Fast-Atom Bombardment and Tandem Mass Spectrometry of Macrolide Antibiotics

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Molecular weights of macrolide antibiotics can be determined from either $(M + H)^+$ or $(M + \text{Met})^+$, the latter desorbed from alkali metal salt-saturated matrices. The ion chemistry of macrolides, as determined by tandem mass spectrometry (MS/MS), is different for ions produced as metallated than those formed as $(M + H)^+$ species. An explanation for these differences is the location of the charge. For protonated species, the charge is most likely situated on a functional group with high proton affinity, such as the dimethylamino group of the amino sugar. The alkali metal ion, however, is bonded to the highly oxygenated aglycone. As a result, the collision-activated dissociation spectra of protonated macrolides are simple with readily identifiable fragment ions in both the high and low mass regions but no fragments in the middle mass range. In contrast, the cationized species give complex spectra with many abundant ions, most of which are located in the high mass range. The complementary nature of the fragmentation of these two species recommends the study of both by MS/MS when determining the structure or confirming the identity of these biomaterials. (*J Am Soc Mass Spectrom* 1994, 5, 151–158)

The macrolides are a class of antibiotics that consist of 12-, 14-, or 16-membered lactone aglycones with one or more pendant sugars, at least one of which is usually an amino sugar (structures 1, 2, 3, and 4) [1]. Erythromycin A is probably the most well-known member of this class as it is the drug of choice for treatment of upper and lower respiratory tract infections and mycoplasma pneumonia [2]. All macrolides exhibit strong activity against gram-positive bacteria and mycoplasma by inhibiting protein synthesis in the target organism [3].

Mass spectrometric techniques such as electron ionization (preceded by sample derivatization) [4], laser desorption [5], chemical ionization [6], and fast-atom bombardment (FAB) [7] have been used to determine macrolide antibiotics. Determination of macrolides by FAB is not sensitive because the hydrophilic nature of the pendant sugars limits the surface activity of these and other glycosidic compounds [8] in polar matrices. For carbohydrates in general, addition of a nonpolar chain [9] as well as selection of an appropriate matrix [10] can serve to increase the sputtering efficiency.

Pramanik et al. [11] reported enhanced abundances of molecular species by the addition of alkali salts to

FAB matrices. Aduru and Chait [12] also reported that the sodium-cationized species of oligosaccharides and glycoconjugates dominate the spectrum generated by ^{252}Cf plasma desorption when sodium chloride was added to the sample. Spectra obtained without added sodium chloride show no molecular ions and are dominated by fragment ions. The formation of cationized permethylated monosaccharides upon FAB [13] was also reported. Structural information can be obtained by collisional activation of the metallated species of disaccharides and oligosaccharides [14, 15], permethylated oligosaccharides [16], and glycosides [17]. Structural studies are also informative for alkali-metal-cationized fatty acids [18], alkali-metal-cationized peptides [19] and fatty acid esters [20], alkaline-earth-metal-cationized peptides [21], and triterpenoids [22] by using collisional activation. Röhlgen et al. [23] first observed structurally informative differences between the decompositions of the $(M + \text{Li})^+$ and $(M + H)^+$ species of small alcohols, aldehydes, and ketones, and a detailed picture of metal ions in mass spectrometry can be found in a recent review [24].

In this article we explore the effects of different matrices on the FAB mass spectrum of eight macrolide antibiotics. Several matrices along with their alkali-metal-salt-saturated counterparts were used to demonstrate a method for verification of the molecular weights of the antibiotics. More important, the fragmentations of the cationized species $(M + \text{Met})^+$

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Structure 1

(where Met refers to the metal species) were found to be significantly different from those of $(M + H)^+$. These differences motivated an investigation of the opportunities for determining structure by collisional activation of both the $(M + H)^+$, and $(M + \text{Met})^+$ ions. The emphasis of this study then is those differences and their implications with respect to metal-ion interactions with large, multifunctional molecules.

