BIOSYNTHESIS OF ANTIBIOTICS OF THE VIRGINIAMYCIN FAMILY, 2.[1] ASSIGNMENT OF THE ^{13}C-NMR SPECTRA OF VIRGINIAMYCIN M_1 AND ANTIBIOTIC A2315A

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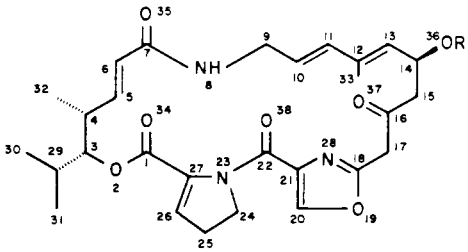
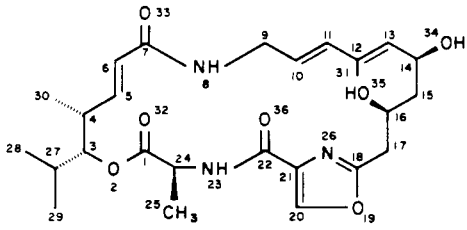
Abstract.—As part of a biosynthetic study using ^{13}C-labeled precursors, the ^{13}C-nmr spectra of the antibiotics virginiamycin M_1 and A2315A have been analyzed. Signal assignments were based on specific proton decoupling, chemical shift and multiplicity analysis, carbon-carbon couplings of antibiotic biosynthesized from [1,2-$^{13}C_2$]acetate and L-[U-$^{13}C_3$]-serine, and comparison with model compounds.

Virginiamycin M_1 (**1**) is a polyunsaturated cyclic peptolide antibiotic obtained from a number of different source microorganisms. It has variously been named as mikamycin A, ostreogrycin A, pristinamycin IIA, streptogramin A, PA 114 A1, and vernamycin A (1), but the name virginiamycin M_1 has been accepted as the one having precedence (2). Its structure was elucidated by a combination of chemical and spectroscopic techniques (3,4), and has been confirmed by an X-ray crystal structure (5). The antibiotic A2315A (**2**) differs from virginiamycin M_1 in the presence of a D-alanine unit in place of the dehydroproline unit of the latter and in the oxidation level of the C-16 oxygen function (6).



1 R=H

3 R=Ac



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In connection with our studies on the biosynthesis of these antibiotics (7), we needed to make complete assignments of the ^{13}C-nmr spectra of virginiamycin M_1 and antibiotic A2315A. Assignments of the spectra of both antibiotics have been published (8), but these assignments were made primarily on the basis of comparisons between the spectra of related molecules and, thus, were open to doubt. For this reason, almost one-half of the published assignments had to be modified in the light of our work.

Virginiamycin M_1 shows signals for 28 carbons in its ^{13}C-nmr spectrum (figure 1 and table 1). The assignments indicated in table 1 were made on the basis of the following considerations.

Initial assignments of groups of signals to corresponding groups of carbons were made on the basis of peak multiplicities (see table 1) and simple chemical shift considerations, with alkyl carbons assigned to the range 0-90 ppm, olefinic carbons to the

[1]For Part 1, see reference (7).

range 120-150 ppm, and carbonyl carbons to the range 150-200 ppm. Individual assignments of carbon resonances within these groups were then made primarily with information derived from the specific frequency proton decoupling experiments reported in table 1. Inasmuch as the ^{1}H-nmr spectrum of virginiamycin M_1 (figure 2) has been completely assigned (4), these experiments provide unambiguous assignments of most of the resonances in the ^{13}C spectrum.

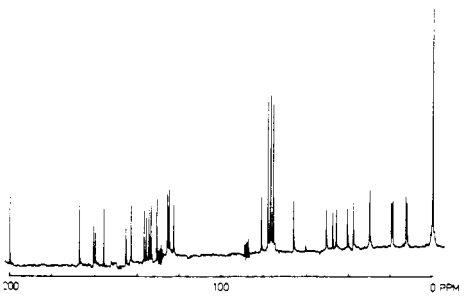

FIGURE 1. ^{13}C-nmr spectrum of virginiamycin M_1.

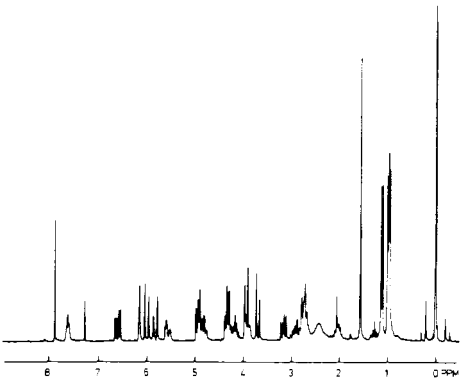

FIGURE 2. ^{1}H-nmr spectrum of virginiamycin M_1.

