of a transition-metal complex or the direct reaction of a transition-metal complex with a free nitrene, has been previously reported for the synthesis of a metal–nitrene complex. From the aforementioned results, showing that porphyrin iron–nitrene bonds do exist and are formed by an O_2 and iron porphyrin dependent oxidation of a 1,1-dialkylhydrazine, it seems likely that the cytochrome P-450 complexes formed by an O_2- and NADPH-dependent metabolic oxidation of 1,1-dialkylhydrazines also involve an iron-nitrene bond as proposed previously.[8]

Acknowledgments. We are grateful to Dr. J. P. Battioni for a gift of TPP-d_{25}, Dr. I. Morgenstern-Badarau for EPR and magnetic susceptibility measurements, Dr. J. Mispelter for ^1H NMR (temperature dependence) measurements and fruitful discussions, and Professor V. Ullrich for stimulating discussions at the origin of this study.

Registry No. 1, 6130-92-3; **2**, 82281-71-8; **3**, 42053-22-5; **4**, 66337-86-8; Fe^{III}(TPP)Cl, 16456-81-8; Fe^{II}(TPP), 16591-56-3; Fe^{II}(TPP)$(Py)_2$, 16999-25-0.

Short Synthesis of Parabactin

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In 1975 Tait[1] isolated two rather unique siderophores from *Paracoccus denitrificans*: N^1,N^8-bis(2,3-dihydroxybenzoyl)-spermidine (II) and N^4-[*N*-(2-hydroxybenzoyl)-L-threonyl]-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine (III), Figure 1a. He demonstrated the former catecholamide to be the biochemical precursor of the latter. Shortly after the isolation and identification of these iron-sequestering agents, Jacobs and Tait[2] were able to show the potential of these catecholamides as therapeutic devices for the treatment of various iron-overload syndromes, e.g., Cooley's anemia.[3,4] Compound II, a tetradentate ligand, removed iron from transferrin, one of the body's iron-binding proteins, substantially better than compound III, a potentially hexacoordinate ligand. Furthermore, both of these catecholamides were more effective at removing iron from this iron-shuttle protein than was desferrioximine, the clinical device currently used in chelation therapy. Unfortunately, because compounds II and III were only accessible in milligram quantities from bacteria, a complete biological evaluation was not possible. However, these findings did spark further interest in this new family of siderophores. Following Tait's discovery, Raymond synthesized a number of catecholamide siderophores and evaluated both the binding stoichiometries as well as the thermodynamics of iron binding.[5-9] However, neither II nor III was actually synthesized until recently.[10-13]

Initially, it seemed peculiar to us that nature should produce a structurally more complicated, less effective iron chelator (III) from a less complicated, more effective chelator (II). However, Neilands[10,11] rather cleverly demonstrated that this unsettling inefficiency could be easily explained by reconsidering Tait's original proof of structure for compound III. He demonstrated that the group fixed to the central nitrogen of the spermidine backbone was not an *N*-(2-hydroxybenzoyl)-L-threonyl moiety but rather a (2-hydroxyphenyl)-4-carboxyl-5-methyl-2-oxazoline

