that of the *trans*-5,6-dihydrodiol, the signal for H_5 in which is shifted downfield 0.7 ppm relative to H_6 due largely to edge-deshielding in the bay region. Methylation of either carbinol results in an expected upfield shift (0.2–0.4 ppm) as confirmed by use of [^{13}C]methyl iodide (see Table II). Signals for the O-methyl groups in the di- and monomethyl ethers clustered tightly in the region of 3.3–3.4 ppm. The 5-methyl ether was readily resolved by analytical, chiral HPLC (5.3% ethanol and 2.7% acetonitrile in hexane at 3.0 mL/min, see arene oxide separations described later) into its early eluting (7.12 min) and late eluting (7.59 min) enantiomers which had mirror-image CD spectra. The spectrum of the early eluting enantiomer on the chiral column was identical in shape and magnitude with that of the more polar (5*R*,6*R*)-*trans*-5-methyl ether on the Zorbax SIL column obtained by addition of methoxide to (-)-chrysene 5(*S*),6(*R*)-oxide (see Figure 1B, Scheme III).

In a further attempt to exclude the possibility that either of the methoxide adducts from chrysene 5,6-oxide had formed by cis addition, chrysene *cis*-5,6-dihydrodiol was methylated as above. In this case, more severe decomposition of the dihydrodiol occurred, and only a small amount (<5% yield) of a single monomethyl ether could be isolated. Comparison of NMR spectra (Table II) with that of the *cis*-5,6-dihydrodiol (4.2 min on HPLC) indicated it was the *cis*-6-methyl ether (2.7 min). It eluted earlier and was well separated from the trans methyl ethers which had retention times identical with the methoxide adducts formed from chrysene 5,6-oxide. The NMR signal (Table II) for the OCH_3 group in the *cis*-6-methyl ether (δ 3.83) is shifted downfield ~0.4 ppm relative to the trans methyl ethers due to edge-deshielding in the bay region. A similar downfield shift for the acetyl group of the *cis*-6-acetate (3.6 min, longer retained isomer due to more polar, axial 5-hydroxyl group) compared to the *cis*-5-acetate (2.7 min) was also observed. Partial acetylation of the *cis*-5,6-dihydrodiol (limited acetic anhydride in pyridine) favored (4:1) esterification of the equatorial 6-hydroxyl group as apparently did methylation. Although the *cis*-5-methyl ether was not isolated and identified among the methylation products, it should elute earlier on HPLC than the *cis*-6-methyl ether which has a more polar, axial hydroxyl group. Thus, the *cis*-5-methyl ether (as well as the *cis*-6-methyl ether) can be excluded as a methoxide adduct of chrysene 5,6-oxide. Of the four possible monomethyl ethers of the *cis*- and *trans*-5,6-dihydrodiols from 7,12-dimethylbenz[*a*]anthracene, the comparable *cis*-6-methyl ether (axial methyl ether and equatorial hydroxyl) was the first to elute under similar HPLC conditions.[18]

Arene Oxide Separations by Chiral HPLC. Analytical separations of enantiomeric arene oxides were achieved on a covalently bonded dinitrobenzoyl-(*R*)-phenylglycine column (0.46 × 25 cm, Regis Chemical Co.). In all cases the elution solvent (3.0 mL/min) was hexane containing 0.33% ethanol and 0.17% acetonitrile: chrysene oxides, (-)-5*S*,6*R* at 11.18 min and (+)-5*R*,6*S* at 11.57 min; benzo[*c*]phenanthrene oxides, (+)-5*S*,6*R* at 7.46 min and (-)-5*R*,6*S* at 7.83 min; 7,12-dimethylbenz[*a*]anthracene oxides, (+)-5*S*,6*R* at 9.87 min and (-)-5*R*,6*S* at 10.20 min. We attribute no particular significance to the fact that the 5*S*,6*R* enantiomers elute first. It should be noted, however, that the order of arene oxide enantiomer elution may reverse when dinitrobenzoyl-(*S*)-leucine columns are utilized.[24]

Synthesis of a Parabactin Photoaffinity Label

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The synthesis of parabactin azide, the first catecholamide siderophore photoaffinity label, is described. Its preparation is predicated on the generation of ethyl 4-azido-2-hydroxybenzimidate. This imidate is coupled with N^1,N^8-bis(2,3-dihydroxybenzoyl)-N^4-threonylspermidine hydrobromide to produce parabactin azide. The photoaffinity label is shown to have the same biological activity as parabactin in stimulating the growth of *Paracoccus denitrificans* when the microorganism is cultured under low iron conditions. Furthermore, parabactin azide is shown to form a gallium(III) complex identical with the parabactin gallium(III) complex as determined by 300-MHz ^{1}H NMR. Finally, ethyl 2-hydroxy-4-nitrobenzimidate hydrochloride, an intermediate in the synthesis of ethyl 4-azido-2-hydroxybenzimidate, is used in the preparation of aminoparabactin which is subsequently attached to an activated sepharose resin to produce a parabactin affinity column.

