SPENOLIMYCIN, A NEW SPECTINOMYCIN-TYPE ANTIBIOTIC

II. ISOLATION AND STRUCTURE DETERMINATION

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(Received for publication September 5, 1984)

A new water-soluble, basic antibiotic has been isolated from the fermentation beers of *Streptomyces gilvospiralis* sp. nov. The structure of the antibiotic has been deduced from spectral studies and confirmed by chemical degradation to spectinomycin. This structure, 3′-*O*-methylspectinomycin-3′,4′-enol ether has led to the name spenolimycin.

In the course of screening for microorganisms producing novel antibiotics, a new streptomycete was discovered which produced a water-soluble basic antibiotic of considerably greater lipophilicity than most typical aminoglycoside or aminocyclitol antibiotics[1]. The antibiotic also showed activity against several aminoglycoside-resistant bacteria. As a result, the producing organism was fermented on a multiliter scale for isolation and structure elucidation. *Klebsiella pneumoniae* ATCC 8045 was chosen as the assay organism.

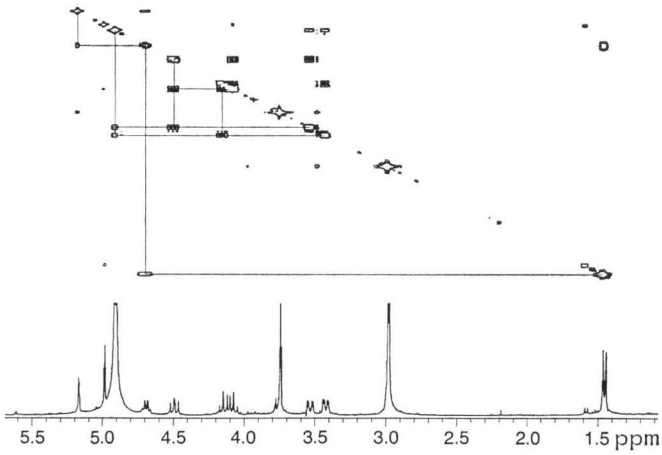
Isolation

The antibiotic activity could be absorbed from the clear filtered fermentation broth, adjusted with mineral acid to pH 7, onto Amberlite IRC-84 resin in the ammonium form. Considerable purification was obtained by eluting the activity from the resin with a gradient eluant from water to 0.1 N ammonium hydroxide. Active fractions were neutralized with dilute acetic acid and lyophilized. A similar chromatography over carboxymethyl Sepharose resulted in the separation of two active components. The first eluate contained the major activity and was further purified by chromatography over Fractogel, conversion to the sulfate salt and a final chromatography over octadecyl-capped silica gel to yield a pure antibiotic, which we have subsequently named spenolimycin. The minor bioactive component, eluted from the carboxymethyl Sepharose after spenolimycin was identified as spectinomycin by comparison of its ^{1}H NMR spectrum with that of an authentic sample.

Structure Determination

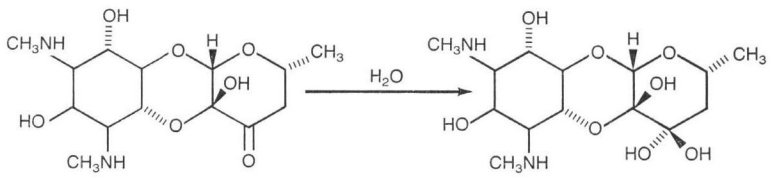
The mass spectrum of spenolimycin was measured with both fast atom bombardment (FAB) and electron impact (EI) ionizations. The FAB positive ion spectrum shows an MH^+ peak at 347 daltons and an MNa^+ peak at 369 daltons. The MH^+ peak was mass-matched at 347.1847 (theory 347.1818) corresponding to $C_{15}H_{26}N_2O_7$ for the molecular formula, and this was supported by mass matching of the molecular ion $M^{+\cdot}$ at 346.1741 (theory 346.1740) in the electron impact spectrum. A major peak at 141 daltons in the FAB spectrum was mass matched at 141.0532 and assigned the molecular formula $C_7H_9O_3$ (theory 141.0552). A major peak at 207 daltons corresponds to a protonated cyclitol portion with the formula $C_8H_{19}N_2O_4^+$.

