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J. Nat. Prod., **1991**, 54 (4), 1003-1008• DOI:
10.1021/np50076a013 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 54, No. 4, pp. 1003-1008, Jul-Aug 1991

NMR STUDIES OF DIMERIC 2,3-DIHYDROXY-*N*-BENZOYL SERINE

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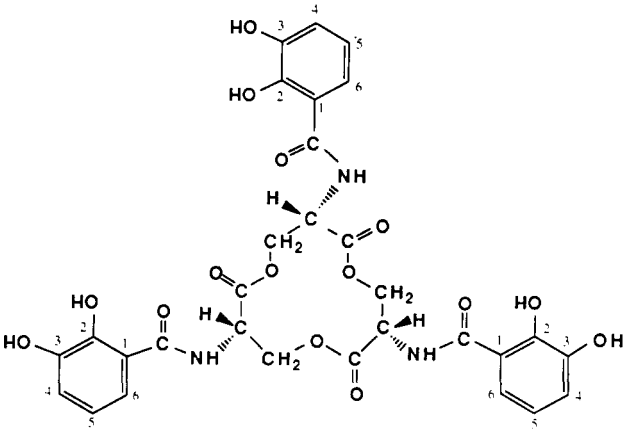
ABSTRACT.—The ^{1}H- and ^{13}C-nmr spectra of the dimeric hydrolysis product **3** of enterobactin [**1**] were compared in deuterated Me_2CO and deuterated DMSO with those of **1**. 2D nmr techniques were used to make or confirm resonance assignments. The ^{1}H nmr of the gallium chelate of **3** is also presented.

Enterobactin [**1**], also known as enterochelin (1), is a high affinity iron chelator that is produced by many Gram-negative bacteria during periods of iron stress (2). Hydrolysis products of **1**, which have a lower iron affinity, were originally considered only as necessary metabolic by-products for bacterial ferric-enterobactin iron release (3). An alternative to this "esterase" mechanism, proposed by Hider *et al.* (4), is based on the identification of iron(II) enterobactin, and suggests that hydrolysis is merely a consequence of an enzymatic ferric-ferrous redox reaction. Although these hydrolysis products have been studied very little as siderophores themselves, it has been demonstrated that they may supply iron to *Escherichia coli* (5). Because cellular uptake of ferric-enterobactin depends upon the stereochemistry around the iron atom and not upon the triester backbone (6), it is possible that the hydrolysis products of **1** are not simply end products as they were once believed to be, and further investigation is warranted.

The ^{1}H-nmr spectra of enterobactin and its hydrolysis products in Me_2CO (1,7), the ^{13}C-nmr spectra of **1** and its monomeric hydrolysis product **2** and DMSO (8), and both the ^{1}H and ^{13}C-nmr spectra of the gallium(III) complex of **1** have been previously studied (5). This paper uses more recent technology to consolidate these nmr studies and to examine the nmr spectra of dimeric 2,3-dihydroxy-benzoyl serine [**3**].

Table 1 compares the ^{1}H-nmr spectra in Me_2CO-d_6 and in DMSO-d_6 at 300 MHz. The resonance assignments were made by 2D homonuclear shift-correlated (COSY) nmr and agree with the literature assignments (1). The complexity of the ^{1}H nmr of **3** in Me_2CO is a consequence of the diastereotopic protons on the serine β-carbons and



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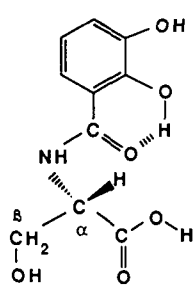
[1]Deceased 20 April 1991.



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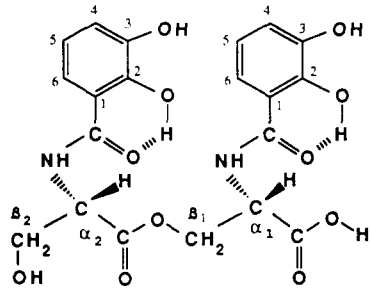

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their magnetic non-equivalence in Me_2CO-d_6. Because the difference in the methylene proton chemical shifts is less pronounced in the "free alcohol" serine of **3** (upfield resonances) and is nonexistent in **1**, one might be tempted to theorize that these differences are due to rapid rotation of the "CH_2-OH" group. However, the two serine β-protons of **1** are also unresolved in Me_2CO, yet have separate chemical shifts in DMSO (2,8), while the spectrum of **3** in DMSO is greatly simplified. In nmr studies of parabactin (9,10), and agrobactin A (9), a "duplicity" of signals based on solvent changes was attributed to the existence of the free ligand in more than one possible conformation. It is possible that the conformations of **1**, **2**, and **3** also depend upon the solvent, as well as differing from one another.

