I (A) and natural pyochelin I (B) in $CDCl_3$. The peak at 7.27 ppm is due to $CHCl_3$ contamination of the solvent. The broad peaks in panel A at 3.60 and 4.02 ppm are due to contaminating solvents. Spectra were obtained on a Nicolet NTC-360 spectrometer.

pyochelin II demonstrated the following shifts: (i) for the H-4′ proton, a triple doublet at 5.08 ppm moved to a quartet at 4.92 ppm; (ii) for the H-2″ proton, a doublet at 4.52 ppm moved to a doublet at 4.58 ppm; (iii) for the H-4″ proton, a double doublet at 3.67 ppm moved to a triplet at 4.10 ppm; and (iv) for the N-3″ methyl protons, a singlet at 2.59 ppm moved to a singlet at 2.49 ppm. A minor change in the methyl ester protons could also be observed. The methyl ester protons in pyochelin I methyl ester yielded a peak at 3.75 ppm, while those of pyochelin II methyl ester yielded a peak at 3.77 ppm. Most ^{1}H NMR spectra of pyochelin displayed a combination of signals from both pyochelins I and II, most likely due to our inability to obtain spectra before the two forms of pyochelin interconverted. (Fig. 2 and 3). The ratio of signals at 2.49 ppm and 2.59 ppm in each spectrum serves as a rough estimation of the purity of the pyochelin form being analyzed. For example, Fig. 3B shows a large peak at 2.49 ppm and a very small peak at 2.59 ppm, indicating high purity of pyochelin II. On the other hand, Fig. 3A shows a much more substantial peak at 2.59 ppm and accompanying peaks at 3.67, 4.52, and 5.08 ppm, which demonstrate the presence of pyochelin I in this pyochelin II sample. The reasons for the appearance of the two species of pyochelin are currently being investigated.

The identity of the synthetic products at R_fs 0.35 and 0.40 with pyochelin I and II, respectively, was established by comparison of spectral data (FAB mass spectrometry, ^{1}H NMR, and absorbance spectra), chromatographic behavior during TLC and HPLC, and chemical reactivity with the spray reagents as described above. Both synthetic products were found to have the same molecular weight (m/z 325, M + H) by low-resolution FAB mass spectrometry, which was

