

ISOLATION AND STRUCTURAL IDENTIFICATION OF NINE AVILAMYCINS

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Avilamycin is an antibiotic complex produced by cultures of the organism *Streptomyces viridochromogenes*, strain NRRL 2860. These compounds belong to the orthosomycin family of antibiotics. Structural composition of the major avilamycins and several minor avilamycins are known. Nine additional minor avilamycins, designated F through N, have been isolated via semi-preparative silica gel or reverse-phase high performance liquid chromatography with final purification using a reverse-phase column loading solvent switching technique. ¹H NMR and mass spectroscopy, negative ion fast atom bombardment (neg FAB) and electron impact, were used to structurally identify the avilamycins. All of the compounds were microbiologically active and similar in structure to other known avilamycins.

Using classical degradation studies^{1,2)} avilamycin was shown to be a member of the orthosomycin family of antibiotics, (flambamycin³⁾ and the everninomicins⁴⁾). Structural characterization of avilamycins A, B and C was first reported by WRIGHT⁵⁾, in an addendum, and later published by KELLER-SCHIERLEIN^{6,7)}. The structures of four additional avilamycins D₁, D₂, E and A' have recently been reported (personal communication, A. P. RAUN, Eli Lilly and Company with Ciba-Geigy, March, 1981). All of the above avilamycins are derivatives of A with changes at the C-45 linkage and/or the C-56 ketone adduct. The purpose of this study was to determine the structure of several additional minor components present in analytical standards of avilamycin and extracts of the fermentation product. Several unique modifications of avilamycin were found.

¹H NMR Studies

¹H FT-NMR spectra of the avilamycins were recorded on a Bruker WM-250 spectrometer at 250 MHz. The samples, 600~1,400 μg, were dissolved in 0.5 ml of acetone-*d*₆ solvent and run at ambient temperature (~23°C) with internal TMS as reference. Difference NOE (nuclear Overhauser effect) spectra were obtained by subtracting free induction decays accumulated with the decoupler off-resonance from similar accumulations with particular resonances irradiated, followed by Fourier transformation of the difference signals.

Mass Spectroscopy Studies

A VG Instrument, model ZAB-3F, was used to obtain negative ion fast atom bombardment (neg FAB) data. Avilamycins F, G and H were dissolved in thioglycerol and I, J, K, L, M and N were dissolved in 5 parts dithiothreitol and one part dithioerythritol, prior to analysis.

A Hewlett Packard 5985 GC-MS system with a direct insertion probe was used to obtain EI (electron impact) spectra. Thermal desorption of the sample was achieved prior to the maximum probe temperature of 200°C.

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Isolation and Chromatographic Separation of the Avilamycins

The avilamycins were isolated from an avilamycin standard. The standard was prepared by extraction of the dried fermentation mycelia with acetone followed by decolorization and the addition of water to form crystals of avilamycin. The crystals were filtered and processed through a second acetonitrile-water re-crystallization then filtered and dried (personal communication, J. M. EDMUNDOWICZ, Eli Lilly and Co.). The initial fractionation of the avilamycin was accomplished by normal or reverse-phase chromatography. Partial resolution of the avilamycins was achieved by loading 100~500 mg of the standard in 3% 2-propanol in chloroform onto a semi-preparative Woelm silica gel, 32~63 μm , column (11 mm \times 100 cm). The column was developed at a flow rate 9 ml/minute with 600 ml of 3% 2-propanol in chloroform followed by 900 ml of 10% 2-propanol in chloroform. Alternatively a semi-preparative Michel-Miller column (37 mm \times 35 cm), ACE Glass Incorporated, packed with C-18 column material, carbon content 21%, was used to obtain partial resolution of the avilamycins. About 250 mg of the standard was loaded onto the column in 55% methanol in 0.04 M $(\text{NH}_4)_2\text{HPO}_4$ pH 7.0 buffer. A step gradient of 55, 60 and 62% methanol in buffer was used to fractionate the avilamycins.

Final purification was accomplished by loading 100~200 ml of the sample, dissolved in 30% methanol in pH 7.0 buffer, with a M45 pump onto a semi-preparative 10.6 \times 250 mm IBM C-18 column. Using a pump switching technique, through a Rheodyne 7120 injection valve, the avilamycins were resolved with a 50~75% methanol gradient in pH 7.0 buffer developed by a 1084B Hewlett Packard HPLC system.

The eluted avilamycins were monitored at a wavelength of 286 nm. Eluates containing the desired avilamycins were collected, diluted in pH 7.0 buffer and reloaded onto the semi-preparative

Table 1. Neg FAB mass spectroscopy fragmentation pattern of avilamycins.

