SYNTHESIS, SOLUTION CONFORMATION, AND MICROBIOLOGICAL PROPERTIES OF SPERMIDINE CATECHOLAMIDE SIDEROPHORES

BY

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DEDICATION

The patience, understanding, and continual encouragement from my parents, Mr. and Mrs. Jerrold J. Kline, in large measure account for this moment in my career. It is to them that I proudly dedicate this dissertation.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENTS</th>
<th>iii</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>vi</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>I  INTRODUCTION AND BACKGROUND</td>
<td>1</td>
</tr>
<tr>
<td>Iron in Man</td>
<td>1</td>
</tr>
<tr>
<td>Iron in Microorganisms</td>
<td>3</td>
</tr>
<tr>
<td>Iron Overload in Man</td>
<td>7</td>
</tr>
<tr>
<td>Potential of Catecholamide Iron Chelators as Therapeutic Iron Clearing Devices</td>
<td>12</td>
</tr>
<tr>
<td>II FLEXIBLE SYNTHESIS OF POLYAMINE CATECHOLAMIDES</td>
<td>15</td>
</tr>
<tr>
<td>Introduction</td>
<td>15</td>
</tr>
<tr>
<td>Synthesis</td>
<td>21</td>
</tr>
<tr>
<td>Experimental</td>
<td>28</td>
</tr>
<tr>
<td>III OCTADECENTATE CATECHOLATE LIGANDS AS ACTINIDE CHELATORS</td>
<td>69</td>
</tr>
<tr>
<td>Introduction</td>
<td>69</td>
</tr>
<tr>
<td>Synthesis</td>
<td>74</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>76</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>81</td>
</tr>
<tr>
<td>IV SYNTHESIS AND SOLUTION CONFORMATION OF PARABACTIN AND ITS GALLIUM(III) CHELATE</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>88</td>
</tr>
<tr>
<td>Experimental</td>
<td>90</td>
</tr>
<tr>
<td>Synthesis of Parabactin, Enantioparabactin, and the Homo and Nor Homologs of Parabactin</td>
<td>108</td>
</tr>
<tr>
<td>Results</td>
<td>119</td>
</tr>
<tr>
<td>Discussion</td>
<td>138</td>
</tr>
<tr>
<td>V PARABACTIN-MEDIATED IRON TRANSPORT IN PARACOCCUS DENITRIFICANS</td>
<td>171</td>
</tr>
<tr>
<td>Introduction</td>
<td>171</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>172</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Results</td>
<td>177</td>
</tr>
<tr>
<td>Discussion</td>
<td>196</td>
</tr>
<tr>
<td>VI CONCLUSION</td>
<td>237</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>239</td>
</tr>
<tr>
<td>BIOGRAPHICAL SKETCH</td>
<td>242</td>
</tr>
</tbody>
</table>
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SYNTHESIS, SOLUTION CONFORMATION, AND MICROBIOLOGICAL PROPERTIES OF SPERMIDINE CATECHOLAMIDE SIDEROPHORES

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Major Department: Medicinal Chemistry

The synthesis of a large number of polyamine catecholamides is described. Among the catecholamides synthesized is the microbial iron chelator, parabactin, and many of its analogs.

The synthesis of the first of a novel class of actinide ligands is described as the preliminary work in the development of a whole class of octadentate catecholamides. The results indicate the octadentate catecholamide ligands are excellent sequestering agents of the actinides plutonium and americium.

High field 300 MHz $^1$H NMR spectroscopy was used to examine the solution dynamics of parabactin, and the solution conformation and stereochemistry of the gallium(III) chelate of parabactin. Parabactin was found to exist in solution as a mixture of interconverting conformers. The gallium(III) chelate of parabactin was shown to exist exclusively as the $\Lambda$-cis coordination isomer. Furthermore, there exists two diastereomeric forms of the $\Lambda$-cis parabactin-Ga(III) chelate that differ only in the disposition of the spermidine backbone of the siderophore.
The parabactin-mediated iron uptake system of the soil bacterium *Paracoccus denitrificans* was examined by using labeled ferric parabactin and other analogs including its enantiomer, ferric enantioparabactin. Parabactin functions as an "iron taxi" in the delivery of iron to the microorganism, with release of siderophore-bound iron presumably taking place at the outer cell surface and the ligand never penetrating the organism. The parabactin-mediated iron transport system of *Paracoccus denitrificans* was also shown to be stereospecific. The synthetic enantiomer of parabactin, enantioparabactin, was unable to supply iron to the microorganism.
CHAPTER I
INTRODUCTION AND BACKGROUND

Iron in Man

Iron is an absolutely essential element for almost all forms of living organisms (1-4). The reason for the importance of this metal is related to its versatility as a biological catalyst. In humans, iron is found at the active centers of the biomolecules responsible for oxygen storage and transport, myoglobin and hemoglobin (5). Iron is also present in the heme-containing proteins that function as electron carriers, the cytochromes (6). The metal is also found in a large number of nonheme-containing enzymes including various oxidoreductases, dehydrogenases, and dehydrases (6).

In man, the majority of the body's iron is bound to the porphyrin rings of hemoglobin. This heme-containing protein found in erythrocytes is responsible for picking up oxygen in the lungs and carrying it to tissues via the circulatory system. Approximately 65% of the 4 g of iron present in a 75 Kg man is found as hemoglobin (5). Another 6% of the body's iron exists in the form of another oxygen-binding protein, myoglobin, which acts as a cellular oxygen storage site (5).

About 25% of the iron present in man is bound to the two iron-storage proteins, ferritin and hemosiderin (5). The iron-storage proteins provide the body with a means of storing surplus iron in a nontoxic form that can be released as required. Ferritin is thought to consist of a protein shell surrounding an internal core of ferric...
hydroxyphosphate (7). The exact structure of hemosiderin is unknown, but is thought to be similar to ferritin. It is believed that up to 5000 iron atoms occupy a single molecule of ferritin or hemosiderin (7). Although the iron-binding storage proteins are widely distributed throughout the body in mammals, these proteins are mainly found in the parenchymal cells of the liver, the reticuloendothelial cells in the spleen, and the bone marrow (7).

At physiological pH in an oxidizing environment such as human blood, the predominant oxidation state of iron is the iron(III) state. Since ferric hydroxide is quite insoluble ($K_{sp}=10^{-38}$) (8), it would seem that the body is faced with a difficult task of circulating this essential, but very insoluble metal throughout the system. The problem of low solubility is circumvented by the use of an iron-binding transport protein that shuttles the metal from the iron storage sites to the various tissues. The iron shuttle protein, transferrin, has a molecular weight of about 80,000 daltons, and binds two molecules of ferric ion tightly but reversibly (3,7,9). Transferrin is found in the serum and is also present in the various extracellular fluids in the body (7). Because transferrin passes freely out of the blood vessels into the extravascular fluids, it has the ability to deposit or remove iron from almost any body tissue.

Iron enters the body by absorption from food in the small intestine (7). The heme-iron containing foods such as red meat are the most effective sources of dietary iron (7). However, only a small proportion of the iron in food is absorbed by the intestine; the usual intake of iron is less than 1 mg per day (7). Despite the exceptionally low dietary intake of iron in humans, iron balance is maintained due to the
extremely efficient recycling of the iron already present in the body. Very little of the body's iron store is excreted, less than 1 mg per day; the remaining 4000 mg of iron are simply recycled (7).

The red blood cells contain the vast majority of the iron present in humans (5). Normally, red blood cells survive for about 120 days in the bloodstream. Senescent red blood cells are removed from the circulation by the reticuloendothelial cells in the spleen, and the iron is removed from hemoglobin to be recycled. This iron can then be stored in the reticuloendothelial cells, but is more frequently picked up by transferrin and shuttled to the erythropoietic cells of the bone marrow (5). Transferrin also shuttles iron from the main iron storage site, the liver, and from the intestine where the metal is absorbed, to the bone marrow where the iron is incorporated into the hemoglobin of developing red blood cells.

As can be seen, the body possesses a highly regulated system to deal with the storage and transport of iron. The danger of toxicity resulting from storing large amounts of ferric ion is overcome by storing the metal as an insoluble hydroxyphosphate complex that is buried within a protein sheath, in the form of ferritin. The free metal is too insoluble to freely travel through the blood and other body fluids to the sites where it is needed. This problem is dealt with by having the iron-transport protein transferrin chelate and solubilize ferric ion and transport it to the tissues.

**Iron in Microorganisms**

In addition to being an essential nutrient to humans, iron also plays a major role in the microbiological world. The involvement of iron in the cytochrome and nonheme enzymes in the respiratory chain
of aerobic and facultative anaerobes underscores the importance of this element to energy metabolism in microorganisms (2,3). Iron is also involved in the hydroperoxidases, catalases, and peroxidases in addition to playing a role in DNA biosynthesis (2).

Iron is the second most abundant metal found on the earth's surface, and is only outranked by aluminum, silicon, and oxygen in terms of the total amount of any element found in the earth's crust (2, 10). It has been reported that iron represents about 5% of an average soil sample (2). Considering that this essential metal is so prevalent in the environment, it is a paradox that it is exceedingly difficult for the microbes to acquire iron. The reason for this apparent contradiction is directly related to the extreme insolubility of iron at pH values near neutrality. In aqueous solutions at or above neutrality, ferric ion is most commonly found as its hydroxide. Due to the extreme insolubility of ferric hydroxide, the equilibrium concentration of ferric ion at a pH of 7 is only about $10^{-18}$ M (11). The concentration of iron required to support microbial growth lies in the range of $5 \times 10^{-8}$ to $1 \times 10^{-6}$ M, at least ten powers of ten higher than the concentration of available soluble iron (12).

However, the microbes have dealt with this apparently insurmountable problem very effectively over the millenium. Microorganisms produce and excrete into the external cell environment lowmolecular weight (500 to 1000 daltons), virtually ferric ion specific ligands that sequester exogenous ferric ion and facilitate the transport of the biologically essential metal into the cell. These microbial iron chelators are referred to as siderophores (2,10-13), a term derived from the greek word meaning iron-carrying. Because these compounds are responsible
for iron acquisition, the biosynthesis of the siderophores is strictly controlled by the iron nutritional status of the cell (2). In conditions of low iron availability, which normally prevail in the environment, microorganisms produce and excrete sometimes very large amounts of the siderophores into the surrounding medium. For example, the yeast *Rhodotorula pilimanae* excretes up to 10 g/l of the siderophore rhodotorulic acid (Figure 1) (11).

Two main classes of siderophores exist, the hydroxamates and the catecholamides (2,3,10). To date, a far greater number of hydroxamic acid-containing siderophores have been isolated and characterized than catecholamides. Fungi generally seem to produce siderophores of the hydroxamate variety, while bacteria are known to produce both hydroxamate as well as catecholate siderophores (2). The vast majority of siderophores, both hydroxamate and catecholamide, are hexadentate ligands. The hexadentate siderophores contain three sets of bidentate ligands, each capable of chelating the metal in the form of a five-membered ring (14). These microbial iron chelating agents form very stable octahedral, high-spin iron(III) complexes with formation constants on the order of $10^{30}$ to $10^{50}$ (2,9-11).

The prototype of the catecholamide siderophores is enterobactin, the cyclic trimer of 2,3-dihydroxybenzoyl serine, found in all enteric bacteria studied to date (Figure 1) (15,16). Enterobactin is the strongest ferric ion chelating agent known; at physiological pH, the iron(III)-enterobactin formation constant has been calculated to be $10^{52}$ (17). An interesting way to look at what this unimaginable number means has been recently presented. It has been calculated that at pH 7, the concentration of unchelated hexaaquoiron(III) in an aqueous
Figure 1. Structures of the Siderophores a) Enterobactin, and b) Rhodotorulic acid.
solution which contains 10 μM enterobactin and 1 μM iron is $10^{-30}$ μM (18). This means there would exist only one unchelated ferric ion in every $10^{12}$ liters of water (12).

Much less is known about the catecholamide siderophore parabactin, first isolated by Tait in 1975 from iron-depressed cultures of the soil bacterium Paracoccus denitrificans (Figure 2) (19). Parabactin was the first spermidine catecholamide siderophore isolated. Shortly after the discovery of parabactin, another catecholamide siderophore containing a spermidine backbone, agrobactin, was isolated from the plant pathogen Agrobacterium tumefaciens (20). Tait had originally isolated three iron-binding catechol containing compounds from cultures of Paracoccus denitrificans, which he referred to as "Compounds I, II, and III" (19). Tait correctly determined the structures of compounds I and II to be 2,3-dihydroxybenzoic acid and $N^1,N^8$-bis(2,3-dihydroxybenzoyl)spermidine, respectively. Compound III was reported by Tait to have the structure depicted in Figure 3. However, later it was shown that Tait's original structure elucidation of compound III was incorrect; the siderophore's true structure is shown in Figure 2 (21). Tait was also able to show that the microorganism synthesized compound II from compound I, and compound III from compound II, L-threonine, and salicylic acid (19).

Iron Overload in Man

As mentioned earlier, iron is conserved in the body (7). Less than 1 mg from about 4 g of iron present in humans is lost from the body each day (5,7). This small amount of iron that is lost is due to the sloughing off of the intestinal epithelium rather than from a true excretion mechanism (7). In fact, no major excretory pathway for iron exists in man (22). Since normal individuals absorb only minute quantities of iron from food and water each day, this economical policy of
Figure 2. Structures of the Spermidine Catecholamide Siderophores Parabactin (R=H), and Agrobactin (R=OH).
Figure 3. Structures of the Catechol Compounds Reported by Tait as a) "Compound I", b) "Compound II", and c) "Compound III".
Iron regulation is very beneficial for maintaining adequate amounts of this vital element. However, in certain disease states this conservation of iron proves to be a liability. Abnormal increases in either oral or parenteral iron cannot be balanced by corresponding losses since no major excretory pathway for iron exists in humans. Thus, the inevitable result is an increase in the total body iron content.

In certain syndromes, iron accumulates in the body in such large quantities that tissue damage results (23). These disorders are referred to as the primary and secondary hemochromatoses; they are somewhat rare disorders, and are very often fatal (23). The disorder classified as primary hemochromatosis is due to an inherited defect in iron metabolism resulting in an inappropriately increased mucosal absorption of iron (23). Primary hemochromatosis is easily treated by periodic venesection; usually, about 500 ml of blood are removed weekly (23).

The secondary hemochromatoses pose a substantially more complicated problem (23). The term secondary hemochromatosis is most often used in reference to patients with iron overload secondary to anemia. Patients suffering from severe forms of anemia require repeated blood transfusions for survival (23). As the normal lifespan of a red blood cell is only about 120 days, the iron from the old transfused red cells is eventually removed by the reticuloendothelial cells of the spleen and stored in the parenchymal cells of the liver or in the reticuloendothelium (5,24). Unfortunately, the efficient iron-recycling system of the body ensures that little of the iron introduced into the body via the transfused red cells is ever excreted (24). The result is an increase in the total amount of iron stored in the body, which eventually reaches toxic levels. This excess iron is initially accumulated in the liver and spleen, and
eventually is deposited in the myocardium and endocrine organs; death usually results from cardiac hemosiderosis (22,23). The most common form of this type of anemia is β-thalassemia (22). This is an inherited disorder in which patients are unable to properly manufacture their own hemoglobin, and therefore require repeated blood transfusions (23). These iron-loading anemias, as they are referred to, are quite fatal (23). Children with β-thalassemia can usually be kept in good health through the first decade of life with regular blood transfusions (22). However, the vast majority of them die from the toxic effects of iron overload in the second or third decade (23).

Unlike primary hemochromatoses, the iron-loading anemias are much more difficult to treat. Obviously, this excess iron cannot be removed by phlebotomy in the case of secondary hemochromatosis; the patients are anemic to begin with. Therefore, other means of removing the large amounts of the metal are needed. To date, the most effective means of promoting the excretion of iron from patients suffering from the iron-loading anemias is chelation therapy. The iron chelating agent desferrioxamine (DFO) is currently the most widely employed therapeutic agent for the treatment of secondary hemochromatoses (5,22). This iron chelating agent is a siderophore isolated from Streptomyces pilosus (23). It has been shown that DFO is able to promote significant increases in urinary iron excretion in iron overloaded patients (23).

Although DFO can promote the excretion of a small amount of the excess iron present in thalassemic patients, there are several drawbacks to the use of DFO in iron chelation therapy. Firstly, DFO is not orally effective. In addition, since the chelator has such a short half-life of clearance from the blood (5-10 minutes), it must be administered as a subcutaneous or intravenous infusion typically over a 12 hour period,
six days a week (23,25). This poses a major inconvenience at the very least to patients who must receive this drug chronically. In addition, extensive DFO chelation therapy has not yet been shown to prolong the life of, or even prevent cardiac disease in, thalassemic patients (22). Intensive DFO chelation therapy is also quite expensive. Unfortunately, countries that have the highest incidence of thalassemia are least likely to have patients that can afford this type of treatment. There is clearly the need for a less expensive, more convenient therapeutic device that could be used to treat the iron-loading anemias.

Potential of Catecholamide Iron Chelators as Therapeutic Iron Clearing Devices

Shortly after the isolation of the spermidine catecholamides from Paracoccus denitrificans, workers began to examine the possibility of using these compounds in iron chelation therapy (26-28). Most of the preliminary studies with the catecholamides had been quite promising.

One of the earliest studies was conducted by Jacobs and coworkers, in which the ability of Tait's Compound II and Compound III (parabactin) to remove iron from human transferrin in vitro was examined (26). Although the total amount of the body's supply of iron that exists bound to transferrin at any one time is less than 1%, this shuttle protein represents a natural target for iron chelation therapy. Transferrin is most prevalent in the serum; however, the protein has an almost unlimited access to all of the various tissues in the body. It is continuously supplied with iron from the reticuloendothelium and the parenchymal cells of the liver, the two main iron storage sites in the body. Therefore, even if an iron chelator were unable to access the storage iron directly, it would nevertheless come into contact with transferrin as long as the chelator could be absorbed into the serum (29). If an iron chelating agent could remove iron from transferrin, the protein would
be resupplied with iron from the stores, and eventually the storage iron would be depleted (29). In this way, an iron chelating device could remove iron from storage sites indirectly, without coming in contact with the iron storage tissues directly.

Jacobs and coworkers were able to show that both compound II and parabactin were better than DFO in removing $^{59}$Fe from labeled human transferrin (26). With the chelators present at a concentration of 1 mM it was found that after 6 hours the amount of iron removed from serum containing transferrin by compound II, parabactin, and DFO were 36%, 18%, and 5% respectively (26). These initial results were very encouraging in showing the potential of the catecholamides as iron clearing devices.

Chang cells, cultured liver cells, were used as an in vitro model for studying the relative abilities of compound II, parabactin, and DFO to act as iron clearing devices (27). Chang cells take up iron and incorporate it into ferritin, just as the liver parenchymal cells do in vivo (27). Since the liver parenchyma constitutes the largest store of iron in the body and is the site of considerable tissue damage in hemochromatosis, a chelator that could remove iron from the liver would be of considerable importance. The abilities of compound II, parabactin, and DFO to inhibit $^{59}$Fe incorporation into ferritin, to inhibit $^{59}$Fe uptake, and to inhibit ferritin synthesis were studied (27). All three of these parameters were decreased in the presence of the chelators, but compound II and parabactin reduced cellular iron uptake considerably better than DFO (27). Compound II also proved to be better than DFO at inhibiting ferritin synthesis in Chang cells (27). Chang cells that were prelabeled with $^{59}$Fe were used to determine if the chelators could diffuse into and out of the cells (27). Both parabactin and compound II
were significantly better than DFO at removing the $^{59}$Fe from the cells (27). This result reinforced the idea that these catecholamides may be useful iron-clearing devices.

The ability to remove iron from animals is a crucial test of proposed iron-clearing compounds. One of the methods for determining this ability is the iron-overloaded rat model of hemochromatosis (28). In this assay, rats are administered large quantities of iron labeled with $^{59}$Fe, and the $^{59}$Fe excretion in the urine and feces is monitored (28). Several of the catecholamide siderophores have been tested with this model, both orally and parenterally (28). It was found that compound II was as effective as DFO in increasing the total iron excreted (28). In addition, while oral administration of DFO had no effect on iron excretion, compound II given orally caused a significant increase in the urinary iron output (28).

In these studies catecholamide iron chelators proved to be more effective than DFO in removing iron from transferrin, from Chang cells, and from rats. These initial findings prompted us to pursue a program of synthesizing catecholamide iron chelators for possible use as therapeutic iron chelating devices.
CHAPTER II
FLEXIBLE SYNTHESIS OF
POLYAMINE CATECHOLAMIDES

Introduction

In recent years there has been a great deal of effort focused on the synthesis of polyamine catecholamide iron chelators (30-41). There are many reasons for the stimulation of interest in this particular area. First, the polyamine catecholamides are closely related structurally to microbial iron chelators, the siderophores (3,13). The synthetic siderophore analogs have proven to be useful tools in the study of the mechanisms of siderophore-mediated iron uptake in microorganisms (12). By preparing a series of siderophore analogs whose structures differ slightly from one another, the sensitivity of the microbial iron uptake system to changes in the siderophore's structure can be evaluated (12).

There also exist more practical applications of the synthetic polyamine catecholamides. The lack of an effective iron clearing device to treat the iron overload syndromes is probably responsible for the vast majority of recent interest in polyamine catecholamide iron chelators (42). Several catecholamide iron chelating agents have been prepared during the past few years that show some potential to be useful therapeutic devices for iron chelation therapy (26,28,31).

Another application of synthetic catecholamide compounds concerns the need for the development of ligands that have the ability to preferentially chelate actinides - for example, plutonium (43-48). The
current devices used to treat individuals that have been exposed to the transuranium elements are not very effective at removing the contaminating metal from the body without also promoting the excretion of other essential metals, such as zinc (46).

Two main classes of catecholamide siderophores are known. The first class is really only comprised of a single compound, enterobactin (Figure 1) (15,16). The first total synthesis of enterobactin was reported by Corey in 1977 (49). Since that time a large number of enterobactin analogs have been synthesized, all of which essentially replace the labile cyclic triester platform of the siderophore with some other less labile backbone (36,38,40,50).

The other main class of catecholamide siderophores are the spermidine catecholamide siderophores parabactin and agrobactin (19,20) (Figure 2). Soon after the isolation of parabactin in 1975, several groups began programs aimed at completing the total synthesis of this microbial iron chelator, as well as synthesizing a number of its analogs (33,40,41). So far, Raymond has synthesized a number of catecholamides that are similar to parabactin and agrobactin in that they have the linear triamine spermidine as their backbone (40,47,50). These linear catecholamides are all essentially variations of one simple synthetic scheme - to acylate all three of the amine groups of spermidine with the same 2,3-dihydroxybenzoic acid derivative. Unlike these synthetic catecholamides which contain three identical acyl groups at each of the three spermidine nitrogens, parabactin contains a different substituent at the N1* position of its spermidine backbone other than the two 2,3-dihydroxybenzoyl groups at the terminal nitrogens.
Until only recently, the total synthesis of parabactin had eluded researchers working in the field (32). The reason for the difficulty in preparing this siderophore is not immediately apparent when examining the structure of the compound. Figure 4 shows the most obvious synthetic disconnections that would be made in designing the synthesis of parabactin. Figure 5 shows the resulting products of these synthetic disconnections. All of the compounds in Figure 5 are commercially available, and the process of simply linking these units together might seem to be a trivial task at first thought. However, a major stumbling block exists in the synthesis of parabactin that has hindered the successful synthesis of this compound for years - the selective acylation of spermidine. Clearly, an attempt at the selective acylation of the terminal amino groups of spermidine with an appropriately protected 2,3-dihydroxybenzoic acid derivative would result in a mixture of mono, di- and triacylated adducts. Nevertheless, such fatuous attempts at achieving N¹,N⁸-bis-acylation of spermidine are surprisingly frequent in the literature. For example, attempted N¹,N⁸-bis-acylation of spermidine with 2,3-dihydroxybenzoic acid derivatives result in yields typically around 14%, with the isolation of desired product from complex mixtures requiring time-consuming procedures (52).

Other workers have attempted to improve on N¹,N⁸-bis-acylation of spermidine by employing bulky, sterically hindered acylating agents. However, such approaches have not resulted in a great deal of success. For example, it was recently attempted to prepare the N¹,N⁸-bis-acyl spermidine adduct of 2,3-bis(benzoyloxy)benzoic acid and spermidine (41). The above condensation provided a mixture of products that required chromatography and offered the desired product in only "about 50% yield" (41).
Figure 4. Obvious Synthetic Disconnections of Parabactin.
Figure 5. Compounds Resulting from Disconnections in Figure 4.
The above examples clearly underscored the necessity of developing a synthetic scheme that could allow for the selective $N^1,N^8$-bis-acylation of spermidine. Surprisingly enough, there are only three reagents available to allow for the selective acylation of spermidine. The first of these synthetic schemes offers $N^4,N^8$-di-t-butoxycarbonyl spermidine as a reagent for introducing an acyl group at the $N^1$ position of spermidine (53). The reagent is available in three steps in a 49% yield, but is only useful in instances where it is not necessary to differentiate between the nitrogens at $N^4$ and $N^8$.

Another reagent has recently been prepared that has two different protecting groups attached to the $N^4$ and $N^8$ nitrogens of spermidine, $N^4$-tosyl-$N^8$-phthaloyl spermidine (54). One could theoretically employ this reagent to selectively effect the acylation of the terminal nitrogens of spermidine in route to the synthesis of parabactin and its analogs. However, the eight steps required for the synthesis of this protected spermidine, and the conditions required for the removal of the protecting groups, do not render this reagent particularly suitable for the synthesis of parabactin.

The most recent of the three methods takes advantage of a transiently protected spermidine. The condensation of spermidine with formaldehyde produces 1-(4-aminobutyl)hexahydropyrimidine which can then be bis-acylated (55). Deprotection involves cleavage of the hexahydropyrimidine ring, which affords the $N^1,N^8$-bis-acyl spermidine. Although this technique would be the most favorable of the three in terms of being applicable to the synthesis of spermidine siderophores such as parabactin, it suffers the drawbacks of not being able to attain bis-acyl norspermidine derivatives as well as requiring somewhat harsh conditions to effect the ring opening.
It was clear that there existed a need to develop a reagent that would allow for the selective bis-acylation of the primary amino groups of spermidine while at the same time keeping the secondary nitrogen protected. The development of such a reagent that allowed for the selective $N^1,N^8$-bis-acylation of spermidine, and its homo and nor homologs, is discussed in this chapter. Using this protected spermidine, a large number of polyamine catecholamides were synthesized.

**Synthesis**

The boundary conditions set for developing a reagent that would enable the selective $N^1,N^8$-bis-acylation of spermidine required that the scheme be composed of a short number of steps which proceed in high yield from relatively inexpensive starting materials. Additionally, it was required that the eventual deprotection of the secondary amine could be effected under mild conditions so that there would be minimal restrictions on what acyl groups could be attached to the $N^1$ and $N^8$ positions of the spermidine derivative. Another condition of developing an $N^4$-blocked spermidine was that the scheme must be applicable to the homo and nor homologs of spermidine.

Perhaps the most obvious approach to developing a protected spermidine would be to begin with spermidine itself and try to selectively introduce the protecting group to the $N^4$ position. However, using this approach there exists the original problem of selectively introducing a substituent to one of the amino groups of spermidine, in this case a protecting group at $N^4$. The approach that was taken was to develop a reagent that would allow for the differentiation of the primary and secondary amine groups of spermidine by having the secondary nitrogen protecting group "built in" to the molecule. The reagent decided on was the $N^4$-benzyl derivative of spermidine (33).
This simple scheme begins with the inexpensive reagents benzylamine and acrylonitrile in a cyanoethylation reaction to afford 2-cyanoethyl benzylamine in high yield (Figure 6). This amine is then alkylated with 4-chlorobutyronitrile in refluxing butanol using potassium carbonate as the base in high yield. The cyano groups of the resulting di-nitrile are then reduced using lithium aluminum hydride in the presence of aluminum trichloride in diethylether, again in good yield. Recently, it was found that by using W-2 Raney nickel as the catalyst it was possible to reduce the cyano groups of the di-nitrile to amino groups via catalytic hydrogenation without debenzyllating the secondary nitrogen. This modification of the above scheme for obtaining N'-benzylspermidine has greatly enhanced the synthetic route since the reduction now proceeds quantitatively and eliminates the elaborate workup conditions required when using lithium aluminum hydride.

One of the major advantages of the above scheme is its ability to be extended to the homo and nor homologs of N'-benzylspermidine (Figure 6). Benzylamine is used as the starting material for the preparation of all three benzylspermidine derivatives. By simply altering the reaction conditions of the cyanoethylation of benzylamine, bis-cyanoethylation can be effected. Thus, heating benzylamine and excess acrylonitrile in a sealed tube for several days in the presence of hydroquinone yields the bis-cyanoethylation adduct, bis-N-(3-cyanopropyl)benzylamine, in a 95% yield. In the absence of hydroquinone typical yields were only 15-30% with large amounts of polymeric side products. The scheme is also easily extended to yield the homo derivative of N'-benzylspermidine. Di-alkylation of benzylamine can be effected in a 70% yield with 4-chlorobutyronitrile. Reduction of the homo or nor bis-nitriles is then conducted in the usual manner providing the homo and nor homologs
Figure 6. Synthesis of Secondary N-benzyltriamines.
of benzylspermidine. Using the above scheme the selective acylation of the primary amino groups of spermidine as well as its homo and nor homologs can be accomplished.

Each of the secondary N-benzylated amines of Figure 6 may now be bis-acylated with an acylating agent. An excess of 2,3-dimethoxybenzoyl chloride was used to acylate benzylspermidine, and its homologs in methylene chloride using triethylamine as a base to produce the bis-catecholamides in excess of 95% yield. The excess acyl chloride is removed by adding the acyl halide scavenger 3-(dimethylamino)propylamine to the reaction mixture prior to an acid wash. The bis-acyl spermidine remains in the organic phase when the reaction mixture is then washed with dilute hydrochloric acid, while the adduct formed between the acyl chloride scavenger and 2,3-dimethoxybenzoyl chloride, as well as triethylamine, extracts into the aqueous phase. In this manner, the bis-acylation proceeds at near quantitative yields with no need for chromatography of the bis-adduct. The terminal bis-acyl adducts are then quantitatively debenzylated in acetic acid over a palladium chloride catalyst, the product of hydrogenolysis not requiring a chromatography step.

Thus, the bis-acyl spermidine, N1,N8-bis(2,3-dimethoxybenzoyl)spermidine, and its homo and nor homologs can now be used to attach any of a large number of acyl groups to the compound's secondary nitrogen. In fact, N1,N8-bis(2,3-dimethoxybenzoyl)spermidine has been a critical reagent for the synthesis of a large number of catecholamides including parabactin analogs, the siderophore parabactin itself, and was also used to prepare the first of a new class of octadentate catecholamide ligands.
In the synthesis of the parabactin analogs, the secondary nitrogen \( N^1,N^8 \)-bis(2,3-dimethoxybenzoyl)spermidine was acylated with either 2-hydroxyhippuric acid, \( N-(2,3\text{-dimethoxybenzoyl}) \)glycine, \( N-(2,3\text{-dimethoxybenzoyl}) \)-4-aminobutyric acid or \( N-(2,3\text{-dimethoxybenzoyl}) \)-\( \beta \)-alanine. While 2-hydroxyhippuric acid is commercially available, the other N-acyl amino acids had to be synthesized. All three were prepared by reacting the \( N \)-hydroxysuccinimidyl active ester of 2,3-dimethoxybenzoic acid with the appropriate amino acid (Figure 37). The active ester was generated in dioxane at 15°C by coupling 2,3-dimethoxybenzoic acid with \( N \)-hydroxysuccinimide using dicyclohexylcarbodiimide (DCC). The dicyclohexylurea was removed by filtration and an aqueous bicarbonate solution of the amino acid was added to the filtrate. In this manner the three \( N-(2,3 \text{ dimethoxybenzoyl}) \)amino acids were obtained in 80-90% yields. These three acids were used to acylate the secondary nitrogen of \( N^1,N^8 \)-bis-(2,3-dimethoxybenzoyl)spermidine and its homologs, using DCC as the condensing agent (Figure 38).