TABLE 1. ^{1}H-nmr Chemical Shifts of Catecholates in ppm.

Proton	Solvent	Compound			
		1	**2**	**3**[a,c]	**3**[b,c]
C_α-H	Me_2CO	5.11	4.78	4.81	5.10
	DMSO	4.90	4.49	4.60	4.79
C_β-H_1	Me_2CO	4.73	4.06	4.02	4.58
	DMSO	4.40	3.80	3.76	4.50
C_β-H_2	Me_2CO	—	—	4.06	4.92
	DMSO	4.65	—	—	—
H-4	Me_2CO	7.02	7.01	7.00	7.01
	DMSO	6.98	6.93	6.93	6.94
H-5	Me_2CO	6.74	6.77	6.75	6.75
	DMSO	6.74	6.71	6.70	6.71
H-6	Me_2CO	7.21	7.36	7.33	7.34
	DMSO	7.35	7.37	7.33	7.37
NH	Me_2CO	8.39	8.05	8.20	8.28
	DMSO	9.11	8.82	8.88	9.02
o-OH	Me_2CO	11.95	12.47	12.17	12.31
	DMSO	11.63	11.60	11.65	11.82
m-OH[d]	Me_2CO	—	—	—	—
	DMSO	9.42	9.37	9.35	9.35

[a]Free alcohol serine.

[b]Free acid serine.

[c]Chemical shifts of aromatic, amide, and phenolic protons in the dimer were listed in order of increasing chemical shift, and do not indicate which serine each proton is associated with.

[d]Resonances for meta phenolic protons were not observed in Me_2CO.

Using heteronuclear shift correlated (HETCOR) nmr, the ^{1}H-nmr assignments above were subsequently used in the assignment of the ^{13}C-nmr resonances (Table 2). As with **1** and **2**, α carbons resonate upfield from the β carbons in **3** due to greater shielding of serine α carbons (8). The HETCOR of the aliphatic region confirms that the upfield β carbon and the downfield α carbon belong to the free alcohol serine. The cross peaks for the magnetically non-equivalent β_1 protons were too weak to be observed in Me_2CO but could be observed in DMSO where the two protons are more nearly equivalent.

TABLE 2. ^{13}C-nmr Chemical Shifts of Catecholates in ppm.

Carbon	Solvent	Compound				
		Theoretical	**1**	**2**	**3**[a,c]	**3**[b,c]
C_α	Me_2CO	—	53.2	56.1	56.1	52.5
	DMSO	—	51.3	55.2	55.1	51.4
C_β	Me_2CO	—	65.5	61.9	62.5	64.8
	DMSO	—	63.4	61.0	60.6	63.5
CO_2	Me_2CO	—	169.9	171.0	170.7	170.9
	DMSO	—	169.4	171.6	169.8	170.1
CONH	Me_2CO	—	169.8	170.4	170.2	170.5
	DMSO	—	168.9	168.4	168.4	168.9
C-1	Me_2CO	118.0	115.1	115.0	114.4	115.2
	DMSO	117.2	115.2	116.0	115.4	115.8
C-2	Me_2CO	143.6	147.1	146.7	147.1	147.1
	DMSO	143.7	148.4	148.3	148.1	148.6
C-3	Me_2CO	143.6	145.7	145.1	144.9	145.0
	DMSO	143.7	146.1	146.0	145.9	146.0
C-4	Me_2CO	120.2	120.0	119.7	119.7	119.8
	DMSO	119.4	119.2	118.8	118.8	119.0
C-5	Me_2CO	121.1	119.6	119.1	119.3	119.4
	DMSO	120.1	118.4	118.2	118.5	118.5
C-6	Me_2CO	121.1	118.3	118.4	118.2	118.4
	DMSO	120.1	118.2	118.5	118.2	118.5

[a]Free alcohol serine.

[b]Free acid serine.

[c]Chemical shifts of aromatic and carbonyl carbons in the dimer were listed in order of increasing chemical shift, and do not indicate which serine each carbon is associated with.

Resonance assignments for the aromatic and carbonyl carbons were made based on literature values (8), as well as upon theoretical chemical shifts (Table 2). These were calculated based on methyl-2,3-dihydroxybenzoate in CCl_4 as a model (8), and utilized additivity rules (11) as well as known solvent effects on phenolic carbons (12). The specific chemical shifts assignments for C-4–C-6 of compound **1** were not previously made because their resonances were too close to be distinguished (8). The aromatic region of the HETCOR spectra, however, permits these assignments to be made. The aromatic ^{13}C-nmr resonance assignments for all four compounds, in each solvent, were made in this manner (Table 2). Differences were not significant between the two aromatic rings of **3**, however, so the two chains could not be distinguished.

