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Structure Elucidation of Everninomicin-6, a New Oligosaccharide Antibiotic, by Chemical Degradation and FAB-MS Methods

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The structural characterization of a new oligosaccharide antibiotic, Everninomicin-6 (EV-6), is described. Detailed fast-atom bombardment mass spectrometry (FAB-MS) studies along with NMR and chemical degradation methods were conducted to elucidate the structure of EV-6. The effects of the use of various matrices, including salt addition, on the quality of the FAB-MS were explored. The use of 3-nitro benzyl alcohol, dimethyl sulfoxide (DMSO), and NaCl produced the best results: an intense sodiated molecular ion plus structurely informative fragmentation. FAB-MS yields information providing the complete sugar sequence information for everninomicins, which is quite valuable to the elucidation of the structure of this complex oligosaccharide antibiotic. In addition, the results of accurate mass work with the molecular ion are consistent with the assigned structure. The use of electrospray ionization mass spectrometry (ESI-MS) and ESI-MS/MS for the study of EV-6 was investigated and was found to produce an abundant molecular ion with limited structural information. These results revealed that EV-6 resembled EV-D quite closely except for the absence of the nitrosugar and the replacement on ring g of the $-CH_2OCH_3$ group with a $-CH_2OH$ group. (J Am Soc Mass Spectrom 1997, 8, 1134–1140) © 1997 American Society for Mass Spectrometry

Everninomicins constitute an important group of oligosaccharide antibiotics, isolated from the fermentation broth for *Micromonospora carbonaceae* [1], that are highly active against gram-positive bacteria including methicillin resistant *staphylococci* and vancomycin resistant *enterococci*. Everninomicin-D (EV-D) is the main component of the above fermentation broth and was the first member of this family of complex oligosaccharide antibiotics to be extensively studied and have its structure elucidated. EV-D has since been used as a template for the characterization of other everninomicin components as well as other oligosaccharides such as flambamycin, curamycin, and avilamycin [2]. The structures of everninomicin B, C, D, everninomicin-2, and 13-384 components 1 and 5 were reported earlier [2–6].

With the increasing growth of drug resistant strains of bacteria, a major effort is being directed towards the reevaluation of the efficacy of existing oligosaccharides and to the identification of new potential oligosaccharide antibiotics. It is, therefore, important to characterize quickly all EV components isolated from the fermentation broth as part of any ongoing process to identify potential antibiotic pharmaceuticals. Component 13-384-1 (Ziracin, Schering-Plough, Kenilworth, NJ) is the most promising drug candidate and is undergoing extensive clinical trials to determine its activity against both sensitive and resistant strains. A minor antibiotic component, isolated from the fermentation producing everninomicin-B, was designated everninomicin-6 (EV-6). We now describe the structural determination of this novel component by chemical degradation methods, NMR methods, and fast-atom bombardment (FAB)–mass spectrometry [7].

Experimental

Short column chromatography [8] of the crude antibiotic complex gave in some fractions (50% acetone/benzene) pure EV-6 **1** as a crystalline solid (acetone/*n*-hexane), $C_{57}H_{84}O_{31}Cl_2$ melting point (m.p.) 233–35 °C, γ_{max} 1730 cm^{-1} (ester), FAB-MS m/z 1357 $[M + Na]^+$. Methylation of EV-6 with diazomethane gave a monomethyl ether **2**, m.p. 247–50 °C (acetone/*n*-hexane), $C_{58}H_{86}O_{31}Cl_2$, $[\alpha]_D^{26}$ + 4.3° ($CHCl_3$), FAB-MS m/z 1371 $[M + Na]^+$. ^{13}C NMR spectrum of **2** showed signals at 120.4 and 119.7 ppm attributable to the orthoester carbon atoms [5].

Mild acid hydrolysis of **2** in a two-phase system [4] provided methyl-everninomicin-6_1 **4** as a major product and methyl-everninomicin-6_0 **6** as a minor product. Both **4** and **6** on treatment with methanolic diazometh-

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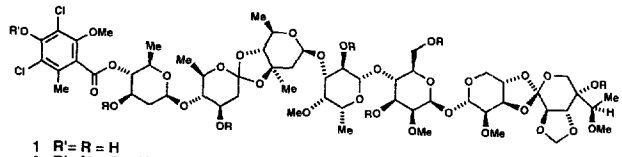

