Pergamon

Tetrahedron Vol. 50, No. 33, pp. 9865-9872, 1994

0040-4020/94 $7.00+0.00

0040-4020(94)00573-7

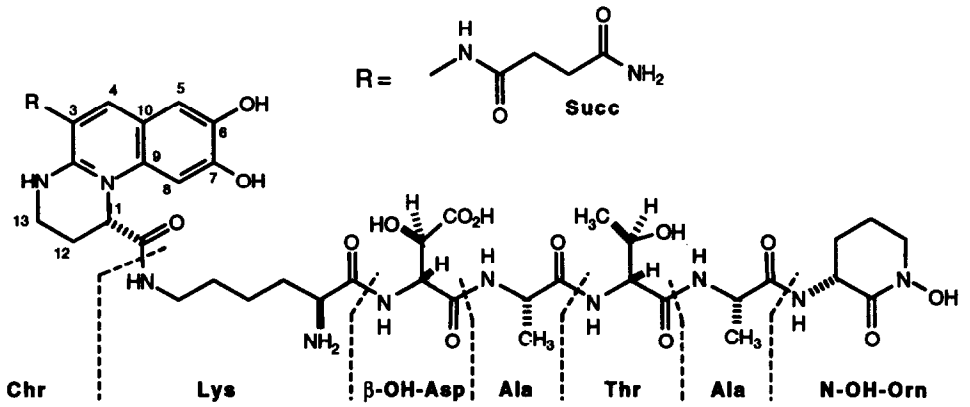
Biosynthesis of the Pseudobactin Chromophore from Tyrosine

Brian Nowak-Thompson and Steven J. Gould*

Department of Chemistry, Oregon State University
Corvallis, OR 97331-4003

Abstract: Biosynthetic studies have shown the incorporation of DL-[2,3,3-$^2H_3$]-tyrosine into the chromophore of pseudobactin, a siderophore produced by *Pseudomonas fluorescens* B10. A subsequent feeding using DL-[2',5',6'-$^2H_3$]-3,4-dihydroxyphenylalanine showed no incorporation of the label, suggesting that oxidation of the aryl ring occurs after the incorporation of tyrosine. The ^{13}C NMR assignments of pseudobactin are also reported herein.

The iron-binding, fluorescent pigments produced by a number of *Pseudomonas* species[1, 2] have attracted attention for their possible role in the biological control of a number of plant pathogens[3-7]. These metabolites are secreted by many Pseudomonads when cultures are deprived of available forms of iron[8]. The first of these metabolites to be completely characterized structurally was pseudobactin[9], **1**, which was isolated from *Pseudomonas fluorescens* B10. Its structure was solved by X-ray crystallography, and it has since become the model structure for a number of related *Pseudomonas* metabolites, collectively referred to as the pyoverdins. Numerous pyoverdins have since been isolated and characterized[1, 10, 11], and they all contain a peptide linked to an aromatic chromophore responsible for the fluorescence. Although there is considerable novelty amongst the amino acid structures making up the peptide portion, their primary precursors are generally recognizable. We have focused our work on the origin of the chromophore, which was more ambiguous[12].



1

Because pseudobactin is well characterized and is representative of the pyoverdins as a whole, we have chosen it as the focus of our biosynthetic studies. We assumed that phenylalanine, tyrosine, or dihydroxyphenylalanine (DOPA) would be a direct precursor to the chromophore. During the course of our work, a report of *in vitro* and *in vivo* studies with mutants of *Ps. putida* indicated that phenylalanine rather than tyrosine was the direct precursor[13], while a different group reported new metabolites from *Ps. fluorescens* ATCC 13525[14] and *Ps. aptata* [15], the ferribactins, that suggested the involvement of tyrosine rather than DOPA. We now report direct feeding experiments with deuterium-labeled tyrosine and DOPA that demonstrate the direct incorporation of the former but not the latter.