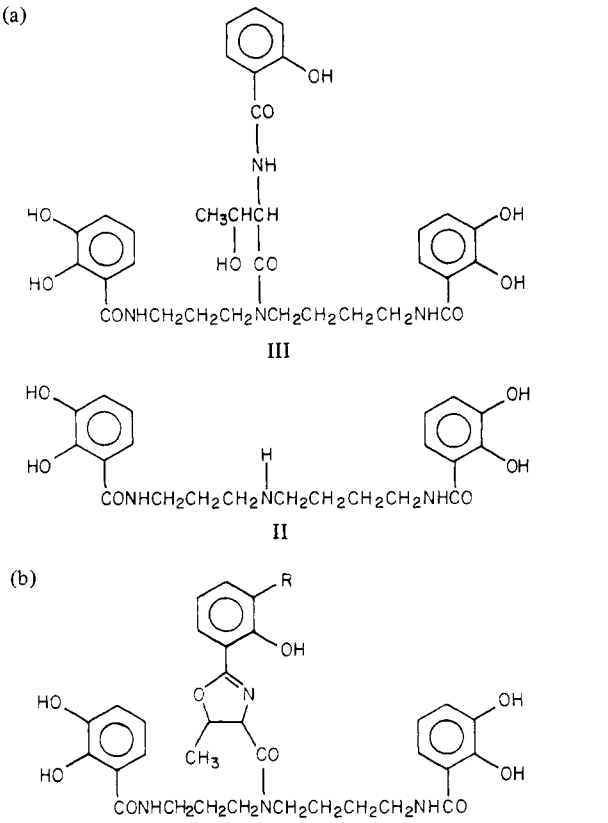

Figure 1. (a) Siderophores N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine (II) and N^4-[*N*-(2-hydroxybenzoyl)threonyl]-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine (parabactin A) (III). (b) Parabactin (R = H); agrobactin (R = OH).

(Figure 1b) and that this oxazole ring was opened to the threonyl compound under the acidic conditions of Tait's isolation.[10] In the oxazoline or closed form, compound III represents a hexacoordinate Fe(III) ligand that, in fact, binds Fe(III) tighter than either compound II or the open form of compound III. These findings stimulated our interest in the total synthesis of compound III in its oxazoline form.

In earlier studies, we were able to develop several syntheses of compound II and several analogues, as well as of compound III analogues.[12-15] We have since shown these catecholamides to be potent iron chelators and effective at removing iron from iron-overloaded animals.[13,16] Now, we report on the first synthesis of the closed form of compound III, parabactin. This procedure allows for the generation of not only parabactin in high yield but also of the corresponding homo- and norspermidine homologues.

The synthesis begins with N^1,N^8-bis(2,3-dimethoxybenzoyl)-spermidine (**1**), a very versatile reagent for the generation of spermidine catecholamides.[15] This compound is coupled with *N*-carbobenzoxy-L-threonine via the *N*-hydroxysuccinimide ester. Compound **1** is reacted with *N*-carbobenzoxy-L-threonine and the condensing reagents dicyclohexylcarbodiimide and *N*-hydroxysuccinimide in the presence of triethylamine in tetrahydrofuran (0 °C) to produce the secondary N^4-acylated product, N^4-(*N*-carbobenzoxy-L-threonyl)-N^1,N^8-bis(2,3-dimethoxybenzoyl)-spermidine (**2**), in 90% crude yield. This threonyl amide was easily purified by silica gel chromatography eluting with 5% MeOH in EtOAc;[17] ^1H NMR (60 MHz in $CDCl_3$) δ 1.16 (3 H, CH_3), 1.58