Experimental

Eight different macrolide antibiotics were studied by using FAB and tandem mass spectrometry (MS/MS). The eight antibiotics are depicted in structures 1, 2, 3, and 4. All the antibiotics were obtained from fermentation broths and isolated at Schering-Plough Research. The antibiotics, which were used without further purification, were dissolved in dimethyl sulfoxide (DMSO) to a concentration of approximately 1 mg/mL. A small volume of solution, generally about 1–2 μL ,

Structure 3

was syringed onto a copper or stainless steel probe tip coated with approximately 1 μL of matrix. Matrices that were found to be most appropriate for desorbing protonated species were 3-nitrobenzyl alcohol (3-NBA), dithiothreitol/dithioerythreitol (5:1 mixture) (DTT/DTE), and thioglycerol. The metal-cationized species were generated from matrix solutions saturated with LiI, NaCl, and KCl. To slow matrix evaporation and/or degradation during desorption, < 1 μL of glycerol was added to the probe tip when either 3-NBA or thioglycerol or their salt solutions were used as the matrix. All matrix reagent materials were obtained from Aldrich Chemical Co. (Milwaukee, WI) and were used without further purification.

One instrument used in this study was the VG Analytical (Manchester, England) ZAB-T. A brief description of this instrument was published recently [25]. The instrument is a prototypal BEBE geometry mass spectrometer that comprises a Nier-Johnson reverse-geometry design for MS-I and an unusual reverse-geometry Mattauch-Herzog type spectrometer for MS-II. MS-II is equipped with an inhomogeneous electrostatic analyzer (ESA) and an array detector.

The instrument is equipped with a cesium ion gun that was operated at 25 kV to desorb the antibiotics by the liquid secondary ion mass spectrometry method. Collisional activation experiments were performed with an accelerating voltage of 8 kV and with the collision cell floated at 4 kV. Helium was used as the collision gas and was added to the collision cell at a level that suppressed the selected ion beam by approximately 80%. This suppression level yields optimal structural information while maintaining the necessary sensitivity. The ion of interest was selected at a resolution of 1000 (10% valley definition) by MS-I. MS-II was operated with object and image slits closed sufficiently so that the final slit was fully illuminated, giving a slight rounding of the shoulders of the ion signal, to maximize ion transmission. The instrument calibration in the MS/MS mode identified calibrant peaks to an accuracy of ± 0.2 u.

The other instrument was a Kratos MS-50 triple analyzer (Kratos Analytical, Manchester, UK), which was described previously [26]. Ions were desorbed by bombardment with 6-keV argon atoms. The collision-activated dissociation (CAD) spectra are nearly identical to those taken with the ZAB-T except the resolution is poorer and there is some discrimination against low mass ions.

Results and Discussion

FAB Mass Spectra

The macrolide antibiotics were desorbed as protonated, $(M + H)^+$, and cationized, $(M + Li)^+$, $(M + Na)^+$, $(M + K)^+$, species, and full mass spectra were acquired primarily to verify molecular weights. The matrix that gives satisfactory abundances of both molecular ions and fragments is DTT/DTE or DTT/DTE saturated with the appropriate alkali metal salt. Fragment ion abundances are less when 3-NBA or its salt solution is used as a matrix, but molecular ion abundances are comparable to those observed when DTT/DTE is used. Matrices such as triethanolamine (TEA) or hexafluorobutyric acid (HFBA) give poor results, and $(M + H)^+$ ions are of low abundance, although desorption from alkali metal salt-saturated TEA yields adequate quantities of $(M + Met)^+$. Generally, the abundance of $(M + Met)^+$ species is somewhat greater when produced from the alkali-metal-cationized antibiotic than that of $(M + H)^+$ ions from matrices containing no salt. Similar observations were made recently by Aduru and Chait [12].

The results of experiments to determine the analytical detection limit of macrolides by using FAB mass spectrometry and 3-NBA as a matrix indicate that a 1-pmol sample is sufficient to give a molecular $(M + H)^+$ ion for single ion monitoring. By using a 1-pmol sample of erythromycin A, 1a, a signal-to-noise ratio (S/N) of 20 was achieved. Reducing the analyte concentration to 0.1 pmol lowers the S/N to approxi-

mately 2:1. Detection limits of 1–10 pmol are typical for oligosaccharides in polar matrices [27]. Similar results were achieved for $(M + Met)^+$ species upon FAB.

