# $^{1}$H-NMR and $^{13}$C-NMR Spectral Assignments of Spiramycins I and III

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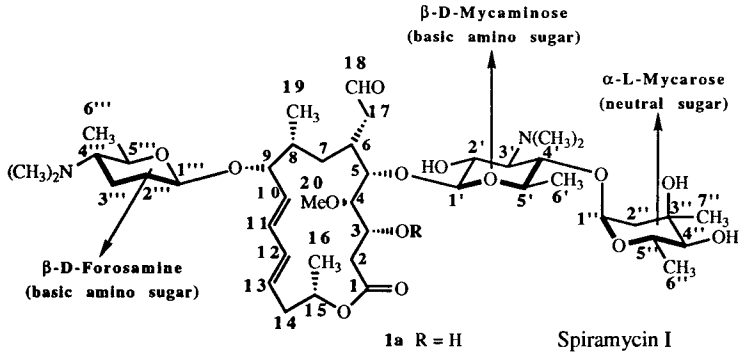
## INTRODUCTION

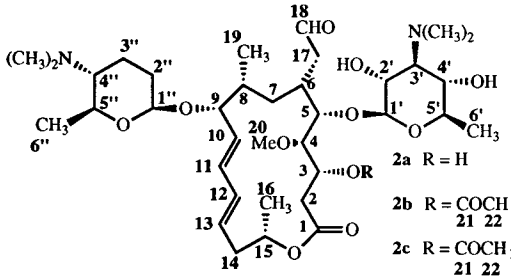
The spiramycins (1a–1c) are 16-membered macrolide antibiotics produced by the bacteria *Streptomyces ambofaciens* (1) (Scheme I). This series of compounds shows gram-positive antibacterial activity as well as antimicrobial activity against both *Toxoplasma gondii* and *Cryptosporidium,* two protozoans which can result in potentially fatal opportunistic infections associated with AIDS (2,3). Our interest in the spiramycins has been the production of spiramycin derivatives through chemical and biochemical modifications of the basic spiramycin chemical structure. To prove unambiguously the structure of any derivatives, a complete structural understanding of the spiramycins themselves was necessary. After a thorough search of the literature, we found various NMR structural characterizations of these compounds. Unfortunately, many of the structural aspects of the spiramycins are reported incorrectly or not at all. We have made the complete NMR spectral assignments of spiramycins I and III based on our interpretation of the NMR techniques of DEPTGL, HETCOR, HMBC, and COSY.




1a R = H Spiramycin I

1b R = $COCH_3$ (21 22) Spiramycin II

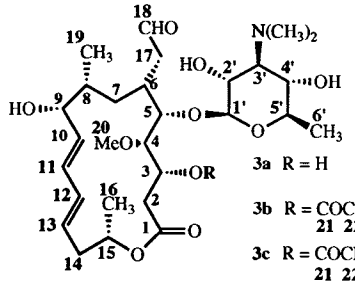
1c R = $COCH_2CH_3$ (21 22 23) Spiramycin III



2a R = H Neospiramycin I

2b R = $COCH_3$ (21 22) Neospiramycin II

2c R = $COCH_2CH_3$ (21 22 23) Neospiramycin III



3a R = H Forocidin I

3b R = $COCH_3$ (21 22) Forocidin II

3c R = $COCH_2CH_3$ (21 22 23) Forocidin III

**Scheme I**

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## Spectral History

To understand fully the spectral assignments of the spiramycins, it is necessary to review chronologically the history of these compounds.

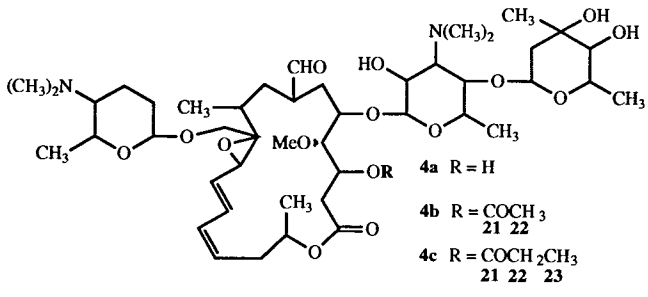
Initial characterization of the spiramycins established spiramycins I, II, and III as an alcohol, an acetate, and a propionate, respectively (4). Mild acid hydrolysis of these compounds yielded the products mycarose sugar and neospiramycins (2), whereas after vigorous acid hydrolysis the sugars mycarose and forosamine are obtained along with the forocidins (3). Even more drastic acid treatment of forocidins leads to the hydrolysis of the mycaminose sugar from the aglycone. From early spectral experiments the spiramycin aglycone was believed to contain a methoxyl group, two methyl groups, a conjugated diene, a lactone, a carbonyl group (aldehyde), and an epoxide. After exhaustive reductive degradation and hydrolysis and reattachment of the mycarose, mycaminose, and forosamine sugars to the lactone, the first incorrect spiramycin structure was reported (5) (**4a–4c**). Catalytic reduction of forocidin along with mass spectral and elemental data helped determine the chain size of the aglycone nucleus, while a comparison of the oxidation products of forocidins with neospiramycins and spiramycins indicated the presence of an allylic alcohol in forocidin similar to that in magnamycin B. This in turn ruled out the presence of an epoxide (6). Early $^1$H-NMR data (5) revealed the presence of an aldehyde peak at 9.6 ppm and, with the absence of the epoxide group, unfortunately resulted in the proposal of a second set of incorrect structures (**5a–5c**).