The signal at 12.2 ppm appears as a singlet on irradiation of the methyl protons at 1.10 ppm, while the signal at 12.7 ppm appears as a singlet on irradiation of the methyl protons at 1.55 ppm. These facts indicate clearly that the signal at 12.2 ppm is assignable to a methyl group at position 32, and that at 12.7 ppm to the group at position 33. Similarly, the signal at 145.4 ppm appeared as a sharp singlet on irradiation of the oxazole proton at 7.89 ppm, identifying it as that of the oxazole carbon at position 20.

Although the specific frequency proton decoupling experiments summarized in table 1 served to elucidate the assignments of most of the carbons of virginiamycin M_1, there were still uncertainties in some assignments, particularly those involving the vinyl carbons 6, 10, 11, 13, and 26. These ambiguities were resolved by a study of the spectrum of virginiamycin M_1 monoacetate and by the analysis of coupling constants in virginiamycin M_1 biosynthesized from [1,2-$^{13}C_2$]acetate.

The ^{13}C chemical shifts of allyl alcohols and their acetates have been studied by Wenkert (9), who showed that acylation shifts are generally systematic and predictable for these compounds. The ^{13}C-nmr spectrum of virginiamycin M_1 monoacetate (**3**) was very similar to that of virginiamycin M_1 itself, with most resonances occurring within 0.3 ppm of those of the corresponding carbons in the underivatized antibiotic. The resonances assigned to C-11, -12, and -13, however, were shifted by -0.8, $+2.1$, and -4.3 ppm, respectively, in the acetate as compared to the alcohol, consistent with the shifts observed by Wenkert in similar situations (9). These observations thus established the assignments of C-11, -12, and -13, and, by elimination, C-6.

Confirmation of the assignments in table 1 was obtained by the analysis of one-bond ^{13}C-^{13}C coupling constants in virginiamycin M_1 biosynthesized from [1,2-$^{13}C_2$]-acetate. Although this analysis requires the availability of biosynthetically labeled material, it requires no assumptions beyond the obvious one that atoms showing one-bond ^{13}C-^{13}C coupling are adjacent to each other. Thus, the C-4 carbon, which was assigned to the resonance at 37.6 ppm by the techniques already described, was shown to be coupled to a carbon resonating at 143.1 ppm, which must then be the C-5 carbon.

TABLE 1. ^{13}C chemical shift assignment of virginiamycin M_1.

Carbon number	^1H-nmr shift of attached protons[a]	^{13}C-nmr assignment[a]	Multiplicity[b]	^{13}C-nmr peaks appearing as singlets on irradiation at indicated proton frequency[c]												$^1J_{C-C}$ Hz[d]
				1.10	1.55	2.70	2.94	3.77	4.15	4.85	5.50	5.88	5.96	6.16	7.84	
1	—	160.9	s													
29	2.00	30.1	d		*											
30	0.95	18.9[e]	q	**												
32	0.95	19.6[e]	q	**												
3	4.98	81.5	d							*						
4	2.70	37.6	d			**	*									42.7
32	1.10	12.2	q	**												
5	6.60	143.1	d													42.0
6	5.96	125.3	d									**	**	**		64.9
7	—	167.6	s													65.2
9	4.15	40.5	t					*	**							
10	5.55	126.1	d								**	*	*			
11	5.83	133.7	d								*	**	**	*		
12	—	134.7	s													[g]
33	1.55	12.7	q		**											[g]
13	4.92	131.0	d							*						48.0
14	4.82	66.0	d							**						48.0
15	3.12, 2.76	47.7	t			*	**									40.0
16	—	200.7	s													40.5
17	3.70, 3.95	45.7	t		*	*	*	**	*							60.7
18	—	156.2	s													60.3
20	7.89	145.4	d												**	
21	—	136.1[f]	s													
22	—	160.0	s													
24	4.35	50.5	t						*							
25	2.80	29.9	t			**	*									
26	6.15	122.7	d										*	**		
27	—	137.2[f]	s													

[a]In ppm downfield from TMS in $CDCl_3$.

[b]from reference (8).

[c]Signals due to carbons not bearing protons have been omitted from this section. These appeared as sharp singlets in most of the spectra.

[d]Carbon-carbon coupling observed when [1,2-$^{13}C_2$] acetate was incorporated into virginiamycin M_1.

[e,f]These assignments may be reversed.

[g]Coupling between carbons 9 and 10 was observable, but the coupling was not first order, and the coupling constant was not determined.

*broadened singlet.

**sharp singlet.

Likewise, the C-6 carbon at 125.3 ppm was coupled to the C-7 carbonyl carbon at 167.6 ppm; the C-11 carbon at 133.7 ppm was coupled to the C-12 carbon at 134.7; the C-14 carbon at 66.0 ppm was coupled to the C-13 carbon at 131.0 ppm; the C-15 carbon at 47.7 ppm was coupled to the C-16 carbonyl carbon at 200.7 ppm; and the C-17 carbon at 45.7 ppm was coupled to the C-18 carbon in the oxazole ring at 156.2.