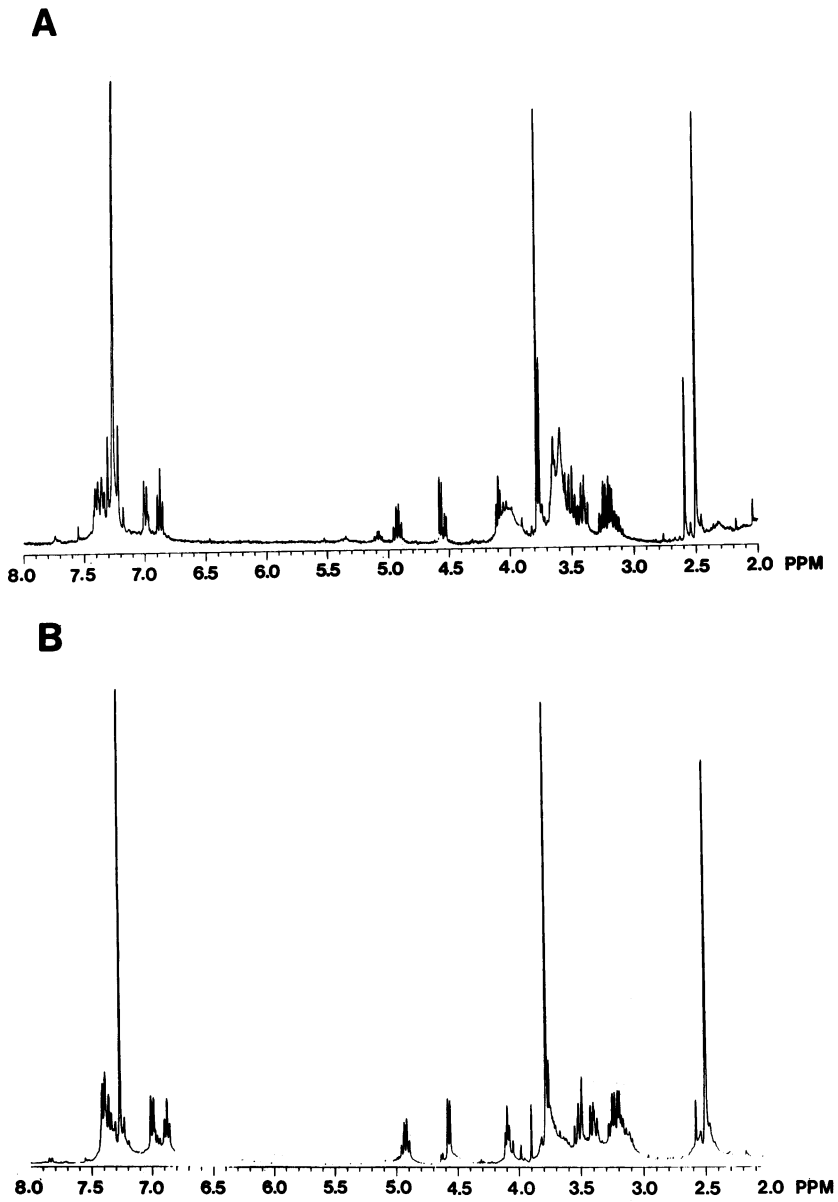

FIG. 3. ^1H NMR spectra of the methyl esters of synthetic pyochelin II (A) and natural pyochelin II (B) in $CDCl_3$. The peak at 7.27 ppm is due to $CHCl_3$ contamination of the solvent. The broad peaks in panel A at 3.60 and 4.02 ppm are due to contaminating solvents. Spectra were obtained on a Nicolet NTC-360 spectrometer.

identical to that of natural pyochelin. The ^1H NMR spectra of the methyl esters of the synthetic products were essentially identical to those of the methyl esters of the two species of natural pyochelin (Fig. 2 and 3). Analysis of the synthetic products by TLC demonstrated the spontaneous interconversion of the two products, a property characteristic of natural pyochelin. This spontaneous interconversion was also observed during HPLC analysis on C-18 reversed-phase columns. With the isocratic elution described in Materials and Methods, synthetic pyochelin eluted as two separate peaks at 12.2 and 14.5 min, identical to those displayed by natural pyochelin.

The product at R_f 0.20 was found by high-resolution FAB mass spectrometry to agree with the same molecular formula ($C_{14}H_{16}N_2O_3S_2$) as pyochelin (M + H for $C_{14}H_{16}N_2O_3S_2$: calculated, 325.0709; found, 325.0695). This product reacted with ammoniacal silver nitrate reagent and $FeCl_3$ in the same manner as pyochelin did. However, the ^1H NMR spectrum of the methyl ester of this product (Fig. 4) was significantly different from those of pyochelins I and II (Fig. 2 and 3, respectively). Neopyochelin eluted as only one peak during HPLC analysis, at 13.4 min. Although this product appeared to have the same chemical structure as pyochelin, it was not identical to pyochelin, and we have designated it neopyochelin. Neopyochelin has not been detected in culture supernatants of *P. aeruginosa* and would appear to be an unnatural isomeric form of pyochelin.

Biological activity of synthetic pyochelin. The ability of synthetic pyochelin to promote bacterial growth in iron-depleted medium containing transferrin was compared with that of natural pyochelin. The bacterial strain used to test the activity of pyochelin was IA1, a PAO1 derivative defective

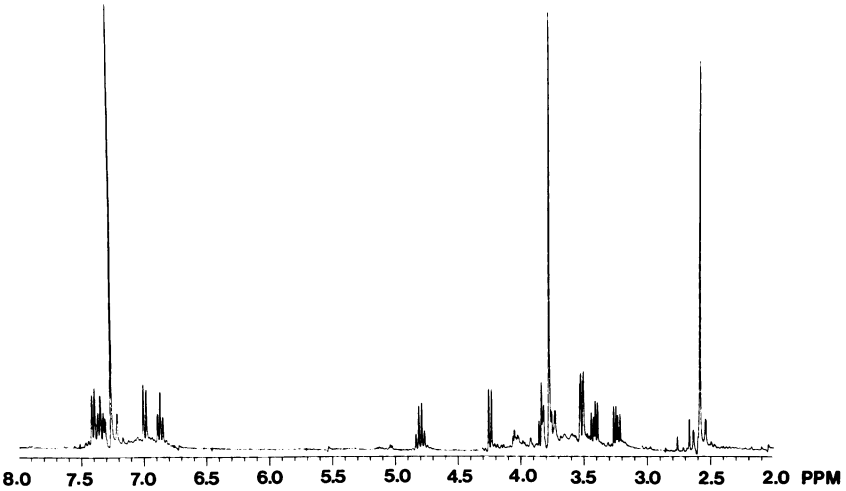

FIG. 4. ^1H NMR spectrum of neopyochelin methyl ester in $CDCl_3$. The peak at 7.27 ppm is due to $CHCl_3$ contamination of the solvent. The spectrum was obtained on a Nicolet NTC-360 spectrometer.

in the production of both pyochelin and pyoverdin (2). IA1 was chosen for this study to ensure that growth promotion would be dependent upon the addition of compounds acting as siderophores and not upon siderophores produced during the assay.