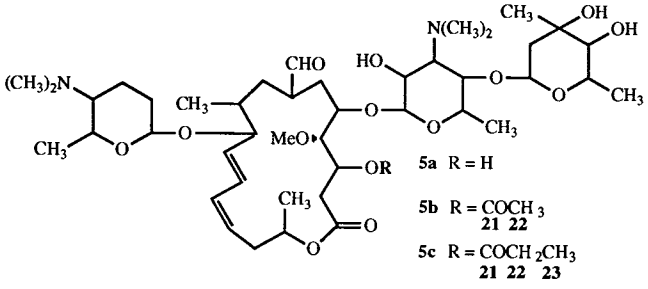
Due to their structural similarities, leucomycin $A_3$ (**6b**), isolated from *Streptomyces kitasatoensis*, was used as a model for the spiramycin structural characterizations. The leucomycins had been shown to possess an α, β, γ, δ unsaturated γ-methoxy acid system, a β configuration of the mycaminosidic linkage, and an α configuration of the mycarosidic linkage (Table I) (7). $^1$H-NMR characteristics of the pi system and the aldehydic proton of the spiramycins (8) were found to be similar to those of the leucomycins and so assigned. The linkage of the mycaminose sugar, being adjacent to the $CH_2CHO$ group and γ to the aldehyde, was confirmed by Woodward's method, as it was applied to the magnamycins. A comparison of the α and β configurations of the free *O*-methoxy sugars (9) confirmed the position of the mycarose residue at position C-4′, not position C-2′, of mycaminose, with the stereochemistry of the aglycone being β to mycaminose and mycarose being α to mycaminose. The leucomycin stereochemistry was confirmed through the oxidation of leucomycin to magnamycin B followed by acid hydrolysis to demycarosyl leucomycin $A_3$ (10). In addition, an allylic rearrangement of the 9-OH to 13-OH was observed

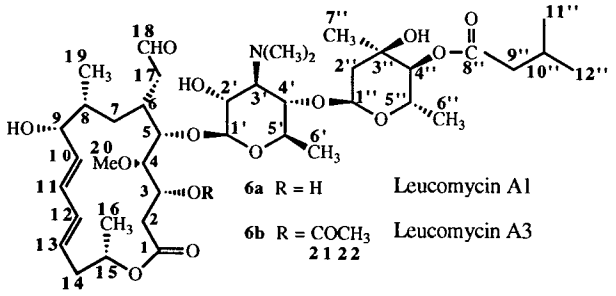
Table I. $^1$H-NMR Chemical Shift Assignments of Leucomycins (6,7,13,14)[a]

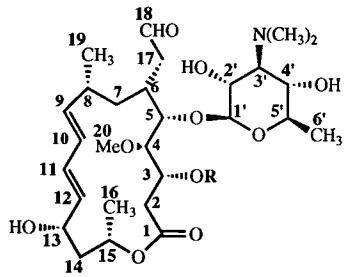
| Proton no. | Leucomycin $A_3$ (3-OAc) | Leucomycin $A_1$ (3-OH) |
|---|---|---|
| $CH_3$'s | ~1.00 | |
| 8 CH | — | |
| | (4.2) | |
| 9 CH-O | 4.05 dd | |
| | (4.2, 8.9) | |
| 10 CH= | 5.60 | |
| | (8.9, 15.4) | |
| 11 CH= | 6.66 | 6.2–6.3 |
| | (10.0, 15.4) | |
| 12 CH= | 6.05 | |
| | (10.0, 15.2) | |
| 18 CHO | 9.73 s | 9.87 s |
| 20 $CH_3$-O | 3.47 s | |
| 22 OAc | 22.2 s | |
| 1′ CH-O | 4.30 d | |
| | (7.4) | |
| 3′N($CH_3$)$_2$ | 2.49 s | |
| 1″ CH-O | ~5.10 | |
| 4″ CH-OH | 4.80 d | |
| | (10.0) | |

[a] Assignments are a composite of information from the references indicated.



