Structure and Behavior of Spermidine Siderophores

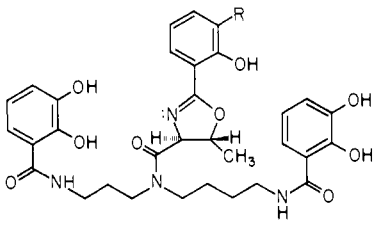
T. Peterson, Karl-Erik Falk, Sally A. Leong, Melvin P. Klein, and J. B. Neilands*[1]

Contribution from the Department of Biochemistry and the Laboratory for Chemical Biodynamics, University of California, Berkeley, California 94720. *Received February 19, 1980*

Abstract: The proposed structures of the microbial iron transport compounds (siderophores) agrobactin and parabactin were confirmed by synthesis of a hydrolysis product, agrobactin A. The unusual stability of the 2-oxazoline ring of the siderophores was shown to arise from electronic effects contributed by the *o*-hydroxy substituent. The duplicate NMR spectra of agrobactin and parabactin were demonstrated to originate from cis-trans isomerization around the tertiary amide bonds.

Siderophores, defined as microbial iron transport agents, are produced commonly by both prokaryotic and eukaryotic unicellular species.[2] Most siderophore ligands may be classed as either hydroxamic acids or catechols and the formation constants for iron(III) are generally at least of the order of 10^{30}.

The properties of the prototypical tricatechol siderophore, enterobactin, have been studied in detail, and its synthesis has been reported.[3] In contrast, less is known about the two more recently discovered catechol-type siderophores based on spermidine, agrobactin (**1**, R = OH) from *Agrobacterium tumefaciens*[4] and parabactin (**1**, R = H) from *Paracoccus denitrificans*. Parabactin was originally described as "compound III"[5] and assigned the constitution now accorded parabactin A (**2**, R = H).[6] Subsequent



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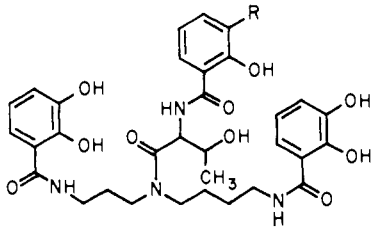

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isolation of the closely related agrobactin and its hydrolysis to the open-chain form, agrobactin A (**2**, R = OH),[4] led to the detection of the oxazoline ring in "compound III".[6] At physiological pH both agrobactin and parabactin form six-coordinate complexes with Fe(III) in which the metal ion is linked to the distal catechols and to the 2-(2-hydroxyphenyl)oxazoline with an affinity virtually identical with that of enterobactin,[7] the siderophore with the largest known formation constant with ferric ion.[8] The spermidine siderophores have been shown to be highly potent in the deferration of mammalian cell lines and are potentially useful for chelation therapy.[9]

In the present report we confirm the proposed structure of agrobactin and, by extension, parabactin, through synthesis of agrobactin A (**2**, R = OH). Two features of the siderophores warranted additional study, namely, the exceptional stability of the oxazoline ring to acid hydrolysis and the duplicate NMR[10] resonance pattern. The preparation of certain model oxazolines and their comparison with the natural products showed the hydrolysis of the oxazolium nucleus to proceed at a rate critically dependent upon the particular substitution in the phenyl ring attached to the 2 position. Examination of the NMR spectra of the siderophores and of a synthetic symmetrical analogue of agrobactin A, together with a study of the temperature dependence of the chemical shifts, enabled assignment of the doubled resonance to cis-trans isomerization around the tertiary amide nitrogen.