IA1 was added to assay tubes containing GMM-transferrin medium with and without natural and synthetic pyochelin, and growth was measured as a function of A_{600} (Fig. 5). The poor growth capability of IA1 was demonstrated by the slow onset of detectable growth. The growth-promoting ability of both compounds was obvious when growth of IA1 with the compounds was compared with that of IA1 alone. Bacteria

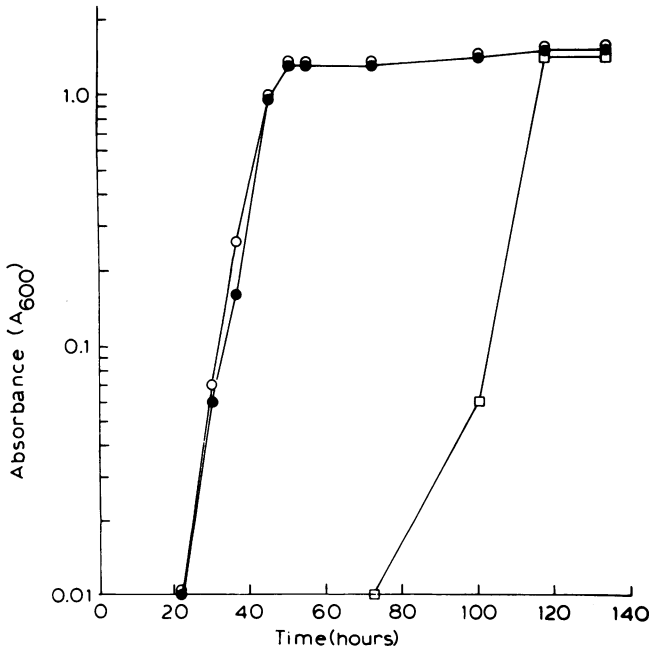

FIG. 5. Growth of strain IA1 in the presence of natural and synthetic pyochelin. Assays contained natural pyochelin at 3.75 μg/ml (○), synthetic pyochelin at 3.75 μg/ml (●), or no additions (control) (□). Tubes containing GMM-transferrin medium were inoculated with 5×10^3 bacteria per ml and incubated at 37°C.

in tubes receiving pyochelin reached the stationary phase approximately 70 h before those in the control tube. There were no significant differences between the growth curves generated by cultures receiving natural and synthetic pyochelin. Identical activities of natural and synthetic pyochelin were also found in iron transport assays. Additions of these compounds to transport assays containing strain PAO1 at $^{55}FeCl_3$ demonstrated that the natural and synthetic pyochelins at R_f 0.35 yielded 3.75 and 3.41 pmol of ^{55}Fe accumulated per mg of bacteria per min, respectively. Similar quantities were accumulated in the presence of natural and synthetic pyochelin II and also when strain IA1 was used in place of strain PAO1. These values were significant in comparison to the 0.62 pmol of ^{55}Fe taken up in the absence of added siderophere. Neopyochelin also stimulated transport, as it yielded 2.19 pmol of ^{55}Fe transported per mg of bacteria per min.

DISCUSSION

Bacterial siderophores have often been the target of attempted syntheses due to their biological activities and interactions with iron. Among those synthesized are aerobactin, agrobactin, arthrobactin, enterobactin, mycobactin, parabactin, and schizokinen (3).

The synthesis of pyochelin presented here consisted of three steps from salicylonitrile, L-cysteine, and L-*N*-methylcysteine. The most difficult step in the synthesis was the formation of aldehyde 4. Several methods for aldehyde synthesis (8, 20, 23, 29, 35) were unsuccessful; the chromium- and aluminum-containing reagents used for this purpose may have been inappropriate due to the chelating abilities of the synthetic intermediates. Furthermore, the heterocyclic and phenolic groups of carboxylic acid 3 and alcohol 5 may have interfered with the action of reagents used to generate the aldehyde intermediate. The only reagent we found to be successful in generating aldehyde 4 was thexylborane (9). Although the aldehyde was produced, it was exceedingly labile in our hands. It has previously been reported that *N*-protected α-amino aldehydes are unstable (45), and the inability to generate aldehyde 4 in high yield was the major reason for the low overall yield of pyochelin. Condensation of the unfractionated thexylborane reduction mixture with L-*N*-methylcysteine yielded pyochelins I and II

and neopyochelin. The overall yield of synthetic pyochelin obtained in this study, approximately 5%, is certainly below the yield achieved in other siderophore syntheses (3). However, the development of synthetic reagents capable of generating aldehyde 4 in high yield would make pyochelin one of the siderophores most easily synthesized.