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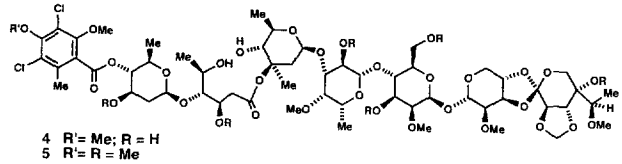

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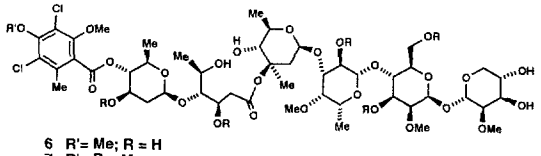

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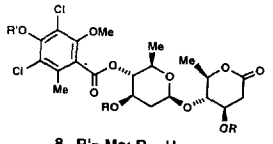

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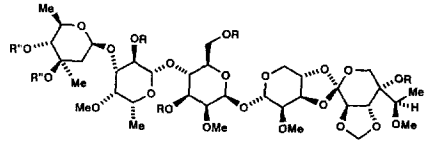

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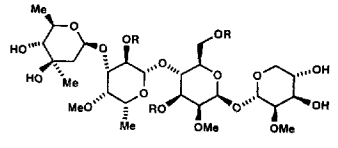

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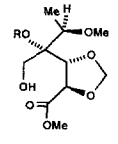

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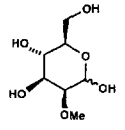

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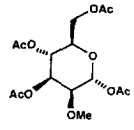

17

ane underwent ready cleavage [4] to provide **8** as a common product, olgose-6 **10** and evertetrose-6 **13**, respectively. The lactone **8**, m.p. 200 °C, $C_{22}H_{28}Cl_2O_{10}$ was found to be identical by NMR, mass spectrometry, and thin-layer chromatography (TLC) to a degradation product of everninomicin-2 [5, 9, 10]. This indicated that the structural variation in EV-6 existed in the olgose-6 **10** portion of the molecule.

Olgose-6 **10** is a crystalline compound, m.p. 200–202 °C, $C_{34}H_{56}O_{21}$ $[\alpha]_D^{26}$, −13.3° ($CHCl_3$). The proton magnetic resonance (PMR) spectrum of olgose-6 closely resembled that of olgose-D except the lack of one O-methyl group in the former. Treatment of olgose-6 **10** with methanolic *p*-toluene sulfonic acid [4] gave evertetrose-6 **13** and an ester **15**. The ester **15** was found identical (NMR, mass spectrometry, TLC) to the same degradation product isolated from everninomicins B [2], D [4], and 2 [5]. Permethylation of olgose-6 gave a compound **11** m.p. 192–194 °C, $[\alpha]_D^{26}$ −13.3° ($CHCl_3$), identical (NMR, mass spectrometry, TLC) to permethylated olgose-D [4]. It now remained to show the position of the four O-methyl groups in olgose-6 by isolation of the individual sugars.

Evertetrose-6 **13**, m.p. 174–176 °C, $C_{27}H_{48}O_{17}$, $[\alpha]_D^{26}$ −23° ($CHCl_3$), showed in the PMR spectrum (pyridine) two secondary methyls, one tertiary methyl, three O-methyl groups, and four anomeric protons at δ4.85 (*d*,J_{aa} 7 Hz), δ5.25 (*s*, *w*/2 *J* 2.5), δ5.3 (*dd*, J_{ae} 2 Hz, J_{aa} 8 Hz) and δ5.67 (*d*, *J* 2 Hz). Prolonged aqueous hydrolysis of evertetrose-6 **13** gave D-curacose, evermicose, 2-O-methyl-D-xylose [11], and 2-O-methyl-D-mannose **16** [12], a sugar previously not isolated from everninomicins. Acetylation of **16** with pyridine-acetic anhydride yielded 1,3,4,6-tetra-O-acetyl-2-0-methyl-α-D-mannopyranose **17**, m.p. 135–6 °C (acetone/*n*-hexane), $C_{15}H_{22}O_{10}$, $[\alpha]_D^{26}$ +51.4° ($CHCl_3$). The PMR spectrum ($CDCl_3$) of **17** had expected signals at δ6.22 (*d*, J 2.2 Hz, H_1), 3.65 (*dd*, J 2.2 and 3.0 Hz, H_2), 5.23 ($J_{3,4}$ 9 Hz, $J_{3,2}$ 3.0 Hz, H_3), 5.40 ($J_{3,4}$ $J_{4,5}$ 9.0 Hz, H_4), 4.04 (*m*, H_5), 4.29 (*J* 12 and 4.5 Hz, H_6), and 4.11 (*J* 12 and 2 Hz, H_6).