Introduction

The fact that iron plays a critical role in the growth of microorganisms is certainly well established.[1-3] An "excess" of the metal causes a fulminant growth of many microorganisms while iron deprivation can substantially slow or even halt growth.[4-6] Because of the poor solubility of iron in aqueous solution, (K_{sp} = 10^{-38} M)[7] at the pH which most bacteria grow, microbes have developed a rather sophisticated apparatus for solubilizing and incorporating the metal.[8-10] They produce low-molecular-weight virtually ferric ion specific ligands which tightly chelate iron and assist in its transport into the cell. These ligands are typically either hydroxamates[11] as exemplified by desferrioxamine[12] or catecholamides[13] as exemplified by enterobactin[14] and the linear catecholamide, parabactin.[15] Both desferrioxamine and parabactin form extremely tight complexes with iron, with formation constants, K_f, of 10^{31} M^{-1} and 10^{48} M^{-1} respectively.[16,17] Two

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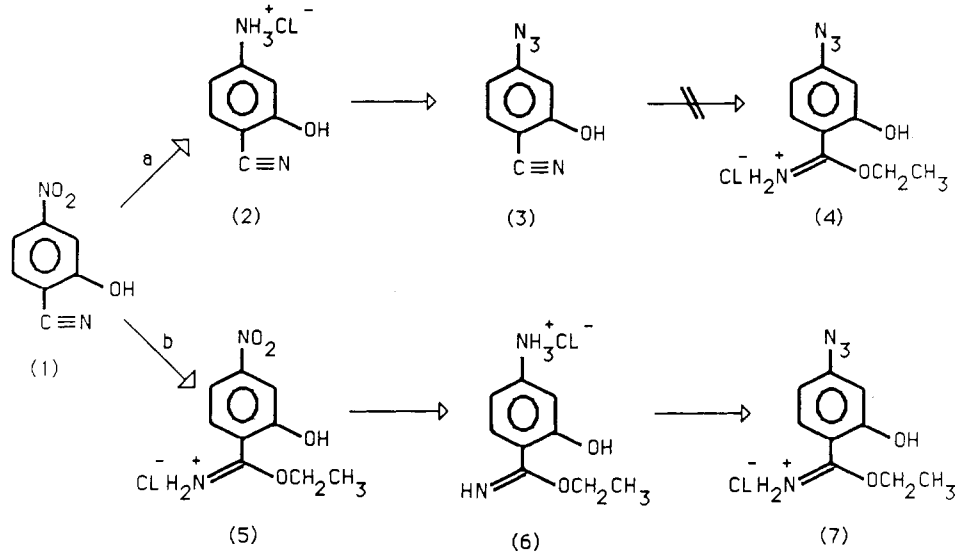
Scheme I



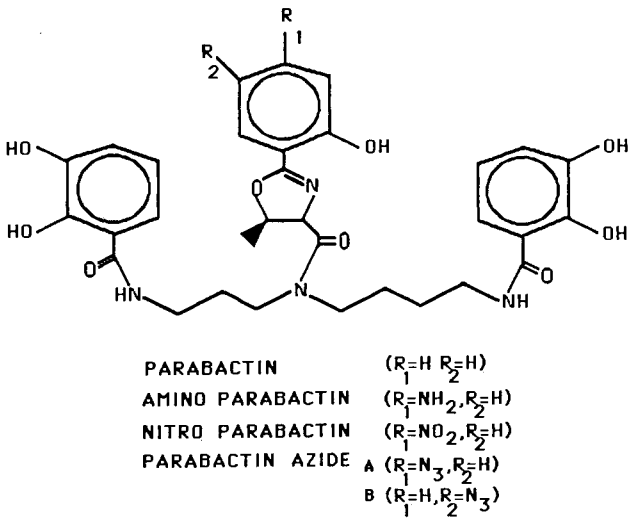


Figure 1.

of the questions of current interest in this field relate to how and at which cellular level, e.g., membrane, the organism removes the metal from such a tight complex for further utilization.[18-22]

In a previous publication we determined that the microorganism *Paracoccus denitrificans* removes the iron from its siderophore, parabactin (Figure 1), at the membrane level.[17] At this point we decided to try to isolate the membrane receptor responsible for siderophore binding and iron removal. It is clear that a parabactin photoaffinity label and a parabactin affinity column represent excellent tools for receptor isolation. Neither polyamine catecholamide siderophore photoaffinity labels nor affinity columns had previously been explored.

Any modifications made on parabactin of course must leave parabactin unaltered in terms of its ability to both bind iron and support microbial growth under limited iron access conditions. In this paper we describe the synthesis of both a parabactin photoaffinity compound and an affinity chromatography column. Furthermore, we demonstrate that the photoaffinity label binds gallium(III) just as parabactin does and that it stimulates the growth of *Paracoccus denitrificans* as effectively as parabactin.

Results and Discussion

We chose to consider parabactin azides A and B (Figure 1) as our photoaffinity system because (1) aryl azides, the photochemical precursors to aryl nitrenes, have been successfully utilized in the past to irreversibly label a variety of biological systems;[23,24] (2) in these systems the photoaffinity functionality, the azide, is far enough removed from the ligand's chelating groups so as not to alter their metal binding properties; and (3) as the last step of the synthesis involves fixing the central aromatic ring to N^1,N^8-bis(2,3-dihydroxybenzoyl)-N^4-threonylspermidine (I),[25] the photoaffinity label does not have to sustain unwanted reaction conditions involved in the synthesis of I.