7 13-OH Demycarosyl Leucomycin A3

Scheme I. Continued.

during acid hydrolysis (11,12). The absolute structure of the 13-OH demycarosyl leucomycin $A_3$ was elucidated by X-ray analysis, which indirectly confirmed the stereochemistry of all of the spiramycin and leucomycin asymmetric centers (13). The conformation of the 9-OH position was determined later as β (R) through the use of the benzoate or Mill's rule (11,14). IR and NMR studies comparing the natural leucomycin $A_3$ and 9-epileucomycin $A_3$ later confirmed the absolute configuration of the 9-OH proton as α (S) (23). To date, only a few of the $^1$H-NMR assignments for 9-OH demycarosyl leucomycin $A_3$ have been made, with most of the structural data based on a combination of NMR, IR, CD, and X-ray analysis of the intramolecularly rearranged macrolide 13-OH demycarosyl leucomycin $A_3$ (7). For example, the downfield shift of the 1 – C = O (carbonyl) signal in the case of spiramycin I was justified by hydrogen bonding between the 3 – OH and the 1 – C = O, which was established using IR (15).

The initial $^{13}$C-NMR assignments of spiramycin III and other 16-membered macrolides were made with some ambiguity by comparing the signals to those obtained for the free α and β sugars and the aglycone with the help of SFORD experiments (16). Some of the conflicting assignments were clarified using INEPT experiments (17), which led to the first $^{13}$C assignments of the spiramycins (18); however, no supporting data have been published. In addition, no $^1$H-NMR spectral assignments of the spiramycins have been made to date with the exception of the olefinic protons.

Since our interest in the chemistry of the spiramycins required a complete and explicit spectral characterization of these compounds, we report here the first unambiguous $^1$H-NMR and $^{13}$C-NMR assignments of spiramycins I and III.

## MATERIALS AND METHODS

Although the spiramycins are marketed commercially (Sigma Chemical Co.) as a mixture of spiramycins I, II, and III, we have not been able to detect any significant amounts of spiramycin II in the mixture. Separation of the commercial mixture of spiramycins was chromatographed using a modified column chromatography procedure [$CHCl_3$:MeOH:10% $NH_4OH$ (bottom layer) as eluting solvent at the ratio 7:1:1] (19). Spiramycin I ($R_f$ = 0.65), 80%, is easily separated from spiramycin III ($R_f$ = 0.75), 20%. Column fractions were monitored by TLC using $CHCl_3$:MeOH:10% $NH_4OH$ (bottom layer) as the mobile phase at the ratio 2:1:1 [spiramycin I ($R_f$ = 0.8) and spiramycin III ($R_f$ = 0.9)]. Spiramycins were visualized using UV (254 nm) and *p*-anisaldehyde spray reagent.

NMR spectra were obtained in $CDCl_3$ and $C_6D_6$ on a Varian VXR-300 spectrometer operating at 300 MHz for proton and at 75 MHz for carbon. A 150-mg sample each of spiramycin I and spiramycin III was dissolved separately in 0.5 mL of deuterated chloroform ($CDCl_3$) to obtain the 1-D proton, 1-D carbon, APT, DEPTGL, 2-D COSY ($^1$H–$^1$H correlation experiment), and 2-D HETCOR ($^1$H–$^{13}$C correlation experiment) spectra, all of which were phase modulated experiments. The APT, DEPTGL (20), COSY (90°) (21), and HETCOR (22) experiments were performed using the standard Varian software.

The $^1$H-NMR spectrum was obtained with double precision using a spectral width of 4100 Hz using 30K data points, a 45° pulse angle, a 3.66-sec acquisition time, a 2.0-sec relaxation delay, and a total of 16 transients. With double precision, data are obtained using a 32-bit integer format instead of the standard 16-bit format, thus enabling the acquisition of $^1$H-NMR spectrum for 16 transients with a better signal-to-noise ratio. In the absence of double precision, acquisition of the $^1$H-NMR spectrum (using 32K data points and a 3.996-sec acquisition time) is automatically aborted after a maximum number of transients (five repetitions) is obtained. The $^{13}$C-NMR spectrum was obtained using a spectral width of 16,502 Hz using 32K data points, a 45° pulse angle, a 0.9-sec acquisition time, a 3.0-sec relaxation delay, Waltz modulated proton decoupling, and a total of 250 transients.

The COSY spectrum was obtained using a narrowed 2358-Hz spectral width in both dimensions, a 90° acquisition pulse angle, a 0.217-sec acquisition time, a 1.0-sec relaxation delay, 256 increments, each with five repetitions in the second frequency domain, and pseudo-echo-shaped data processing, giving a total acquisition time of 38.0 min. Double precision was not used for the COSY experiment and therefore the number of repetitions was set at five, which is the maximum possible. The HETCOR spectrum was obtained using a 9785-Hz spectral width in the 1-D (F2-carbon) dimension and 2358 Hz in the 2-D (F1-proton) dimension, a 90° acquisition pulse angle, a 0.105-sec acquisition time, a 1.0-sec relaxation delay, 64 increments, each with 1000 repetitions in the second frequency domain, broadband modulated proton decoupling, and pseudo-echo-shaped data processing, giving a total acquisition time of 20 hr. Similarly, identical spectra were obtained using deuterated benzene ($C_6D_6$) as solvent.