Experimental Section[11]

Isolation of Siderophores. Agrobactin (*N*-[3-(2,3-dihydroxybenzamido)propyl]-*N*-[4-(2,3-dihydroxybenzamido)butyl]-2-(2,3-dihydroxyphenyl)-*trans*-5-methyloxazoline-4-carboxamide, **1**, R = OH), mp 108-112 °C, was obtained from *A. tumefaciens* as previously described.[4] Parabactin (*N*-[3-(2,3-dihydroxybenzamido)propyl]-*N*-[4-(2,3-dihydroxybenzamido)butyl]-2-(2-hydroxyphenyl)-*trans*-5-methyloxazoline-4-carboxamide, **1**, R = H), mp 114-117 °C, was prepared by growth of *P.* (formerly *Micrococcus*) *denitrificans* in the succinate-ammonium chloride medium used by Tait,[5] without addition of molybdate. Both siderophores were crystallized from ethyl acetate-hexane. Brown oxidation products, when present, were removed by chromatography on silicic acid in benzene-ethyl acetate.[5] Residual ethyl acetate was eliminated by lyophilization, after precipitation of the siderophores from methanol-water.

Chemical Synthesis. Oxazolines. The synthesis of 2-phenyl-4-carbomethoxy-5-methyl-2-oxazoline (**3**) was achieved by published methods,[12] using cold $SOCl_2$ to cyclize *N*-benzoyl-L-threonine methyl ester. The structure of the product was confirmed by NMR; $J_{4,5}$ was 10.2 Hz, characteristic of protons cis to one another in a five-membered ring.[13]

The synthesis of 2-(2-hydroxyphenyl)-2-oxazoline (**4**) followed a similar route. Salicyl chloride was used to acylate 2-aminoethanol, affording 2-salicylamidoethanol in 65% yield. Five grams (27 mmol) of this amide in 10 mL of $CHCl_3$ was added slowly with stirring and cooling at 0 °C to a solution of 9 mL of $SOCl_2$ in 25 mL of $CHCl_3$. Upon standing for 3 h at 0 °C, a brown solid appeared. The solvent and excess $SOCl_2$ were evaporated and the residue treated with 10 g of $NaHCO_3$ in cold water. The oil that formed was extracted into ether, dried with $MgSO_4$, evaporated to an oil, and distilled in vacuo, bp 108-114 °C (0.5 mm). The

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(10) Abbreviations: NMR, nuclear magnetic resonance; DMF, dimethylformamide; THF, tetrahydrofuran; Me_2SO, dimethyl sulfoxide; TLC, thin layer chromatography; DCC, dicyclohexylcarbodiimide; UV, ultraviolet; IR, infrared; CDI, carbonyldiimidazole; Bz, benzyl; Me_4Si, tetramethylsilane.

(11) Microanalyses by Chemistry Department, University of California, Berkeley. Melting points (uncorrected) were determined in capillaries. Thin layer plates were examined under ultraviolet light or sprayed with 1% $FeCl_3$.

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clear distillate yellowed slightly in the receiver and solidified overnight. The yield was 3 g (65%). A portion was purified by recrystallization from chilled CH_3OH: mp 37–40 °C; NMR (CCl_4) δ 11.4 (1 H, broad s, phenol OH), 6.6–7.8 (4 H, m, aromatic), 3.7–4.8 (4 H, m, oxazoline ring CH_2); IR 1642 cm^{-1} (C = N stretch, lit.[14] 1647 cm^{-1}). Anal. ($C_9H_9NO_2$) C, H, N. Portions were also converted to the picrate, mp 163–164 °C, and the hydrochloride, mp 147–148 °C (lit.[15] mp 159 °C). Anal. ($C_9H_{10}NO_2Cl$) C, H, N, Cl.

Catechol Conjugates. 2,3-Dibenzyloxybenzoic Acid (5). 2,3-Dihydroxybenzaldehyde (Sigma Chemical Co.) was benzylated[16,17] and oxidized[18] by literature methods, mp 122–124 °C (lit.[16] 120 °C) after recrystallization from C_2H_5OH/H_2O, yield 70–80%.