Avilamycin	Fragments $(\text{M}-\text{H})^-$ (m/z)							
	H ^a	G+H ^b	G ^a	F	E	D	C	B
A	1,401	1,229	1,169	1,000	810	650	508	378
F	1,353	— ^c	—	952	762	602	460	330
G	1,415	1,243	1,183	1,000	810	650	508	378
H	1,367	—	—	966	776	616	474	344
I	1,387	1,215	1,155	1,000	810	650	508	378
J	1,387	1,215	1,155	986	810	650	508	378
K	1,417	1,245	1,185	1,016	826	650	508	378
C	(1,403) ^d	(1,229)	(1,201)	(1,000)	(810)			
L or No. 1 ^e	1,387	1,229	1,169	1,000	810	650	508	378
No. 2	1,387	1,215	1,155	986	796	650	508	378
M or No. 2	1,387	1,215	1,155	986	796	650	508	378
No. 3	1,387	1,215	1,155	986	810	650	508	378
N or No. 3	1,387	1,215	—	986	810	650	508	377
No. 2	1,387	1,215	—	986	796	650	508	377

^a Fragmentation pattern of avilamycin breaking at each ring (Fig. 5); H is the deprotonated molecular weight $(\text{M}-\text{H})^-$, G is the deprotonated molecular weight of rings A through G.

^b Fragmentation pattern including A through G and part of ring H (see Fig. 5).

^c Fragment nondetectable in mass spectra.

^d Avilamycin K contained a small concentration of avilamycin C which chromatographically is eluted right behind K on the HPLC column.

^e Two compounds were present in the avilamycin L isolate, compound No. 1 was named L, compound No. 2 was named M (see M fraction).

Table 2. ^1H NMR data (acetone- d_6).

Group(s)	Avilamycins (ppm)									
	A	F	G	H	I	J	K	L	M	N
CH_3CH (15, 21, 28, 35, 51, 52, 58) ^a	1.00~	1.00~	1.00~	1.00~	1.00~	1.00~	1.00~	1.00~	1.00~	1.00~
CH_3 (tertiary) (27)	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40
CH_3 (60)	2.31	2.30	2.31	2.25	2.31	2.29	2.31	2.31	2.29	2.30
				or						
CH_3 (8)	2.36	2.48	2.35	2.25	2.35	2.34	2.35	2.33 ^c	2.33	2.34
				or						
CH_3OCH_2 (43)	3.30	3.28	3.29	3.28	3.30	—	3.30	3.29	3.29	3.29
CH_3O (34)	3.54	3.52	3.53	3.51	3.54	3.52	3.53	3.54	3.52	3.52, 3.53 ^d
CH_3O (41)	3.55	3.53	3.55	3.53	3.55	3.53	3.55	3.58	3.58 ^b	3.56
CH_3OAR (7)	3.88	—	3.88	3.84	3.88	3.86	3.88	3.85	3.87	3.87
Ar-H (2 or 6)	—	6.50	—	6.71	—	—	—	—	—	—

^a Number in parenthesis represents carbon position, see Fig. 3.

^b Estimate, no integration print out.

^c Diminished signal.

^d Doublet due to the presence of two compounds, one with the C-35 missing, and the second compound with the C-41 missing.

column for future purification. The purified isolates were extracted from the column eluent into AR grade chloroform which was then evaporated to dryness. The samples were stored at 8°C until analyzed by ^1H NMR and mass spectroscopy.