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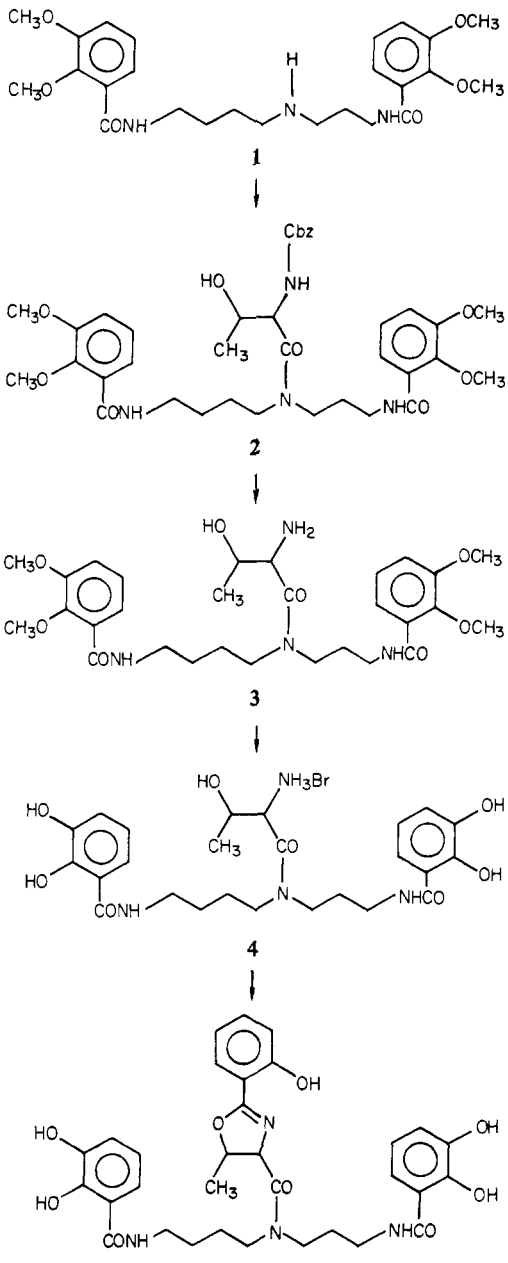
(6 H, CH_2), 3.42 (8 H, CH_2) 3.56 (12 H, OCH_3), 4.0–4.60 (3 H, 2CH, OH), 4.82 (2 H, CH_2), 5.85–6.30 (1 H, NH), 6.62–7.70 (11 H, Ar), 7.70–8.40 (2 H, N–H). The carbobenzoxy protecting group of **2** was next removed quantitatively by hydrogenolysis over $PdCl_2$ in methanol/HCl for 24 h, generating N^4-L-threonyl-N^1,N^8-bis(2,3-dimethoxybenzoyl)spermidine (**3**). The methanol was removed under vacuum, the residue dissolved in water, and the aqueous mixture washed with chloroform. The water was removed under vacuum, and the residue was dissolved in chloroform and washed with aqueous sodium carbonate. The compound was purified on silica gel eluting with 5% methanol in chloroform.[18] The most revealing ^{1}H NMR features at 60 MHz in $CDCl_3$ were the absence of the benzyl methylene at δ 4.82 and the simplicity of the aromatic region. The envelope of complex peaks extending from 6.62 to 7.70 in **1** corresponding to 11 aromatic protons, was simplified to two signals, one at δ 6.70–7.10 (4 H) and one at δ 7.28–7.66 (2 H). The low-field signal corresponds to the ortho protons of the 2,3-dimethoxybenzoyl groups and the high-field signal to the meta and para protons. The methyl protecting groups of **3** were removed quantitatively by reacting the amide with BBr_3 in CH_2Cl_2 at 0 °C for 6 h, and the product was hydrolyzed with methanol, generating the free catechol N^4-L-threonyl-N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine·HBr (**4**). The product was chromatographed on Sephadex LH-20 eluting with 20% ethanol in benzene.[19] At 60 MHz the most outstanding ^{1}H NMR feature was the absence of the OCH_3 signal at δ 3.56.

The final and most critical step in the procedure involved the coupling of 2-hydroxybenzimido ethyl ether[20] with **4**. In designing the synthesis of parabactin, it was clear that a consideration of both the stereochemistry and acid sensitivity of the oxazoline ring system was critical. Neilands was able to show that the hydrogens of the oxazoline ring system were trans to each other. It was also clear from his work that the oxazoline ring was acid labile. This meant that the synthesis must employ a stereospecific procedure as well as a sequence in which there were no steps involving acid after the introduction of the oxazoline ring. In an earlier study, Elliott demonstrated that benziminoethyl ether could be coupled with DL-threonine ethyl ester to generate a *trans*-4-carboethoxy-2-phenyl-5-methyl-Δ^2-oxazoline.[21] In a series of model studies, we determined that 2-hydroxybenzimino ethyl ether could be condensed with DL-serine·HCl methyl ester in the presence of catechol to produce the corresponding 4-carbomethoxy-2-hydroxyphenyl-Δ^2-oxazoline in 80% yield. This implied that the catechols of **4** would not interfere with the desired condensation, a feature that was critical to our scheme. The actual reaction was carried out in 78% yield by refluxing **4** and 2-hydroxybenzimino ethyl ether in methanol for 24 hours and the product purified on Sephadex LH-20 (20% ethanol in benzene).[22] The synthesis is summarized in Scheme I. The specific rotation of the siderophore is $[\alpha]^{25}_D$ 98 ± 2° (*c* 3.0, methanol). The structure of the final product was verified by a high-field ^{1}H NMR study.