A final assignment was possible in the light of an additional biosynthetic experiment. It has previously been shown that serine is incorporated into the oxazole ring of virginiamycin M_1, with carbons 20, 21, and 22 deriving from this precursor (7). Incorporation of [1-^{13}C] glycine into virginiamycin M_1 would be expected to yield [1-^{13}C] serine *via* the known serine hydroxymethyltransferase system (10). This experiment resulted in labeling the resonance at 160.0 ppm (11), indicating that this resonance can be assigned to the C-22 carbon.

These data enabled the assignments in table 1 to be made, with the only uncertainty being the assignment of the C-21, C-27, and C-30, C-31 pairs.

Assignment of the ^{13}C-nmr spectrum of antibiotic A2315A (**2**) (figure 3) followed similar lines to those described for virginiamycin M_1. The ^{1}H-nmr spectrum (figure 4) of this antibiotic has been assigned previously (6), and these assignments were confirmed by analysis of the 200 MHz ^{1}H-nmr spectrum of the antibiotic with the aid of spin decoupling measurements. Assignments of the carbon types were made by the INEPT pulse sequence method (12,13) to complement the assignments previously reported (8). The results of this study were clear in most cases, but were ambiguous in the assignment of the resonances at 134.5 and 134.7 ppm. The INEPT method, using the pulse sequence described in the experimental section to reveal only signals from methine carbon atoms, gave a signal *inter alia* for a carbon at 134.58 ppm. This signal did not correlate exactly with either of the two carbons whose resonances appear at 134.49 and 134.67 ppm (recorded in table 2 as 134.5 and 134.7 ppm). However, analysis of the proton-coupled spectrum in this region indicated clearly that the upfield signal at 134.5 ppm was a singlet and the downfield signal was a doublet, thus allowing us to make the assignments of carbon type indicated in table 2. The reason for this discrepancy is presumably because the INEPT method depends on $J_{C\text{-}H}$, and the sequence was not optimized for vinylic carbons.

Actual assignment of the resonances in the ^{13}C-nmr spectrum of A2315A was then accomplished by the knowledge of carbon type taken in conjunction with chemical shift considerations, specific frequency proton decoupling experiments, and measurement of one-bond ^{13}C-^{13}C couplings in material biosynthesized from [1,2-$^{13}C_2$] acetate and L-[U-$^{13}C_3$]serine. These data are summarized in table 2, and the resulting assignments,

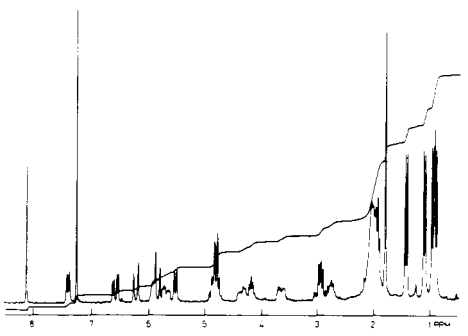

FIGURE 3. ^{13}C-nmr spectrum of antibiotic A2315A.

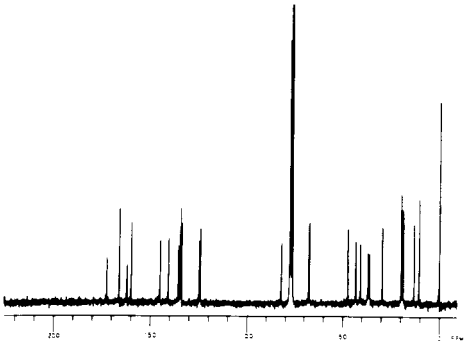

FIGURE 4. ^{1}H-nmr spectrum of antibiotic A2315A.

TABLE 2. ^{13}C chemical shift assignment of antibotic A2315A.

Carbon number	^{1}H-nmr shift of attached protons[a]	^{13}C-nmr assignment[a]	Carbon type[b]	^{13}C-nmr peaks appearing as singlets on irradiation at indicated proton frequency[c]														$^{1}J_{C-C}$ Hz[d]	
				0.95	1.10	1.43	1.79	2.00	2.80	2.90	3.00	4.20	4.80	5.50	5.70	5.84	6.58	8.10	d	e
1	—	172.6	C																	
27	2.00	29.6	CH_2				**	**												
28	0.95	18.6[g]	CH_3	**																
29	0.95	19.7[g]	CH_3	**																
3	4.80	82.3	CH										*							
4	2.80	36.8	CH						**	*	*								35	
30	1.10	10.5	CH_3		**															
5	6.60	145.1	CH														*		40	
6	5.84	124.4	CH													**			65	
7	—	166.3	C																63	
9	3.65,4.35	40.7	CH_2																	40
10	5.70	124.7	CH											*	**	*				40
11	6.22	134.7[h]	CH'																h	
12	—	134.5	C'																h	
31	1.80	13.1	CH_3				**	*												
13	5.52	134.0[h]	CH																h	
14	4.80	67.4	CH										**						40	
15	2.00	43.2	CH_2				**	**											35	
16	4.20	67.7	CH									**							40	
17	2.95	36.1	CH_2						*	**	**								56	
18	—	162.2	C																58	
20	8.10	140.7	CH																**	71
21	—	135.6	C																	70,80
22	—	160.1	C																	80
24	4.80	47.3	CH										**							
25	1.45	19.1	CH_3			**														