***N*-(2,3-Dibenzyloxybenzoyl)-L-threonine (6).** **5** (6.0 g, 18 mmol) and 3.2 mL of oxalyl chloride (36 mmol) in 10 mL of dry C_6H_6 and 1 drop of dry DMF (as catalyst) was stirred for 1 h and evaporated to an oil, which was taken up in 2 mL of THF and added to a vigorously stirred solution of 3.0 g of L-threonine (25 mmol) and 6 g of K_2CO_3 (43 mmol) in 50 mL of H_2O, at room temperature. After 20 min the clear solution was washed once with $(C_2H_5)_2O$, then acidified to pH 2 with concentrated HCl, extracted into ethyl acetate, dried with $MgSO_4$, and evaporated to an oil which soon solidified: yield 5.5 g (70%) after recrystallization from ethyl acetate/hexane; mp 136–138 °C; NMR (Me_2SO-d_6) 9.0 (1 H, d, amide NH), 7.1–8.0 (13 H, m, aromatic), 5.3 (4 H, 2 close s, benzyl CH_2O), 4.9 (1 H, m, β-CH), 4.7 (1 H, d of d, α-CH), 1.3 (3 H, d, γ-CH_3). Anal. ($C_{25}H_{25}NO_6$) C, H, N.

***N*-(2,3-Dibenzyloxybenzoyl)glycine (7)** was prepared in the same way: yield 50% after recrystallization from $CHCl_3$/hexane; mp 122–123 °C; NMR ($CDCl_3$) δ 9.9 (1 H, broad s, COOH), 9.3 (1 H, t, amide NH), 7.9–8.5 (13 H, m, aromatic), 5.8 (4 H, apparent s, benzyl CH_2), 4.6 (2 H, d, glycine CH_2). Anal. ($C_{23}H_{21}NO_5$) C, H, N.

***N*^{1},*N*^{10}-Bis(2,3-dibenzyloxybenzoyl)spermidine (8).** *N*,*N*'-Carbonyldiimidazole (3.6 g, 22 mmol) was dissolved in 10 mL of $CHCl_3$ and 7.8 g of **5** (23 mmol) added in portions, allowing the CO_2 evolution to subside between additions. After the mixture was stirred for 20 min, the acylimidazole was added to 1.23 g of spermidine (8.4 mmol) and 3 mL of triethylamine (22 mmol) in 10 mL of $CHCl_3$. After standing overnight the solution was diluted with 50 mL of $CHCl_3$ and washed thrice with 20 mL of 1 N HCl to remove imidazole and triethylamine. The product, although charged, remained in the organic phase. Any emulsions were centrifuged to separate the layers. The $CHCl_3$ solution was then washed thrice with K_2CO_3 solution to remove the excess acid, dried over K_2CO_3, and evaporated. The residual oil was taken up in CH_2Cl_2 and applied to a column of Bio-Sil A, 200–400 mesh, 50 mL bed volume (Bio-Rad Lab), in $CHCl_3$, washed with 100 mL of $CHCl_3$, and eluted with 20:1 $CHCl_3$–CH_3OH (400–600 mL). Fractions pure by TLC in 10:1 $CHCl_3$–CH_3OH (Brinkmann Sil G plates) were pooled and evaporated to a light yellow oil. The yield, about 50%, was improved by rechromatography of impure fractions. Acidification of the K_2CO_3 wash precipitated the excess **5** in nearly pure form.

That the free amino group is secondary was confirmed by use of dinitrofluorobenzene;[19] the ratio of absorptions at 350 and 390 nm was 0.81, characteristic of secondary amines. NMR (CCl_4/Me_2SO-d_6): δ 8.0 (2 H, m, amide NH), 7.0–7.7 (26 H, m, aromatic), 5.0 (8 H, two close s, benzyl CH_2O), 3.2 (4 H, m, CH_2 next to amide N), 2.3 (4 H, m, CH_2 next to amine N), 1.3 (6 H, m, CH_2 not next to N).