The HMBC (heteronuclear multiple-bond correlation) (24) spectrum was obtained on a 150-mg sample of spiramycin III dissolved in 0.5 mL of deuterated benzene ($C_6D_6$) on a Bruker AMX 500 spectrometer operating at 500 MHz for proton and a 125.7 MHz for carbon with 60° sine bell squared in both dimensions. The data were acquired over a 31,250-Hz spectral width in the F2-carbon dimension and 6097 Hz in the F1-proton dimension, a 90° acquisition pulse angle, a 0.168-sec acquisition time, and a 2.0-sec relaxation delay optimized for an average two- or three-bond coupling constant of 7 Hz with 512 $t_1$ increments, each with 2048 repetitions in the F2 domain and one order of zero filling in the F1 domain.

## RESULTS AND DISCUSSION

With our initial interest in the spiramycins came the realization that many of the NMR spectral data reported for these compounds were rather misleading as well as being scattered piecemeal throughout the scientific literature. Before pursuing any chemical or biochemical modifications of the spiramycins, it was necessary to obtain explicit carbon and proton NMR spectral assignments of each of these compounds in which we had an interest. To provide a complete spectral summary, we report the $^1$H-NMR (Table III) and $^{13}$C-NMR (Table II) assignments for spiramycins **(1a)** and III **(1c)** in $CDCl_3$ and $C_6D_6$.

Table II. $^{13}$C-NMR Chemical Shift Assignments of Spiramycins

| Carbon no. | | SP I[18] ($CDCl_3$) | SP I ($CDCl_3$) | SP I ($C_6D_6$) | SP III ($CDCl_3$) (16)[a] | SP III ($CDCl_3$) | SP III ($C_6D_6$) |
|---|---|---|---|---|---|---|---|
| 1 | C=O | 174.1 | 173.8 | 173.7 | 169.9 | 169.9 | 169.9 |
| 2 | $CH_2$ | 37.8 | 37.5 | 38.6 | 37.2 | 37.2 | 37.6 |
| 3 | CH−O | 68.3 | 68.0 | 68.9 | 71.7 | 68.7 | 69.4 |
| 4 | CH−O | 85.3 | 85.0 | 85.8 | 77.8[h] | 84.7 | 85.3 |
| 5 | CH−O | 79.3 | 78.9 | 80.3 | 84.5[h] | 77.7 | 78.4 |
| 6 | CH | 30.6 | 30.2 | 31.3 | 28.9[c] | 28.9 | 29.5 |
| 7 | $CH_2$ | 30.7 | 30.5 | 32.2 | 30.2[c] | 30.1 | 30.4 |
| 8 | CH | 31.8 | 31.4 | 32.5 | 31.9 | 31.8 | 33.2 |
| 9 | CH−O | 78.7 | 78.5 | 78.9 | 79.7 | 79.6 | 80.4 |
| 10 | CH= | 128.6 | 128.2 | 130.0 | 126.7 | 126.6 | 128.2 |
| 11 | CH= | 134.6 | 134.4 | 134.0 | 135.4 | 135.3 | 135.1 |
| 12 | CH= | 132.8 | 132.5 | 133.2 | 132.3[d] | 132.2 | 132.7 |
| 13 | CH= | 131.0 | 130.8 | 130.9 | 131.9[d] | 131.9 | 132.3 |
| 14 | $CH_2$ | 42.0 | 41.7 | 41.7 | 40.6[e] | 41.0 | 41.1 |
| 15 | CH−O | 69.2 | 68.9 | 69.2 | 68.9[f] | 69.1 | 68.9 |
| 16 | $CH_3$ | 20.1 | 19.9 | 20.2 | 20.3 | 20.3 | 20.4 |
| 17 | $CH_2$ | 43.3 | 42.9 | 43.9 | 42.5[e] | 42.4 | 42.9 |
| 18 | C=O | 202.7 | 202.7 | 202.3 | 201.3 | 201.3 | 200.7 |
| 19 | $CH_3$ | 15.3 | 15.0 | 16.0 | 15.4 | 15.4 | 16.2 |
| 20 | $CH_3$−O | 61.8 | 61.5 | 61.4 | 62.4 | 62.4 | 62.1 |
| 21 | C=O | | | | 173.8 | 173.8 | 174.0 |
| 22 | $CH_2$ | | | | 27.7 | 27.6 | 28.1 |
| 23 | $CH_3$ | | | | 9.0 | 8.9 | 9.2 |
| 1′ | CH−O | 103.9 | 103.6 | 104.6 | 104.0 | 103.9 | 104.5 |
| 2′ | CH−OH | 71.7 | 71.4 | 72.1 | 69.2[f] | 71.7 | 71.8 |
| 3′ | CH−N | 68.8 | 68.5 | 69.3 | 69.2[f] | 68.7 | 69.1 |
| 4′ | CH−O | 75.0 | 74.6 | 75.6 | 74.9 | 74.7 | 75.1 |
| 5′ | CH−O | 73.1 | 72.8 | 73.2 | 73.0 | 73.0 | 73.0 |
| 6′ | $CH_3$ | 19.0 | 18.8 | 19.2 | 19.0[g] | 19.0 | 19.1 |
| 3′N | $(Me)_2$ | 42.0 | 41.8 | 42.2 | 42.0 | 42.0 | 42.1 |
| 1″ | CH−O | 96.4 | 96.1 | 96.9 | 96.4 | 96.3 | 96.7 |
| 2″ | $CH_2$ | 40.9 | 40.6 | 41.3 | 41.0 | 40.8 | 41.2 |
| 3″ | C | 69.4 | 69.2 | 69.6 | 69.5 | 69.4 | 69.5 |
| 4″ | CH−OH | 76.4 | 76.2 | 76.8 | 76.4 | 76.3 | 76.7 |
| 5″ | CH−O | 66.0 | 65.7 | 66.5 | 66.1 | 66.0 | 66.6 |
| 6″ | $CH_3$ | 18.3 | 18.0 | 18.8 | 18.3[g] | 18.2 | 18.8 |
| 7″ | $CH_3$ | 25.4 | 25.1 | 26.0 | 25.4 | 25.4 | 26.0 |
| 1‴ | CH−O | 100.2 | 99.9 | 100.3 | 100.3 | 100.0 | 100.6 |
| 2‴ | $CH_2$ | 31.3 | 31.0 | 31.9 | 31.2 | 31.2 | 31.9 |
| 3‴ | $CH_2$ | 18.5 | 18.2 | 18.5 | 18.3 | 18.5 | 18.5 |
| 4‴ | CH−N | 64.8 | 64.6 | 65.4 | 64.9 | 64.8 | 65.3 |
| 5‴ | CH−O | 73.8 | 73.5 | 74.0 | 73.7 | 73.7 | 73.8 |
| 6‴ | $CH_3$ | 19.0 | 18.7 | 19.4 | 18.7[g] | 18.9 | 19.5 |
| 4‴N | $(Me)_2$ | 40.7 | 40.4 | 40.8 | 41.0 | 40.6 | 40.7 |

