Synthesis and Solution Structure of Microbial Siderophores

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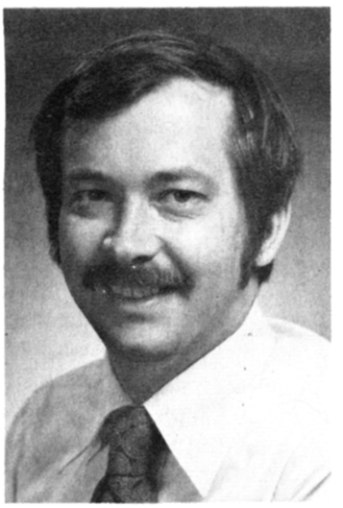

Professor Raymond J. Bergeron, Jr., was born in Fitchburg, MA, in 1945. He received his A.B. in Chemistry from Clark University in 1968 and his Ph.D. from Brandeis in 1972. He next spent 2 years as a National Institutes of Health Postdoctoral Fellow at Harvard University and in 1974 joined the chemistry faculty at the University of Maryland. As an Assitant Professor at the University of Maryland, he was awarded a Sloan Foundation Fellowship. In 1977, Dr. Bergeron was a visiting scientist at the National Institutes of Health and shortly thereafter moved to the University of Florida. Dr. Bergeron is currently a Professor of Medicinal Chemistry at the University of Florida, with a joint appointment in the Chemistry Department. His research interests include polyamines, metal chelators, total synthesis, and membrane transport.

I. Introduction

Except for perhaps the lactobacilli, life without iron is essentially unknown.[1] It is not surprising that a metal which composes 5% of the earth's crust and can serve as both an electron source as well as an electron sink would find such a useful role in metabolic processes. A member of the first transition triad characterized by its $3d^6,4s^2$ ground state configuration, iron has oxidation states varying from −2 up to +6.[2] However, the predominant oxidation states that exist under biological conditions are the +2 and +3 oxidation states, oxidation states which are very sensitive to both pH and ligation.[3] It is the equilibrium between these two oxidation states that nature has taken such effective advantage of. Iron has been shown to play a critical role in most biological redox processes, serving as the prosthetic group in a variety of redox proteins, e.g., the cytochromes, oxidases, catalase, ribonucleotide reductase, citric acid cycle enzymes, etc. This account will focus on the synthesis and solution structure of iron chelator systems which microbes use to access this metal, namely siderophores. We will not discuss the thermodynamics or kinetics of iron binding in this review as this is best left to other experts in this phase of these systems. Furthermore, we will not discuss the synthesis of every siderophore or model system generated but will focus on representative cases largely avoiding what was discussed in a previous review by W. Wierenga.

Microbial iron chelators fall into two major structural families—the catecholamides and the hydroxamates.[4] The catecholamides consist of both cyclic tricatechols as exemplified by enterobactin[5] and the linear catecholamides, as represented by parabactin.[6] The hydroxamates encompass a rather large collection of structurally different systems including ferrichromes,[7] rhodotorulic acid,[8] citrate,[9] myobactin,[10] fusarinine, and the ferrioxamine[12] systems. In fact, siderophores of one kind or another can be found in virtually every imaginable taxonomic group of microbe. Bacteria produce siderophores of both hydroxamate and catechol types, although no catechol siderophores have yet been isolated from eukaryotic microbes.[13] Some of these microorganisms produce the siderophores in incredible quantities. For example, the ferrichromes associated with fungi such as penicilliae, aspergilli, and neurospora[14] occur at 0.1 M concentrations in the fermentation media. In fact, desferrioxamine B, which is the currently accepted therapeutic device for treatment of iron overload, is prepared commercially by fermentation even though the drug has been synthesized. Yeast, though not unusually effective in the biosynthesis of siderophores, produces rhodotorulic acid in almost unprecedented concentrations[15] of 1 g/L at stationary phase. We could of course go on to enumerate in some detail the vast number of organisms explored in terms of their siderophore production. However, this has been very well described in several excellent reviews by Professor Neilands of Berkeley.[4]

Current interest in siderophores is associated with three major frontiers in iron metabolism: (1) the continued interest in the mechanism by which iron is actually transported into various cell types;[16] (2) the role which iron plays in infection;[17] (3) the demand for a suitable therapeutic iron chelator for the treatment of patients with both primary and secondary hemochromatosis.[18]

It is probably best to discuss briefly the first two issues in an integrated fashion. It has often been suggested that iron plays an essential role in the virulence of many pathological organisms.[17] Microbial growth

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rates have been clearly associated with iron concentration in the media, with most microorganisms requiring something in excess of 10 μM iron for growth. Any method of depriving microbes of this essential metal results in clear growth suppression. One of the most thought-stimulating observations was made by Boland, who demonstrated the bacteriostatic properties of lactoferrin, a potent iron-binding protein found in rather substantial concentrations in mother's milk.[19] It is clear then that an understanding of how pathogens accumulate or access iron would be useful in the design of bacteriostatic agents. In fact we have shown in our laboratories that parabactin, a *Paracoccus denitrificans* siderophore which cannot be utilized by many other organisms, suppresses the growth of many species of bacteria and fungi.[19] Of course, any study of the mechanism by which siderophores facilititate iron transport will ultimately demand an understanding of their solution conformation as well as the solution conformation of their iron chelates. Frequently an organism's utilization of an iron chelate is very sensitive to the stereochemistry of the complex. With such information in hand it is likely that the siderophore could be rationally modified so as to still bind iron tightly but not to be utilizable by the microorganism. The use of these siderophores as bacteriostatics, etc., is of course predicted on the idea that they are accessible either by fermentation processes or by synthesis. We will focus on synthetic accessibility.

The importance of synthetic siderophores is well exemplified by the work focused on the development of the therapeutic devices for the treatment of iron-overload diseases. A number of iron chelators modeled after microbial chelators have been considered as devices for the removal of iron from patients suffering from hemochromatosis. Unfortunately, animal studies using enterobactin and models thereof were very disappointing. This iron sequestor demonstrated a rather substantial toxicity, a toxicity which was later associated with the chelator's ability to deliver iron to the animal's natural flora resulting in sepsis and eventually the animal's death.[20] Of course this should not have come as much of a surprise as this is precisely what the chelator does under normal circumstances—stimulates microbial growth. In fact this very same problem of bacterial infection occurred, although to a less serious degree, in patients subjected to desferrioxamine therapy.[21] This underscores the importance of being able to synthesize modified microbial chelators that still bind iron tenaciously but cannot be utilized by the microorganism, e.g., enantioenterobactin. We will begin then with an account of the synthesis of a number of classic microbial iron siderophores and several corresponding model systems. These synthetic discussions will be followed by a brief description of our current understanding of the solution structure of these natural product chelators.

II. Catecholamide Siderophores

The single most studied of all the catechol chelators is enterobactin, a macrocyclic tris(catecholamide) (Figure 1). This macrocycle was isolated independently by two groups[5,22] and has since been shown to be the tightest iron(III) chelator known to man. The ligand forms high-spin hexacoordinate octahedral complexes

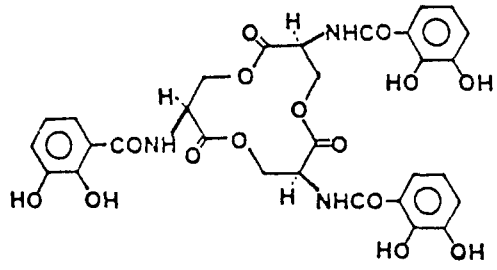
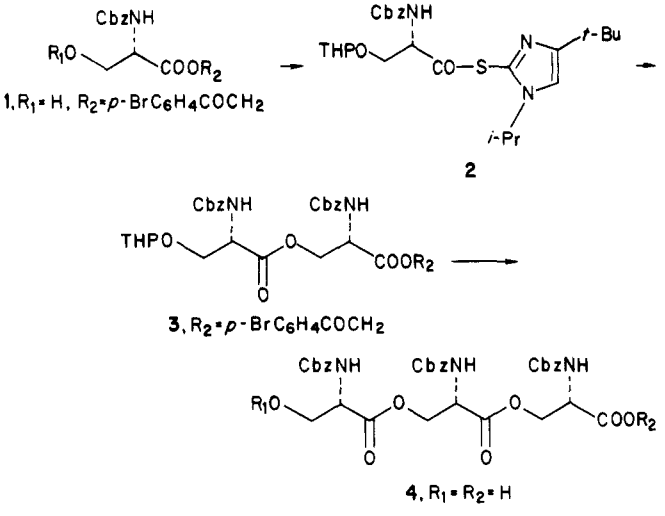


Figure 1. Enterobactin.

SCHEME I. Key Intermediates in the Synthesis of Enterobactin



with iron(III) with a formation constant[23] of 10^{52}. This macrocycle was first synthesized by E. J. Corey using the double activation method for closure to the macrocyclic lactone (Scheme I). [A note of caution is in order: Compound numbers are used with reference to specific schemes and are not carried throughout the paper. Thus, 1 in Scheme I is not the same as 1 in Scheme II, etc.] The two most essential components of this scheme are the *p*-bromophenacyl ester of *N*-benzyloxycarbonyl-L-serine (1) and the *O*-tetrahydropyranyl-L-*N*-benzyloxycarbonyl-L-serine thioester 2. The thioester 2 was condensed with the protected serine 1 in very good yield to produce the ester 3. The phenacyl protecting group of 3 was next removed reductively with zinc and acetic acid. The carboxyl group of the product was then converted to the corresponding thioester by reaction with bis(4-*tert*-butyl-1-isopropylimidazol-2-yl) disulfide and triphenylphosphine in benzene. The resulting adduct was subsequently coupled with 1. The triserine product was first divested of its tetrahydropyranyl groups by exposure to acetic acid and methanol in THF to produce the corresponding hydroxy compound. Next the *p*-bromophenacyl group was removed by treatment with zinc and acetic acid. The resulting intermediate 4 was cyclized by conversion in situ to the corresponding 4-*tert*-butyl-1-isopropylimidazol-2-yl thioester, and the benzyloxycarbonyl protecting groups were removed by hydrogenolysis. In the last step the hydrogenolysis product, the triamine hydrochloride was reacted with 2,3-dihydroxybenzoyl chloride in the presence of triethylamine in tetrahydrofuran to generate enterobactin. The overall yield of this 12-step synthesis is difficult to evaluate as numbers are provided only up to and including the cyclization. At this point the yield is ap-

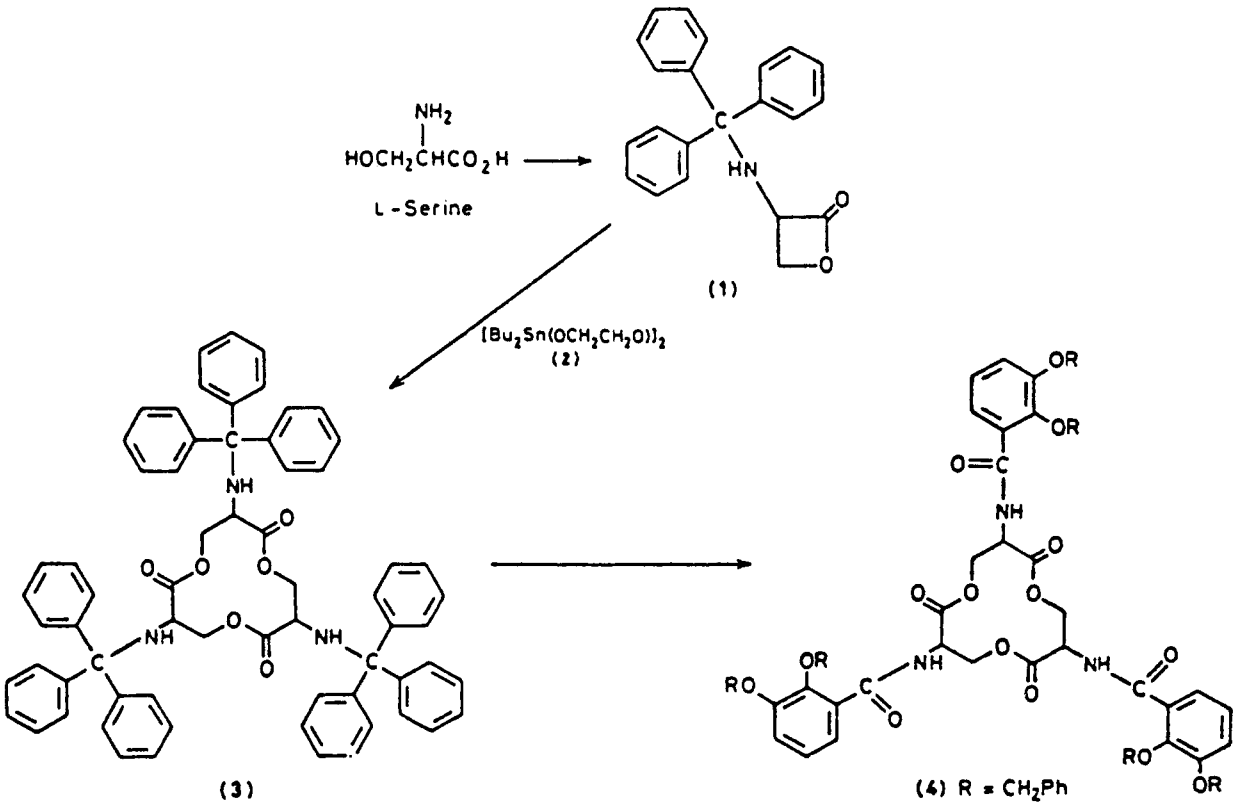


Figure 2. Template synthesis of enterobactin.

proximately 16%. It is clear of course that this does not offer opportunities for synthesis of such material on large scale but it certainly does offer the opportunity for the production of enantioenterobactin simply by replacement of the L-serine by D-serine. A similar synthesis of enantioenterobactin was described by Rastetter,[25] the only major difference in the two approaches being that Rastetter chose somewhat different protecting groups for the carboxyl function of serine and the hydroxyls of catechol. Methylanthraphenone, a photochemically labile protecting group, was used to mask the carboxyl, and introduction of the 2,3-dihydroxybenoyl moieties employed 2,3-bis(benzyloxy)benzoyl chloride as the acylating agent. The benzyl protecting groups were later removed under hydrogenolysis conditions. Other than this, conceptually, the generation of this serine macrocycle itself was essentially identical with Corey's procedure. Enantioenterobactin was the target system which was accessed in this 12-step synthesis, again in an overall yield around 20%.