Synthesis of Parabactin Azide and Aminoparabactin. There was a precedent for synthesizing aryl azide imidates in high yield using the commercially available 4-aminobenzonitrile. In a model experiment, we synthesized ethyl 4-azidobenzimidate[26] and successfully condensed it with the parabactin precursor (I) to yield the corresponding parabactin analogue. The stability of the azide imidate to the condensation reaction conditions and its ease of synthesis did not suggest there would be any problems in generating ethyl 4-azido-2-hydroxybenzimidate. However, this was not the case.

As the last step in the synthesis of parabactin involves coupling of ethyl 2-hydroxybenzimidate with I, it is clear that this imidate represents the optimum target for fixation of the azide functionality. The standard protocol for the synthesis of aryl azide imidates involves conversion of an aryl amino nitrile to the corresponding azide.[26,27] The amine to azide step generally involves reaction of the amine in concentrated aqueous acid with $NaNO_2$ followed by addition of NaN_3. The intermediate diazonium compound is generally not isolated. We attempted this sequence with 2-hydroxy-4-nitrobenzonitrile (1) (Scheme I). Reduction

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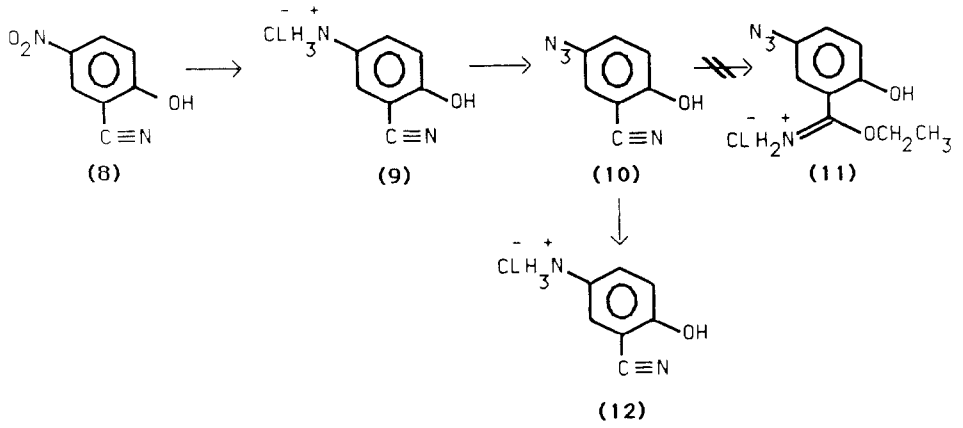
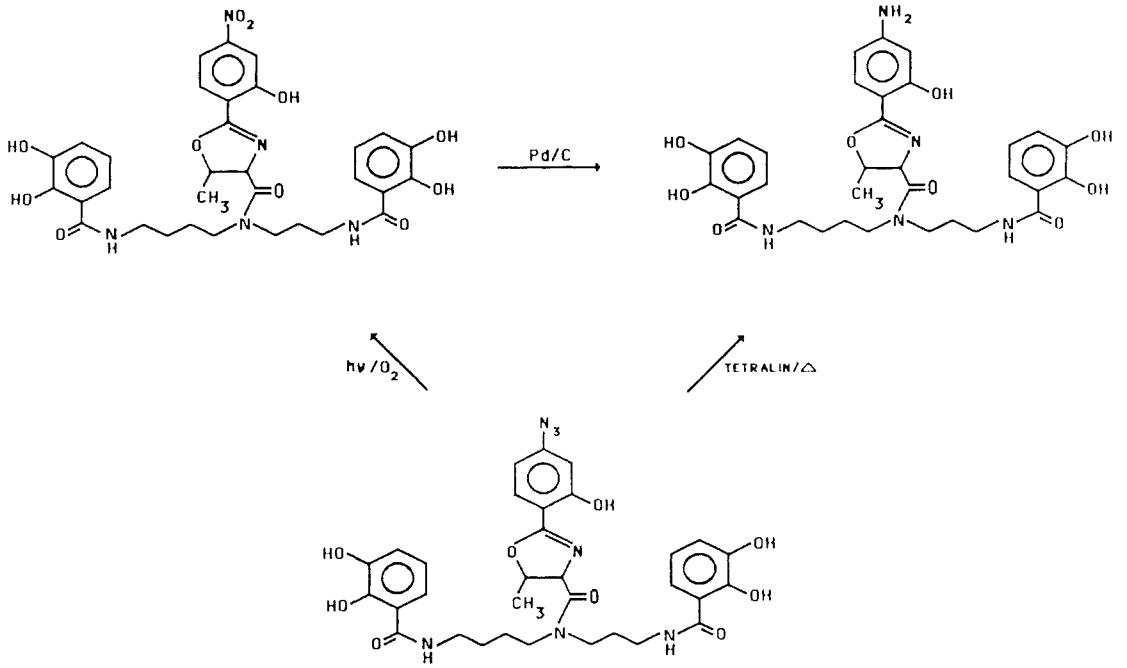
Scheme II