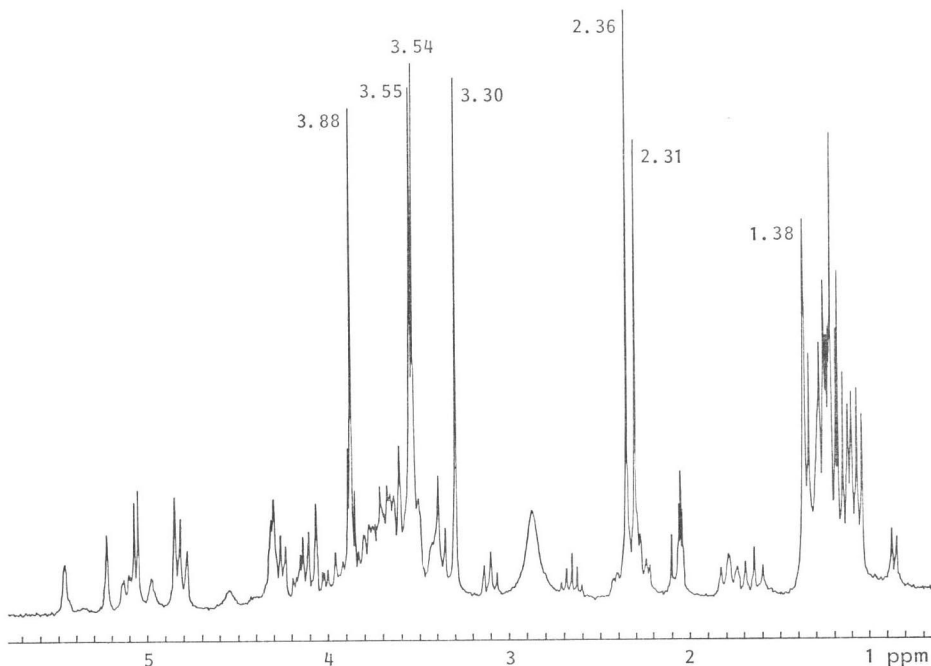
Results and Conclusions

Structural identification of the avilamycins was aided by comparing the neg FAB mass spectral fragmentation data and the ^1H NMR data to the data for avilamycin A and other known avilamycins. See Tables 1 and 2. Structural changes in the dichloroisoeverninic acid ring (A), predicted by the neg FAB mass spectrum, were verified by the EI mass spectrum. Fig. 1 shows the 250 MHz ^1H NMR spectrum of avilamycin. Key assignments used can be found in Table 2 for avilamycin A.

The structurally unknown avilamycins F through N were designated *via* the sequence of their isolation and identification, which along with the known avilamycins A, B, C, D₁ and E are shown in the HPLC of avilamycin standard 293-1M6-155 (Fig. 2). Avilamycins F, G and H were first separated from the other avilamycins using silica gel chromatography. They were then purified by the reverse-phase HPLC solvent switching technique. Avilamycins I and J were first fractionated using the semi-preparative C-18 column and were then purified *via* the column/solvent switching technique. Avilamycins K, L, M and N were isolated by loading about 300 mg of the standard onto the semi-analytical IBM column and were then resolved *via* the gradient elution-solvent switching technique.

Avilamycin A

The structure of avilamycin A is shown in Fig. 3. The ^1H NMR data (Table 2) for avilamycin A were similar to the ^1H NMR data reported by OLLIS *et al.*³⁾ for flambamycin an antibiotic of similar structure. Assignments in the ^1H NMR spectrum for the following functional groups were OCH_3

Fig. 1. ^1H NMR spectrum of avilamycin in acetone- d_6 (250 MHz).

(singlets, 3.88, 3.55, 3.54 and 3.30 ppm); one aromatic methyl and one methyl ketone (singlets, 2.36 and 2.31 ppm, respectively) and a tertiary methyl (singlet, 1.38 ppm).

The molecular ion region of the neg FAB spectrum of avilamycin A is shown in Fig. 4 with a molecular weight of 1,402 depicted by the deprotonated molecular ion $(\text{M}-\text{H})^-$ of m/z 1,401. Analysis of the complete spectrum revealed the fragmentation pattern shown in Fig. 5.

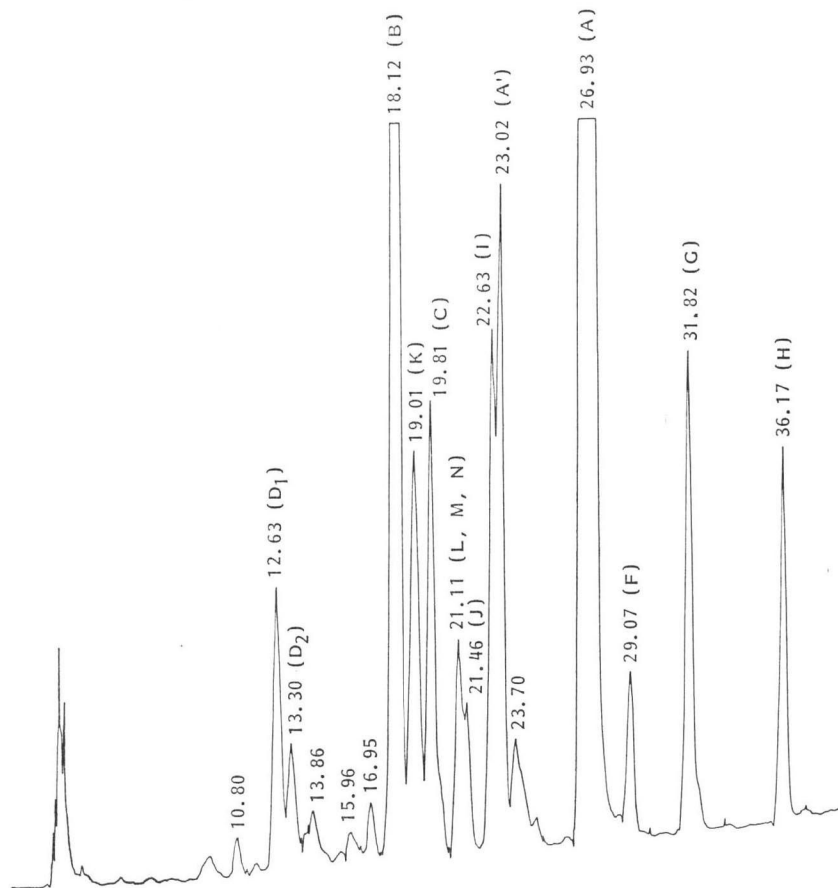
EI mass spectrum of avilamycin A is shown in Fig. 6. The compound yielded fragment ions of 233 and 235 (3:2) which represent the A ring containing two chlorine atoms cleaved at the carbonyl group, $\text{C}_6(\text{OH})(\text{CH}_3)(\text{OCH}_3)\text{Cl}_2\text{C}\equiv\text{O}^+$, as shown in Fig. 5.