CAD Mass Spectra: Determination of Optimal Collisional Activation Conditions

The CAD spectra of erythromycin A, 1a, the most familiar of the macrolide antibiotics (structure 1), yield information about the structure of the protonated molecule, but high levels (50–80%) of collisional activation are required to generate sufficient fragmentation for identification purposes (Figure 1). Metastable ion decompositions of $(erythromycin\ A + H)^+$ generate abundant high mass ions that result from elimination of water and detachment, accompanied by hydrogen transfer, of the nonamino sugar, cladinose, from the C-3 position, but fragment ions in the low mass range are nearly nonexistent. Low mass ions are observed, however, as the number of collisions accompanying activation increases. Most prominent is the amino sugar fragment. To obtain the most structurally complete data, sufficient helium must be added to suppress the $[M + H]^+$ by 50–80%.

Structure Determination By Using Collisional Activation

The chemistry observed upon the collisional activation of the protonated or metallated forms of 1, 2, 3, and 4 is general even though the aglycone portions of the molecules are different. Most of the fragmentation is centered at the sugar components of the molecules, either as elimination of portions of the sugars or as production of fragments for which the charge is retained by the sugar. There are, however, distinct differences in the fragmentation processes that protonated and metallated antibiotics undergo upon colli-

Figure 1. Effect of precursor beam suppression on fragmentation processes of the $(M + H)^+$ of erythromycin A, 1a. Spectra were acquired with a three sector tandem mass spectrometer. Spectra A, B, C, and D were taken after 0%, 20%, 50%, and 80% precursor beam suppression, respectively.

sional activation. We wish to highlight these differences.

The antibiotics studied contain from one to three saccharide units, at least one of which is an amino sugar. These saccharides are attached to the aglycone to give a variety of structure classes of antibiotics: containing one saccharide unit, containing two individual saccharide units individually attached to the aglycone ring, containing a disaccharide unit, containing three saccharide units individually attached to the aglycone, or containing a disaccharide and a monosaccharide. To illustrate the chemistry observed upon collisional activation, the data are presented for compounds in increasing order of saccharide complexity.

The simplest CAD spectrum for this series of compounds is that of protonated rosamycin, **2** (see Figure 2). With only one pendant saccharide entity, rosamycin does not have many channels for decomposition and, thus, primarily gives protonated dehydro desosamine, the amino sugar ion of m/z 158. The ion of m/z 174 indicates that glycosidic bond cleavage also occurs on the aglycone side of the bond. Elimination of the elements of ketene from the ion of m/z 158 gives that of m/z 116. No ions of significant abundance are observed above m/z 174. The chemistry exhibited by the $(M + H)^+$ ion of rosamycin is consistent with protonation of the high proton affinity dimethylamino entity of the pendant desosamine. As no fragmentations occur from the lactone ring, the proton appears to be locked into position on the amino sugar.

The collisionally activated fragmentations of the metallated species are much more complex. Ions are formed by the elimination of the sugar unit, a sugar fraction, and the aglycone as seen in the CAD spectrum of $(\text{rosamycin} + \text{Na})^+$ (Figure 2). The ion of m/z 475 is formed upon cycloreversion of desosamine to eliminate $\text{C}_7\text{H}_{15}\text{NO}$. Cleavage of the glycosidic bond

on the sugar side accompanied by hydrogen transfer to either the saccharide or the glycosidic oxygen occurs to give the ions of m/z 445 and 447, respectively. Similar reactions on the aglycone side of the glycosidic bond produce the ions of m/z 429 and 431. Fragment ions of lower mass result from degradations of the aglycone. The ion of m/z 387, as the most abundant fragment, likely results from the elimination of ketene from the aldehyde side chain of the m/z 429 ion. No assignment has been made for the ion of m/z 352. A low abundance ion of m/z 158 for (dehydro desosamine) $^+$ is also observed. The ion of m/z 196 is likely the Na-containing analog of the ion of m/z 174 observed in the CAD spectrum of the $(M + H)^+$, and the ion of m/z 23 is Na^+ . Losses of small neutrals, such as methane, water, ethane, and both dimethylamine and methanol, occur to give ions of m/z 588, 586, 574, and 543, respectively.