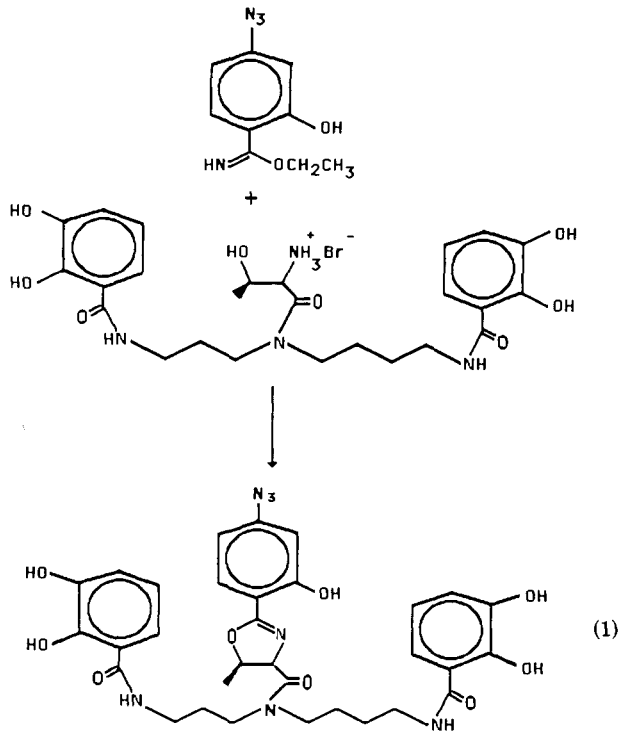
Scheme III



of 1 to the corresponding amine (2) utilizing iron and hydrochloric acid proceeded in 50% yield. Conversion of the amino nitrile (2) to the azide (3) by treatment with aqueous $NaNO_2$ and concentrated HCl followed by exposure of the diazonium compound to NaN_3 proceeded in 80% yield. However, we were unable to convert 3 to the imidate (4) by treatment with anhydrous ethanolic hydrogen chloride even in a variety of solvents. A similar sequence was effected on 2-hydroxy-5-nitrobenzonitrile (8) (Scheme II). Again the amino compound (9) was obtained in 50% yield and conversion of this to the azide (10) proceeded in 80% yield. However, attempted conversion of this azide to the imidate (11) by treatment with ethanolic hydrochloric acid, again in a variety of solvents resulted in the amine (12). This collapse of the azide (10) to the amine under acid conditions can probably be attributed to the para relationship of the azide functionality to the phenolic hydroxyl. This instability suggested the azide imidate (11) (Scheme II) might not be a suitable intermediate in the synthesis of parabactin azide. The alternative path b (Scheme I) was considered. The nitrile (1) was converted to the nitroimidate (5) in 80% yield by treatment with anhydrous ethanolic hydrochloric acid. We next determined that the nitro group in imidate (5) could be reduced to the corresponding amine quantitatively by hydrogenation over palladium. The imidate was left intact and the compound fortunately did not polymerize. Next the imidate (6) was taken to the azide (7) in 30% yield by treatment with isoamyl nitrite and acetic acid in ethyl acetate followed by exposure to NaN_3. The low yield is probably associated with the hydrochloride's poor solubility in ethyl acetate. Finally, ethyl 4-azido-2-hydroxybenzimidate (7) (Scheme I) was reacted with N^1,N^8-bis(2,3-dihydroxybenzoyl)-N^4-threonylspermidine hydrobromide in refluxing methanol to produce parabactin azide (eq 1) in 76% isolated yield.

All reactions after production of the amino compounds 2 and 6 (Scheme I) and 9 (Scheme II) were carried out in minimal light. The azide of interest, ethyl 2-hydroxy-4-azidobenzimidate, when run on TLC and exposed even briefly to a UV lamp for identification, quickly turned yellow. Parabactin azide itself must also be handled cautiously with regard to light exposure.

Several lines of evidence were utilized as proof of structure for the azide. It is important to point out that because of the compounds thermal and light sensitivity, elemental analysis was unobtainable. The azide inevitably decomposed in the hands of the analytical companies. A comparison of the 300 MHz ^1H NMR of parabactin and parabactin azide showed the two compounds to be essentially identical, including duplication of signals. However, as expected, the aromatic region was different.



(1)

In order to assign the aromatic ring protons of the azide to specific ^1H NMR signals, we found it practical to compare the 300-MHz ^1H NMR of the parabactin and parabactin azide gallium complexes. In the spectra of the parabactin gallium(III) chelate, the proton meta to the hydroxyl in the central aromatic ring is found to be a triplet centered at 7.21 ppm. However in the spectra of the parabactin azide gallium(III) complex, the signal at 7.21 ppm is absent. These data indicate that the compound at hand is a parabactin derivative, substituted on the central aromatic ring meta to the hydroxyl. The IR (KBr) of parabactin azide showed the characteristic absorption at 2110 cm^{-1} for N_3. However, the signal for a diazonium functionality can also occur at essentially the same wavelength.

Additional proof of structure was obtained from the pyrolysis and photolysis of parabactin azide (Scheme III). The thermal decomposition of parabactin azide was carried out in boiling tetralin at 207 °C. This resulted in a number of decomposition products including aminoparabactin. (The synthesis of which is described below.) This arises from first loss of nitrogen leading to the unstable nitrene, which then abstracts hydrogen from the solvent. The product had an R_f on silica gel identical with synthetic aminoparabactin and the same retention time on HPLC.

