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The Structural Elucidation of Polyene Macrolide Antibiotics by Mass Spectrometry. Nystatin, Amphotericin B and Pimaricin

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Abstract Complete mass spectra of the pertrimethylsilylated derivatives of three high molecular weight polyene macrolide antibiotics are reported for the first time. The fragmentation pathways which are proposed have been corroborated by the stable isotope derivatives d_9-TMS and d_3-acetyl. Accurate mass measurements and metastable transitions confirm the proposed fragmentation mechanisms in the low mass range. Nystatin—the macrolide of highest molecular weight in this study—expels several TMSOH molecules and a TMS radical. Amphotericin B underwent extensive rearrangements preliminary to eliminating a series of TMSOH molecules. An apparent equilibrium between the keto and ketal forms of amphotericin B facilitated the rearrangements. Pimaricin fragmented in a manner parallel to that of the other two macrolides. The sugar portion of the molecules dominated the fragmentation in the low mass region in the spectra of all compounds. The transfer of a TMS group from the sugar amine to the glycosidic oxygen was observed when the amino sugar was eliminated from the intact molecule. The resulting sugar ion then expelled ammonia.

Introduction

POLYENE macrolides constitute a diverse and extensive class of compounds containing many membered lactones of a polyhydroxylic nature of high molecular weight.[1–3] These polyenic antifungal antibiotics are produced by the *Streptomycetes* species of microorganisms; about 50 polyene macrolides are known.[4] The structural elucidation of these interesting compounds has occupied many laboratories for the past two decades. Mass spectrometry has not been employed extensively in the elucidation of the intact molecule due to the high molecular weight and the large number of functional groups present.

Filipin is the least complicated (and for that reason perhaps the most studied) polyene macrolide. Djerassi,[5,6] Ceder,[7] Golding and Rickards[8,9] have elaborated the structure of filipin. Mass spectrometry was employed recently in studies of the peracetylated[10,11] and permethylated[12] derivatives of filipin.

Other polyene macrolides studied include lagosin,[13] oligomycin,[14,15] nystatin,[16-19] amphotericin B[20-22] and pimaricin.[23-32] The use of mass spectrometry in these earlier studies was limited to determine molecular weights. No other mass spectral details were given.

Nystatin, the first polyene macrolide discovered, was isolated in 1950 by Hazen and Brown from *S. noursei*.[33] The molecular composition and weight of the compound are $C_{47}H_{75}NO_{17}$ and 925.5035. The chromophore is composed of a tetraene and a diene. The 38-membered lactone ring has the following substituents: the amino sugar mycosamine (3-amino-3,6-dideoxy-D-mannopyranose), one carboxyl, one keto and eight hydroxyl groups. Chong and Rickards in 1970 completed the structural elucidation of nystatin.[19]

Amphotericin B is produced by *S. nodosus* and was first isolated by Vandeputte *et al.* in 1956.[34] The molecular composition and weight are $C_{47}H_{73}NO_{17}$ and 923.4879. Like nystatin, it is composed of a 38-membered ring with the same substituents. In contrast to nystatin, however, the chromophore of amphotericin B is a conjugated, all-*trans* heptaene. The three dimensional structure of *N*-iodoacetylamphotericin B was elucidated by Mechlinski, *et al.* in 1970 by means of X-ray analysis of a single crystal.[35] The keto function at C-13 was shown to form a hemiketal with the hydroxyl group at C-17.

Pimaricin, produced by *S. natalensis*, was isolated in 1958 by Struyk, *et al.*[36] The molecular composition and weight of pimaricin are $C_{33}H_{47}NO_{13}$ and 665.3047. Pimaricin is composed of a 26-membered lactone ring. The chromophore is a tetraene plus a diene. The latter is in conjugation with the lactone carbonyl group. The substituents on the ring include three hydroxyl groups, one keto group, one epoxide and the mycosamine sugar. Meyer established the position of the sugar[31] and Golding *et al.*[32] elucidated the structure of this macrolide antibiotic.

Interest in the chemistry of polyene macrolides[37] was enhanced due to reports that a wider range of biochemical effects had been discovered in addition to the known antifungal activity; these effects include increased membrane permeability,[38] a high degree of association with cholesterol[39] and the ability to decrease prostatic swelling.[40]

As we have been studying the chemistry and mass spectrometry of some antibiotics[41] and other high