One of the more recent methods of accessing enterobactin comes from Shanzer's laboratories[56] and employs tin as a metal template (Figure 2). Tritylated L-serine is cyclized to its β-lactone 1 by using 4-(dimethylamino)pyridine and diisopropylcarbodiimide. The lactone is next cyclized to the enterobactin skeleton 3 by using the stannoxane 2. The trityl protecting groups were removed with HCl in ethanol and the product was acylated with the nitrophenyl ester of 2,3-bis(benzyloxy)benzoic acid to produce 4. Finally, the *O*-benzyl protecting groups were removed under hydrogenolysis conditions to produce the target system enterobactin. The scheme represents both economy in steps and a new potentially very useful application of the template concept.

Because of enterobactin's remarkable iron-binding properties a number of groups saw fit to consider the synthesis of enterobactin model systems. The first of these analogues was a carbocyclic system also synthesized by Corey.[26] *all-cis*-Cyclodecane-1,5,9-triol was converted to the corresponding tris(azide) by first tosylating the triol and next reacting this with sodium azide in dimethylformamide. The resulting tris(azide) was next reduced catalytically to the corresponding triamine and this was acylated with the acetonide of 2,3-dihydroxybenzoyl chloride. Finally, the catechol isopropylidene protecting groups were removed by heating the tris(amide) with acetic acid and water. This final hexacoordinate ligand was shown to bind iron tenaciously and also to serve as an iron transport device in microbial systems.

The next synthetic enterobactin analogue was reported simultaneously by Neilands and Raymond: 1,3,5-tris[((2,3-dihydroxybenzoyl)amino)methyl]-benzene.[27,28] The design of this isosteric equivalent of enterobactin was built on the idea that the 1,3,5-benzylic platform would effectively mimic the rigidity conferred upon the 2,3-dihydroxybenzoyl moieties by enterobactin's 12-membered cyclic triester. Furthermore, at the time that the synthesis of this analogue was undertaken, workers were particularly enchanted with the prospect that these systems would not be subject to the kind of hydrolysis that enterobactin could sustain under physiological conditions. Enterobactin's macrocyclic serine backbone would clearly be subject to either aqueous hydrolysis or nonspecific serum esterase promoted hydrolysis when given as a therapeutic to animals. Both of these problems would render this system essentially useless as a therapeutic for the treatment of iron overload. At any rate, the Neilands synthesis of this enterobactin analogue was accomplished very effectively by coupling 1,3,5-tris(aminomethyl)benzene trihydrochloride with 2,3-bis(benzyloxy)benzoyl chloride affording the triacylated amine in 81% yield. The

benzyl protecting groups were easily removed under hydrogenolysis conditions in ethanolic acetic acid with palladium in 90% yield. In was not too surprising to find that this system was a very effective iron chelator as well as capable of delivering iron to a number of *E. coli* mutants enterobactin matrix.[29]

Raymond's scheme for the synthesis of this system differs from Neilands' procedure in two major areas: access of the carbocyclic platform and the protecting groups used to protect the catechol hydroxyls. Neilands' procedure for the synthesis of 1,3,5-tris(aminomethyl)benzene trihydrochloride employed the catalytic reduction of 1,3,5-benzene-tri-L-doxamine. Raymond's procedure, on the other hand, is initiated by the reaction of "trimzoyl" chloride with concentrated ammonium hydroxide to produce the corresponding amide followed by reduction of this amide with diborane and THF to generate the corresponding triamine.[27] Unlike the Neilands synthesis Raymond's employs 2,3-dimethoxybenzoyl chloride as the acylating agent, a reagent which is more easily accessible and less likely to present problems associated with the sterically bulky benzyl protecting groups. Furthermore, once the triamine is acylated, the methyl protecting groups can be easily removed with boron tribromide and methylene chloride, resulting in excellent yields of the desired product. One of the major concerns regarding this model system is that the platform provided by the aromatic ring is so much smaller than the one provided by the serine backbone of the natural siderophore, enterobactin. In keeping with this concern Raymond synthesized a system which is sterically at least more like the enterobactin molecule, the *N,N',N''*-tris(2,3-dihydroxybenzoyl) derivative of 1,5,9-triazacyclotridecane.[27] This system was generated from the condensation of 2,3-dimethoxybenzoyl chloride and the parent triazacyclodecane followed by removal of the methyl protecting groups with BBr_3 and methylene chloride. The parent macrocycle was generated by the reaction of *N,N',N''*-tris(*p*-toluenesulfonyl)-1,5,11-triazadecane dianion with 1,3-ditosylpropane. The three *p*-toluenesulfonyl protecting groups of the resulting macrocycle were removed with concentrated sulfuric acid. The initial tris(*p*-toluenesulfonyl) compound is obtained by exhaustive tosylation of spermidine.

Finally, the Rastetter group considered both a cyclic imido analogue of the enterobactin and an acyclic enterobactin model system.[25] Although the attempted synthesis of a macrocyclic imido analogue of enterobactin failed, the acyclic imido analogue was synthesized. It is interesting that the iron(III) acylic imido analogue and the enterobactin Fe(III) complex CD curves are essentially identical. Furthermore, the thermodynamics of iron(III) binding revealed the equilibrium for this analogue of enterobactin was very similar to the equilibrium of the formation of the enterobactin iron(III) complex.[5] The stability constant for the linear iron(III) complex was calculated[5] to be 10^{46}.

Synthesis of the linear enterobactin analogue was a rather lengthy 22 steps.[25] For details of the synthesis readers are encouraged to see the original reference. The key intermediate 1 shown in Scheme II is a tripeptide with three internal carbobenzyloxy protected nitrogens and a terminal *tert*-butoxycarbonyl protected

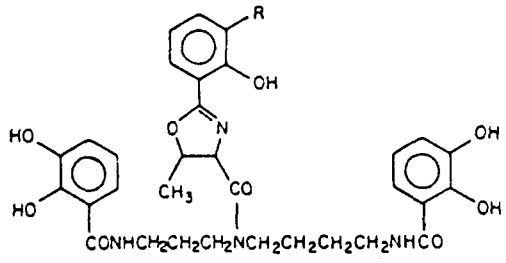
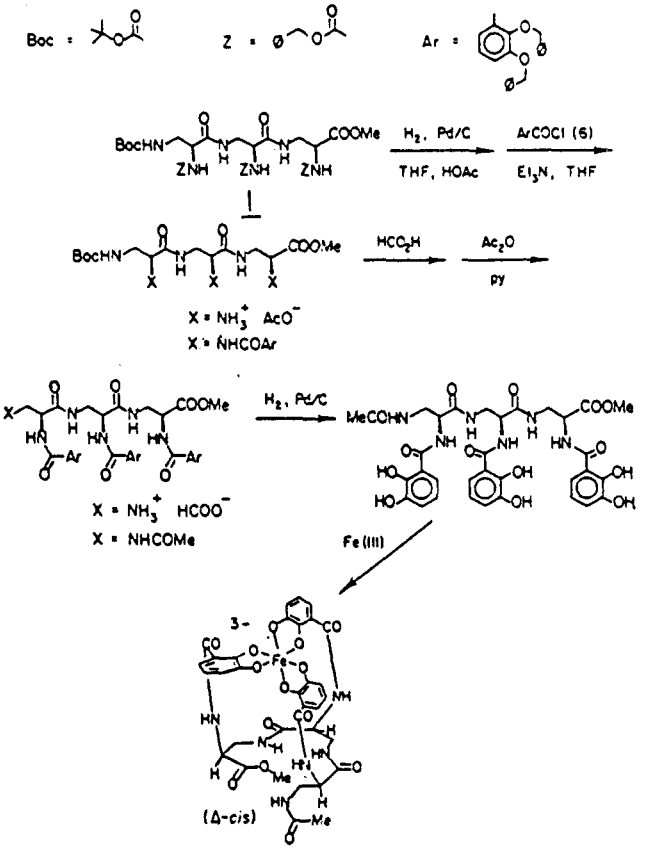


Figure 3. Agrobactin (R = OH) and parabactin (R = H).

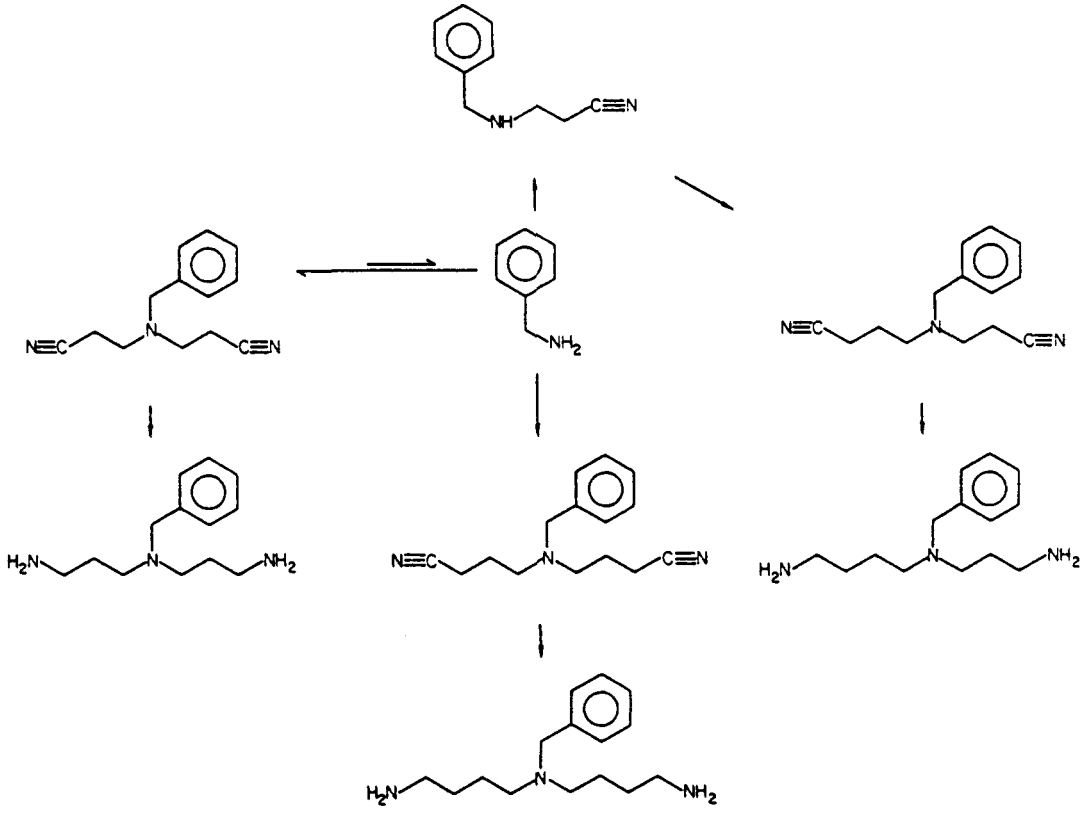
SCHEME II. Key Intermediates in the Synthesis of Acyclic Enterobactin Analogue



nitrogen. This intermediate was first hydrogenolyzed to remove the carbobenzyloxy protecting groups followed by acylation of the free nitrogens with 2,3-bis(benzyloxy)benzoyl chloride. The *tert*-butoxycarbonyl protecting group was next removed with formic acid followed by acetylation of the free nitrogen and finally the catechol benzyl protecting groups were removed under hydrogenolysis conditions resulting in the linear tris(catechol) enterobactin analogue.