The photochemical decomposition of aromatic azides in the presence of oxygen has recently been investigated.[28,29] One of the products, the corresponding nitro derivative, was shown to be formed by the trapping of triplet aryl nitrenes by triplet oxygen. With the successful synthesis of nitroparabactin (see below), the photochemistry of parabactin azide in the presence of oxygen could be evaluated. The initial photolysis experiments were run using dilute solutions (10^{-4} M) of parabactin azide in acetonitrile or benzene under a steady stream of oxygen. Unfortunately, this resulted in the formation of intractable tars and complete decomposition of the azide as detected by HPLC. However, on a change of the solvent to acetone, the azide was extremely stable and after photolysis for 24 h under a stream of oxygen, several products were detected by HPLC. Indeed, a major product had an identical retention time as synthetic nitroparabactin; however, attempts at distinguishing this from parabactin failed. The photodecomposition product was therefore isolated using preparative HPLC. The isolated compound was then subjected to high-field NMR and the aromatic region compared to both synthetic nitroparabactin and parabactin. Utilizing ^1H NMR difference spectroscopy, it was shown that the photodecomposition product was identical with nitroparabactin.

Finally, the most significant proof of structure was obtained using FAB mass spectroscopy. A mass peak at 661 was observed corresponding to the intact azide along with a peak at 635, arising from loss of molecular nitrogen and proton abstraction, corresponding to aminoparabactin. It should be noted that attempts at simple CI and EI mass spectroscopy were unsuccessful in observing a molecular ion. We regard the accumulated evidence as proof of structure for parabactin azide.

The ease of access and stability of the nitro imidate (**5**) (Scheme I) encouraged us to generate aminoparabactin (Figure 1) for use in the production of a parabactin affinity chromatography column. The nitro imidate (**5**) was condensed with I again in refluxing methanol in 85% yield, and the resulting nitro parabactin was reduced to the corresponding amino compound by hydrogenation over palladium (eq 2).

Synthesis of Parabactin Affinity Column. Aminoparabactin was reacted with activated CH-sepharose 4B, an *N*-hydroxysuccinimide-activated acyl Sephadex, in aqueous methanol–HEPES buffer. The reaction was run for 24 h at room temperature and the resulting matrix washed exhaustively with coupling buffer. The washing was continued until the eluant was negative to ferric chloride. A radioactive assay employing $^{59}FeCl_3$ was used to determine the amount of ligand coupled to the resin. Gamma counting of the $^{59}Fe^{3+}$ chelated to the aminoparabactin, which binds in a 1:1 stoichiometry, indicates a 20% coupling efficiency of aminoparabactin with the resin.

^1H NMR Study of Parabactin Azide Gallium Complex. In an earlier study we established that the parabactin gallium complex was largely one isomer Λ cis 3,4.[30] Later evidence from our laboratories showed that indeed the small amount of what we thought to be Λ cis 4,3 was only a contamination.[31] When the 300-MHz ^1H NMR of the gallium(III) parabactin azide complex is compared with that of the parabactin gallium(III) complex, the spectra are essentially identical except for the aromatic ring protons associated with the azide aromatic ring. This, of course, suggests the conformation of both the parabactin azide gallium complex and the parabactin gallium complex are the same.

Microbial Growth Stimulation. The key issue in the design of a parabactin photoaffinity label is that it be recognized by the *Paracoccus denitrificans* parabactin receptor. The simplest way to ascertain whether or not the modified parabactin is utilized by this receptor is simply to measure its ability to stimulate the microorganism's growth in a low iron environment. Consequently, both parabactin and parabactin azide were compared in their ability to stimulate the growth of *Paracoccus denitrificans* in a minimal iron media (Figure 2). As is clear from this figure, both parabactin and parabactin azide stimulate microbial growth to essentially the same degree.

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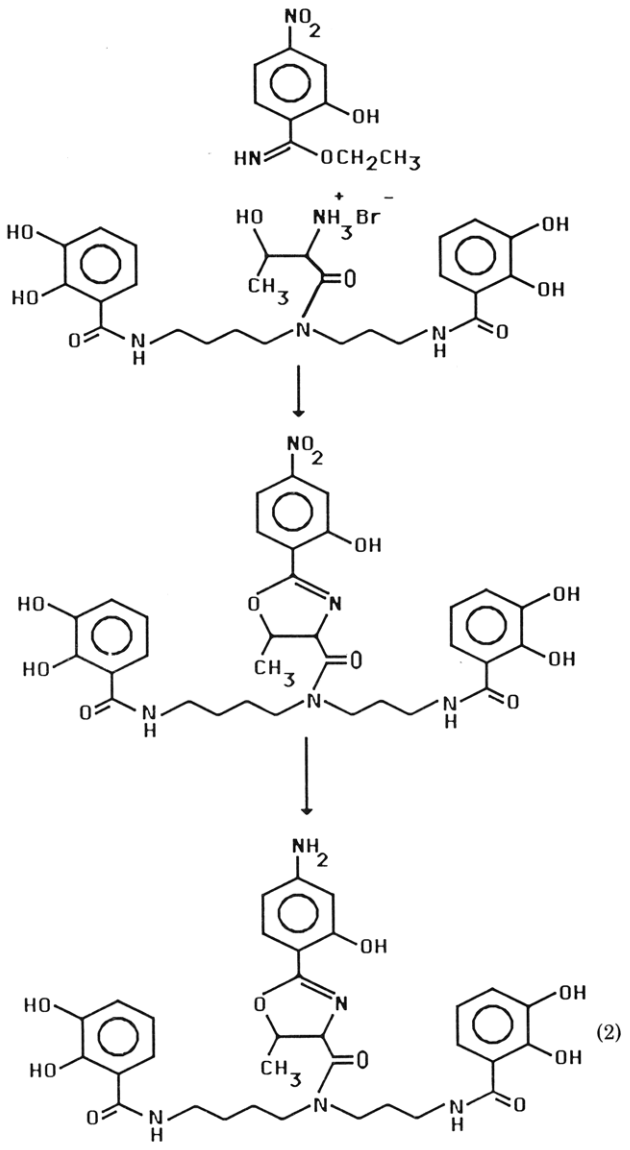