RESULTS AND DISCUSSION

Structural Analysis of Pseudobactin

Biosynthetic feeding experiments using stable isotopes require unambiguous assignment of the ^{1}H- and ^{13}C NMR spectra. Unfortunately, previously reported ^{1}H NMR assignments contained ambiguous assignments for the aromatic protons attached to C-5 and C-8 of the chromophore[16]. In an effort to clarify this, purified pseudobactin was obtained for NMR analysis using a fermentation and isolation protocol developed in our laboratory[17]. The observed proton chemical shifts obtained in D_2O (400 MHz) were sensitive to sample pH and optimal spectral resolution was obtained for samples between pH 6.5 - 7.0. Several broad, overlapping upfield resonances were also present in the ^{1}H NMR spectrum and attempts to increase spectral resolution by dissolving samples in either dimethyl sulfoxide (DMSO-d_6) or methanol or by using elevated temperatures (50 °C, D_2O or DMSO-d_6) were unsuccessful. Nonetheless, using 2D NMR techniques, we have clarified the assignments for the C-5 and C-8 aromatic protons and also report herein the ^{13}C NMR assignments for all non-carbonyl resonances of pseudobactin. We have also confirmed previously reported ^{1}H NMR assignments for the remainder of the structure using magnitude[18, 19] and phase sensitive[20, 21] COSY experiments.

The ^{13}C NMR assignments for all proton bearing carbons were made from observed cross peaks obtained with a heteronuclear correlation NMR[22] (HETCOR) experiment. This information was also used to confirm proton assignments derived from the COSY experiments. The assigned proton and carbon chemical shifts are presented in Table 1.

Table 1. ^{13}C and ^{1}H NMR Assignments of Pseudobactin.

^{13}C	^{1}H*	Assignment
154.9	—	Chr C-7
149.8	—	Chr C-2
145.1	—	Chr C-6
139.6	7.77 (s)	Chr C-4
133.0	—	Chr C-9
117.0	—	Chr C-3
114.7	—	Chr C-10
113.7	7.00 (s)	Chr C-5
101.0	6.91 (s)	Chr C-8
72.8	4.54 (d)	β-OH-Asp-β
67.7	4.12 (5)	Thr - β
59.7	4.32 (d)	Thr - α
57.8	5.51 (bs)	Chr CH-11
57.3	4.90 (d)	β-OH-Asp-α
53.9	4.07 (t)	Lys - α
52.3	3.66 (mult)	N-OH-Orn- δ
50.9	4.45 (mult)	N-OH-Orn- α
50.8	4.43 (q)	Ala_1 - α
50.5	4.36 (q)	Ala_2 - α
39.9	3.28 (mult)	Lys - ε
35.8	3.70 (mult)	Chr CH-13
35.8	3.30 (mult)	Chr CH-13
31.3	2.78 (t)	Succ-CH_2-
31.2	1.83 (mult)	Lys - β
30.5	2.71 (t)	Succ-CH_2-
28.5	1.54 (bs)	Lys - δ
27.3	1.75 (mult)	N-OH-Orn- β
27.3	1.98 (mult)	N-OH-Orn- β
22.8	2.64 (bd)	Chr CH-12
22.8	2.41 (bs)	Chr CH-12
22.2	1.31 (bs)	Lys - γ
20.7	1.96 (mult)	N-OH-Orn- γ
19.3	1.22 (d)	Thr - γ
17.3	1.41 (d)	Ala_2 - β
17.1	1.43 (d)	Ala_1 - β

* Multiplicities are indicated in parenthesis ().

Both the ^{1}H and the ^{13}C chemical shifts for the aromatic portion of the chromophore were completely assigned using a LR HETCOSY experiment[23, 24]. Cross peaks correlated each of the proton-bearing carbons to several quaternary carbons and established the relative positioning of all aromatic carbons. The observed correlations are outlined in Figure 1.

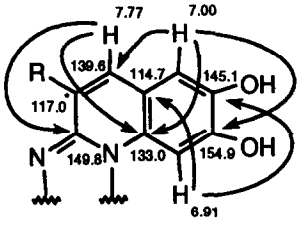


Figure 1. Long range HETCOSY assignments for the pseudobactin chromophore. The δ 117.0 ^{13}C assignment was made by default.