Fig. 1. Chemical shift correlated 2D (COSY) NMR spectrum of spenolimycin in D_2O at 360 MHz.



The ^1H NMR spectrum of spenolimycin measured in deuterium oxide in a 2D correlated spectroscopy (COSY) experiment is shown in Fig. 1. This spectrum was measured at ambient temperature at 360 MHz. The COSY results indicate a definite coupling for a peak hidden under the large HOD peak at δ 4.9. In a similar spectrum measured at 300 MHz, the HOD peak was displaced to reveal a triplet at δ 4.88 with a coupling of slightly less than 3 Hz. A splitting of this magnitude is seen in each of the pairs of doublets at δ 3.42 and δ 3.54 which, from their chemical shifts, are assigned to protons attached to carbons bearing nitrogens. The COSY experiment shows that the other (larger) coupling of these two protons is to the protons at δ 4.15 and δ 4.5, respectively. These latter two protons are each coupled to the single proton at δ 4.07. This 6 spin system defines a diamino cyclitol of the *myo* stereochemistry. Taken in conjunction with the mass spectral results and the appearance of two 3-proton singlets δ 2.97 and δ 2.99, assigned as *N*-methyl signals, this evidence is diagnostic for the presence of actinamine as portion of the structure of spenolimycin.

Only two naturally-occurring antibiotics are known to contain actinamine as a structural moiety; spectinomycin[2~4] (**1**) and 3′-*R*-dihydrospectinomycin[5,6] (**2**).* Clearly, from its ^1H NMR spectrum, spenolimycin is different from each of these antibiotics. However, not only does the presence of actinamine suggest a structural relationship but other aspects of the ^1H NMR spectrum of spenolimycin resemble those of the spectinomycins. A singlet at δ 4.98 is characteristic of the anomeric proton of these antibiotics where C-2′ of the sugar is involved in a stable quaternary hemiketal. The spectrum also



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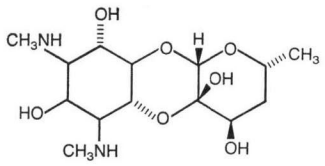
* We have chosen to use the aminoglycoside-type numbering system rather than that more commonly used with spectinomycin in which compound **2** would be described as 4-*R*-dihydrospectinomycin.



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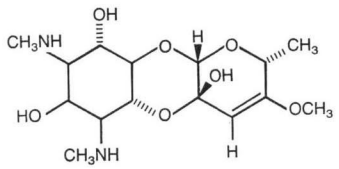
contains a *C*-methyl doublet at δ 1.46 coupled to a one proton signal at δ 4.69 typical of the C-5 and C-6 fragment of the sugar. However, unlike the situation in the other spectinomycins, the signal assigned to the proton at C-5′ of the sugar of spenolimycins shows only a small (about 1 Hz) coupling to a proton resonating as a broad singlet at δ 5.17. This is indicative of a very different chemical environment in the C3′-C4′ region of the sugar to that in the known spectinomycins. The remaining signal in the ^{1}H NMR of spenolimycin is a three proton singlet at δ 3.75 which is assigned to a methoxyl group attached to an sp^2 carbon.

These observations are consistent with two possible structures, **3** and **4**.