Nature's answer to the above linear tris(catechol) model of enterobactin can be seen in a number of natural product catecholamide siderophores with a spermidine backbone. The first of the spermidine catecholadmide systems identified, parabactin, was isolated by Tait from *Paracoccus denitrificans*[29] and is shown in Figure 3. Tait's initial studies indicated that the biological precursor to parabactin was N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine, a ligand also effective at binding iron. The initial structural assignment for parabactin, however, was incorrect. The group fixed to the N^4 nitrogen was originally thought to be *N*-(2-hydroxybenzoyl)-L-threonyl, but was later shown to be 2-(2-hydroxyphenyl)carboxyl-5-methyl-2-oxazoline.

SCHEME III. Synthesis of Protected Polyamine Reagents for Selective Acylation

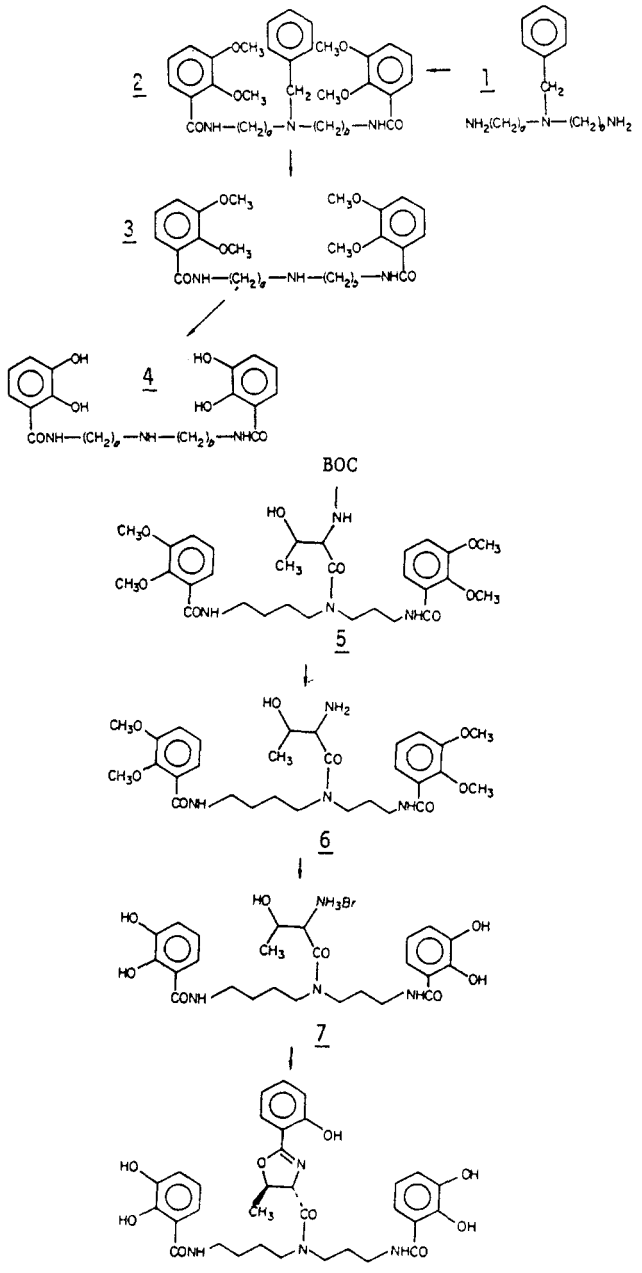


Neilands was able to prove the existence of an oxazoline ring in parabactin by the use of both nuclear magnetic resonances and a comparison of ultraviolet spectra[30,31] of parabactin with that of model compounds he synthesized.[24,31]

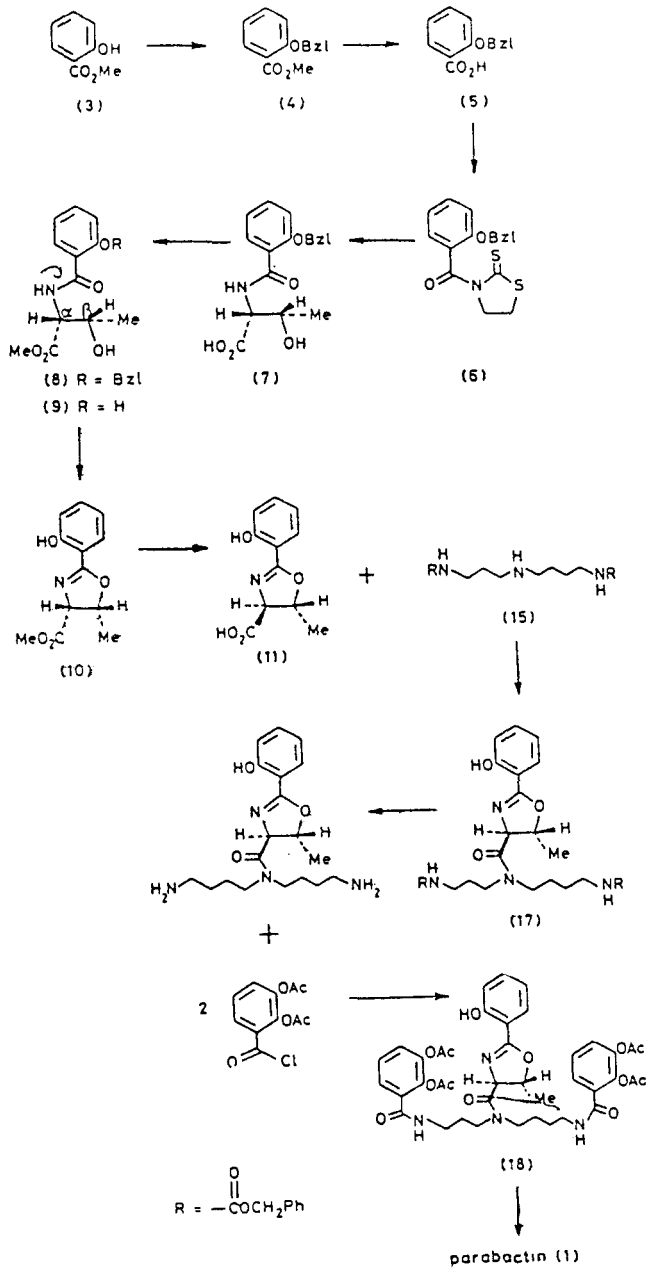
The most outstanding structural difference between enterobactin and parabactin is, of course, the lack of symmetry in parabactin, a feature which immediately complicates the synthesis. The structural features to be considered in the design of a parabactin synthesis are the N-terminal 2,3-dihydroxybenzoyl group, the central oxazoline ring, and, of course, the spermidine backbone. All three nitrogens of the polyamine are acylated in this system, suggesting that any synthetic design of this siderophore must consider methods of differentiating between the two terminal primary amino nitrogens and the central secondary amino nitrogen. In addition, the synthetic sequence must also deal with the possible configurational isomers of the oxazoline ring. The stereochemistry of this five-membered ring was shown to be trans, arising from the cyclization of the appropriate L-threonyl derivative about a one-carbon synthon.

The synthesis of parabactin and its homologues and analogues developed in our laboratories was predicted on the development of a group of secondary N-protected triamines.[32,33] These reagents, as shown in Scheme III, are easily accessible in high yield and make it possible to access either parabactin itself or its norspermidine or homospermidine analogues. The synthesis of all three of the protected polyamines begins with benzylamine. Benzylamine can be first biscyanoethylated with acrylonitrile and the symmetrical nitrile reduced with W-2 Raney nickel to the corresponding N^4-benzylnorspermidine system. Alternatively, benzylamine can be bisalkylated with 4-chlorobutyronitrile and this symmetrical nitrile reduced with W-2 Raney nickel to produce the N^5-benzylhomospermidine. Finally, the N^4-benzylspermidine reagent itself is made by first cyanoethylating benzylamine followed by alkylation of the resulting N^4-benzylproprionitrile with 4-chlorobutyronitrile. The asymmetrical bis(nitrile) is now reduced with W-2 Raney nickel to the desired reagent. These systems can be acylated with 2,3-dimethoxybenzoyl chloride and the secondary N-benzyl group removed by hydrogenolysis. At this point one can next proceed either to parabactin or its homologues or to the production of its biological precursor or its homologues (Scheme IV).[37,39] Simple demethylation of the methylcatecholamide intermediate **3** with BBr_3 in methylene chloride provides parabactin's biological precursor or its homologues **4**. In moving on to parabactin itself and its analogues, the tetramethoxy compounds **3** are first reacted with the N-hydroxysuccinimidyl ester of N-*tert*-butoxycarbonylated threonine. The resulting amide **5** is next stripped of its *tert*-butoxycarbonyl protecting group by brief exposure to trifluoroacetic acid followed by removal of the methyl protecting groups with boron tribromide in methylene chloride. This intermediate **7** is now reacted with 2-hydroxybenzimido ethyl ether to produce either parabactin or its homologues depending on which of the benzylated polyamines was used for the starting material. Parabactin obtained in this way was identical with the natural product in terms of its infrared, high-field ^1H NMR, and optical rotation spectra. There have been a large number of siderophore model systems developed in this laboratory and elsewhere, predicted on the polyamine backbone. This was generally accomplished by fixing the appropriate functionality to the tetramethoxy system **3** (Scheme IV) in one step followed by treatment with BBr_3. A number of the

SCHEME IV. Total Synthesis of Parabactin and Its Analogues



SCHEME V. Alternative Synthesis of Parabactin

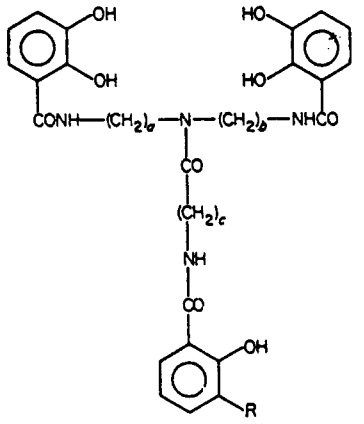


hexacoordinate ligands synthesized in this fashion are indicated in Table I.

In contrast to the efficiency and flexibility in this synthesis is the procedure described by Nagao and Fujita (Scheme V). The synthesis requires some 14 steps with an overall yield of 3%. The previous procedure proceeds in 69% overall yield and requires only four steps from N^1,N^8-bis(2,3-dimethoxybenzoyl)spermidine, an extensively utilized intermediate in the synthesis of catecholamide chelators. Although the Nagao procedure does offer an additional example of the use of sulfur-containing leaving groups, it certainly does not represent facile access to iron chelators. The synthesis begins with methyl 2-hydroxybenzoate (3) which is benzylated to the corresponding ether 4 prior to hydrolysis of the methyl ester to produce the acid 5. This compound is reacted with 1,3-thiazolidine-2-thione and dicyclohexylcarbodiimide in the presence of 4-(dimethylamino)pyridine to afford 3-(2-(benzyloxy)-benzoyl)-1,3-thiazolidine-2-thione (6). This was reacted with threonine to acylate the amino acid resulting in 7. One of the points the authors advertize is the chemoselectivity of this step. Of course, this kind of selectivity is hardly new as it can be easily accomplished with *N*-hydroxysuccinimide esters.[57] The intermediate 7 was debenzylated to the phenolic ester 9 and cyclized with $SOCl_2$ to the oxazoline 10. This compound was next epimerized at C_α to provide the trans isomer 11. This intermediate was next condensed with N^1,N^{10}-bis(benzyloxycarbonyl)spermidine in the presence of phenylphosphoramide to produce 17. The N^1,N^{10}-acyl groups were next removed with acid and the product acylated with 2,3-diacetoxybenzoyl chloride to give 18 and this intermediate deacylated, providing "crude parabactin." It is this kind of circuitous route that future workers should be encouraged to circumvent.