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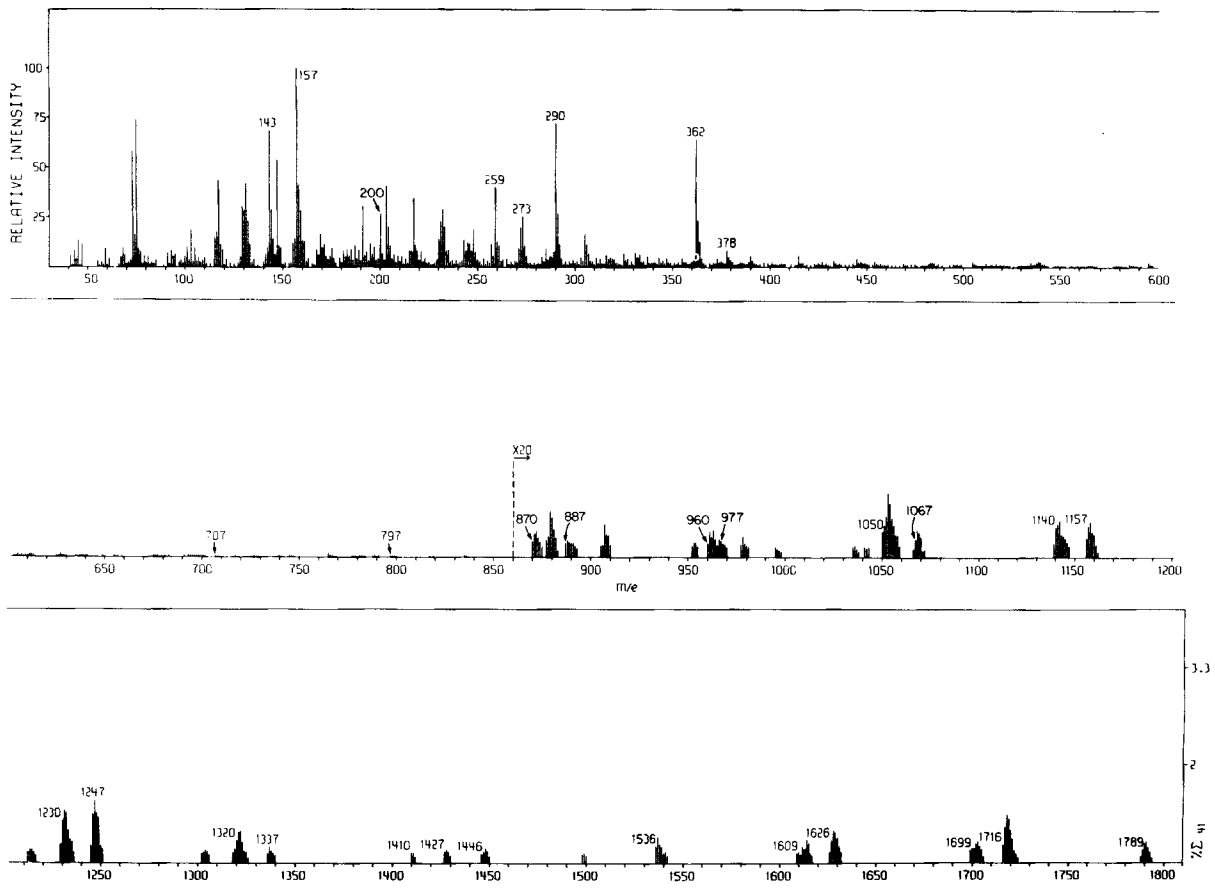


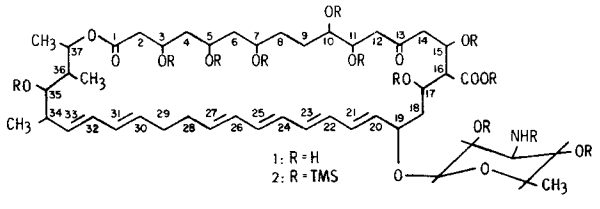
FIG. 1. Low resolution mass spectrum of per-TMS nystatin (**2**).

molecular weight compounds, we extended our research to include nystatin, amphotericin B and pimaricin. The purpose of this paper is to report the mass spectral data of these macrolides as their per-TMS and *N*-acetyl-*O*-per-TMS derivatives.

Results and discussion

Nystatin (**1**)

The structure of nystatin is **1** and of per-TMS nystatin **2**. The mass spectrum of **2** is given in Fig. 1.



The molecular ion of **2** is represented by the cluster of ions around *m/e* 1789.†

The electron ionization induced fragmentation of **2** is straightforward and four modes of fragmentation occur:

† The calculated relative intensities of the ion clusters are complicated by the observance of losses of H· and H_2 for some of the ions. Therefore, the isotopic pattern obtained for these ions is the result of two to three superimposed ion clusters and may differ from the relative intensities calculated for this molecular composition.

(1) The loss of a TMS group leads to the ion (cluster) at *m/e* 1716. Three molecules of TMSOH are eliminated from *m/e* 1716 to form the ions at 1626, 1536 and 1446. (On the original oscillographic recording, the loss of up to nine TMSOH moieties could be accounted for, but these ions were of rather low abundance).

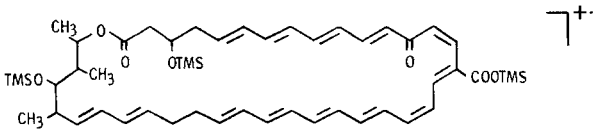
(2) The loss of the amino sugar portion—with retention of the glycosidic oxygen by the aglycone—produces the $[M - 362]^+$ ion at *m/e* 1427. This ion loses in succession eight molecules of TMSOH to yield the ions at *m/e* 1337, 1247, 1157, 1067, 977, 887, 797 and 707.

(3) The expulsion of the neutral sugar moiety forms the $[M - 379]^{\ddagger}$ ion at *m/e* 1410. The required hydrogen atom for this process probably arises from C-18 to produce an ion where the conjugation is extended. Up to six molecules of TMSOH are then eliminated from this ion to form the series of ions at *m/e* 1320, 1230, 1140, 1050, 960 and to produce finally the highly conjugated ion *a* at *m/e* 870. The strain inherent in an all-*trans* polyene system may prohibit the loss of the two remaining TMS groups as TMSOH moieties.[37]

(4) The loss of one and two molecules of TMSOH from $[M]^{\ddagger}$ generates the ions at *m/e* 1699 and 1609, respectively.

Scheme 1 rationalizes the genesis of the ions in the low mass region of Fig. 1. The ions at *m/e* 378 and 362 are due to the mycosamine portion—with and without

the glycosidic oxygen—respectively. The large ion at *m/e* 290 is due to the loss of the aglycone and $(CH_3)_2$—Si=CH_2 to form ion *b'*. Accurate mass measurement confirms the elemental composition of



ion a, m/e 870

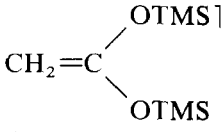
this ion. A metastable transition relates the ion at *m/e* 273 to that at *m/e* 290. Accurate mass measurement shows that NH_3 is expelled. The resulting ion (*c*, *m/e* 273) is stabilized by the conjugated diene system. Finally, the loss of TMSOH from ion *b* yields the ion at *m/e* 200.

Fragmentation of the substituted ketene produced by opening of the lactone ring yields the ions at *m/e* 143, 157, 259 and 273 (see Scheme 2). The ion at *m/e* 273 has the same elemental composition and the same number of TMS groups as ion *c* (Scheme 1), and therefore cannot be distinguished by accurate mass measurements or d_9-TMS labelling.