Since *Ps. fluorescens* has been shown to produce at least five similar siderophores[17], there was concern as to the identity of the compound under study. Although the LR HETCOSY experiment was successful for assignment of the aromatic chromophore, it did not provide sufficient data for ^{13}C assignments of the amide carbonyls and precluded the use of NMR for obtaining the peptide sequence. Attempts to complete the NMR assignments for pseudobactin using correlation spectroscopy of long range couplings[25] (COLOC) or the inverse detected heteronuclear multiple-bond correlation (HMBC) NMR[26] method were unsuccessful.

FAB mass spectrometry provided partial sequence information, but the relationship of the alanine and the threonine residues was ambiguous. However, electrospray ionization mass spectrometry yielded a series of N-terminal fragment ions which confirmed the isolation of pseudobactin (Table 2).

Table 2. N-Terminal Fragment Ions Observed for Pseudobactin

N-Terminal Fragment	m/z
Chr-Lys-(β-OH-Asp)-Ala-Thr-Ala-(N-OH-Orn)	989
Chr-Lys-(β-OH-Asp)-Ala-Thr-Ala-	788
Chr-Lys-(β-OH-Asp)-Ala-Thr-	687
Chr-Lys-(β-OH-Asp)-Ala-	616
Chr-Lys-(β-OH-Asp)-	485
Chr-Lys-	357

Incorporation of Tyrosine as a Pseudobactin Precursor

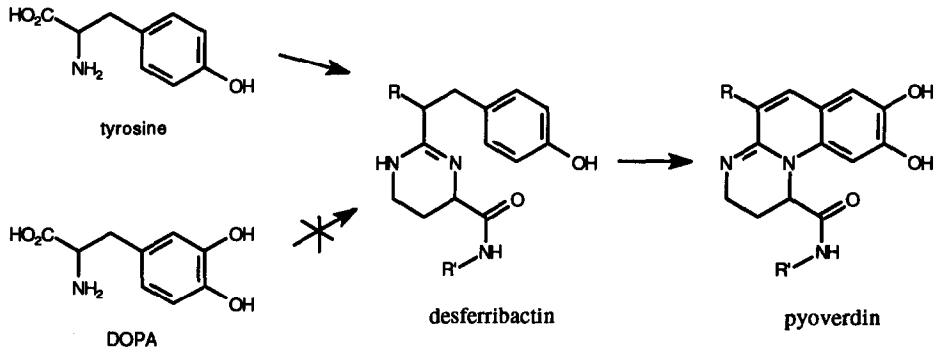
[3-^{14}C]-Tyrosine was fed to cultures of *Ps. fluorescens* B10 in order to test it as a pseudobactin precursor. HPLC analysis of the culture broth indicated that 12.2 mg had been produced. To minimize mass loss, the fermentation broth was treated with $Fe(NO_3)_3$ and 6.9 mg of ferric pseudobactin was isolated. Authentic ferric pseudobactin was added, and the combined material was recrystallized from methanol and water to constant specific radioactivity (3.1×10^4 dpm mg^{-1}). The observed incorporation

of label into ferric pseudobactin was 3.7%.

A similar feeding experiment utilizing deuterium labelled tyrosine was performed to examine the specificity of tyrosine incorporation. [2,3,3-$^2H_3$]-Tyrosine was prepared as previously reported[27]. Portions of the deuterated sample were administered twice during the fermentation, at 14 and 16 hours after inoculation. A control experiment used unlabelled tyrosine, and production levels of pseudobactin were similar in both cultures (approx. 35 mg L^{-1}). NMR analysis required isolation of the desferri-siderophore.

A total of 13 mg of pure pseudobactin was isolated and analyzed by ^2H NMR. It was expected that one of the three deuterium labels would have been retained if the tyrosine had been specifically incorporated into the chromophore. *t*-Butanol was used as an internal reference both for chemical shift and to quantify deuterium enrichment. A deuterium resonance was indeed observed at 7.77 ppm, the chemical shift of the C-4 hydrogen. Comparison of the integrals for the *t* -butanol and pseudobactin signals indicated a 13 % enrichment over natural abundance.