The only N-acyl amino acid that was not attached to the bis-acyl spermidines via DCC was 2-hydroxyhippuric acid. This compound was activated with trifluoroacetic anhydride. It has been shown that when 2-hydroxyhippuric acid is reacted with trifluoroacetic anhydride, the active acylating agent is not the mixed anhydride but rather, 2-(2-trifluoroacetoxyphenyl)-5-oxazolone (30,34). It is likely that this oxazolone is generated through the mixed anhydride.

The last step of the synthesis involved deprotection of the catechol protecting groups using boron tribromide (BBr₃) in methylene chloride. It was found that the dimethoxy protecting group was an ideal masking group for the catechols in this synthesis. Not only was
Figure 7. Synthesis of N-(2,3-Dimethoxybenzoyl)amino Acids.
Figure 8. Overview of Flexible Synthesis of Polyamine Catecholamides.
the starting material commercially available, therefore eliminating the need of protecting the catechol group of 2,3-dihydroxybenzoic acid, but the O-methyl groups are taken off quantitatively using BBr₃. It was found that the acidic conditions involved in the hydrolytic workup of the BBr₃ reaction did not adversely affect the catecholamides.

**Experimental**

All reagents were purchased from Aldrich Chemical Co. and were used without further purification. Unless otherwise specified, sodium sulfate was used as a drying agent. Melting points were taken on a Fisher-Johns apparatus and are uncorrected. Preparative thin-layer chromatography was done on 20 x 20 cm silica gel plates obtained from Analtech Co. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals Co. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. Samples for ¹H NMR were prepared in d-chloroform; chemical shifts are given in parts per million relative to an internal tetramethylsilane standard unless stated otherwise. The spectra were recorded on a Varian T-60 spectrometer. Samples for IR spectra were prepared in potassium bromide unless stated otherwise and the spectra were recorded on a Beckman IR 4210 spectrophotometer.

**Bis-N-(3-Cyanopropyl)benzylamine (1)**

A solution of 4-chlorobutyronitrile (19.30 g, 0.186 mol) in 100 ml dry 1-butanol was added dropwise over 2 hours to a stirred mixture of benzylamine (10.30 g, 96 mmol), anhydrous sodium carbonate (30.48 g, 288 mmol) and potassium iodide (5.66 g, 34 mmol) at 115°C. After refluxing an additional 20 hours under a nitrogen atmosphere, the mixture was allowed to cool to room temperature and was filtered; then, the salts were washed well with diethyl ether. The combined filtrate and
washings were extracted 3 x 100 ml with 3N hydrochloric acid and 2 x 100 ml water. The acid and water extracts were combined and washed 2 x 100 ml with ether, made basic with sodium carbonate and extracted 3 x 100 ml with ether. The resulting ethereal solution was dried, filtered, evaporated and distilled to give (1): 15.71 g (70%); b.p. 166° (0.05 mm). An analytical sample was purified on silica gel, using 25% petroleum ether in diethyl ether as the eluent. ¹H NMR: δ 1.45-2.08 (m, 4H), 2.10-2.77 (m, 8H), 3.50 (s, 2H), 7.22 (s, 5H).

Anal. calcd. for C₁₅H₁₉N₃: C, 74.65; H, 7.94; N, 17.41. Found: C, 74.83; H, 8.06; N, 17.45.

N⁵-Benzylhomospermidine (2)

A solution of aluminum chloride (5.05 g, 38 mmol) in 100 ml anhydrous diethyl ether was added to lithium aluminum hydride (1.44 g, 38 mmol) in 300 ml anhydrous diethyl ether. The mixture was stirred under a nitrogen atmosphere for 15 minutes followed by the dropwise addition of (1) (4.16 g, 17 mmol) in 50 ml anhydrous diethyl ether. After stirring the reaction mixture an additional 15 hours, it was cooled to 0°C and quenched with 200 ml aqueous 30% potassium hydroxide (w/v). The contents of the reaction vessel were loaded into a continuous extraction apparatus and extracted with diethyl ether for 48 hours. The resulting solution was dried, filtered, evaporated, and distilled: yield: 2.79 g (65%); b.p. 185°C (0.075 mm). ¹H NMR: δ 1.12 (s, 4H), 1.23-1.63 (m, 8H), 1.93-2.74 (m, 8H), 3.40 (s, 2H), 7.10 (s, 5H).

Anal. calcd. for C₁₅H₂₇N₃: C, 72.24; H, 10.91; N, 16.85. Found: C, 72.15; H, 11.10; N, 16.76.

N⁴-Benzyl-N¹,N⁸-bis(2,3-methylenedioxybenzoyl)spermidine Hydrochloride(3)

A solution of N⁴-benzylspermidine (33) (0.396 g, 1.68 mmol) and 1,8-bis(dimethylamino)naphthalene (0.750 g, 3.50 mmol) in 150 ml
methylene chloride (CH₂Cl₂) was cooled to 0°C under nitrogen. Dropwise addition of 2,3-methylenedioxybenzoyl chloride (56) (0.630 g, 3.41 mmol) in 100 ml methylene chloride was completed over a one hour period, and the reaction mixture was allowed to warm slowly to room temperature. After 16 hours, the reaction mixture was cooled to 0°C, washed with 3 x 10 ml ice-cold 3% (w/v) aqueous hydrogen chloride (HCl), and 3 x 10 ml ice water. The organic layer was then dried, filtered, and the filtrate evaporated. The product was dissolved in a minimum amount of methylene chloride and precipitated with several volumes of diethyl ether yielding 0.902 g (94% crude yield) of the desired product as a white, crystalline, hygroscopic solid.

An analytical sample was dissolved in methanol and sodium methoxide added to a pH of 11. After stirring the mixture for 30 minutes, the methanol was evaporated in vacuo. The residue was taken up in methylene chloride and washed with water. The organic layer was then dried, filtered, and the filtrate evaporated. Chromatography of the resulting amine on silica gel, using 8% methanol/chloroform as the eluent afforded a brown oil: ¹H NMR: δ 1.30-2.00 (m, 6H), 2.20-2.73 (m, 4H), 3.10-3.68 (m, 4H), 3.82 (s, 2H), 5.78 (s, 2H), 5.85 (s, 2H), 6.57-7.63 (m, 13H).

Anal. calcd. for C₃₀H₃₃N₃O₆: C, 67.78; H, 6.28; N, 7.90. Found: C, 67.70; H, 6.25; N, 7.78.

N¹N₈-bis(2,3-Methylenedioxybenzoyl)spermidine (4)

A solution of N°-benzyl-N¹,N₈-bis(2,3-methylenedioxybenzoyl)spermidine hydrochloride (3) (0.867 g, 1.53 mmol) in 45 ml glacial acetic acid was prepared and palladium chloride (0.103 g, 0.581 mmol) added. The reaction was allowed to proceed at room temperature until hydrogen was no longer taken up. The mixture was then filtered, and the filtrate evaporated. The residue was then dissolved in 35 ml
absolute methanol and the pH adjusted to pH 11 with sodium methoxide and evaporated. The resulting solid was taken up in 25 ml methylene chloride. This solution was washed with 2 x 25 ml cold water, dried, and was filtered. The filtrate was then evaporated to give 0.660 g (98% crude yield) of product, a brown oil.

An analytical sample was purified on silica gel, using 3% ammonium hydroxide/chloroform as the eluent: \(^1H\) NMR: \(\delta\) 1.40-2.02 (m, 7H), 2.42-2.90 (m, 4H), 3.20-3.78 (m, 4H), 5.92 (s, 4H), 6.60-7.68 (m, 1H).

Anal. calcd. for C\(_{23}\)H\(_{27}\)N\(_3\)O\(_6\)·H\(_2\)O: C, 60.25; H, 6.16; N, 9.17.

Found: C, 59.94; H, 5.96; N, 9.15.

\(N^4\)-[N-(2-Hydroxybenzoyl)glycyl]-\(N^1, N^8\)-bis(2,3-methylenedioxybenzoyl)-spermidine (5)

Trifluoroacetic anhydride (4.46 g, 21.24 mmol) was added to a suspension of N-(2-hydroxybenzoyl)glycine (0.402 g, 2.06 mmol) in 10 ml methylene chloride, and the mixture was refluxed at 45°C for 2 hours. The solution was evaporated in vacuo and the N-(2-trifluoroacetoxybenzoyl)glycyl trifluoroacetic anhydride was redissolved in 15 ml methylene chloride. Upon cooling this mixture to -78°C, 1,8-bis(di-methylamino)naphthalene (0.634 g, 2.96 mmol) in 10 ml methylene chloride was added, followed by the dropwise addition of \(N^1, N^8\)-bis-(2,3-methylenedioxybenzoyl)spermidine (0.653 g, 1.48 mmol) in 30 ml methylene chloride. The reaction mixture was allowed to warm to room temperature under nitrogen. After 45 hours, the solution was washed with cold 3% (w/v) aqueous HCl, dried, filtered, and the filtrate evaporated. The residue was then dissolved in methanol and the pH adjusted to 9 by the addition of sodium methoxide. After stirring the solution under nitrogen for 30 minutes, methanolic HCl was added at 0°C to give a pH of approximately 3. The resulting solution was
filtered and the filtrate evaporated. The residue was chromatographed on silica gel eluting with 5% methanol/ethyl acetate. This purification procedure resulted in 816 mg (89% yield) of the product - a white crystalline solid. \( ^1H \) NMR: \( \delta \) 1.30-2.30 (m, 6H), 3.42 (m, 8H), 4.17 (s, 2H), 6.00 (s, 4H), 6.53-7.77 (m, 13H), 11.99 (s, 1H).

Anal. calcd. for C\(_{32}\)H\(_{39}\)N\(_2\)O\(_3\): C, 62.13; H, 5.54; N, 8.96. Found: C, 61.95; H, 5.59; N, 8.90.

\( N^4-[\text{N-(2-Hydroxybenzoyl)glycyl}]-N^1,N^8\)-bis(2,3-dihydroxybenzoyl)spermidine (6)

To a solution of \( N^4-[\text{N-(2-hydroxybenzoyl)glycyl}]-N^1,N^8\)-bis(2,3-methylenedioxybenzoyl)spermidine (5) (0.230 g, 0.370 mmol) in 20 ml methylene chloride was added boron tribromide (0.50 ml, 5.29 mmol) dropwise under nitrogen, and the reaction vessel was cooled to 0°C. The mixture was allowed to warm slowly to room temperature. After 22 hours, 20 ml cold water were added dropwise with vigorous stirring. After stirring the mixture an additional 2 hours, the crude product was collected by filtration, washed thoroughly with water, and was dissolved in methanol. The solvent was then evaporated to yield 0.210 g (95% crude yield) of the desired product - a white solid.

An analytical sample was preadsorbed on Sephadex LH-20 and eluted with an ethanol/benzene gradient (5:50% v/v). \( ^1H \) NMR (CD\(_2\)Cl\(_2\)): \( \delta \) 1.32-3.28 (m, 6H), 3.04-3.72 (m, 8H), 4.22 (s, 2H), 6.48-8.12 (m, 15H), 12.10 (s, 1H), 12.74 (s, 1H), 13.09 (s, 1H).

Anal. calcd. for C\(_{30}\)H\(_{34}\)N\(_4\)O\(_9\)·2H\(_2\)O: C, 57.14; H, 6.07; N, 8.88. Found: C, 57.20; H, 5.67; N, 8.77.

\( N^4\)-Benzyl-N\(^1\),N\(^8\)-bis(2,3-dimethoxybenzoyl)spermidine Hydrochloride (7)

A solution of \( N^4\)-benzylspermidine (8.60 g, 36.5 mmol) and triethylamine (8.87 g, 87.7 mmol) in 500 ml methylene chloride was stirred
at 0°C under nitrogen. Dropwise addition of 2,3-dimethoxybenzoyl chloride (57) (15.39 g, 76.7 mmol) in 100 ml methylene chloride was completed over one hour, and the reaction mixture allowed to warm slowly to room temperature. After 18 hours, the reaction mixture was cooled to 0°C and 3-dimethylaminopropyl amine (58) (10 ml, 79.5 mmol) in 100 ml methylene chloride was added slowly. After stirring the reaction mixture for 2 hours, the reaction vessel was again cooled to 0°C, 150 ml ice-cold 3N HCl added, and the mixture was stirred an additional 15 minutes. The organic phase was washed 3 x 100 ml with ice-cold 3N HCl, dried, filtered, and evaporated to 21.45 g (98% yield) of product—a white, hygroscopic solid.

An analytical sample was dissolved in methanol and sodium methoxide was added to obtain pH 11. After stirring this mixture for 30 minutes, the mixture was evaporated in vacuo. The resulting solid was redissolved in methylene chloride, washed with water, dried, filtered, and evaporated. Silica gel chromatography (10% methanol/chloroform) of the resulting amine gave a tan oil. ^1H NMR: δ 1.32-1.98 (m, 6H), 2.22-2.68 (m, 4H), 3.13-3.82 (m, 6H), 3.83-3.90 (s, 12H), 6.78-8.05 (m, 13H).

Anal. calcd. for C_{32}H_{41}N_{3}O_{6}·H_{2}O: C, 66.07; H, 7.45; N, 7.22. Found: C, 66.30; H, 7.18; N, 7.19.

N^-Benzyl-N^1,N^7-bis(2,3-dimethoxybenzoyl)-bis(3-aminopropyl)amine Hydrochloride (8)

Preparation and purification of (8) was in the same manner as (7) (97% yield). ^1H NMR: δ 1.55-2.03 (m, 4H), 2.27-2.72 (m, 4H), 3.22-3.68 (m, 6H), 3.67 (s, 6H), 3.72 (s, 6H), 6.77-7.95 (m, 13H).

Anal. calcd. for C_{31}H_{39}N_{3}O_{6}: C, 67.74; H, 7.15; N, 7.64. Found: C, 67.64; H, 7.20; N, 7.47.
**N₅-Benzyl-N¹,N₇-bis(2,3-dimethoxybenzoyl)bis-(4-aminobutyl)amine Hydrochloride (9)**

Preparation and purification of (9) was in the same manner as (7) (97% yield). ¹H NMR: δ 1.48-1.85 (m, 8H), 2.20-2.65 (m, 4H), 3.08-3.63 (m, 6H), 3.77 (s, 12H), 6.78-8.02 (m, 13H).

Anal. calcd. for C₆₈H₇₇N₉O₁₈: C, 68.61; H, 7.50; N, 7.27. Found: C, 68.43; H, 7.39; N, 7.12.

**N¹,N₇-bis(2,3-Dimethoxybenzoyl)spermidine (10)**

To a solution of (7) (7.31 g, 12.2 mmol) in 50 ml glacial acetic acid was added palladium chloride (0.5 g, 2.8 mmol). The reaction was stirred at room temperature until hydrogen was no longer taken up. The reaction mixture was then filtered and evaporated, and the residue was dissolved in 50 ml methanol. The solution was adjusted to pH 11 with sodium methoxide. After stirring this mixture for 30 minutes, it was evaporated in vacuo. The resulting solid was redissolved in 100 ml methylene chloride and this solution was washed with 2 x 50 ml cold water, dried, and filtered, and the filtrate evaporated to give 5.65 g (98% yield) of desired product - a light tan oil.

An analytical sample was purified by silica gel chromatography (10% methanol/chloroform). ¹H NMR: δ 1.38-2.02 (m, 7H), 2.45-2.87 (m, 4H), 3.18-3.70 (m, 4H), 3.83 (s, 12H), 6.78-8.37 (m, 8H).

Anal. calcd. for C₂₅H₃₅N₃O₆: C, 63.41; H, 7.45; N, 8.17. Found: C, 63.55; H, 7.33; N, 8.89.

**N¹,N₇-bis(2,3-Dimethoxybenzoyl)bis-(3-aminopropyl)amine (11)**

Preparation and purification was in the same manner as (10), (98% yield). ¹H NMR: δ 1.63-2.12 (m, 4H), 2.53-2.95 (m, 4H), 3.27-3.83 (m, 5H), 3.85 (s, 12H), 6.90-8.25 (m, 8H).
Anal. calcd. for C_{2}\textsubscript{4}H_{3}\textsubscript{3}N_{3}O_{6}\cdot H_{2}O: C, 60.36; H, 7.39; N, 8.80. Found: C, 60.10; H, 7.29; N, 8.56.

N\textsuperscript{1},N\textsuperscript{2}-bis(2,3-Dimethoxybenzoyl)bis-(4-aminobuty1)amine (12)

Preparation and purification was in the same manner as (10), (99% yield). \textsuperscript{1}H NMR: δ 1.30-1.87 (m, 8H), 2.30 (s, 1H), 2.48-2.82 (m, 4H), 3.10-3.60 (m, 4H), 3.80 (s, 12H), 6.73-8.33 (m, 8H).

Anal. calcd. for C_{26}H_{37}N_{3}O_{6}: C, 64.31; H, 7.27; N, 8.65. Found: C, 64.46; H, 7.20; N, 8.86.

N\textsuperscript{8}-[N-(2-Hydroxybenzoyl)glycyl]-N\textsuperscript{1},N\textsuperscript{8}-bis(2,3-dimethoxybenzoyl)spermidine (13)

Trifluoroacetic anhydride (5.17 g, 24.6 mmol) was added to a suspension of 2-hydroxyhippuric acid (1.53 g, 7.9 mmol) in 35 ml methylene chloride, and the resulting mixture stirred under nitrogen for 2 hours. The solution was evaporated in vacuo and the N-(2-trifluoroacetoxybenzoyl)glycyl trifluoroacetic anhydride redissolved in 35 ml methylene chloride. After cooling this mixture to -78°C, triethylamine (2.0 g, 19.8 mmol) in 20 ml methylene chloride was added, followed by the dropwise addition of (10) (3.10 g, 6.5 mmol) in 25 ml methylene chloride. The mixture was allowed to warm slowly to room temperature. After 40 hours, the reaction vessel was cooled to 0°C, and its contents were washed with 3 x 30 ml ice cold 3% (w/v) aqueous HCl, dried, filtered, and the filtrate evaporated. The residue was dissolved in 100 ml methanol and the pH was adjusted to 9 with sodium methoxide under nitrogen. After stirring this mixture for 30 minutes, methanolic HCl was added at 0°C to obtain a pH of 3. The solution was evaporated in vacuo. The residue was redissolved in 100 ml methylene chloride and this solution was washed with 2 x 75 ml cold water, dried, filtered, and the filtrate was evaporated. The residue was chromatographed on
silica gel (5% methanol/ethyl acetate) yielding 4.05 g (95% yield) of the product - a white solid. $^1$H NMR: δ 1.33-2.13 (m, 6H), 3.08-3.70 (m, 8H), 3.71-4.03 (m, 12H), 4.03-4.32 (d, 2H), 6.48-8.42 (m, 13H), 12.12 (s, 1H).


$N^4$-[N-(2-Hydroxybenzoyl)glyclyl]-N$^2$,N$^7$-bis(2,3-dimethoxybenzoyl)bis-(3-aminopropyl)amine (14)

Preparation and purification was in the same manner as (13), (93% yield). $^1$H NMR: δ 1.30-2.87 (m, 4H), 3.10-3.77 (m, 8H), 3.87 (d, 12H), 4.17 (s, 2H), 6.53-8.40 (m, 13H), 12.12 (s, 1H).

Anal. calcd. for C$_3$H$_4$N$_4$O$_3$: C, 62.25; H, 6.33; N, 8.80. Found: C, 62.06; H, 6.41; N, 8.70.

$N^4$-[N-(2-Hydroxybenzoyl)glyclyl]-N$^2$,N$^9$-bis(2,3-dimethoxybenzoyl)bis-(4-aminobutyl)amine (15)

Preparation and purification was in the same manner as (13), (95% yield). $^1$H NMR: δ 1.30-2.22 (m, 6H), 3.27-4.24 (m, 6H), 4.40-4.86 (s, 2H), 6.42-8.19 (m, 13H).

Anal. calcd. for C$_3$H$_4$N$_4$O$_3$: C, 63.24; H, 6.67; N, 8.43. Found: C, 63.16; H, 6.72; N, 8.39.

$N^4$-[N-(2-Hydroxybenzoyl)glyclyl]-N$^4$,N$^7$-bis(2,3-dihydroxybenzoyl)bis-(3-aminopropylamine) (16)

Preparation and purification was in the same manner as (20), (95% yield). $^1$H NMR (d$_6$-acetone): δ 1.40-2.22 (m, 6H), 3.27-4.24 (m, 6H), 4.40-4.86 (s, 2H), 6.42-8.19 (m, 13H).

\[ N^\text{-}[N-(2\text{-Hydroxybenzoyl})\text{glycyl-N}^1, N^2\text{-bis}(2,3\text{-dihydroxybenzoyl})\text{bis-4-aminobutylamine (17)} \]

Preparation and purification was in the same manner as (20), (94% yield). \(^1H\) NMR (d\(_6\)-acetone): \(\delta\) 1.41-2.20 (m, 8H), 3.25-4.25 (m, 8H), 4.45-4.87 (s, 2H), 6.41-8.25 (m, 13H).

Anal. calcd. for \(C_{31}H_{36}N_9O_9\): C, 61.18; H, 5.96; N, 9.21. Found: C, 61.38; H, 6.02; N, 9.11.

2,3-Dimethoxybenzoyl Glycine (18)

Following the procedure of Van Brussel and Van Sumere (59), a mixture of 2,3-dimethoxybenzoic acid (2.21 g, 12.1 mmol) and \(N\)-hydroxysuccinimide (1.68 g, 14.6 mmol) in 35 ml dioxane was stirred at 15°C under nitrogen. Dropwise addition of dicyclohexylcarbodiimide (3.02 g, 14.6 mmol) in 30 ml dioxane was completed over 30 minutes, and the reaction was allowed to warm slowly to room temperature. After 13 hours, the reaction mixture was filtered and the precipitate was washed with 30 ml of cold dioxane. A mixture of glycine (1.21 g, 16.1 mmol) and sodium bicarbonate (1.35 g, 16.1 mmol) in 50 ml water was added to the filtrate and the resulting mixture was stirred at room temperature. After 27 hours, the solvent was reduced to one third its original volume in vacuo. Concentrated HCl was added to this mixture at 0°C to a pH of 2 and the resulting solid collected by filtration. Recrystallization of the solid from water yielded 2.64 g (91% yield) of the desired product: m.p. 134.0-134.5°C. \(^1H\) NMR (CD\(_3\)OD): \(\delta\) 3.83 (s, 3H), 3.88 (s, 3H), 4.10 (s, 2H), 6.95-7.63 (m, 3H).

Anal. calcd. for \(C_{11}H_{13}NO_5\): C, 55.23; H, 5.48; N, 5.89. Found: C, 55.40; H, 5.61; N, 5.81.
\[ \text{N}^\text{Na}-[\text{N-(2,3-Dimethoxybenzoyl)glycyl}]\text{-N}^\text{1},\text{N}^\text{8}-\text{bis}(2,3\text{-dimethoxybenzoyl})\text{-spermidine (19)} \]

To a solution of (18) (335 mg, 1.4 mmol) and (10) (616 mg, 1.3 mmol) in 15 ml dry methylene chloride under nitrogen was added DCC (270 mg, 1.3 mmol) in 10 ml dry methylene chloride. After 48 hours the reaction was filtered, the precipitate was washed with 5 ml methylene chloride, and the filtrate was evaporated. Chromatography on silica gel (chloroform/benzene/methanol) (10:10:1) afforded 723 mg (80%) of (19) as a white solid. \( ^1\text{H NMR: } \delta \text{ 1.43-2.20 (m, 6H), 3.10-3.68 (m, 8H), 3.83 (m, 18H), 4.23 (d, 2H), 6.77-9.17 (m, 12H).} \)

Anal. calcd for C\(_{36}\)H\(_{46}\)N\(_4\)O\(_{10}\): C, 62.23; H, 6.67; N, 8.06. Found: C, 62.09; H, 6.67; N, 7.96.

\[ \text{N}^\text{Na}-[\text{N-(2,3-Dihydroxybenzoyl)glycyl}]\text{-N}^\text{1},\text{N}^\text{8}-\text{bis}(2,3\text{-dihydroxybenzoyl})\text{-spermidine (20)} \]

To a 1 M solution of boron tribromide in methylene chloride (19 ml, 19 mmol) and dry methylene chloride (30 ml) at 0°C was added (19) in methylene chloride (30 ml) dropwise, under nitrogen. The reaction mixture was allowed to warm slowly to room temperature. After 14 hours, the reaction vessel was cooled to 0°C, and ice cold water (40 ml) was added slowly with vigorous stirring. After continued stirring of this mixture for 2 hours, the crude product was collected by filtration and washed with cold water. The resulting solid was purified on Sephadex LH-20 (20% ethanol/benzene) yielding 0.79 g (90%) of product as a white solid. \( ^1\text{H NMR (d}_6\text{-acetone): } \delta \text{ 1.47-2.20 (m, 6H), 3.19-4.30 (m, 8H), 4.25-4.81 (s, 2H), 6.42-8.31 (m, 12H).} \)

Anal. calcd for C\(_{30}\)H\(_{34}\)N\(_4\)O\(_{10}\): C, 59.01; H, 5.61; N, 9.18. Found: C, 58.90; H, 5.83; N, 9.01.
\textbf{N}^1,\textbf{N}^9\text{-bis(2,3-Dihydroxybenzoyl)bis-(4-aminobutyl)amine Hydrobromide (21)}

To a solution of boron tribromide (6.39 g, 25.5 mmol) in 50 ml methylene chloride at 0°C was added (10) (1.13 g, 2.3 mmol) in 45 ml methylene chloride dropwise under nitrogen. The mixture was allowed to warm slowly to room temperature. After 15 hours, the reaction mixture was cooled to 0°C and 75 ml of ice-cold water was added slowly with vigorous stirring. After continued stirring for 2 hours, the crude product was collected by filtration and washed well with water and methylene chloride. The resulting solid was crystallized from methanol to give 1.10 g (93% yield) of the desired product.

Anal. calcd. for C_{22}H_{30}N_{3}O_{6}Br: C, 51.57; H, 5.90; N, 8.20. Found: C, 51.75; H, 6.0; N, 7.95.

\textbf{N}^1,\textbf{N}^7\text{-bis(2,3-Dihydroxybenzoyl)bis-(3-aminopropyl)amine Hydrobromide (22)}

Preparation and purification was in the same manner as (21), (93% yield).

Anal. calcd. for C_{20}H_{20}N_{3}O_{6}Br: C, 49.60; H, 5.41; N, 8.68. Found: C, 49.77; H, 5.40; N, 8.42.

\textbf{N}^1,\textbf{N}^8\text{-bis(2,3-Dihydroxybenzoyl)spermidine Hydrobromide (23)}

Preparation and purification was in the same manner as (21), (94% yield). Spectral characteristics were identical to those reported in the literature (31).
Figure 9. NMR Spectrum of Bis,N-(3-Cyanopropyl)benzylamine (1)
Figure 10. NMR Spectrum of N<sup>5</sup>-Benzylohomospermidine (2).
Figure 11. NMR Spectrum of $N^6$-Benzy1-$N^1,N^8$-bis(2,3-methylenedioxybenzoyl)spermidine Hydrochloride (3).
Figure 12. NMR Spectrum of \( N^1, N^8 \)-bis(2,3-Methylenedioxybenzoyl)spermidine (4).
Figure 13. NMR Spectrum of N'-[N-(2-Hydroxybenzoyl)glycyl]-N¹,N¹-bis(2,3-methylenedioxybenzoyl)spermidine (5).
Figure 14. NMR Spectrum of $N^4-[N-(2$-Hydroxybenzoyl)glycyl]$-$N^4,N^8$-bis(2,3-dihydroxybenzoyl)$-$spermidine (6).
Figure 15. NMR Spectrum of N^4-Benzyl-N^1,N^8-bis(2,3-dimethoxybenzoyl)spârmidine Hydrochloride (7)
Figure 16. NMR Spectrum of $N^1$-Benzyl-$N^1,N^7$-bis(2,3-dimethoxybenzoyl)-bis(3-aminopropyl)amine Hydrochloride (8).
Figure 17. NMR Spectrum of N<sup>5</sup>-Benzyl- N<sup>1</sup>, N<sup>9</sup>-bis(2,3-dimethoxybenzoyl) bis-(4-aminobuty1)-amine Hydrochloride (9)
Figure 18. NMR Spectrum of $N^{1}, N^{8}$-bis(2,3-dimethoxybenzoyl)spermidine (10).
Figure 19. NMR Spectrum of N₁,N₇-bis(2,3-Dimethoxybenzoyl)bis-(3-aminopropyl)amine 11.
Figure 20. NMR Spectrum of $N^1,N^2$-bis(2,3-dimethoxybenzoyl)bis(4-aminobutyl)amine (12).
Figure 21. NMR Spectrum of $N^\alpha$-[N-(2-Hydroxybenzoyl)glycyl]-$N^1,N^8$-bis(2,3-dimethoxybenzoyl)-spermidine (13)
Figure 22. NMR Spectrum of $N^4-[N-(2$-Hydroxybenzoyl)glycyl]$-$N^1,N^7$-bis(2,3-dimethoxybenzoyl)-bis-(3-aminopropyl)amine (14)
Figure 23. NMR Spectrum of $N^4-[N-(2$-Hydroxybenzoylglucyl)]-$N^1,N^9$-bis(2,3-dimethoxybenzoyl-bis-(4-aminobutyl)amine (15).
Figure 24. NMR Spectrum of $N^4$-[N-(2-Hydroxybenzoyl)glycyl]-$N^1,N^7$-bis(2,3-dihydroxybenzoyl)bis-(3-aminopropylamine) (16)
Figure 25. NMR Spectrum of \( N^+\cdot N^\circ\cdot N\cdot (\text{2-Hydroxybenzoyl} \cdot \text{Glycyl} \cdot N^+\cdot N^\circ\cdot N\cdot \text{bis}(\text{2,3-dihydroxybenzoyl}) \cdot \text{bis-4-aminobutylamine}) \).
Figure 27. NMR Spectrum of $N^a-[N-(2,3$-dimethoxybenzoyl)glycyl]$-$N^1,N^a$-bis(2,3-dimethoxybenzoyl)spermidine (19).
Figure 28. NMR Spectrum of $N^1,N^8$-bis(2,3-Dihydroxybenzoyl)spermidine Hydrobromide (23).
Figure 29. IR Spectrum of Bis-N-(3-Cyanopropyl)benzylamine (I).
Figure 30. IR Spectrum of N⁵-Benzylhomospermidine (2).
Figure 31. IR Spectrum of $N^4$-[N-(2-Hydroxybenzoyl)glycyl]$\cdot N^3, N^8$-bis(2,3-methylenedioxybenzoyl)spermidine (5).
Figure 32. IR Spectrum of N'-Benzy1-N, N,N'-bis(2,3-dimethoxybenzoyl) spermidine hydrochloride (2).
Figure 33. IR Spectrum of N'-Benzyl-N\textsubscript{1},N\textsubscript{7}-bis(2,3-dimethoxybenzoyl)-bis(3-aminopropyl)-amine Hydrochloride (8).
Figure 34. IR Spectrum of N⁵-Benzyl-N¹,N⁹-bis(2,3-dimethoxybenzoyl)bis-(4-aminobutyl)-amine Hydrochloride (9).
Figure 35. IR Spectrum of $N^1,N^8$-bis(2,3-Dimethoxybenzoyl)spermidine (10).
Figure 36. IR Spectrum of N\textsuperscript{14}-[N-(2-Hydroxybenzoyl)glycyl]-N\textsuperscript{1},N\textsuperscript{8}-bis(2,3-dimethoxybenzoyl)-spermidine (13).
Figure 37. IR Spectrum of 2,3-Dimethoxybenzoyl Glycine (18).
CHAPTER III
OCTADENTATE CATECHOLAMIDE LIGANDS
AS ACTINIDE CHELATORS

Introduction

The potential biological hazards associated with the nuclear fuel cycle and nuclear military systems has rapidly increased along with their development. Unfortunately, the methods for dealing with such potential radiation hazards have not paralleled the expanding nuclear industry. Of the actinides used in nuclear systems plutonium is the element associated with the greatest potential for lethal radiation damage. Plutonium is one of the most toxic substances known (43,46). The high toxicity of plutonium in man is a result of both its radioactivity and its similarity to iron (60,61). Although plutonium is able to exist in each of the oxidation states from III to VI in aqueous solution (43,60), it is believed that the metal exists almost exclusively as plutonium (IV) at physiological pH in vivo. The charge to radius ratio of plutonium (IV) is quite similar to that of iron(III) (444 to 460 e/μm) (62). And, as expected, much of the chemistry of the two metals is very similar, including their coordination chemistry. Both Fe(III) and Pu(IV) are considered to be hard acids (46). The metals both display a high positive charge, small ionic size, and high acidity. In fact, the similarities between plutonium(IV) and iron(III) are so great the body cannot effectively discriminate between the two metals. The coordination chemistry of the two metals is similar enough that many of the bioorganic ligands present in the body that are normally
quite specific for iron(III) will also chelate plutonium(IV) (63). Like Fe(III), Pu(IV) is bound by the human serum transport protein transferrin (64). Transferrin shuttles the plutonium throughout the body, storing a large amount of the metal in the parenchymal cells of the liver where it is bound to the iron storage protein ferritin (65,66). Once plutonium is absorbed by the body, a large amount of the actinide is transported to the bone marrow and the reticuloendothelial tissue of the spleen (46). Like Fe(III), once Pu(IV) makes its way into the deep iron storage sites of the body, it is very difficult to remove (43,46).