In a second approach to the synthesis of spermidine catecholamide chleators we chose not to consider N^1,N^8-bis(2,3-dimethoxybenzoyl)sperimidine as the intermediate in the synthesis of a tris(catecholamide)

TABLE I

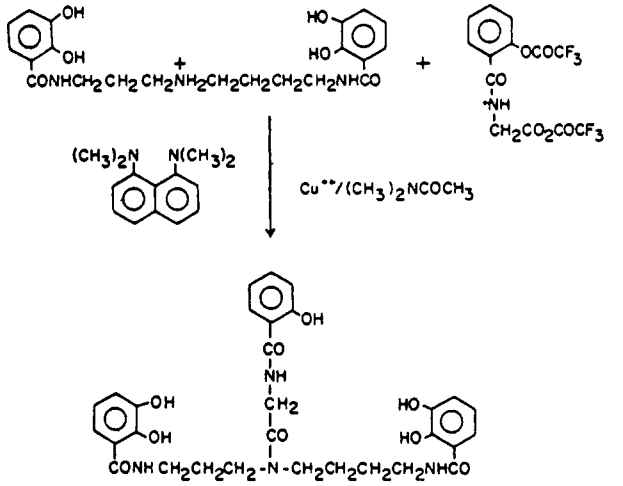


a	b	c	R
3	4	1	H
3	3	1	H
4	4	1	H
3	4	1	OH
3	4	2	OH
3	4	3	OH

chelator but, rather, decided to evaluate a biomimetic synthesis. The biomimetic synthesis was based on the observation made by Tait that N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine compound (II) is the biological precursor to parabactin.[34] The issue is of course how the microorganism selectively acylates the polyfunctional catecholamide at its secondary nitrogen. The acylation could have been carried out by an enzyme that simply supplies the kind of steric selectivity that allows it only to access the secondary nitrogen. Alternatively, the catechol hydroxyls could have been protected in some way by metal chelation. One might even imagine that the metal could serve as a template in bringing the synthons together. A number of possibilities come to mind regarding protection of the catechols through metal chelation, the first of which is the formation of a copper chelate. Copper(II) cations are well-known to form stable square-planar complexes with tetracoordinate ligands. We were able to show by using Job's plots that copper(II) did indeed form 1:1 complexes with compound II. We were further able to demonstrate that these were very tight pH sensitive complexes with formation constants in excess of 10^{30}. The thermodynamic parameters for these complexes were measured by using potentiometric titration techniques and are described in detail elsewhere.[35] Finally, that the catechol groups were indeed involved in chelation was demonstrated by a ^{1}H NMR study of the copper(II)–compound II complex. When copper(II) was added to a basic solution of compound II the aromatic protons were seen to quickly broaden into the baseline. This broadening occurred at very low copper(II)/ligand ratios and long before any broadening was observed in the protons associated with the spermidine backbone.

With these observations in hand we were encouraged to attempt the selective acylation of the copper chelate. The tetracoordinate ligand was reacted with copper acetate in dimethylacetamide in the presence of N^1,N^8-bis(dimethylamino)naphthalene, producing a green solution which was treated with an activated ester of hippuric acid. After several hours at 0° C, the reaction mixture was acidified to destroy the copper complex (Scheme VI). The secondary N-hippurylated

SCHEME VI. Biomimetic Synthesis of Parabactin Analogue

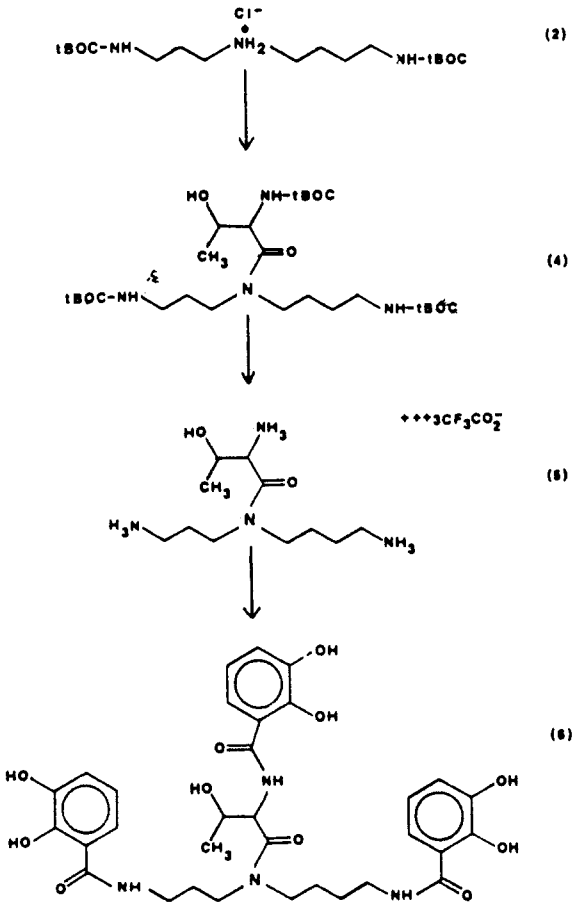


compound was isolated with no indication of any concomitant O-acylation. Attempted acylation of the tetracoordinate ligand in the absence of copper resulted in a mixture of products associated with O-acylation.

All of the syntheses of the spermidine catecholamide ligands described above employed schemes in which the central nitrogen was functionalized last, procedures in which the catechol groups were protected, thus requiring subsequent deprotection in the last steps. In the synthesis of agrobactin A we chose a somewhat different "inside out" approach. The central nitrogen was acylated in the early stages of synthesis. This demanded the development of reagents in which the terminal nitrogens of the triamines were protected.[37] The secondary N-benzylated triamines described above in Scheme III were reacted with the reagent BOC-ON, (*tert*-butoxycarbonyloximino)-2-phenylacetonitrile, in order to introduce *tert*-butoxycarbonyl protecting groups at the primary nitrogens. The benzyl protecting group of each of these bis(BOC)triamines was then removed under hydrogenolysis conditions to produce the corresponding terminal bis(BOC) polyamine derivatives. With these reagents it was now possible to secondary-N-acylate the triamines selectively. Agrobactin A as well as the homospermidine analogue of agrobactin A were prepared by using these reagents.[39] The bis(butoxycarbonyl)spermidine reagent was first reacted with the *N*-hydroxysuccinimidyl ester of N-*tert*-butoxycarbonylated threonine to produce the secondary N-threonylated spermidine compound in 91% yield as shown in Scheme VII. This tri-*tert*-butoxycarbonylated system, when briefly exposed to trifluoroacetic acid, resulted in the corresponding deprotected triamine in 97% yield. Next, the three nitrogens were acylated with the *N*-hydroxysuccinimidyl ester of 2,3-dihydroxybenzoic acid to produce agrobactin A, the hydrolysis product of the natural chelator agrobactin, in 75% yield. The overall yield of this procedure is certainly reasonable at 66%. The same procedure can of course be followed by using either homo- or norspermidine starting materials.

Neilands' synthesis of agrobactin A employed 2,3-bis(benzyloxy)benzoyl chloride and spermidine as starting materials. The terminal nitrogens of the triamine are acylated in 50% yield with the acid chloride. The diamide was next coupled with *N*-[2,3-bis(ben-

SCHEME VII. Synthesis of Agrobactin A



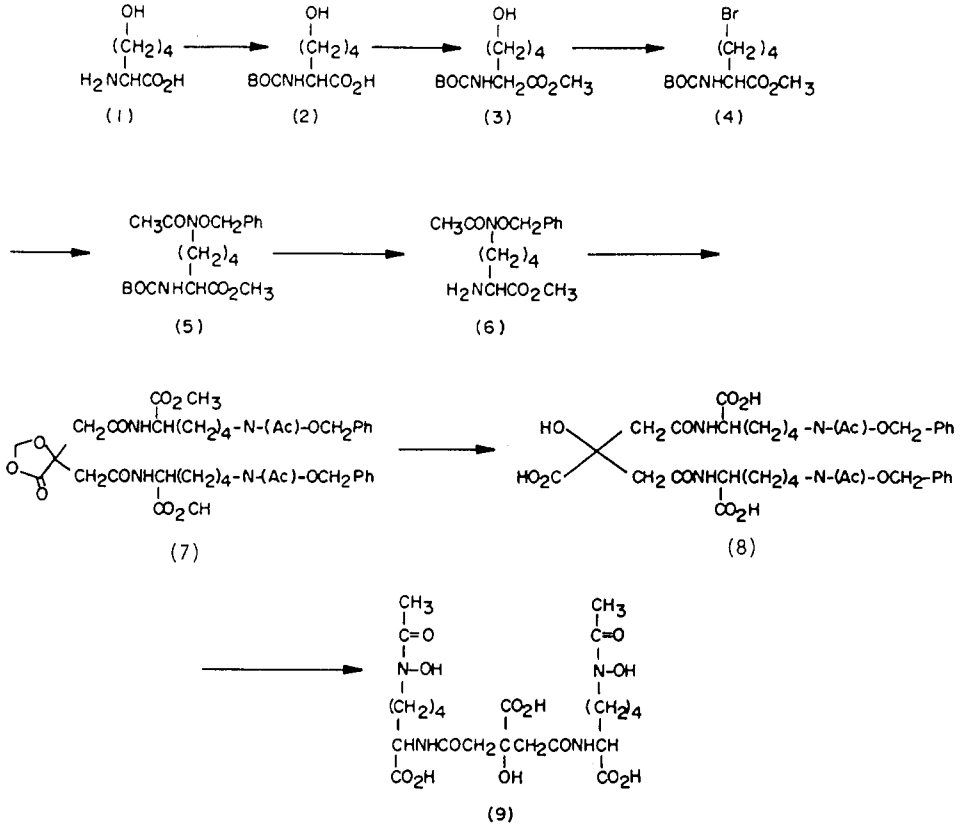
zyloxy)benzoyl]threonine using dicyclohexylcarbodiimide and the product debenzylated to produce agrobactin A in 45% yield. The threonyl compound was generated in 70% yield by coupling 2,3-bis(benzyloxy)benzoyl chloride with threonine. This represents an overall yield of 16%.

A variety of other linear tris(catecholamide) iron chelators have been synthesized; the schemes involve only simple addition of protected 2,3-dihydroxybenzoyl groups to the appropriate triamine followed by deprotection of the catechol groups. Although the chemistry involved in the synthesis of these tris(catecholamides) does not warrant extended discussion, it is worthwhile at this point to consider some of the more useful protected 2,3-dihydroxybenzoic acid synthons. These synthons can be divided into two groups, those which do not have the catechol protected and those which do. The first group of the synthons includes 2,3-dihydroxybenzoic acid (DHB) itself[38] and the *N*-hydroxysuccinimidyl ester of 2,3-dihydroxybenzoic acid.[38] The second group includes among others 2,3-(methylenedioxy)benzoic acid,[32] 2,3-dimethoxybenzoic acid,[26] 2,3-(sulfinyldioxy)benzoyl system,[8] 2,3-diacetoxybenzoic acid,[32] and the 2,3-dibenzyloxy system.[28] The first family of synthons is of course plagued with the expected problem, self-condensation, a situation which is quite variable depending on how the acid is activated as well as on the nucleophile adding to it. Attempted condensation of 2,3-dihydroxybenzoic acid itself with spermidine via condensing agents such as dicyclohexylcarbodiimide have not generally resulted in high yields of selectively acylated products. However, we have found that the *N*-hydroxysuccinimidyl ester of 2,3-dihydroxybenzoic acid is an excellent reagent for the introduction of the DHB function at nitrogens. The reagent itself is easy to prepare in very high yield, is crystalline, and can be stored on the shelf indefinitely.[38] The hydroxyl protected reagents of course can be condensed with the appropriate nitrogen, again either as the corresponding acid chloride, the *N*-hydroxysuccinimidyl ester, or the carboxylic acid in the presence of a condensing agent. In choosing one of the protected DHB reagents, one must consider the following four factors: (1) the ease with which the protecting group can be introduced; (2) the ease with which the protecting group can be removed; (3) the compatibility of the group with the chemistry to be carried out on the system (i.e., are there any conditions likely to cause unwanted protective-group removal); and (4) the conditions required for the protective-group introduction or removal must be compatible with the chemistry of the rest of the molecule. In terms of protective-group introduction the preparation and purification of the methylenedioxy and dibenzyloxy compounds is somewhat laborious. The compound 2,3-dimethoxybenzoic acid is, in fact, commercially available, and 2,3-diacetoxybenzoic acid is very easily prepared[32] and easily purified by reaction of the parent acid with acetic anhydride in sulfuric acid. Finally, the 2,3-sulfinyldioxy protecting group is very easy to prepare.[26] In fact, by reacting 2,3-dihydroxybenzoic acid with thionyl chloride under reflux conditions one obtains 2,3-(sulfinyldioxy)benzoyl chloride, i.e., both the protected and activated 2,3-dihydroxybenzoic acid derivative. Both the methylenedioxy[8] and the methyl protecting[18] groups can be removed in very high yield and with great facility by using boron tribromide and methylene chloride. Other strong Lewis acids have also been shown to promote at least partial deprotection. The benzyl protecting group is removed by hydrogenolysis over a palladium catalyst under a hydrogen atmosphere, preferably in acetic acid as a solvent. Again, strong acid conditions have been shown to promote the cleavage of the benzyl protecting group, although mixtures of products are obtained. Once these ether-protected DHB's have been fixed to a nitrogen, exposure of the system to a strong acid should be avoided lest partial cleavage of the protecting groups occur. The acetyl protecting groups are very base labile and have been typically removed by treatment with sodium methoxide and methanol followed by an acid workup. However, extended exposure to aqueous acid also results in some hydrolysis. Obviously, then, once 2,3-diacetoxybenzoyl groups have been fixed to the nitrogen the system cannot be exposed to base or strong aqueous acid. The 2,3-(sulfinyldioxy)benzoyl group is easily removed simply by exposure of the product to warm water. Because of its extreme lability it has been avoided by some workers.[20]