Bis(3-(2,3-dibenzyloxybenzamido)propyl)amine (9) was prepared in the same way from bis(3-aminopropyl)amine (Aldrich Chemical Co.). The NMR was similar but had only four protons at δ 1.3.

***N*^{1},*N*^{10}-Bis(2,3-dibenzyloxybenzoyl)-*N*^{5}-(*N*-(2,3-dibenzyloxybenzoyl)-L-threonyl)spermidine (10) (Hexabenzyl agrobactin A).** **8** (1.56 g, 2 mmol), 0.86 g of **6** (2.2 mmol), 0.35 mL of triethylamine (2.5 mmol), and 0.3 g of *N*-hydroxysuccinimide (2.5 mmol) were dissolved in 3 mL of dry THF and 0.5 g of DCC (2.4 mmol) in 0.5 mL of dry THF added. The flask was purged with dry N_2, sealed, and stirred magnetically for 2 days. The solid was removed by filtration and washed with THF, and the filtrate and washings were diluted with 20 mL of ethyl acetate, washed with 1 N HCl, 1 N NaOH, and saturated NaCl, dried with $MgSO_4$, and evaporated to an oil. The oil was dissolved in CH_2Cl_2 and applied to a column of BioSil A in CH_2Cl_2 (50 mL bed volume). The column was washed with 200 mL of $CHCl_3$, then with $CHCl_3$ containing 1, 2, and 5% 2-propanol (200 mL each). The bulk of the product came off in 2% 2-propanol. Fractions pure by TLC were pooled and evaporated to an oil: yield 35%; NMR ($CDCl_3$) δ 9.5 (1 H, broad d, central amide NH), 8.6 (2 H, br, outer amide NH), 7.2–8.4 (39 H, m, aromatic), 5.6 (12 H, s, benzyl CH_2O), 4.3–4.6 (2 H, m, threonine α- and β-CH), 3.2–3.8 (8 H, m, CH_2 next to amide N), 0.9–1.9 (9 H, m + d, CH_2 not next to N, also threonine γ-CH_3).

Coupling the acylthreonine **6** to the central nitrogen of **8** failed when CDI, or DCC alone, was used as condensing agent, probably owing to the bulky nature of the reagents involved.

***N*,*N*-Bis(2,3-dibenzyloxybenzamidopropyl)-(2,3-dibenzyloxybenzoyl)glycinamide (11)** was also made in the same way from **7** and **9**: NMR ($CDCl_3$) δ 8.4 (1 H, broad, central amide NH), 8.2 (2 H, broad, outer amide NH), 7.2–8.0 (39 H, m, aromatic), 5.4 (12 H, 2 close s, benzyl CH_2O), 4.4 (2 H, d, glycine CH_2), 3.4–3.9 (8 H, m, CH_2 next to N), 1.7–2.3 (4 H, m, CH_2 not next to N).

***N*^{1},*N*^{10}-Bis(2,3-dihydroxybenzoyl)-*N*^{5}-(*N*-(2,3-dihydroxybenzoyl)-L-threonyl)spermidine (Agrobactin A, 2, R = OH). 10** (600 mg, 0.5 mM) was catalytically hydrogenated over 100 mg of 10% Pd on charcoal (Aldrich Chemical Co.) in 10 mL of THF for 12 h and filtered from the catalyst, and the solvent was evaporated and replaced with 15 mL of benzene–ethyl acetate (2:1). A column of 10 g of silica (Mallinckrodt, 100 mesh) was prepared in the same solvent. The crude product was applied and eluted with 15 mL of the same solvent; colored impurities remained adsorbed to the column. The eluate was evaporated and the product slowly crystallized from ethyl acetate–hexane to yield 150 mg (45%) of white crystals, R_f in 4:1 $CHCl_3$–CH_3OH on silica gel 0.50. Anal. ($C_{32}H_{38}N_4O_{11}$) C, H; N: calcd, 8.56; found, 8.48.