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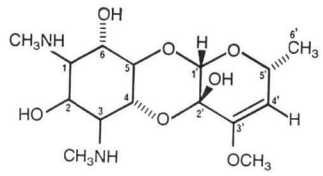

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The ^{13}C NMR spectrum of spenolimycin is listed in Table 1 together with literature[7] values for spectinomycin measured under comparable conditions. The assignments can be made by analogy with these values for spectinomycin, but are on much a stronger basis from the 2D heteronuclear chemical shift correlation shown in Fig. 2. Two quaternary carbons, not evident in this 2D experiment, are seen at δ 89.4 and δ 150.3 in a single pulse experiment. Using the empirical rules for substituent additivity effects[8] the positions for each of the two olefinic carbons can be calculated for the two possible structures. The results are shown in Table 2. The large disagreement between observed and calculated values must be ascribed to the unusual substitution pattern around C2′. These values could then be seen as favoring structure **4** allowing for a reasonable agreement between the calculated value (92.4) and the observed value (89.0) for C4′. Structure **4** was also strongly favored by biogenetic considerations when it is born in mind that spectinomycin (**1**) was also isolated from the same fermentation. The small coupling between H-5′ and the olefinic proton may, at first glance, seem to favor the allylic orientation in structure **3**, but, an examination of Dreiding models indicates that the dihedral angle between H-4′ and H-5′ in structure **4** is very close to 90° in keeping with a small coupling between these two protons. Thus, both structures **3** and **4** would be accommodated by the ^{1}H NMR data. We

Table 1. Comparison of ^{13}C NMR spectrum of spenolimycin with published spectrum of spectinomycin.

Spenolimycin pD 5.2	Spectinomycin hydrate pD 4.6[7]	Assignment
61.8	62.5	C1
60.5	60.7	C2
59.2	59.5	C3
66.0	66.5	C4
71.4	70.7	C5
66.5	66.9	C6
31.3	31.8	NCH_3
31.3	31.3	HCH_3
95.1	94.4	C1′
89.4	94.4	C2′
150.3	92.6	C3′
102.9	42.3	C4′
70.0	69.2	C5′
21.3	20.5	C6′
55.8		OCH_3

Fig. 2. ^{1}H-^{13}C 2D NMR chemical shift correlation map (CSCM) of spenolimycin in D_2O at 360/90.8 MHz.

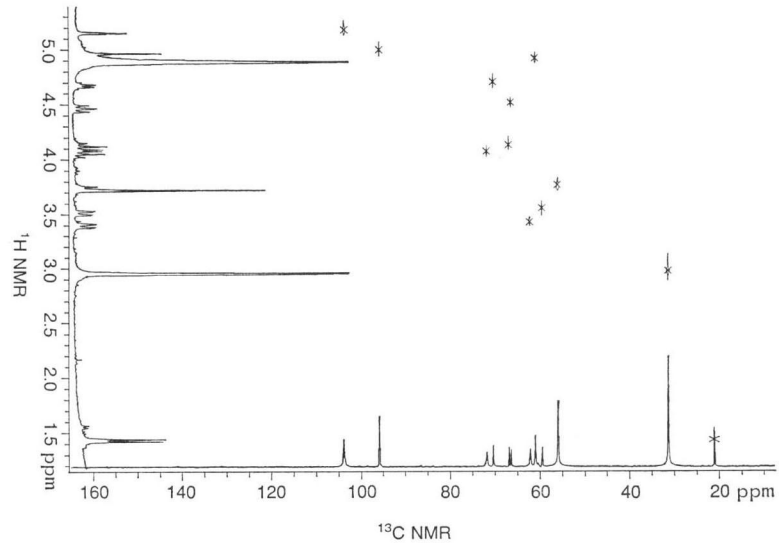

Table 2. Comparison of the ^{13}C NMR chemical shifts for the olefinic carbons of spenolimycin with empirical values for olefinic carbons in structures **3** and **4**.

	C3′	C4′
Calculated value structure **3**	99.4	160.4
Calculated value structure **4**	167.4	92.4
Actual values for spenolimycin	89.0	
	150.3	

attempted a number of Nuclear Overhauser Effect (NOE) experiments to distinguish between these structures, but the results were inconclusive.