In all of the mass spectra presented, abundant ions are formed by the interaction of remote TMS groups. For example, the following ion structures are associated with the *m/e* values for the protium (d_0) and the deuterium (d_9) derivatives:[42]

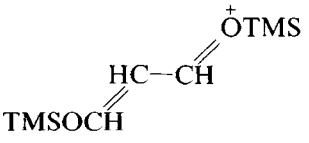
$[(CH_3)_3—Si]^+$
d_0: *m/e* 73
d_9: *m/e* 82

$[(CH_3)_3—Si—\overset{+}{O}=Si(CH_3)_2]$
d_0: *m/e* 147
d_9: *m/e* 162



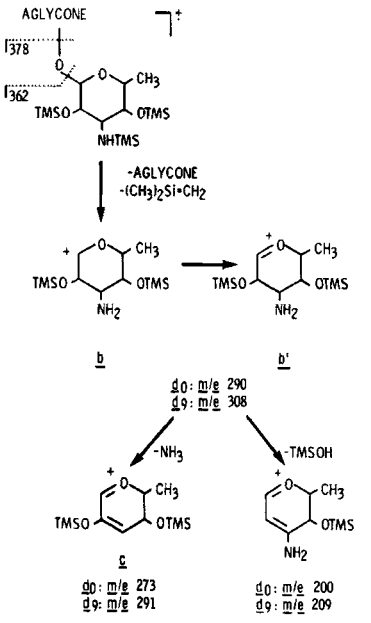
d_0: *m/e* 204
d_9: *m/e* 222

$[(CH_3)_2—Si=O]^{+\cdot}$
d_0: *m/e* 75
d_9: *m/e* 81

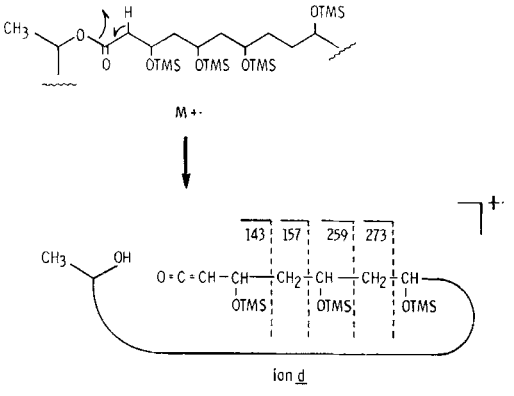


d_0: *m/e* 217
d_9: *m/e* 235

The fragmentation pattern of nystatin—the compound of highest molecular weight studied here—is therefore relatively straightforward and uncomplicated. The majority of the ion current is accounted for by the loss of neutral molecules, while the lesser part is due to cleavage products. The apparent driving force behind



SCHEME 1.



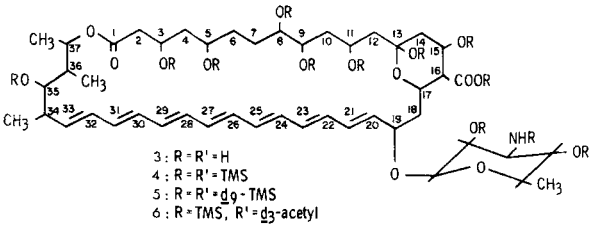
(The m/e values given are for d_0-TMS derivatives)

SCHEME 2.

most of the fragmentation processes is the extension of the conjugated polyene system. This energetically favorable process is also facilitated by the stability of the leaving groups—TMSOH and the amino sugar.

Amphotericin B (**3**)

The structure of amphotericin B is given as **3**, the per-TMS derivative as **4**, the per-d_9-TMS derivative as **5** and the *N*-d_3-acetyl-*O*-per-TMS derivative as **6**. The mass spectra of **4** and **5** are presented in Figs. 2 and