To establish the connectivity (location) of the C16 and C49 ortho ester linkages, a well established degradation sequence previously applied to EV-B and EV-D [2, 4] was followed. Permethylation of Me-EV-6 **2** with MeI/NaH in tetrahydrofuran provided **3**. Two-phase aqueous hydrolysis of **3** gave **5** and **7**, which were separated by chromatography on silica gel column. Treatment of the ester **5** with methanolic diazomethane yielded **9** and **12**, which were identical by NMR, mass spectrometry, and TLC to the corresponding products obtained from permethylated EV-2 [5]. The degradation product **12** was also identical by NMR, mass spectrometry, and TLC to the product obtained by the same method with EV-D [4]. Treatment of the more polar ester **7** with methanolic diazomethane, as above, gave **14**, which was identical (NMR, mass spectrometry, TLC) to the product isolated from EV-D. Thus, the site of the attachment of the two orthoester linkages in EV-6 is established to be the same as in EV-2 and EV-D. The FAB mass spectral data that further confirmed our structural assignments are discussed below.

Mass Spectrometry

FAB mass spectra were obtained on the JEOL JMS-HX110A mass spectrometer equipped with a FAB gun

using xenone gas: the source was operated at an accelerating voltage of 10 kV and the FAB gun at an accelerating voltage of 3 kV and emission current of 10 mA. Liquid secondary ion mass spectrometry (LSIMS) work was obtained on a VG-ZAB-SE mass spectrometer operated at 8 kV equipped with a cesium ion gun operated at 25 kV. A few crystals of EV-6 was dissolved in 1–2 μL of DMSO and a few crystals of NaCl were usually added to this solution. The DMSO solutions were mixed with 1 μL of various matrices: glycerol, thioglycerol, glycerol/thioglycerol, 3-nitro-benzyl alcohol (3NBA), and 3NBA + NaCl, with the last matrix producing the best results. A thin layer of the above solution was then applied to the FAB probe and introduced into the instrument. For the peak matching experiment, a cyclic peptide, with a molecular formula of $C_{63}H_{96}N_{12}O_{21}$, was added to the EV-6 solution as the reference compound. This peak matching experiment was performed at a mass resolving power of 5000. Electrospray studies were performed on a Sciex API III triple quadrupole mass spectrometer. About 20 μL methanol solution (1 μg/μL) of EV-6 was introduced by direct infusion or via a short C_{18} column [4.6 mm inner diameter (i.d.) × 1 cm] using 95% acetonitrile/5% H_2O with 0.03% trifluoroacetic acid. An orifice voltage of 40 V was applied for the direct infusion ESI analysis. For tandem mass spectrometry experiments, an orifice voltage of 70 V and a collision energy of 76 eV with collision gas (Ar) thickness of 290 were used. The sample was introduced at a flow rate of 5 μL/min into the mass spectrometer with a nebulizing gas flow at 0.6 L/min.

Results and Discussion

Everninomicins are essentially nonvolatile oligosaccharide compounds that do not yield molecular ion information by electron ionization (EI), chemical ionization (CI), and direct chemical ionization (DCI) mass spectrometry. Plasma desorption, laser desorption, and electrospray yielded molecular ions but with little structurally informative fragmentation. FAB-MS yields abundant ions plus detailed fragmentation data delineating the structure of all everninomicin components.

Positive ion electrospray ionization (ESI) of everninomicin yielded strong molecular ion data but produced only limited structurally useful fragmentation. The ESI-MS spectrum displayed abundant ions at m/z 827, 701, and 365 that involved cleavage of the sugar ether linkages, thus providing partial insight into the sugar sequence of the right portion of EV-6 as taken from the central ortho ester group. These ions correspond to the FAB-produced sodiated ions of m/z 849, 723, and 387 observed in the FAB spectrum (see Figure 3). Discussion of the FAB data is provided later. However, the ESI spectrum did not provide clear structural information defining the left portion of the molecule. It was also found that ESI was hindered by a problem that both ortho ester groups in everninomicin undergo hydrolysis during sample introduction, thus producing a complex spectrum.

