STRUCTURAL ELUCIDATION OF MINOR COMPONENTS IN THE ANTIMYCIN A COMPLEX BY MASS SPECTROMETRY

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Mass spectrometry has been employed in the study of the antimycin A complex. This has been necessary for two reasons. On one hand, a complete fragmentation scheme is proposed based upon electron ionization, chemical ionization, and accurate mass determinations of the ions in the mass spectra of the antimycin A complex and four simple chemical derivatives. On the other hand, a more rapid qualitative and semiquantitative analysis is shown. Incidental to these findings is the interesting fact that the amide and lactone bonds undergo gas phase ammonolysis.

The antimycin A complex has found use because of its specific inhibition of certain enzyme mechanisms in the electron transport system[1,2] and in fish management. DUNSHEE, *et al.*, first crystallized the complex in 1949.[3] In 1961, two groups elucidated the structure of the antimycin A complex by means of chemical degradation.[4,5] The structure arrived at is given in Scheme 1 (Structure **I**). In addition to two R_3 groups (butyl, hexyl) and two R_4 groups (C_3, C_4), the presence of other isomeric minor constituents was mentioned.[6b]

SCHILLING, *et al.* have devised methodology for the analysis of the various components of antimycin A, based upon pyrolytic gas liquid chromatography.[6a] These authors attempted to employ gas chromatography. However, due to the thermal instability of the molecule, only pyrolytic products were found. It was interesting that an analytical scheme was developed, based on the three major peaks produced by pyrolyzing each compound. While it is true that counter current distribution was amenable for analysis,[7] 500 transfers were necessary and limitations were found for this technique whenever applied to the purity of a single component. Therefore a real need existed for a more rapid and more reliable method for qualitative and semiquantitative analysis of the components of antimycin A. SCHILLING, *et al.* proposed six antimycins (A_0 (a-d), A_5, A_6) in addition to the four known ones (A_1, A_2, A_3, A_4).[6a]

ENDO and YONEHARA developed gas chromatographic assays for the more thermally stable trimethylsilyl derivatives of the antimycin A compounds.[6b] After treatment of the antimycin A complex with mild alkali, blastymic acid and a series of antimycinones were produced. GLC analysis of the antimycinones (and the ketoacids produced by vigorously boiling the antimycinones with alkali) yielded structural elucidation information concerning the R- substituents. Other workers have transformed antimycin A with hog kidney acylase,[8] synthesized antimycin A_3[9] and separated the complex by counter current distribution.[10] While all of these methods contributed to the unraveling of the antimycin A complex, we thought that it would be quite useful to have a more direct method, involving no degradation steps, for obtaining a qualitative and semiquantitative