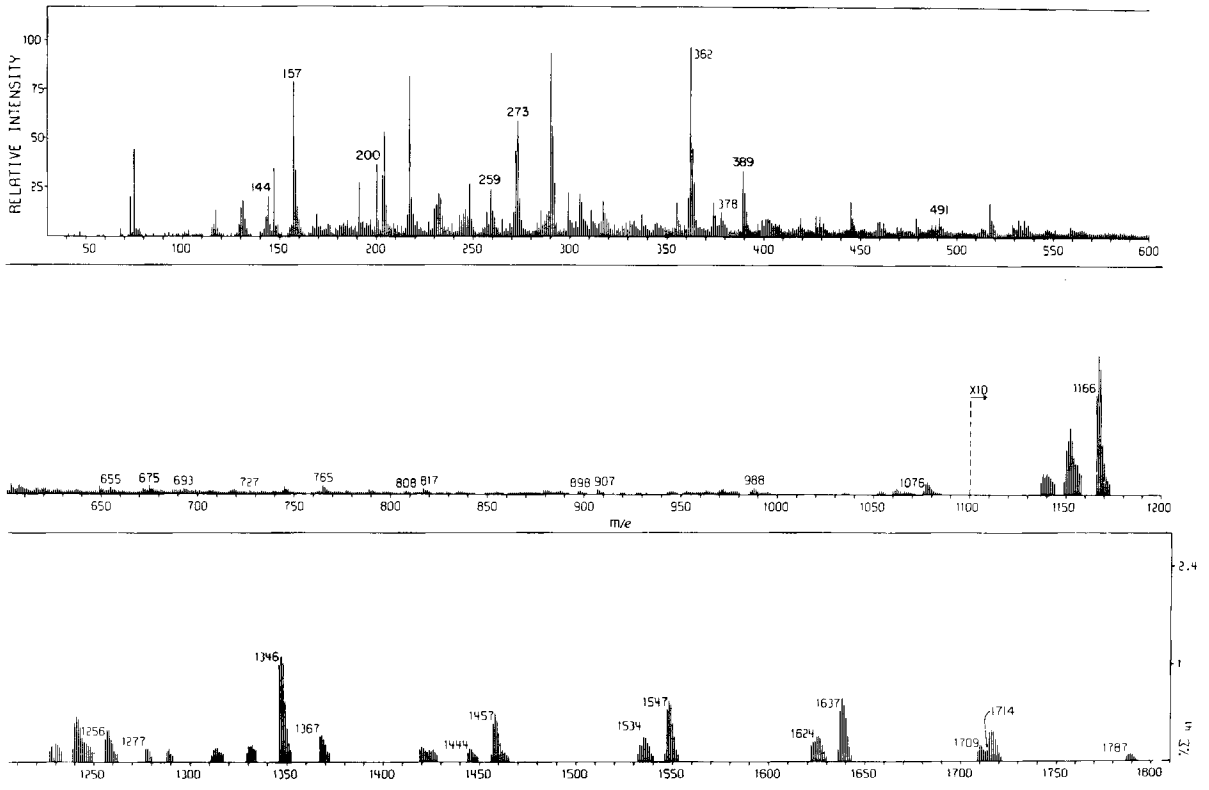


FIG. 2. Low resolution mass spectrum of per-TMS amphotericin B (**4**).

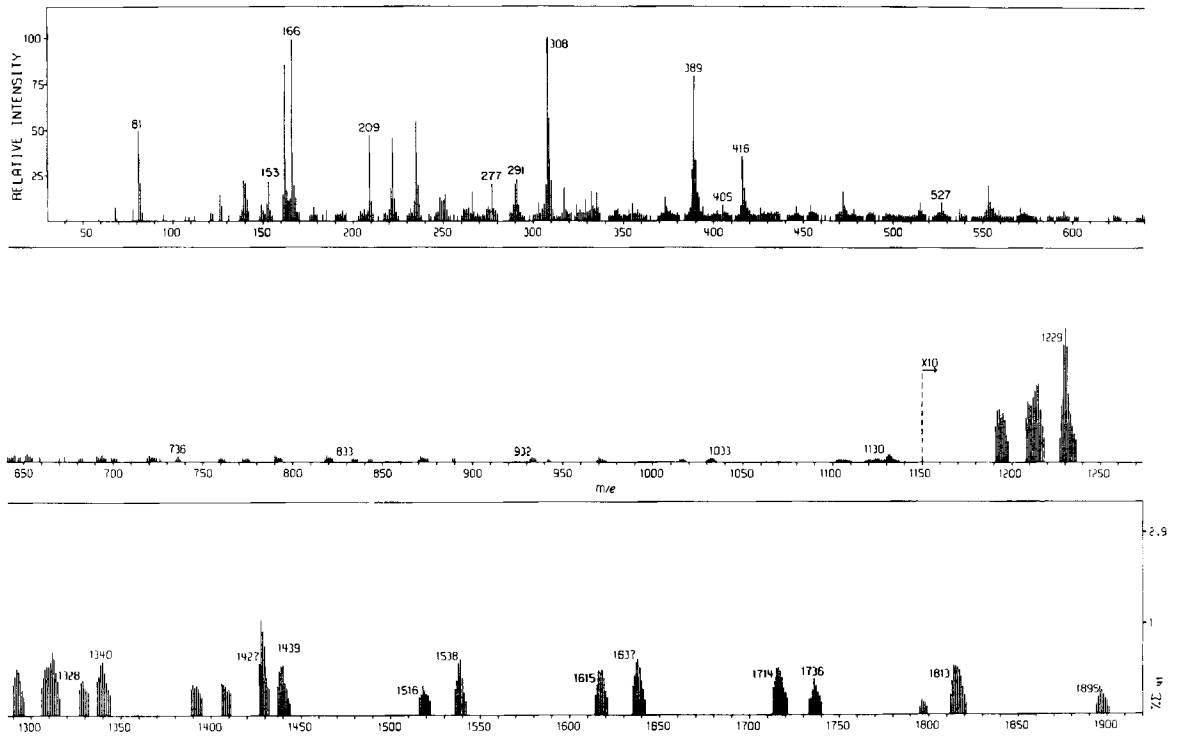
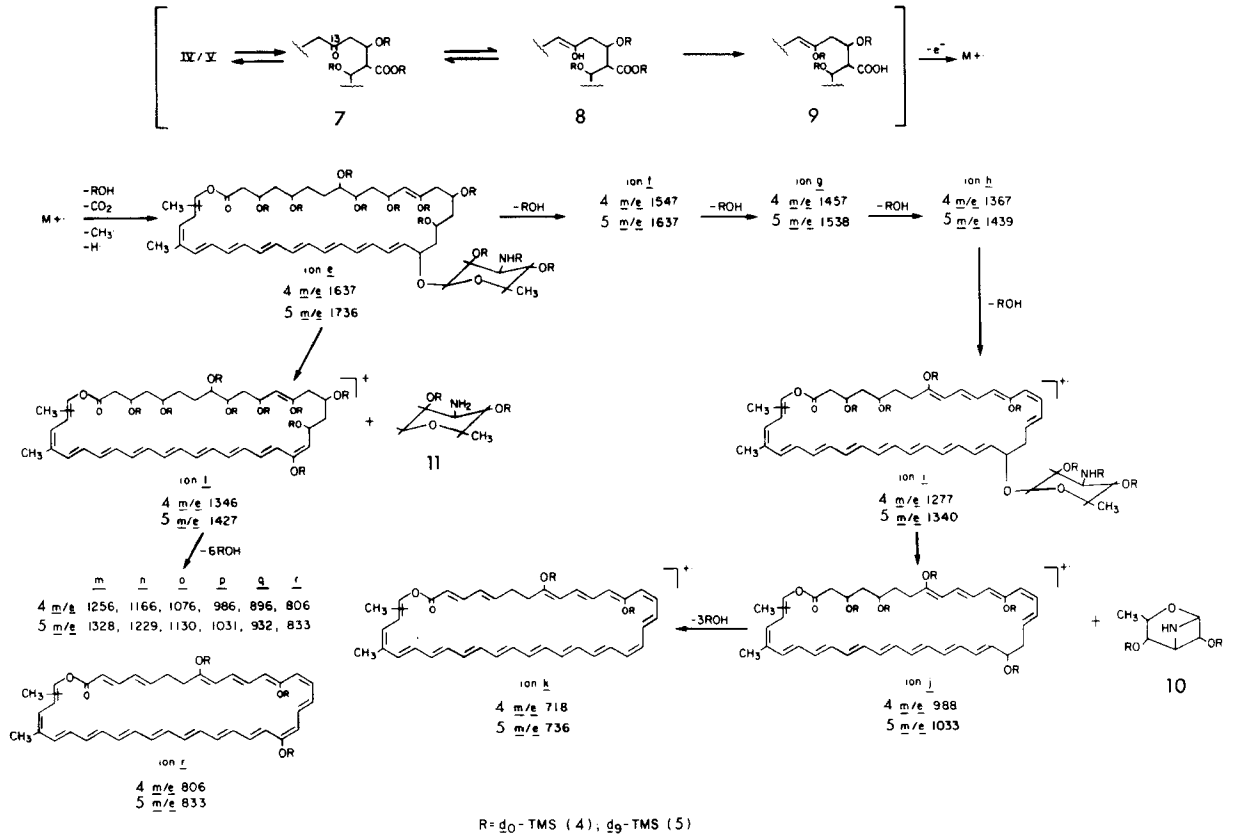