[a] Signals bearing the same superscript may have interchangeable assignments.

### The Aglycone Portion of the Molecule

The aldehydic carbon at ~200.0 δ in spiramycins has been assigned to the aldehydic carbonyl C-18 as it shows a cross-peak in the HETCOR spectrum to the singlet at ~9.6 δ assigned to the aldehydic proton H-18. A weak cross-peak between H-18 and the methylenes H-17a and H-17b (in case of the methylene protons "a" being designated to the signal more downfield and the other "b") indicated by the COSY explains the multiplicity (broad singlet) of the aldehydic proton. Both the methylene protons which appear as multiplets show cross-peaks to C-17 in the HETCOR spectrum and to H-6 in the COSY spectrum. This helps to locate accurately the position of the methine proton H-6 and carbon C-6 from the COSY and HETCOR spectra, respectively.

When the position C-3 consists of a hydroxyl group (as in spiramycin I) instead of an ester (as in spiramycin III), hydrogen bonding between the hydroxyl and the lactone carbonyl C-1 was used to explain a downfield shift of C-1 to 174.0 ppm (in spiramycin I), which otherwise appears at 169.0 ppm (in spiramycin III). The signal at 174.0 ppm in spiramycin III was assigned to the ester carbonyl C-21. HMBC experiments (average two- or three-bond coupling constant of 5–7 Hz) (24) performed on spiramycin III in $C_6D_6$ confirmed that the more downfield carbonyl signal (174.0 ppm) is that of the ester carbonyl at C-21 since it

Table III. $^1$H-NMR Chemical Shift Assignments of Spiramycins[a]