The aromatic ^{13}C chemical shifts are an indicator of solvent effects and substituent interactions (13). Since differences in the aromatic ^{13}C chemical shifts among the three compounds were small, the observed solvent effects may be considered constant for the 2,3-dihydroxybenzamide portion of the molecule. Therefore, intramolecular hydrogen bonding between the amide carbonyl and the ortho phenol was demonstrated in each

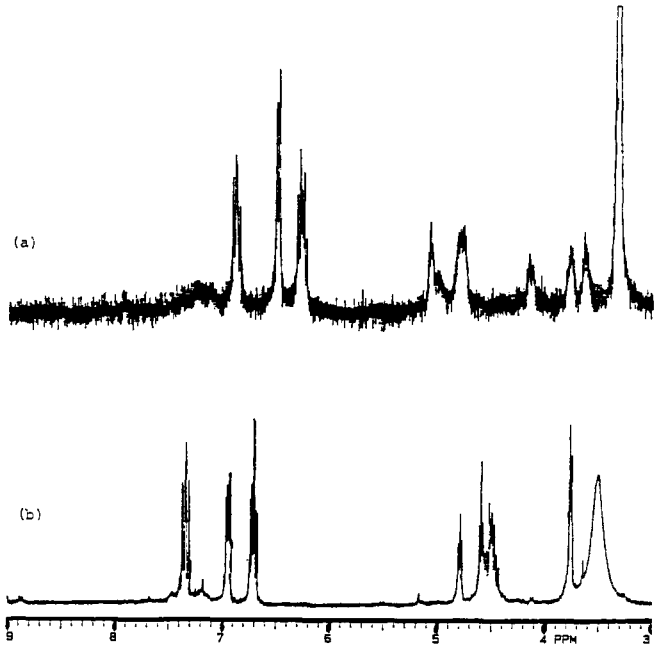


FIGURE 1. ^{1}H-nmr spectra of the gallium complex of **3** (a), and metal-free **3** (b) in DMSO-d_6.

compound by both the upfield shifts of the C-1 resonances and the downfield shifts of the C-2 resonances relative to the predicted values. In DMSO, a stronger solvent-phenol hydrogen bond occurs as a result of the higher basicity of DMSO than Me_2CO (12), and at the expense of the intramolecular hydrogen bond. This can be seen by the smaller upfield shift of the C-1 resonance and the large upfield shift of the amide carbonyl, yet the larger downfield shift of the C-2 resonance. Hydrogen bonding was similarly observed at the C-3 phenol, possibly to the C-3 phenol oxygen as well as to solvent, but the smaller chemical shift differences indicate that these are weaker than the carbonyl-phenol hydrogen bond (12).

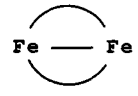
As in similar studies (8,14,15), because of line-broadening problems associated with iron(III) complexes, the gallium(III) complex of each catechol derivative was prepared in DMSO. Care was taken to dissolve the gallium(III) complexes at the same concentration as the free ligands above, which were kept low to minimize intermolecular interactions that occur with phenols (16). The spectrum of **1** agreed with literature results (8). Monomer **2** (data not shown) resulted in a complex spectrum, probably due to the superimposed spectra of the two or more metal-ligand combinations that were suggested by O'Brien *et al.* (3). The effects of metal binding on the ^{1}H resonances of **3** (Figure 1) are comparable with those of **1** (Table 3). For example, the β protons show a greater difference in chemical shifts in the metal complex, and the α proton resonances are shifted downfield. While this suggests similar conformational changes, the dimer has only two metal binding sites per molecule. Whether **3** forms a dimer-metal complex [$(Dimer)_3Fe_2$] similar to **4**, as in the case of rhodotorulic acid (17,18), rather than **5** is not known. However, two lines of evidence suggest that this is the case. First, the two stereochemically non-equivalent dimer units in **5** conceivably might produce a complex spectrum of one dimer superimposed on one of the other, yet a simple spectrum was observed. In addition, receptor binding of iron-bound siderophores is known to depend upon the configuration about the metal (19), and while the Δ-cis isomer is

TABLE 3. ^{1}H-nmr Chemical Shifts of Catecholates and their Gallium Complexes.[a]