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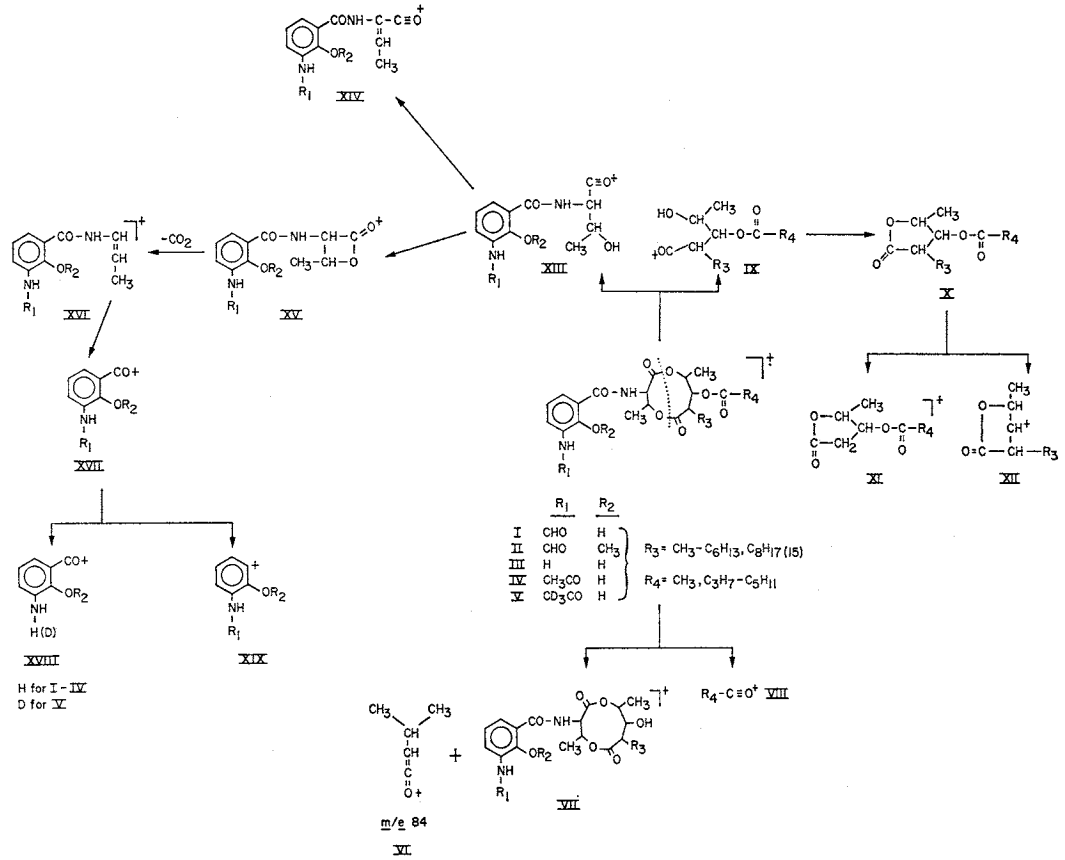
Scheme 1.



analysis of the antimycin A complex.

Accordingly, we set up two goals for this study. On one hand, we thought that it was imperative to thoroughly understand the complete fragmentation scheme of antimycin A. This is necessary, because it was found that some fragments carried information as to the alkyl and acyl substituents on the lactone. It was necessary to employ both low resolution and high resolution mass spectral techniques towards this end. In addition, simple chemical modifications to the aromatic ring substituents proved to be useful.

On the other hand, it was necessary to obtain information concerning the molecular ion region. When dealing with a series of homologous compounds, the mass spectra contain peaks differing by 14 mass units in the molecular ion region. In those cases, one must confirm that these ions are molecular ions, and not potential fragments arising from long, aliphatic chains. Thus, we employed a useful adjunct to electron ionization (EI) mass spectrometry known as chemical ionization (CI).[11] In CI, the pressure in the ion source is increased with a gas (methane, butane, or ammonia), a series of fast ion-molecule reactions occurs, and the vaporized molecule of interest (antimycin A) is ionized by the ion-molecule reaction products. CI processes involve much less energy and fragmentation is usually minimized. (In more complex biological compounds, however, fragmentation may still occur.) However, the molecular ion region is now usually clearly defined by the appear-

ance of adduct ions. With ammonia, for example, $(M+H)^+$ and $(M+NH_4)^+$ ions are produced. Thus, each molecular ion in a complex mixture can be determined.

Materials and Methods

The antimycin A complex was obtained from Nutritional Biochemicals Co., Cleveland, Ohio and was used as received.

Acetic anhydride-d_6 was purchased from Merck Canada.

Derivatives were formed as follows: *O*-methylation-diazomethane treatment; deformylation-dissolve antimycin in methanol, treat with concentrated HCl[5]; acetylation-methanol: acetic anhydride (4:1) for 3 hours.

Low resolution electron ionization (EI) mass spectra were obtained on an LKB 9000 instrument (70 eV ionizing voltage, 3.5 kV accelerating voltage, 60μA trap current, direct introduction probe temperature=135°C, source temperature=270°C). High resolution EI spectra were obtained on a CEC 21-110B instrument (70 eV ionizing voltage, 8.4 kV accelerating voltage, 100μA trap current, direct introduction probe temperature=200°C). All ion currents were integrated on an Ilford Q2 photoplate. The accurate masses (for each ion in all of the spectra) were obtained by normal computer methods to within three millimass units (mMU), unless otherwise noted.