FIG. 3. Low resolution mass spectrum of per-d_9-TMS amphotericin B (**5**).

3, respectively. In Fig. 2, the molecular ion of **4** is represented by the cluster of ions at *m/e* 1787. This cluster of ions shifts 108 a.m.u. (12 × 9) to *m/e* 1895 in the mass spectrum of **5** in Fig. 3. The molecular ion for compound **6** is accordingly found at *m/e* 1760 (spectrum not shown).

Loss of a TMS group from $[M]^{+\cdot}$ yields the ion at *m/e* 1714 for **4**, *m/e* 1813 for **5** and *m/e* 1687 for **6**. Expulsion of 78 a.m.u. from $[M]^{+\cdot}$, which is observed in the spectrum of **4** to give rise to the ion at *m/e* 1709, is most probably due to the loss of C_6H_6 from the heptaene system. The corresponding ion for



SCHEME 3.

5 unfortunately is not clearly distinguished because the ion cluster for $[M\text{-}d_9\text{-TMS}]^+$ overlaps this mass region.

One of the major fragmentations that **4** undergoes is to expel 150 a.m.u. to form ion *e* (Scheme 3) at *m/e* 1637. This ion shifts to *m/e* 1736 in Fig. 3, indicating that one TMS group is lost in the 150 a.m.u. moiety. A plausible route to form $[M - 150]^+$ is given in Scheme 3. This process requires that the molecular ion be formed from the equilibrium involving structures **7**, **8** and **9**. This sequence involves an opening of the ketal ring (**7**), followed by enolization (**8**) and transfer of the carboxylic TMS group to the newly formed C-13 hydroxyl group to form **9**.

Ion *e* is then formed by eliminating TMSOH, CO_2, CH_3· and H· (or CH_4) from $[M]^+$ However, it is also possible that ion *e* is formed by losing CO_2 directly from the lactone,[43] followed by loss of TMSOH, CH_3· and H·.

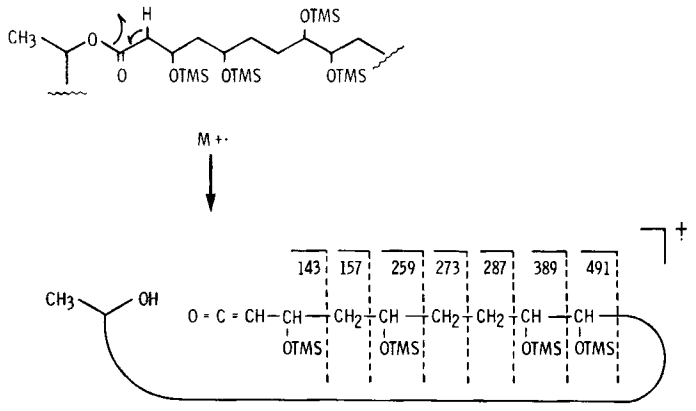
While it is usually energetically unfavorable to lose two radicals from an ion radical, sufficient energy to overcome this barrier is provided by the extension of the conjugated polyene system. The CH_3· lost is not from a TMS group (no corresponding shift in the spectrum of **5**, Fig. 3, was observed), but most likely originates from either C-36 or C-37.

Ion *e* eliminates up to four molecules of TMSOH in succession to form ions *f*, *g*, *h* and *i* at *m/e* 1547, 1457, 1367 and 1277, respectively (for **5** these ions are observed at *m/e* 1637, 1538, 1439, 1340). Ion *i* undergoes rearrangement to transfer the amine TMS group to the glycosidic oxygen to form ion *j* at *m/e* 988 and the neutral sugar moiety **10** (for **5**: *j* = 1033). Ion *j* loses three more TMSOH molecules to produce ion *k* at *m/e* 718 (for **5**: *k* = 736). This highly conjugated ion is similar to the corresponding species found in the mass spectrum of permethylated filipin.[12]

On the other hand, ion *e* directly eliminates a neutral sugar molecule (**11**), with concomitant transfer of the amine TMS group to the aglycone to form ion *l* at *m/e* 1346 (for **5**: *l* = 1427). This process involves the transfer of two hydrogen atoms probably from C-18 and C-19 to the amino sugar. One hydrogen atom rearranges to the amine group (from which TMS is transferred), while the other rearranges to C-1 of the sugar. Ion *l* loses up to six TMSOH to form ions *m*, *n*, *o*, *p*, *q* and *r* at *m/e* 1256, 1166, 1076, 986, 896 and 806, respectively (for **5**: *m/e* 1328, 1229, 1130, 1031, 932, 833).