Avilamycin F

A molecular weight of 1,354, $(\text{M}-\text{H})^- = 1,353$, was obtained by neg FAB mass spectrometry which suggested a loss of m/z 48 or the equivalent of a CH_2 group and chlorine. The neg FAB fragmentation products, when compared to the avilamycin A fragmentation products, Table 1, Fig. 5, showed a consistent decrease of 48 mass units throughout the ring fragmentation pattern which suggested the decrease in molecular weight was in the A ring.

^1H NMR spectral data of F, Table 2, showed the absence of the methoxy functional group at 3.88 ppm (C-7) on the A ring. The retention of the aromatic methyl (C-8) on the A ring was confirmed by the singlet signal at 2.48 ppm. An aromatic proton resonance (singlet) was found at 6.50 ppm indicating the presence of a proton on A ring at either C-2 or C-6. ^1H NMR NOE studies suggested that the chlorine was present at C-6 with a proton at C-2 (see Results for H). An EI mass spectrum was run to verify the change in the A ring. A fragment ion of m/z 185 was obtained, 48 mass units less than the 233 for the intact A ring thus confirming the presence of a hydroxyl group at the C-3 position and the loss of a chlorine at the C-2 carbon position of the A ring.

Fig. 2. HPLC of avilamycin standard 293-1M6-155.



Retention time	Area	Area (%)	
10.80	1,133	0.082	
12.63	16,180	1.168	Avilamycin D ₁
13.30	5,909	0.427	Avilamycin D ₂
15.96	986	0.071	
16.95	1,978	0.143	
18.12	139,900	10.099	Avilamycin B
19.01	31,950	2.306	Avilamycin K
19.81	29,040	2.096	Avilamycin C
21.11	12,020	0.868	Avilamycins L, M and N
21.46	7,018	0.507	Avilamycin J
22.63	25,480	1.839	Avilamycin I
23.02	35,940	2.594	Avilamycin A'
23.70	11,060	0.798	
26.93	1,009,000	72.833	Avilamycin A
29.07	10,810	0.780	Avilamycin F
31.82	27,420	1.979	Avilamycin G
36.17	19,530	1.410	Avilamycin H

Avilamycin G

¹H NMR spectral data of G, when compared to avilamycin A (Table 2), showed both compounds contained the same similar groups. A molecular weight of 1,416, (M-H)⁻=1,415, was obtained

Fig. 3. Structure of avilamycin A.