It was necessary to study the EI and CI mass spectra of the following series of compounds-underivatized antimycin A (**I**), *O*-methyl antimycin A (**II**), deformylated antimycin A (**III**), deformylated, *N*-acetylated antimycin A (**IV**) and deformylated, *N*-d_3 acetylated antimycin A (**V**).

Low resolution chemical ionization (CI) mass spectra were obtained on the above CEC 21-110B, modified for high pressure work, as described.[12] Methane and ammonia were employed as reagent gases for CI.

With methane as reagent gas, satisfactory spectra were also obtained. However, a very interesting complexity arose out of the use of this gas. As we are dealing with a homologous series of compounds, some of the adduct ions of methane [$(M+CH_5)^+$ and $(M+C_2H_5)^+$] overlapped the molecular ions and, in addition, differed by 14 and 28 mass units, as the homologs do. Therefore, we do not present the CI spectra with methane.

Results and Discussion

Table 1 contains the accurate masses for the ions I-XIX of Scheme 1 for the four derivatives.

Fig. 1 is the low resolution EI mass spectrum of the underivatized antimycin A complex (**I**). Scheme 1 contains the fragmentation pattern for compounds **I**~**V**. From the previous structural elucidation work, it is known that the molecular ion (m^+) of the molecule containing R_3=butyl and R_4=butyl should be at *m/e* 520 and of R_3=hexyl and R_4=butyl at *m/e* 548. However, from Fig. 1, one observes peaks at *m/e* 492, 506, 534 and 562 corresponding to R_3=ethyl, propyl, pentyl and heptyl (all for R_4=butyl). The ion at *m/e* 478 may be due to R_3=methyl, or due to the overlap caused by the loss of structure **VI** in the scheme. This point will be resolved below. The ions at *m/e* 408, 422, 436, 450, 464 and possibly 478 are due to the loss of R_4CO from the molecular ion. This was verified by accurate mass measurements; only ions containing eight oxygen atoms were found. This set of ions also defines R_3 as being ethyl through heptyl, inclusive. The two intense ions at *m/e* 418 and 446 correspond to the loss of water from ion VII for the more abundant compounds where R_3=butyl or hexyl respectively.

Up to this point, we have assumed R_4 to be a butyl group as previously reported. However, accurate mass measurement shows that the ion XI, (*m/e* 200.1056) contains the moiety $C_6H_7O_4$ in addition to an alkyl side chain (C_4). From the antimycin structure, a four oxygen fragment can be produced either from the intact dilactone ring or by breaking the lactone ring and including the

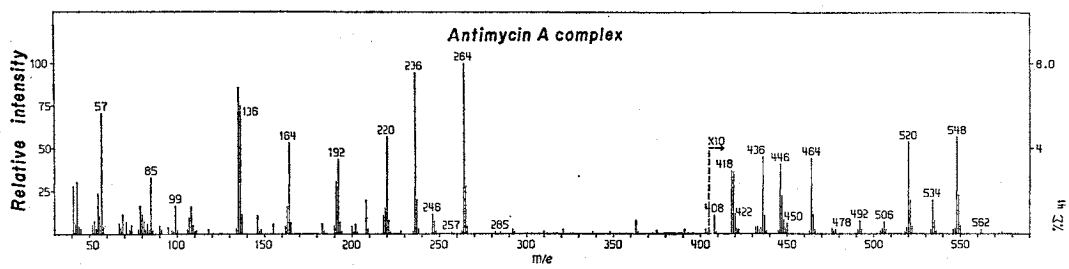
Table 1.