The current treatment for exposure to plutonium involves the administration of the chelating agent DTPA (Figure 38) (67,68). Although DTPA has been shown to facilitate the excretion of plutonium if the chelator is administered very shortly after exposure to the actinide, it is not a very specific ligand for plutonium (69,70). The agent DTPA chelates and facilitates the excretion of a large number of the metal ions found in the body, including cobalt, magnesium, and zinc. In fact, it is the tendency of DTPA to chelate and clear zinc that is responsible for the toxic effects when DTPA is administered over a long period of time (69,70). Clearly, there exists the need for the development of a more specific sequester of Pu(IV) to treat individuals that have been exposed to the transuranium elements such as plutonium.

Based on the similarities of plutonium(IV) and iron(III), it would appear that catecholamide ligands might be able to form stable complexes with the metal. Catecholamides have indeed been shown to chelate Pu(IV) and other actinides very effectively (43-48). However, in order to effect the specific chelation of plutonium(IV), a ligand of the appropriate denticity and coordination geometry would have to be constructed. Unlike
Figure 38. Structure of Diethylenetriaminepentate Acetic Acid (DTPA).
Fe(III), which forms octahedral coordination compounds with catecholamides, the actinide(IV) catecholate complexes have been reported to exist in the geometry of a trigonal-faced dodecahedron (44).

Several octadentate catecholamide ligands have recently been synthesized by Raymond that have both cyclic and linear tetraamine backbones (45,46). It was found that the synthetic catecholamide ligands which possessed a large degree of freedom in their polyamine platforms were more effective chelating agents (45). Thus, the linear octadentate catecholamides - for example, N1,N5,N10,N14-tetra(2,3-dihydroxybenzoyl)tetraazatetradecane (LICAM) - were judged better actinide chelators than the catecholamides with cyclic platforms (45).

With the above information in mind, the synthesis of the first of a novel class of octadentate catecholamide ligands was undertaken. This new class of catecholamide ligands is referred to as octadentate "H-shaped" ligands, referring to the shape of the polyamine backbone of the catecholamides (Figure 39). Each of the four arms of the "H-shaped" ligands has a 2,3-dihydroxybenzoyl group attached to it. It is hoped that the new octadentate "H-shaped" ligands will have the flexibility of the linear octadentate chelators, but also possess the ability to encapsulate the metal without a large degree of internal rotation, something that the simple linear octadentate ligands cannot offer.

The development of this new class of octadentate catecholamides was undertaken in the hope that systems capable of environmental decontamination as well as biological decontamination could be achieved.
Figure 39. Structure of the First of a New Class of Catecholamide Octadentate Ligands (PLUTO).
Synthesis

The synthesis of the "H-shaped" catecholamide ligands was designed such that the length of each of the four arms hooking the four catecholate groups together, as well as the central tether linking the two spermidine backbones of the ligands, could be shortened or lengthened (Figure 40). In this way, it is hoped that the geometry of the ligands can be tailored to allow for the preferential chelation of one actinide over other metals.

The synthesis of the first octadentate "H-shaped" ligand was accomplished by linking together two molecules of $N^1,N^8$-bis(2,3-dimethoxybenzoyl)spermidine with glutaryl dichloride in high yield. The methyl protecting groups were then removed with BBr$_3$, yielding the free octadentate catecholamide again in high yield.

Of course, the synthetic scheme can be applied to the homo and nor homologs of $N^1,N^8$-bis(2,3-dimethoxybenzoyl)spermidine, and by using the acid chloride of any dicarboxylic acid (oxalic, succinic, glutaric, etc.), a large number of octadentate catecholamides can be synthesized. From the above description it can be seen that a number of octadentate ligands, in which both of the spermidine backbones present in the molecule are the same length, could be synthesized. It will also be possible to synthesize catecholamide ligands in which each of the two polyamine chains in the ligand are of a different length. For example, by first acylating the homo derivative of $N^1,N^8$-bis(2,3-dimethoxybenzoyl)-spermidine with glutaric anhydride, followed by linking the resulting monoglutaramide with the nor derivative of $N^1,N^8$-bis(2,3-dimethoxybenzoyl)spermidine, a ligand with both a nor and homo spermidine backbone can be prepared. It will be possible to eventually prepare a large
Figure 40. Synthesis of Catecholamide "H-shaped Ligands."
number of octadentate "H-shaped" catecholamide ligands that possess slightly different dimensions from one another. It is hoped that by lengthening or shortening the various appendages of the ligand, it will be possible to prepare ligands that specifically chelate plutonium over any other competing metal ions that may be present. Furthermore, the synthetic scheme described offers an opportunity to covalently attach an octadentate catecholamide ligand to a polymeric support. For example, N-(carbobenzyloxy)glutamic acid could be used to link two molecules of N₁,N₈-bis(2,3-dimethoxybenzoyl)spermidine together. After removal of the carbobenzyloxy protecting group, the free amino group could serve as a synthetic handle to covalently link the ligand to a matrix. Removal of the methyl protecting groups of the ligand that is now attached to the resin would generate a polymeric form of an octadentate catecholamide ligand. Such a device may be useful in the decontamination of actinide-tainted water.

Materials and Methods

Materials

All reagents were purchased from Aldrich Chemical Co. and, except where noted, used without further purification. Unless otherwise specified, sodium sulfate was used as a drying agent. Resins (20-50 mesh) were supplied by Rohm and Haas. Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. Boiling points are also uncorrected. Unless otherwise indicated, ¹H NMR chemical shifts are given in parts per million downfield from an internal tetramethylsilane standard. The spectra were recorded on a Varian T-60 spectrometer. Elemental analyses were performed by Galbraith Laboratories. Preparative thin-layer chromatography was done on Analtech 20 x 20 cm silica gel GF plates.
Experimental

Bis-\([\text{N}^1,\text{N}^8-\text{bis}(2,3-\text{dimethoxybenzoyl})\text{spermidine}]\)glutaramide (24)

A mixture of \(\text{N}^1,\text{N}^8-\text{bis}(2,3-\text{dimethoxybenzoyl})\text{spermidine}\) (1.00 g, 2.11 mmol) and triethylamine (0.64 g, 6.33 mmol) in \(\text{CH}_2\text{Cl}_2\) (40 ml) was cooled to 0°C under nitrogen. A solution of glutaryl dichloride (0.16 g, 0.95 mmol) in \(\text{CH}_2\text{Cl}_2\) (20 ml) was added dropwise over 10 minutes. After 16 hours, the mixture was washed with 3% (w/v) aqueous HCl (3 x 30 ml) and water (3 x 30 ml); the organic phase was then dried, filtered, and the filtrate evaporated. Purification by silica gel chromatography (10% methanol/ethyl acetate) yielded 0.95 g (96%) of product - a white solid. \(^1\text{H NMR } (\text{CDCl}_3): \delta 1.38-2.11 \text{ (14 H)}, 2.18-2.51 \text{ (4H)}, 3.01-3.61 \text{ (16H)}, 3.64-3.98 \text{ (24H)}, 6.74-8.31 \text{ (16H)}.\)

Anal. calcd. for \(\text{C}_{55}\text{H}_{74}\text{N}_6\text{O}_{14}\div C, 63.32; H, 7.15; N, 8.06. \) Found: C, 63.09; H, 7.12; N, 7.97.

Bis-\([\text{N}^1,\text{N}^8-\text{bis}(2,3-\text{dihydroxybenzoyl})\text{spermidine}]\)glutaramide (25)

To a 1 M solution of BBr\(_3\) in \(\text{CH}_2\text{Cl}_2\) (25.0 ml, 25 mmol) and dry \(\text{CH}_2\text{Cl}_2\) (30 ml) at 0°C was added bis-\([\text{N}^1,\text{N}^8-\text{bis}(2,3-\text{dimethoxybenzoyl})\text{spermidine}]\)-glutaramide (1.05 g, 1.01 mmol) in \(\text{CH}_2\text{Cl}_2\) (30 ml) dropwise under nitrogen. The reaction mixture was allowed to warm slowly to room temperature. After 16 hours, the reaction vessel was cooled to 0°C, and ice cold water (40 ml) was added slowly to the mixture with vigorous stirring. After continued stirring of the mixture for 2 hours, it was filtered and the resulting solid washed with cold water. The solid was dissolved in methanol and then evaporated, and the process repeated several times. Purification on Sephadex LH-20 (20% ethanol/benzene) yielded 0.8 g (85%) of product - a white solid. \(^1\text{H NMR } d_6\)-acetone:
δ 1.35-2.18 (14H), 2.25-2.75 (4H), 3.02-3.75 (16H), 6.45-7.42 (12H), 7.95-8.52 (4H).

Anal. calcd. for C_{47}H_{58}N_{14}·2H_{2}O: C, 58.38; H, 6.46; N, 8.69.

Found: C, 58.54; H, 6.55; N, 8.43.

Absorption Studies

The catecholamides N¹-[N-(2,3-dihydroxybenzoyl)butyryl]-N¹,N⁸-bis-(2,3-dihydroxybenzoyl)spermidine (30) (GABA), PLUTO, and LICAM (LICAM was supplied by Dr. Kenneth Raymond, Department of Chemistry, University of California, Berkeley) were tested for their ability to sequester plutonium(IV) and americium(III) from aqueous solutions after the ligands had been adsorbed on macroreticular resins. These determinations were performed by Dr. James Navratil's group at Rockwell International, Golden, Colorado using the following procedures.

The solubilities of the catecholamides in various reagent grade solvents were tested by mixing 50 mg of each catecholamide with 10 ml of solvent at ambient temperatures (22-24°C). The samples were visually inspected after one hour. Samples showing no dissolution were left overnight and reinspected the next day.

The catecholamides were dissolved in methanol and mixed with a macroreticular resin. The XAD resins are nonionic macroreticular polystyrene-divinyl benzene materials. The methanol was allowed to slowly evaporate. The loaded resin was washed with -20 column volumes of distilled water and air-dried. The amount of ligand absorbed by the resin was determined by the weight differences of the resin before and after contact with the ligand.

For the study of sequestering of americium and plutonium, two aqueous solutions were prepared: synthetic buffered solutions and actual
Figure 41. NMR Spectrum of Bis-[\(\text{N}^1,\text{N}^8\)-bis(2,3-Dimethoxybenzoyl)spermidine]glutaramide (24).
Figure 42. NMR Spectrum of Bis-[N°,N°-bis(2,3-Dihydroxybenzoyl)spermidine]glutaramide (25).
Rocky Flats plutonium process waste adjusted to various pH levels. Standard buffers were prepared according to the National Bureau of Standards procedure, and plutonium and/or americium were added from purified, concentrated actinide stock solutions in dilute HCl to prepare the synthetic waste samples. Process waste samples were adjusted to appropriate pH with 12 M nitric acid (HNO₃). All the solutions were filtered through Whatman 42 paper prior to analysis.

Weighed amounts of XAD-4 macroreticular resin containing the octa-coordinate catecholamide (PLUTO), were added to 10 ml of buffered or waste solution and equilibrated overnight on a rotary mixer. After the solution was separated from the resin by filtering through Whatman 42 paper, it was analyzed for americium and plutonium. In the americium analysis by gamma spectroscopy on a Canberra 80 multichannel analyzer, the bulk aqueous sample was run before and after contact with the sequester. This minimized sampling error. Precision and accuracy were determined by running standards with samples. Three readings of either sample or standard were made. The average precision is ± 3%, while the average accuracy of the standard with independently determined value is ± 10%. Plutonium was analyzed on a Nuclear Measurement Corporation proportional counter with an argon/methane atmosphere. Aliquots of 250 μl from either a standard or the test solution were affixed to stainless steel planchets and run. For low steel (<10⁻⁶ g/l) samples, 1000 μl aliquots were used. The precision and accuracy from simultaneously run standards are ± 6% and ± 9%, respectively.

Results and Discussion

It was originally desired to examine the ability of the catecholamides to sequester the actinides plutonium and americium by partitioning
the ligands between an organic solvent and aqueous solutions containing the actinides. However, the synthetic catecholamides were found to be generally quite insoluble in water-imiscible organic solvents such as carbon tetrachloride, diisopropylbenzene or kerosene. Only LICAM and GABA were found to be somewhat soluble in octanol. Of these two ligands, only GABA was judged soluble enough in both water and octanol to allow a partition experiment to be conducted.

GABA was partitioned between a solution of octanol and an aqueous process waste solution at pH 6.6 which contained plutonium and americium. The decontamination factors, which represent the ratio of the initial actinide concentration to that concentration after contact with the ligand, for GABA in the partition experiment are presented in Table 1. The ability of the hexadentate catecholamide ligand, GABA, to chelate plutonium(IV) is evident by the fairly large plutonium decontamination factor of 390, which corresponds to a removal of 97% of the plutonium from the aqueous phase. In addition to being a good sequester of plutonium, it can be seen that GABA shows a large degree of specificity towards the chelation of plutonium over americium. The americium decontamination factor for the GABA partition experiment was only 18, which corresponds to only 29% of the americium removed from the aqueous layer.

Since the octadentate catecholamides were not soluble in water-imiscible solvents, partition experiments could not be conducted. Instead, the ligands were adsorbed onto XAD-4 resin, and the resin washed with an aqueous solution of the actinides. Table 1 compares the plutonium and americium decontamination factors for resins coated with the hexadentate ligand GABA, and the octadentate ligand LICAM. As
Table 1.
Preliminary Adsorption Study - Sequestering of Am and Pu from Filtered Waste at pH 6.6

<table>
<thead>
<tr>
<th>Waste pH 6.6</th>
<th>Pu g/ℓ in Raffinate</th>
<th>Am g/ℓ in Raffinate</th>
<th>Decon. Factor Per G. Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waste (filtered)</td>
<td>5.4 x 10^-4 g/ℓ Pu</td>
<td>9.8 x 10^-6 g/ℓ Pu</td>
<td>2.9 x 10^-5 g/ℓ Am</td>
</tr>
<tr>
<td>Decontamination factor of filtration</td>
<td>55</td>
<td>9.8 x 10^-6 g/ℓ Pu</td>
<td>1.4 x 10^-7 g/ℓ Am</td>
</tr>
<tr>
<td>LIGAND (Vehicle)</td>
<td>gLigand</td>
<td>Pu g/ℓ in Raffinate</td>
<td>Decon. Factor Per G. Ligand</td>
</tr>
<tr>
<td>GABA(Octanol)</td>
<td>0.078</td>
<td>3.3 x 10^-7</td>
<td>390</td>
</tr>
<tr>
<td>GABA(XAD 4)</td>
<td>0.073</td>
<td>7.2 x 10^-7</td>
<td>45</td>
</tr>
<tr>
<td>LICAM(XAD 4)</td>
<td>0.017</td>
<td>1.3 x 10^-6</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.9 x 10^-8</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.6 x 10^-8</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.1 x 10^-7</td>
<td>16</td>
</tr>
</tbody>
</table>

Decon. Factor = (Actinide Content in Feed) / (Actinide Content in Raffinate) / wt. ligand
expected, the octadentate catecholamide chelator LICAM displayed a greater plutonium decontamination factor than the hexadentate catecholamide ligand GABA. This is understandable since plutonium(IV) is an octacoordinate metal ion. The americium decontamination factors for the two ligands were both rather low compared to the plutonium decontamination factors. It should be pointed out that the reason for the much smaller decontamination factors obtained when the ligands are coated onto the resin compared to the octanol-water partition experiment is due to the large amount of ligand used in the resin experiments. It has been determined that by using less ligand, larger decontamination values are obtained in the resin experiments.

Table 2 shows the data from a study examining the ability of XAD resin coated with PLUTO to remove plutonium and americium from two different types of actinide solutions as a function of pH. As can be seen from the table, the optimum pH region for the removal of both actinides is in the pH range 6 to 7. While the plutonium removal was greatest at pH 6 in process waste solutions, the removal of plutonium from standard buffer solutions was quite effective over a large pH range of 2 to 6. It should be noted that, although the plutonium and americium decontamination factors appear to vary considerably in some instances, the actual difference in the amount of metal removed may be quite small. For example, when the standard americium buffer at pH 6 is exposed to the PLUTO-XAD resins system, a plutonium decontamination factor of 677 is obtained. This decontamination factor corresponds to the removal of 94% of the plutonium from the standard solution. At pH 7 a substantially smaller americium decontamination factor of 300 is obtained. However, a decontamination factor here of 300 means that 94% of the metal has been removed from the buffer, the same amount removed at pH 6.
Table 2.
Sequestering of Plutonium and Americium from Aqueous Solutions with PLUTO (octadentate catecholamide) on XAD-4 Resin

<table>
<thead>
<tr>
<th>pH</th>
<th>Test Solution</th>
<th>Initial Actinide Concentration (g/l)(^a)</th>
<th>Wt (g) PLUTO</th>
<th>Decontamination Factor (per g PLUTO)(^b,c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pu</td>
<td>Am</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Buffer</td>
<td>1.6 x 10(^{-4})</td>
<td>1.5 x 10(^{-7})</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>Waste-II (^d)</td>
<td>1.6 x 10(^{-7})</td>
<td>1.5 x 10(^{-7})</td>
<td>0.064</td>
</tr>
<tr>
<td>3</td>
<td>Buffer</td>
<td>1.1 x 10(^{-4})</td>
<td>1.5 x 10(^{-7})</td>
<td>0.067</td>
</tr>
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<td></td>
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a. Error: Pu ±6%, Am ±3%
b. Decontamination Factor = \(\frac{\text{Actinide Conc. in Feed}}{\text{Actinide Conc. in Raffinate}}/\text{wt. PLUTO}\)
c. D.F. Error: Pu ±12%, Am ±6%
d. Waste-II: Waste spiked with Pu and Am after first filtration and refiltered after pH adjustment
As opposed to the standard plutonium buffer solutions, when process waste is treated with the PLUTO-XAD resin system, there is a significantly different amount of plutonium removed at pH 2 than at pH 6. At pH 6 the plutonium decontamination factor of 300 corresponds to a removal of 95% of the metal, while at pH 2 the decontamination factor of 28 indicates that only 44% of the plutonium has been removed from the process waste solution.

Like plutonium, the greatest amount of americium removed from the two solutions by the PLUTO-XAD system occurred at pH 6-7. With few exceptions, the ability of the PLUTO-XAD resin system to remove americium from these solutions represents a bell-shaped curve where the removal of metal at very high or very low pH values was substantially poorer than at pH values around neutrality. For example, 81% of the americium in the standard buffer solution was removed by the PLUTO-XAD resin system at pH 7, while only 2% and 44% are removed at pH values of 2 and 12, respectively.

It should be mentioned that the results of Table 2 represent experiments done on two different batches of PLUTO. The even numbered pH series was done on the first batch of the ligand while the odd numbered pH series was conducted on a different batch of the ligand. It is possible that some of the differences observed among the data are due to batch differences. However, the differences may be also a result of metal and pH-dependent hydrolysis of plutonium and americium at pH values near neutrality.

Despite the uncertainties regarding the nature of the metal ion species present in the solutions at different pH values, PLUTO has been shown to be effective in removing americium and plutonium from aqueous solutions of the actinides. These results suggest that the octadentate
catecholamide H-shaped ligands may be useful in the removal of actinides from patients exposed to the metals, as well as being effective devices to serve in environmental actinide decontamination.
CHAPTER IV
SYNTHESIS AND SOLUTION CONFORMATION OF PARABACTIN AND ITS GALLIUM(III) CHELATE

Introduction

In 1975 Tait isolated a catecholamine iron chelator, which he referred to as "Compound III," from iron-depressed cultures of *Paracoccus denitrificans* (19). Compound III was shown to consist of a spermidine backbone with a 2,3-dihydroxybenzoyl group at each of the two terminal nitrogen atoms. The central N\(^4\) position of the spermidine chain was proposed to be acylated with an N-salicyl-L-threonine moiety. Later, Neilands was able to show that Tait's original proposed structure of compound III was incorrect (21). Based on the ability of compound III to form a hydrochloride salt with a concomitant red shift in its ultraviolet absorption spectrum, which is characteristic of oxazoline ring systems, it was concluded that the proposed salicyl threonine group was actually a 2-hydroxyphenyl-threonyloxazoline ring (21). The true structure of the natural catecholamide Tait has isolated is now referred to as parabactin A (Figure 3).

Since the isolation of parabactin, a great deal of interest has been focused on the biological and physical properties of this siderophore and its ferric chelate (19,21,41). It has recently been determined that the formation constant for the iron(III) complex of parabactin at physiological pH is 10\(^{4.8}\), making parabactin one of the strongest ferric ion chelators known to date (71). The coordination of iron(III) by parabactin is believed to take place with the siderophore encapsulating
the metal ion to form an octahedral coordination complex (72). Unlike some other catecholam ide siderophores, parabactin chelates iron(III) with oxygen as well as nitrogen atoms serving as the ligating groups. Para¬bactin is believed to chelate ferric ion via the five phenolic oxygens, as well as the oxazoline ring nitrogen of the threonyloxazoline ring (72).

In addition to the physical properties of the metal chelate of parabactin, the properties of the free ligand, mainly its solution conformation, have been the subject of considerable interest (32,41). A high field $^1$H NMR study of parabactin and agrobactin, a siderophore isolated from Agrobacterium tumefaciens (Figure 2), was undertaken which provided some insight about the solution behavior of these siderophores (41). It was observed that the peaks in the spectra of the siderophores were present in duplicate and that, upon heating, the duplicate signals coalesced into single peaks. Two theories were offered to explain the behavior of the NMR spectra of parabactin and agrobactin. The first involved a rotation of the oxazoline ring moiety about the alpha-carbon and carbonyl-carbon single bond that results in two interconvertible conformers of the siderophore (73). The second explanation suggested that the duplicate signals were a result of a simple cis-trans isomerization of the $N^4$ amide bond (41).

Part of the reason the solution conformation of the catecholamide siderophores has received so much attention is because of the exceptionally high formation constants of their metal chelates. It was thought that one of the reasons for the large ferric ion formation constants of these ligands may be due to a similar solution conformation of both ligand and chelate. Another reason for studying the solution behavior
of the siderophores in general is concerned with the role these iron chelating compounds play in microbial iron transport.

Evidence has been presented recently that shows certain microorganisms have the ability to discriminate between various ferric siderophore chelates (74-81). While some ferric chelates are transported and utilized by a microorganism, others are not - presumably because receptor proteins on the outer membrane surface of the microorganism can only recognize certain siderophore iron complexes (77,80). The exact nature of how microorganisms can discriminate between ferric siderophore complexes is unclear. It is believed that recognition may depend on the configuration of various groups of the ligand or, perhaps, on the configuration of the chelating groups about the metal center itself (12, 72,77).

It was desired to ultimately evaluate the specificity of the ferric parabactin iron transport system in Paracoccus denitrificans. However, before the specificity of the microbial iron uptake system of Paracoccus denitrificans could be understood, the solution conformation and stereochemistry of the complex formed between parabactin and trivalent metal ions needed to be examined in more detail. This was accomplished via 300 MHz $^1$H NMR spectroscopy. To avoid line broadening by the paramagnetic ferric ion, gallium(III) was used to examine the parabactin metal complex.

**Experimental**

All reagents, with the exception of (L)-N-tert-butoxycarbonylthreonine (Sigma Chemical Co.) and gallium(III)nitrate.9 H$_2$O (Alfa Co.) were purchased from Aldrich Chemical Co. and were used without further purification. Sodium sulfate was used as a drying agent. Melting points were
taken on a Fisher-Johns apparatus and are uncorrected. Preparative thin-
layer chromatography was done on 20 x 20 cm silica gel plates obtained
from Analtech Co. Sephadex LH-20 was purchased from Pharmacia Fine
Chemicals Co. Optical rotations were measured with a Perkin-Elmer model
141 polarimeter. Elemental analyses were performed by Galbraith Labora-
tories, Knoxville, Tenn., or Atlantic Microlab Inc., Atlanta, Ga. Pro-
ton NMR spectra were obtained on a Nicolet Instrument Corp. NT-300 spec-
trometer and NIC-1180 E data system. Probe temperature was determined
using test samples of ethylene glycol. Computer simulation and curve
analysis/deconvolution programs used were included in the NMCFT software
package provided by Nicolet Technology Corp. Resolution enhancement,
when necessary, was performed by apodization of the FID by a double ex-
ponential multiplication followed by zero filling. Samples for the de-
termination of temperature of coalescence were prepared by dissolving
5-10 mg of the compound in 500 µl d$_6$-DMSO. Coalescence temperatures,
± 1°C, were then determined by observing the coalescence of the beta-
decoupled gamma methyl signals. The coalescence temperatures were meas-
ured both on heating and cooling cycles and activation energies, ± 0.2
kcal/mol, subsequently calculated by the method of Gutowsky and Cheng
(82). Chemical shifts are reported downfield from an external DSS stan-
dard. By dissolving a sample of 5 mg of parabactin in 500 µl d-chloro-
form (CDCl$_3$) in a 5 mm O.D. NMR tube, d$_6$-DMSO/CDCl$_3$ titrations were
carried out. Additions of d$_6$-DMSO were made directly into the NMR tube
via a microliter syringe, and spectra recorded after mixing the sample.
In this manner, spectra were recorded at d$_6$-DMSO concentrations (volume
percent) of 0%, 2%, 5%, 20%, 30%, 40%, and 50%. pH measurements were
obtained with an Ingold microelectrode. Gallium chelates were prepared
by adding a slight excess of gallium(III) nitrate to an aqueous solution of the catecholamide at -pH 10 and immediately adjusting the pH to -7.4 under N₂. Water was removed in vacuo after allowing the mixture to stir overnight at room temperature.

**(L)-N-Hydroxysuccinimidido-N-tert-butoxycarbonylthreoninate (26)**

To a solution of (L)-N-tert-butoxycarbonylthreonine (2.96 g, 13.5 mmol) and N-hydroxysuccinimide (1.63 g, 14.2 mmol) in dry tetrahydrofuran (THF) (100 ml) at 0°C was added DCC (2.97 g, 14.4 mmol) in THF (100 ml). The mixture was allowed to warm slowly to room temperature with continued stirring under N₂. After 14 hours, the mixture was filtered and the DCU washed with THF (25 ml). The filtrate was evaporated and the residue crystallized from diethyl ether to yield 3.84 g (90%) of (26) as white crystals: mp 134-135°C, [α]²⁴_D -33.7 ± 0.7 (C=2.7 EtOAc) (lit. mp 134-135°C, [α]²⁵_D - 33.3 (EtOAc)) (83); ¹H NMR (CDCl₃): δ 1.25 (d, 3H), 1.47 (s, 9H), 2.78 (s, 4H), 3.89-4.98 (m, 3H), 6.38 (m, 1H).

**(L)-N⁴-[N-tert-Butoxycarbonylthreonyl]-N₁,N⁸-bis(2,3-dimethoxybenzoyl)spermidine (27)**

A solution of N¹,N⁸-bis(2,3-dimethoxybenzoyl)spermidine (10) 2.50 g, 5.28 mmol) in DMF (150 ml) was cooled to 0°C. A solution of (26) (1.75 g, 5.54 mmol) in DMF (50 ml) was added at once and the mixture allowed to warm slowly to room temperature. After 48 hours the solvent was evaporated. The residue was dissolved in CH₂Cl₂ (100 ml), washed with cold 3% (w/v) aqueous HCl (3 x 30 ml) and cold water (10 x 30 ml). The organic phase was then dried, filtered, and the filtrate evaporated. Silica gel chromatography (5% methanol/ethyl acetate) yielded (27) as a hygroscopic, white solid: 2.99 g (84%); ¹H NMR (CDCl₃): δ 1.13 (d, 3H), 1.40 (s, 9H), 1.50-2.13 (m, 6H), 3.07-3.63 (m, 8H), 3.83 (s, 12H), 4.17-4.57 (m, 3H), 5.33-5.60 (d, 1H), 6.77-8.27 (m, 8H).
Anal. calcd. for C₃₄H₅₀N₄O₁₀: C, 60.52; H, 7.47; N, 8.30. Found: C, 60.60; H, 7.38; N, 8.45.

(L)-N¹'-Threonyl-N¹,N⁸-bis(2,3-dimethoxybenzoyl)spermidine (28)

A solution of (27) (1.70 g, 0.25 mmol) in trifluoroacetic acid (TFA) (40 ml) was stirred at room temperature for 35 min. The solvent was then evaporated and the residue dissolved in cold CH₂Cl₂ (100 ml) and washed with ice cold 30% (w/v) aqueous sodium carbonate (3 x 50 ml). The organic phase was then dried, filtered, and the filtrate evaporated. Purification on silica gel (10% methanol/chloroform) provided 1.38 g (95%) of (28) as a hygroscopic, white solid. ¹H NMR (CDCl₃): ′H 1.10-1.20 (d, 3H), 1.43-2.00 (m, 6H), 2.94-4.08 (25H), 6.84-8.25 (m, 8H).

Anal. calcd. for C₂₉H₄₂N₄O₈: C, 60.61; H, 7.37; N, 9.75. Found: C, 60.50; H, 7.36; N, 9.62.

(L)-N⁴'-Threonyl-N¹,N⁸-bis(2,3-dihydroxybenzoyl)spermidine Hydrobromide (29)

To a 1 M stirred solution of BBr₃ (20 ml, 20.0 mmol) in dry CH₂Cl₂ (30 ml) at 0°C was added (28) (0.83 g, 1.44 mmol) in CH₂Cl₂ (30 ml) dropwise under nitrogen. The reaction mixture was allowed to warm slowly to room temperature. After 12 hours the reaction vessel was cooled to 0°C, and ice cold water (15 ml) was added dropwise with vigorous stirring. The resulting suspension was allowed to warm to room temperature with continued stirring over one hour and the product collected by filtration. The residue was dissolved in methanol and evaporated, this process being repeated several times. Chromatography on Sephadex LH-20 (20-40% ethanol/benzene) gave 0.78 g (90%) of (29) as a white solid. ¹H NMR (CD₃OD): ′H 1.10-1.37 (d, 3H), 1.43-2.23 (m, 6H), 3.13-3.80 (m, 8H), 3.83-4.40 (m, 3H), 6.33-7.33 (m, 6H).
Anal. calcd. for C$_{25}$H$_{35}$N$_4$O$_8$Br: C, 50.09; H, 5.88; N, 9.35. Found: C, 49.98; H, 5.96; N, 9.27.