Spenolimycin was hydrogenated over 5% rhodium on carbon with the expectation that an analysis of the couplings to the protons of the methylene group generated would distinguish conclusively between structure **3** and **4** for spenolimycin. The crude hydrogenation product was converted into carbobenzoxy derivatives, and these were separated by chromatography over silica gel. In addition to the expected two epimeric *N,N′*-dicarbobenzoxydihydro derivatives, *N,N′*-dicarbobenzoxyspectinomycin was isolated. This product of cleavage of the enol ether establishes the structure and absolute stereochemistry of spenolimycin as **4**. It would, of course, be possible for spenolimycin to exist in any of the five forms which can arise from the keto function at C-2′ free or in hemiketal formation with either the 4*R* hydroxyl group or the 6*S* hydroxyl group of actinamine. The weak UV absorption at 264 nm (ε 470) may be taken as evidence of a small contribution from the K-band of the free C2′ oxo function; however, the ^{13}C NMR spectrum indicates the presences of only one predominant hemiketal form. Drieding models of the hemiketal form shown in **4** superimpose on a model of spectinomycin **7** with the oxygen of the *O*-methyl group of **4** between the two 3′ oxygen of spectinomycin hydrate and with only slight differences in the position of C-4′ and C-5′ for these two structures. It is not surprising then that spenolimycin (**4**) should exhibit somewhat improved antimicrobial activity to that of spectinomycin (**1**)[9].

Experimental

General Procedures

NMR spectra were obtained with a General Electric QE 300 MHz or a Nicolet WB-360 MHz wide-

bore spectrometer in D_2O solutions with TSP as an external standard (δ 0 ppm) or in $CDCl_3$ solutions with TMS as an internal standard. The 2D proton-carbon chemical shift correlation (CSCM) experiment was perfomed on the Nicolet WB-360 spectrometer using a 5 mm C-13 probe and a model 1280 computer. The CSCM pulse sequence employed for the experiment was essentially that of FREEMAN[10] modified to deliver a composite 180° pulse[11]. Ninety degree pulse widths of 9 μseconds for carbon and 25 μseconds for hydrogen were used. Fixed delays around the acquisition pulse (Δ_1 and Δ_2) were set to 3.3 and 1.7 mseconds, respectively, with phase-cycling to provide the equivalent of quadrature data in both dimensions. The COSY pulse sequence used a 90° pulse of 12 μseconds.

Mass spectra were determined on a Kratos MS-50 spectrometer. High resolution measurements were made with 10,000 resolving power. UV spectra were determined with a Cary 219 spectrophotometer. IR spectra were measured using a Perkin Elmer Model 521 grating spectrometer. Optical rotations were measured in 1 dm tubes using a Perkin Elmer Model 241 polarimeter.

Isolation of Spenolimycin

The fermentation broth of *Streptomyces gilvospiralis* sp. nov. was adjusted to pH 7 with dilute HCl and filtered through Celite. The clear filtrate (94 liters) was charged onto a column (1.5 liters bed volume) of Amberlite IRC-84 (NH_4^+ form), and the column was eluted with a gradient from H_2O (3 liters) to 0.1 M ammonium hydroxide solution (6 liters). Fractions active against *Klebsiella pneumoniae* ATCC 8045 were combined, neutralized with dilute acetic acid and lyophilized. The white, solid residue was digested in H_2O and chromatographed over a column (2.5×90 cm) of CM-Sepharose (Pharmacia) in the ammonium form. Antibiotic activity was eluted with a gradient from H_2O (2 liters) to 0.1 N ammonium hydroxide solution (3 liters). Two major bioactive bands were eluted. The fractions from each band were combined, neutralized with acetic acid and lyophilized to yield crude spenolimycin 3.9 g and spectinomycin 1.17 g. The spenolimycin was further purified by chromatography of 1 g portions over a column (2.5×90 cm) of Fractogel in 0.05 M pH 6.9 ammonium acetate solution. Active fractions from four columns were pooled, lyophilized and converted to the sulfate salt with dilute sulfuric acid. The sulfate salt was further purified by chromatography over a column (1.5×70 cm) of Baker bonded phase octadecyl reverse phase silica gel (40 μm) eluted with H_2O (250 ml). Active fractions were combined and lyophilized to yield spenolimycin sulfate 0.9 g, $[\alpha]_D^{22}$ +24° (*c* 0.9, CH_3OH - H_2O, 1 : 1).