Ion	No. of Carbons	Underivatized	Deformylated	Deformylated acetylated	Deformylated d_3-acetylated
I~V	$R_3+R_4=7$	506.2275(+1.1)		520.2410(−1.0)	523.2610(−0.1)
	$R_3+R_4=8$	520.2429(+0.8)	492.2470(−0.2)	534.2596(+1.8)	537.2767(+0.2)
	$R_3+R_4=9$	534.2551(−2.7)		548.2726(−0.7)	551.2895(−2.7)
	$R_3+R_4=10$	548.2733(−0.1)		562.2875(−1.6)	565.3053(−2.5)
VI		84.0574(−0.5)	84.0580(+0.1)	84.0568(−0.7)	84.0558(−1.7)
VII	$R_3=4$	436.1843(−0.2)		450.1981(−2.1)	453.2176(−1.4)
	$R_3=5$	450.1959(−4.3)			
	$R_3=6$	464.2128(−3.1)		478.2314(−0.1)	481.2482(−2.2)
VIII		85.0640(−1.4)	85.0651(−0.3)	85.0669(+1.6)	85.0655(+0.2)
IX	$R_3+R_4=8$	257.1738(−1.5)		257.1736(−1.7)	257.1740(−1.3)
	$R_3+R_4=9$	271.1897(−1.2)		271.1894(−1.6)	271.1890(−1.9)
	$R_3+R_4=10$	285.2060(−0.6)		285.2046(−2.0)	285.2050(−1.6)
XI	R_4=methyl	158.0582(+0.3)		158.0568(−1.2)	158.0557(−2.2)
	R_4=ethyl				
	R_4=propyl	186.0879(−1.3)		186.0881(−1.1)	186.0878(−1.4)
	R_4=butyl	200.1056(+0.8)	200.1043(−0.5)	200.1042(−0.7)	200.1046(−0.3)
	R_4=pentyl	214.1178(−2.7)		214.1177(−2.8)	214.1187(−1.8)
	R_4=hexyl				
XII	R_3=methyl	113.0587(−1.5)	113.0600(−0.2)	113.0593(−1.0)	113.0589(−1.3)
	R_3=ethyl	127.0744(−1.5)	127.0763(+0.4)	127.0752(−0.7)	127.0739(−2.0)
	R_3=propyl	141.0903(−1.3)	141.0917(+0.1)	141.0923(+0.7)	141.0881(−3.4)
	R_3=butyl	155.1070(−0.2)	155.1070(−0.2)	155.1070(−0.2)	155.1054(−1.8)
	R_3=pentyl	169.1224(−0.5)	169.1215(−1.4)	169.1209(−2.0)	169.1204(−2.5)
	R_3=hexyl	183.1374(−1.1)	183.1370(−1.5)	183.1365(−2.0)	183.1375(−1.0)
	R_3=heptyl				
	R_3=octyl	211.1671(−2.7)			211.1678(−2.0)
XIII		265.0789(−3.6)	237.0853(−2.2)	279.0962(−1.9)	282.1120(−4.9)
XIV		247.0711(−0.8)	219.0752(−1.8)	261.0861(−1.5)	264.1033(−3.0)
XV		264.0750(+0.4)	236.0785(−1.2)	278.0903(+0.1)	281.1089(−0.2)
XVI		220.0818(−2.9)	192.0882(−1.7)	234.0983(−2.1)	237.1151(−4.2)
XVII		164.0357(+0.9)	136.0396(−0.3)	178.0509(+0.5)	181.0678(−1.4)
XVIII		136.0392(−0.7)	136.0396(−0.3)	136.0392(−0.7)	137.0457(−0.4)
XIX		136.0392(−0.7)	108.0450(±0)	150.0532(−2.3)	154.0823*(+0.2)

* additional H

The number in parentheses is the difference, in millimass units, between that mass corresponding to the elemental composition of the ion fragment and the given, found mass.

Fig. 1. EI Spectrum—Underivatized antimycin A complex.



R_4-ester group. The former possibility requires $C_{10}H_{10}O_4+R_8$. Therefore, structure **XI** is the only possible structure. For the data for ion XI in Table 1, we saw ions that correspond to R_4= methyl, propyl, butyl and pentyl in a ratio (as integrated on the photoplate) of 9:11:71:9. This finding agrees well with previous workers who found predominantly R_4=butyl.