In the course of analyzing the 300-MHz ^{1}H NMR spectrum of synthetic parabactin, two structural features of the natural product observed by Neilands must be kept in mind—the trans hydrogens of the oxazoline ring and the conformer populations.[11] Although Neilands did not provide a comparison of the parabactin and parabactin A ^{1}H NMR spectra, he did provide a rather exhaustive analysis of the agrobactin and agrobactin A spectra. This information has made it possible to determine whether or not the observed parabactin ^{1}H spectrum was indeed that of the oxazoline system. A comparison of the methine and methyl signals of agrobactin with its open-form agrobactin A in 10:1 $CDCl_3$/

Scheme I



Me_2SO-d_6 points out some rather revealing differences. The α-proton multiplet of agrobactin is centered at δ 4.6 with a $J_{\alpha,\beta}$ = 6.8 Hz while the α-proton multiplet of agrobactin A is centered at δ 5.0 with a $J_{\alpha,\beta}$ = 2.4 Hz. The β-proton multiplets of the open and closed forms are centered at δ 4.2 and 5.4, respectively. The methyls of agrobactin and agrobactin A are centered at δ 1.4 with $J_{\gamma,\beta}$ = 6.4 Hz and δ 1.2 with $J_{\alpha,\beta}$ = 6.1 Hz, respectively. Finally, what is most notable about the α and γ protons is that their signals exist "in duplicate". This was attributed to the siderophore existing in separate conformations separated by an 18 kcal/mol energy barrier.[11] Clearly, very similar differences and/or similarities can be expected for the parabactin and parabactin A systems. This expected extension is strongly supported by the remarkable similarities in the agrobactin and parabactin oxazoline ring system spectra. The reported parabactin numbers for the α, β, and γ hydrogens are δ 4.6 (J = 6.8 Hz), δ 5.3 and 1.4 (J = 6.4 Hz), respectively.

Our 300-MHz ^{1}H NMR spectrum of synthetic parabactin in 10:1 $CDCl_3$/MeSO-d_6 (Figure 2) 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 several conformers.

(17) Anal. Calcd for $C_{37}H_{48}N_4O_{10}$: C, 62.70; H, 6.83; N, 7.90 Found: C, 62.59; H, 6.86; N, 7.80.
(18) Anal. Calcd for $C_{29}H_{42}N_4O_8$: C, 60.61; H, 7.37; N, 9.75. Found: C, 60.50; H, 7.36; N, 9.62.
(19) Anal. Calcd for $C_{25}H_{35}N_4O_8Br$: C, 50.09; H, 5.88; N, 9.35. Found: C, 49.98; H, 5.96; N, 9.27.
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(22) Anal. Calcd for $C_{32}H_{36}N_4O_9$: C, 61.93; H, 5.85; N, 9.03. Found: C, 61.93; H, 5.94; N, 8.96.