ESI tandem mass spectrometry studies provided information partially defining the portion to the right of the central ortho ester of EV-6 but failed to provide an ion series for the left portion of the molecule. However, this technique did produce an abundant ion of m/z 233 that involved the aromatic moiety; this ion was observed to increase by the expected 14 mass units to m/z 247 for the mono-methyl ether of EV-6 (compound **2**). Irregardless of which ion was used in the tandem mass spectrometry experiment for EV-6 $[M + H]^+$ or $[M + Na]^+$, the same ions (m/z 849, 723, 485, and 359) were observed in the spectrum. These ions all contain Na^+ and involve the right portion of the EV-6 molecule. The ions of m/z 849 and 723 are observed in both FAB (see Figure 3) and regular ESI, but the m/z 485 and 359 ions were not observed by either of these techniques, and involved small fragments of the molecule generated from the middle of the sugar backbone (the m/z 485 involved rings E–G and the m/z 359 involved rings F and G).

In summary, we obtained partial structural information corresponding to the right portion of EV-6 but were unable to define the left portion of the molecule using ESI-MS. In addition, fragmentation was more complex because some EV-6 hydrolyzed during the experiment. It will be shown below that the positive ion FAB-MS provides more complete information that is useful for the structural characterization of this new oligosaccharide antibiotic, EV-6.

FAB spectra of EV-6 were initially obtained by using a 50:50 glycerol/thioglycerol (gly/thio) matrix and produced a very weak protonated molecular $[M + H]^+$ ion at m/z 1335 and a very low-abundance sodiated molecular ion at m/z 1357 (Figure 1): it should be noted that everninomicins did not yield molecular ions when desorbed from a straight glycerol matrix, although fragment ions of interest were observed. The cationized low-abundance m/z 1357 ion was due to the presence of trace amounts of Na salt in the sample. Although the results of these initial FAB studies were comparatively limited in value, some structural insight could be obtained. The mass spectrum showed fragment ions at m/z 233, 345, and 363 that partially define the portion of the EV-6 molecule to the left of the central ortho ester and an ion at m/z 827 formed by the right portion of the molecule.

Much better results have since been obtained with 3NBA and NaCl doped 3NBA as the matrices. In earlier structural studies with thermolabile organic molecules, natural products, and various components of everninomicin antibiotics, we and other groups [4, 6, 7, 13–17] demonstrated the importance of salt addition in FAB-MS for enhancement of their spectra. This technique of using NaCl in LSIMS and FAB yielded strongly enhanced sodiated molecular ions plus detailed sugar sequencing information. The addition of NaCl to the gly/thio matrix produced abundant sodiated molecular