| Proton no. | SP I ($CDCl_3$) | SP I ($C_6D_6$) | SP III ($CDCl_3$) | SP III ($C_6D_6$) |
|---|---|---|---|---|
| 2a | 2.55 dd (10.9, 14.5) | 2.60 (10.8) NR | 2.64 dd (10.7, 13.2) | 2.71 (10.8) NR |
| 2b | ~2.14 | 2.09 (3.7) NR | ~2.19 | 2.08 (3.3) NR |
| 3 | 3.68 d (10.9) | 3.90 d (10.8) | 5.06 d (10.7) | 5.42 d (10.8) |
| 4 | 2.96 d (8.9) | 2.99 d (8.2) | ~3.14 | 3.11 d (9.1) |
| 5 | ~3.98 | 4.32 (8.2) NR | 3.75 d (9.1) | ~4.16 |
| 6 | ~2.20 | ~2.55 | ~2.09 | ~2.50 |
| 7a | ~1.40 | 1.75 NR | ~1.28 | 1.72 NR |
| 7b | 0.90 NR | 1.13 NR | 0.88 NR | 1.18 NR |
| 8 | 1.82 NR | 2.20 NR | 1.84 NR | 2.16 NR |
| 9 | ~3.92 | 4.34 dd (3.7, 9.2) | 3.88 dd (3.2, 9.7) | 4.23 dd (3.5, 9.6) |
| 10 | 5.65 dd (9.5, 15.1) | 5.82 dd (9.2, 15.2) | 5.53 dd (9.7, 15.0) | 5.93 dd (9.6, 15.1) |
| 11 | 6.20 dd (10.6, 15.1) | 6.27 dd (10.6, 15.2) | 6.48 dd (10.6, 15.0) | 7.02 dd (10.0, 15.1) |
| 12 | 5.98 dd (10.6, 14.8) | 5.94 dd (10.6, 14.9) | 5.97 dd (10.6, 14.6) | 6.20 dd (10.0, 14.0) |
| 13 | 5.53 ddd (3.4, 11.0, 14.8) | 5.53 ddd (4.0, 10.9, 14.9) | 5.65 ddd (3.1, 10.8, 14.6) | 6.09 ddd (3.3, 10.0, 14.0) |
| 14a | ~2.44 | 2.30 NR | ~2.36 | 2.05 NR |
| 14b | ~1.98 | 1.92 NR | ~1.98 | 1.96 NR |
| 15 | 5.25 m (3.3, 6.4, 8.1) | 5.13 m (3.2, 6.3, 9.7) | 4.93 (3.3, 6.3) NR | 5.05 m (3.2, 6.2, 9.8) |
| 16 | 1.18 d (6.4) | 1.05 d (6.3) | 1.16 d (6.3) | 1.04 d (6.2) |
| 17a | 2.68 m (1.2, 9.8, 11.2) | 3.05 (7.9) NR | 2.72 (7.3, 11.2) NR | ~2.99 |
| 17b | ~2.26 | ~2.48 | ~2.27 | ~2.36 |
| 18 | 9.78 s | 9.98 s | 9.56 s | 9.66 s |
| 19 | 0.86 d (6.6) | 1.18 d (5.9) | 0.89 d (6.5) | 1.26 d (6.1) |
| 20 | 3.38 s | 3.32 s | 3.43 s | 3.19 s |
| 22 | | | 2.50 dq (7.7, 9.7) | 2.82 dq (7.5, 9.3) |
| 23 | | | ~1.13 | 1.34 t (7.5) |
| 1′ | 4.36 d (7.6) | 4.62 d (7.6) | ~4.31 | 4.41 d (7.5) |
| 2′ | 3.40 dd (7.6, 10.3) | 3.64 dd (7.6, 10.1) | ~3.46 | 3.49 dd (7.5, 10.0) |
| 3′ | ~2.33 | ~2.57 | ~2.42 | ~2.30 |
| 4′ | 3.13 d (9.0) | 3.23 d (9.2) | ~3.18 | 3.15 d (9.2) |
| 5′ | 3.15 dq (6.1, 9.0) | 3.15 dq (6.1, 9.2) | ~3.16 | 2.64 |
| 6′ | ~1.12 | 1.20 d (6.1) | ~1.11 | 1.02 d (6.2) |
| 3′N($Me)_2$ | 2.36 s | 2.53 s | 2.40 s | 2.44 s |
| 1″ | 4.94 d (3.2) | 4.92 d (3.2) | 4.97 d (3.2) | 4.82 d (3.0) |

Table III. Continued

| Proton no. | SP I ($CDCl_3$) | SP I ($C_6D_6$) | SP III ($CDCl_3$) | SP III ($C_6D_6$) |
|---|---|---|---|---|
| 2″a | 1.90 (14.2) NR | 1.88 dd (3.2, 14.0) | 1.93 (14.5) NR | ~1.80 |
| 2″b | 1.63 dd (3.5, 14.2) | ~1.40 NR | 1.63 dd (3.5, 14.5) | ~1.35 |
| 4″ | 2.81 d (9.7) | 2.96 d (9.5) | 2.85 d (9.8) | 2.94 d (9.6) |
| 5″ | ~3.96 | 4.25 dq (6.1, 9.5) | 3.96 dq (6.1, 9.8) | ~4.13 |
| 6″ | 1.16 d (6.1) | 1.48 d (6.1) | 1.20 d (6.1) | 1.47 d (6.1) |
| 7″ | 1.10 s | 1.28 s | 1.14 s | 1.27 s |
| 1‴ | 4.26 dd (1.3, 8.7) | 4.44 dd (1.7, 9.2) | ~4.31 | 4.33 dd (1.6, 9.4) |
| 2‴a | ~1.72 | 1.82 (12.4) NR | ~1.77 | ~1.75 |
| 2‴b | 1.35 (8.9) NR | ~1.62 | 1.39 (8.9) NR | ~1.65 |
| 3‴a | ~1.69 | 1.55 (3.3, 6.2, 9.5) | ~1.75 | ~1.62 |
| 3‴b | 1.29 (4.48, 8.9) NR | ~1.16 | 1.34 (8.9) NR | ~1.11 |
| 4‴ | ~2.04 | 2.13 (3.5, 9.4) NR | ~2.05 | ~2.10 |
| 5‴ | 3.30 dq (6.2, 9.5) | 3.40 dq (6.2, 9.4) | 3.32 dq (6.3, 9.1) | 3.28 dq (6.3, 9.3) |
| 6‴ | ~1.09 | 1.43 d (6.2) | ~1.11 | 1.40 d (6.3) |
| 4‴N(Me)$_2$ | 2.09 s | 2.02 s | 2.14 s | 1.99 s |
| 3,2′,3″,4″ OH | | ~1.5–2.5 NR | | |