By another pathway, up to three TMSOH are eliminated from $[M - TMS]^+$ at *m/e* 1714 (*m/e* 1813 for **5**) to form the ions at *m/e* 1624, 1534 and 1444 (*m/e* 1714, 1615 and 1516 for **5**).

The base peak in Fig. 2, *m/e* 362, is due to the mycosamine moiety, as shown in Scheme 1. The presence of three TMS groups in this moiety causes this ion to shift 27 a.m.u., to *m/e* 389, in Fig. 3; the corresponding ion for **6** is found at *m/e* 335. The loss of TMSOH from *m/e* 362 is supported by a metastable ion for the transition 362 → 272 in the spectrum of **4**. The abundant ions at *m/e* 290, 273 and 200 arise from the sugar moiety as was described for nystatin in Scheme 1 (for **5** see the ions at *m/e* 308, 291 and 209).



(The m/e values given are for d_0-TMS derivatives)

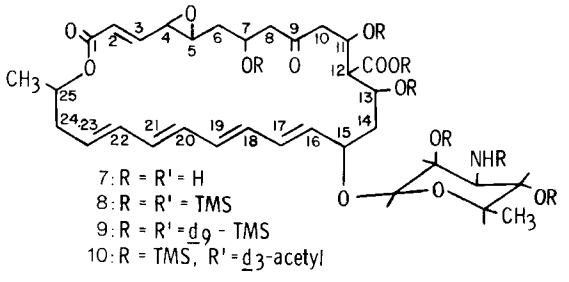
SCHEME 4.

Scheme 4 rationalizes the genesis of the other abundant ions in the low mass range of Fig. 2. As amphotericin B and nystatin are equivalent in structure between C-1 and C-6, the same fragments are observed, as shown in Scheme 4: *m/e* 144 (+H), 157, 259 and 273. In addition, however, the ions indicated in Scheme 4 are found at *m/e* 287, 389 and 491. These ions shift appropriately in the mass spectrum of **5** (Fig. 3) to *m/e* 153, 166, 277, 291, 305, 416 and 527, respectively.

Even though the structures of amphotericin B and nystatin are closely related, their mass spectral fragmentation patterns are quite dissimilar. One explanation for the difference in fragmentation could be due to the hemiketal ring in amphotericin B, which may not be present originally in nystatin. Another important factor to consider is the temperature necessary to evaporate the two compounds. Amphotericin B vaporizes at 180 °C whereas nystatin evaporates at 230 °C. The difference in temperature can lead to quite different fragmentation processes due to subtle structural transformations.

Pimaricin (**12**)

The structure of per-TMS pimaricin is **13**, of per-d_9-TMS pimaricin is **14** and of *N*-d_3 acetyl, *O*-per-TMS



4

pimaricin is **15**. The mass spectra of **13** and **14** are given in Figs. 4 and 5, respectively. The temperature of vaporization for **13** and **14** is 140 °C.

The molecular ion of **13** is represented by the cluster of ions at *m/e* 1169 in Fig. 4. This ion shifts 63 a.m.u. to *m/e* 1232 in Fig. 5, due to the presence of seven TMS groups and is observed at *m/e* 1142 for **15**. A molecule

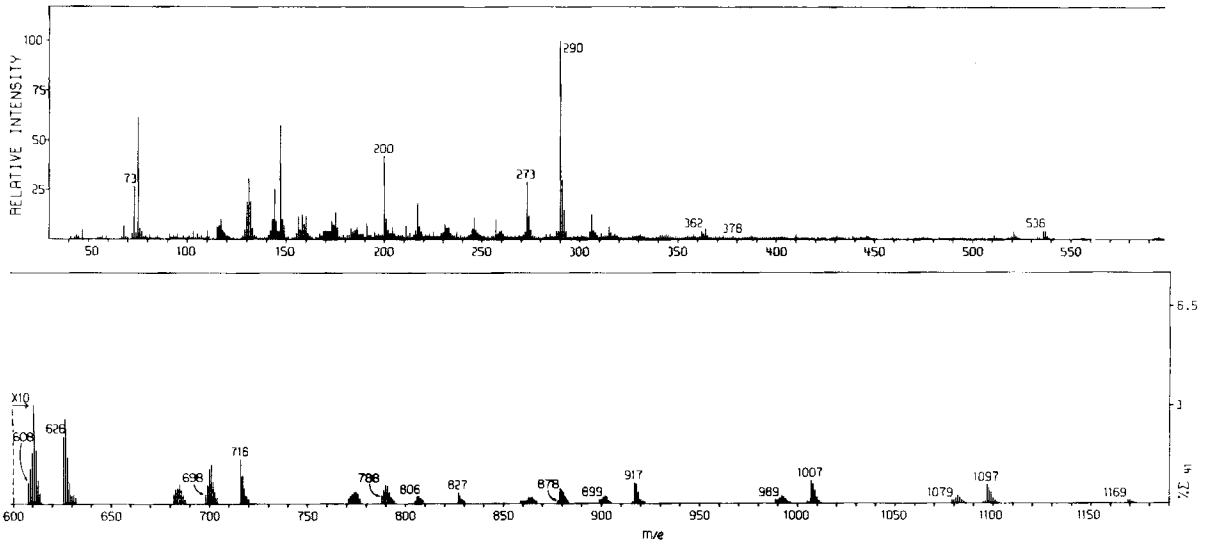


FIG. 4. Low resolution mass spectrum of per-TMS pimaricin (**13**).