The nature of the alkyl side chain (R_3) is corroborated by the presence of ions corresponding to Ion XII. In Table 1, the presence of methyl through hexyl, plus octyl groups is indicated.

We stated above that *m/e* 562 in Fig. 1 was due to R_3=heptyl and R_4=butyl. However, we now see that there is no R_3=heptyl. Thus *m/e* 562 is actually due to the coincidence of two mole-

Fig. 2. CI Spectrum—Underivatized antimycin A complex (NH_3, 0.9 torr).

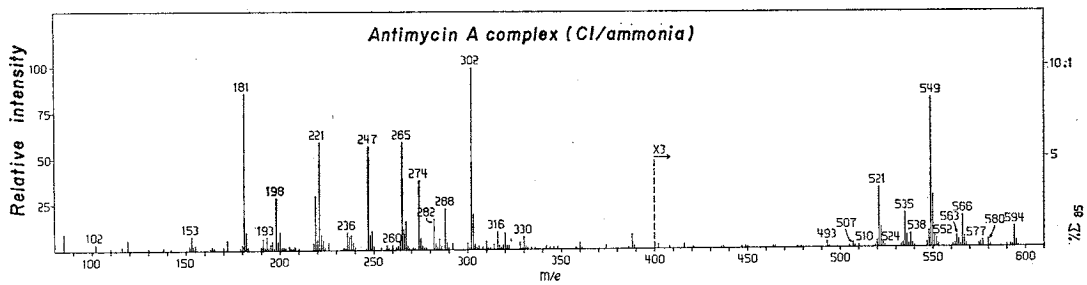


Table 2.

No. of Carbon Atoms (R_3+R_4)	$m/e(M+1)^+$	$m/e(M+18)^+$
5	479	—
6	493	510
7	507	524
8	521	538
9	535	552
10	549	566
11	563	580
12	577	594

cules; R_3=octyl, R_4=propyl and R_3=hexyl and R_4=pentyl.

With these seven possible R_3 groups and these four possible R_4 groups one calculates 28 possible, different molecular components in the antimycin A complex. This analysis yields a mixture more complex than has been reported to date.

At this point in our analysis of such a mixture of homologous compounds, we thought it judicious to obtain the CI spectrum of this complex.[13] A recent paper also reports the use of CI for macrolides.[14]

With ammonia as a reagent gas, an ion corresponding to $(M+H)^+$ and $(M+NH_4)^+$

Table 3.

No. of Carbon atoms			*m/e*	# Possibilities
R_3+R_4	R_3=	R_4=		
5	1	4	478	3
	2	3		
	4	1		
6	1	5	492	4
	2	4*		
	3	3		
	5	1		
7	2	5	506	4
	3	4*		
	4	3		
	6	1		
8	3	5	520	3
	4	4*		
	5	3		
9	4	5	534	4
	5	4*		
	6	3		
	8	1		
10	5	5	548	2
	6	4*		
11	6	5	562	2
	8	3		
12	8	4*	576	1

The asterisks indicate the more abundant components.

would normally be produced for each molecular species, and potential molecular ions are thus "double-checked." (As mentioned above, methane was also employed, but was found to be useless, as there was an overlap of compound ions and adduct ions.)

Fig. 2 contains the CI spectrum of the underivatized antimycin A complex. The ions in the molecular ion region are analyzed in Table 2.

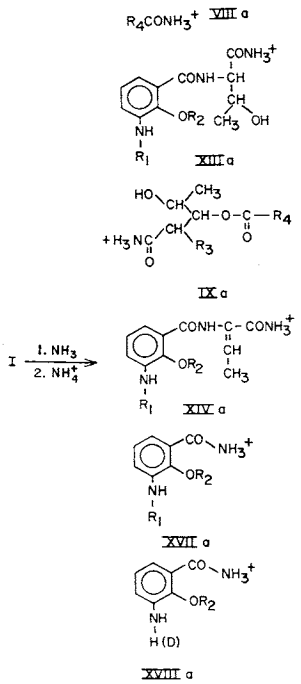
These data in Table 2 show that *molecular* species can exist at *m/e* 478, 492, 506, 520, 534, 548, 562 and 576. Normally, these ions represent eight molecular species. However, we have shown that combinations of R_3 and R_4 are present, and that more than one component occurs at any given *m/e* value. Therefore, we must further analyze the molecular ion region as in Table 3.