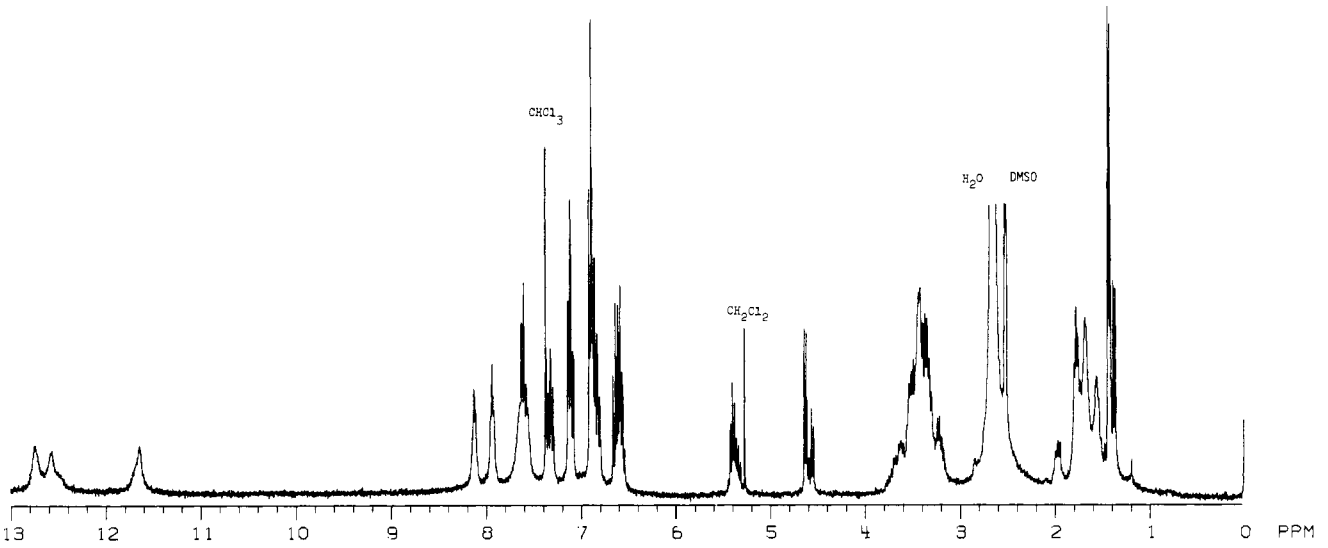

Figure 2. 300-MHz ^{1}H NMR spectrum of 3 mg of parabactin in 0.5 mL of 10:1 $CDCl_3/Me_2SO$-d_6 at 23 °C.

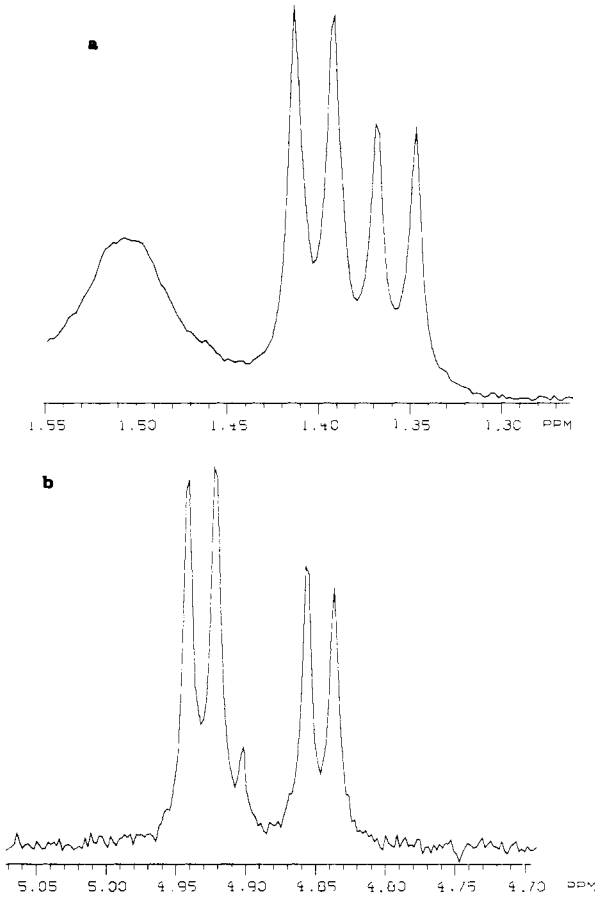

Figure 3. (a) γ-Methyl region of a 300-MHz ^{1}H NMR spectrum of parabactin in Me_2SO-d_6 at 23 °C. (b) α-Methine region of a 300-MHz ^{1}H NMR spectrum of parabactin in Me_2SO-d_6 at 23 °C.

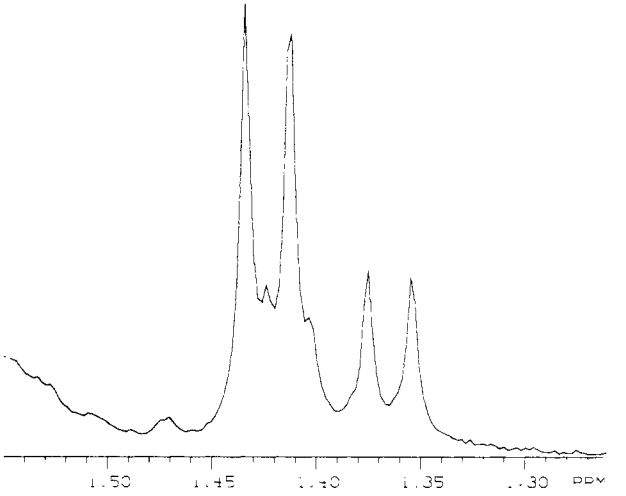

Figure 4. γ-Methyl region of the spectrum in Figure 2.