(L)-(N-[3-(2,3-Dihydroxybenzamido)propyl]-N-[4-(2,3-dihydroxybenzamido)butyl]-2-(2-hydroxyphenyl)trans-5-methyloxazoline-4-carboxamide (Parabactin) (30)

A solution of (29) (0.35 g, 0.59 mmol) and ethyl-2-hydroxybenzimidate (84) (0.11 g, 0.67 mmol) in dry methanol (50 ml) was heated to reflux under nitrogen. After 24 hours the solvent was evaporated and the residue chromatographed on Sephadex LH-20 (20% ethanol/benzene) providing 0.31 g (85%) of (30) as a white solid [α]$^{25}$D 98 ± 2° (C, 3.0, methanol). $^1$H NMR (10:1, CDCl$_3$: d$_6$-DMSO): δ 1.34-1.45 (m, 3H), 1.48-2.03 (m, 6H), 3.11-3.78 (m, 8H), 4.59 (m, 1H), 5.28-5.45 (m, 1H), 6.52-7.71 (m, 10H), 7.89-8.17 (m, 4H), 11.56-12.82 (m, 3H).

Anal. calcd. for C$_{32}$H$_{36}$N$_8$O$_9$: C, 61.93; H, 5.85; N, 9.03. Found: C, 61.93; H, 5.94; N, 8.96.

(L)-(N-[3-(2,3-Dimethoxybenzamido)propyl]-N-[4-(2,3-dimethoxybenzamido)butyl]-2-(2-hydroxyphenyl)trans-5-methyloxazoline-4-carboxamide (31)

A solution of (28) (0.49, 0.85 mmol) and ethyl-2-hydroxybenzimidate (0.148 g, 0.86 mmol) in dry methanol (25 ml) was heated to reflux under nitrogen. After 24 hours the solvent was evaporated, the residue dissolved in CH$_2$Cl$_2$ (50 ml), and washed with cold water (2 x 25 ml). The organic phase was dried, filtered, and the filtrate evaporated. Purification on silica gel (10% methanol/chloroform) provided 0.48 g (83%) of (31) as a white, hygroscopic solid. $^1$H NMR (10:1 CDCl$_3$: d$_6$-DMSO): δ 1.44-1.53 (m, 3H), 1.56-2.13 (m, 7H), 3.53-3.63 (m, 8H), 3.84-3.96 (m, 12H), 4.62-4.70 (m, 1H), 5.17-5.48 (m, 1H), 6.83-7.68 (m, 10H), 7.98-8.38 (m, 2H), 11.56 (s, 1H).
Anal. calcd. for C_{36}H_{44}N_{4}O_{8}: C, 63.89; H, 6.55; N, 8.28. Found: C, 63.75; H, 6.78; N, 8.19.

(L)-N^5-[(N-(tert-Butyloxycarbonylthreonyl)]-N^1,N^9-bis(2,3-dimethoxybenzoyl)-homospermidine (32)

Preparation and purification was in the same manner as (27): (82\% yield); ^1H NMR (CDCl$_3$): δ 1.14 (d, 3H), 1.40 (s, 9H), 1.48-2.14 (m, 8H partially obscured), 3.05-3.64 (m, 8H), 3.82 (s, 12H), 4.15-4.55 (m, 3H), 5.31-5.69 (d, 1H), 6.77-8.26 (m, 8H).

Anal. calcd. for C_{35}H_{52}O_{10}: C, 61.03; H, 7.61; N, 8.13. Found: C, 61.12; H, 7.83; N, 7.98.

(L)-N^5-Threonyl-N^1,N^9-bis(2,3-dimethoxybenzoyl)homospermidine (33)

Preparation and purification of (33) was in the same manner as (28): (95\% yield); ^1H NMR (CDCl$_3$): δ 1.09-1.20 (d, 3H), 1.44-1.99 (m, 8H), 2.95-4.00 (overlapping 25H), 6.84-8.25 (m, 8H).

Anal. calcd. for C_{30}H_{44}N_{4}O_{8}: C, 61.21; H, 7.53; N, 9.52. Found: C, 61.10; H, 7.41; N, 9.30.

(L)-N^5-Threonyl-N^1,N^9-bis(2,3-dihydroxybenzoyl)homospermidine Hydrobromide (34)

Preparation and purification of (34) was in the same manner as (29): (88\% yield); ^1H NMR (CD$_3$OD): δ 1.11-1.40 (d, 3H), 1.40-2.24 (m, 8H), 3.10-3.83 (m, 8H), 3.83-4.40 (m, 3H), 6.34-7.41 (m, 6H).

Anal. calcd. for C_{26}H_{37}N_{4}O_{6}Br: C, 50.90; H, 6.08; N, 9.13. Found: C, 51.01; H, 5.95; N, 9.31.

(L)-(N-[4-(2,3-Dihydroxybenzamido)butyl])-N-[4-(2,3-dihydroxybenzamido)butyl]-2-(2-hydroxyphenyl)trans-5-methyloxazoline-4-carboxamide (Homoparabactin) (35)

Preparation and purification of (35) was in the same manner as (30): (82\% yield); ^1H NMR (10:1, CDCl$_3$:d$_6$-DMSO): δ 1.30-1.44 (d, 3H), 1.50-2.02 (m, 8H), 3.00-3.77 (m, 8H), 4.61 (m, 1H), 5.30-5.45 (m, 1H), 6.52-7.70 (m, 10H), 7.89-8.17 (m, 4H), 11.55-12.82 (m, 3H).
Anal. calcd. for C\textsubscript{33}H\textsubscript{38}N\textsubscript{4}O\textsubscript{9}: C, 62.45; H, 6.04; N, 8.83. Found: C, 62.53; H, 5.94; N, 8.93.

(L)-N\textsuperscript{4}-[N-(tert-Butoxycarbonylthreonyl)]-N\textsuperscript{1},N\textsuperscript{7}-bis(2,3-dimethoxybenzoyl)norspermidine (36)

Preparation and purification of (36) was in the same manner as (27): (83% yield); \textsuperscript{1}H NMR (CDCl\textsubscript{3}): \textdelta 1.14 (d, 3H), 1.40 (s, 9H), 1.49-2.13 (m, 6H), 3.06-3.65 (m, 6H), 3.82 (s, 12H), 4.16-4.55 (m, 3H), 5.31-5.60 (d, 1H), 6.77-8.24 (m, 8H).

Anal. calcd. for C\textsubscript{59}.H\textsubscript{7}.N\textsubscript{8}: C, 59.99; H, 7.32; N, 8.48. Found: C, 60.10; H, 7.12; N, 8.37.

(L)-N\textsuperscript{a}-Threonyl-N\textsuperscript{1},N\textsuperscript{7}-bis(2,3-dimethoxybenzoyl)norspermidine (37)

Preparation and purification of (37) was in the same manner as (28): (88% yield); \textsuperscript{1}H NMR (CDCl\textsubscript{3}): \textdelta 1.10-1.20 (d, 3H), 1.43-1.98 (m, 6H), 2.94-4.00 (overlapping 23H), 6.82-8.27 (m, 8H).

Anal. calcd. for C\textsubscript{59}H\textsubscript{7}N\textsubscript{8}O\textsubscript{10}: C, 59.99; H, 7.19; N, 9.99. Found: C, 60.23; H, 7.23; N, 8.17.

(L)-N\textsuperscript{a}-Threonyl-N\textsuperscript{1},N\textsuperscript{7}-bis(2,3-dihydroxybenzoyl)norspermidine Hydrobromide (38)

Preparation and purification of (38) was in the same manner as (29): (87% yield); \textsuperscript{1}H NMR (CD\textsubscript{3}OD): \textdelta 1.11-1.39 (d, 3H), 1.40-2.20 (m, 6H), 3.07-3.83 (m, 8H), 3.83-4.38 (m, 3H), 6.34-7.43 (m, 6H).

Anal. calcd. for C\textsubscript{24}H\textsubscript{33}N\textsubscript{4}O\textsubscript{10}Br: C, 49.24; H, 5.68; N, 9.57. Found: C, 49.46; H, 5.77; N, 9.33.

(L)-(N-[3-(2,3-Dihydroxybenzamido)propyl)]-N-[3-(2,3-dihydroxybenzamido)propyl]-2-(2-hydroxyphenyl)trans-5-methyloxazoline-3-carboxamide (norparabactin) (39)

Preparation and purification of (39) was in the same manner as (30): (83% yield); \textsuperscript{1}H NMR (10:1, CDCl\textsubscript{3}:d\textsubscript{6}-DMSO): \textdelta 1.31-1.44 (d, 3H), 1.51-2.00 (m, 6H), 3.10-3.76 (m, 6H), 4.61 (m, 1H), 5.29-5.44 (m, 1H), 6.53-7.71 (m, 10H), 7.89-8.21 (m, 4H), 11.55-12.82 (m, 3H).
Figure 43. NMR Spectrum of (L)-N-Hydroxysuccinimido-N-tert-butoxycarbonylthreoninate (26).
Figure 44. NMR Spectrum of (L)-N°°-[N-tert-Butyloxycarbonylthreonyl]-N°,N°-bis(2,3-dimethoxybenzoyl)spermidine (27).
Figure 45. NMR Spectrum of (L)-N\textsuperscript{4}-Threonyl-N\textsuperscript{1},N\textsuperscript{\textgreek{p}}-bis(2,3-dimethoxybenzoyl)spermidine (28).
Figure 46. NMR Spectrum of (L)-N<sup>n</sup>-Threonyl-N<sup>1</sup>,N<sup>8</sup>-bis(2,3-dihydroxybenzoyl)spermidine Hydrobromide (29).
Figure 47. NMR Spectrum of (L)-\((\text{N-}[\text{3-(2,3-Dihydroxybenzamido)propyl}]\text{N-}[\text{4-(2,3-dihydroxybenzamido)butyl}]\text{2-(2-hydroxyphenyl)trans-5-methylloxazoline-4-carboxamide})\) (Parabactin) (30).
Figure 48. NMR Spectrum of (L)-(N-[3-(2,3-Dimethoxybenzamido)propyl])-N-[4-(2,3-dimethoxybenzamido)butyl]-2-(2-hydroxyphenyl)trans-5-methylloxazoline-4-carboxamide (31).
Figure 49. NMR Spectrum of (L)-N³-[N-(tert-Butoxycarbonylthreonyl)]-N¹,N³-bis(2,3-dimethoxybenzoyl)homospermidine (32).
Figure 50. NMR Spectrum of (L)-N\textsuperscript{a}-Threonyl-N\textsuperscript{1},N\textsuperscript{7}-bis(2,3-dimethoxybenzoyl)norspermidine (37).
Figure 51. IR Spectrum of (L)-N\textsuperscript{4}-[N-tert-Butoxycarbonylthreonyl]-N\textsuperscript{1},N\textsuperscript{8}-bis(2,3-dimethoxybenzoyl)spermidine (27).
Figure 52. IR Spectrum of (L)-(N-[3-(2,3-Dihydroxybenzamido)propyl])-N-[4-(2,3-dihydroxybenzamido)butyl]-2-(2-hydroxyphenyl)trans-5-methyloxazoline-4-carboxamide (Parabactin) (30).
Figure 53. IR Spectrum of (L)-N\textsuperscript{4}-Threonyl-N\textsuperscript{1},N\textsuperscript{7}-bis(2,3-dihydroxybenzoyl)norspermidine Hydrobromide (38).
Anal. calcd. for C_{31}H_{34}N_{0}O_{9}: C, 61.38; H, 5.65; N, 9.24. Found: C, 61.57; H, 5.47; N, 9.22.

Synthesis of Parabactin, Enantioparabactin, and the Homo and Nor Homologs of Parabactin

Since Tait's isolation of parabactin, a considerable amount of effort has gone into the synthesis of this siderophore (4,32). A large number of polyamine catecholamides have been prepared by several groups in recent years (30,41), but the synthesis of parabactin was only recently accomplished (32). It was desired to synthesize parabactin according to the scheme presented in Figure 54. In this manner, the corresponding homo and nor homologs of the siderophore could also be obtained. Using the scheme for the selective acylation of spermidine (Figure 8), it would be possible to synthesize parabactin by attaching the 2-hydroxyphenylthreonyloxazoline ring moiety to the N^{4} position of N^{1},N^{8}-bis(2,3-dimethoxybenzoyl)spermidine. However, one of the major problems associated with the synthesis of parabactin was the acid lability of the oxazoline ring. Attaching an intact 2-hydroxyphenylthreonyloxazoline ring to the N^{4} position of N^{1},N^{8}-bis(2,3-dimethoxybenzoyl)spermidine followed by demethylation of the catechol groups could be expected to result in the hydrolysis of the oxazoline ring during the acidic workup of the demethylation reaction. Therefore, it was decided to construct the threonine oxazoline ring as the last step in the synthesis of parabactin, after the catechol protecting groups have been removed.

The first step in the synthesis of parabactin involved attaching an appropriately protected L-threonine moiety to the N^{4} position of N^{1},N^{8}-bis(2,3-dimethoxybenzoyl)spermidine. The N-hydroxysuccinimide active
Figure 54. Synthesis of Parabactin.
Figure 54. (Continued)
ester of a protected L-threonine derivative was chosen as the acylating agent because of the ease of its formation, and the high yields usually associated with preparation and condensation of N-hydroxysuccinimide active esters (85). Additionally, it was thought that attaching a fairly large group such as an N-protected threonine to the already somewhat hindered secondary nitrogen of N1,N8-bis(2,3-dimethoxybenzoyl) spermidine would best be accomplished with a relatively small activating group such as N-hydroxysuccinimide. It was decided to use the t-butoxy-carbonyl (t-BOC) group for the protecting group of the amino function of L-threonine. The two major advantages of the t-BOC group for protecting amino groups of amino acids are that the t-BOC group lowers the susceptibility for racemization of the α-proton during condensation, and the t-BOC protecting group is very easily removed under mild conditions (85). The N-hydroxysuccinimide active ester of N-t-BOC-L-threonine was prepared in 90% yield by reacting N-t-BOC-L-threonine with dicyclohexylcarbodiimide and N-hydroxysuccinimide in THF. The active ester was then condensed with N1,N8-bis(2,3-dimethoxybenzoyl)spermidine to form (27) in an 84% yield using dimethylformamide as the solvent. The t-BOC group of (27) was then removed by a brief exposure to trifluoroacetic acid to yield (28) in a 95% yield. The removal of the methyl protecting groups of (28) was accomplished with boron tribromide in methylene chloride to yield the catecholamide (29).

The final step in the synthesis of parabactin required the cyclization of the threonine moiety into a heterocyclic oxazoline ring. Tait had demonstrated the configuration of the threonine moiety of the natural product to be the L-configuration (19). Neilands later supported this finding by showing that the alpha and beta protons of the oxazoline ring
were trans to one another (41). In the last step of the synthesis it was critical to effect the cyclization of the oxazoline ring while maintaining the stereochemical integrity of the threonine moiety. This requirement posed somewhat of a problem since the most common method of preparing oxazolines from alpha-beta amino alcohols involves first the acylation of the amino group with a carboxylic acid derivative followed by exposing the adduct to thionyl chloride (86). Besides the harsh reaction conditions employed, cyclization via this method proceeds to yield cis-oxazoline derivatives of threonine. Application of this scheme would, of course, lead to the L-allothreonine derivative of parabactin, if anything at all. One could attempt to circumvent this problem by using the much more costly N-t-BOC-D-threonine in the initial stages of the synthesis. Cyclization of the N-salicyl-D-threonine derivative of (29) with thionyl chloride would yield the D-allothreonine derivative of parabactin. Epimerization of this compound could be attempted with base.

A more practical approach to this problem was accomplished by cyclizing (29) with 2-hydroxybenzimidido ethyl ether (84). This imidate was prepared by reacting 2-hydroxybenzonitrile with ethanol and HCl. The hydrochloride of the resulting imidate was converted to the free imino ester with bicarbonate. Thus, 2-hydroxybenzimidido ethyl ether was reacted with (29) in refluxing methanol yielding parabactin in an 85% yield. The configuration of the L-threonine moiety is unaffected by this condensation.

In addition, the corresponding homo and nor homologs of parabactin were also prepared. Since recent evidence has suggested that microbial iron uptake systems may be stereospecific (73,77), the enantiomer of parabactin, enantioparabactin, was synthesized. The synthesis of
Figure 55. 300 MHz 1H NMR Spectrum of Parabactin in 10:1 d-CHCl3, d6-DMSO.
Figure 56. 300 MHz $^1$H NMR Spectrum of the Gamma Methyl Group of Parabactin in $d_6$-DMSO.
Figure 57. 300 MHz $^1$H NMR Spectrum of the Alpha Methine of Parabactin in $d_6$-DMSO.
Figure 58. 300 MHz $^1$H NMR Spectrum of the Gamma Methyl Group of Parabactin in 10:1 d-CHCl$_3$ d$_6$-DMSO.
Figure 59. 300 MHz $^1$H NMR Spectrum of the Alpha Methine of Parabactin in 10:1 d-$\text{CHCl}_3$ 
d$_6$-DMSO.
enantioparabactin was accomplished using N-t-BOC-D-threonine instead of N-t-BOC-L-threonine in the above synthesis.

Results

Conformational Isomerism of the Free Ligand

The 300 MHz $^1$H NMR spectrum of synthetic parabactin in 10:1 CDCl$_3$/d$_6$-DMSO (Figure 55) was very similar to the tabulated NMR data reported by Neilands of the natural product. The NMR spectrum of synthetic parabactin also suggested the presence of more than one conformer.

It should be pointed out that the $^1$H NMR spectrum of parabactin is extremely sensitive to solvent and temperature changes e.g., when run in d$_6$-DMSO the spectrum is substantially simpler than when run in CDCl$_3$ or 10:1 CDCl$_3$/d$_6$-DMSO. When the $^1$H NMR of parabactin is run in d$_6$-DMSO at 23°C, the γ methyl i.e., the methyl fixed to the oxazoline ring, corresponds to four lines - two sets of doublets, one centered at δ 1.40 and one centered at δ 1.36 with $J_{\alpha,\beta} = 6.3$ Hz (Figure 56). In d$_6$-DMSO at 23°C the α-methine signals are centered at δ 4.89 and consist of five lines (Figure 57). On a change of solvent to 10:1 CDCl$_3$/d$_6$-DMSO in order to avoid the line-broadening problems associated with low-temperature induced viscosity changes with d$_6$-DMSO, the spectrum revealed several additional lines at 23°C. The α-methine multiplet was now centered at δ 4.59 although it still consisted of five lines. However, the γ-methyl signal consisted of six lines, three sets of doublets with identical coupling constants (Figure 58). Furthermore, when the sample is cooled to -13°C, the five lines of the α-methine become six lines, three sets of doublets (Figure 59), with identical coupling constants ($J = 6.5$ Hz). The γ methyls are not nearly as sensitive to cooling. When the β-methine is decoupled, the five α-methine lines collapse to three lines just as the six γ-methyl lines collapse to three lines.
The remainder of the spectrum, when taken in 10:1 CDCl₃/d₆-DMSO, is as expected. The six internal methylene protons of the spermidine backbone are in a δ 2.03-1.48 envelope while the eight amide methylene protons are under a δ 3.78-3.11 envelope. The β-proton signal is a complex envelope extending from δ 5.45 to 5.28. The aromatic proton signals consist of five well-separated envelopes: δ 6.52-6.67 (2H), 6.78-6.92 (4H), 7.06-7.15 (2H), 7.27-7.35 (1H), 7.51-7.71 (1H). Finally, the NH and OH protons are as described by Neilands with the NH and nonhydrogen-bonded OH protons at δ 7.89-8.17 and the hydrogen-bonded protons at δ 11.56-12.82.

**Stoichiometry of Chelate**

The stoichiometry of the parabactin-gallium chelate was determined by titrating a basic solution of parabactin in D₂O with Ga(NO₃)₃·9 H₂O in D₂O and observing the increase in integrated intensity of the gamma-methyl signal of the resulting complex. Figure 60 shows a plot of the integrated intensity of the gamma methyl signal of the parabactin-gallium complex against the [gallium]/[parabactin] ratio. The break in the linear plot at a [gallium]/[parabactin] ratio of 1.0 is consistent with parabactin forming a 1:1 complex with gallium.

**Amide Protons**

In d₆-DMSO four distinct amide NH resonances are observable for the parabactin-gallium chelate, Figures 61 and 62 show a set of large peaks centered at 11.04 and 10.78 ppm, and a smaller pair of signals at 11.11 and 10.33 ppm. The two peaks comprising each set integrate equally, while a comparison of the total integrated area between the two sets reveals a 3:1 ratio. All four of these amide NH resonances consist of a set of fairly well-resolved doublets, each multiplet
Figure 60. Titration of Parabactin with Gallium(III) in D₂O.
Figure 6. 300 MHz $^3$H NMR Spectrum of the Amide Region of the Gallium(III) Chelate of Parabactin in $d_6$-DMSO.
Figure 62. 300 MHz $^1$H NMR Spectrum of the Amide Region of the Gallium(III) Chelate of Parabactin in D$_2$O.
arising from separate splitting by the two geminal protons on adjacent carbon atoms. By irradiating the appropriate individual methylene protons, the coupling constants for each amide signal were obtained (Table 3). Finally, the temperature dependence of the four amide proton chemical shifts was examined in d<sub>6</sub>-DMSO, and the slopes of the linear chemical shift vs temperature plot for each amide NH is summarized in Table 3.

Unlike the <sup>1</sup>H NMR spectrum of the parabactin-gallium complex in d<sub>6</sub>-DMSO where four distinct amide NH signals were observed, the spectrum of the chelate in D<sub>2</sub>O revealed only a single amide proton. This single amide NH resonance is a well-resolved doublet of doublets corresponding to an AMX spin system with J<sub>ax</sub> = 8.4 Hz and J<sub>mx</sub> = 4.3 Hz. Like the amide protons observed in d<sub>6</sub>-DMSO, this signal occurs at an unusually low field for amide protons, 10.62 ppm. Integration of this multiplet shortly after preparation of the sample indicated only 0.7 protons. The signal completely disappears after two or three days in D<sub>2</sub>O.

**Oxazoline Ring Protons: Alpha, Beta, and Gamma Protons**

The gamma methyl protons of the parabactin gallium chelate in D<sub>2</sub>O at 22°C appear as a sharp doublet centered at 0.62 ppm with a J<sub>Y,B</sub> of 6.2 Hz. The alpha and beta methine signals occur at similar shifts and are almost completely obscured by the HOD peak. However, on heating the sample to 30°C, the HOD resonance moves upfield revealing a complex multiplet centered at 4.79 ppm. This multiplet is simplified into two overlapping AB quartets when the gamma methyl peak is decoupled. A computer simulation was utilized to extract the chemical shifts and coupling constants of the A and B members comprising the two AB quartets. It was thus determined that the chemical shift of the alpha methylene
Table 3.
Amide NH Proton Data for the Parabactin-Gallium(III) Chelate

<table>
<thead>
<tr>
<th>NMR solvent</th>
<th>chemical shift, ppm</th>
<th>slope of plot of chemical shift vs temperature (ppm/°C)(10^3)</th>
<th>coupling constants, Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>(d_6)-DMSO</td>
<td>11.11</td>
<td>0.80</td>
<td>3.9, 8.3</td>
</tr>
<tr>
<td></td>
<td>11.04</td>
<td>0.37</td>
<td>3.7, 6.7</td>
</tr>
<tr>
<td></td>
<td>10.78</td>
<td>0.22</td>
<td>3.8, 7.1</td>
</tr>
<tr>
<td></td>
<td>10.33</td>
<td>0.18</td>
<td>3.2, 7.8</td>
</tr>
<tr>
<td>(D_2O)</td>
<td>10.62</td>
<td>not measured</td>
<td>4.3, 8.4</td>
</tr>
</tbody>
</table>
proton is 4.82 ppm in the minor set and 4.80 ppm in the major set of quartets. Likewise, the chemical shift of the beta methine proton was determined to be 4.78 ppm and 4.76 ppm, for the minor and major sets respectively, while $J_{\alpha,\beta}$ is 2.5 Hz for both sets of AB quartets.

The parabactin gallium chelate's $^1$H NMR spectrum in $d_6$-DMSO reveals the $\gamma$-methyl group as a pair of partially overlapping doublets (Figure 63). Decoupling the beta methine resonance afforded the chemical shift values of 1.05 and 1.09 ppm for the minor and major gamma methyl signals respectively, while computer simulation provided the coupling constant $J_{\alpha,\beta} = 6.4$ Hz for both doublets. At 22°C in $d_6$-DMSO the alpha methine appears as a pair of doublets. The minor doublet is located at 5.40 ppm with $J_{\alpha,\beta} = 2.3$ Hz, while the major doublet is found slightly upfield at 5.37 ppm with the same $J_{\alpha,\beta}$ as the minor doublet. The beta methine proton emerges as a complex multiplet which is simplified into two doublets upon irradiation of the gamma methyl resonance. The chemical shift of these two doublets is 5.22 and 5.19 ppm, corresponding to the minor and major sets, respectively.

**External Methylene Protons**

The signals falling in the region between 3.0 and 4.4 ppm in the $^1$H NMR spectrum of the parabactin-gallium chelate in $D_2O$ are assigned to those eight methylene protons of the spermidine chain which are adjacent to nitrogen, protons on C(1), C(3), C(5), and C(8). Examination of the signals in this area reveals several well-resolved multiplets (Figure 64). The nonequivalence of the geminal methylene protons is very clear. There is enough separation between the signals such that double resonance experiments could provide a detailed assignment of this region complete with geminal and vicinal coupling constants. Irradiation of
Figure 63. 300 MHz $^1$H NMR Spectrum of the Gamma Methyl Group of the Gallium(III) Chelate of Parabactin in $d_6$-DMSO.
Figure 64. 300 MHz $^1$H NMR Spectrum of the External Methylene Region of the Gallium(III) Chelate of Parabactin in D$_2$O.
the long amide NH signal in D₂O led to a partial collapse of the multiplets at 3.96 and 3.27 ppm. These two peaks can therefore be assigned to the methylene protons on one of the terminal methylene carbons C(1) or C(8); however, differentiation between the two possibilities cannot be made from these data alone. In the spectrum of the gallium chelate in which all amide protons have been completely replaced with deuterons, the peaks at 3.96 and 3.27 ppm appear as two apparent quartets, each proton split once by a nonequivalent geminal proton and again, by one of the two vicinal protons on the adjacent carbon. Irradiation of the apparent quartet at 3.96 ppm reduced the multiplet at 3.27 ppm into a doublet with a $J_{\text{vic}}$ of 8.8 Hz. Decoupling the peak at 3.27 ppm reduced the signal of 3.96 ppm to a doublet with $J_{\text{vic}}$ of 5.7 Hz. Decoupling the multiplet at 4.26 ppm produced a simplification of the overlapping resonance at 3.09 ppm (Figure 65). This second pair of geminal protons was found to be separated by 360 Hz. This irradiation provided a single vicinal coupling constant of 4.4 Hz for the upfield portion of the overlapping peaks at 3.09 ppm. Decoupling the region at 3.09 ppm reduced the multiplet at 4.26 ppm into a doublet of doublets, corresponding to vicinal coupling constants of 3.4 and 10.5 Hz. A third set of geminal methylene protons was discovered by irradiating the upfield portion of the overlapping set of peaks corresponding to three protons at 3.6 ppm, reducing the downfield side of the resonance at 3.1 ppm into an apparent triplet. The apparent triplet was really two doublets of equal vicinal coupling constant of 5.5 Hz. Due to the extensive overlapping of the resonances in the 3.6 ppm region, no further information regarding vicinal coupling constants would be obtained. However, process of elimination dictates the two remaining geminal methylene protons yet
Figure 65. External Methylene Region of Parabactin-Ga(III) in D₂O (above). Irradiation of Multiplet at 4.26 ppm Yields Simplication of Multiplet at 3.09 ppm.
to be assigned must lie in the downfield portion of this overlapping region. Figure 66 shows the geminal relationships between the eight external methylenes obtained by the above decoupling experiments.

Close examination of the multiplets comprising the external methylene proton region reveals the presence of a second, smaller set of methylene signals. Such duplicity of proton resonances in the spectrum of the parabactin-gallium chelate has been encountered in all previous regions described so far; however, the presence of such additional signals, presumably arising from a second parabactin-chelate species, is not quite so obvious in this region of the $^1$H NMR spectrum. Inspection of this spectral region (Figure 64) indicates the second set of signals are not really duplications of the major set of multiplets simply shifted to a lower or higher field, although a rough chemical shift correlation between individual minor and major multiplets does appear to exist. No quantitative assignment involving correlation of minor or major signals was undertaken due to the poor resolution of these signals. In fact, even the most qualitative analysis of the multiplicity in the smaller set of signals was precluded due to the general lack of resolution in the smaller multiplets and their partial overlap with the major external protons' resonances. However, an integration of the multiplets comprising this region demonstrates the presence of a second chelate species quite clearly. The integration of the multiplets in this region is summarized along with chemical shifts for both major and minor species in Table 4. An extensive study of the smaller set of external proton multiplets, although desirable, was not critical to the evaluation of the parabactin-gallium chelate's solution conformation since a large amount of information still could be obtained by studying the major set of resonances.
Figure 66. Geminal Relationships of All Eight External Methylene Protons of Parabactin-Ga(III) in D$_2$O.
Table 4.
"External" Methylene Proton Data for the Parabactin-Gallium(III) Chelate

<table>
<thead>
<tr>
<th>chemical shift, ppm</th>
<th>integration, protons</th>
<th>vicinal coupling constants, Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.26</td>
<td>0.7</td>
<td>10.5, 3.4</td>
</tr>
<tr>
<td>4.05</td>
<td>0.15</td>
<td>-</td>
</tr>
<tr>
<td>3.96</td>
<td>0.7</td>
<td>5.7</td>
</tr>
<tr>
<td>3.84</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>3.45-3.75</td>
<td>2.6</td>
<td>-</td>
</tr>
<tr>
<td>3.27</td>
<td>1.1</td>
<td>8.8</td>
</tr>
<tr>
<td>3.14, 3.09</td>
<td>1.7</td>
<td>5.5, 4.4</td>
</tr>
<tr>
<td>2.99</td>
<td>0.8</td>
<td>-</td>
</tr>
</tbody>
</table>

*Unable to measure
Internal Methylenes and Double Resonance Difference Spectroscopy

In D$_2$O, protons on the internal carbons of the spermidine chain, specifically C(2), C(6), and C(7) fall in a region between 2.2 and 1.6 ppm which integrates out to six protons (Figure 67). Although the complexity of overlapping peaks in this region rendered analysis by conventional double resonance experiments all but useless, considerable information was gained from the difference spectra obtained by irradiation of external methylene proton multiplets. Double resonance difference spectroscopy (87,88) was utilized to obtain the approximate chemical shifts for the six overlapping internal methylene protons. Difference spectra were generated by subtraction of the spectra obtained during irradiation of individual external protons from spectra in which the decoupling frequency was set off resonance. In this way, resonances of all internal methylene protons not coupled to the irradiated external methylene proton will cancel out in the spectrum subtraction. Coupling between an internal methylene proton and the irradiated external methylene proton will be indicated by the presence of a large perturbation in the difference spectrum. The center of these perturbations represents the chemical shift of the internal methylene proton coupled to the irradiated external methylene proton. Thus, from the major perturbations in the difference spectra, approximate chemical shifts were elucidated for all six internal methylene protons and are listed in Table 5. An example of the technique is shown in Figure 68 where irradiation of the external proton multiplet at 4.26 ppm leads to a single major perturbation in the difference spectrum centered at 2.03 ppm. This indicates the external methylene proton at 4.26 ppm is coupled to the internal methylene proton at 2.03 ppm.
Figure 67. Internal Methylene Region of the Parabactin-Ga(III) Chelate in D2O.
Table 5.
Double Resonance Difference Spectral Data for Parabactin-Gallium(III) Chelate in D$_2$O

<table>
<thead>
<tr>
<th>$f_2$, ppm</th>
<th>ppm of perturbation in resultant difference spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.26</td>
<td>2.03</td>
</tr>
<tr>
<td>3.96</td>
<td>1.98</td>
</tr>
<tr>
<td>3.67</td>
<td>2.11</td>
</tr>
<tr>
<td>3.55</td>
<td>1.77 1.63</td>
</tr>
<tr>
<td>3.27</td>
<td>2.03</td>
</tr>
<tr>
<td>3.11</td>
<td>1.98 1.78 1.63</td>
</tr>
</tbody>
</table>
Figure 68. Example of Double Resonance Difference Spectroscopy Where the External Methylene Multiplet at 4.26 ppm Has Been Decoupled.
Although exact chemical shift values cannot be elucidated, it is still possible to determine by inspection if two perturbations in the difference spectra occur at different frequencies. For example, Figure 69 shows that, although one might have difficulty in determining the center of the perturbations in spectra e and f, it is clear that the two perturbations do occur at two different frequencies.