Thus, by means of mass spectrometry the number of possible compounds (28) is reduced to 23. That this is a small reduction of possibilities is due to the eight observable molecular ions each containing 1-4 molecular species.

Other Derivatives Studied

In order to confirm the ions in Scheme 1, advantage was taken of the mass spectral "shift" technique by simple chemical modifications of the aromatic ring substituents. The following compounds were subjected to mass spectrometry: *O*-methyl antimycin A (**II**), deformylated antimycin A (**III**), deformylated *N*-acetylated antimycin A (**IV**), and deformylated *N*-d_3-acetylated antimycin A (**V**). Due to space limitations, the mass spectra of **II**~**V** will not be presented. However these four spectra corroborate completely all of the peaks in Scheme 1.

Scheme 2.



Further corroboration of the ions given in the scheme came from an interesting gas phase reaction. As noted by ENDO and YONEHARA[6b], mild alkali opens the lactone ring producing blastymic acid and the antymicinones. With ammonia as a CI reagent gas, we noticed ammonolysis occurring, with the production of amides, which were then protonated to form the ions VIIIa, IXa, XIIIa, XIVa, XVIIa, and XVIIIa (Scheme 2). All of these ions in Fig. 2, at *m/e* 102, 274, 282, 264, 181 and 153, respectively (for $R_3+R_4=8$ carbons), further corroborate the analysis of the lactone alkyl and acyl groups. This ammonolysis reaction has also been reported for proline dipeptides.[15]

Other R-groups

Close scrutiny of all of the CI spectra reveals that for $R_3+R_4=12$ carbon atoms, a small ion occurs two mass units lower. As R_4 is predominantly butyl, R_3 could be a cyclic structure or a monoene.

Conclusions

From this study, one can conclude that EI and CI mass spectrometry have significant advantages to offer as a more rapid and more reliable method for qualitative and semiquantitative

analysis of the antimycin A complex. This report has shown that, in addition to the ten known components,* thirteen further components are present.

The following semi-quantitative results can be presented, based upon the ratios of R_4= C_1:C_3:C_4:C_5=9:11:71:9, and [R_3+R_4]=(7:8:9:10) Carbon atoms =1.25:38.5:13:47-

The sum of these percentages equals 87 %. That this is less than 100 % is due to the lower sensitivity to ion detection on an emulsion as compared to electrical detection. The latter method has detected ions corresponding to 5, 6, 11 and 12 carbon atoms for [R_3+R_4]. These four values make up most of the remaining percentage.

[R_3+R_4] No. of carbon atoms	R_3	R_4	%
7	6	1	0.1
	2	5	0.1
	4	3	0.1
	3	4	0.9
8	3	5	4
	5	3	4
	4	4	27
9	8	1	1.7
	6	3	1.7
	5	4	9
	4	5	1.7
10	6	4	33
	5	5	4

All of the findings presented here agree with previous chemical findings. In addition, some other minor components have been found. We feel that the procedures outlined in this paper could offer some advantages to workers investigating antibiotics composed of components that may be extremely difficult, or virtually impossible, to separate. As illustrated with the antimycin A complex, these mass spectral procedures may be more rapid and more reliable qualitative and semiquantitative methods.

Acknowledgments

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* The following antimycin A components have been summarized in reference 6a:

Component	Number of Carbons			Component	Number of Carbons		
	alkyl (R_3)	acyl (R_4)	(R_3+R_4)		alkyl (R_3)	acyl (R_4)	(R_3+R_4)
A_0 a.	6	6	12	A_2	6	4	10
b.	4	7	11	A_3	4	5	9
c.	8	4	12	A_4	4	4	8
d.	7	5	12	A_5	2	5	7
A_1	6	5	11	A_6	2	4	6

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