Although this result clearly indicated that tyrosine was incorporated, the timing of the incorporation had not been established. Since incorporation may have occurred only after oxidation to DOPA, a second feeding study using deuterium labelled DOPA was carried out. Initial attempts to prepare [2,3,3-$^2H_3$]-DOPA by pyridoxal catalyzed exchange[27] resulted in substantial decomposition and the material recovered was only modestly exchanged at the β-position (<50%). However, acid catalyzed deuterium exchange afforded high yields of DL-[2',5',6'-$^2H_3$]-DOPA, which was then administered to *Ps. fluorescens* cultures. Isolation and ^2H NMR analysis of pseudobactin obtained from this experiment failed to show a ^2H enrichment at either C-5 or C-8. Thus, the labelled DOPA was not incorporated into the pseudobactin chromophore. Calculations based on the intensity of the *t*-butanol signal, the signal-to-noise ratio, and the amount of pseudobactin produced indicated that even a 0.05% incorporation of labelled DOPA would have been detected.



Scheme 1

Our results, taken in conjunction with the isolation of the pyoverdin-desferribactin pairs[14, 15], reveal that tyrosine is the primary precursor involved in the biosynthesis of the pyoverdin chromophores, and suggest that oxidation of the aryl ring to the catechol moiety occurs at some point after condensation of the tyrosine and diaminobutanoic acid residues (Scheme 1).

EXPERIMENTAL

Structural Analysis of Pseudobactin

NMR spectra were acquired at ambient temperature either on a Bruker AC 300 or AM 400 spectrometers. Purified samples were dissolved in D_2O and then adjusted to pH 6.5-7.0 with either 2% DCl or 2% NaOD. Spectra were referenced to either HOD (4.85 ppm) or acetone (2.22 ppm).

COSY (AC 300). Data were acquired under the following parameters: D0 = 3 μsec, D1 = 1.0 sec, IN = 452 μsec, SI1 = 512 word, SI2 = 1024 word, SW1 = 1106 Hz, SW2 = 2212 Hz, NE = 256, NS = 8. Data were multiplied by a standard sine-bell window function prior to the Fourier transform.

Phase sensitive COSY (AM 400). Phase sensitive data were acquired using a water suppression pulse sequence (COSYPDHG.AUR) under the following parameters: D0 = D3 = 3 μsec, D1 = 2.5 sec, IN = 162 μsec, S1 = S2 = 25L, D4 = 2 msec, SI1 = SI2 = 2048 word, SW1 = 1543 Hz, SW2 = 3086 Hz, NE = 512, NS = 32. Data were multiplied by a squared sine-bell window shifted by $\pi/3$ prior to the Fourier transform.

HETCOR (AM 400). Data were acquired under the following parameters: D0 = 3 μsec, D1 = 1.25 sec, D3 = 3.7 msec, IN = 156 μsec, S1 = 0H, S2 = 15H, D4 = 1.85 msec, SI1 = 256 word, SI2 = 16384 word, SW1 = 1599 Hz, SW2 = 15151 Hz, NE = 128, NS = 256. The data were multiplied by a squared sine-bell window shifted by $\pi/3$ prior to the Fourier transform.

LR HETCOSY (AM 400). Data were acquired under the following parameters: D0 = 3 μsec, D1 = 1.5 sec, D2 = 3.7 m sec IN = 353 μsec, S1 = 0H, SI1 = 512 word, SI2 = 8192 word, SW1 = 1414 Hz, SW2 = 16666 Hz, NE = 256, NS = 256. Data were multiplied by a squared sine-bell window shifted by $\pi/3$ prior to the Fourier transform.

Mass Spectrometry. Fast Atom Bombardment mass spectrometry of pseudobactin was performed on a Kratos MS-50. The sample was introduced to the ionization chamber in a 3:1 thioglycerol:glycerol matrix and fragmented by bombardment with an 8 keV Xe gas source. Electrospray Ionization Mass Spectrometry of pseudobactin was performed on a Sciex API-III Triple-Quadrapole mass spectrometer. The sample was dissolved in an acetonitrile:water:acetic acid solution (50:50:0.1) and introduced directly by loop injection. The samples were analyzed by tandem mass spectrometry (MS/MS) using a He collision gas of 45 eV.