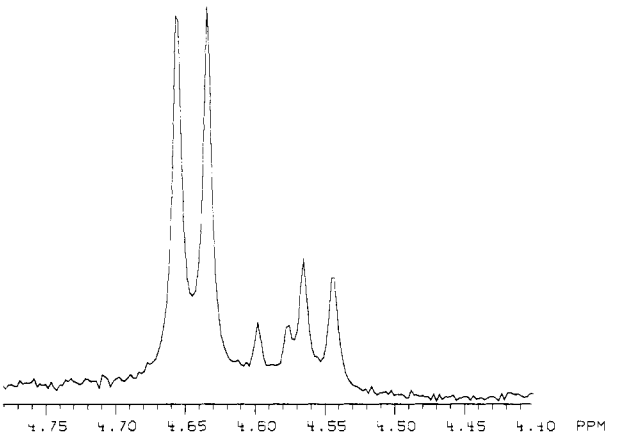

Figure 5. α-Methine region of a 300-MHz ^{1}H NMR spectrum of parabactin in 10:1 $CDCl_3/Me_2SO$-d_6 at −13 °C.

It should be pointed out that the ^{1}H NMR of parabactin is extremely sensitive to solvent and temperature changes, e.g., when run in Me_2SO-d_6 the spectrum is substantially simpler than when run in $CDCl_3$ or 10:1 $CDCl_3/Me_2SO$-d_6. In fact, the simplicity of the spectrum in Me_2SO-d_6 is almost deceiving, and investigators must be cautioned to use the same solvent and temperature when comparing results. When the ^{1}H NMR of parabactin is run in Me_2SO-d_6 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 $J_{\gamma,\beta}$ = 6.3 Hz (Figure 3a). In Me_2SO-d_6 at 23 °C the α-methine signals are centered at δ 4.89 and consist of five lines (Figure 3b). On a change of the solvent to 10:1 $CDCl_3/Me_2SO$-d_6 in order to avoid the line-broadening problems associated with low-temperature-induced viscosity changes with Me_2SO-d_6, 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 fine lines. However, the γ-methyl signal now consists of six lines, three sets of doublets with identical coupling constants (Figure 4). Fur-

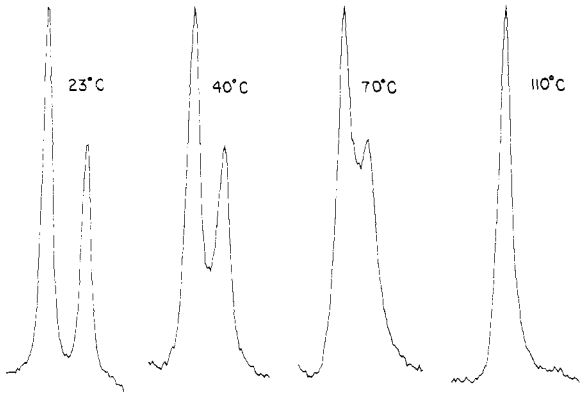

Figure 6. Effect of increasing temperature on the γ-methyl region of the β-decoupled 300-MHz ^{1}H NMR spectrum of parabactin in Me_2SO-d_6.

thermore, when the sample is cooled to −13 °C, the five lines of the α-methine become six lines, three sets of doublets (Figure 5), 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. Furthermore, and as expected under these decoupling conditions when the sample is observed at higher temperatures, e.g., 110 °C in Me_2SO-d_6, the three lines of the α methine and the two lines of the γ methyls appear as single lines (Figure 6). Determination of the exact coalescence temperature and Arrhenius energy is under study. These results are, of course, in complete accord with the idea of different conformers.[11,23] Because of the complexity of the β-methine signals, temperature experiments were not very revealing, although decoupling experiments clearly demonstrated its coupling to the α-methine and γ-methyl protons. We do not understand yet precisely how the temperature-dependent ^{1}H NMR spectrum of parabactin relates to its various conformers; however, this is currently under investigation.