III. Hydroxamate and Related Siderophores

The major functional difference between hydroxamate siderophores and the catecholamides is related to environmental iron concentration.[18] The hydroxamates are generated by the microorganism in a high-iron environment while the catecholamide's "backup" system is activated when iron concentrations are low. The catecholamide chelators typically bind iron far more

SCHEME VIII. Key Intermediates in the Synthesis



tightly than hydroxamates. We will begin our discussion of siderophore hydroxamate synthesis with aerobactin,[39] an iron chelator isolated from *Aerobacter aerogenes*. The major hurdle in the construction of this molecule is in the production of N^6-acetyl-N^6-hydroxylysine (Scheme VIII). The synthesis begins with ξ-hydroxynorleucine, a previously prepared amino acid. This hydroxy amino acid was first converted to the corresponding N-*tert*-butoxycarbonylated compound by reaction with di-*tert*-butyl dicarbonate in THF/water, and the resulting amino acid **2** was next converted to the corresponding methyl ester **3**. The hydroxyl group of this system was next brominated with triphenylphosphine and carbon tetrabromide, resulting in **4**, followed by alkylation of the halide with *O*-benzyl acetohydroxamate in anhydrous acetone in the presence of potassium carbonate and potassium iodide to produce **5**. The *tert*-butoxycarbonyl protecting group was next removed by brief exposure of the alkylated product to trifluoroacetic acid and the resulting salt converted in the presence of sodium carbonate to the free amine **6**. This free amine was next coupled with anhydromethylenecitryl chloride to provide the expected diamide **7** in excellent yield. Finally, the anhydromethylenecitric acid derivative was deprotected by brief exposure to sodium hydroxide and water/THF followed by an acid workup, resulting in **8**. The final product aerobactin (**9**) was generated by reductive removal of the benzyl protecting groups. The overall reaction sequence required nine steps and proceeded in about 35% overall yield. Similar thinking went into the synthesis of both schizokinen and arthrobactin.[40] These natural ferric ionophores differ only in the length of the methylene backbone: $n = 3$ for schizokinen and $n = 5$ for arthrobactin (Figure 4). Conceptually, the syntheses of both schizokinen and arthrobactin begin

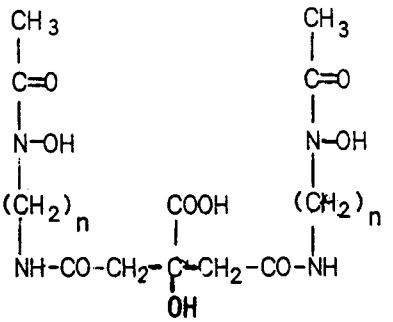
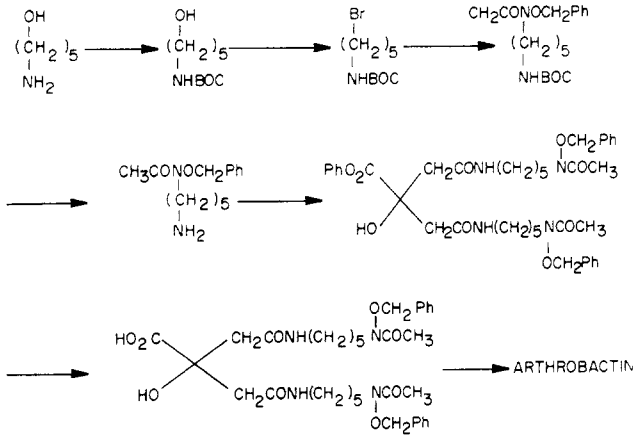


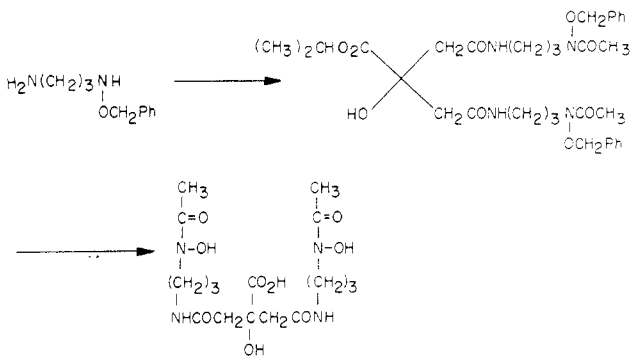
Figure 4. Schizokinen ($n = 3$), arthrobactin ($n = 5$).

in the same place. Either the C-3 or the C-5 amino alcohols are first N-*tert*-butoxycarbonylated, and the corresponding alcohols are converted to the halides by reaction with methylphosphine and carbon tetrabromide. These halides are next alkylated with the anion of *O*-benzyl acetohydroxamate. At this point the similarity between the two syntheses disappears. Arthrobactin, the ionophore with the longer of the two methylene bridge systems, is synthesized by first removing the *t*-BOC protecting group by brief exposure of the last described arthrobactin intermediate to trifluoroacetic acid. This amine salt is then reacted with bis(*p*-nitrophenyl) ester activated, benzyl ester protected citric acid to produce the corresponding diamine compound. This intermediate is reacted with sodium hydroxide in THF to remove the benzyl ester followed by exposure to catalytic reduction over palladium to remove the remaining benzyloxy protecting groups (Scheme IX). The rather unorthodox cleavage of the benzyl ester was required because attempted removal of the three benzyl protecting groups by a single hydrogenolysis in methanol resulted in the corresponding methyl esters. Attempted conversion to the final

SCHEME IX. Total Synthesis of Arthrobactin



SCHEME X. Key Intermediates in the Synthesis of Schizokinen

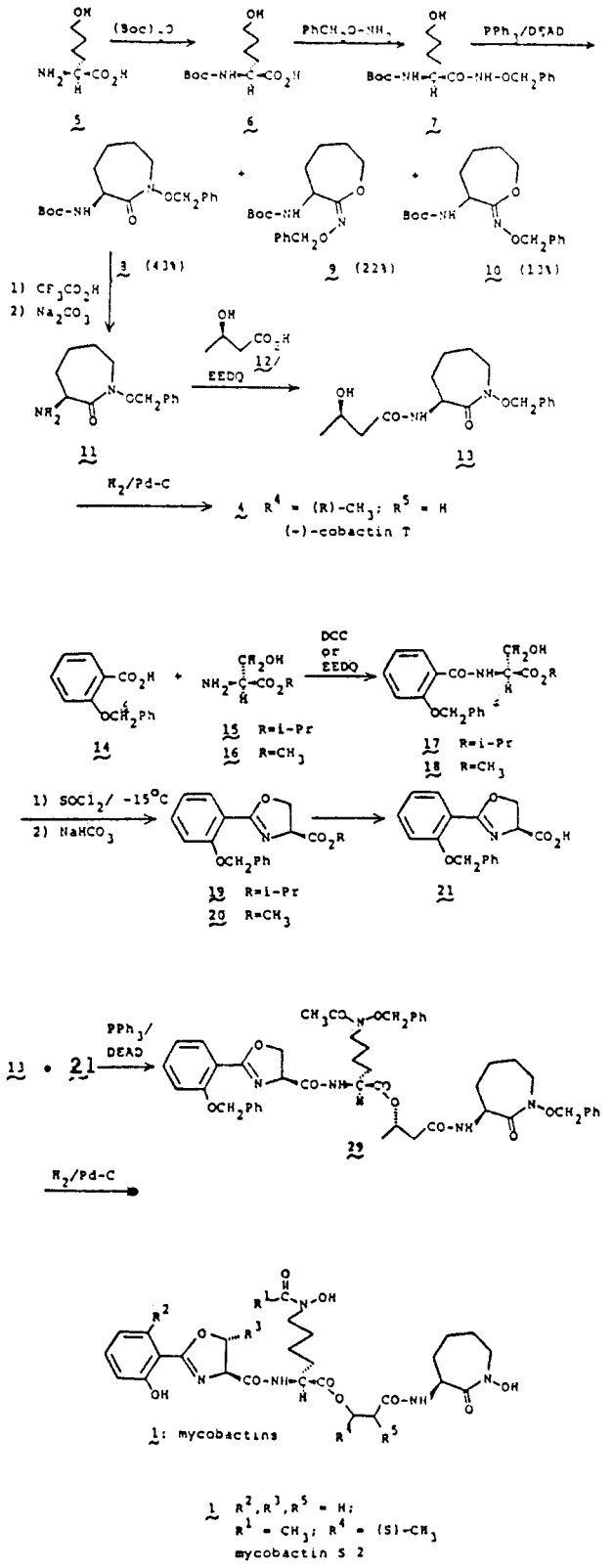


products in THF/water resulted in cyclized analogues, e.g., arthrobactin A or simply mixtures of undesired material.

The synthesis of schizokinen followed a somewhat different route. The compound 1-amino-3[(benzyloxy)amino]propane was coupled with the bis(*p*-nitrophenyl) ester of citric acid. However, in this instance the benzyl ester of Scheme IX was replaced by the more bulky isopropyl group. After coupling, acetic anhydride was added prior to acetylating the hydroxylamine nitrogen. The final protective group cleavage, treatment with base followed by hydrogenolysis, was identical with the arthrobactin case, giving schizokinen (Scheme X).

The mycobactins[41] incorporate not only the hydroxamate functionality but also the salicyloxyloxazoline system. Although the synthetic scheme for the synthesis of mycobactin S2 incorporates many of the synthetic devices described above for the hydroxamates, the differences in the overall picture certainly merit some discussion. The synthesis (Scheme XI) begins with ξ-hydroxynorleucine (**5**) which is reacted with di-*tert*-butyl dicarbonate to protect the α-nitrogen followed by reaction of the carboxyl group with *O*-benzylhydroxylamine to produce the corresponding acyl derivative **7**. The amide is now cyclized with triphenylphosphine and diethyl azodicarboxylate although the desired lactam (**8**) is produced in only 43% yield. The lactam is then exposed to trifluoroacetic acid, promoting loss of the *tert*-butoxycarbonyl protecting group followed by a basic workup to generate the expected amine **11**. The amine nitrogen is then condensed with *d*-β-hydroxybutyric acid by using a slight excess of *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ), resulting in the first of the key intermediates,

SCHEME XI. Total Synthesis of Mycobactin S2



benzyl-protected cobactin **13**. The next major component of this sequence focuses on the mycobactic acid segment of the molecule. This synthesis begins with coupling of serine with the *p*-nitrophenyl-activated ester of *O*-benzyl-protected salicylic acid. The resulting amine **15** or **16** is then coupled to the benzyl-protected hydroxamate by using EEDQ in choroform. This product was cyclized by treatment with thionyl chloride followed by exposure to aqueous base and then mineral

acid to produce the corresponding carboxylic acid 21. The acid is finally condensed with the benzyl-protected cobactin by using triphenylphosphine in diethyl azodicarboxylate (DEAD) followed by hydrogenolysis to remove all of the benzyl protecting groups, resulting in the production of mycobactin S2.

We recognize the fact that we have not described all of the syntheses focused on the generation of siderophores but rather have dealt with what we regard as representative examples of some of the more important systems. However, for further materials, readers can see the article by Wierenga.[58]

IV. Solution Structure of Siderophores

Having looked at some of the more relevant synthetic schemes as they apply to the production of microbial iron chelators, we will now move on to a description of the solution structure of several of the ligands and their metal complexes. The solution structures of the systems are very relevant to the biological functions of these systems.