Incorporation of DL-[3-^{14}C]-Tyrosine

An aqueous solution of DL-[3-^{14}C]-tyrosine (3.21×10^7 dpm, sp. act.= 15.6 mCi mmol^{-1}) was sterile-filtered into a 12 h culture (200 mL) contained in a 1 L Erlenmeyer flask[17]. Ferric pseudobactin (6.9 mg) was isolated[17] 42 h after inoculation. Unlabelled ferric pseudobactin (20.1 mg) was subsequently added to the radioactive sample and the combined sample was recrystallized from methanol and water to a constant specific activity (3.1×10^4 dpm/mg, std. dev.= ± 2.8 %). The purified sample corresponded to a single peak detected by HPLC[17] at λ_{max}= 254 and 400 nm.

Preparation of DL-[2,3,3-$^2H_3$]-Tyrosine

A mixture of DL-tyrosine (906 mg, 5.0 mmol), pyridoxal hydrochloride (102 mg, 0.5 mmol), $Al_2SO_4 \cdot 18\ H_2O$ (83 mg, 0.125 mmol), and pyridine (210 μL, 2.5 mmol) in 50 mL D_2O was treated as described[27].

The reaction was then quenched with H_2O (5 L) containing 3-methylpyrazole (274 μL, 5.0 mmol), concentrated HCl (416 μL, 5 mmol HCl), and sodium oxalate (134 mg, 1.0 mmol). The resultant solution was pH 3.5. The quenched reaction mixture was applied to a column (2.7 x 3.0 cm) of Dowex 50W-X4 (H^+ form, 50 - 100 mesh) ion exchange resin. The charged resin was then washed with deionized H_2O (100 mL). Deuterated tyrosine was eluted from the column with aqueous 3 M HCl (40 mL). The acidic eluant was collected and adjusted to pH 7 with concentrated NH_4OH, and a flocculent precipitate formed upon cooling. Recrystallization from ethanol and water afforded pure deuterated tyrosine (377 mg, 42% overall yield, mp 324-325 °C dec.).

^1H NMR (300 MHz, D_2O): δ 6.79 (dd, 1 H, J = 2, 6 Hz), 6.48 (dd, 1 H, J = 2, 6 Hz), 3.93 (dd, 0.06 H), 2.81 (d, 0.35 H, J = 15 Hz), 2.75 (d, 0.35 H, J = 15 Hz). Deuterium enrichment was determined to be 94% and 65% at the α-C and β-C, respectively.

Incorporation of DL-[2,3,3-$^2H_3$]-Tyrosine

The deuterated tyrosine (280 mg) was suspended in sterile water (14 mL) and fed to 7 cultures (300 mL) contained in 2 L Erlenmeyer flasks at 14 h and 16 h after inoculation. As a control, unlabelled tyrosine (40 mg suspended in 2 mL H_2O) was fed to a single culture using the same procedure. Work up of the cultures 42 h after inoculation yielded 13 mg of pure pseudobactin from the labelling experiment. The purified sample corresponded to a single peak detected by HPLC[17] at λ_{max}= 254 and 400 nm.

The purified pseudobactin was dissolved in deuterium depleted water (approx. 1 mL) and lyophilized; this was repeated two more times. The lyophilized sample was then dissolved in deuterium depleted water (approx. 400 μL) containing *t*-butanol (20 μL) and analyzed by ^2H NMR spectroscopy[28]: (61 MHz, H_2O): δ 7.77 (pseudobactin, C-4), 4.41 (HOD), 1.27 (*t*-BuOH).