Proton	Compound					
	1		**3**			
	Ligand[b]	Chelate[c]	Ligand		Chelate	
			Ser1[d]	Ser2[e]	Ser1[d]	Ser2[e]
C_α-H	4.90 (4.94)	5.16 (5.14)	4.59	4.79	4.82	5.07
C_β-H_1	4.40 (4.41)	3.86 (3.80)	3.76	4.50	3.64	4.15
C_β-H_2	4.65 (4.66)	5.12 (5.07)	3.76	4.50	3.78	4.76
H-4	6.98 (6.98)	6.51 (6.44)	6.93	6.95	6.49	6.49
H-5	6.74 (6.73)	6.30 (6.13)	6.70	6.71	6.25	6.29
H-6	7.35 (7.34)	6.89 (6.84)	7.32	7.35	6.86	6.90

[a]Numbers in parenthesis refer to values from Hancock *et al.* (5).
[b]Ligand refers to the metal-free catecholate.
[c]Chelate refers to the Ga^{3+} complex.
[d]Free alcohol serine.
[e]Free acid serine.

known to be selectively taken up by bacterial cells (20), the cyclic-triester backbone of **1** is of little or no importance (6). The inability of **2** to support bacterial growth (5) may reflect a trans configuration for the metal complex which could not bind to the receptor, but the ability of **3** to support growth implies a cis configuration, which is more likely with **4** than with **5**.



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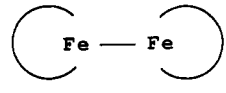

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The ^{13}C nmr of the dimer-metal complex (data not shown) only revealed the aromatic carbons C-4–C-6, which were all shielded, as expected (8). Llinàs *et al.* (8) explained that higher concentrations are required to see the ^{13}C-nmr spectra of the gallium complex of **1**. Because there are only half as many of each aliphatic carbon per catechol moiety in a solution of **3**, a spectrum with a similar signal-to-noise ratio would require even higher concentrations, where intermolecular interactions are more likely.

EXPERIMENTAL

Enterobactin was prepared by the method of Shanzer and co-workers (21,22), and the monomeric and dimeric hydrolysis products were prepared by the method of Rastetter *et al.* (23). ^{1}H-nmr spectra of these samples agreed with literature spectra (1,7).

Fab mass spectra were performed by dissolving the sample in glycerol and generating ions at 8 kV using xenon as the reagent gas. Each sample resulted in the expected molecular ion.

All nmr spectra were recorded on a Varian XL-300 Fourier transform spectrometer operating at 299.945 MHz and 75.43 MHz for ^{1}H and ^{13}C, respectively, at 20 ± 1°. Proton and carbon 90° pulses were

calibrated at 25.3 and 10.7 μsec, respectively. Chemical shifts are reported in ppm downfield relative to TMS. Samples of 25 mg were dissolved in 0.35 ml of either Me_2CO-d_6 or DMSO-d_6 (Sigma Chemical Co., St. Louis, MO), and were recorded in 5-mm tubes.

For each ^{1}H-nmr spectrum, a data size of 16K data points was collected over a sweep width of 4000 Hz, with a total of 200 transients. A pulse width of 12.6 μsec was used. A line broadening of 0.5 Hz was applied by apodization of the free induction decay prior to Fourier transformation. For ^{13}C-nmr spectra, 30K data points were collected over a sweep width of 16,502 Hz, with a total of 4000 transients. A line broadening of 0.5 was used.

Standard pulse sequences were used for both phase sensitive COSY (24) and HETCOR (25–27) experiments. For COSY experiments, a 2-sec delay was allowed between each scan, the coupling constant was optimized for $J = 8$ Hz, 2K data points were collected over a sweep width of 4000 Hz, and 4 transients were accumulated for each 512 time increments in the F_1 dimension. The spectral width in both dimensions was the same. The resulting 128 × 2048 data point matrix was zero-filled and Fourier-transformed to a 2K × 2K matrix.

For HETCOR experiments a 1-sec delay was allowed between each scan, and the coupling constant ($^1J_{CH}$) was optimized for $J = 140$ Hz. Spectral widths of 4000 Hz and 16,502 Hz were selected in F_1 and F_2, respectively. The acquisition involved 48 transients for every 256 time increments, with a total acquisition time of about 4 h. An initial data matrix (256 × 1024 points) was zero-filled and Fourier-transformed to a 512 × 2048 matrix.

ACKNOWLEDGMENTS

The authors are grateful to Mr. Richard Dvorak for obtaining fab mass spectra. This research was supported by the Graduate College, University of Illinois at Chicago.

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Received 21 November 1990