Discussion

Conformational Isomerism of the Free Ligand

Parabactin's $^1$H NMR spectrum showed three sets of gamma-methyl doublets in 10:1 CDCl$_3$/d$_6$-DMSO at 23°C. The alpha methine multiplet in this solvent is characterized by five lines at 23°C which increase to six lines, three sets of doublets, upon cooling to -13°C. The alpha methine multiplet in d$_6$-DMSO revealed five lines at 23°C, presumably three sets of doublets with two of the doublets partially overlapping. It appears that parabactin exists in at least three different isomeric species in the solvents examined.

Although the included data cannot define the exact nature of this isomerism, certain deductions can be made concerning its origin. All three of the isomers exhibit $\alpha,\beta$ coupling constants of approximately 6.8 Hz consistent with trans coupling. Therefore, the possibility that one of the isomers is a cis-oxazoline ring is unlikely (20,41). It is equally unlikely that one of the three isomers is the product of oxazoline ring hydrolysis resulting in an open form of parabactin due to the well-established differences in chemical shifts and coupling constants of the alpha, beta and gamma protons in the open and closed forms of the siderophore (41). The evidence clearly suggests that the siderophore exists as three different, interconverting conformers (32,41).
Figure 69. Double Resonance Difference Spectra Generated by Subtraction of Spectra Obtained During Irradiation of Individual External Methylene Protons from Spectra in Which the Decoupling Frequency Was Set Off Resonance. Arrows Indicate Chemical Shifts of Internal Methylene Protons. \( F_2 \) set at: a, 3.11; b, 3.27; c, 3.55; d, 3.67; e, 3.96; f, 4.26, ppm.
Two suggestions as to the origin of the conformational isomerism resulting in at least two major solution conformers involve: 1) an approximate 180° rotation about the N°-carbonyl carbon-alpha carbon (73) bond, and 2) -180° rotation about the N°-carbonyl carbon tertiary amide bond in a classical cis-trans amide bond isomerism (41). As expected, the long homologue of parabactin displayed no duplicity in its 1H NMR spectrum.

An attempt was made to evaluate the various intramolecular interactions responsible for establishing the energy barrier that must be overcome for interconversion between the conformers. As previously noted, the 1H NMR spectrum of parabactin is extremely sensitive to the particular solvent used. Changes in both chemical shift and conformer population occur upon relatively small change in the solvent. This situation is typified by the addition of small amounts of d6-DMSO to a CDCl3 solution of parabactin. Two sets of gamma methyl signals exist in approximately a 7 to 1 ratio in CDCl3. Addition of small amounts of d6-DMSO to the sample results in a steady decrease in this ratio until the spectrum of parabactin in neat d6-DMSO shows the gamma-methyl peaks in an approximate 1 to 1 ratio. Such a solvent dependency on conformer population suggests that intramolecular hydrogen bonding plays a role in determining the interconversion between conformational isomers. Since CDCl3 has relatively poor hydrogen bond donor and acceptor abilities, a large degree of intramolecular hydrogen bonding could be expected to occur in CDCl3 solutions of the siderophore; thus, the conformer population in this solvent would be largely influenced by intramolecular hydrogen bonds. The addition of a good hydrogen bond acceptor, DMSO, would compete for hydrogen bond donor sites, leading to a disruption of intramolecular hydrogen bonding. The disruption of the weaker intramolecular hydrogen
bonds by d$_6$-DMSO could explain the change in conformer population observed during the titration.

The magnitude of the internal rotational energy barrier that must be overcome for interconversion between the conformers of parabactin was determined by measuring the coalescence temperature, $T_c$, of the two gamma-methyl signals in d$_6$-DMSO. Thus, the energy of activation ($E_a$) for the interconversion between conformers can be calculated by the method of Gutowsky and Cheng (82). Any intramolecular hydrogen bonding present will result in an increase in this energy barrier since interconversion between conformers is likely to require the breaking of some of these bonds. To elucidate the contribution to the rotational energy barrier by the hydrogen bond donating ability of the catechol groups, a tetramethylated derivative of parabactin was synthesized and its $T_c$ measured. Methylation of the catechols will eliminate hydrogen bond donation by these groups. As expected, elimination of the hydrogen bonding component to the internal rotational barrier resulting from hydrogen bonding by catechol groups resulted in a markedly lower coalescence temperature. The tetramethylated derivative of parabactin displayed a coalescence temperature of 82°C ± 1 which corresponded to an activation energy, $E_a$, of approximately 19.3 ± 0.2 kcal/mole. The exact coalescence temperature of parabactin's gamma-methyl groups was found to be greater than the operating limits of our probe, preventing an accurate measurement of $T_c$ and thus, $E_a$. It was reported earlier that at temperatures around 110°C the beta decoupled gamma-methyl signal of parabactin could be observed as a single peak (32). Although the beta decoupled gamma methyl signals of the conformers are merging into one peak, the true coalescence point has not yet been achieved at this
temperature. This is evidenced by the unsymmetrical nature of the beta decoupled gamma signal. An examination of the nondecoupled spectra of the two gamma methyl doublets better demonstrates this point, where it can be seen that the two gamma doublets are not yet completely superimposed on each other at 110°C. However, the fact that coalescence is beginning at 110°C allows us to roughly estimate the lower limit of $T_c$ and hence, $E_a$, of rotation. At 110°C the peak width at half-height, $\delta v$, is 4.5 Hz which corresponds to an $E_a$ of rotation of 21 kcal/mole. Of course, since $E_a$ varies directly with temperature and inversely with $\delta v$, further increases in temperature with concomitant decreases in $\delta v$ will lead only to an increase in the value of $E_a$, rotation. Thus, the estimate on $E_a$ for conformer interconversion of 21 kcal/mole is certainly the lower limit of the true activation energy.

If the assumption that the hydrogen bonding contribution of the catechol groups to the conformational barrier has been removed without introducing any other large perturbations is valid, then it is obvious that the hydrogen bond donating capacity of these catechol groups plays an important role in conformational isomerism of parabactin. The author wishes to emphasize that the relative comparison of $E_a$'s was required; therefore, total line shape analysis was not necessary.

**Amide Protons**

In $d_6$-DMSO the amide protons of the free catecholamide parabactin overlap in a narrow, complex multiplet between 9.2 and 9.4 ppm, while the NH signals of the parabactin-gallium chelate are considerably more deshielded and appear over a wider range between 10.2 and 11.3 ppm. The difference in frequency between the two amide NH signals comprising the major set, 0.26 ppm, and the minor set, 0.78 ppm, both exceed the
separation in frequency between amide protons of the free ligand. These observations clearly indicate the parabactin-gallium chelate is present as two species, in which a considerable difference in magnetic environments of the amide protons exists between these species.

The extreme downfield shifts experienced by the amide NH protons on going from parabactin to its gallium chelate, up to 1.9 ppm, and the fairly large distance between the metal center and amide NHs suggest some phenomenon beyond the metal charge is contributing to the net deshielding effect of these protons. It may be that a perturbation occurs in the electronic state of the amide bonds upon metal chelation resulting in a decreased electron density on the amide hydrogens.

The coordination of gallium could result in an electron delocalization in the 2,3-dihydroxybenzamido groups, maximizing conjugation between the aromatic ring and the carbonyl carbon p-orbitals. This would decrease the importance of the amide resonance form responsible for maintaining the planarity of the amide linkage, rendering rotation about the C-N amide bond more feasible (89). Examination of the various possible structures of the gallium chelate using CPK molecular models indicates a preference for the adoption of a nonplanar amide bond, where the carbonyl group maintains planarity with the aromatic ring, but omega (90) is significantly altered from its normal angle of 180°. Yan has calculated that the electron density on the amide hydrogen decreases as an amide bond is rotated out of its planar configuration towards 90° (91). The deshielding of the amide NH protons that would result from such a decrease in electron density might help explain, in part, the large downfield shifts experienced by the amide protons of parabactin on formation of its gallium chelate.
The most striking feature in the $^1$H NMR spectrum of the parabactin-gallium chelate when observed in D$_2$O is the presence of only one detectable amide proton signal. The fact that three of the four amide protons are extremely quick to undergo exchange in D$_2$O, while a fourth is so slow to exchange, clearly indicates at least one amide NH has limited solvent accessibility. This may be a result of steric shielding, or intramolecular hydrogen bonding, or both.

Table 3 lists the slopes of the chemical shift vs temperature plot of the four amide NH hydrogens in d$_6$-DMSO. Amide hydrogens that are less accessible to the solvent due to intramolecular hydrogen bonding or are sterically buried display smaller slopes in such plots than amide protons that are able to interact with the solvent (92-94). All four of the amide NH protons of the parabactin-gallium chelate suggest limited solvent accessibility, as their slopes are smaller than that of N-methylacetamide, which showed a slope of 6 (°C/ppm)$^{103}$. The chemical shift vs temperature data suggest that, within each of the two sets of amide NH resonances, one of the N-H protons should exchange with the solvent more rapidly than the other. The low field resonance of the major set exhibits a slope of 0.37 (°C/ppm)$^{103}$ while the high field peak of the same set exhibits a slope of 0.22. Comparing the slopes of the signals comprising the minor set of NH hydrogens reveals a very large temperature dependence on the chemical shift of the low field signal of 0.80 (°C/ppm)$^{103}$, indicating a relatively large degree of interaction with the solvent. The high field peak of the minor set exhibits a slope almost four times smaller, 0.18 (°C/ppm)$^{103}$. From the slopes of the chemical shift vs temperature plot, it is apparent that all four of the amide NH hydrogens exhibit restricted interaction with the solvent, with the high
field signal of each set experiencing the least solvent accessibility. The proposal that the high field amide NH hydrogens experience the greatest restricted interaction with the solvent is confirmed by the addition of a small amount of D$_2$O to a d$_6$-DMSO sample of the parabactin-gallium chelate. All four amide signals persist for several hours with the peaks at 10.78 and 10.33 taking the longest to undergo complete exchange with D$_2$O.

In trying to ascertain whether or not intramolecular hydrogen bonding is responsible for the slow exchange of these amide protons, it is tempting to consider relative chemical shifts of the four amide protons in d$_6$-DMSO. The varying degree of deshielding experienced by the amide protons in d$_6$-DMSO evidenced by fairly large differences in their chemical shifts, suggests an approach to determine whether the restricted amide NH interaction with the solvent is a result of intramolecular hydrogen bonding. However, although it is accepted that intramolecular hydrogen bonding leads to low field $^1$H NMR shifts, any attempt at distinguishing the extent of intramolecular hydrogen bonding associated with each amide NH by their relative chemical shifts must be undertaken with extreme caution. Any deshielding contribution to the chemical shift of the amide protons due to intramolecular hydrogen bonding must be considered relative to those amide protons that are hydrogen bonded with the solvent. Since DMSO is a good hydrogen bond acceptor, hydrogen bonding between the solvent and those protons not involved in intramolecular hydrogen bonds could lead to a greater deshielding of those solvent exposed protons than protons involved in weak intramolecular hydrogen bonds (93-95).
External Methylene Protons

From CPK molecular model building it was obvious that the structure of the parabactin-gallium chelate would be rather inflexible, and severe restrictions in the rotation of the methylene units of the spermidine backbone could be expected. This was verified by the gallium chelate's $^1$H NMR spectrum. The geminal protons on each of the four external carbon atoms of the spermidine backbone, C(1), C(3), C(5), C(8) all exhibited markedly different chemical shifts (Figure 64). The difference in frequency separating geminal pairs was observed to be greater than 120 Hz for three of the four external methylenes with the largest frequency difference around 360 Hz. This strongly suggests a high degree of rigidity exists in the methylene backbone of the gallium chelate compared to that of the free ligand.

The external methylene protons of the parabactin gallium chelate are spread out over a larger area than the same external methylene protons of the free ligand in $d_6$-DMSO, or of the penta-anion of parabactin in $D_2O$. This is an indication that the individual methylene protons in the gallium complex are experiencing substantially different magnetic environments than those of the corresponding protons of the polyanion or free ligand. Upon chelation of gallium, the severe rotational restrictions imposed on the spermidine chain serves to "freeze" the molecules' conformation such that individual geminal protons on each methylene carbon now find themselves in different magnetic environments resulting in different chemical shifts.

If the spermidine chain methylene carbons were free to rotate in the gallium chelate as they are in the free ligand, the chemical shift of the methylene protons would reflect the average of the different
magnetic environments experienced by the various rotamers. The chemical shifts of the external methylene protons of the gallium complex in D$_2$O fall over a 1.3 ppm area between 3.1 and 4.4 ppm, whereas the same protons of the penta-anion fall between 3.3-3.8 ppm, over a much smaller area, 0.5 ppm. If one averages the two chemical shift values between each of the four geminal pairs of external methylene protons in the gallium complex in D$_2$O, the range of these averaged chemical shifts is 3.3 to 3.7 ppm, almost identical to the range of the methylene protons of the penta-anion in D$_2$O. In other words, the chemical shifts of geminal pair of protons at 4.3 and 3.1 ppm would average out to 3.7 ppm. Likewise, the geminal pair at 3.5 and 3.1 ppm would average out to 3.3 ppm. The upfield and downfield chemical shifts experienced by geminal protons on the external carbons upon metal chelation could thus be a result of the chelate's relatively "frozen" structure. Such a rigid structure would place the individual methylene protons in shielding or deshielding regions of the adjacent amide groups or adjacent C-C bonds and result in exaggerated chemical shifts between geminal pairs.

The $^1$H NMR data of the parabactin gallium chelate point to inflexibility of the spermidine backbone. The data further suggest that the propyl chain of the spermidine backbone is more restricted to internal C-C rotation than the butyl chain. The difference in frequency between geminal protons on the propyl chain, 360 and 210 Hz, far exceeds the difference between geminal protons on the butyl chain, 120 and 45 Hz. This is consistent with a situation where increased internal C-C rotation by the butyl chain results in an increased averaging out of the chemical shifts of the geminal protons. In addition, upon warming a D$_2$O sample of the parabactin-gallium chelate to 40°C, the only external methylene
signals to undergo a detectable change in chemical shift are the multiplets of 3.55 and 3.60 ppm. These butyl chain protons correspond to the geminal pair on either C(5) or C(8). Additionally, the intramolecular hydrogen bond between the propyl amide proton and the N\textsuperscript{*} carbonyl oxygen would certainly be expected to help in "freezing" the propyl side of the spermidine chain. While the butyl methylene chain can undergo a limited amount of internal rotation, CPK molecular models clearly show an extremely rigid propyl chain.

The well-resolved multiplets of the external methylene protons offer an opportunity for the elucidation of dihedral angles between the hydrogens of the spermidine chain via an application of the Karplus equation. In the past few years a number of Karplus curves have been proposed to correlate the angular dependences of the three bond homonuclear coupling constants $^3J_{\text{NHCH}}$ and $^3J_{\text{CHCH}}$ (96-98). Establishing a relationship between $^3J_{\text{CHCH}}$ and torsional angles is, however, complicated by the fact that $^3J$ is not only dependent on the dihedral angle between the two protons, but also on the electronegativity and orientation of adjacent substituents present in addition to the C-H angles and length of the HC-CH bond (96). Therefore, attempts at quantitatively relating torsional angles with coupling constants frequently requires a particular treatment for each type of chemical system examined. Notwithstanding these difficulties, three Karplus equations were employed in an attempt to determine the approximate dihedral angles of the propyl chain by $^3J_{\text{NHCH}}$ and $^3J_{\text{CHCH}}$.

The equation used to determine dihedral angles between the propyl chain amide hydrogen and adjacent protons of C(1) was

$$^3J(\theta) = 6.55 \cos^2 \theta - 1.55 \cos \theta + 1.35 \quad (97)$$
while the following equations were used to calculate the CH-CH dihedral angles between protons on C(1)-C(2) and C(3)-C(2)

\[ ^3J(\theta) = 10.0 \cos^2 \theta - 1.0 \cos + 2.0 \]  

(99)  

\[ = 9.4 \cos^2 \theta - 1.4 \cos + 1.6 \]  

(100)  

Throughout the Karplus analysis, referral was made to CPK models to eliminate unlikely choices of \( \theta \) generated by the equations.

In the elucidation of the orientation of the propyl methylene chain it was found that Karplus equation (1) provided a relatively clear distinction between the possible calculated values of the dihedral angles. These calculated dihedral angles led to an orientation of the propyl methylene chain that was very similar to the orientation suggested by the CPK molecular models.

A combination of the calculated dihedral angles and the orientation of the propyl chain suggested by CPK models enables the assignment of pro-R and pro-S methylene protons. The validity of the assignment of the absolute configuration at each propyl chain carbon, and thus the Karplus equation used to help elucidate this assignment can be checked by referring to the double resonance difference spectral data. This verification can be carried out since the double resonance difference spectra reveals which of the individual, nonequivalent protons on each of the propyl chain carbons are coupled to, or not coupled to, adjacent protons. It was found that the assignment of the absolute configuration at each propyl chain based on Karplus equation (1) did not agree with the results of the double resonance difference experiments.

For example, Karplus equation (1) and the CPK models suggest the orientation depicted in Figure 70 to exist about the C(3)-C(2) bond. From equation (1), it was determined that proton \( H_1 \) (4.26 ppm) is
<table>
<thead>
<tr>
<th></th>
<th>J, Hz</th>
<th>Angle Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₁ - H₃</td>
<td>10.5</td>
<td>13°</td>
</tr>
<tr>
<td>H₁ - H₄</td>
<td>3.4</td>
<td>109°</td>
</tr>
<tr>
<td>H₂ - (either H₃ or H₄)</td>
<td>4.4</td>
<td>116°</td>
</tr>
</tbody>
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Figure 70. Orientation About the C(3)-C(2) Bond Suggested by Karplus Equation (1).
coupled to adjacent $H_3$ ($J_{H_3H_3} = 10.5$ Hz, $13^\circ$), and to adjacent $H_4$ ($J_{H_3H_4} = 3.4$ Hz, $109^\circ$). With a coupling constant of $4.4$ Hz, $H_2$ is coupled to an adjacent proton, $H_3$ or $H_4$. From equation (1), this coupling constant corresponds to a dihedral angle of $116^\circ$. From Figure 70, it can be seen that $H_2$ must therefore be coupled to $H_3$. The double resonance difference spectra show that, when $H_3$ is irradiated, a single large perturbation is observed in the difference spectra in the internal methylene proton region at $2.03$ ppm. This large perturbation is, of course, caused by the elimination of a large coupling constant, which corresponds to $J_{H_3H_3} = 10.5$ Hz. The double resonance difference spectra also show that irradiation of proton $H_2$ ($3.09$ ppm) results in a single perturbation at $1.98$ ppm. If the hypothesis that $H_2$ is coupled to $H_3$ (as indicated by Karplus equation (1)) is correct, then the irradiation of $H_2$ would have caused a perturbation at $2.03$ ppm, not $1.98$ ppm.

Because of large errors, equation (2) did not even allow for the distinction between possible calculated values of dihedral angles. Thus, the orientation of the propyl chain using this equation could not be accomplished either. It is clear that neither Karplus equation (1) or equation (2) is applicable to the propyl chain methylenes of the parabactin-gallium complex. The reason for this is likely related to the role electronegativity plays in coupling.

**Internals and Double Resonance Difference Spectroscopy**

Double resonance difference spectroscopy provided the chemical shifts of all six internal methylene protons in addition to revealing which external multiplets are coupled to individual internal resonances (Figure 69). However, the information available from the double resonance difference spectra is not limited to chemical shift and vicinal
coupling information alone, but leads directly to the differentiation between those resonances belonging to protons on the propyl chain of the spermidine backbone from those protons on the butyl side.

The separate irradiation of two non-geminal methylene proton multiplets resulting in a single major perturbation at identical chemical shifts in the internal methylene region of their difference spectra can lead to only one conclusion: the two external methylene protons, and the internal methylene proton to which they are both coupled, all lie on the propyl chain of the spermidine backbone. This is exemplified in Table 5 and Figure 71 where it can be seen that irradiation of the two external methylene proton multiplets at 4.26 and 3.27 ppm both produce a single major perturbation in their difference spectra centered at 2.03 ppm. Thus, external protons resonating at 4.26 and 3.27 ppm as well as protons geminal to them, at 3.09 and 3.96 ppm, are all assigned to the protons belonging to the two external methylene carbons of the propyl side of the spermidine chain, while those multiplets located at 3.7, 3.6, 3.55 and 3.14 ppm must all belong to the external protons on the butyl side. Since the lone amide NH proton in D₂O is coupled to protons at 3.96 and 3.27 ppm, this amide NH is therefore on the propyl side of the spermidine backbone. This, of course, allows the assignment of those multiplets at 3.96 and 3.27 ppm to the geminal protons on C(1) while the peaks at 4.26 and 3.09 ppm must then correspond to the geminal pair on C(3).

**Stereochemistry**

Because of the unsymmetrical nature of the 2,3-dihydroxybenzoyl groups of parabactin, its gallium chelate can adopt either a cis or trans geometry. Furthermore, two different configurations of the chelating groups about the metal are possible. These are the Λ and Δ optical
Figure 71. Double Resonance Difference Spectra of Figure 69. Arrows Show Perturbations at Identical Chemical Shifts When Peaks at 3.27 and 4.26, ppm, are Decoupled.
isomers corresponding to a left or right handed "coordination propeller" about the metal atom (Figures 72,73). From CPK molecular model building it is clear that the trans geometrical coordination isomers are precluded on grounds of steric restraints; however, both of the cis optical isomers $\Lambda$ and $\Delta$ are structurally feasible (Figures 74,75). The situation is further complicated by the possibility of additional geometrical isomerism arising from the two unsymmetrical methylene units on either side of the $N^4$ nitrogen, which comprise the spermidine backbone. In other words, both optical isomers $\Lambda$ and $\Delta$ can exist in two different geometrical isomeric forms due to the asymmetry in the spermidine backbone. Figure 76 shows the two geometrical isomeric forms in which the $\Lambda$ cis optical isomers can exist.

In the first form, octahedral coordination positions 1 and 2 are occupied by the catecholamide group fixed to the propyl chain of the spermidine backbone, while coordination positions 3 and 4 are occupied by the catecholamide moiety attached to the butyl methylene chain. We will refer to this configuration as the $\Lambda$-cis-3,4 configuration. A second configuration of the $\Lambda$ cis optical isomer exists when coordination positions 1 and 2 are occupied by the catecholate of the butyl chain while the propyl chain catecholate occupies positions 3 and 4, just the opposite orientation of the above. This configuration is referred to as the $\Lambda$ cis-4,3 configuration. The two geometrical coordination forms of each optical isomer can be envisioned as interconvertible by an approximate 180° rotation of the spermidine backbone about the $N^4$-CO bond any time prior to the complexation by any two of the three chelating groups in the molecule. An analogous situation of course exists for the $\Delta$ optical isomer, leading to the formation of two diastereomeric $\Delta$ cis coordination isomers.
Figure 72. Representation of Λ Coordination Isomer.
Figure 73. Representation of $\Delta$ Coordination Isomer.
Figure 74. CPK Model of the Parabactin-Gallium(III) Chelate as the A Coordination Isomer, the Left-Handed "Coordination Propeller."
Figure 75. CPK Model of the Parabactin-Gallium(III) Chelate as the $\Delta$ Coordination Isomer, the Right-Handed "Coordination Propeller."
Figure 76. Representation of Λ-cis Coordination.
The asymmetry of ligands possessing chiral carbons leads to the formation of diastereomeric metal complexes in which one may be formed more favorably than others or sometimes, to the exclusion of all others. Such ligand stereospecificity is exemplified in the case of enterobactin, a catecholamide siderophore whose three L-serine units which make up its backbone impart such an asymmetric bias upon chelation that the \( \Delta \) optical coordination isomer is formed to the exclusion of its \( \Lambda \) diastereomer (11). From the above, one might expect the formation of the parabactin-metal complex also to result in the preference of one optical coordination isomer over the other. Neilands first obtained evidence to support this by comparing the CD spectrum of parabactin's iron-complex with the spectrum of the enterobactin-iron chelate, known to exist as the \( \Delta \) optical isomer (72). From the CD spectra of the two metal chelates, Neilands postulated that parabactin exists predominantly in the form of the \( \Lambda \) optical isomer (72). If Neilands' proposal that the parabactin metal chelate exists predominantly as the \( \Lambda \) optical isomer is correct, one would expect to encounter mainly, if not exclusively, isomers of the \( \Lambda \) \text{cis} variety, either the \( \Lambda \) \text{cis}-3,4 and/or the \( \Lambda \) \text{cis}-4,3 diastereomers. As discussed below, our \( ^1 \text{H} \) NMR data are in complete agreement with this expectation.

In \( d_6 \)-DMSO, the gamma methyl signal of parabactin appears as two doublets centered at 1.9 ppm, while the two gamma methyl signals of the parabactin-gallium chelate in the same solvent now occur further upfield at 1.0 ppm (Figure 77). This upfield shift of 0.9 ppm is easily understood after considering the different possible magnetic environments the gamma methyl protons can experience on formation of either \( \Delta \) \text{cis} or \( \Lambda \) \text{cis} optical coordination isomers. Inspection of CPK molecular models of the gallium chelate reveals that, on formation of the \( \Lambda \) \text{cis} optical
FREE LIGAND

Ga(III) CHELATE

Figure 77. 300 MHz $^1$H NMR Spectrum of the Gamma Methyl Region of Parabactin (Left), and Parabactin-Ga(III) (Right) in $d_6$-DMSO.
coordination isomer, the gamma methyl group is located directly in the shielding zone of one of the aromatic rings (Figure 78). In fact, both the $\Lambda$ cis-3,4 and $\Lambda$ cis-4,3 diasteromers possess a geometry in which the gamma methyl hydrogens are located in the shielding zone of an aromatic ring. Conversely, the gamma methyl group of the $\Delta$ cis optical isomer is not in the shielding zone of the aromatic and would not be expected to be deshielded relative to the $\Lambda$ cis coordination isomer (Figure 79). Therefore, the chemical shift of the gamma methyl protons strongly suggest the chelating groups around the gallium ion adopt the configuration of a left-handed coordination propeller, the $\Lambda$ form. These findings are in complete agreement with Neilands' initial proposal that the parabactin-iron chelate exists predominantly as the $\Lambda$ optical isomer. In fact, our studies reveal the $\Lambda$ optical isomer exists to the exclusion of any $\Delta$ coordination isomers. Therefore, the observation of two species in the $^1$H NMR spectrum of the chelate is not the result of a $\Lambda$-$\Delta$ isomerization. Instead, the $^1$H NMR data strongly support the expectation based on the stereochemical considerations discussed previously; that the gallium-chelate exists as a mixture of the two diastereomeric $\Lambda$ cis-coordination isomers.

Inspection of CPK models reveals that intramolecular hydrogen bonding can occur between the N$^4$ carbonyl oxygen and either the amide NH proton of the propyl chain or the amide NH of the butyl chain in the parabactin-gallium chelate. The question as to how the hydrogen bonding network can be assigned in each of the diastereomers, $\Lambda$ cis-3,4 or $\Lambda$ cis-4,3, will now be considered.

It was determined from double resonance difference spectral studies that the amide NH proton (10.62 ppm, D$_2$O) most resistant to exchange in
Figure 78. CPK Model of the Parabactin-Gallium(III) Chelate as the A Cis 3,4-diastereomer. Protons "A" Belong to the Gamma-Methyl Group and are Positioned in the Shielding Zone of a Nearly Aromatic Ring. Proton "B" Represents the Propyl Amide NH Proton.
Figure 79. CPK Model of the Parabactin-Gallium(III) Chelate as the $\Delta$ Cis 3,4-diastereomer Protons "A" Belong to the Gamma-Methyl Group and are not Positioned in the Shielding Zone of any Nearby Aromatic Rings. Proton "C" Represents the Butyl Amide NH Proton.
D$_2$O was the NH hydrogen belonging to the propyl chain amide in the major isomeric species. This proton corresponds to the signal at 10.78 ppm in d$_6$-DMSO. Integration of this proton or its "sister" resonance (11.04 ppm) and the external methylene proton multiplets that are coupled to it indicates that whichever diastereomer they correspond to exists in a 3:1 ratio over the minor isomer. That the "sister" peak, (11.04), which integrates out to the same area as the 10.78 ppm signal, corresponds to the butyl amide proton of the same molecule is further supported by the δ vs T plots. As expected, the chemical shift of the butyl amide is more sensitive to temperature changes than the propyl amide hydrogen. This is in keeping with its solvent accessibility.

Molecular models indicate that the major isomer must be the Λ cis-3,4 diastereomer as this is the only low energy system in which the central amide carbonyl can be hydrogen bonded to the propyl amide hydrogen. Similar considerations indicate that the minor component, the Λ cis-4,3, is so constrained that the butyl amide hydrogen is now hydrogen bonded to the central carbonyl oxygen. The butyl amide hydrogen of the Λ cis-4,3 isomer (10.33 ppm), like the propyl amide hydrogen of the Λ cis-3,4 isomer (10.62 ppm), display a smaller δ vs T slope and are much slower to exchange with D$_2$O in DMSO than the corresponding nonintramolecularly hydrogen bonded amide hydrogens.

This analysis results in the assignment of the upfield peaks of each set of amide NH signals as those forming the tightest intramolecular hydrogen bonding. This seems a little strange upon first consideration. However, other factors could be operating in the case of parabactin's gallium chelate, such as deshielding by the solvent of protons able to form intermolecular hydrogen bonds. Or, perhaps differing degrees of
nonplanarity of the terminal amide linkages result in the observed differences in chemical shift of the amide signals.

**Homochelate, Diastereomer Interconversion, A-A Racemization**

It is proposed that the two sets of signals observed in the $^1$H NMR spectrum of the parabactin-gallium chelate are due to the presence of two $\Lambda$ cis coordination diastereomers. Rotation of the spermidine chain about the $N^\alpha$-CO amide bond prior to chelation could result in the two diastereomeric chelates. The difference in the orientation of the spermidine chain of the two diastereomeric chelates results in differences in the magnetic environments of the protons of the chelate and hence, the presence of duplicate signals. Of course, if the spermidine chain was symmetrical, rotation about the $N^\alpha$-CO bond prior to chelation would result in two identical conformers, and only one set of NMR signals would be expected. In order to confirm the hypothesis that the duplicate NMR signals are the result of two $\Lambda$ cis diastereomers, the long symmetrical homologue of parabactin was synthesized and its gallium chelate was studied. Of course, one has to make the assumption that the long homologue of parabactin will display the same bias in metal chelation that parabactin shows. If the duplicate $^1$H NMR signals of the parabactin gallium chelate are indeed the result of two diastereomeric $\Lambda$ cis coordination isomers, then the $^1$H NMR spectrum of the long symmetrical chelate will display a single set of peaks. Conversely, if the duplicate $^1$H NMR signals of the parabactin gallium chelate are due to a mixture of $\Lambda$ cis and $\Delta$ cis optical isomers, then the long homologue chelate's spectrum would also show duplicate signals.