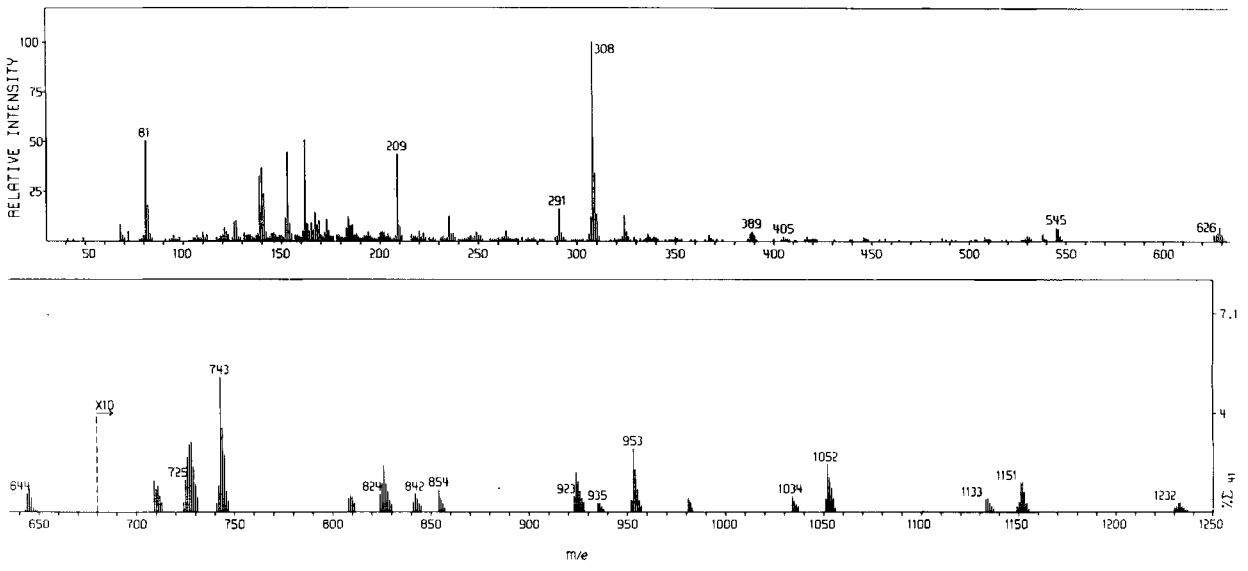


FIG. 5. Low resolution mass spectrum of per-d_9-TMS pimaricin (**14**).

of $(CH_3)_2$—Si=CH_2 is eliminated from $[M]^{+\cdot}$ to produce the $[M - 72]^+$ ion found at *m/e* 1097 for **13** and the $[M - 80]^+$ ion at *m/e* 1151 for **14**. The loss of a TMS group from $[M]^{+\cdot}$ (which was the dominant process in nystatin and amphotericin B) is a minor process in the spectra of **13** and **14**. However, in the spectrum of **15**, an $[M - TMS]^+$ ion is observed at *m/e* 1069. In **13**, the $[M - 72]^+$ ion loses up to three TMSOH molecules to form the ions at *m/e* 1007, 917 and 827 (in **14**: *m/e* 1052, 953 and 854). Three molecules of TMSOH are also lost from $[M]^{+\cdot}$ to form the ions at *m/e* 1079, 989 and 899 in the mass spectrum of **13** (for **14**: *m/e* 1133, 1034 and 935).

In a manner analogous to the fragmentation of amphotericin B, the mycosamine moiety is lost from pimaricin as **11** (see Scheme 3) to yield ion *s* at *m/e* 878 in Scheme 5 (for **14**: *m/e* 923). Ion *s* loses up to three TMSOH molecules to produce the ions at *m/e* 788, 698 and finally, ion *t* at *m/e* 608 (for **14**: 824, 725 and 626).

The mycosamine moiety can also be eliminated from $[M]^{+\cdot}$ as **16** as shown in Scheme 6 to produce ion *u* at *m/e* 806 (*m/e* 842 for **14**). Ion *u* then loses up to three TMSOH molecules to give rise to the ions at *m/e* 716. 626 and 536—ion *v* (for **14**: *m/e* 743, 644 and 545). The more stable form of *v* is represented by *v'* wherein the conjugated polyene system is extended in the enol.

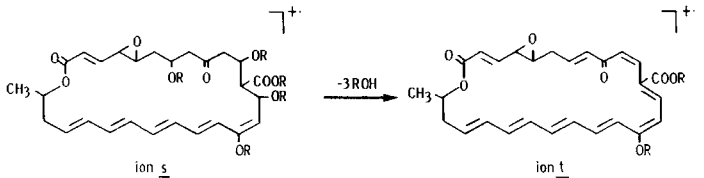
Because pimaricin predominantly loses the mycosamine moiety as the neutral molecules **11** and **16**, the ions due to the sugar at *m/e* 362 and 378 for **13**; *m/e* 389 and 405 for **14**; and *m/e* 335 and 351 for **15** are of relatively lower abundance, as seen in Figs. 4 and 5. Scheme 1 again rationalized the genesis of the ions at *m/e* 290, 273 and 200 for **13** and *m/e* 308, 291 and 209 for **14**, respectively.