[a] Values in parentheses represent the observed coupling constants in the 1-D $^1$H-NMR ~, proton chemical shift from center of 2-D HETCOR experiment; a and b, the methylene ($CH_2$) protons, "a" being more downfield than "b"; NR, the proton signal was either partially overlapped or unresolved. s, singlet; d, doublet; t, triplet; dd, doublet of doublets; ddd, doublet of doublet of doublets; dq, doublet of quartets; m, multiplet.

couples with the proton H-3, the methylenes H-22, and the methyl H-23. On the other hand, the second signal (169.0 ppm) was assigned to the lactone carbonyl C-1 since it couples strongly with the methylenes H-2a and H-2b and weakly with H-3. In the case of spiramycin III the methylenes H-22 appear as a two-proton doublet of quartets showing a cross-peak into the adjacent methyl protons H-23, which appear as a triplet.

The olefinic proton H-11 appears as a clear doublet of doublets showing cross-peaks in the COSY spectrum to the adjacent olefinic protons H-10 and H-12, which also appear as a doublet of doublets, thereby identifying the remaining doublet of doublet of doublets in the olefinic region as proton H-13. The proton H-13 in turn couples to each of the methylene protons H-14a and H-14b, which appear as multiplets. By virtue of the cross-peaks shown by each of the olefinic protons (H-10, H-11, H-12, and H-13) into the four carbon signals in the olefinic region, we have assigned the olefinic carbons (C-10, C-11, C-12, and C-13). Therefore, any previous ambiguities associated with the assignment of C-12 and C-13 for spiramycin III (16) have thus been clarified. The methylene protons H-14a and H-14b show cross-peaks to C-14 in the HECTOR spectrum, and differentiating the methylene carbons C-14 and C-17 in the case of spiramycin III[16] was straightforward with the aid of the COSY spectrum.

There are five downfield methine carbons, at positions C-3, C-4, C-5, C-9, and C-15. The downfield methine proton multiplet, assigned to H-15 with the help of the COSY spectrum, shows a weak coupling to the methylenes H-14a and H-14b (indicated above as coupling to H-13) and a strong coupling to the methyl doublet H-16. From their respective cross-peaks in the HETCOR spectrum, one can easily determine the carbons C-15 and C-16. The methine proton H-9 appears as a doublet of doublets, showing a strong cross-peak to H-10 and a weak one to H-8 in the COSY spectrum (visible only in $CDCl_3$, and not $C_6D_6$, as solvent) and a cross-peak in the HETCOR spectrum which identifies C-9.

Proton H-6 coupled to the methylenes H-17a and H-17b as indicated above in both the solvent systems. In $CDCl_3$ as solvent, H-6 shows a weak cross-peak in the COSY spectrum to only one of the methylene protons H-7a and not H-7b and does not show cross-peaks into the doublet for the methine proton H-5. In contrast, using $C_6D_6$ as solvent, H-6 shows a weak cross-peaks in the COSY spectrum to a doublet for the methine proton H-5 and the methylene protons H-7a and H-7b. The methylene protons H-7a and H-7b appear as multiplets. Also, proton H-5 shows a cross-peak to the methine proton doublet H-4, which in turn couples into H-3. The methine H-3 is coupled to one of the methylene protons H-2a, more strongly than H-2b. Therefore we have

assigned the methine carbons C-3, C-4, C-5, and C-15 unambiguously (16). Both of the pairs of methylene protons, H-7a and H-7b as well as H-2a and H-2b, show cross-peaks to C-7 and C-2, respectively, in the HETCOR spectrum. Any ambiguity between the methine carbon C-6 and the methylene carbon C-7 in the case of spiramycin III (16) has been clarified using the DEPTGL spectrum.