As expected, the $^1$H NMR spectrum of the long homologue's gallium chelate, in $D_2O$ and $d_6$-DMSO, shows only one set of resonances (Figure 80).
Figure 80. 300 MHz $^{1}J_{	ext{H,H}}$ NMR Spectrum of the Amide NH Protons of Homoparabactin in $d_{6}$-DMSO.
This of course lends further support to the suggestion that the duplicity in parabactin's gallium chelate spectrum is the result of the presence of two $\Lambda$ cis diastereomers. The observation of the single gamma methyl doublet at an upfield shift of 1.0 ppm $d_6$-DMSO is indicative that the long homologue forms the $\Lambda$ cis coordination isomer. It is interesting that alteration of the backbone chain length by one methylene did not change the preference of the ligand in adopting the $\Lambda$ optical isomer stereospecifically.

The $^1$H NMR data strongly suggest the presence of two $\Lambda$ cis diastereomeric coordination isomers. However, the question of whether they are interconverting remains to be addressed. It seems unlikely that the two diastereomeric chelates would undergo a rapid rate of interconversion in view of: 1) the high formation constants typical of $\text{Ga}^{+3}$ catecholamide complexes, on the order of $10^{30}$, and 2) the interconversion would require the total dissociation of the two catecholate groups, in addition to multiple bond rotations which would amount to a fairly large expenditure of energy. However, if the two diastereomeric chelates do exist as two rapidly interconverting species, then heating should result in a coalescence of the duplicate signals in the $^1$H NMR spectrum.

A sample of the parabactin-gallium chelate in $d_6$-DMSO was heated over the range of 20°-130°C and, although the difference between the two gamma methyl signals decreased with increasing temperature, they never coalesced. In fact, none of the signals present in duplicate in the $^1$H NMR spectrum of the chelate coalesced over the temperature range of 20°-130°C. The decrease in separation between the two gamma doublets was a result of both signals moving downfield by approximately 20-30 Hz during heating, the upfield doublet moving slightly more downfield than
the downfield doublet. It should be pointed out that the gamma methyl signal of the homoparabactin-gallium chelate also displayed a chemical shift dependency on temperature of this same magnitude. Also, after allowing the parabactin-gallium sample to cool to room temperature after heating to 130°C, it appeared identical to that which was taken prior to heating. The data cannot rule out the possibility of the eventual coalescence between the two diastereomeric coordination isomers. However, the difference between the two gamma methyl signals was still 7.3 Hz at 130°C, which would make the eventual T_c (if one exists) extremely high. It is the belief of the author that the parabactin-gallium chelate exists as two separate, extremely low interconverting diastereomers.

An additional point of interest brought out by the 1H NMR data concerns the rate of racemization of the \( \Lambda_{\text{cis}} \) chelate. It is strongly suggested that the \( \Lambda_{\text{cis}} \) gallium chelate is formed exclusive of the \( \Delta_{\text{cis}} \) complex. The data also suggest that the \( \Lambda_{\text{cis}} \) chelate racemizes to the \( \Delta_{\text{cis}} \) complex very slowly, if at all. Racemization to the \( \Delta \) chelate would of course be easily detected by NMR in the form of additional signals appearing - specifically, the appearance of a gamma methyl signal downfield to the gamma doublet of the \( \Lambda_{\text{cis}} \) chelate. Even after standing in \( D_2O \) or \( d_6\)-DMSO for several weeks, no additional observable 1H NMR signals were detected. Additionally, heating a solution of the chelate in \( d_6\)-DMSO to 130°C did not result in any new signals.

The spermidine catecholamide siderophore, parabactin, appears to form a 1:1 complex with gallium(III). The 1H NMR data strongly suggest the absolute configuration of the parabactin-gallium chelate is \( \Lambda_{\text{cis}} \). Upon complexation with gallium, parabactin appears to form the \( \Lambda_{\text{cis}} \) chelate to the exclusion of the \( \Delta_{\text{cis}} \) coordination isomer. Furthermore,
the \( ^{\Lambda} \text{cis} \) gallium complex appears to exist in a 3:1 ratio of two diastereomeric forms, \( ^{\Lambda} \text{cis} \ 3,4 \) and \( ^{\Lambda} \text{cis} \ 4,3 \), which differ only in the disposition of the spermidine backbone. A -180° rotation about the \( N^4-C(O) \) bond by the ligand any time prior to chelation will lead to the two diastereomeric coordination isomers upon chelation; thus, the conformation equilibrium existing in the free ligand may dictate diastereomer ratios in the metal chelate. This hypothesis lends support to Neilands' proposal that the equilibrium between the two major conformers in the spermidine siderophores results from a \textit{cis-trans} isomerization about the \( N^4-C(O) \) amide bond. The study suggests that in the solvents examined, the free ligand parabactin exists as three interconverting conformational isomers. Data strongly suggest that the internal rotational barrier that must be overcome for interconversion of these conformers is partially dependent upon intramolecular hydrogen bonding. In particular, the hydrogen bond donor ability of the catechol groups appears to play a major role in the conformational equilibrium of the ligand.

The elucidation of the stereochemistry of parabactin's gallium chelate will aid in the evaluation of metal-parabactin-receptor specificity in \textit{Paracoccus denitrificans}. 
CHAPTER V
PARABACTIN-MEDIATED IRON TRANSPORT IN PARACOCCUS DENITRIFICANS

Introduction

The concentration of ferric ion required to support the growth of most microorganisms lies in the range of $5 \times 10^{-8}$ to $1 \times 10^{-6}$ moles per liter (11). Due to the extreme insolubility of ferric hydroxide at physiological pH, the equilibrium concentration of this ion is reported to be only about $10^{-18}$ moles per liter (11).

The problem of first solubilizing the extremely insoluble metal and then transporting this essential nutrient into the cell has been dealt with very effectively by the microbes. Microorganisms excrete large quantities of low molecular weight ferric ion complexing agents, siderophores, into the external cell medium that are able to chelate iron(III) and deliver the metal to the organism (1-4,10). The siderophores belong to what has been called the "high affinity" transport system of microbial iron uptake (72). Another system, referred to as the "low affinity" transport system, is relatively inefficient and less well understood (2). It is believed that the low affinity iron transport process is in operation primarily when the concentration of iron in the cell environment is relatively high (2). At lower iron concentrations, such as those usually present in the environment, the high affinity system is responsible for the introduction of ferric ion to the microorganism (2).
Although it has been known for some time that microbial iron sequestering agents exist, and are responsible for supplying iron to the cells, many questions have only very recently begun to be answered. For example: 1) What is the actual mechanism of siderophore-mediated iron uptake in microorganisms - is the intact metal chelate transported into the cell, or does the siderophore simply deliver the metal to the outer membrane to be transported into the microorganism by another system? 2) What is the degree of specificity of the siderophore-mediated iron uptake system - will any iron chelate able to supply iron to the microorganism? 3) Once the microorganism is presented with a siderophore metal complex, how does the cell remove the iron? 4) Is the recognition of a ferric siderophore and subsequent transport and/or release of the metal from the chelate dependent on one specific coordination isomer? Using the soil bacterium, *Paracoccus denitrificans*, the answers to some of these questions were examined and are presented in this chapter.

**Materials and Methods**

**General**

All liquid culture media components were purchased from Fisher Scientific Co. The ethylenediamine-di(O-hydroxyphenyl) acetic acid (EDDA) was from Sigma Chemical Co. The $[^{55}\text{Fe}]$ferric chloride ($^{55}\text{FeCl}_3$) (25 Ci/g, in 0.5 M hydrochloric acid) and the Aquasol-II scintillation fluid were purchased from New England Nuclear Corp. The Chelex-100 resin was obtained from Bio-Rad Laboratories. Both the trypticase soy agar and trypticase soy broth were purchased from BBL Microbiology Systems. Filters used in the transport assays were from Schleicher & Schuell, and were 0.45 μm pore diameter.
Culture Media and Glassware

All water used was distilled in a Mega-pure still (Corning) and passed through a deionizing cartridge (Sybron-Barnstead) prior to use. All glassware was rinsed with 3N hydrochloric acid and rinsed well with water.

Liquid culture medium contained 13.5 g sodium succinate, 4.0 g potassium phosphate monobasic (KH₂PO₄), 4.9 g sodium phosphate dibasic (Na₂HPO₄), 1.6 g ammonium chloride (NH₄Cl), 0.2 g magnesium sulfate heptahydrate (MgSO₄·7H₂O), and 4.5 μmol manganese sulfate (MnSO₄) per liter of water. The pH was adjusted to 7.0 with 5M sodium hydroxide (NaOH). The "chelex-treated" media were prepared as follows. The culture medium components required for preparing 1 l of liquid culture media, minus the divalent metals, were dissolved in approximately 100 ml water and passed through a column containing 94 g chelex-100 that had been packed and washed with a total of 1 l of water. After approximately 950 ml of the culture medium eluent from the chelex column had been collected, the divalent metals were added and the pH adjusted as previously described. The "transport medium" contained the above components minus the NH₄Cl, and was also passed through a chelex column as described above.

It was determined by atomic absorption (CH2M Hill Laboratories) that the liquid culture medium contained 2.0 μM total iron, while the transport medium contained 0.3 μM total iron.

Bacterial Strains

Paracoccus denitrificans was obtained from Dr. J.B. Neilands, Department of Biochemistry, University of California, Berkeley, and was maintained on trypticase agar plates. Individual colonies of Paracoccus
Paracoccus denitrificans were inoculated into 20 ml trypticase soy broth in 300 ml Klett flasks and incubated with rotary shaking for 24 hours at 30°C. Inoculations were then made into the liquid culture medium at 40 μl broth per 10 ml culture media.

**Preparation of Labeled Chelates**

Stock solutions of labeled chelates were prepared by mixing 0.05 to 0.1 μmol of ligand in 200 μl methanol and adding 200 μl of an aqueous ferric nitrate solution (prepared by the dilution of Aldrich iron atomic absorption standard solution). A 100 μl solution of $^{55}$FeCl$_3$ was then added to give 1 μCi $^{55}$Fe per 0.033 μmol ligand, and the pH adjusted to 10 with 0.01 N NaOH. The ligand and total amount of iron were mixed in molar proportions of 1.1 to 1, respectively, which produced a slight excess of ligand to insure chelation of all the labeled iron. After the mixture was allowed to stand under nitrogen for 10 minutes, it was evaporated at room temperature with a stream of nitrogen and redissolved in transport medium to a final concentration of 30 μM and stored at 10°C.

Chelates labeled with $[^3]$H]parabactin were prepared as above, except that the molar proportion of ligand to metal was 1:1. The gallium(III) chelate of $[^3]$H]parabactin was also prepared in this manner, using an aqueous solution of gallium nitrate instead of ferric nitrate.

**Growth Studies**

The effect of various ligands and their ferric chelates on the growth of Paracoccus denitrificans was determined by monitoring the Klett reading of the liquid culture with time.

Iron chelates were prepared as in the preparation of labeled chelates and added to Klett flasks in liquid culture medium. The free ligands were added in methanol to the inside of empty, sterile Klett flasks,
followed by evaporation of the methanol by a stream of nitrogen before the addition of liquid culture medium.

Transport Assays

Individual colonies of Paracoccus denitrificans were inoculated into 20 ml trypticase soy broth and incubated at 30°C with rotary shaking. After 24 hours, 200 μl of the cell broth mixture were added to 50 ml of chelex-treated culture medium and incubated with rotary shaking at 30°C. Cells were harvested after 48 hours by centrifugation (1100 g for 15 minutes) at room temperature, washed twice with transport medium, and resuspended in fresh transport medium to a Klett reading at 95. It was determined by plating out the resuspended cells on trypticase soy agar plates that a Klett reading of 25 corresponded to a cell density of $10^9$ colony forming units per ml. A total of 29 ml of resuspended cells was added to each 300 ml Klett flask. Cell suspensions were allowed to incubate with rotary shaking at 30°C for 15 minutes before stock solutions (1 ml) of labeled chelates were added (final concentration 1 μM, specific activity 0.033 μCi/ml). For experiments where the effect of sodium azide on transport was studied, an aqueous solution of the metabolic inhibitor was added at this stage to give 5 mM sodium azide in a total of 1 ml transport media, and the Klett flask allowed to incubate 15 minutes with rotary shaking at 30°C before labeled chelate was added. Aliquots (0.5 ml) of cell suspensions were removed at regular intervals, immediately diluted with cold transport medium (5 ml), and then rapidly filtered through Schleicher & Schuell BA85 filters. The filters had been soaked overnight in a 1 mM unlabeled chelate solution, and then rinsed once with transport medium (5 ml) before filtering the cell suspensions. After rinsing the collected
cells on the filters twice with cold transport medium (5 ml), the filters were allowed to air-dry. The filters were then dissolved in ethylene glycol monomethyl ether (1 ml), and counted in a Packard Tri-carb 460 CD liquid scintillation counter after 6 ml Aquasol-II were added. Control values of labeled chelates adsorbed to the filters in the absence of cells were determined and subtracted from the values obtained with cells. Update of labeled chelates by Paracoccus denitrificans is presented as "% uptake," which indicates the percentage of the total amount of added label that has been taken up by the cells.

Reversal of Iron Starvation

Trypticase soy agar was prepared containing 1 mg EDDA per ml of agar. It was found that when EDDA was added at less than 1 mg per ml agar, a large degree of background growth resulted. At concentrations too much greater than 1 mg/ml EDDA, all bacterial growth was completely suppressed. The EDDA was deferrated by the method described by Rogers (102). After sterilization, the EDDA-containing agar was allowed to stand for 24 hours at 4°C to permit the chelation of all adventitious iron present. After 24 hours the agar was remelted and allowed to cool to 43°C, at which point 1000 colony forming units per ml of Paracoccus denitrificans (from a 24 hour trypticase soy broth) were added. Then, 25 ml of the bacteria-seeded agar were dispensed into petri dishes. Methanolic solutions (10 µl) of the various ligands were added to 6 mm filter paper discs. After allowing the methanol to evaporate, the filter papers were applied to the solidified agar and the plates were incubated at 30°C. The diameter of growth around the filter paper discs was measured at various intervals.
Results

Growth Studies

The effect of added siderophore or siderophore iron chelates on the growth of liquid cultures of *Paracoccus denitrificans* was studied by monitoring the Klett reading of the liquid cultures over time.

Ferric citrate has been reported to be an effective iron transporting agent in various microorganisms (103-105). To compare the ability of ferric citrate and ferric parabactin in promoting the growth of *Paracoccus denitrificans*, solutions of ferric citrate and ferric parabactin were added to low-iron media in separate flasks at the time of inoculation from a trypticase soy broth solution into growth medium. Figure 81 compared the stimulatory effect of the two ferric chelates on the growth of *Paracoccus denitrificans*. It can be seen that the addition of ferric citrate at the time of inoculation results in a stimulation of growth from the very beginning. The lag time between inoculation and measurable increases in Klett units is significantly shorter for flasks with ferric citrate present in the low-iron medium than controls. Conversely, there is no effect on the lag time or initial growth of the cultures when ferric parabactin has been added up to 12-14 hours after inoculation. After 12-14 hours, there is a marked increase in Klett units of the ferric parabactin containing flasks compared to controls; the increase in Klett readings eventually surpasses the maximum value obtained when ferric citrate has been added.

It was desired to compare the effects on cell growth of adding ferric parabactin, as well as free parabactin, to liquid cultures of *Paracoccus denitrificans*. If the microorganism was unable to utilize the ferric parabactin chelate, there would be no effect on the growth of
Figure 81. Effect on Growth of Paracoccus denitrificans by the Addition of Ferric Citrate ▼; Ferric Parabactin ○; Control △.
the bacteria for as long as it could not use the iron complex. However, if a metal chelator was added to the cell medium that could not be utilized, the chelating agent would tie up any available iron in the buffer and suppress the growth by a process of iron starvation. Figure 82 shows the Klett reading vs time curves of an experiment that compares the effects on the growth of the addition of increasing concentrations of both free ligand and siderophore-iron complex. At the time of inoculation of a 24 hour trypticase soy broth culture into low-iron medium, parabactin was added to separate flasks at 2 μM and 6 μM, and incubated at 30°C. When parabactin was added at a concentration of 2 μM, there are essentially no effect on the growth of the microorganism until 18 hours, when a slight stimulation occurred. Parabactin added at a concentration of 6 μM resulted in a marked attenuation of growth for the first 24 hours, followed by a slight stimulation of growth compared to controls. When the ferric chelate of parabactin was added, a stimulation of the growth resulted after about 11 hours for both 2 μM and 6 μM added chelate. The chelate at 6 μM displayed a greater degree of stimulation than added ferric parabactin at 2 μM.

Figure 83 shows the Klett unit vs time graph of an experiment in which free ligand parabactin was added to liquid cultures after they had been allowed to grow for 16 hours after inoculation. The addition of free ligand at this time resulted in a stimulation of the growth of the organism compared to the control flasks, in which no ligand was added. Transport of Labeled Siderophore Metal Chelates

Cell suspensions of Paracoccus denitrificans, growth under iron-deficient conditions, were tested for their ability to transport $[^{55}\text{Fe}]$-ferric parabactin, $[^{3}\text{H}]$ferric parabactin, the Ga(III) chelate of $[^{3}\text{H}]$-
Figure 82. Effect on Growth of Paracoccus denitrificans by the Addition of 2 μM Ferric Parabactin •; 6 μM Ferric Parabactin ○; 2 μM Parabactin □; 6 μM Parabactin ■; Control △.
Figure 83. Effect on Growth of Paracoccus denitrificans by the Addition of 5 μM Parabactin Δ; Control ○; After Allowing Bacteria to Grow for 16 Hours Before Addition of Compound.
parabactin, and $[^{55}\text{Fe}]$ferric enantioparabactin, all at 1.0 μM. To follow the transport of siderophore-bound iron, resuspended cells were presented with 1 μM $[^{55}\text{Fe}]$ferric parabactin at 30°C and pH 7. When the bacteria were presented with $[^{55}\text{Fe}]$ferric parabactin, a fairly rapid incorporation of the $^{55}\text{Fe}$ label into the cells occurred (Figure 84). Within the first minute of the assay approximately 10% of the $^{55}\text{Fe}$ label was taken up by the cells, followed by a steady increase of iron uptake until the uptake vs time curve leveled off at about 50% of label taken up after 60 minutes. To monitor the fate of the ligand of the ferric siderophore complex, the labeled siderophore $[^{3}\text{H}]$ferric parabactin was added to separate flasks of the same batch of harvested cells. Contrary to the observed uptake of $^{55}\text{Fe}$, no appreciable transport of tritium labeled ligand occurred over the entire course of the experiment. However, similar to the uptake of $^{55}\text{Fe}$ from $[^{55}\text{Fe}]$ferric parabactin, in which there was an initial 10% uptake of label, there was also an initial 10% uptake of the $^{3}\text{H}$ label of $[^{3}\text{H}]$ferric parabactin. However, no additional uptake of tritium label occurred throughout the experiment (Figure 84).

Separate experiments compared the ability of suspended cells to transport $[^{55}\text{Fe}]$ferric parabactin and $[^{3}\text{H}]$ferric parabactin at 30°C and 4°C. It should be pointed out here that both the absolute rate of transport, and the total amount of incorporation of radiolabeled metal siderophore chelate into cells varied from experiment to experiment. Therefore, uptake of $[^{55}\text{Fe}]$ferric parabactin was always determined in each experiment in order to compare the transport behavior of the other metal chelates being examined to ferric parabactin.
Figure 84. Uptake of $^{55}\text{Fe}$Ferric Parabactin $\bigcirc$; and $^{3}\text{H}$Ferric Parabactin $\bigtriangleup$, by Paracoccus denitrificans.
The data in Figure 85 are a comparison of the ability of Paracoccus denitrificans to transport $[^{55}\text{Fe}]$ferric parabactin and $[^{3}\text{H}]$ferric parabactin at both 30°C and 4°C. In contrast to the rapid uptake of $^{55}\text{Fe}$ label from $[^{55}\text{Fe}]$ferric parabactin at 30°C, there is no appreciable transport of labeled metal at 4°C. Instead, there was a steady baseline value of approximately 7% of the label that remained cell-associated throughout the entire course of the assay. The uptake of the tritium label of $[^{3}\text{H}]$ferric parabactin was the same at 30°C as it was at 4°C. A constant amount of $^{3}\text{H}$ label, approximately 7%, was found to be associated with the cells at both 30°C and 4°C. This small amount of cell-associated $^{3}\text{H}$ label was bound to the cell by the time the first aliquot of the assay was taken, at one minute. Thus, as can be seen in Figure 85, the transport curves of $[^{55}\text{Fe}]$ferric parabactin and $[^{3}\text{H}]$ferric parabactin at 30°C, $[^{3}\text{H}]$ferric parabactin at 4°C, are all essentially identical.

Figure 86 compares the transport of the Ga(III) chelate of $[^{3}\text{H}]$parabactin with the transport of $[^{55}\text{Fe}]$ferric parabactin, both at 30°C. The uptake of labeled iron from ferric parabactin shows the typical transport curve - steady uptake of $^{55}\text{Fe}$ followed by a plateau after about 60 minutes. However, there is essentially no uptake of the Ga(III) complex of $[^{3}\text{H}]$parabactin. Its transport curve closely resembles the uptake curve of $[^{3}\text{H}]$ferric parabactin; essentially, a constant amount of $^{3}\text{H}$ label remains cell-associated at all times.

In order to determine whether Paracoccus denitrificans displays any stereoselectivity or stereospecificity in its ferric parabactin transport system, $[^{55}\text{Fe}]$ferric parabactin and its enantiomer, $[^{55}\text{Fe}]$ferric enantioparabactin, were compared for their ability to supply the organism
Figure 85. Uptake of $^{55}\text{Fe}$Ferric Parabactin at 30°C ○; 0°C □; $^{3}\text{H}$Ferric Parabactin at 30°C △; $^{3}\text{H}$-Ferric Parabactin at 0°C ▽, by Paracoccus denitrificans.
Figure 86. Uptake of $^{55}$Fe Ferric Parabactin $\odot$; and the Gallium(III) Chelate of $^{3}$HParabactin $\triangle$, by Paracoccus denitrificans.
with labeled iron. As can be seen from Figure 87, enantioparabactin proved to be far inferior in its ability to donate $^{55}$Fe to the microorganism. When [$^{55}$Fe]ferric parabactin and [$^{55}$Fe]ferric enantioparabactin were examined for their ability to supply bacteria with iron, an equivalent amount of label was associated with the resuspended cells within the first minute of the assay, approximately 5%. From that point, the $^{55}$Fe label delivered to the cells by [$^{55}$Fe]ferric enantioparabactin only very slowly increased to a maximum value of about 10% over 120 minutes. By this time, the uptake curve for [$^{55}$Fe]ferric parabactin had already leveled off at a value of about a 40% intake of $^{55}$Fe. The extremely slow incorporation of labeled metal of [$^{55}$Fe]ferric enantioparabactin lies very close to the range of experimental error; therefore, it is not known for certain whether an extremely slow increase of $^{55}$Fe is actually occurring.

It was originally planned to examine the specificity of the ferric siderophore transport system of Paracoccus denitrificans by monitoring the uptake of labeled metal donated to the organism by various synthetic parabactin analogs, as well as other iron chelating agents. The $^{55}$Fe metal chelates of a large number of catecholamides listed in Table 6 were prepared and added to separate cell suspensions of bacteria. After incubation of the labeled metal chelates with cell suspensions for 90 minutes at 30°C, the cells were collected in the usual manner, and the amount of $^{55}$Fe donated to the microorganism by the various ligands determined. Table 6 represents the apparent ability of various catecholamides to donate $^{55}$Fe to Paracoccus denitrificans. The data are presented as the % total amount of cell-associated label at 90 minutes relative to the amount of total cell-associated label supplied as
Figure 87. Uptake of $^{55}$Fe Ferric Parabactin $\bigcirc$; and $^{55}$Fe Ferric Enantioparabactin $\triangle$, by Paracoccus denitrificans.
Table 6.
Relative Ability of Synthetic Catecholamides to Supply *Paracoccus denitrificans* with $^{55}$Fe

<table>
<thead>
<tr>
<th>Compound</th>
<th>% $^{55}$Fe Uptake Relative to Parabactin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enantioparabactin</td>
<td>10</td>
</tr>
<tr>
<td>Homoparabactin</td>
<td>105</td>
</tr>
<tr>
<td>Norparabactin</td>
<td>100</td>
</tr>
<tr>
<td>L-Parabactin A</td>
<td>95</td>
</tr>
<tr>
<td>&quot;Tait's Compound II&quot;</td>
<td>115</td>
</tr>
<tr>
<td>Ethyl-2,3-dihydroxybenzamide</td>
<td>130</td>
</tr>
<tr>
<td>Ib</td>
<td>110</td>
</tr>
<tr>
<td>II</td>
<td>100</td>
</tr>
<tr>
<td>III</td>
<td>105</td>
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<td>IV</td>
<td>95</td>
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<td>V</td>
<td>95</td>
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<tr>
<td>IV</td>
<td>90</td>
</tr>
<tr>
<td>VII</td>
<td>105</td>
</tr>
<tr>
<td>VIII</td>
<td>110</td>
</tr>
</tbody>
</table>

*a* Three equivalents of ligand used  
b* Compounds I-IV defined in figure 88
Figure 88. Synthetic Parabactin Analogs.
Table 7.
Ability of Iron Chelators to Reverse Iron Starvation When Present at 150 Nanomoles

<table>
<thead>
<tr>
<th>Compound</th>
<th>Growth, mm diameter after 47 hours</th>
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</thead>
<tbody>
<tr>
<td>Parabactin</td>
<td>60</td>
</tr>
<tr>
<td>Enantioparabactin</td>
<td>37</td>
</tr>
<tr>
<td>L-Parabactin A</td>
<td>26</td>
</tr>
<tr>
<td>&quot;Tait's Compound II&quot;</td>
<td>17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Catechol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>--</td>
</tr>
<tr>
<td>2,3-Dihydroxybenzoic acid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>--</td>
</tr>
<tr>
<td>Ethyl-2,3-Dihydroxybenzamide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>--</td>
</tr>
<tr>
<td>DFO</td>
<td>--</td>
</tr>
<tr>
<td>Citric acid</td>
<td>22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>I</td>
<td>39</td>
</tr>
<tr>
<td>II</td>
<td>34</td>
</tr>
<tr>
<td>III</td>
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<td>VI</td>
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<tr>
<td>VII</td>
<td>50</td>
</tr>
<tr>
<td>VIII</td>
<td>31</td>
</tr>
</tbody>
</table>

<sup>a</sup> Faint growth
<sup>b</sup> Three equivalents of ligand used
Table 8.
Ability of Catecholamides to Reverse Iron Starvation at Very Low Concentrations

<table>
<thead>
<tr>
<th>Ligand</th>
<th>amount present, nmols</th>
<th>diameter of growth at varying intervals after incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>42h</td>
</tr>
<tr>
<td>Parabactin</td>
<td>0.5</td>
<td>30</td>
</tr>
<tr>
<td>Enantiopara-</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>bactin</td>
<td>Parabactin A</td>
<td>-</td>
</tr>
<tr>
<td>Parabactin</td>
<td>0.1</td>
<td>26</td>
</tr>
<tr>
<td>Enantiopara-</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>bactin</td>
<td>Parabactin A</td>
<td>-</td>
</tr>
<tr>
<td>Parabactin</td>
<td>0.05</td>
<td>23</td>
</tr>
<tr>
<td>Enantiopara-</td>
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<td>-</td>
</tr>
<tr>
<td>bactin</td>
<td>Parabactin A</td>
<td>-</td>
</tr>
<tr>
<td>Compound</td>
<td>54h</td>
<td>66h</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Parabactin</td>
<td>23</td>
<td>36</td>
</tr>
<tr>
<td>Enantioparabactin</td>
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<td>L-Parabactin A</td>
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<td>Enantioparabactin A</td>
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<td>--</td>
</tr>
<tr>
<td>Norparabactin</td>
<td>--</td>
<td>25</td>
</tr>
<tr>
<td>Homoparabactin</td>
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<td>&quot;Tait's Compound II&quot;</td>
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<td>--</td>
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<tr>
<td>&quot;Tait's Compound II&quot;a</td>
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<td>--</td>
</tr>
<tr>
<td>Tait's Compound II&quot;b</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Ethyl-2,3-dihydroxy-</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>benzamideb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>II</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>III</td>
<td>--</td>
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<td>VII</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>VIII</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IX</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

a. 1.5 equivalents of ligand tested  
b. 3 equivalents of ligand tested
[\textsuperscript{55}Fe]ferric parabactin. It is apparent from Table 6 that all but one of the ligands possess the ability to deliver \textsuperscript{55}Fe to the microorganism in a capacity essentially identical to that of parabactin. The one exception is enantioparabactin, which was only able to supply the cells with 10\% of the amount of iron that parabactin was able to deliver to the cells after 90 minutes. It is very likely that the numbers presented in Table 6 all represent essentially the same ability to deliver iron; in other words, there is a fairly large expected standard error associated with the numbers of Table 6.

Reversal of Iron Starvation of Paracoccus denitrificans

Another method used to test the ability of iron chelators to deliver iron to a microorganism involves the capacity of the ligand to reverse iron starvation induced by an excess of ethylene diamine di(0-hydroxy-phenyl)acetic acid (EDDA) (106). EDDA is an iron chelating agent whose ferric complex is unable to supply iron to microorganisms. Therefore, when EDDA is added in large excess to the agar media, it ties up all iron in a form that cannot be used by the microorganism. Ligands to be tested for their ability to supply iron to the microorganism are applied to filter paper discs and set on solidified EDDA-containing agar plates that have been seeded with the microorganism. The ligand will diffuse out from the paper discs into the surrounding agar and compete with EDDA for ferric ion. If the microorganism is able to utilize the iron chelate of the test ligand, a halo of cell growth around the paper disc will result.

Tables 7, 8 and 9 show the result of experiments conducted to examine the ability of various ligands to supply iron to Paracoccus denitrificans. Table 7 shows the diameters of the zones of bacterial growth resulting from the various ligands tested; each ligand was present at
150 nanomoles on each filter paper disc. The results show that parabactin displayed the largest diameter of growth, 60 mm, when plates were scored after incubation at 30°C for 48 hours. All of the synthetic parabactin analogs tested showed the ability to promote growth when present at 150 nanomoles, but none of the diameters of growth approached 60 mm as in the case of parabactin. Compound II showed a very faint halo of growth, while catechol, 2,3-dihydroxybenzoic acid, ethyl-2,3-dihydroxybenzamide, and desferrioxamine were all completely ineffective. Since the amount of ligand applied to each filter paper disc - 150 nanomoles - was fairly large, it was thought that the chelates might be presenting iron to the bacteria by mechanisms other than the high affinity ferric parabactin transport system.

In an attempt to distinguish the high affinity transport from any other transport mechanisms that may be operating - for example, slow diffusion of a ferric siderophore chelate across the outer membrane, an experiment was conducted where the amount of ligand was decreased dramatically. Table 8 shows the abilities of parabactin, enantioparabactin, and parabactin A to reverse EDDA-induced iron starvation in Paracoccus denitrificans. By working at extremely low ligand amounts, such as 0.05 nanomoles, the differences in the abilities of the chelates to supply iron to the bacterium are amplified. Table 8 shows that parabactin is far superior to enantioparabactin in supporting growth of this organism, which is particularly evidenced by a lack of growth around the enantioparabactin disc until the growth on the parabactin-containing plates at 0.05 nanomoles had completely covered the surface of the plate. The titration in Table 8 also demonstrates the marked difference in the abilities of enantioparabactin and parabactin A to deliver iron to the microorganism. As opposed to Table 7, where enantioparabactin and
parabactin A gave rise to growth diameters of 37 and 26 mm respectively, Table 8 shows that parabactin A, up to 0.5 nanomoles, is completely unable to support any detectable growth over the entire course of the experiment. Table 9 shows the ability of a large number of parabactin analogs to reuse iron starvation at 0.1 nmols. Of all the compounds tested, only three were able to stimulate bacterial growth - parabactin, nonparabactin and homoparabactin. Although both the homo and nor homologs of parabactin were able to support bacterial growth, it is clear from Table 9 that parabactin is slightly superior to nonparabactin, and both are far superior to homoparabactin in stimulating growth.