The predominant fragmentations of $(M + \text{Na})^+$ indicate that the likely location of the sodium ion is on the aglycone. Neutral fragments of the saccharide, the saccharides themselves, and portions of the macrocycle are eliminated, leaving the sodium attached to the remaining structure. Only the low abundance ion of m/z 196 indicates any sodium ion interaction at the saccharide. With the metal ion locked into position on the aglycone entity, fragmentations occur remote to the charge site [28]. A contrasting observation was made for the $(M + \text{Na})^+$ species of steroid glycosides: The sodium cation attaches to the aglycone when one sugar is present, but on a saccharide when the steroid glycoside has more than one saccharide [17].

Antibiotics Containing Two Saccharide Units

The principal process that occurs upon CA of the protonated erythromycin A, **1a**, (Figure 3) involves

Figure 2. FAB CAD spectra of $(M + H)^+$ (of m/z 582) and $(M + Na)^+$ (of m/z 604) ions of rosamycin, **2**. The spectra were obtained with a four sector tandem mass spectrometer. Explanations for the location of the proton and metal ions are given in the text.

Figure 3. FAB CAD spectra of $(M + H)^+$ (of m/z 734) and $(M + K)^+$ (of m/z 772) ions of erythromycin A, 1a. The spectra were obtained with a four sector tandem mass spectrometer. Explanations for the location of the proton and metal ions are given in the text.

cleavage at the glycosidic linkage to the nonamino sugar to give the Y ion of m/z 576, according to the nomenclature suggested by Domon and Costello [29]. An ion of m/z 576 is also produced as a result of ion source reactions. Its CAD reactions produce the same products that are seen in the $(M + H)^+$ spectrum below m/z 576. The ion of m/z 558 may result from cleavage of the glycosidic bond on the aglycone side (Z ion) or from the elimination of water from the ion of m/z 576. Two sequential water losses follow. Additional fragments are formed via cleavage of either side of the glycosidic oxygen of the amino sugar with accompanying hydrogen transfers, generating the B, C - 2H, and C ions of m/z 158, 174, and 176, respectively. According to the proposed nomenclature [29], the C ion designation is a shortened version of C + 2H. Abiding by the proposed nomenclature, we assign the C ion as that of m/z 176 whereas the more abundant m/z 174 ion is a C - 2H ion. The C - 2H is more abundant than C for amino sugars because the protonation has presumably occurred at the amine and cleavage follows as a charge-remote H-transfer from the amino sugar to the departing neutral macrolide. The $^{0.2}A$ ion of m/z 116 is formed either in a one-step process from $(M + H)^+$ or in a two-step elimination of C_2H_2O from the m/z 158 ion (or $C_2H_2O_2$ from the m/z 174 ion). Analogous results were obtained upon chemical ionization of other macrolide antibiotics [13]. Eliminations of small molecules from the backbone of the aglycone ring or from the framework of either sugar species do not occur to any measurable extent.

One of the most significant fragment ions produced by decomposition of (erythromycin A + K) $^+$ in the source or upon collisional activation is that of m/z 643, generated via loss of 129 u. Peak-matching experiments were performed on the source-produced fragment, at a mass resolution of greater than 30,000,

Scheme I

to establish the exact mass to be 643.3120, which fits to within 0.5 ppm the atomic composition of $C_{30}H_{52}O_{12}K^+$. This ion can be formed upon elimination of a $C_7H_{15}NO$ entity via a cycloreversion ($^{1.5}X$ ion) of the amino sugar desosamine. In this way, a formyl group remains attached to the glycosidic oxygen. The ion of m/z 642 is formed in an analogous manner by elimination of $C_7H_{14}O_2$ from cladinose.