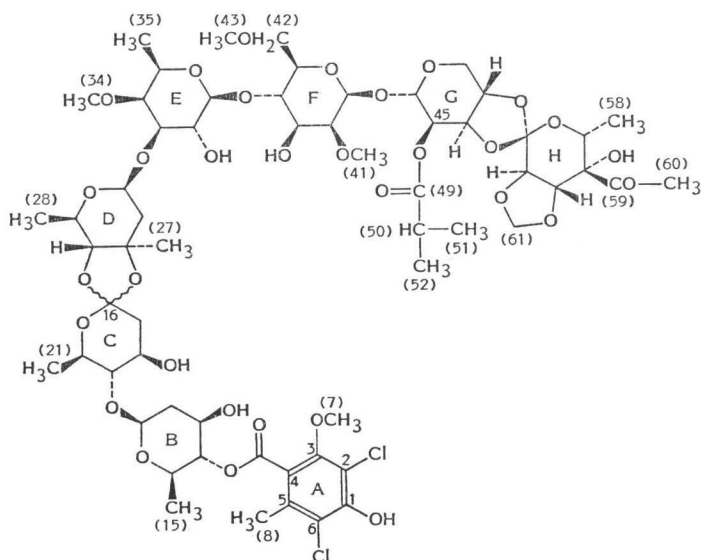
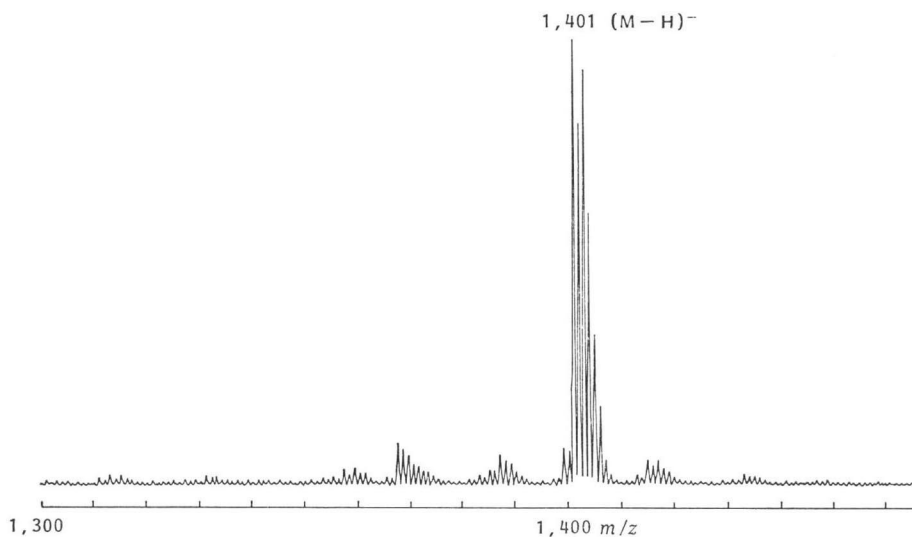


Fig. 4. Neg FAB mass spectrum of avilamycin A.



by neg FAB mass spectroscopy. The additional 14 amu suggested the addition of a CH_2 group to an avilamycin like structure. Examination of the neg FAB fragmentation data of avilamycins A and G, Table 1 and Fig. 5, showed identical fragmentation patterns of A through the F rings for both compounds. Data including the G and G+H rings were m/z 14 units higher for avilamycin G, 1,183 versus 1,169 and 1,243 versus 1,229, when compared to avilamycin A. These data indicated that the CH_2 addition was at the R_1 ester linkage to ring G.

Avilamycin H

A molecular weight of 1,368, $(\text{M}-\text{H})^- = 1,367$, was obtained by neg FAB mass spectrometry

Fig. 5. Neg FAB fragmentation pattern of avilamycin A.
* Observed in positive EI spectrum.