The major interest in the stereochemistry of siderophores and their metal chelate complexes is closely aligned with the idea that only those chelates or chelators of the proper geometry will promote delivery of iron to the microorganisms.[25] It was demonstrated by Raymond that the desferriferrichrome–chromium(III) complex in solution was composed solely of the Δ isomer.[42] This chromium complex was taken up at the same rate as ferrichrome was by *Uestalago sperageno*.[43] This uptake behavior of both ligands was interpreted in the following way. As the chromic complex of desferriferrichrome is kinetically inert and was found to be transported by *Uestalago*, the Δ optical isomer must be at least one of the biologically active isomers. Furthermore, since the uptake rate of the ferrichrome and chromic desferriferrichrome complexes are similar, both probably share the same transport system. Finally, it was thought unlikely that this specific iron transport system could rely on isomerization or partial dissociation of the complex during transport through the membrane.

The solution geometry of the siderophores and their corresponding iron chelates has been the subject of numerous spectroscopic investigations, most of which have employed either NMR[44-46] or circular dichroism[41-47] as the spectroscopic technique. One of the initial concerns regarding studies of the coordination isomers of biological iron transport compounds was the kinetic lability of these high-spin ferric complexes. In order to overcome this potential problem workers considered the synthesis, isolation, and characterization of the corresponding chromium(III) complexes. In an early study the chromic complex of tris(*N*-methyl-L-menthoxyacetohydroxamato)chromium(III) was resolved into four isomers, Λ-cis, Δ-cis, Λ-trans, and Δ-trans isomers, and characterized. A comparison of the circular dichroism spectra of these isolated models with the circular dichroism spectra of chromic desferrichrome and chromic desferriferrichrysin revealed that the solution geometry of the chromic siderophore complexes was essentially all Δ-cis. This selectivity in the formation of specific coordination isomers was also observed with desferriferrioxamine B–chromium(III) complexes.[49] There are in fact five possible enantiomeric geometric isomers which ferrioxamine B can form with chromium(III). However, chromatographic studies reveal that the chromic complexes consist of only two geometrical isomers, which have been resolved, a cis and a trans isomer. Which of the trans isomers was produced will, of course, have to await X-ray crystallographic studies.

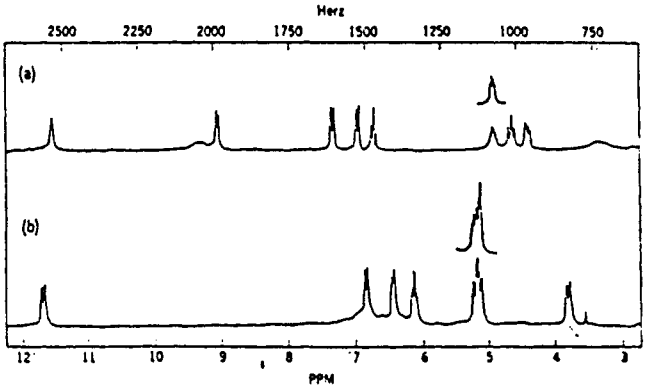


Figure 5. 220-MHz ^1H NMR spectra of enterobactin (a) and Ga^{3+}–enterobactin (b) dissolved in $(CD_3)_2SO$ at ~45°. Chemical shifts are referred to internal Me_4Si and are listed in Table I. Inserts above each spectrum show the α-proton resonance after the amide has been exchanged with D_2O. See Table II for the magnitudes of the proton spin-spin coupling constants (*J*). The broad resonance at ca. 3.4 ppm is due to residual water.

Similar solution conformational studies were carried out on the corresponding catecholamide–iron complexes. For example, it can be seen from molecular models that two diastereomers are possible for the ferric enterobactin complex, Λ-cis and Δ-cis. Because of the optical center found in the ligand, these are not simple mirror images but diastereomers. However, only one chromic complex of enterobactin was observed, a complex whose optical activity was even greater than that of the resolved chromium(III)–tris(catecholate) complex, forcing one to conclude that one isomer of the two possible isomers predominates.[52] The comparison of the chromium(III)–enterobactin CD spectra with the CD spectra of the Λ isomer and Δ isomer of the chromium(III)–tris(catecholate) complexes of chromodesferriferrichrome clearly shows that the enterobactin complex is of the Δ-cis absolute configuration. Furthermore, because the ferric complex is known to have a net optical activity, one isomer must also predominate in ferric enterobactin. This isomer of ferric enterobactin has also been assigned as the Δ isomer on the basis of the following: (a) the ionic radii of iron(III) and chromium(III) are equal to within 0.03 Å, and the salts $K_3[M(K_{at})_3]\cdot 1.5\ H_2O$ (M = Fe, Cr) are isostructural; (b) both the chromium–enterobactin complex and the iron–enterobactin complex have identical chromatographic properties.[52]

A second and far more informative technique applied to the studies of the enterobactin–metal complex conformation is that of nuclear magnetic resonance spectroscopy. The investigations were carried out on the gallium(III) complex to avoid the line-broadening problems associated with the paramagnetic properties of the iron(III) complex.[46] The 220-MHz ^1H NMR spectra of enterobactin and its gallium chelate dissolved in dimethyl sulfoxide are shown in Figure 5, and the corresponding chemical shifts and coupling constants are indicated in Table II and Table III. It is clear that there are rather substantial differences in the coupling

TABLE II. ^{1}H NMR Chemical Shifts[a]

proton	enterobactin	Ga^{3+}–enterobactin
	Benzoyl	
o,p-CH	7.34, 6.98	6.84, 6.44
m-CH	6.73	6.13
o-OH	11.56	
m-OH	9.34	
	Seryl	
$C_\alpha H$	4.94	5.14
$C_\beta H_1$	4.66	5.22
$C_\beta H_2$	4.41	3.80
NH	9.06 (−4.73)	11.72 (−1.32)

[a] The ^{1}H NMR chemical shifts (parts per million), referred to internal Me_4Si, of enterobactin and its Ga^{3+} chelate in $(CD_3)_2SO$ at ~45° and 220 MHz. $C_\beta H$ and $C_\beta H_2$ shifts refer to β protons in the order they resonate in scanning from low to high field and do not imply absolute assignment or correspondence between the free and metal-bound siderochrome. The slopes of the amide NH chemical shift vs. temperature linear plots are shown in parentheses in units of ppm/°C × 10^3.

TABLE III. Proton–Proton Spin Couplings[a]

doublet	enterobactin	Ga^{3+}–enterobactin
o–m, p–m	8.02, 7.75	7.36, 6.41
o-OH	none	
m-OH	none	
α–β_1	8.06	unresolved
α-β_2	4.19	unresolved
β_1-β_2	10.80	10.68
α-N	6.52	9.83

[a] J coupling constants for the proton spin–spin interactions in enterobactin and its Ga^{3+} complex. The values are in hertx and were directly measured on expanded-scale spectra. The aryl o–m and p–m splittings in the chelate might be underestimated because of broadening of the resonances.

constants as well as the chemical shifts of enterobactin's protons once chelated with gallium. In the enterobactin spectra one observes two low-field signals, one at 11.57 ppm and the other at 9.34 ppm. The very lowest field signal was assigned to the ortho catechol proton of the 2,3-dihydroxybenzoyl moiety largely on the supposition that it would be the most strongly hydrogen bonded because of its proximity to the carbonyl group and would thus be expected to be strongly deshielded. Because of its intramolecular hydrogen bonding it would also be expected to exchange very slowly and thus be associated with a narrower line than the m-hydroxyl, which because of its ability to exchange would be associated with a broader resonance line. This strong hydrogen bonding between the o-hydroxyl and the amido carbonyl is partially responsible for a rather substantial downfield shift of the amide hydrogen. Amide hydrogens are usually observed between 7.5 and 8.4 ppm in dimethyl sulfoxide while the enterobactin amide hydrogen is observed at 9.08 ppm. Part of this rather low-field shift of this amide hydrogen has also been attributed to ring current deshielding from the benzenoid group as the amide proton is only 3.6 Å from the ring center on the equatorial plane.

Consideration of the coupling constant data in conjunction with CPK model building provided substantial information regarding the solution structure of the free ligand. Application of the modified Karplus equations to the coupling data provided the dihedral angles between the α and β protons. When viewed from the α end of the C_α–C_β axis the $\Theta_{\alpha\beta_a}$ is equal to 42°, and the $\Theta_{\alpha\beta_b}$ is equal to 16° (summarized diagrammatically in

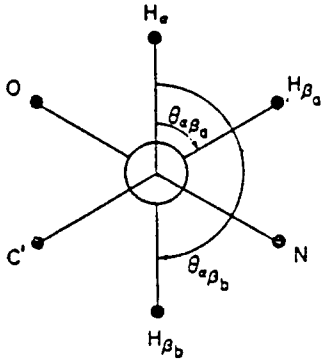


Figure 6. Dihedral angles (θ) between the α and β protons. For illustrative purposes the Newman projection shown here depicts a fully staggered conformation ($\theta_{\alpha\beta} = 60°$. $\theta_{\alpha\beta_b} = 180°$) that does not correspond to either enterobactin or its Ga^{3+} complex. Angles for the latter are given in the text and in the legends to Figure 4 and 7, respectively. It should be noted that the value for the conventional dihedral angle χ is directly given by $\theta_{\alpha\beta_b}$.

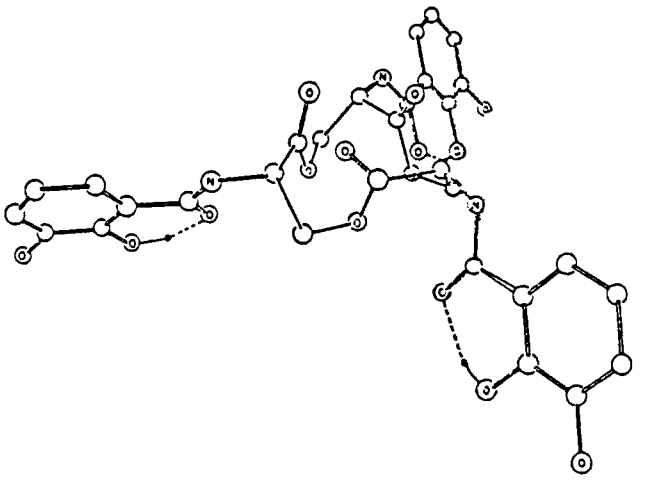


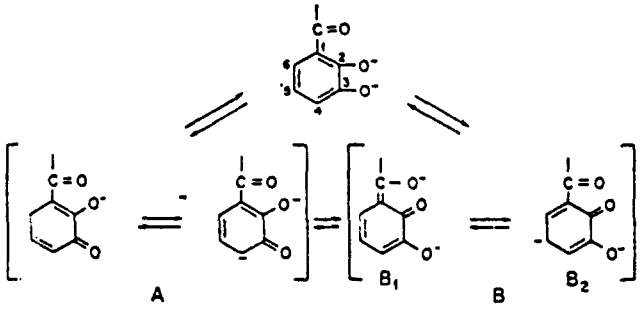
Figure 7. Molecular model for enterobactin dissolved in dimethyl sulfoxide. The cycle ester backbone atoms and bonds are shown in heavier lines. No hydrogen atoms are depicted except for the strongly H-bonded ortho phenolic group. Assuming planarity of the benzoyl amide moiety (ω = 180°), the three dihedral angles that characterize the model are $\phi \simeq 60°$, $\psi \simeq 100°$, and $\chi \simeq 162°$.

Figure 6). Furthermore, the $\Theta_{NH\text{-}C_\alpha H}$ angle was determined to be approximately 0°. Finally, on the basis of the observation that the chemical shift differences between the resonances of the two protons are very similar in the free ligand, it is suggested that the orientation of carbonyls of the ester linkages and the amide bonds should be such that the anisotropic shifts they generate in enterobactin's β-protons should be similar and small. On the basis of these observations the proposed structure of the free ligand is indicated in Figure 7.

In the gallium(III) chelate of enterobactin each of the three catechol moieties is associated with a single negative charge. By observing the difference in chemical shifts of the aromatic ring protons in the free and bound ligand it is possible to comment on the charge distribution. On going from enterobactin to its gallium(III) complex the aryl proton resonance that shifts the most ($\Delta\sigma$ = −0.6 ppm) is the meta triplet attached to C-5 rather than the C-6 ortho ($\Delta\sigma$ = −0.5 ppm) or C-4 para ($\Delta\sigma$ = −0.54 ppm) proton doublets. The overall shifts indicate loss in aromaticity as would be expected for the semiquinoid tautomer resulting in this charge shift. Scheme XII indicates the possible tautomers associated

Figure 8. Origins of restricted rotation in amide bond of enterobactin.