FAB mass spectra, ^{1}H NMR, optical spectra, HPLC, and TLC data, along with the chemical behavior of the synthetic pyochelins I and II, all indicated identity with natural pyochelin. Neopyochelin is closely related to pyochelin and is most likely stereoisomeric at one or more of the three assymetric centers present in pyochelin. The unique behavior of pyochelin, demonstrating two interconvertible species, remains unexplained at this time. The ^{1}H NMR spectra would indicate that some conformational or isomeric difference involving the 3-methylthiazolidine ring results in the two forms of pyochelin. The use of CPK space-filling models of the pyochelin structure indicates difficulty in rotation about the C-4′–C-2′′ bond due to the large atomic radius of the thiazolidine sulfur atom. There is also the possibility of rapid epimerization about the C-2′′ atom due to opening and closing of the thiazolidine ring via Schiff base intermediates (33), but this must be viewed as unlikely due to the high stability of *N*-methyl-4-thiazolidinecarboxylic acids (25). Additional investigations into the stereochemistry and absolute structure of pyochelin are currently under way.

Our observation of pyochelins I and II has been supported by a recent investigation into the structure of a pyochelin-zinc complex (18) which also reported two forms of pyochelin. Despite extensive spectral analyses, structural differences between the two forms were not elucidated (18). A similar phenomenon of a single siderophore existing as two forms has been reported previously in the case of agrobactin (32, 34). ^{1}H NMR spectra of agrobactin demonstrate a splitting of peaks similar to that observed in the ^{1}H NMR spectra of pyochelin. The split peaks are similarly associated with the heterocyclic rings in the two siderophores. A *cis-trans* isomerization with respect to the 4′-oxazoline amide group in agrobactin was proposed as the basis of this two-form phenomenon (34). However, a report on the crystal structure of agrobactin indicated that one form of agrobactin was observed (21). This report further suggested that the proposed *cis-trans* isomerization was unlikely due to the large energy of activation required. The two forms of pyochelin and agrobactin may be due to structural aspects of the 2-oxazoline/thiazoline rings which have yet to be identified.

Pyochelin is unique and is one of the smallest known siderophores; however, it shares structural features with other siderophores. Pyochelin contains a 2-thiazoline ring which is similar to the 2-oxazoline systems present in other siderophores such as agrobactin, mycobactin, parabactin, and vibriobactin (22, 30). Agrobactin, mycobactin, parabactin, and vibriobactin all possess an *o*-hydroxyphenyl derivative bonded to the C-2 position of the 2-oxazoline ring. Pyochelin possesses a similar structural feature, an *o*-hydroxyphenyl group bonded to the C-2 position of the 2-thiazoline ring. It is also interesting that several siderophores contain hydroxylated benzoate derivatives amide bonded to α-amino groups of the similar amino acids threonine and cysteine. The amide bonds are "hidden" in these siderophores by the 2-oxazoline and 2-thiazoline ring systems. Agrobactin, mycobactin, parabactin, and vibriobactin all possess threonine, while pyochelin contains cysteine. A recent report on the characterization of anguibactin, a siderophore of *V. anguillarum*, indicated strong similarities between this siderophore and pyochelin (1). The structural similarities of all these siderophores invite speculation about the relatedness of the genes encoding their respective biosynthesis pathways.

The biological activity of pyochelin was demonstrated by using the siderophore-deficient mutant IA1 in growth assays and PAO1 in transport assays. The similarity of the growth curves in Fig. 5 and the small amounts of natural and synthetic pyochelin required to stimulate growth in the assays indicate similar activities between the natural and synthetic products. No differences between the biological activities of pyochelins I and II could be determined, possibly because of the rapid interconversion of the two species of pyochelin. The biological activity of the synthetic product indicated that it possesses the necessary stereochemistry to interact with the pyochelin-binding protein of *P. aeruginosa* (42, 43). However, the finding that neopyochelin has residual biological activity when compared with pyochelin raises questions about the ability of the pyochelin transport system to discriminate between pyochelin and structurally similar compounds. We are currently synthesizing analogs of pyochelin in an attempt to determine the range of molecular structures recognized by the pyochelin transport system.

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