Anal Calcd for $C_{15}H_{26}N_2O_7 \cdot H_2SO_4$: C 40.53, H 6.35, N 6.30, O 39.60, S 7.21
Found: C 39.90, H 6.61, N 6.64, O 39.62, S 7.22

UV λ_{max} H_2O 264 nm (ε 470), shoulder 318 nm (ε 100).

IR ν_{max} (KBr) 1674, 1232 cm^{-1}.

Mass, ^1H NMR, and ^{13}C NMR spectra as described in the figures, text and Table 1.

Reduction of Spenolimycin

Spenolimycin sulfate (26 mg) in H_2O (2 ml) was passed through a column (2 ml) of Amberlite AG1 X2 (OH^-). The eluate was lyophilized and the spenolimycin free base obtained was digested in MeOH (10 ml). The solution was shaken over 5% rhodium on carbon (20 mg) under 3 atmospheres of hydrogen for 20 hours. Catalyst was filtered off and a TLC of the filtrate indicated multiple ninhydrin positive spots of similar Rf. Solvent was removed and the residue was digested in H_2O (1 ml) and acetonitrile (1 ml) and treated with triethylamine (0.02 ml). The mixture was cooled to −5°C and *N*-(benzoyloxycarbonyloxy)succinimide (37 mg) was added. The mixture was stirred and allowed to come to room temperature. After being stirred for 5 hours the mixture was diluted with H_2O (15 ml) and extracted with $CHCl_3$ (4×10 ml). The combined chloroform extracts were concentrated to an oil (28.3 mg). This was chromatographed over a column (0.9×30 cm) of Woelm, Kiesel Gel G 32~63 μm (about 7.6 g) packed and loaded in benzene and eluted with a mixture of benzene (460 ml) and isopropanol (40 ml). Approximately 2 ml fractions were collected. Fractions 22, 23 and 24 were combined and concentrated to yield a clear oil (2.9 mg), $[\alpha]_D^{22}$ −4.6° (*c* 0.14, CH_3OH). The ^1H NMR of which was identical with that of *N,N'*-dibenzyloxycarbonylspectinomycin prepared from an authentic sample of spectinomycin, $[\alpha]_D^{22}$ −10.7° (*c* 8.0, CH_3OH).

Addendum in Proof

After the completion of this work we became aware of U.K. Patent Application GB 2,127,019A, published April 4, 1984, inventors H. SAKAKIBARA, M. AWATA, S. SATOI, N. MUTOH, M. TAKADA and M. HAYASHI of Toyo Jozo Kabushiki Kaisha, Japan.

This application claims an antibiotic, acmimycin, of the same structure as that described here for spenolimycin. Although the stereochemistry for acmimycin is not defined in this patent and some minor differences in quoted physical constants exist, the close correspondence of the carbon magnetic resonance data suggest that acmimycin and spenolimycin are the same antibiotic. The producing organisms *Streptomyces gilvospiralis* (strain AB634D-177) and *Streptomyces* strain AC 4559 clearly differ from one another based on their carbon source utilization patterns and on the color of their aerial mycelium. *S. gilvospiralis* can assimilate arabinose, fructose, mannitol, raffinose, and rhamnose while strain AC 4559 cannot. The aerial mycelium of *S. gilvospiralis* is yellowish white to pale yellow on most media (ISP 2, 3, 4 and 5 and nutrient agars); strain AC 4559 is white to dusty aqua on the same media.

Acknowledgments

The authors thank Dr. J. HOCHLOWSKI and Dr. M. NUSS, Abbott Laboratories, Prof. L. MITSCHER, University of Kansas and Prof. S. HANESSIAN, University of Montreal for helpful discussions; Dr. L. SEIF, Abbott Laboratories for hydrogenations and Ms. J. HOOD, Abbott Laboratories for microanalyses.

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