***N*,*N*-Bis(2,3-dihydroxybenzamidopropyl)-(2,3-dihydroxybenzoyl)-glycinamide (12)** was made in the same way by hydrogenating **11**, yield 50%.

Both **2** (R = OH) and **12** did not have sharp melting points but decomposed above 110 °C, evolving a gas. They were characterized by their UV, IR, and NMR spectra. Synthetic **2** (R = OH) was judged identical with agrobactin A[4] obtained by mild acid hydrolysis of agrobactin by UV spectra and by TLC in three ratios of $CHCl_3$–CH_3OH. Despite several attempts, parabactin A (**2**, R = H; similarly synthesized, details not shown) could not be crystallized in our hands. It was therefore characterized only by its UV spectrum and by chromatographic comparison with authentic material obtained by mild acid hydrolysis of parabactin.[6] The two were identical by all accounts (R_f in 4:1 $CHCl_3$–CH_3OH on silica gel 0.55). Experiments with the crude material revealed that it was an inferior iron chelator,[7] and no further attempts at crystallization were made. Anal. ($C_{29}H_{32}N_4O_{10}$) C, H; N: calcd, 9.39; found, 9.28.

Determination of Rates of Hydrolysis for Oxazolines. A small amount of the oxazoline under investigation was dissolved in 3:1 C_2H_5OH–H_2O and diluted in the same solvent until the maximum optical density, as measured in a Beckman Model 25 spectrophotometer, at 25 °C, was 1.3–1.6 over the 210–250-nm range. A control ("no acid") spectrum is taken; then the cuvette, containing 2 mL of solution, was treated with 20 μL of concentrated HCl (final concentration 0.1 N) and a new spectrum taken immediately and at appropriate intervals thereafter, noting the elapsed time. The rise and fall of the new peak were followed until no further change occurred. A wavelength at which large spectral changes occurred was selected and the absorbance plotted vs. time on semilog paper. The absorbance at infinite time was estimated graphically if not obvious from the raw data; with proper choice of this constant straight lines were obtained, indicating first-order kinetics.

NMR Spectroscopy. High-resolution ^1H NMR spectra were recorded on a custom-built[20] spectrometer employing a Bruker 6.3 T magnet and a Nicolet 1180 computer located at the Laboratory of Chemical Biodynamics, University of California, Berkeley, and on a Bruker WH 270 spectrometer located at the Department of Biochemistry and Biophysics, University of Göteborg. All data were obtained by using the pulsed Fourier transform method, and the decoupling and saturation transfer experiments were performed by using a gated homonuclear decoupler. T_1 values were calculated from the spectra following a standard 180°–τ–90° pulse sequence and the *J*-resolved two-dimensional spectrum[21] from a series of 90°–τ–180° sequences. Routine spectra were recorded on a Varian EM-360 spectrometer. The chemical-shift values are given relative to Me_4Si.

Results and Discussion

Hydrolysis of Oxazolines. When agrobactin or parabactin is treated with dilute acid, the UV spectrum undergoes a charac-

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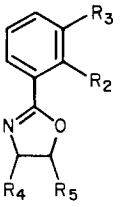
Table I. Rate of Hydrolysis of Various Oxazolines[a]



compd	R_2	R_3	R_4	R_5	λ_{max}, nm	$t_{1/2}$, min
agrobactin (1, R = OH)	OH	OH		CH_3	277	360
parabactin (1, R = H)	OH	H		CH_3	254	324
3	H	H	$COOCH_3$	CH_3	258[b]	3
4	OH	H	H	H	253	350
13	OH	OH	H	H	273	1860
14	OCH_3	OCH_3	$COOCH_3$	CH_3	263	6
15	OBz	OBz	$COOCH_3$	CH_3	267	7
16	OCH_3	H	H	H	254	950
ethyl iminobenzoate	H	H			260[b]	10

[a] Rates were measured in 3:1 ethanol-water, 0.1 N in HCl at 25 °C. Oxazolines 13-16 were formed in solution by the methods similar to those used for synthesis of 3 and 4. [b] Shoulder.

teristic red shift[6] and in the presence of water slowly collapses as the oxazoline ring opens. We found this spectral behavior typical of 2-oxazolines and other imino ethers.