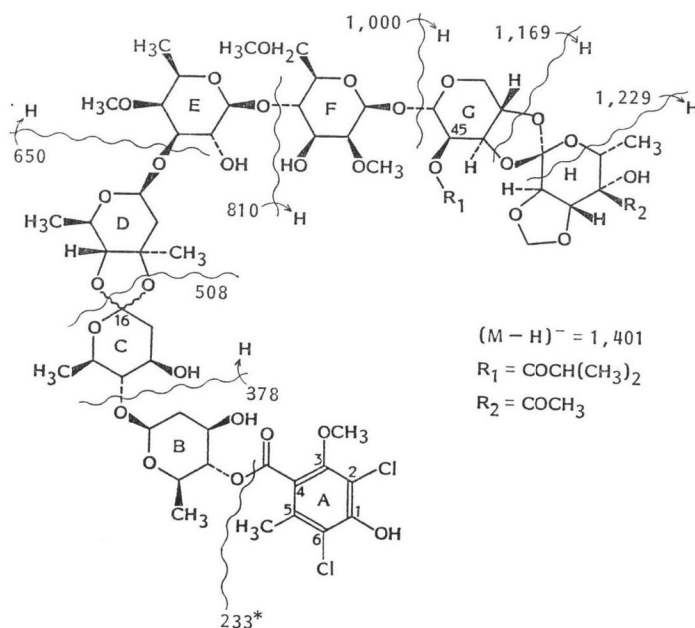
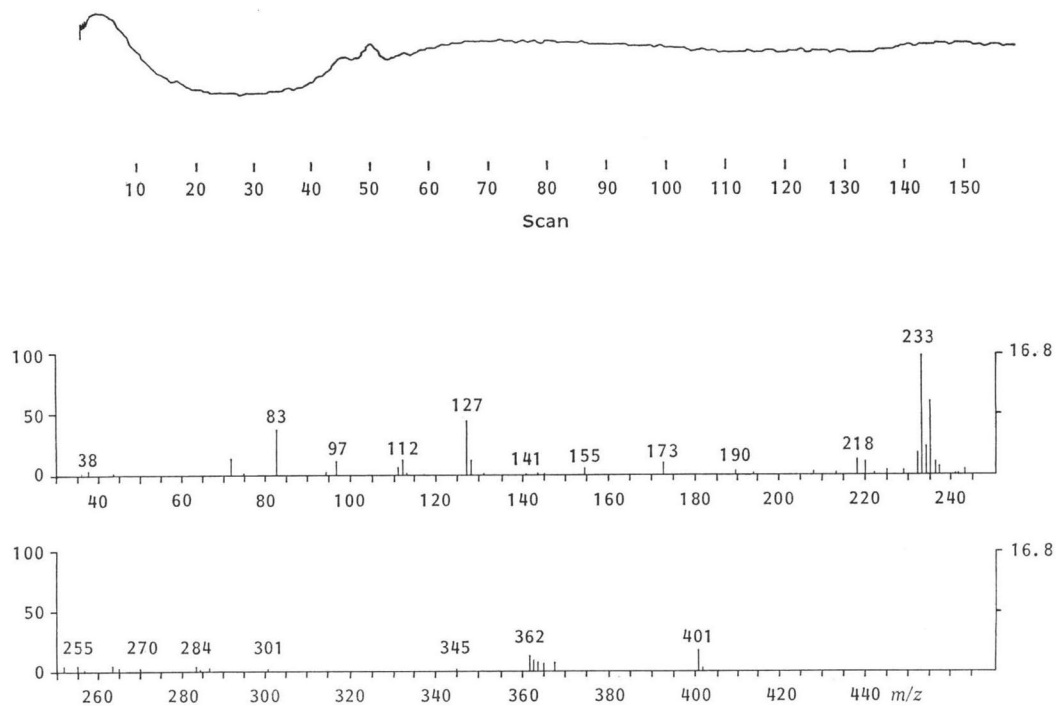


Fig. 6. EI mass spectrum of avilamycin A.



which suggested the loss of a chlorine on the A ring of avilamycin A. Examination of the neg FAB fragmentation data of avilamycins A and H, Table 1 and Fig. 5, showed the loss of m/z 34 throughout the fragmentation pattern for rings A through G which suggested the loss of a chlorine in the A ring.

An EI mass spectrum was run to verify the change in the A ring. A fragment ion of m/z 199 was obtained, 34 mass units less than the 233 for the intact A ring. This confirmed the replacement of a chlorine atom with a proton in the A ring.

^1H NMR spectral data of H, when compared to avilamycin A (Table 2), showed both compounds were similar but with differences in the A ring. ^1H NMR NOE studies were run to determine the structure of the dechloro compound. Irradiation of the OCH_3 (C-7) followed by subtraction of the normal spectrum (NOE difference) did not give a signal intensity enhancement of the aromatic proton, which suggested the chlorine was still present at C-2. Correspondingly, irradiation of the CH_3 (C-8) followed by subtraction of the normal spectrum gave a positive intensity enhancement at 6.71 ppm. These data confirmed the replacement of the chlorine at C-6 with a proton.

Avilamycin I

^1H NMR spectral data of I (Table 2) were similar to the spectrum of A and G, which indicated the three compounds contained similar groups. A molecular weight of 1,388, $(\text{M}-\text{H})^- = 1,387$, was obtained by neg FAB mass spectrometry, which suggested the loss of a CH_2 when compared to avilamycin A. Examination of the neg FAB fragmentation data showed that I was consistent with avilamycin A from the A ring through the F ring (Table 1, Fig. 5) but was m/z 14 less at the G ring 1,155 *versus* 1,169 and 1,215 *versus* 1,229. These data predict that the R_1 side chain has lost a CH_2 group and now has the same R_1 side chain as avilamycin A'.

Avilamycin J

A molecular weight of 1,388, $(\text{M}-\text{H})^- = 1,387$, was obtained by neg FAB mass spectroscopy, which when compared to the molecular weight of avilamycin A suggested the loss of a CH_2 . Examination of the fragmentation data showed J to have the same fragmentation data as avilamycin A from the A ring through E ring (Table 1). All fragmentation data for avilamycin J when compared to avilamycin A was 14 amu less from F through the H rings. These data suggested the loss of a CH_2 at C-41 or C-43 (see Fig. 3) of the F ring.

^1H NMR spectral data of J confirmed the presence of three of the four methoxy groups, singlets at 3.52 and 3.53 ppm (C-34 and C-41) along with the aryl methoxy, 3.86 ppm, at C-7. Missing from the spectrum was the 3.28 ppm singlet indicating the absence of the C-43 methoxy group.