SCHEME XII. Tautomeric Forms of the 2,3-Dihydroxybenzamido Dianion



with the dianion of the catechol moiety. Of the possibilities, the tautomer of B (B_1) seems the most reasonable. The B set, of course, places a large part of the charge in the meta position, whereas the A set places a large part of its charge in the ortho and para positions. The increased charge on the meta proton is in agreement with the fact that it sustains the largest field shift. Furthermore, of the two possible B tautomers, it seems likely that B_1 is the most reasonable structure as it places one of its negative charges on the carbonyl which might be easily solvated by a counter ion. This B_1 structure may well help explain the rather unusual downfield shift of the NH resonance on going from enterobactin to its gallium chelate, $\Delta\sigma$ = +2.66 ppm. This deshielding might reflect a change in the electronic state of the amide bond. In tautomer B_1, it can be seen that the amide carbonyl resonance responsible for amide planarity is of somewhat less importance (Figure 8). It should be clear that a great deal of structural information regarding the catecholamide ligands and their complexes is available from ^{1}H NMR.

A second catecholamide iron chelator system studied by using this technique was that of parabactin and its corresponding gallium(III) chelate.[53] This ligand is structurally substantially more complicated than enterobactin, a complexity which is clearly reflected on its ^{1}H NMR spectra (Figure 9). The spectra of the free ligand can be separated into five regions. From 1 to 2 ppm represents the signals corresponding to the oxazoline methyl protons as well as the spermidine methylene protons not connected directly to the nitrogen. The region between 3 and 4 ppm represents all the protons flanking the nitrogens. The signals at 4.4 and 5.5 ppm result from the α and β oxazoline protons, respectively. The region between 6.4 and 8.2 ppm encompasses the aromatic protons and the low-field signals from 11.4 to 13.0 ppm include the catechol hydroxyl protons.

What is most interesting about the 300-MHz ^{1}H NMR spectra of parabactin is the duplicity of signals, duplicity which is very sensitive to both temperature and solvent changes. Figure 10 is an example of parabactin's ^{1}H NMR solvent sensitivity. Less polar solvent systems result in more complex spectra. Figure 11 demonstrates the temperature dependence of parabactin's ^{1}H NMR. This spectral duplicity has been attributed by both Neilands and us to the existence of the free ligand in a number of different conformations.[54] Furthermore, it has been suggested that the duplicity of signals resulting from various conformer populations is associated with the symmetry of the polyamine chain, i.e., the spermidine backbone. This of course implies that replacement of the spermidine backbone by homo- or norspermidine should simplify the NMR spectra because of the introduction of symmetry in the system. Indeed we have shown that homoparabactin, i.e., parabactin with a homospermidine backbone as opposed to a spermidine backbone, results in the expected spectra. The spectra is lacking in the complications introduced by conformational factors associated with the asymmetry of the spermidine chain. The precise nature of the conformer populations in the free ligand still remains somewhat of a puzzle although we are currently investigating the situation. However, it is clear that the conformer populations are separated by an energy barrier in excess of 21 kcal/mol as determined by coalescence temperature studies. In addition to simple steric interactions associated with rotation of the oxazoline moiety about the amide bond, it seems likely that at least some of the conformational immobility responsible for spectral duplicity should be associated with hydrogen-bonding phenomena. This seems reasonable in view of the fact that the molecule contains numerous hydrogen-bond donors and acceptors and therefore any number of hydrogen-bond networks are possible. Consequently one would expect that

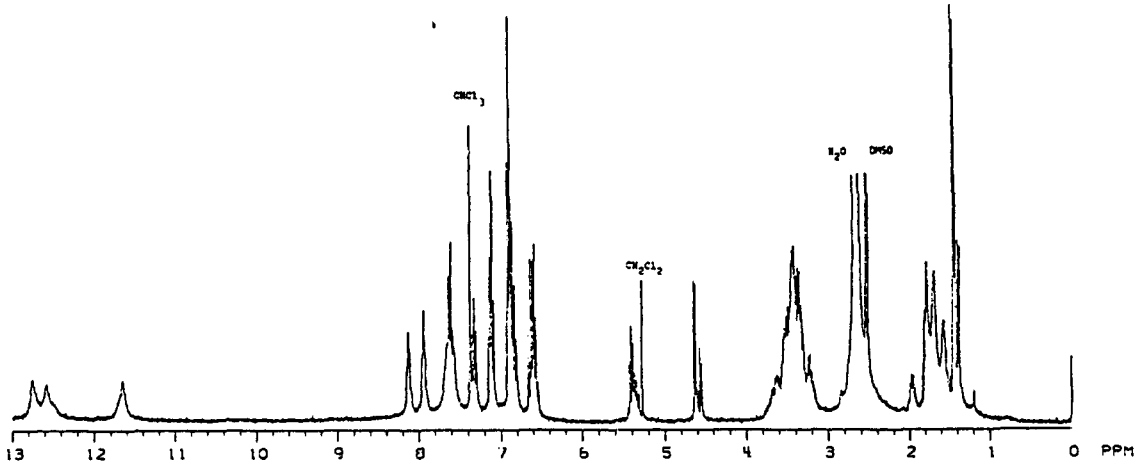


Figure 9. ^{1}H NMR of parabactin in 10:1 chloroform:dimethyl sulfoxide.

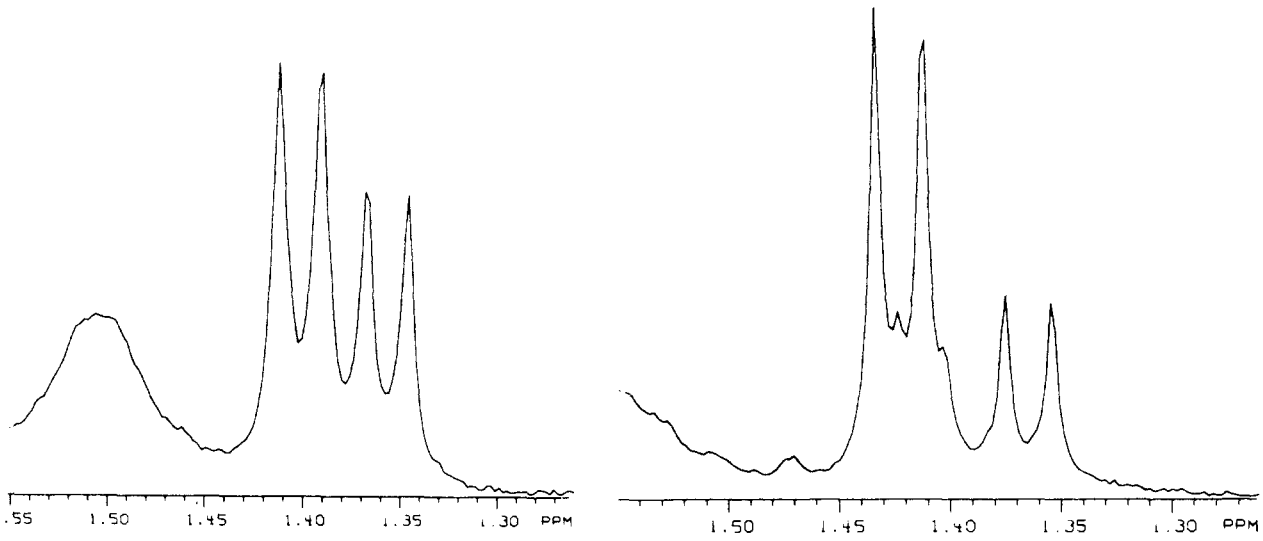


Figure 10. γ-Methyl region of a 300-MHz ^1H NMR spectrum of parabactin: in Me_2SO-d_6 at 23 °C (left); in 10:1, $CDCl_3/Me_2SO$ (right).

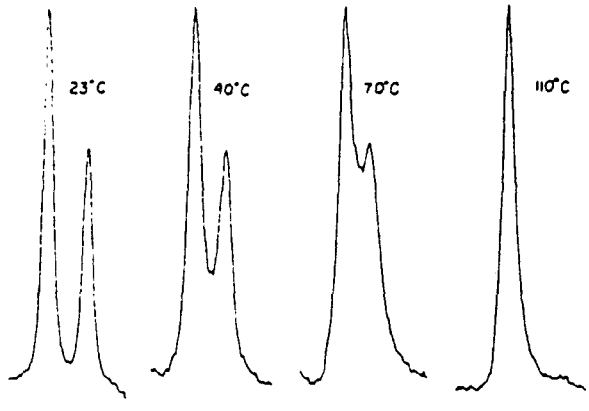


Figure 11. Effect of increasing temperature on the γ-methyl region of the β-decoupled 300-MHz ^1H NMR spectrum of parabactin in Me_2SO-d_6.

the methylation of the catechol groups would diminish the energy barriers between various conformer populations. We, in fact, found this to be the case as in tetramethoxyparabactin that coalescence occurred at 87° C corresponding to a conformational energy barrier of around 18 kcal/mol, some 3 kcal lower than the corresponding catechol compound.

We also observed similar kinds of spectral duplicity in the gallium(III) complex of parabactin. Furthermore, chelation of the metal by the ligand generated substantial changes in the ^1H NMR of the ligand. Many of the methylene signals became very clearly separated. Because of this separation it was possible to ascertain substantial detail regarding the solution structure of the chelate.

In principle there are a rather large number of possible isomers of the ligand–metal complex. Superficial considerations indicate either the Λ or the corresponding Δ isomer could exist in either the cis or trans forms. The trans forms are unrealistic in terms of strain. It should be recalled that the Λ and Δ forms are not enantiomers but rather diastereomers and therefore should exhibit different physical and spectral properties. There is an added complication for each of the potential Δ-cis and Λ-cis stereoisomers.

The asymmetry of the spermidine backbone makes it possible in principle to arrange this backbone relative to the central oxazoline moiety about the metal center in different ways. Because of the trans relationship of the amide and methyl groups on the oxazoline ring it is clear that revolution about the N–CO bond will produce two different isomers. One isomer could be described as that which has the 3-butyl carbon of spermidine on the same side of the plane as the methyl, while the corresponding isomer is just the opposite. This asymmetry provides for some rather interesting hydrogen-bonding possibilities.

With all of the gallium coordination sites filled let us now consider the structural possibilities in terms of how the central amide oxygen would be hydrogen bonded to either of the amide hydrogens of the terminal amides. One arrangement allows for the propyl amide to be on the same side of the plane as the oxazoline amide carbonyl, making it possible to form an eight-membered hydrogen-bonded ring between the central carbonyl and the hydrogen of the propyl amide. Revolution about the central amide bond by 180° places the butyl segment of the spermidine chain on the same side as this central amide carbonyl. It is now possible to form a nine-membered hydrogen-bonded ring between the terminal amide hydrogen and the central carbonyl. This rotation about the central amide bond of course can be effected with the Λ as well as the Δ isomer. Consideration of the CPK space-filling models of these isomers indicates that the most notable difference between the Λ set and the Δ set is the fact that in the Λ set of isomers the methyl group fixed to the oxazoline ring is in the shielding zone of one of the catechol moieties whereas in the Δ isomer it is considerably removed from this shielding zone. So, then, both the Λ-cis and the Δ-cis isomer systems have two possible isomers with regard to the orientation of the asymmetrical spermidine backbone. We will refer to the systems in which the propyl amide hydrogen is hydrogen bonded to the central carbonyl as the 3,4-systems and to the systems with the butyl amide hydrogen hydrogen bonded to the central carbonyl as the 4,3-systems.

The NMR evidence regarding the diastereomer populations is quite clear. The 300-MHz ^1H NMR spectra of the gallium(III) chelate of parabactin indicated the existence of one major isomer. Double resonance difference spectra, decoupling experiments, and deuterium exchange studies suggested that the predominant iso-

mer was one in which the propyl chain of the spermidine backbone was hydrogen bonded via its amide hydrogen to the central carbonyl of parabactin. This information coupled with the substantial shielding of the oxazoline methyl on complexation with gallium(III) strongly suggests that the Λ-cis-3,4 isomer is the major isomer.

It is interesting that the duplicity in NMR signals seen in the free parabactin NMR is also observed in one of its analogues, agrobactin A. The spectra of this spermidine catecholamide can be divided into two regions, the upfield portion above 6.0 containing the resonances from the spermidine backbone and the threonine residue and the downfield portion containing the aromatic phenolic and amide resonances. The high-field end of the spectrum is characterized by a pair of doublets centered at δ 1.2 which integrates to three protons and was assigned by the appropriate decoupling experiments to the γ-methyl of threonine. The envelope between δ 1.5 and 2.1 integrates to six protons and originates from the three internal methylene groups of the spermidine backbone while the four external methylene groups adjacent to the amides are responsible for the eight-proton envelope observed from δ 3.15 to 3.75. The final set of peaks in the upfield region of the spectra includes an extensively split multiple at δ 4.18 and an apparent doublet of doublets at δ 5.02, both integrating to one proton each. These have been assigned to the β- and α-methylenes, respectively, again by the appropriate decoupling experiments. Additionally, a broad hump observed at δ 4.7 results from the threonly hydroxyl which disappears on exchange with D_2O. The downfield region of the spectrum lying between δ 6.6 and 7.4 integrates to nine protons and is assigned to the aromatic protons. The terminal amides are located at δ 8.03 and 8.24 while the threonyl amide is located between them at δ 8.09. The phenolic protons lie the furthest downfield and like the threonyl hydroxyl protons undergo exchange at room temperature, resulting in somewhat broad and undefined signals at very low temperature. At −45 °C, however, the rate of exchange is considerably slower, resulting in sharper signals at approximately δ 12.0 and 13.0.