The first synthetic oxazoline in our hands (**3**) opened two orders of magnitude faster than the siderophores. A review of the literature revealed that rapid hydrolysis is to be expected. To determine the cause of the anomalous stability of the siderophores, a number of substituted 2-phenyl-2-oxazolines were synthesized (details given for **4**; others were made similarly) and examined under standard conditions (Experimental Section). The results are shown in Table I. It can be seen that all compounds with R_2 = OH are essentially as stable as agrobactin or parabactin. Since the first compounds studied (**14** and **15**) contained R_2 = R_3 = methoxy or benzyloxy and were labile, we felt unable to consider the effect purely due to a resonance contribution of oxygen nonbonding electrons with the π system. The subsequent synthesis and examination of **16**, in which the methoxy group is unhindered and can lie coplanar with the benzene ring, demonstrated that alkoxy groups can confer stability if n-π orbital overlap is possible, i.e., when the group is coplanar.[22] When R_2 = OH, the small size of this group allows coplanarity even when R_3 is not hydrogen.

The *o*-hydroxyl therefore has two functions in spermidine siderophores: as a metal ligand atom and to confer stability against hydrolysis upon the oxazoline ring. We estimate the half-life of agrobactin at pH 7 to be 10^6 min. A similar instance of resistance to acid hydrolysis has been reported for salicylglycine,[23] indicating that the phenomenon is not limited to imino ethers.

Syntheses. The syntheses of agrobactin A and the symmetrical analogue **12** employed 2,3-dibenzyloxybenzoic acid as a key reagent. This protected catechol does not suffer from the oxidative degradation characteristic of catechols at high pH. The 2,3-dibenzyloxybenzoic acid, as the acid chloride, coupled smoothly to the amino acids threonine and glycine and, via CDI, to the primary amino groups of spermidine and its symmetrical homologue, bis(3-aminopropyl)amine. Next, DCC was used to link the appropriate acylamino acid and diacylpolyamine to afford the protected product. Deprotection by hydrogenolysis completed the synthesis of the catechol conjugates. A mixture melting point with synthetic and naturally derived agrobactin A[4] was not attempted since the latter was never obtained entirely free from agrobactin.

Characterization. The IR spectra of synthetic agrobactin A (**2**, R = OH), prepared either by synthesis or by hydrolysis of agrobactin, and the symmetrical glycine analogue (**12**) were similar to each other and to the native siderophores agrobactin (**1**, R = OH) and parabactin (**1**, R = H). The spectra were in fact so similar that the original aim of the IR study, namely, the distinction between oxazoline and open, or A, forms, was frustrated. The oxazoline C=N stretching band, at 1642 cm^{-1}, lies among the amide C=O bands and precise assignment is not possible. Bands common to all four compounds appeared near 1640, 1595, 1550, 1485, 1460, 1375, 1330, 1265, 1176, 1072, and 840 cm^{-1}.