Avilamycin K

Molecular weight of 1,418, $(\text{M}-\text{H})^- = 1,417$, and 1,404, $(\text{M}-\text{H})^- = 1,403$, were obtained by neg FAB mass spectrometry, which when compared to the molecular weight of avilamycin A suggested the addition of a molecule of oxygen to form avilamycin K. The 1,404 molecular weight, a minor component of the isolate, suggested the addition of two protons to avilamycin A. Examination of the fragmentation data (Table 1) showed K to be consistent with avilamycin A fragmentation data from A ring through D ring; however, rings E through H all showed the addition of 16 amu. The data suggested the major compound had the addition of oxygen at the C-35 position of the E ring. The minor component of the isolate was similar to the fragmentation pattern of A, except for the H ring, where two protons were added to give the final molecular weight of 1,404. Review of the earlier work confirmed the 1,404 compound to be avilamycin C or the addition of two protons at C-59 to form the secondary alcohol. Avilamycin C elutes immediately after K, see Fig. 2, and therefore must have been a minor contaminate of the K isolate.

^1H NMR spectral data of K (Table 2) confirmed the presence of all four methoxy groups, singlets at 3.53 and 3.55 ppm (C-34 and C-41) and singlets at 3.88 and 3.30 ppm (C-7 and C-43). The lack of an additional or enhanced signal in the 3.53~3.55 ppm range (aryl methoxy functional group response) confirmed the hydroxylation of the C-35 carbon and not the presence of an additional methoxy function group.

The isolation of avilamycins L, M and N proved to be a chromatographic challenge. The "purified" fraction chromatographed as a sharp single peak. The initial mass spectroscopy data along with ^1H NMR spectral data indicated the isolate was composed of several avilamycins. Chromatography of the isolate using a very moderate methanol gradient failed to resolve the avilamycins. During the gradient separation, the peak was split into three chromatographic fractions (No. 1, No. 2 and No. 3), the front, middle, and tail portion of the chromatographic peak. Each fraction was examined by neg FAB mass spectroscopy.

Avilamycin L

From fraction No. 1 a molecular weight of 1,388, $(\text{M}-\text{H})^- = 1,387$, was obtained by mass spectroscopy which when compared to avilamycin A, suggested the loss of the CH_2 (14 amu). Examination of the data showed that two avilamycins were present in the sample. Fragmentation data (Table 1) showed both compounds were identical to avilamycin A in rings A through D. At E ring, two sets of fragmentation data appeared, one fragment was similar to avilamycin A and the second fragment was 14 amu less or a loss of CH_2 at the E ring. Fragmentation data of rings E through G contained both sets of data, one consistent with avilamycin A and the other set 14 amu less. At H ring the second compound lost 14 amu resulting in identical mass weights for both compounds.

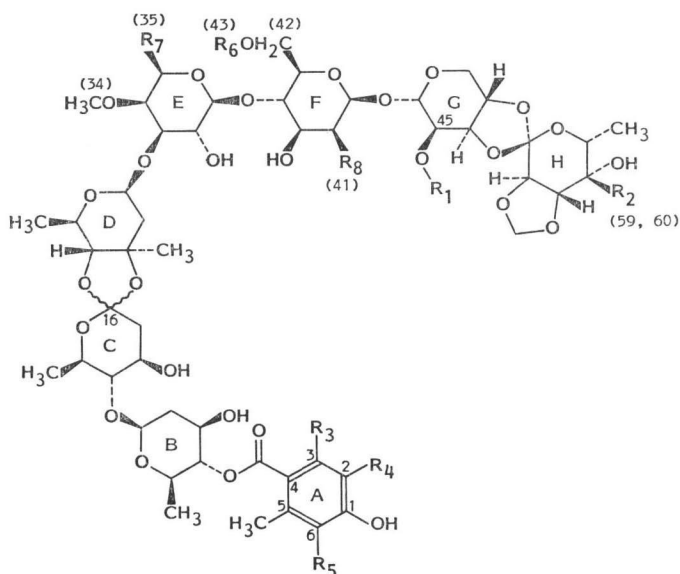
The loss of CH_2 from H ring could have occurred at one of two positions, the secondary methyl (C-58) or the acetyl methyl (C-60) (see Fig. 3). ^1H NMR spectrum showed a depletion of the acetyl carbon singlet in the 2.31~2.35 ppm range. The lack of resolution in the 1.00~1.40 ppm region of the ^1H NMR spectrum prevented confirmatory presence of the C-58 methyl group. The partial depletion of the singlet for the acetyl carbon (C-60) is consistent with the knowledge that two compounds existed in the fraction. The second compound in this fraction was also present in fraction No. 2, middle portion of the chromatographic peak, and was named avilamycin M.