The assignment of H-8 has been based on its weak cross-peaks into H-9, H-7b, and a methyl group H-19. No cross-peak has been observed between proton H-8 and proton H-7a. The methoxy methyl carbon shows a clear cross-peak in the HETCOR spectrum to a distinct singlet around 3.3 ppm for the methyl protons H-20.

### The Sugar Portion of the Molecule

Of the three anomeric (methine) sugar protons H-1′, H-1″, and H-1‴, we can clearly identify the doublet H-1′, which shows a strong cross-peak to the adjacent methine proton H-2′, whereas both H-1″ and H-1‴ show two cross-peaks, coupling to adjacent methylene protons H-2″ and H-2‴, respectively, in the COSY spectrum. Proton H-2′ appears as a doublet of doublets coupling into the methine H-3′, which in turn couples weakly to its adjacent methine proton H-4′. The proton H-4′ appears as a doublet coupling into the methine proton H-5′, which in turn couples strongly to the methyl proton doublet H-6′. Using the cross-peaks in the HETCOR spectrum corresponding to each of the protons, we can clearly identify the carbon (C-1′, C-2′, C-3′, C-4′, C-5′, and C-6′) signals, and the previously encountered ambiguities in assigning C-2′ and C-3′ for spiramycin III (16) have thus been clarified.

In the COSY spectrum, only one of the pairs of sugar methylenes H-2‴a and H-2‴b shows cross-peaks into another pair of methylene protons, H-3‴a and H-3‴b, while H-2″a and H-2″b (appears as a clear doublet of doublets in $CDCl_3$ and is less defined in $C_6D_6$ as solvent) do not, this being reasonable since C-3″ is a quaternary carbon. This information allows for the differentiation of protons H-1″ and H-1‴. Proton H-1″ appears as a doublet in $CDCl_3$ and $C_6D_6$, whereas H-1‴ appears as a doublet of doublets in $CDCl_3$ (not resolved in SP III) and $C_6D_6$ as solvent. Of the remaining downfield carbon signals, the assignments of C-1″ and C-1‴ are obvious from the cross-peaks observed in the HETCOR spectrum.

The two pairs of methylene protons H-2‴a/H-2‴b and H-3‴a/H-3‴b show cross-peaks into C-2‴ and C-3‴, respectively, in the HETCOR spectrum. The methylene protons H-3‴a and H-3‴b show cross-peaks into the methine H-4‴ which couple to the methine proton multiplet H-5‴, which in turn couples to a doublet for the methyl protons H-6‴. Identification of the carbons C-4‴ and C-5‴ was simplified using the HETCOR spectrum. The pair of dimethyl groups of protons 3′-N(Me)$_2$ (~δ 2.4) and 4‴-N(Me)$_2$ (~δ 2.0) appears as clear singlets and show cross-peaks in the HETCOR spectrum to their corresponding carbons, which appear at 42.0 and 40.5 ppm, respectively.

The remaining downfield methine proton multiplet has been assigned to H-5″, which shows cross-peaks into the doublet for the methyl protons H-6″ and to the methine proton doublet H-4″. Again, identification of the carbons C-4″ and C-5″ was simplified using the HETCOR spectrum. The ambiguity in differentiating the methyl carbons C-6′, C-6″, and C-6‴ identified by cross-peaks in the HETCOR spectrum has been clarified using the COSY spectrum. The methyl protons H-7‴ appear as a singlet and show a cross-peak in the HETCOR spectrum to carbon C-7‴ (~25.5 ppm).

From the integration of the $D_2O$ exchanged $^1$H-NMR spectrum, the hydroxyl protons 3-OH (spiramycin I only), 2′OH, 3″-OH, and 4″-OH were found to be between 1.5 and 2.5 ppm; however, their exact chemical shifts have yet to be determined. The methylene protons have been designated "a" and "b" based on their chemical shifts, but their stereochemistry, either as α and β or as pro-R or pro-S, is unknown.

### Solvent Effects

Pronounced solvent effects on the $^1$H-NMR spectrum of spiramycins I and III have helped identify and determine the coupling constants of some protons from one solvent system when unclear in the other. For example, the protons H-2a, H-17b, and H-2″n are more distinct in $CDCl_3$ as solvent, while the protons H-2b and H-3‴a and the methyl groups are more distinct in $C_6D_6$ as solvent. Interestingly, weak cross-peaks between protons H-9 and H-8, H-6, and H-7b, and H-6 and H-5 in the COSY spectrum were observed only when using $C_6D_6$ as solvent, and not in $CDCl_3$.

With the renewed interest in the spiramycins and their pharmacological activities, it is imperative that conclusive and unambiguous structural assessments of these compounds be made. We are currently investigating the $^1$H-NMR assignments of the spiramycins specifically to determine the stereochemistry (α and β) of the methylene protons (a and b), the exact chemical shifts (δ or ppm), and the coupling constant (*J*) values. We also hope to explain some of the odd multiplicities, as in the case of protons H-3, H-4, H-5, and H-1″, with the help of molecular modeling studies.

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