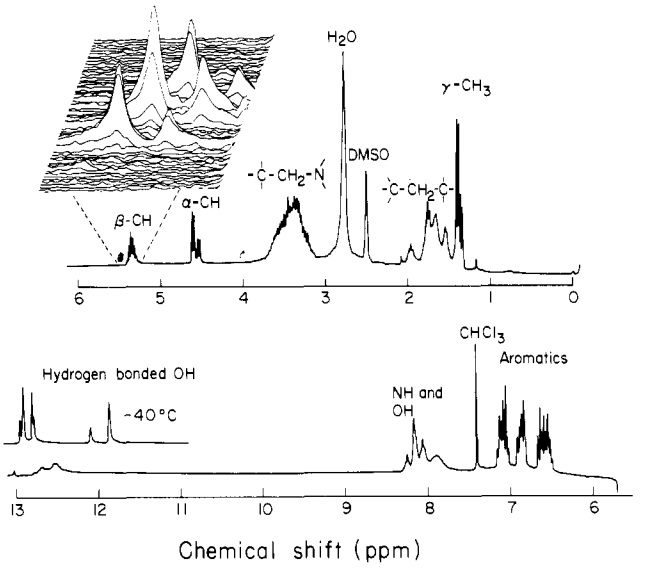


Figure 1. A 270-MHz ^1H NMR spectrum of 5 mg of agrobactin in 0.5 mL of $CDCl_3$-Me_2SO-d_6 (10:1) at 22 °C. No exponential multiplication or other time domain treatment has been applied.

In order to determine the characteristic absorptions of the oxazoline system, the IR spectra of **4** and its hydrochloride were compared with the above spermidine siderophores. The common bands near 1642, 1375, 1265, and 1072 cm^{-1} were also present in the spectrum of **4**. Protonation shifted the frequency of C=N stretching only slightly from 1642 to 1630 cm^{-1}, as previously noted[24] in other imino ethers.

The UV spectra of synthetic **2** (R = OH) and **12** were identical with those of other 2,3-dihydroxybenzamides and, in particular, with those of authentic agrobactin A. Their specific absorptions agreed well with predictions based on the molecular weight and known absorptivity of dihydroxybenzamides (ϵ_{315} 3.2 × 10^3 mol in C_2H_5OH). A 2:1 mixture of a dihydroxybenzamide and the oxazoline **4** accurately mimicked the spectrum of parabactin, including its behavior in acid media.[6]

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Table II. Chemical Shifts and Coupling Constants

	agrobactin	agrobactin A	parabactin
		Threonine	
NH		7.9	
		$J_{NH\alpha}$ = 7.9 Hz	
α	4.6	5.0	4.6
	$J_{\alpha\beta}$ = 6.8 Hz	$J_{\alpha\beta}$ = 2.4 Hz	$J_{\alpha\beta}$ = 6.8 Hz
β	5.4	4.2	5.3
	$J_{\beta\gamma}$ = 6.4 Hz	$J_{\beta\gamma}$ = 6.1 Hz	$J_{\beta\gamma}$ = 6.4 Hz
γ	1.4	1.2	1.4
		Spermidine	
NH	8.0-8.3	7.8-8.3	7.8-8.3
CCH_2C	1.5-2.0	1.5-2.0	1.5-2.0
CCH_2N	3.2-3.7	3.2-3.7	3.2-3.7
		Aromatic	
H	6.5-7.1	6.5-7.2	6.5-7.6
OH (not H bonded)	7.8-8.3	7.8-8.3	7.8-8.3
OH (H bonded)	12.5-13.0	12.5-13.0	11.7-13.0

The synthetic open forms of the oxazoline-containing siderophores agrobactin and parabactin were shown by their UV spectra and chromatographic behavior to be identical with material obtained by mild acid hydrolysis of the corresponding native siderophore. Since the opening of the oxazoline ring in acid is well understood, the structures of agrobactin and parabactin are now conclusively established.

Cis-Trans Isomerization. A 270-MHz ^1H NMR spectrum of agrobactin (**1**, R = H), dissolved in a 10:1 $CDCl_3$-Me_2SO mixture, is shown in Figure 1. The groups of peaks are assigned as indicated in the figure. From decoupling experiments the threonine α, β, and γ protons are found to be 4.6, 5.4, and 1.4 ppm. The coupling constants are given in Table II and correspond to a trans configuration. Furthermore, the peaks were present in duplicate, a feature that is most easily seen for the α and γ doublets; it is also evident in the inserted two-dimensional presentation of the β multiplet. The details of two-dimensional NMR spectroscopy have been reviewed elsewhere.[21] The aromatic protons were found at 6.5-7.1 ppm and no further assignments within that group have been attempted. The NH resonances at ~8 ppm are coupled to protons within the group at 3.2-3.7 ppm, which in turn are coupled to the resonances at 1.5-2.0 ppm. These groups were accordingly assigned to CCH_2N and CCH_2C of the spermidine, respectively. Finally, the hydroxyl protons of the catechols were identified with saturation transfer from the water signal and the hydrogen-bonded protons were assigned on the basis of chemical shift. These signals, when recorded at lower temperature, also show a doubling of the resonances with the same ratios as those from the α, β, and γ protons. The non-hydrogen-bonded hydroxyl protons overlapped with the NH resonances but their identification was aided by their various exchange rates, different relaxation times, and the fact that only the NH protons showed a coupling pattern.