Avilamycins M and N

From fractions No. 2 and No. 3 a molecular weight of 1,388, $(\text{M}-\text{H})^- = 1,387$, was obtained by mass spectroscopy which when compared to avilamycin A, suggested the loss of a CH_2 (14 amu) in the avilamycin structure. Examination of the mass spectroscopy fragmentation data indicated two avilamycins were present in both fractions. Avilamycin M, see compound No. 2 in avilamycin L discussion, and a third compound avilamycin N were both identical with the fragmentation pattern of avilamycin A from the A ring through the D ring. However at the E ring, for both fractions, two sets of fragmentation data appeared, one similar to A and other fragment 14 amu less indicating the loss of a CH_2 group. At F ring the fragmentation data were identical $(\text{M}-\text{H})^- = 986$ for both fractions, indicating the loss of CH_2 from compound No. 3 or avilamycin N. The fragmentation patterns for both compounds were identical in rings G and H which gave a net loss of CH_2 for both compounds when compared to avilamycin A (Table 1).

^1H NMR spectra for both fractions are summarized in Table 2. The loss of a CH_2 in ring E to form avilamycin M can occur at one of two carbon positions, C-34 or C-35. The ^1H NMR response at

Fig. 7. Structure of avilamycins.



Avilamycins	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈
A	COCH(CH ₃) ₂	COCH ₃	OCH ₃	Cl	Cl	CH ₃	CH ₃	OCH ₃
A'	COCH ₂ CH ₃	H	OCH ₃	Cl	Cl	CH ₃	CH ₃	OCH ₃
B	COCH ₃	COCH ₃	OCH ₃	Cl	Cl	CH ₃	CH ₃	OCH ₃
C	COCH(CH ₃) ₂	CH(OH)CH ₃	OCH ₃	Cl	Cl	CH ₃	CH ₃	OCH ₃
D ₁	H	COCH ₃	OCH ₃	Cl	Cl	CH ₃	CH ₃	OCH ₃
D ₂	COCH ₃	CH(OH)CH ₃	OCH ₃	Cl	Cl	CH ₃	CH ₃	OCH ₃
E	H	CH(OH)CH ₃	OCH ₃	Cl	Cl	CH ₃	CH ₃	OCH ₃
F	COCH(CH ₃) ₂	COCH ₃	OH	H	Cl	CH ₃	CH ₃	OCH ₃
G	COC ₄ H ₉	COCH ₃	OCH ₃	Cl	Cl	CH ₃	CH ₃	OCH ₃
H	COCH(CH ₃) ₂	COCH ₃	OCH ₃	Cl	H	CH ₃	CH ₃	OCH ₃
I	COCH ₂ CH ₃	COCH ₃	OCH ₃	Cl	Cl	CH ₃	CH ₃	OCH ₃
J	COCH(CH ₃) ₂	COCH ₃	OCH ₃	Cl	Cl	H	CH ₃	OCH ₃
K	COCH(CH ₃) ₂	COCH ₃	OCH ₃	Cl	Cl	CH ₃	CH ₂ OH	OCH ₃
L	COCH(CH ₃) ₂	CHO	OCH ₃	Cl	Cl	CH ₃	CH ₃	OCH ₃
M	COCH(CH ₃) ₂	COCH ₃	OCH ₃	Cl	Cl	CH ₃	H	OCH ₃
N	COCH(CH ₃) ₂	COCH ₃	OCH ₃	Cl	Cl	CH ₃	CH ₃	OH

3.52 ppm in both fractions indicates the presence of C-34 methoxy functional group in avilamycin M.

Since two compounds are present in both fractions, with and without the methyl group at C-35, one would expect a shift in the C-34 methoxy functional group response for each compound. Such a doublet does exist in the second fraction at 3.52 and 3.53 ppm for the C-34 methoxy group.

The loss of a CH₂ in the F ring to form avilamycin N can occur only at C-41 or C-43. Diminished (fraction No. 2) or absent (fraction No. 3) is the methoxy functional group (3.56~3.58) at C-41 which indicated the loss of the CH₂ from the methoxy group attached at C-41 on the ring. Correspondingly, in both fractions the presence of the methoxy group at C-43 was indicated by the strong signal at 3.29 ppm in both fractions. Signals of equal intensity for both compounds were also observed for the aryl methoxy functional group (C-7) at 3.87 ppm. The structure assignments for all avilamycins are shown in Fig. 7.

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