Of particular interest, however, is the duplicity of signals originating from the threonyl moiety. Figure 10 indicates that, although the predicted coupling patterns do exist for the threonyl segment of the molecule, they again exist in a ratio of approximately 2:1 in a 10:1 mixture of $CHCl_3$–Me_2SO-d_6. Likewise, the β-methine also exhibits an additional set of signals. However, the α-methine does not show duplicity of lines but rather has the expected doublet of doublets as one might anticipate. This observation is of course different from that observed for parabactin or agrobactin, where duplicate signals are observed for the α-methine. Because the β- and γ-methyl protons are located further out on a threonyl side chain, i.e., closer to the terminal 2,3-dihydroxybenzoyl groups than the α-methine, on rotation about the central amide bond they see more significant changes in magnetic environment. The α-methine has a more "internal" location and moves through a relatively smaller area upon rotation and at greater distances from the aromatic ring and sees little or not change in its environment, resulting in only one observable signal. In Figure 12 the inner and outer

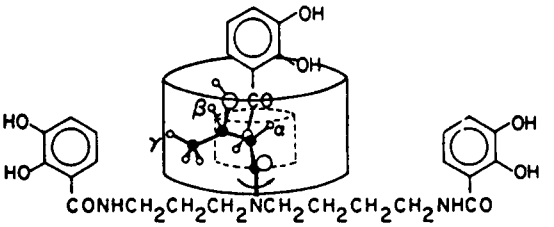


Figure 12. Diagram illustraing the approximate sweep volumes of the α- and γ-protons of agrobactin A on rotation about the central amide.

cylinders represent the sweep volumes of the α-methine and γ-methyl groups, respectively. The γ-methyl group and β-methine lie in close proximity to the terminal aromatic rings. They can easily be influenced by anisotropic effects; however, since these effects decrease rapidly with increasing distances, the α-methine is likely to be too distant from the aromatic rings. Furthermore, the cylinders are cut in half by a plane passing through the central nitrogen perpendicular to the spermidine backbone. It can easily be seen that corresponding points in either half are at different intermolecular distances from the aromatic rings due to the asymmetry of the spermidine chain. Accordingly, these point will also experience different magnetic fields; therefore, a proton existing in a conformation lying in the left half will likely result in a different signal as compared to its counterpart that lies in the right half of the cylinder. Although this model does not consider all the possible orientations of the spermidine backbone and aromatic rings, a recent X-ray crystallographic study of agrobactin does suggest a conformation within a nearly linear spermidine backbone that is similar to that depicted in the model. Finally, like the situation for homoparabactin, the NMR of homoagrobactin A predictably shows no signs of spectral duplicity. However, unlike the situation observed with parabactin, methylation of the catechol hydroxyls of agrobactin A raises rather than lowers the coalescence temperature observed for the threonyl methyl, thus raising the energy of activation or interconversion of the conformers. In fact, the energy of activation calculated for agrobactin A itself is 18.2 ± 0.15 kcal/mol while the energy of activation calculated for tetramethyl agrobactin A is in excess of 21 kcal/mol. This has been attributed to a loss of importance of a hydrogen-bonding network in fixing stability to any one conformer and the increased importance of simple steric factors in regulating energies of activation. Unfortunately, there are no current data on the effects of gallium(III) chelation on the NMR of this ligand.

We have looked briefly at the application of two spectroscopic techniques for determination of the solution conformations of siderophores. We did not consider the NMR studies of hydroxamates simply because of the limited material available.

V. Conclusion

The synthetic procedures described in this review fall into two categories: (1) those which offer the opportunity of synthesizing the ligands in reasonably large quantitites with some facility and (2) those which are practical for the generation of only small quantities of the systems of interest. The first group of systems

include many of the model systems described by Neilands and Raymond and Corey as well as the spermidine catecholamide systems generated in these laboratories and several of the simpler hydroxamates described by Miller. The second group of chelators entail the analogues of parabactin synthesized by the Nagao procedure as well as a number of the hydroxamates. This is of course a critical issue only if one considers the potential application of these ligands to a variety of problems such as treatment of iron-overload diseases or as potential bacteriostatic devices. However these potential uses should encourage investigators to consider the development of more efficient schemes for some of the ligands described in the review. The synthetic challenges provided by the siderophores are only limited by the investigator's imagination and by the numbers of new microbial chelators discovered every year. Finally, it is clear that with spectroscopic techniques available, particularly nuclear magnetic resonance, information regarding the solution structure of the chelates will now be more easily accessible. This of course will allow for tremendous advances regarding the stereoselectivity that various microorganisms display in their siderophore transport systems.

VI. References

(1) Neilands, J. B. *Struct. Bonding (Berlin)* **1970**, *11*, 145.
(2) Cotton, F. A.; Wilkinson, G. "Advanced Inorganic Chemistry"; Wiley-Interscience: New York, 1980; p 744.
(3) Cooper, S. R.; McArdle, J. V.; Raymond, K. N. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3551.
(4) Neilands, J. B. *Annu. Rev. Biochem.* **1981**, *50*, 715.
(5) Pollack, J. R.; Neilands, J. B. *Biochem. Biophys. Res. Commun.* **1970**, *38*, 989.
(6) Peterson, T.; Falk, K.; Leong, S.; Klein, M. P.; Neilands, J. B. *J. Am. Chem. Soc.* **1980**, *102*, 7715.
(7) Rogers, S.; Neilands, J. B. *Biochem. J.* **1950**, *3*.
(8) Carrano, C. J.; Raymond, K. N. *J. Am. Chem. Soc.* **1978**, *100*, 5371.
(9) Gibson, F.; Magrath, D. I. *Biochim. Biophys. Acta* **1969**, *192*, 175.
(10) Snow, G. A. *Bacteriol. Rev.* **1970**, *34*, 99.
(11) Emery, T. *Biochemistry* **1965**, *4*, 1410.
(12) Schwarzenbach, J.; Schwarzenbach, B. *Helv. Chim. Acta* **1963**, *46*, 1390.
(13) Neilands, J. B. In "Development of Iron Chelators for Clinical Use"; Martell, A. E., Anderson, W. F., Badman, D. G., Eds.; Elsevier/North Holland: New Yor, 1980.
(14) Winkelmann, G. *FEBS Lett.* **1979**, *97*, 43.
(15) Carrano, C. J.; Raymond, K. N. *J. Bacteriol.* **1978**, *128*, 169.
(16) Neilands, J. B. *Annu. Rev. Biochem.* **1981**, *50*, 715.
(17) Bullen, J. B. *Rev. Infect. Dis.* **1981**, *3*, 1127.
(18) Martell, A. E.; French Anderson, W.; Badman, D. G. In "Development of Iron Chelators for Clinical Use"; Martell, A. E., Anderson, W. F., Badman, D. G., Eds.; Elsevier/North-Holland: New York, 1980.
(19) Reuter, B.; Brock, J. H.; Steel, E. D. *Immunology* **1975**, *28*, 83.
(20) Grady, R. W.; Jacobs, A. "Development of Iron Chelators of Clinical Use"; Martell, A. E., Anderson, W. F., Badman, D. G., Eds.; Elsevier/North Holland: New York, 1980; p 189.
(21) Jones, R. L.; Peterson, C. M.; Grady, R. W.; Kumbaraci, T.; Cerami, A. *Nature (London)* **1977**, *267*, 63.
(22) O'Brien, I. G.; Gibson, F. *Biochim. Biophys. Acta* **1970**, *215*, 393.
(23) Avdeef, A.; Sofen, S.; Bregante, T. L.; Raymond, K. N. *J. Am. Chem. Soc.* **1978**, *100*, 5362.
(24) Corey, E. J.; Bhattacharyya, S. *Tetrahedron Lett.* **1977**, 3919.
(25) Rastetter, W. Erickson, T. J.; Venuti, M. C. *J. Org. Chem.* **1981**, *46*, 3579.
(26) Corey, E. J.; Hurt, S. D. *Tetrahedron Lett.* **1977**, 3923.
(27) Weitl, F. L.; Raymond, K. N. *J. Am. Chem. Soc.* **1979**, *101*, 2728.
(28) Venuti, M. C.; Rastetter, W. H.; Neilands, J. B. *J. Med. Chem.* **1979**, *22*, 123.
(29) Tait, G. H. *Biochem. J.* **1975**, *146*, 191.
(30) Peterson, T.; Neilands, J. B. *Tetrahedron Lett.* **1979**, 4805.
(31) Peterson, T.; Falk, K.; Leong, S. A.; Klein, M. D.; Neilands, J. B. *J. Am. Chem. Soc.* **1980**, *102*, 7715.
(32) Bergeron, R. J.; McGovern, K. A.; Channing, A. M.; Burton, P. S. *J. Org. Chem.* **1980**, *45*, 1589.
(33) Bergeron, R. J.; Stolowich, N. J.; Kline, S. J. *Synthesis*.
(34) Bergeron, R. J.; Burton, P. S.; Kline, S. J. McGobern, K. A. *J. Org. Chem.* **1981**, *46*, 3712.
(35) Bergeron, R. J.; Burton, P. S.; Kline, S. J.; McGovern, K. A. *J. Org. Chem.* **1981**, *46*, 4524.
(36) Bergeron, R. J.; Streiff, R. R.; Burton, P. S.; McGovern, K. A.; St. Onge, E. J. *J. Med. Chem.* **1980**, *23*, 1130.
(37) Bergeron, R. J.; Kline, S. J.; Solowich, N. J. *Synthesis*.
(38) Bergeron, R. J.; Kline, S. J.; Stolowich, N. J. *J. Org. Chem.* **1983**, *48*, 3432.
(39) Maurer, P. J.; Miller, M. J. *J. Am. Chem. Soc.* **1982**, *104*, 3096.
(40) Lee, B. H.; Miller, M. J. *J. Org. Chem.* **1983**, *48*, 24.
(41) Maurer, P. J.; Miller, M. J. *J. Am. Chem. Soc.* **1983**, *105*, 240.
(42) Leong, J.; Raymond, K. N. *J. Am. Chem. Soc.* **1974**, *96*, 6628.
(43) Leong, J.; Neilands, J. B.; Raymond, K. N. *Biochem. Biophys. Res. Commun.* **1974**, *60*, 1066.
(44) Llinas, M.; Klein, M. P.; Neilands, J. B. *Int. J. Pept. Protein Res.* **1972**, *4*, 157.
(45) Llinas, M.; Kelin, M. P.; Neilands, J. B. *J. Mol. Biol.* **1972**, *68*, 265.
(46) Llinas, M.; Wilson, D. M.; Neilands, J. B. *Biochemistry* **1973**, *12*, 3836.
(47) Leong, J.; Raymond, K. N. *J. Am. Chem. Soc.* **1974**, *96*, 1758.
(48) Leong, J.; Raymond, K. N. *J. Am. Chem. Soc.* **1974**, *96*, 6628.
(49) Leong, J.; Raymond, K. N. *J. Am. Chem. Soc.* **1975**, *97*, 293.
(50) Isied, S. S.; Kuo, G.; Raymond, K. N. *J. Am. Chem. Soc.* **1976**, *98*, 1763.
(51) Abu-Dari, K.; Raymond, K. N. *J. Am. Chem. Soc.* **1977**, *99*, 2003.
(52) Tufano, T. P.; Raymond, K. N. *J. Am. Chem. Soc.* **1981**, *103*, 6617.
(53) Bergeron, R.; Kline, S. J. *J. Am. Chem. Soc.*, in press.
(54) Bergeron, R. J.; Kline, S. J. *J. Am. Chem. Soc.* **1982**, *104*, 4489.
(55) Eng-Wilmat, D. L.; van dor Helm, D. J. *J. Am. Chem. Soc.* **1980**, *102*, 7710.
(56) Shanzer, A.; Libman, J. *J. Chem. Soc., Chem. Commun.* **1983**, 847.
(57) Nagao, Y.; Miyasaka, T.; Hagiwura, Y.; Fujita, E. *J. Chem. Soc., Perkin Trans. 1* **1984**, 183.
(58) Wierenga, W. "The Total Synthesis of Natural Products"; Vol. 4, Chapter 3, pp 274–286.