A comparison of the NMR parameters for three similar compounds is given in Table II. Parabactin (**1**, R = H) gives of course very similar parameters to agrobactin (**1**, R = OH), except for the presence of the additional aromatic proton. Agrobactin A (II, R = OH) exhibits distinctive coupling constants and chemical-shift values in the threonine fragment, indicating a different conformation than when an oxazoline ring is present. Possibly this is related to the observation that the iron(III) complexes of the two siderophores, from criteria acquired by both circular dichroism and bacterial feeding experiments,[7] appear to form coordination isomer with chirality opposite to that displayed by the open, or A series, compounds.

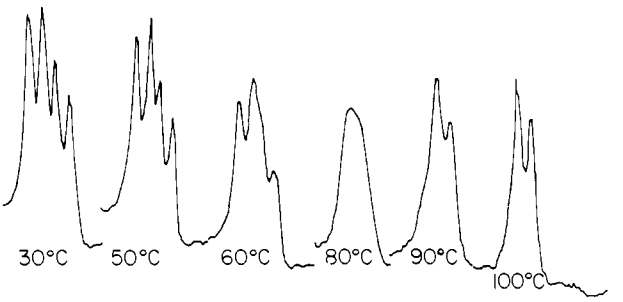

Figure 2. High-field portion of the spectrum in Figure 1 at various temperatures showing the collapse of the γ-methyl duplicate signals.

The rate of exchange between the two isomeric forms was investigated as a function of temperature. The required temperature range, however, precluded $CDCl_3$ as solvent and this part of the study was therefore performed in Me_2SO. The interpretation of the data is also facilitated by the fact that the ratio of the isomeric forms is closer to unity in this solvent. The result is shown in Figure 2 and it is seen that the coalescence temperature for the γ-CH_3 group is ~75 °C. With the measured chemical-shift difference of 13 Hz between the two forms a value of 18 kcal/mol can be calculated for the activation energy at that temperature, a value fully compatible with the barrier for a cis-trans isomerization of an amide.[25] This value was confirmed via an experiment in which the β multiplet was irradiated in order to give γ singlets, a case which is easier to interpret. The reason that the protons on the threonine residue are sensitive to the isomer forms can be attributed to the unsymmetrical nature of the spermidine chain. A synthetic compound with a symmetrical polyamine chain gave, as expected, only one signal from protons on the amino acid residue. The resonances on the N substituents will of course also show duplicate signals, although the latter are extensively overlapped.

We therefore conclude that the NMR data presented here are consistent with a picture of the molecule existing in two isomeric forms, a cis-trans mixture with respect to the amide group. The exchange rate at normal temperatures is rather slow and at 75 °C it is 30 s^{-1}. At present we have no information that would make it possible to assign the peaks to the different forms, but is should be noted that when the acyl residue is larger than formyl the most favored conformation seems to be with the bulkier N substituent cis to the carbonyl oxygen.[25] Our data also suggest the existence of three internal hydrogen bonds probably involving the three *o*-hydroxyls.

Note Added in Proof: The molecular and crystal structure of agrobactin has been determined by D. van der Helm, Chemistry Department, University of Oklahoma, and will be reported in a separate paper.

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