Other fragment ions produced by collisional activation of potassium-erythromycin A (see Figure 3) result from cleavages and rearrangements involving intact saccharide frameworks. Y ions of m/z 612 and 614 and of m/z 613 and 615 and Z ions of m/z 596, 598, 597, and 599 are produced by the elimination of cladinose and desosamine, respectively. The ion of m/z 511 likely results from concurrent cycloreversions at both sugars. The ion of m/z 439 is produced by two sequential or concurrent cleavages, one of an X and the

other a Y cleavage. The ion of m/z 423 is the potassiumated core aglycone. Decomposition of cationized erythromycin A also involves elimination of portions of the aglycone ring. For example, the ions of m/z 381 and 353 likely result from the sequential elimination of the elements of ketene and water from the m/z 423 ion.

The spectra of erythromycin A cationized with other alkali metal ions are comparable to that of $(M + K)^+$, showing similar ion abundances and suggesting metal-independent fragmentation. Röllgen et al. [30] obtained analogous results for cationized monosaccharides and first suggested that high energy CAD for such species is independent of the identity of the metal ion. Others, however, report small differences that depend on the size of the alkali metal, but the glycosides studied do not contain a macrocycle as an aglycone [17, 24]. The macrocycle may be sufficiently large to coordinate strongly K^+ as well as Na^+ and Li^+ .

The fragment ions observed upon collisional activation of the $(M + H)^+$ and $(M + K)^+$ ions of four other antibiotics, mycinamicin II, 4, desmycosin, 3a, megalalosamine, 1b, and 23-O-desmycosinyltylosin, 3c, are summarized in Tables 1 and 2. The protonated macrolides decompose similarly as protonated erythromycin A and give simple CAD mass spectra; most of the fragment ions are of low mass and characteristic of the amino sugar. The data support tight location of the charge on an amino sugar. The $(M + Met)^+$ species of the macrolides fragment in a similar manner as that of cationized erythromycin A; that is, many abundant fragments are clustered in the high mass region of their CAD spectra. These arise from cleavages around the glycosidic bonds to eliminate the saccharides; the metallated aglycone ring remains intact. Often, the only low mass ion is the alkali metal itself.

Antibiotics Containing Three Saccharide Units

Two of the macrolide antibiotics studied here contain three saccharide units. Megalomycin A has the same structure as megalalosamine but with an additional neutral sugar, mycarose, at C-3 of the aglycone ring (see structure 1c). The two amino sugars do not cause significant changes in the fragmentations, which are similar to those of erythromycin A. Decomposition of protonated megalomycin A occurs principally via the two established routes: elimination of the mycarose (to give the m/z 733 ion) and formation of a B-ion of m/z 158 from either desosamine or megosamine. In addition, either of the amino sugars can be protonated, making elimination of the other amino sugars a facile process to produce the ion of m/z 720. Elimination of both mycarose and one of the amino sugars results in the ion of m/z 576; this is the same species observed in the CAD spectrum of fragments from protonated erythromycin A.

The metallated megalomycin A also fragments as expected. Elimination of either mycarose or one of the amino sugars results in two sets of X-, Y-, and Z-ions. In addition, two saccharides are eliminated via $^{1,5}X$ cleavages, and the final product is abundant. The loss of all three saccharides also occurs to produce a potassiumated aglycone of m/z 407.

Tylosin has a 16-membered aglycone ring with a disaccharide unit of mycaminose and mycarose at C-5 and the monosaccharide mycinose attached at C-14 (see structure 3b). Consistent with the other macrolide antibiotics, $(tylosin + H)^+$ principally decomposes by cleavage of the mycaminose glycosidic bond on the amino sugar side to form the ion of m/z 174 (Figure 4). A second decomposition process occurs via hydrogen transfer and cleavage of the mycaminose-mycarose

Table 1. Fragment ions observed in the CAD spectra of protonated macrolide antibiotics containing two sugars*

Antibiotic	Amino sugar			Nonamino sugar		
	Sugar	$^{0,2}A$	B	C-2H	Sugar	$^{1,5}X$
Mycinamicin II, 4	Desosamine	116	158	174	Mycinose	552/554
		(12)	(100)	(7)		(2) (3)
Desmycosin, 3a	Mycaminose	132	174	190	Mycinose	596/598
		(15)	(100)	(5)		(2) (2)
Megalalosamine, 1b	Desosamine	116	158	174		574/576 [†]
		(4)	(100)**	(4)**		(5) (1)
	Megosamine	132				
		(14)				
23-O-desmycosinyl tylosin, 3c	Mycaminose	132	174	190	Mycarose	598
		(19)	(100)	(5)		(22)

* Mass-to-charge ratio values, relative abundance in percent in parentheses.