**Discussion**

**Mechanisms of Siderophore-Mediated Microbial Iron Transport**

Although siderophores have been known to play a major role in the microbial iron uptake system for nearly thirty years now, it has been only recently that the molecular mechanisms of iron transport have been elucidated (78,80,107). Typically, microbial iron transport systems are examined by adding a radiolabeled ferric siderophore to a cell suspension and measuring the incorporation of radioactivity into the cells (74,75,80). To monitor the transport of both metal and ligand of ferric siderophore complexes, double-label experiments in which both components of the siderophore-metal chelate are radiolabeled are typically conducted (78,80). Early work with such double-label experiments in bacteria and fungi has revealed two basic mechanisms for siderophore mediated uptake of iron by microorganisms (103).

The more prevalent of the two processes, known as the "iron shuttle" mechanism, involves the transport of the intact siderophore-iron complex into the microorganism, followed by intracellular dissociation of the metal-ligand complex (107). The first step of the iron shuttle is
proposed to begin with a temperature-independent binding of the siderophore-metal chelate to a specific membrane receptor protein on the outer cell surface (78, 103). The initial metal chelate-receptor binding is followed by the movement of intact siderophore-iron complex into the cell, bound perhaps by certain membrane transport proteins. Once inside the microorganism the iron chelate is dissociated, providing the cell with the biologically essential metal. After the intracellular release of siderophore-bound iron, the subsequent step in the iron shuttle mechanism usually involves expulsion of deferrated ligand from the microorganism back into the extracellular medium where it may be used for another round of iron transport (75). A fairly common variation of the iron shuttle mechanism occurs in which the siderophore is degraded intracellularly, and ligand degradation products, rather than intact ligand, are then released into the medium (108).

The prototype of the iron shuttle mechanism is the transport of the trihydroxamate siderophore-iron chelate, ferrichrome, by the deferri-ferrichrome-producing fungus Ustilago sphaerogena (75). Emery, using ferrichrome labeled with $^{14}$C and $^{59}$Fe, was the first to study the uptake of ferrichrome by this organism (75). Uptake of the $^{59}$Fe label of $[^{59}\text{Fe}]$-ferrichrome by cell suspensions of Ustilago was rapid and nearly complete by the end of one hour. When the transport process was followed by monitoring uptake of $[^{14}\text{C}]$ferrichrome, Emery observed that during the first 45 minutes of the assay, the incorporation of $^{14}$C label occurred at a rate equal to the rate of uptake of $[^{59}\text{Fe}]$ferrichrome. However, after the initial uptake of $[^{14}\text{C}]$ferrichrome, no further accumulation of the $^{14}$C label was observed; instead, counts were slowly released from the mycelium back into the medium. The slow loss of $^{14}$C label leveled
off after about 1.5 hour to a value about 50% of the maximal \([^{14}\text{C}]\text{ferri-chrome}\) incorporated into the cells. Subsequent experiments by Emery showed that the \(^{14}\text{C}\) label expelled by the cells represented unmodified deferriferrichrome. In a separate experiment the stable aluminum(III) chelate of \([^{14}\text{C}]\text{deferriferrichrome}\), \([^{14}\text{C}]\text{alumichrome}\), was observed to be taken up by mycelium at a rate almost identical to the transport rate of \(^{59}\text{Fe}\) labeled ferrichrome. However, when \([^{14}\text{C}]\text{alumichrome}\) uptake was followed, no reappearance of \(^{14}\text{C}\) label in the medium was observed. The absence of \(^{14}\text{C}\) label reappearing in the culture medium after initial uptake of \([^{14}\text{C}]\)-alumichrome can be explained by the fact that the mycelium lacks a mechanism to remove the metal from the aluminum chelate of alumichrome; therefore, the intact aluminum complex remains intracellular. As is generally true for both siderophore mediated iron uptake mechanisms, it was found that no uptake of free ligand, \([^{14}\text{C}]\text{deferriferri-chrome}\), by \textit{Ustilago} occurred (75). Other examples of siderophores operating via the iron shuttle mechanism include schizokinen in \textit{B. megaterium} (78), aerobactin in \textit{A. aerogenes} (78), and enterobactin in \textit{E. coli} (105).

Workers have given the second basic mechanism of siderophore mediated iron uptake in microorganisms the term "iron taxi" (11). The simpler of the two mechanisms, the iron taxi mechanism requires only a transient association between the siderophore-metal complex and a specific outer membrane receptor. The salient feature of the iron taxi mechanism involves donation of metal ion by the ligand to the external membrane receptor, without the siderophore ever penetrating the cell. Like the iron shuttle, the iron taxi scheme is proposed to begin with a temperature-independent binding of siderophore-metal chelate to a specific outer
membrane receptor. The iron is then thought to be unloaded at this binding site to an iron-binding acceptor compound at the membrane surface which then facilitates the transfer of iron into the cell. The short-lived association of siderophore-metal complex with the outer membrane receptor could then be terminated by the replacement of the metal-free ligand by another siderophore-iron complex molecule, which would presumably possess a greater affinity for the specific membrane binding site. The siderophore remains extracellular at all times in the iron taxi mechanism, allowing the siderophore to repeatedly deliver iron to the cell membrane. Examples of iron uptake via the iron taxi sequence are rhodotorulic acid in *Rhodotorula pilimanae* (107), ferric exochelins in *Mycobactin smegmatis* (109,110), and ferrichrome A in *Ustilago sphaerogena* (103).

Carrano and Raymond studied the iron uptake process in the yeast *Rhodotorula pilimanae* by following the double-labeled chelate \(^{59}\text{Fe}\)-ferric\(^{3}\text{H}\)rhodotorulic acid (107). In their studies, a steady uptake by yeast suspensions of \(^{59}\text{Fe}\) from the double-labeled chelate was observed over a five hour period. However, no appreciable incorporation of the \(^{3}\text{H}\) label of the siderophore chelate was ever observed which was interpreted to mean that no penetration of siderophore metal chelate into the cell was occurring (107).

The "iron shuttle" and "iron taxi" are the two basic mechanisms of siderophore-mediated iron uptake by microorganisms. The two schemes are unlikely to be all-inclusive, but represent two means of iron uptake which differ on one basic note: whether the complex penetrates the microorganisms followed by internal metal release, or whether the iron chelate simply releases the metal at the cell surface without ever entering the microorganism.
Although the results do not allow for a totally unequivocal assignment of the iron uptake mechanism in \textit{Paracoccus denitrificans}, it appears likely that parabactin operates by the iron taxi mechanism in this organism. When $[^{55}\text{Fe}]$ferric parabactin was added to cell suspensions of \textit{Paracoccus denitrificans} at 30$^\circ$C, a fairly rapid uptake of the $[^{55}\text{Fe}]$ label was observed, which leveled off after about one hour. When the same experiment was conducted at 4$^\circ$C, no appreciable uptake of $[^{55}\text{Fe}]$-ferric parabactin was observed.

When $[^{3}\text{H}]$ferric parabactin was added to cell suspensions at 30$^\circ$C, there was a rapid binding of small amounts (<10%) of the tritium label to the cells within the first minute of the experiment. However, no additional uptake of label occurred over the entire course of the transport assay. Subsequent experiments showed that this small amount of tritium label of $[^{3}\text{H}]$ferric parabactin associated with the cells at 30$^\circ$C was equal to the amount of cell association of the same label at 4$^\circ$C. Since there was an equivalent amount of cell association of $[^{3}\text{H}]$ferric parabactin at both 30$^\circ$C and 4$^\circ$C, it can be concluded that the small amount of cell-association represents some form of binding of the label at the cell surface, and not the actual transport of parabactin into the cell.

In cell suspensions at 4$^\circ$C, the labels of both $[^{55}\text{Fe}]$ferric parabactin and $[^{3}\text{H}]$ferric parabactin were bound in equal quantities within the first minute of the experiment. Binding of the two labeled components of ferric parabactin in equal amounts strongly suggests that at 4$^\circ$C, it is the intact siderophore metal complex that is bound to the cell surface. The observed binding of ferric parabactin to the cell surface at 4$^\circ$C could represent a temperature-independent initial phase of iron
uptake, in which the complex is locked to a specific siderophore receptor site at the membrane surface and awaits further processing. Of course, one cannot rule out that the observed binding of ferric-parabactin at 4°C is due to some form of nonspecific cell association of the iron chelate with the cell surface, or at least a combination of the two binding events.

To date, most of the siderophore-mediated iron uptake systems in bacteria and fungi studied appear to operate via active transport mechanisms (111,112). The various microbial iron uptake systems examined have shown a wide range of susceptibilities in their responses to various metabolic poisons such as sodium azide, sodium cyanide, p-nitrophenol, etc (111,112). In the case of *Paracoccus denitrificans*, it was found that up to 5 mM sodium azide had no pronounced effect on the transport of \([^{55}\text{Fe}]\)-ferric parabactin, although the absence of transport at 4°C was quite clear.

It cannot be taken for granted that microbial iron-uptake systems are always enzyme-mediated events requiring the input of energy. A classic example of an energy-independent siderophore-mediated iron uptake process occurs in *Mycobacterium smegmatis* (113-116). Mycobactin S, produced by *Mycobacterium smegmatis*, is an extremely hydrophobic iron chelating compound that resides in large quantities (up to 10% cell dry weight) throughout the thick lipophilic cell envelope (115). Because of its lipoidal character, mycobactin is not released to the external cell medium but, rather, it is confined to the cell membrane where it functions as a transmembrane iron transporting device (114,115). Mycobacteria excrete exochelins into the external medium for the purpose of chelating and solubilizing exogenous iron (110). The ferric exochelins are thought to donate their iron to mycobactin at the outer
membrane surface by a simple ligand exchange mechanism (11). Ferric mycobactin, an uncharged lipophilic complex, is then free to diffuse from the outer membrane surface to the membrane-cytoplasm interface where iron is released to iron acceptors in the cytoplasm. Both mycobactin and ferric mycobactin are free to equilibrate throughout the microbial membrane; it is the release of mycobactin-bound iron to the cytoplasm that provides the thermodynamic sink to drive the equilibrium towards transport of iron into the cell. Thus, the iron uptake system in Mycobacterium smegmatis operates via a facilitated diffusion process (114,115). Although the internal release of mycobactin-bound iron to the cytoplasm is likely to be enzyme mediated, the actual iron loading of the bacterial cell via metal donation by ferric exochelins to mycobactin is an energy-independent process which has been shown to occur readily at 4°C (115).

The rapid uptake by cell suspensions of the $^{55}$Fe label of $[^{55}\text{Fe}]$-ferric parabactin at 30°C, but no appreciable incorporation of the labeled metal at 4°C, provides insight towards the siderophore-mediated iron uptake system in Paracoccus denitrificans. It is most likely that the specific binding of the ferric parabactin chelate to an outer membrane receptor is a temperature-independent event. Since it appears that parabactin acts as an iron taxi in Paracoccus denitrificans, the release of the siderophore-bound iron must soon follow the binding of the chelate to a specific outer membrane receptor. It has been reported that the removal of iron from parabactin by Paracoccus denitrificans involves enzymatic reduction of the ferric ion to ferrous ion (19). Following reduction, parabactin would presumably release its ferrous ion to endogenous Fe(II) acceptors by a ligand exchange process. The reduction of ferric to ferrous ion, and the ensuing metal exchange processes, would
not likely be very sensitive to temperature, and therefore would be expected to take place at temperatures such as 4°C.

It was found that when cell suspensions of Paracoccus denitrificans were chilled to 4°C, all transport of labeled metal ceased. Therefore, it is likely that the translocation of iron, bound to ferrous ion acceptors at or near the cell surface, is the event that is shut down when the temperature is lowered. The transfer of iron from the outer membrane to the cell cytoplasm might take place by active transport, facilitated diffusion, or by the formation of an ionic pore in the membrane.

Lowering the temperature of the cell suspensions to 4°C would be expected to change the fluidity of the cell membrane (117). It is the fluidity, due to thermal agitation of the lipid hydrocarbon tails, that provides the means whereby molecules can diffuse through the interior of a membrane. Once the temperature is brought below the phase transition of the membrane, the hydrocarbon tails are thought to align themselves into a closely packed hexagonal array (117). This closely packed structure will severely hinder the ability of a molecule, an iron carrier in this case, to diffuse through the membrane interior. Therefore, if the translocation of the once parabactin bound iron into the cell cytoplasm was mediated by either an active transport or facilitated diffusion event, both of these processes would be shut down since they both rely on diffusion of a molecule through a lipid bilayer. It is not possible to differentiate between an active transport of a facilitated diffusion mechanism of iron transport from the data. However, if the translocation of iron from the outer cell membrane to the cytoplasm was dependent on the passage of the ferric ion through an ion channel, transport of $^{55}$Fe at 4°C would have been unaltered. This is because a channel-forming
molecule does not have to diffuse back and forth through the membrane like the carrier molecule would have to do. Therefore, it appears that the translocation of iron from the cell outer membrane to the cell cytoplasm, occurs by an iron carrier molecule rather than through an ion pore that bridges the exterior and interior surface of the cell membrane.

It may be argued that parabactin is actually operating as an iron shuttle, as opposed to an iron taxi, and that an extremely rapid expulsion of deferrated ligand follows the transport of ferric parabactin into the cell. A scenario in which a siderophore-metal chelate is transported into the cell, followed by free ligand expulsion at a greater rate, could explain the lack of observable uptake of $[^{3}\text{H}]-\text{labeled ferric parabactin}$. However, the results obtained by incubating cell suspensions with the Ga(III) complex of $[^{3}\text{H}]$parabactin strongly suggest against the possibility of such a rapid iron shuttle mechanism operating. No uptake of $[^{3}\text{H}]$parabactin Ga(III) was observed when the Ga$^{+3}$ chelate was supplied to cell suspensions of Paracoccus denitrificans at 30°C. In fact, there was only a small amount of tritium label associated with the cells - in an amount equal to the quantity of $[^{3}\text{H}]$ferric parabactin found cell-associated when $[^{3}\text{H}]$ferric parabactin was added to cell suspensions at 30°C. This small amount of cell association of $[^{3}\text{H}]$ferric parabactin has already been attributed to surface binding, and not to real transport of label into the cell. Initial studies by Tait suggested that release of iron from ferric parabactin by Paracoccus denitrificans was accomplished by a reductase mechanism in which the parabactin-bound iron is reduced from ferric to ferrous ion (19). Although the chemistries of Fe(III) and Ga(III) are similar, it should be noted that, unlike iron, there is
no stable +2 oxidation state for gallium (103). Therefore, microorganisms which utilize a reductase mechanism to remove iron from siderophores would be unable to reduce and hence, remove gallium from a siderophore-gallium(III) complex. If parabactin were functioning as an iron shuttle, the gallium(III) chelate of parabactin would have been transported into the cell in a similar manner as ferric parabactin. However, once the metal chelate was inside the cell, the internal removal of siderophore-bound gallium, via reduction, would not occur. Thus, if Paracoccus denitrificans were operating via an iron shuttle mechanism, one would have observed an initial uptake of Ga(III)-[3H]parabactin at a rate similar or identical to the uptake of [55Fe]ferric parabactin. In addition, since the microorganism could not then remove the gallium from parabactin, the tritium label of Ga(III)-[3H]parabactin would remain associated with the cells. Of course, no uptake of Ga(III)-[3H]parabactin was observed, thus supporting the view that parabactin functions as an iron taxi in Paracoccus denitrificans.

Iron Release Mechanisms

The role that siderophores play in the acquisition of iron by bacteria and fungi has been fairly well established in recent years (10, 11, 72). It is understood that siderophores are responsible for the chelation and solubilization of exogenous ferric ion, and facilitate the transport of this metal into the microorganism (10, 11, 72). Lately, mechanisms of iron transport have been studied in detail. However, the mechanisms responsible for the release of siderophore-bound iron to the microorganism are not yet fully evaluated (10). Indeed, considerable controversy exists concerning how and under what circumstances siderophores transfer their iron to the microbial cell (10, 11).
It is understood that iron must be released from ferric siderophores to become metabolically useful to the microorganism (16). Therefore, mechanisms must exist whereby the cell is able to efficiently remove the metal from the siderophore-iron chelate, and prevent the re-formation of the complex. In view of the exceedingly high formation constants of ferric siderophores (typically between $10^{30}-10^{50}$), the removal of iron from these ligands would appear to be a formidable task (11). The possible means of removing iron from ferric siderophores would most likely involve the following: 1) simple ligand exchange, 2) reduction of siderophore-bound iron from ferric to ferrous ion, 3) modification or destruction of the ligand, 4) protonation of the metal chelate, 5) any combination of the above events. The direct transfer of siderophore-bound iron to other ligands seems quite unlikely in view of the extraordinarily large formation constants of the ferric siderophores.

Although siderophores typically have very large formation constants for the binding of iron(III), it has been recognized for some time that these ligands have only weak affinity for iron(II) (3,10). The marked difference in the stabilities of the iron(III) and iron(II) chelates has long implicated reduction of siderophore-bound ferric ion to the ferrous state as a possible means of metal release from these iron chelators (3,10). With the siderophore-bound iron in the iron-(II) state, removal by endogenous ferrous ion chelators would become more favorable (3,10). Indeed, it has been shown that the redox potential for the ferric ion of various ferric hydroxamate siderophores falls within the range of known biological redox systems (118). Microorganisms should theoretically be able to reduce the iron(III) of the trihydroxamate siderophores to the iron(II) state.
Other workers have demonstrated that ferric siderophore reductase activity, which catalyzes the transfer of siderophore-bound iron from hydroxamate siderophores to various ferrous ion acceptors, exists in the cell extracts of numerous systems, both bacterial and fungal (119-125). Arceneaux examined the ability of cell-free extracts of *Bacillus megaterium* to reduce the iron complex of schizokinen, the hydroxamate siderophore produced by this organism (124). Typical of almost all the examples of ferric siderophore reductase activity, reduction of siderophore-bound iron was observed only when NADH or NADPH was supplied as the reductant in *Bacillus megaterium* (124). This finding is significant considering schizokinen operates via the iron shuttle mechanism; therefore, release of siderophore-bound iron could be expected to take place in the cytoplasm, after the intact chelate has been transported across the microbial membrane (126,127). Other examples of microorganisms demonstrating such reductase activity towards ferric chelates of various hydroxamate siderophores include: *Mycobacterium smegmatis* (113), *Neurospora crassa* (125), *Aspergillus fumigatus* (125), and *Bacillus subtilis* (121).

A mechanism involving enzymatic reduction of siderophore-bound iron followed by metal donation to endogenous ferrous ion acceptors is a process that has inherent advantages over other possible methods of iron removal. In addition to reduction being energetically economic, siderophores typically have relatively little affinity for ferrous ion: therefore, the metal can be more easily removed from the iron(II) chelate. As the deferrated siderophore would be unchanged by the action of an iron reductase, the unmodified ligand could return to the external cell medium to serve in another round of iron transport. Because of
these advantages of iron release via ferric siderophore reductase action, it is generally believed that enzymatic reduction plays an integral role in iron release for the vast majority of microbial iron transport systems (118-125). It would not be surprising to find that all microorganisms utilize reduction of iron(III) to iron(II), at least in conjunction with other methods, to achieve the removal of siderophore-bound iron. The stated examples of ferric siderophore reductase activity found in cell-free extracts of various microorganisms leave no doubt that the reductive release of siderophore-bound iron is an enzyme-catalyzed process in these examples. However, whether or not these particular enzymatic reductions represent the actual physiological method of siderophore-bound iron removal \textit{in vivo} is far from conclusive.

In the previous paragraphs, examples were given in which ferric siderophore reductase activity has been observed in cell-free extracts of a number of microorganisms. It is proposed that, following iron release, unchanged free ligand is then reextracted into the external cell medium to serve in another cycle of iron transport. Such a scenario in which a siderophore is used again and again to deliver iron to the cell has been called the "European Approach" to microbial iron transport (11).

There are examples, however, of siderophores that transport a single molecule of ferric ion into a microorganism, and are then degraded rather than used again (108,128). The catecholamide siderophore enterobactin, produced and utilized by \textit{E. coli}, is the most well known example of such a "use once and throw away" system of iron transport (129). In \textit{E. coli}, only a single ferric ion is transported by a single molecule of enterobactin before the ligand is hydrolyzed intracellularly by the bacterium
Microbial iron transport systems in which the siderophore is used only once, such as enterobactin in *E. coli*, are referred to as the "American Approach" to microbial iron transport (11). An examination of the so-called "American Approach" to microbial iron transport offers insight to siderophore-bound iron release mechanisms other than the simple ferric siderophore reductase system previously described. The chemical alteration of a siderophore by the microorganism so that the ligand is no longer an effective chelator for ferric ion may represent an alternative to the reductive release mechanisms of siderophore-bound iron, or at least offer a second major mechanism of iron release that utilizes modification of the ligand in addition to reduction of bound iron. Another example of a siderophore that is hydrolyzed internally by a microbial esterase is triacetylfusigen, a trihydroxamate siderophore of the fusigen family occurring in fungi of the genus *Penicillium* (130). The hydroxamate groups in triacetylfusigen are hooked together via ester linkages, as in enterobactin. It was reported that the enzyme responsible for intracellular hydrolysis of triacetylfusigen plays an integral role in iron release, so that iron transport and release in *Penicillium sp.* is analogous to the enterobactin transport system in *E. coli* (129).

The apparent waste of metabolic energy in iron transport system such as enterobactin in *E. coli* leads one to believe that the destruction of the ligand must be an essential step in the iron uptake mechanism (11). Workers have proposed that, in the case of enterobactin, the hydrolysis of ligand is required to effect the release of iron from the enterobactin-iron complex (118). As opposed to the relatively high redox potential of the ferric hydroxamate siderophores, the redox
potential of ferric enterobactin at physiological pH is well out of the range of known biological reductants (118). Therefore, for a long time it was generally accepted that enzymatic reduction of the intact catecholamide-iron chelate cannot be the initial step in iron removal (118). Conversely, the product of ferric enterobactin hydrolysis, ferric-tris-(dihydroxybenzoyl)serine, has a redox potential well within the range of known physiological reducing agents (129). It had been long thought that hydrolysis of the intact ferric enterobactin complex must be a prerequisite for iron release; the subsequent step in the iron removal mechanism would presumably involve reduction of bound iron(III) to iron(II) (11,129). In fact, an enterobactin esterase has been isolated; however, there exists considerable controversy concerning whether the substrate for the enzyme is the intact ferric siderophore or free ligand (131,132). Of course, if the substrate for the enterobactin esterase proved to be the ferric enterobactin chelate, this would imply the need for estrolytic degradation of the intact chelate prior to metal removal. Conversely, if the free deferrated ligands proved to be the only substrate of the esterase, then metal removal would have preceded ligand degradation and the theory that prior hydrolysis of the chelate was required for metal removal would be severely damaged.

Evidence has recently been presented that indicates the esterase responsible for hydrolyzing enterobactin is not essential for the uptake of iron. Various synthetic enterobactin analogs which lack the ester linkages and are, therefore, less susceptible to esterase activity have been synthesized (38,133). The ferric chelates of these enterobactin analogs have been shown to be as effective as ferric enterobactin in promoting the growth of E. coli and Bacillus subtilis (120). It has also
been found that cell-free extracts of *Bacillus subtilis* are able to
catalyze the reductive removal of iron from the various synthetic en-
terobactin analogs (120). It should be noted here that additional exam-
pies exist in which ferric siderophore reductase activity has been ob-
served towards various natural catecholamide siderophores, including
ferric parabactin in *Paracoccus denitrificans* (19) and ferric agrobactin
in *Agrobacterium tumefaciens* (119). The ferric catecholamides, both
synthetic enterobactin analogs and natural spermidine catecholamide
siderophores, would all display similar negative redox couples to en-
terobactin but, of course, would lack the hydrolytic lability. The ex-
amples of both the natural and synthetic catecholamide iron chelates
serving as substrates for various ferric siderophore reductases provide
additional support that microorganisms possess a means of circumventing
the exceptionally low redox couple of ferric catecholamides other than
prior hydrolysis of the metal chelate.

Although hydrolysis of the intact metal chelate may not be a pre-
requisite for the release of iron from siderophores such as enterobactin
and triacetylfusigen, modification of the ligand during the iron assimil-
lation process could be very beneficial to the cell, especially micro-
organisms operating under the iron shuttle mechanism. In the iron shut-
tle mechanism of iron uptake, intact siderophore-metal chelate is trans-
ported into the microorganism. Following internal removal of sidero-
phore-bound iron, the accumulation of deferrated siderophore in the cy-
toplasm could adversely affect the cell since free ligand could now
compete with the cell for iron. Intracellular modification of the
siderophore so that it is no longer an effective chelator of ferric ion
would relieve the problem of competition for iron by deferrated ligand
in the cytoplasm. In other words, modification or degradation of the deferrated ligand would provide the microorganism with the thermodynamic drive that allows iron to remain unbound to the siderophore within the cell cytoplasm. It is likely that many microorganisms operating via the iron shuttle mechanism utilize both mechanisms—iron reduction and ligand modification—to effect and maintain intracellular release of siderophore-bound metal.

Like the above examples of enterobactin in \textit{E. coli} and triacetyl-fusigen in \textit{Penicillium sp.}, in which an iron shuttle mechanism of iron uptake is in operation, ferrichrome also works via the iron shuttle mechanism in \textit{E. coli} (80,134). However, unlike the two above examples where an ultimate degradation of the ligand molecule occurs, ferrichrome is not destroyed by \textit{E. coli} during the iron transport process (129). It was previously believed that ferrichrome released its iron to \textit{E. coli} after being transported into the cell, followed by the ligand being reexcreted unchanged into the cell medium to be used in another round of iron transport (80). However, Braun recently showed that ferrichrome is indeed modified intracellularly during iron transport, to a form which does not act as an effective iron chelator (134). It was proposed that one of the N-hydroxy groups of deferriferrichrome was acetylated, giving rise to a far less effective iron chelator (134). This example of a siderophore operating in the iron shuttle mechanism appears to represent a more energetically economical process compared to enterobactin or triacetyl-fusigen, in which the ligand is completely degraded. It is not known whether the acetylation of ferrichrome occurs during or after iron removal. The acylated deferriferrichrome is then expelled into the extracellular medium and is not transported into the cell as
long as unmodified deferriferrichrome is present in the environment. Presumably, the native siderophore may be regenerated from the acetylated product by chemical hydrolysis of the labile acetyl group, thus providing the microorganism with original siderophore.

To date, no examples of modification or destruction of ligand have been observed in microorganisms operating via the iron taxi mechanism. Contrary to microorganisms operating the iron shuttle mechanism, cells which utilize the iron taxi, such as Paracoccus denitrificans, would appear not to have the need for the destruction of the ligand since the siderophore never enters the cytoplasm and therefore could not compete with the cell for intracellular iron. Following binding of ferric parabactin to outer membrane receptors, the parabactin-bound iron is presumably removed by reduction followed by chelation by ferrous ion acceptor molecules present at or near the cell surface. The thermodynamic drive for iron donation could be provided by two factors. First, the endogenous ferrous ion acceptor chelate is presumably allowed to penetrate the cell membrane to the cytoplasm, while the charged parabactin would be denied access. Second, soon after metal removal from the siderophore, the free ligand parabactin is displaced from the binding site by another ferric parabactin molecule. Thus, the free ligand parabactin is restricted to the outer membrane binding site and never penetrates the cell membrane where competition for cellular iron in the cytoplasm might occur.

From the above discussions concerning mechanisms of uptake and iron release in microorganisms, it would appear that microbes operating via the iron taxi and utilizing a reductive release of iron system such as Paracoccus denitrificans, have two distinct advantages over microorganisms operating an iron shuttle. First, since there is no accumulation
of free ligand in the cytoplasm, the iron taxi mechanism offers economy in that no expenditure of energy is required to modify or destroy the deferrated siderophore; instead, free ligand may be used repeatedly in the iron transport process. Second, and more importantly, microorganisms using a taxi and reduction system, such as Paracoccus denitrificans, would not be burdened with the transport and assimilation of nonessential elements. For example, aluminum, which is more prevalent in the soil than iron, would be expected to form a stable chelate with parabactin based on the similarities between the two metals. It has already been shown that the aluminum(III)-complexes of a number of siderophores closely resemble their iron(III) chelates in terms of solution conformation and coordination stereochemistry (135,136). Since the Al(III)-parabactin chelate would closely resemble ferric parabactin, the aluminum chelate would therefore bind to the ferric parabactin outer membrane receptors.

If Paracoccus denitrificans were operating via an iron shuttle mechanism, transport of the Al(III) chelate would result, presenting the cell with large amounts of useless aluminum-parabactin complex. However, since aluminum and other nonessential metals which may form chelates with parabactin cannot be reduced to the +2 oxidation state, the metal will not be released from the parabactin chelate at the cell surface as iron is and hence, will be denied entry into the microorganism.

Although the above discussion indicates that prior hydrolysis of metal chelate is not necessary for iron release from catecholamide siderophores, the question still remains as to how the microorganism circumvents the extraordinarily low redox potential of catecholamides such as enterobactin in an iron release mechanism utilizing reduction. Some of the more recent work in the field has shed new light on possible
answers to this question. In recent work, Silver and coworkers examined the Mössbauer spectra of ferric enterobactin at low pH in methanol, and of the iron chelates of simple catechol compounds in aqueous solution (137,138). It was reported that at low pH the ferric complexes undergo protonation with subsequent reduction of bound iron from iron(III) to iron(II). It has been suggested that this behavior is due to a one-electron oxidation of a catecholate moiety which yields a semiquinone radical via an internal reduction scheme (139). It is known that pH and potential gradients can be associated with biological membranes (140,141). Since ferric catecholamide siderophores may experience such gradients in the microbial iron uptake process, a protonated ferric siderophore species might play a significant role in membrane transport and/or iron release. Although the binding of iron(III) by catecholamides has been well documented in aqueous solutions at or above physiological pH, the behavior of ferric-catecholamides at low pH has only recently been looked at (138,142). By examining IR spectra of various tris-catecholamide iron chelates, Raymond and coworkers have proposed that, as the acidity of a solution of ferric catecholamide is increased, three stepwise protonations of the metal chelate occur (139,142). The protonation of a tris-catecholamide iron complex by three equivalents of acid has been shown to switch the mode of metal chelation from catecholate to what has been termed a "salicylate" binding mode (139). In such a salicylate binding mode, the phenolic groups in the meta positions of the 2,3-dihydroxybenzamido functions become protonated, and are not coordinated to the metal ion. Instead, the ferric ion coordination sphere is filled by the ortho phenolic and carbonyl oxygens of the three catecholamide groups of the ligand. Based on the above
information, protonation of the iron(III) chelate of parabactin by two equivalents of acid would presumably give rise to a metal complex which is coordinated via the two catecholamide groups in a salicylate binding scheme. The fifth and sixth points of attachment to the hexacoordinate ferric ion by parabactin would involve the phenolic oxygen and the oxazoline nitrogen in the 2-hydroxyphenyl oxazoline ring. Such a ferric parabactin chelate would possess no net electrical charge, and would most likely be quite lipophilic considering the organic solvent solubility of the free ligand parabactin. The obvious similarities in terms of lipid solubilities between the diprotonated ferric parabactin complex and the ferric mycobactin chelate, which is soluble in organic solvents such as chloroform, may lead one to speculate on the role a lipid soluble iron complex may play in transmembrane iron transport.