(2)

We recognize that further evidence is required in that the parabactin azide may utilize another transport apparatus to provide iron; however, this seems unlikely. This is currently under study.

Experimental Section

All reagents, with the exception of gallium(III) nitrate nonahydrate (Alfa), were purchased from Aldrich Chemical Co. and were used without further purification. Sodium sulfate was employed as a drying agent. Melting points are uncorrected. Activated CH-Sepharose 4B and Sephadex LH-20 were purchased from Pharmacia Fine Chemicals. Proton NMR spectra were recorded on a Nicolet NT-300, 300-MHz instrument or a Varian EM-390 90-MHz instrument. IR spectra were recorded on a Beckman Acculab 1 spectrophotometer. Elemental analyses were performed by Atlantic Microlabs, Atlanta, GA, or Galbraith Laboratories, Knoxville, TN.

Growth Studies. The effects of parabactin and parabactin azide on the growth of *Paracoccus denitrificans* in iron-deficient media were determined. Iron-free ligands (2 μM) were added in methanol to sterile Klett flasks, evaporated to dryness under nitrogen which had been passed through a 0.22 μm millipore filter and redissolved in sterile growth media. The media used was as previously described.[17] Growth rates were determined by monitoring Klett readings vs. time. Standard curves were generated correlating Klett units with colony forming units (CFU) using serial plating techniques. Growth studies were reported as CFUs vs. time.

HPLC was performed on a Rainin Rabbit 4P/HPX System utilizing a C-18 reverse-phase preparatory column. The mobile phase contained 80% acetonitrile/20% H_2O pH = 3.

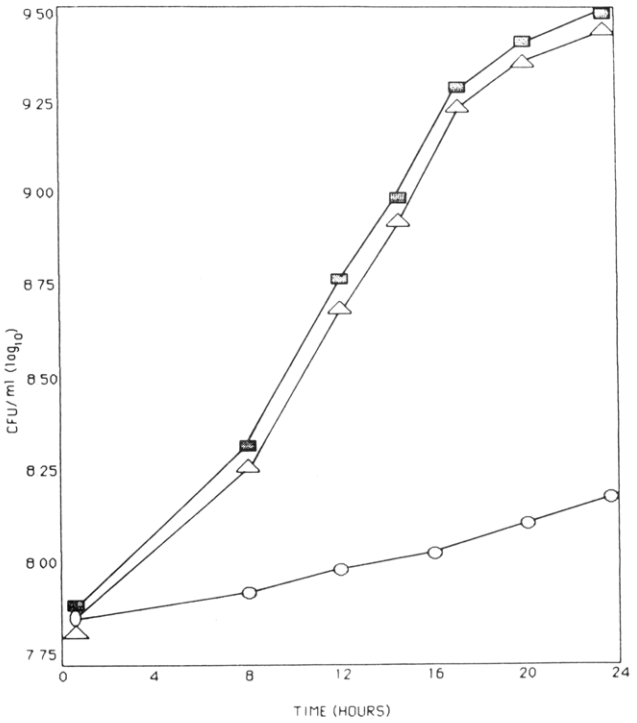

Figure 2. Growth rate of *P. denitrificans* in the presence of parabactin (■) (2μM), parabactin azide (△) (2 μM), or controls (○).

2-Hydroxy-4-nitrobenzonitrile (1) was prepared from 2,4-dinitrophenylacetic acid as previously described.[32]

4-Amino-2-hydroxybenzonitrile Hydrochloride (2). Compound **1** (2.10 g, 12.80 mmol) was dissolved in 200 mL of ethanol containing 5 mL of concentrated HCl. The reaction mixture was heated to reflux, at which point 3.0 g of iron powder was added in portions over a period of 3 h. Upon cooling the ethanol was removed in vacuo and the residue was taken up in water (150 mL) and extracted into ether (5 × 50 mL), dried, and concentrated to afford the crude product. Further purification was effected by chromatography on silica gel, eluting with 10% MeOH/$CHCl_3$ to afford 1.10 g (50%) of product: mp 184–185 °C (lit. 186 °C).[33]