Conclusions

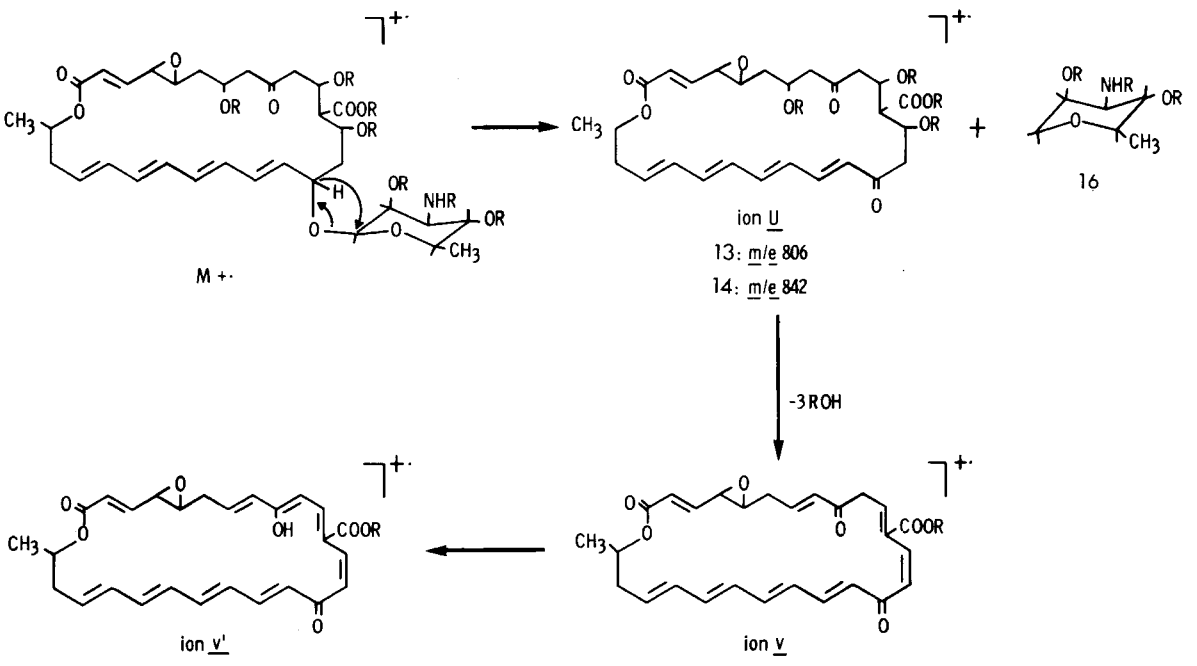
On one hand many similarities exist among the fragmentation patterns of nystatin, amphotericin B and pimaricin. The consecutive losses of TMSOH and the loss of the mycosamine are found from all of the molecular ions and some fragment ions. On the other hand, some very unique fragmentation pathways occur which serve to characterize each polyene macrolide.

(a) The compound of highest molecular weight investigated—nystatin—had the simplest mass spectral fragmentation pattern. Only the loss of TMS, TMSOH and mycosamine was observed.

(b) The only structural differences between amphotericin B and nystatin are a double bond and the position of two hydroxyl groups. However, the fragmentation pattern of amphotericin B is much more



SCHEME 5.



SCHEME 6.

complicated than that of nystatin, where up to three molecules of TMSOH are eliminated from $[M - 73]^+$, whereas in amphotericin B the loss of TMSOH molecules from $[M]^{+\cdot}$ does not occur until ion *e* (Scheme 3) is formed. Labeling with d_9-TMS groups showed that the formation of ion *e* requires the loss of a TMS group.

The loss of the sugar moiety (**11**) from ion *e* to form ion *l* is the second major difference in the spectrum of amphotericin B as opposed to that of nystatin. The loss of the mycosamine moiety involves the simultaneous transfer of the amine TMS group to the glycosidic oxygen. The resulting ion then eliminates six molecules of TMSOH to form the pentadecaene-dienone (*r*), which is still an ion radical.

(c) The molecular ion of pimaricin loses $(CH_3)_2Si{=}CH_2$ and TMSOH directly. The mycosamine sugar is eliminated in two ways. On one hand, the rearrangement product **11** is produced, as in the case of amphotericin B. On the other hand, the neutral molecule **16** is expelled only in pimaricin. The driving force for all of these fragmentation pathways is the energetically favorable extension of the conjugated polyene system and the production of neutral molecules.

In all the mass spectra discussed, the most abundant ions arise from the mycosamine moiety. The intact amino sugar, with and without the glycosidic oxygen, yields ions in the low mass range. After the loss of $(CH_3)_2{-}Si{=}CH_2$ further loss of NH_3 is observed.

The data reported here are the first complete mass spectra of the TMS derivatives of some of the polyene macrolides of high molecular weight. The proposed ion structures have been corroborated by stable isotope labeling, accurate mass measurements and metastable transitions. The data illustrate the analytical use of mass spectrometry in elucidating the structure of these therapeutically important, high molecular weight compounds.

Experimental

Nystatin was purchased from Sigma Chemical Co. (St Louis, Mo.) and was used as received. Samples of amphotericin B and pimaricin were generous gifts from Dr C. P. Schaffner of Rutgers—The State University of New Jersey. The published procedures for forming TMS[44] and acetyl[45] derivatives were followed without modification.

Mass spectra were obtained by means of a direct introduction probe. Low resolution mass spectra were obtained on an Atlas/Varian CH-7 mass spectrometer and high resolution spectra on a DuPont/CEC 21-110B. The instrumental conditions for the CH-7 were: ionizing voltage = 25 eV; ionizing current = 300 μA; accelerating voltage = −2.4 kV; ion source temperature (estimated) = 200 °C. The 21-110B conditions were: ionizing voltage = 70 eV; ionizing current = 100 μA; accelerating voltage = −8.4 kV; ion source temperature = 280 °C. An Ilford Q2 photoplate served as the ion detector in the latter case. Data from the photoplate were obtained by means of a microdensitometer-comparator-computer system described previously.[46]

The theoretical relative abundance data for those ions containing many TMS groups were calculated with the aid of the TPEAKS computer program.[46] The

theoretical values were then compared to the values obtained from the spectra. For example, a calculation for the molecular composition of amphotericin B TMS_{12}—$C_{83}H_{169}NO_{17}Si_{12}$– is presented in Table 1.

TABLE 1

Accurate mass	Relative abundance Theoretical	Found	Nominal mass
1787.962	61%	64%	1787
1788.964	95%	94%	1788
1789.964	100%	100%	1789
1790.965	79%	91%	1790
1791.966	37%	59%	1791

Caution must be exercised whenever *m/e* values are assigned in this mass range because the third peak in a cluster of ions with twelve TMS groups is the most abundant ion, rather than the first peak usually observed with most compounds.

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