The remainder of the spectrum, when taken in 10:1 $CDCl_3$/Me_2SO-d_6, 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. We were unable to run a two-dimensional ^{1}H spectrum, and therefore, further splitting information was not available. The aromatic proton signals consist of five well-separated envelopes: δ 6.52–6.67 (2 H), 6.78–6.92 (4 H), 7.06–7.15 (2 H), 7.27–7.35 (1 H), 7.51–7.71 (1 H). Finally, the NH and OH protons are as described by Neilands with the NH and non-hydrogen-bonded OH protons at δ 7.89–8.17 and the hydrogen-bonded protons δ 11.56–12.82.

The scheme described above represents the first synthetic route for accessing parabactin. This facile sequence offers the siderophore in high yield and can also be applied to generation of the nor- and homospermidine homologues with equal success. We are currently employing ^{1}H nuclear Overhauser effects to determine which conformations of parabactin belong to which set of oxazoline methine doublets. This will make it possible to pinpoint changes required for metal complexation and allow for a detailed examination of the siderophores' solution conformations.

Acknowledgment. We acknowledge the National Institute of Health Institutional grant and the Veterans Administration for support of this research.

Registry No. 1, 78217-75-1; **2**, 82247-45-8; **3**, 82265-49-4; **4**, 82247-46-9; 2-hydroxybenzimino ethyl ether, 82247-47-0; parabactin, 74149-70-5; *N*-carbobenzoxy-L-threonine, 19728-63-3.

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NMR Determination of Site-Specific Deuterium Isotope Effects

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Nuclear magnetic resonance spectroscopists have long anticipated the day when very high-field magnets would be commerically available for obtaining high-resolution deuterium spectra.[1] Aside from a small isotope effect, ^{2}H chemical shifts correspond to ^{1}H shifts. Moreover, the relaxation mechanism of ^{2}H is reasonably well understood. But, more importantly, the relatively low natural occurrence (~0.015%) of ^{2}H precludes scalar coupling between two ^{2}H nuclei from ever being significant in the ^{2}H spectra of nonenriched compounds. Thus, as with other relatively rare isotopes, proton-decoupled ^{2}H spectra of most organic compounds consist of a single resonance for each chemically different nucleus, presuming of course that the magnetic field is large enough that chemically similar resonances can be resolved. Because spin–spin splittings are eliminated in these resonances, there is no problem with the second-order splittings which lead to banding of proton resonances in coupled, closely positioned proton spectra.

While this feature was being explored in a series of methylcyclohexanes, it was observed that the peak intensities of the ^{2}H singlets in most of the molecules corresponded reasonably well within experimental errors to the proton atomic ratios (e.g., see the ^{2}H spectrum of methylcyclohexane in Figure 1a and the quantitative data in Table I). However, the significant deviations from atomic ratios involved more peaks and were considerably larger in 1,1,3-trimethylcyclohexane (see the table and Figure 1b). Special attention is drawn to the low relative intensity of the 5-e peak. The data on peak intensities have been normalized to the number of protons in each molecule. Pending the availability of absolute intensity data, the use of normalized relative intensities focuses on the intensity deviations of each peak or site from the average molecular deuterium concentration. If the isotopic effect is either all depletion or all enhancement, then such a normalization procedure tends to minimize the extent of deviations from normal isotope ratios. A careful study of the T_1's and proton–deuterium NOE's for the two compounds given in Figure 1 indicates that there are no significant differences in relaxation parameters for any of the carbons in the two different molecules. No nuclear Overhauser enhancement of ^{2}H due to ^{1}H irradiation could be detected in either molecule. Therefore transient effects cannot be used to account for variations in the peak intensities from the intramolecular hydrogen ratios. Instead what is being observed are measurable variations in the ^{2}H to ^{1}H isotope ratio at different sites within 1,1,3-trimethylcyclohexane.

Marked isotope effects among the isotopes of hydrogen are of course legion, especially when hydrogen substitution, elimination, or transfer reactions are involved. Furthermore, enzymatic and other biologically significant reactions could be expected to be quite sensitive to such effects. Accordingly, ^{2}H spectra at natural

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