[a]In ppm downfield from TMS in $CDCl_3$.
[b]Determined by the INEPT sequence.
[c]Signals due to carbons not bearing protons have been omitted from this section. These appeared as sharp singlets in most of the spectra.
[d]Carbon-carbon coupling observed when [1,2-$^{13}C_2$] acetate was incorporated into A2315A.
[e]Carbon-carbon coupling observed when L-[U-$^{13}C_3$]serine was incorporated into A2315A.
[f,g]These assignments may be reversed.
[h]See text for discussion of these signals.
[i]Overlapping signals prevented detection of coupling for these resonances.
*broadened singlet.
**sharp singlet.

which were derived therefrom, are also given in that table. The only ambiguities concerned the assignments of the pairs of signals for carbons 28 and 29 and carbons 11 and 13, but these were not critical to our biosynthetic investigations and were, thus, not studied further.

EXPERIMENTAL

SPECTRA.—^{13}C-nmr spectra were recorded at ambient temperature in $CDCl_3$ solutions in 10-mm spinning tubes on a JEOL FX-60Q spectrometer operating at 13.8 Kgauss or on a Bruker WP-200 Spectrometer operating at 46 Kgauss. The typical pulse-width was 7.5 μsec (45° pulse) and the repetition time between pulses was 2.5 sec. All proton resonances were decoupled by a broad band irradiation (1.0 or 2.5 kHz) from an incoherent proton source (59.75 MHz or 200.13 MHz, respectively) for proton noise-decoupled spectra. Specific frequency proton decoupling was carried out by irradiation of the sample with low power coherent radio frequency radiation at a frequency corresponding to the absorption frequency of a specific set of protons in the antibiotic. The INEPT pulse sequence used for the spectrum of A2315A was ^{1}H: 90°(Y)-τ-180°(X)-τ-90°(X)-Δ-[^{1}H] and ^{13}C(obsd): 180°(X)-τ-90°(Y)-Δ-180°(X)-Δ, where $\Delta = \frac{1}{4}J$, $\frac{1}{2}J$, or $\frac{3}{4}J$, and $\tau = \frac{1}{4}J$. For methine carbons the value $\Delta = \frac{3}{4}J$ was used. Chemical shift values are given in parts per million relative to internal tetramethylsilane.

VIRGINIAMYCIN M_1 MONOACETATE.—Virginiamycin M_1 (100 mg) was dissolved in pyridine (4 ml) and acetic anhydride (2 ml) added to the cooled solution. The solution was allowed to stand for 1 h at 0° and was then treated with water (100 ml). The aqueous solution was extracted with chloroform (3 x 10 ml), and the extracts were combined and washed with water, dilute hydrochloric acid, and water, and dried. The crude product was purified by preparative hplc on Lichrosorb RP-8, ¼" x 25 cm, with elution by acetonitrile-water, 1:1. The isolated product had a ^{1}H-nmr spectrum that showed resonances at δ 7.84 (1H, s, 20), 7.75 (1H, m, 8), 6.60 (1H, dd, J = 16, 7 Hz, 5), 6.16 (1H, t, J = 3 Hz, 26), 6.03 (1H, d, J = 16 Hz, 6), 5.90 (1H, dd, J = 16, 2 Hz, 11), 5.8 (1H, m, 14), 5.70 (1H, dt, J = 16, 4 Hz, 10), 5.00 (1H, dd, J = 10, 2 Hz, 3), 4.85 (1H, d, J = 10 Hz, 13), 4.40 (2H, m, 24), 4.28 (2H, m, 9), 3.98 (1H, d, 17), 3.72 (1H, d, 17), 3.30 (1H, dd, J = 10, 10 Hz, 15), 2.9 (1H, m, 15), 2.8 (2H, m, 25), 1.97 (3H, s, 14-OAc), 1.64 (3H, s, 33), 1.12 (3H, d, J = 7 Hz, 32), 0.99 (3H, d, J = 4 Hz, 30), 0.96 (3H, d, J = 4 Hz, 31).

ACKNOWLEDGMENTS

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