More interesting than the possible membrane permeability of such a neutral metal complex is the role a diprotonated ferric parabactin chelate might play in the reductive removal of iron from the complex. The ferric enterobactin redox couple has been measured to be -1.0V at pH 10, but increases as the pH is lowered so that the redox potential of the metal chelate is -0.75V at physiological pH (118). Similarities in redox couples are expected to exist between other catecholamide siderophores and enterobactin (120). Further decreases in pH with a concomitant change in binding of a catecholamide siderophore iron chelate to the salicylate mode would likely shift the redox couple sufficiently to allow for the reduction of siderophore-bound metal by physiological reductants. Alternatively, reduction of siderophore bound iron may take place in the protonated ferric catecholamide via an internal redox reaction in which ferric ion is reduced to ferrous ion, with concomitant oxidation of a catechol moiety to a semiquinone radical.
There indeed is evidence supporting the hypothesis that, in solvents of low dielectric, such as the nonaqueous environment of a membrane, the reduction of siderophore-bound iron takes place by an internal redox reaction (137). Mössbauer studies have shown that the iron in ferric enterobactin and other ferric catecholamide complexes exists in the iron(III) oxidation state in aqueous solution between the pH range of 2 to 10 (139). Spartalian reported that the iron enterobactin chelate in methanol at a pH reading of 7 exists exclusively in the iron(III) state (143). As the acidity of a methanolic solution of a ferric catecholamide is increased, the redox couple will become more and more positive, which essentially stabilizes the ferrous complex (139). This effect was demonstrated recently with Mössbauer spectroscopy of a solution of iron-enterobactin in methanol at low pH (139). It was found that, at a pH meter reading of 1, 45% of the iron in a methanolic solution of iron-enterobactin exists in the ferrous state; the reduction presumably takes place via an internal redox reaction (139). Thus, by a change in the dielectric constant of the medium and/or protonation of the ferric enterobactin chelate, it may be possible for E. coli to reduce the iron of ferric enterobactin without prior hydrolysis of the serine backbone of the siderophore.

The extension of these results to all catecholamide siderophores in general suggests that protonation of catecholamide iron chelates may provide microorganisms with a means of circumventing the extraordinarily low redox couple of ferric catecholamides. Reduction of the protonated ferric catecholamide siderophore may proceed enzymatically since the redox couple of a ferric catecholamide utilizing the salicylate binding scheme would be much higher than as in the
catecholate mode of binding. Alternatively, in solvents of low dielectric, the protonated ferric catecholamide may undergo internal reduction, in which ferric ion is reduced to the ferrous state with concomitant oxidation of a catecholate moiety to a semiquinone radical (137,139). Reduction of the semiquinone radical of the deferrated catecholamide siderophore could easily be envisioned to occur by any one of a number of biological reductants such as ascorbate, citrate or glutathione that are known to be present in microorganisms (144). Silver and coworkers have postulated that glutathione might function as a ferrous ion acceptor in the reductive release mechanism of ferric enterobactin in E. coli (144). Not only is glutathione a good chelator of iron(III), but it is also found in large quantities in many bacteria (144). Silver found that a tenfold molar excess of glutathione was able to remove ~70% of enterobactin-bound iron at pH 3, in a 50% aqueous methanolic solution (144). Thus, it appears that the reductive release of iron from ferric siderophores still represents the most likely method of iron removal by microorganisms. How the microorganism circumvents the inordinately low redox couple of the ferric catecholamides is still unknown, but new information strongly implicates a scheme where protonation of the ferric complex would change the binding mode to one of lower stability and higher redox potential. Reduction of a protonated ferric catecholamide that utilizes the salicylate mode of metal chelation could take place enzymatically since the redox couple of such a chelate is predicted to lie within the range of known physiological reductants. In addition, a mechanism of nonenzymatic reduction that had been long discounted, namely an internal redox reaction between catecholate and bound iron, has been shown to be possible at low pH values in solvents of low dielectric.
Specificity of Siderophore-Mediated Microbial Transport Systems

A fundamental problem that faces microorganisms is how to obtain exogenous hydrophilic nutrients, since they must first be brought across the lipophilic cell membrane. This basic transport problem is amplified in the case of metal cations since they do not possess even a modest degree of lipid solubility. One of the most essential metals, iron, poses an even greater membrane transport problem because of the insolubility of its hydroxide at physiological pH (8). The soil and plant microbes have the doubly difficult task of first solubilizing the insoluble ferric ion and then facilitating the transport of the now water-soluble metal chelate across the hydrophobic membrane. Of course, it is now well known that low molecular weight chelating agents, referred to as siderophores, are responsible for the solubilization and transport of ferric ion into the microbial cells (1-4).

Although it had been known for some time that siderophores played a critical role in microbial iron transport systems, the methods by which these metal chelates were allowed access into the microorganisms became clear only recently (107,134). The initial theory of iron assimilation mediated via ferric siderophore chelates involved the penetration of intact metal-siderophore complex across the microbial cell membrane, in essentially what is now referred to as the iron shuttle mechanism (80). The possibility of an iron taxi mechanism of siderophore-mediated iron transport was not considered until later. It was unknown for some time how the siderophore-metal chelate gained entry into the cell and whether there was any degree of selectivity demonstrated by the microorganism with respect to allowing various chelates to enter the cell.
About the same time the mechanism of siderophore-mediated iron transport was beginning to build interest, several low molecular weight ionophores such as valinomycin and nonactin were being examined for their ability to transport monovalent cations across cell membranes (145). It was found that the mechanism by which compounds such as valinomycin transported monovalent cations involved forming a complex with the cation whereby the cation's hydration shell is essentially replaced with a non-polar, lipid-soluble sheath (145,146). Although some of the ionophore chelates possessed a net electrical charge, it was agreed that the increased lipid solubility of the complex over the hydrated cation was the property responsible for the ability of these compounds to transport cations across cellular membranes (10). The discovery of mycobactin S, the iron chelating compound that exists in the membrane envelope of Mycobacterium smegmatis, suggested that the method of microbial iron transport may operate as in the monovalent cation ionophores: encapsulation of the metal ion with a lipid-soluble sheath (113-116). The ferric mycobactin chelate is an electrically neutral, highly lipophilic complex, shown to transfer iron through the microbial cell membrane by diffusion (114).

Most of the initial siderophores discovered were of the trihydroxamate variety. Upon complexation with ferric ion, the ferric hydroxamate complex would possess no net electrical charge— a situation similar to that of ferric mycobactin. The possibility that the ferric chelates of the hydroxamate siderophores gain entry into the microbial cell by simple diffusion across the cell membrane was initially considered as a possible explanation. One of the earliest siderophores examined for the role which it assumed in microbial iron transport was the hydroxamate siderophore
ferrichrome. Using [$^{14}$C]labeled ferrichrome, it was found that the ferric siderophore chelate was transported into the cell while the metal-free ligand was not accumulated by the microorganism at all (75). Both the free ligand deferriferrichrome and its iron chelate, ferrichrome, are water-soluble neutral compounds with no ionizable groups near physiological pH. Therefore, differences in lipid solubility appeared to be an unlikely factor in the preferential transport of the metal chelate over free ligand. In addition, the transport of ferrichrome by Ustilago was shown to demonstrate many features indicative of an active transport system such as temperature and pH optima, and inhibition by metabolic poisons (75). Other microorganisms also displayed the ability to transport the metal siderophore chelate into the cell while denying access of the free ligand (107). Thus, it became apparent from the transport of ferrichrome by Ustilago, and from other microbial transport systems, that the ferric siderophores generally gained entry into the cell by transport processes other than simple diffusion as was initially expected.

Later, it was considered that differences in solution conformation might exist between the free siderophore and its metal chelate, which would offer the microorganism a means of differentiating between the two substances (75). Early $^1$H NMR studies showed that the metal-free siderophores undergo a dramatic conformational change upon chelation of various trivalent metal ions (98,136). A large degree of conformational flexibility was found to exist in the free ligands, as opposed to the high degree of inflexibility imposed on the molecule upon metal chelation. Such clear-cut differences in the solution conformation between the free ligands and metal chelates were thought to be responsible for...
the differential transport behavior of microorganisms (75, 94, 98, 135, 136).

Even more striking was the ability of certain microorganisms to differentiate between structurally similar iron chelates. In most of the cases examined, a high degree of selectivity existed in the transport of structurally similar metal complexes (12, 79, 107). Such a high degree of selectivity in regard to the transport of various siderophore complexes indicated the presence of a membrane-bound receptor molecule. The recognition and binding of ferric siderophores to specific outer membrane receptors may be due to the presence and/or disposition of various groups on the siderophore. Soon thereafter, the first metal-siderophore binding proteins from the outer membranes of microorganisms were isolated (76).

In addition to the varying degrees of specificity displayed by bacteria and fungi to the transport of ferric siderophores, just recently workers have begun to examine the stereochemistry of ferric siderophore transport (74, 77). Tris-bidentate ligands that chelate metal ions in octahedral coordination complexes can exist as an enantiomeric pair: the \( \Lambda \) and \( \Delta \) coordination isomers (2). Since the ligands are usually achiral molecules, they usually show a preference for one of the particular coordination isomers. Since the \( \Lambda \) and \( \Delta \) coordination isomers are either enantiomeric or diastereomeric, it was thought that a further degree of specificity in the recognition and binding of ferric siderophores to receptor proteins might exist (77). The disposition of the chelating groups in a left or right handed coordination propeller around the metal atom would be another way for the receptors to recognize ferric siderophore chelates. In this case, recognition and binding might
depend on the chirality of the ligating groups about the metal center itself (77).

In 1979, Winkelmann was the first to report on the stereospecific uptake of siderophore-iron chelates in microorganisms (74). Winkelmann examined the uptake of $[^{55}\text{Fe}]$labeled chelates of ferrichrome and enantioferrichrome by the fungi *Neurospora crassa* and *Aspergillus quadricincius*. He found that, while *Neurospora crassa* transported the $^{55}$Fe label of ferrichrome in a manner that displayed typical saturation kinetics, the fungus showed an uptake behavior towards labeled enantioferrichrome that indicated a diffusion-controlled uptake process. Similar results were obtained in the fungus *Aspergillus quadricincius* in which ferrichrome supplied $[^{55}\text{Fe}]$ to the suspended cells in a manner far superior to enantioferrichrome.

In another example of stereospecificity of a microbial iron transport system, Neilands and Rastetter examined the ability of enterobactin and enantioenterobactin to supply iron to *E. coli* (74). The ferric complexes of the isolated natural siderophore and of a synthetic enterobactin which chelates iron in a $\Delta$ complex were able to support the growth of *E. coli*. However, synthetic enantioenterobactin, which forms a $\Lambda$ coordination isomer with iron(III), was unable to support growth. It was proposed that the stereospecificity exemplified by *E. coli* was due to the outer membrane receptor recognizing and binding the $\Delta$ complex, and not the $\Lambda$ coordination isomer. The hypothesis concerning the recognition of ferric enterobactin complexes by virtue of the stereochemistry of the particular coordination isomer was consistent with earlier experiments with a synthetic enterobactin analog. The achiral, carbocyclic enterobactin analog synthesized by Corey was shown to bind iron(III) in a racemic
mixture of \( \Delta \) and \( \Lambda \) coordination isomers (76). The racemic mixture of the ferric chelate of the synthetic carbocyclic analog was shown to be almost exactly half as effective as ferric enterobactin in binding to the isolated outer membrane receptor protein of \textit{E. coli} \cite{76}. Additional evidence to support the supposition that \textit{E. coli} recognizes only ferric catecholamides which form the \( \Delta \) coordination isomers comes from studies examining the ability of ferric parabactin and ferric agrobactin to support the growth of \textit{E. coli}. Both parabactin and agrobactin bind iron-(III) as the \( \Lambda \) coordination isomers. While the ferric chelates of the catecholamide siderophores agrobactin and parabactin were unable to supply \textit{E. coli} with iron, their hydrolysis products - namely agrobactin A and parabactin A - which exist as the \( \Delta \) coordination isomers, were active \cite{72}.

The data presented in this work suggest that \textit{Paracoccus denitrificans} exhibits outer membrane stereospecificity for the iron(III) chelate of parabactin. This is the first report to explore the stereospecificity of microbial iron transport by using both labeled metal and labeled ligand complexes.

Based on high field \( ^1 \text{H} \) NMR studies of the Ga(III) complex of parabactin, and correlations of the CD spectrum of the ferric siderophore with other ferric catecholamides, it has been concluded that ferric parabactin exists in solution as the \( \Lambda \) coordination isomer to the exclusion of the \( \Delta \) isomer \cite{72,147}. The enantiomeric form of parabactin, enantioparabactin, was synthesized in which L-threonine is replaced by D-threonine in the threonyl oxazoline ring of the siderophore. By reversing the chirality of the threonyl oxazoline ring present in parabactin, the bias towards forming only the \( \Lambda \) coordination isomer upon metal
chelation is also reversed. Thus, the enantiomer of parabactin will chelate ferric ion to form the $\Delta$ coordination isomer, the enantiomer of $\Lambda$ ferric parabactin.

Iron transport experiments, which measure the ability of a ligand to deliver $^{55}$Fe to cell suspensions of Paracoccus denitrificans, demonstrated that enantioparabactin was unable to transfer an appreciable amount of labeled metal to the microorganism. It may be that the binding to the specific ferric parabactin outer membrane receptor and/or reduction of the chelate is not possible in the case of ferric enantioparabactin. The inefficiency of enantioparabactin in supplying ferric ion to Paracoccus denitrificans is also demonstrated by comparing the abilities of L- and D-parabactin to reverse the iron starvation caused by the presence of an excess of the iron chelate EDDA (106). The iron chelator EDDA is an agent whose ferric chelate has been found to be unable to furnish iron to all microorganisms tested (106). The capability of parabactin to reverse the EDDA-induced iron starvation is obvious by examining the data presented in Table 8. Parabactin, even in quantities as low as 0.05 nanomoles, was able to reverse the effect of iron deprivation caused by a large excess of EDDA present and thus, support the growth of the bacteria within 42 hours. Enantioparabactin, present at 0.05 nmols, was only able to stimulate a small amount of growth and, by the time any measurable amount of cell growth was evident, the bacterial growth on the plates containing parabactin had essentially covered the entire surface of the agar plate. It is likely that the weak growth in the presence of enantioparabactin is not the result of the transport of iron by the ferric parabactin-mediated iron uptake system. Instead, a slow diffusion of the ferric enantioparabactin chelate across the outer
cell membrane might be occurring which could then provide iron to the microorganism in an unknown manner.

It is also noteworthy to examine the ability of synthetic parabactin A to reverse EDDA-induced iron starvation in cultures of Paracoccus denitrificans. Parabactin A proved to be far inferior than even enantioparabactin in its ability to supply iron to the microorganism. Only after long periods of time, at the highest amounts of ligand tested, was parabactin A able to reverse iron starvation. Again, it is likely that a mechanism other than the high affinity iron uptake system, perhaps the slow diffusion of minute quantities of the ferric parabactin A chelate through the outer cell membrane, is operating here.

The siderophore-mediated iron transport system of Paracoccus denitrificans has been reported to have the opposite chirality requirement as E. coli (72). Iron chelators which form \( \Delta \) complexes are able to support the growth of Paracoccus denitrificans, while ferric-siderophore complexes of the \( \Lambda \) variety are inactive (72). Both enantioparabactin and parabactin A form \( \Delta \) chelates with ferric ion, and both were found to be unable to efficiently reverse EDDA-induced iron deprivation in this work. Parabactin, which coordinates ferric ion in the form of the \( \Lambda \) coordination complex is, of course, very capable of stimulating the growth of Paracoccus denitrificans.

In an effort to examine such a chirality requirement in the ferric-siderophore mediated iron uptake system in Paracoccus denitrificans, the enantiomer of parabactin A was synthesized and tested. The enantiomeric form of parabactin A, enantioparabactin A, would be expected to form the \( \Lambda \) coordination isomer upon chelation of ferric ion. Therefore, if Paracoccus denitrificans used coordination stereochemistry as the sole, or primary means of recognition of ferric chelates,
enantioparabactin A would be likely to support the growth of the microorganism. Conversely, if the microbe used other means of recognizing siderophore metal complexes, enantioparabactin A might be expected to be inactive at low concentrations considering the overall sensitivity of the uptake system observed so far. Table 9 shows that when enantioparabactin A was tested at 0.1 nanomoles, no bacterial growth resulted over the entire course of the experiment. It therefore appears likely that the very specific ferric parabactin iron uptake system in Paracoccus denitrificans uses means other than, or in addition to, coordination stereochemistry to recognize ferric siderophore complexes.

In addition to stereospecificity, the overall sensitivity of the ferric parabactin-mediated iron uptake system in Paracoccus denitrificans was evaluated by examining the abilities of various parabactin analogs to reverse EDDA-induced iron starvation. When present in relatively high amounts most of the iron-chelating compounds tested were able to reverse EDDA-induced iron deprivation and thus stimulate the growth of Paracoccus denitrificans (Table 7). However, when the ligands were present in much lower amounts the specificity of the parabactin-mediated iron uptake system was obvious. At 0.1 nmols the only ligands other than parabactin able to reverse iron starvation were the long and short homologs of parabactin, homoparabactin, and norparabactin, respectively. The data in Table 9 indicate that norparabactin is similar to parabactin in its ability to stimulate bacterial growth. However, it is apparent that the short homolog of parabactin is somewhat less efficient than parabactin itself. This is evidenced by the diameters of the halo of bacterial growth stimulated by the two compounds. At every time interval it can be seen that parabactin promoted a slightly
larger diameter of bacterial growth than norparabactin. Homoparabactin proved to be greatly inferior to both parabactin and norparabactin. The growth promoted by homoparabactin had not occurred until the bacterial growth had completely covered the agar plates containing parabactin or norparabactin.

Thus, it appears that the parabactin-mediated iron transport system of Paracoccus denitrificans is very sensitive to changes in the structure of the siderophore. None of the synthetic tri-catecholamides were able to reverse iron deprivation at low amounts of ligand. The siderophore-mediated iron uptake system of Paracoccus denitrificans is apparently so sensitive that it can effectively distinguish between parabactin and homoparabactin, a difference of only one additional methylene unit in the triamine backbone of the siderophore. However, the distinction between the natural siderophore, parabactin, and its short homolog, norparabactin, is not nearly so great.

Although the stimulation resulting from homoparabactin occurs late in the experiment compared to norparabactin or parabactin, it nevertheless does occur. Although homoparabactin is relatively inefficient compared to parabactin, it is still able to stimulate bacterial growth. Other compounds such as parabactin A and enantioparabactin, which were found to be active when tested in high amounts, were completely inactive when tested at 0.1 nmols.

It is interesting that Paracoccus denitrificans is able to utilize norparabactin, considering that Tait observed that the microbe was able to synthesize the nor homolog of parabactin when cell extracts were supplied with norspermidine in place of spermidine.

Further evidence that the recognition of ferric parabactin by Paracoccus denitrificans occurs by means other than, or in addition to,
coordination stereochemistry is provided by the results obtained with homoparabactin. It has been determined by high field $^1$H NMR spectroscopy that the gallium(III) chelate of homoparabactin exists as the $\Lambda$ coordination isomer to the exclusion of the $\Delta$ isomer (147). It is reasonable to assume that the ferric chelate of parabactin also displays the same preference in coordination stereochemistry. The simple fact that parabactin is very active in reversing the effects of EDDA-induced iron deprivation while homoparabactin is relatively inefficient, clearly indicates that the microorganism uses additional means other than the disposition of chelating groups around the metal center of ferric siderophores for recognition.

The uptake system is extremely sensitive to deviations in the structure of the siderophore, as evidenced by the fact that insertion of a single extra methylene in the triamine chain of parabactin resulted in a far less efficient compound. Thus, the disposition of the spermidine backbone of parabactin appears to be at least one of the factors that determines whether or not a ferric chelate will be recognized by P. denitrificans. This last observation in turn raises questions concerning what possible role might the two diastereomeric forms of the metal complex of parabactin - the $\Lambda$ cis-3,4 and $\Lambda$ cis-4,3 - play in iron transport in this microorganism.

One of the fundamental questions this project was designed to answer was whether all three of the catechol-containing compounds isolated by Tait are true siderophores. In other words, although all three of these catechol compounds have the ability to chelate iron, do they also possess the capability to facilitate the transport of ferric ion into the microorganism?
Tait found that when *Paracoccus denitrificans* was grown under iron deficient conditions, all three of these catechol compounds were excreted in fairly large amounts. By the end of 24 hours of growth in iron-depressed culture media, the microorganism had excreted 48 μM compound I, 105 μM compound II, and 25 μM compound III. The quantities of all three compounds in the culture medium increased by the end of 48 hours of growth to 108 μM compound I, 267 μM compound II, and 207 μM compound III (19). Although the amounts of all three catechol-containing compounds increased between 24 and 48 hours, compound III showed the most dramatic increase, over 800%. It is interesting that compound II, presumably a less effective iron chelator than compound III, is present in the greatest amount throughout the entire course of the experiment.

The obvious question arises, why are all three of these iron-binding compounds excreted in such large quantities when it is known that compounds I and II are less effective ferric chelators than compound III, and that the former compounds are needed by the microorganism for the biosynthesis of the latter? A possible answer was that *Paracoccus denitrificans* may utilize the ferric chelates of all three of these catechol compounds, and that depending on the iron nutritional state of the microorganism, different amounts of the three compounds are excreted. In other words, at the first sign of low iron conditions the bacteria begins producing and excreting compound I and compound II to sequester exogenous iron. If the internal cell iron concentration has not been adequately increased by the first two catechol compounds, it is then necessary to synthesize and excrete a more powerful iron chelator, compound III. Some indication that this scenario was operating was provided by Tait's original work. The concentrations of compound I
and II in the cell medium were initially much greater than compound III, followed by compound III increasing dramatically as the experiment progressed.

This work is the first to examine the ability of all three of the catechol-containing compounds isolated by Tait to function as siderophores by supplying iron to *Paracoccus denitrificans*. The results of the reversal of EDDA-induced iron starvation experiments show quite clearly that of the three catechol compounds excreted by the microorganism only parabactin (compound III) serves as a siderophore to *Paracoccus denitrificans*. At 0.1 nmol only parabactin was able to reverse iron starvation and support growth of the bacteria while compounds I and II were totally inactive even at amounts as high as 150 nmols, while this amount of compound II supported only a very weak halo of growth.

The finding that only one of the three iron-binding compounds excreted by *Paracoccus denitrificans* is able to effectively deliver iron to the microorganism is somewhat surprising. This is especially true considering that greater quantities of the nonutilizable compounds are initially excreted, compared to the utilizable compound, and that the amount of compound II found in the culture media at all times exceeds that of compound III. Just why *Paracoccus denitrificans* excretes an iron chelator whose ferric chelate cannot be efficiently utilized is unknown. This is especially peculiar considering that compound II is needed for the biosynthesis of a useful iron chelator, compound III. Exactly why this microorganism apparently wastes an essential intermediate for a useful compound is left to speculation.

Possible explanations might involve the excretion of large quantities of compound I and II to first solubilize the very insoluble
ferricin, followed by the excretion of parabactin to then capture the solubilized iron via a ligand exchange reaction. Alternatively, compound II may be excreted in large quantities to chelate, and thereby tie up various divalent metal ions that would otherwise compete with the small amount of soluble ferric ion for parabactin.

The inefficiency of parabactin A and other synthetic catecholamides in reversing EDDA-induced iron deprivation is clear. However, in iron transport studies $[^{55}\text{Fe}]$ferric parabactin A was shown to deliver labeled iron to suspended cells of *Paracoccus denitrificans* at a rate essentially identical to ferric parabactin. In addition to parabactin A, every ferric chelate tested with the exception of ferric enantioparabactin was found to supply labeled iron to suspended cells at a rate near or equal to that of ferric parabactin. These findings can be explained by a simple metal exchange reaction taking place during the iron transport assay between endogenous parabactin and the $[^{55}\text{Fe}]$labeled iron chelate being tested. It should be remembered that the cells used in the transport assays were starved for iron prior to harvesting, and therefore were producing and excreting parabactin. Although the harvested cells were washed in an attempt to remove endogenous parabactin, due to the extremely high iron(III) binding constant of the siderophore, its presence in even minute quantities would effectively remove labeled metal from other iron chelates with smaller iron(III) formation constants. Support for the iron-exchange hypothesis comes from a comparison of ferric ion formation constants of some of the iron chelates examined. A synthetic parabactin analog, GABA, has an iron(III) formation constant of $10^{44}$, four powers of ten lower than the iron(III)-parabactin formation constant. Therefore, even minute quantities of endogenous
parabactin remaining in the washed cells should be expected to rob the 
\[^{55}\text{Fe}\]-GABA chelate of labeled metal and then transport the label as the 
\[^{55}\text{Fe}\]\text{ferric parabactin complex. The various catecholamides that were} 
apparently able to supply labeled iron to the bacteria all could be ex-
pected to have formation constants of $10^{14}$ or lower. The only iron 
chelator tested that did not supply labeled metal to the microorganism 
was enantioparabactin. The absence of any detectable iron exchange oc-
curring between \[^{55}\text{Fe}\]\text{ferric enantioparabactin and endogenous parabactin} 
could, of course, be explained in terms of the binding constant of ferric 
enantioparabactin. The enantiomeric form of parabactin would possess 
an iron(III) formation constant identical to parabactin, at least four 
powers of ten greater than the other catecholamides tested. It should 
also be pointed out that various ferric catecholamides that apparently 
delivered \(^{55}\text{Fe}\) to \text{Paracoccus denitrificans} in a fashion identical to 
parabactin were completely unable to support the growth of the organism 
on EDDA-containing plates in amounts of ligand even as high as 150 mmols-
3000 times the amount of parabactin able to reverse EDDA-induced iron 
starvation.

The results presented in this work indicate the microbial iron up-
take system of \text{Paracoccus denitrificans} mediated by parabactin is a 
stereospecific process. The ferric parabactin-mediated iron transport 
system in \text{Paracoccus denitrificans} is the most recent of only a few ex-
amples of a microbial iron uptake mechanism that have been shown to de-
monstrate stereospecificity. Other such systems are ferrichrome/enan-
tioferrichrome in the fungus \text{Aspergillus quadricincius} (74), and the 
ferric enterobactin/ferric enantioenterobactin uptake system in \text{E. coli} 
(77). It appears that the stereospecificity observed in the case of
*Paracoccus denitrificans* is a result of factors other than, or in addition to, recognition of the disposition of the ligating groups about the metal center of the ferric parabactin chelate.

**Stimulation of Growth of Paracoccus denitrificans**

The results presented here suggest that the parabactin-mediated iron transport system in *Paracoccus denitrificans* is induced by a low concentration of utilizable ferric ion in the cell medium. When liquid cultures of *Paracoccus denitrificans* were presented with parabactin or its iron complex, there was no initial stimulation of the growth of the microorganism. Instead, addition of parabactin at the time of inoculation from a nutrient broth to low iron growth medium resulted in a marked suppression of the growth rate compared to controls. A possible explanation is that the added parabactin was chelating any extraneous iron still present in the growth medium, forming a metal complex which the microorganism cannot utilize. Support for this hypothesis comes from comparing the growth rates of separate liquid cultures which contained equal quantities of ferric citrate and ferric parabactin at the time of inoculation. It can be seen from Figure 81 that the addition of ferric citrate resulted in a pronounced increase in the growth rate of the microorganism compared to controls. In contrast, the addition of ferric parabactin had no effect on the growth rate over the first 12-14 hours of the experiment. After 12-14 hours, growth of the cultures containing ferric parabactin was stimulated, and eventually reached a level greater than that of the cultures containing ferric citrate. Further support for this hypothesis comes from Figure 82 which shows the effect of increasing concentrations of parabactin and its ferric chelate on the growth of *Paracoccus denitrificans*. 
Parabactin, added at a concentration of 2 μM, showed absolutely no effect on the rate of growth of the microorganism over the first 18 hours of the experiment. After about 18 hours, the cultures containing 2 μM parabactin showed a slight increase in growth compared to controls. The effect was even more pronounced when 6 μM parabactin were added to liquid cultures at the time of inoculation. A marked attenuation of growth was seen over the first 24 hours, followed by a slight stimulation of total growth compared to controls. This concentration-dependent suppression of growth by parabactin is most likely due to the formation of a metal complex that the bacteria are unable to utilize. This would be the case if the parabactin-mediated iron transport system in Paracoccus denitrificans required induction, presumably by low concentrations of utilizable ferric ion.

The hypothesis that Paracoccus denitrificans cannot initially recognize the iron complex of the very siderophore it will eventually produce is supported by the effect of added ferric parabactin on the growth of the microorganism. Ferric parabactin was added to liquid cultures of the bacteria at the time of inoculation from a nutrient broth in concentrations of 2 μM and 6 μM. During the first ten hours of incubation, the presence of the iron chelate had no effect on the growth rate of the bacteria. However, after ten hours the growth was stimulated by 2 μM ferric parabactin and, to an even greater extent, by 6 μM ferric parabactin. Apparently, growth in the low-iron culture medium results in iron depletion within the cells which triggers the induction of the ferric parabactin iron transport system. By about ten hours after inoculation, the cells are able to recognize and/or assimilate the ferric chelate of parabactin and a marked enhancement of growth results.
Additional support for a scenario in which induction of the ferric parabactin uptake system occurs is offered by Figure 83. In experiments just discussed, parabactin had been added at the time of inoculation into low-iron medium to measure the ability of parabactin to stimulate the growth of liquid cultures of Paracoccus denitrificans. However, the microorganism cannot utilize the ferric chelate of parabactin until the ferric-parabactin transport system is induced. Therefore, parabactin simply deprives the cells from needed iron and the result is attenuation of cell growth. Figure 83 shows the results of an experiment where 5 μM parabactin were added to liquid cultures of the microorganism after the cells had been growing in low-iron medium for 16 hours. Supposedly, by this time, the ferric parabactin iron transport system has been induced and is fully operational. As opposed to attenuation of cell growth when parabactin is added at the time of inoculation, a clear stimulation of growth results when the free ligand is added 16 hours after inoculation.

These results clearly show that parabactin is able to stimulate the growth of Paracoccus denitrificans, presumably by supplying the cells with iron. Both the free ligand and the ferric chelate of parabactin show stimulation of cell growth, but only after the induction of the ferric parabactin iron transport system. The results also indicate that, contrary to the ferric parabactin transport system that needs to be induced, there exists a ferric citrate uptake system that requires little or no time period for its induction.
CHAPTER VI
CONCLUSION

This work has described the successful development of a synthetic scheme that allows for the preparation of a large number of tetradentate, pentadentate, and hexadentate catecholamide ligands. Using this synthetic route, the first total synthesis of the spermidine siderophore, parabactin, has been accomplished in high yield. Parabactin A, enantioparabactin A, enantioparabactin, homoparabactin, and norparabactin were also synthesized.

The extension of the above synthetic route to the development of octadentate catecholamides has furnished the first of a new class of actinide chelators. Early results show that the new class of octadentate catecholamide ligands are excellent sequestering agents of plutonium, and show potential for environmental decontamination systems.

High field 300 MHz $^1$H NMR studies of the solution dynamics of parabactin, and the solution conformation and coordination stereochemistry of its gallium(III) chelate were accomplished. These studies demonstrated that while parabactin exists in solution as a mixture of interconverting conformational isomers, the gallium(III) complex of parabactin does not display a great deal of conformational freedom. The Ga(III) complex was found to exist in the form of the $\Lambda$ cis coordination isomer to the exclusion of the $\Delta$ isomer. Furthermore, the gallium(III) chelate of parabactin exists as a mixture of two diastereomeric forms of the $\Lambda$ cis coordination isomer - what we have called the $\Lambda$ cis-3,4 and $\Lambda$ cis-4,3 isomers. These two coordination stereoisomers differ only in the disposition of the spermidine backbone of parabactin.
Using the catecholamide compounds that were synthesized, and the information concerning the coordination stereochemistry gained from the NMR work, the parabactin-mediated iron transport system of the soil bacterium Paracoccus denitrificans - the microorganism that excretes parabactin - was successfully examined. By using radioactive ferric siderophore complexes, it was determined that parabactin operates via the "iron taxi" mechanism in the delivery of iron to Paracoccus denitrificans. Release of the parabactin-bound ferric ion presumably takes place at the outer cell surface such that the ligand never penetrates the microbial membrane. The parabactin-mediated iron transport system of Paracoccus denitrificans was also shown to be stereospecific. The synthetic enantiomer of parabactin, enantioparabactin, was unable to efficiently supply iron to, or support the growth of, the microorganism. Finally, it was shown that the very selective iron transport system in Paracoccus denitrificans uses factors other than the disposition of chelating functions about the metal center to recognize ferric siderophore chelates.
REFERENCES


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