Preparation of DL-[2',5',6'-$^2H_3$]-3,4-Dihydroxyphenylalanine

To a 2 x 12 cm heavy-walled tube with an attached teflon valve and equipped with a stir bar, DL-3,4-dihydroxyphenylalanine (1.0 g, 5.1 mmol) was dissolved in a solution of deutero-trifluoroacetic acid (10 g, 6 mL, 87 mmol, 99.5 atom % D) and D_2O (1 mL). The reaction tube was repeatedly cooled in a dry ice/acetone bath, evacuated, and filled with argon (3x). The solution was then heated (100 °C) for 24 h, after which it was cooled to room temperature and concentrated DCl (5 drops) was added to the mixture, and the reaction was heated for an additional 14 h. The mixture was then transferred to a round bottom flask and the solvent removed by rotary evaporation and high vacuum.

The brown gummy residue was dissolved in H_2O (3 mL) containing a few milligrams of $Na_2S_2O_5$ and the solution was adjusted to pH 2.4 with dilute aqueous ammonia. After lyophilization, the resulting white powder was then dissolved in H_2O (3 mL) and the solution adjusted to pH 6 with dilute aqueous ammonia. Formation of a white precipitate occurred instantaneously. The sample was cooled to 0 °C, and the light grey powder was recovered by filtration (70% yield). Recrystallization was not required (mp 289 °C dec.).

^{1}H NMR (300 MHz, D_2O) δ 6.85 (s, 0.15 H), 6.81 (s, 0.16 H), 6.68 (s, 0.16 H), 4.28 (dd, 1 H, J = 5, 8 Hz), 3.26 (dd, 1 H, J = 5, 15 Hz), 3.07 (dd, 1 H, J = 8, 15 Hz). Integration of the ^{1}H NMR spectrum indicated 85% deuterium enrichment at each aromatic position.

DL-[2',5',6'-$^2H_3$]-3,4-Dihydroxyphenylalanine Feeding

The deuterated DOPA (266 mg) was dissolved in deionized water (21 mL), and one-third was sterile filtered into 7 cultures (300 mL) contained in 2 L Erlenmeyer flasks at 14 h, 16 h, and 17.5 h after inoculation. Work up of the cultures 42 h after inoculation yielded 29 mg of pure pseudobactin. The purified sample corresponded to a single peak detected by HPLC[17] at λ_{max}= 254 and 400 nm.

The purified pseudobactin was dissolved in deuterium depleted water (approx. 1 mL) and lyophilized; this was repeated two more times. The lyophilized sample was then dissolved in deuterium depleted water (approx. 400 μL) containing *t*-butanol (25 μL) and analyzed by ^{2}H NMR spectroscopy[29]. No deuterium enrichment was observed.

ACKNOWLEDGMENTS

Dr. Joyce Loper (USDA-ARS, Corvallis, OR) is thanked for supplying the organism used in this study, as are Dr. Jeffrey Buyer (USDA-ARS, Beltsville, MD) for providing an authentic sample of ferric pseudobactin, Dr. Douglas Barofsky and Brian Arborgast (Department of Agricultural Chemistry, Oregon State University) for obtaining and interpreting mass spectrometry data, Rodger Kohnert (Department of Chemistry, Oregon State University) for assistance in obtaining NMR data, and Michael Orlov (Department of Chemistry, Oregon State University) for translations of Russian papers. The Bruker AM 400 NMR spectrometer was purchased in part through grants from the National Science Foundation (CHE-8216190) and from the M. J. Murdoch Charitable Trust to Oregon State University. The Bruker AC 300 spectrometer was purchased in part through the grants from the Public Health Service Division of Research Resources (RR 04039-01) and the National Science Foundation (CHE-87123443) to Oregon State University. This work was supported by Public Health Service Research Grant GM 32110 awarded to S.J.G.

REFERENCES AND NOTES

1. Budzikiewicz, H. *Nat. Prod. Chem.* **1988**, *3*, 317-333.
2. Abdallah, M. A. Pyoverdins and Pseudobactins. In: *CRC Handbook of Microbial Iron Chelates*; Winkelmann, G., Ed.; CRC Press: Boca Raton, Fla., 1991; pp. 139-153.
3. Kloepper, J. W.; Leong, J.; Teintze, M.; Schroth, M. N. *Nature (London)* **1980**, *286*, 885-886.
4. Kloepper, J. W.; Schroth, M. N. *Phytopathology* **1981**, *71*, 642-644.
5. Kloepper, J. W.; Schroth, M. N. *Phytopathology* **1981**, *71*, 1020-1024.
6. Buyer, J. S.; Leong, J. *J. Biol. Chem.* **1986**, *261*, 791-794.