** Cannot distinguish between the two amino sugars.

[†] Protonation occurs at one amino sugar; the other amino sugar is eliminated. Fragmentation nomenclature used is that proposed in ref. 29.

Table 2. Fragment ions observed in the CAD spectra of potassiated macrolide antibiotics containing two sugars*

Antibiotic	Amino sugar				Nonamino sugar				Other fragments	Comments
	Sugar	X	Y	Z	Sugar	X	Y	Z		
Mycinamicin II, 4	Desosamine	637 (13)	607 (6)	591 (8)	Mycinoses	620 (19)	590 (7)	574 (< 1)	476, 490 (7), (6) 314 (7)	Elimination of both sugars
Desmycosin, 3a	Mycaminose	665 (85)	635 (58)	619 (38)	Mycinoses	664 (60)	634 (13)	618 (25)	577 (m/z 619-42) (40) 605 (42)	Aldehyde at C-6 position Cleavage on aglycone side of intervening methylene group on mycinose I
Megalalosamine, 1b	Desosamine	642 (26)	612 [†] (7)	598 (3)	—				455, 423 (9), (23) 582 (3)	Elimination of both sugars
23- <i>O</i> -desmycosinyl tylosin, 3c	Mycaminose	Z ₂ 445 (9)	Y ₂ 463 (29)	X ₂ 491 (25)	Mycarose	Z ₁ 618 (14)	Y ₁ 636 (6)	X ₁ 664 (8)	403 (m/z 445-42) (15) 575, 521 (29), (19)	Aldehyde at C-6 position

* Mass-to-charge ratio values, relative abundance in percent in parentheses. In all of the CAD spectra, K⁺ of m/z 39 is the most abundant ion.

[†] An ion of m/z 614 (8%) is also observed and results from hydrogen transfer to the aglycone rather than to the sugar.

Fragmentation nomenclature used is that proposed in ref. 29.

glycosidic bond to eliminate a mycarose entity and form the ion of m/z 772.

Upon collisional activation of (tylosin + K)⁺ (see Figure 4), abundant ions are formed by losses of small neutrals, such as methanol to give the ion of m/z 922. More important, the structurally significant X-, Y-, and Z-ions are all formed in large abundance. The fragmentations can be divided into three classes: elimina-

tion of one saccharide (products from m/z 700 to 850), elimination of two saccharides (products from m/z 550 to 700), and elimination of all the saccharide units (products below m/z 450). In addition to the ions identified in the fragmentation scheme, those of m/z 577 and 372 are also structurally important. They identify the presence of the aldehyde side chain at C-6 and result from the elimination of the elements of ketene

Figure 4. FAB CAD spectra of (M + H)⁺ (of m/z 916) and (M + K)⁺ (of m/z 954) of tylosin, **3b**. The spectra were obtained with a four sector tandem mass spectrometer. Explanations for the location of the proton and metal ions are given in the text.

from the ion of m/z 619 and from the potassiated bare aglycone ring of m/z 414, as was seen for other antibiotics.

Conclusions

In summary, the results obtained here point to generalities about the ion chemistry of macrolide antibiotics. Metallated species fragment in a way that is consistent with an alkali-metal interaction at the aglycone, whereas the protonated species undergo fragmentations remote from the protonated site on an amino sugar. The results establish the advantages of obtaining CAD spectra of both species when the goal is determining the structure or identifying these types of biomaterials.

The macrolide antibiotics also illustrate the need for unit resolution and correct mass assignments in MS/MS. In the case of erythromycin A, for example, the saccharides cladinose and desosamine differ in mass by only 1 u. In addition, cleavages at the glycosidic bond occur with hydrogen transfer to either the sugar or the aglycone, resulting in two ions of 2-u difference. These two factors result in clusters of four ions differing by 1 u in the CAD spectra of the metallated species of erythromycin A. Determination of fragmentation mechanisms clearly requires unit mass resolving power.

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