4-Azido-2-hydroxybenzonitrile (3). Compound **2** (0.90 g, 5.28 mmol) was suspended in 10 mL of ice-cold concentrated HCl. To this cooled solution $NaNO_2$ (0.65 g, 9.4 mmol) in 5 mL of water was added slowly with stirring over a period of 1 h. This was followed by addition of NaN_3 (0.60 g, 9.2 mmol) in 5 mL of water. After stirring for an additional 1 h, the cooled reaction mixture was filtered and the product washed with ice-cold water to yield 0.72 g of **3** in 85% yield. ^1H NMR (10:1 $CDCl_3/Me_2SO$-d_6) δ 6.58 (m, 2 H), 7.44 (d, 1 H); IR (KBr), 3200 (br), 2240 (s), 2110 (s), 1600 (s), 1480 (s), 1285 (s); mp 148–149 °C.

2-Hydroxy-5-nitrobenzonitrile (8) was prepared utilizing published procedures.[34]

5-Amino-2-hydroxybenzonitrile hydrochloride (9) was prepared and purified analogously as **2**; mp 160–162 °C (lit. 162 °C).[35]

5-Azido-2-hydroxybenzonitrile (10) was prepared and purified as described for compound **3**: 82% yield; ^1H NMR (10:1 $CDCl_3/Me_2SO$-d_6) δ 7.03 (m, 2 H), 7.11 (d, 1 H); IR (KBr), 3200 (br), 2230 (s), 2100 (s), 1510 (s), 1260 (s).

Ethyl 2-Hydroxy-4-nitrobenzimidate Hydrochloride (5). Vacuum-dried 2-hydroxy-4-nitrobenzonitrile (**1**) (0.71 g, 4.33 mmol) was suspended in 10 mL of dry absolute ethanol, and dry

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HCl was bubbled through the cooled mixture for 1 h. The reaction mixture was allowed to sit for 48 h at room temperature, at which point the product was recovered by filtration and dried under vacuum to afford 0.90 g (84%) of product: ^{1}H NMR (DMSO-d_6) δ 1.40–1.52 (t, 3 H), 4.54–4.68 (q, 2 H), 7.70–7.75 (d, 1 H), 7.91–8.0 (d, 1 H), 8.04–8.09 (s, 1 H); mp 196–198 °C dec. Anal. Calcd for $C_9H_{11}N_2O_4Cl$: C, 43.83; H, 4.49; N, 11.36. Found: C, 43.75; H, 4.52; N, 11.32.

Ethyl 4-Amino-2-hydroxybenzimidate Hydrochloride (6). To a solution of **5** (2.10 g, 8.51 mmol) in dry absolute ethanol (100 mL) was added 10% palladium on carbom (0.99 g). The solution was stirred under a hydrogen atmosphere for 7 h. The solution was then filtered through a medium (10–15) frit and washed with ethanol. Solvent removal, followed by purification by chromatography on silica gel, eluting with 10% MeOH/$CHCl_3$ afforded product (**6**) in near quantitative yield: ^{1}H NMR (7:3 $CDCl_3$:Me_2SO-d_6) δ 1.54 (t, 3 H), 4.58 (q, 2 H), 6.03 (br, 2 H), 6.28 (d, 1 H), 6.42 (s, 1 H), 7.58 (d, 1 H), 9.41–10.70 (m, 3 H); mp 155–156 °C.

Anal. Calcd for $C_9H_{13}N_2O_2Cl$: C, 49.89; H, 6.05; N, 12.93. Found: C, 49.82; H, 6.08; N, 12.90.

Ethyl 4-Azido-2-hydroxybenzimidate Hydrochloride (7). To a suspension of **6** (0.96 g, 4.43 mmol) in 100 mL of ethyl acetate was added freshly distilled isoamyl nitrite (714 μL, 5.32 mmol) and 75 μL of acetic acid. The cooled mixture was stirred for 3 h, at which time (0.35 g, 5.38 mmol) of NaN_3 was added. After an additional 3 h the resulting reaction mixture was filtered and washed with ethyl acetate. Solvent removal, followed by purification by chromatography on silica gel eluting with 10% MeOH/$CHCl_3$, afforded (0.25 g) product: ^{1}H NMR (Me_2SO-d_6) δ 1.49 (t, 3 H), 4.13 (q, 2 H), 6.48 (d, 1 H), 6.64 (s, 1 H), 7.75 (d, 1 H); IR (KBr) 3020 (br), 2090 (s), 1595 (br), 1510 (br), 1260 (br) 1085 (br).

Nitroparabactin (8). Vacuum-dried ethyl 2-hydroxy-4-nitrobenzimidate (**5**) (0.25 g, 1.19 mmol) and N^1,N^8-bis(2,3-dihydroxybenzoyl)-N^4-(L-threonyl)spermidine hydrobromide[25] (I) (0.59 g, 0.98 mmol) were heated at reflux in dry, degassed methanol (50 mL) for 20 h. The cooled solution was concentrated and dissolved in ethanol and then dry packed on Sephadex LH-20. Column chromatography on LH-20, eluting with 20% EtOH/benzene afforded 0.55 g (85%) product: ^{1}H NMR (10:1 $CDCl_3$/Me_2SO-d_6) δ 1.35–1.51 (2 d, 3 H), 1.51–2.01 (m, 6 H), 3.18–3.72 (m, 8 H), 4.58–4.63 (2 d, 1 H), 5.31–5.48 (m, 1 H), 6.50–6.65 (m, 2 H), 6.81–6.91 (m, 2 H), 7.01–7.15 (m, 2 H), 7.58–7.77 (m, 3 H), 7.81–8.15 (m, 2 H); mass spectrum, m/e 666 (M + 1).