7. Mishaghi, I. J.; Olsen, M. W.; Cotty, P. J.; Donndelinger, C. R. *Soil Biol. Biochem.* **1988**, *20*, 573-574.

8. Stainer, R. Y.; Palleroni, N. J.; Doudoroff, M. *J. Gen. Microbiol.* **1966**, *43*, 159-271.

9. Teintze, M.; Hossain, M. B.; Barnes, C. L.; Leong, J.; van der Helm, D. *Biochemistry* **1981**, *20*, 6446-6457.

10. Demange, P.; Wendenbaum, S.; Linget, C.; Mertz, C.; Cung, M. T.; Dell, A.; Abdallah, M. A. *Biol. Metals* **1990**, *3*, 155-170.

11. Persmark, M.; Frejd, T.; Mattiasson, B. *Biochemistry* **1990**, *29*, 7348-7356.

12. The reader may wish to refer to the following paper regarding the synthesis of the chromophore: Kolasa, T.; Miller, M. J. *J. Org. Chem.* **1990**, *55*, 4246-4255.

13. Maksimova, N. P.; Blagevich, O. V.; Fomichev, Y. K. *Mikrobiologica* **1992**, *61*, 818-823.

14. Linget, C.; Stylianou, D. G.; Dell, A.; Wolff, R. E.; Piémont, Y.; Abdallah, A. *Tetrahedron Lett.* **1992**, *33*, 3851-3854.

15. Budzikiewicz, H.; Schröder, H.; Taraz, K. *Z. Naturforsch* **1992**, *47c*, 26-32.

16. Teintze, M.; Leong, J. *Biochemistry* **1981**, *20*, 6457-6462.

17. Nowak-Thompson, B.; Gould, S. J. *BioMetals* **1994**, *7*, 20-24.

18. Aue, W. P.; Bartholdi, E.; Ernst, R. R. *J. Chem. Phys.* **1976**, *64*, 2229-2246.

19. Nagayama, K.; Kumar, A.; Wuthrich, K.; Ernst, R. R. *J. Mag. Reson.* **1980**, *40*, 321-334.

20. Marion, D.; Wuthrich, K. *Biochem. Biophys. Res. Comm.* **1983**, *113*, 967-974.

21. Rance, M.; Sorensen , O. W.; Bodenhausen, G.; Wagner, G.; Ernst, R. R.; Wuthrich, K. *Biochem. Biophys. Res. Comm.* **1983**, *117*, 479-485.

22. Bay, A.; Morris, G. *J. Mag. Reson.* **1981**, *42*, 501-505.

23. Bleich, H.; Gould, S. J.; Pitner, P.; Wilde, J. *J. Mag. Reson.* **1984**, *56*, 515-517.

24. Sato, Y.; Kohnert, R.; Gould, S. J. *Tetrahedron Lett.* **1986**, *27*, 143-146.

25. Kessler, H.; Griesinger, C.; Zarbock, J.; Loosli, H. R. *J. Mag. Reson.* **1984**, *57*, 331-336.

26. Bax, A.; Summers, J. *J. Am. Chem. Soc.* **1986**, *108*, 2093-2094.

27. LeMaster, D. M.; Richards, F. M. *J. Label. Comp. Radiopharma* **1982**, *XIX*, 639-646.

28. ^{2}H NMR aquisition parameters: SW 1432 Hz, SI = 16K, TE = 333 K, NS = 40,057. Data multiplied by LB = 3.0.

29. ^{2}H NMR aquisition parameters: SW 1432 Hz, SI = 16K, TE = 297 K, NS = 31,694. Data multiplied by LB = 2.0.

(*Received in USA* 18 *April* 1994; *accepted* 21 *June* 1994)