Anal. Calcd for $C_{32}H_{35}N_5O_{11}\cdot 0.5\ H_2O$: C, 56.97; H, 5.38; N, 10.38. Found: C, 57.02; H, 5.38; N, 10.32.

Aminoparabactin (9). To a solution of **8** (0.54 g, 0.80 mmol) in dry absolute ethanol (75 mL), 10% palladium on carbon (0.24 g) was added. The solution was stirred under a hydrogen atmosphere for 6 h. The suspension was then filtered through a medium (10–15) frit and washed with ethanol. Concentration of solvent followed by purification on Sephadex LH-20, eluting with 20% EtOH in benzene, afforded 0.45 g (89%) of product: ^{1}H NMR (10:1 $CDCl_3$/Me_2SO-d_6) δ 1.35–1.51 (2 d, 3 H), 1.55–2.10 (m, 6 H), 3.20–3.71 (m, 8 H), 4.47–4.66 (2 d, 1 H), 4.70–5.01 (m, 2 H), 5.27–5.43 (m, 1 H), 6.13–6.30 (m, 2 H), 6.60–6.79 (m, 2 H), 6.92–7.05 (m, 2 H), 7.16–7.31 (m, 2 H), 7.33–7.46 (m, 1 H), 8.05–8.41 (m, 4 H); mass spectrum, m/e 636 (M + 1).

Anal. Calcd for $C_{32}H_{37}N_5O_9$: C, 60.46; H, 5.87; N, 11.02. Found: C, 60.45; H, 5.83; N, 11.05.

Parabactin Azide (10). Vacuum-dried ethyl 4-azido-2-hydroxybenzimidate (**7**) (0.13 g, 0.63 mmol) and N^1,N^8-bis(2,3-dihydroxybenzoyl)-N^4-(L-threonyl)spermidine hydrobromide (I) (0.30 g, 0.50 mmol) were heated in dry degassed refluxing methanol (50 mL) for 20 h. The solution was concentrated and dissolved in ethanol and then dry packed on Sephadex LH-20. Column chromatography on LH-20, eluting with 15% EtOH in benzene, afforded 0.25 g (76%) of product: ^{1}H NMR (10:1 $CDCl_3$/Me_2SO-d_6) δ 1.41–1.56 (2 d, 3 H), 1.55–2.10 (m, 6 H), 3.21–3.71 (m, 8 H), 4.60–4.71 (2 d, 1 H), 5.35–5.49 (m, 1 H), 6.52–6.73 (m, 4 H), 6.96 (d, 2 H), 7.17 (t, 2 H), 7.63 (t, 1 H), 8.02–8.26 (m, 2 H); IR (KBr) 3350 (br), 2110 (s), 1640 (s), 1265 (s) cm^{-1}; mass spectrum, m/e 661 (M + 1).

Parabactin-Coupled Resin. Dry activated CH-Sepharose 4B resin (Pharmacia Fine Chemicals, Uppsala, Sweden) was swelled in 10^{-3} M HCl (50 mL) for 30 min. The swelled resin was rinsed in 0.1 M HEPES buffer pH 7.5.

A solution of aminoparabactin (67 mg) dissolved in coupling buffer containing CH_3OH (4 mL) and 0.1 M HEPES buffer pH 7.2 (2 mL) was added to 3 mL of the swelled CH-sepharose 4B resin in a sealed glass tube under an argon atmosphere. The tubes were rotated at 12 rpm for 24 h. The resin was then filtered and exhaustively washed with coupling buffer. Unreacted *N*-hydroxysuccinimide ester groups on the resin were then blocked by addition of 1 M ethanolamine hydrochloride (0.1 mL/mL gel) pH 8 for 30 min. The resin was washed with 3 cycles of alternative 0.1 M TRIS–HCl buffer pH 8 (50 mL) and 0.1 M sodium acetate buffer pH 4 (50 mL). A control resin was subjected to identical coupling conditions as above; however parabactin was added in place of aminoparabactin.

To a dilute solution of methanolic $FeCl_3$ (1 mg/mL) was added 250 μL of $^{59}FeCl_3$ (100 000 cpm/100 μL). To 1.0 mL of resin was added 500 μL of the hot iron solution, and the mixture was rotated for 12 h. The resulting purple resin was first washed with coupling buffer (3 × 3 mL) and finally suspended in a 10 μM EDTA solution (3 mL) and rotated for 30 min. The EDTA washings were continued until counts on the control resin were negligible.

In order to determine coupling efficiency, the amount of iron bound to the aminoparabactin coupled resin was measured. Sample radiation of the resin was counted with an automatic gamma counter (LKB-Wilac Ria Gamma 1274, Wallac Oy, Finland) and indicated 20% coupling efficiency.

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