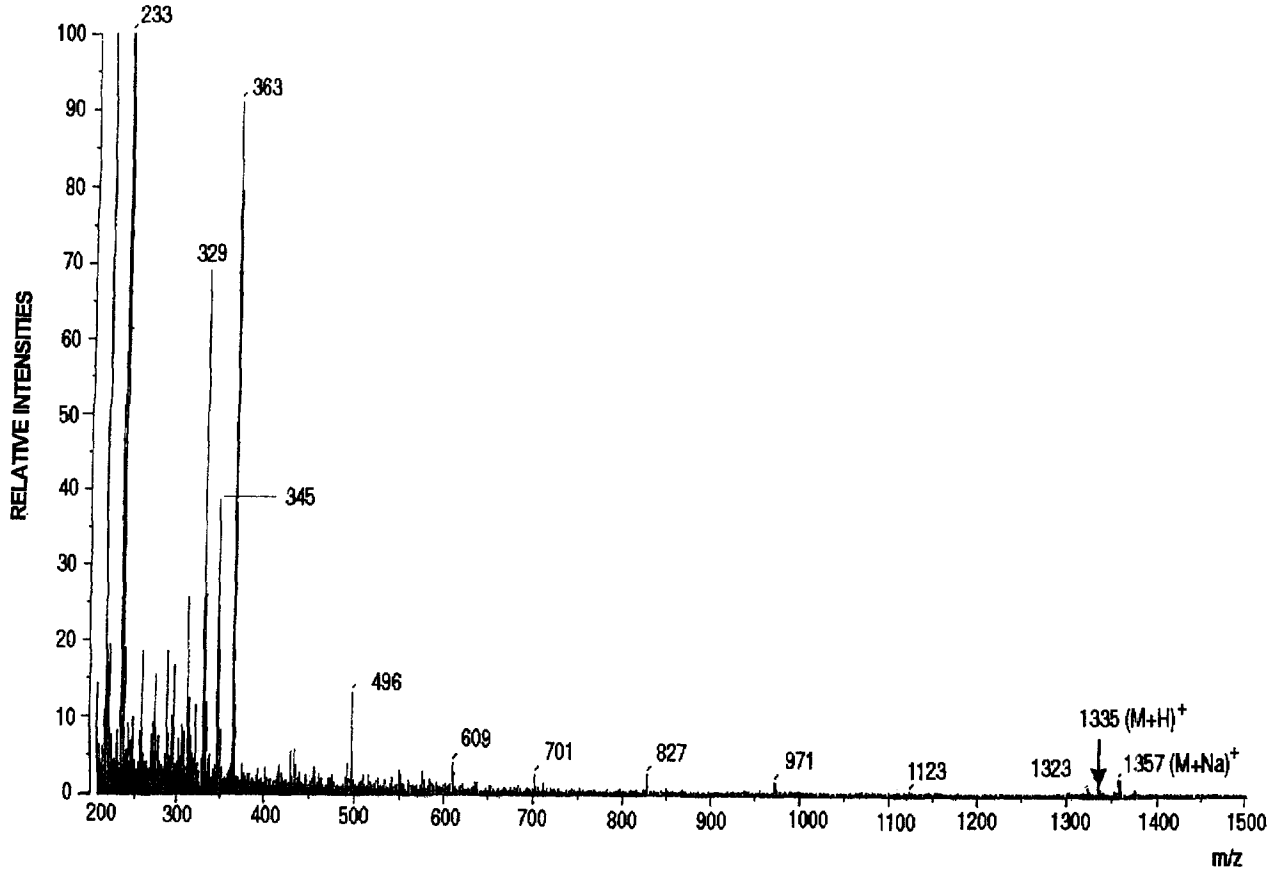

Figure 1. FAB-MS of EV-6 with 50:50 thioglycerol-glycerol as the matrix; trace amounts of Na salt present as a contaminant.

ions and structurally informative fragment ions. Additional fragment ions were observed in this spectrum, arising from the substantial displacement of the aromatic chlorines. The displacement of the aromatic chlorines were also observed when glycerol, thioglycerol, and gly/thio were used as the matrix without the addition of NaCl. These ion series, involving successive displacements of the aromatic chlorines with hydrogen, were reported earlier in several mass spectrometry papers [6, 7, 14] on everninomicin, but were not observed here with 3NBA as the matrix.

The use of DMSO as the solvent and 3NBA as the matrix with NaCl addition produced the best results. We now make a careful evaluation of the FAB spectrum of EV-6 (see Figures 2 and 3) and then compare these results with those obtained for EV-D (Figure 4). EV-D is the main component in the fermentation broth producing this group of oligosaccharides, and its structure has been extensively studied by us, thus making it a good model for comparison. We reached the following conclusions; (a) EV-6 has no nitro-sugar, (b) rings b, c, d, e, f, h, j, and k of EV-6 (Figure 3) have identical compositions and the same sequence as EV-D, and (c) sugar g has a $-CH_2OH$ group in place of the $-CH_2OCH_3$ group of EV-D.

Figure 2 shows the FAB spectra of EV-6 with a 3NBA + NaCl matrix. This matrix resulted in an abundant sodiated molecular ion $[M + Na]^+$ at m/z 1357. Considerable enhancement of the molecular ion with the addition of salt [14] to the FAB matrix was observed for all everninomicins. An insert in Figure 2 provides a blowup of the molecular ion region to illustrate the dichloride isotope pattern of EV-6, ions of m/z 1357, 1359, and 1361. The peak, 18 u higher, that is observed at m/z 1375, is due to the partial hydrolysis of the ortho ester between sugar c and e during the long storage of the sample. An accurate mass of 1357.4283 was obtained for the sodiated molecular ion by peak matching, and this result was in good agreement with an elemental composition of $C_{57}H_{84}O_{31}Cl_2Na$ (calculated, 1357.4271).

The NaCl-doped positive ion FAB of EV-6 produced several series of structurally informative ions consistent with cleavage at successive sugar units in this molecule; Figure 3 illustrates the fragment ions observed in the above spectrum (Figure 2). An important series of ions (of m/z 1023, 893, 751, 591, and 415) involving the portion of the molecule to the right of the central ortho-ester is consistent with successive cleavage at the anomeric carbons of the various sugars [17(a)]. Two other sets of ions (m/z 995, 849, 723, 563, 387, 219 and 1107, 977, 833, 705 and 545) also map the right portion

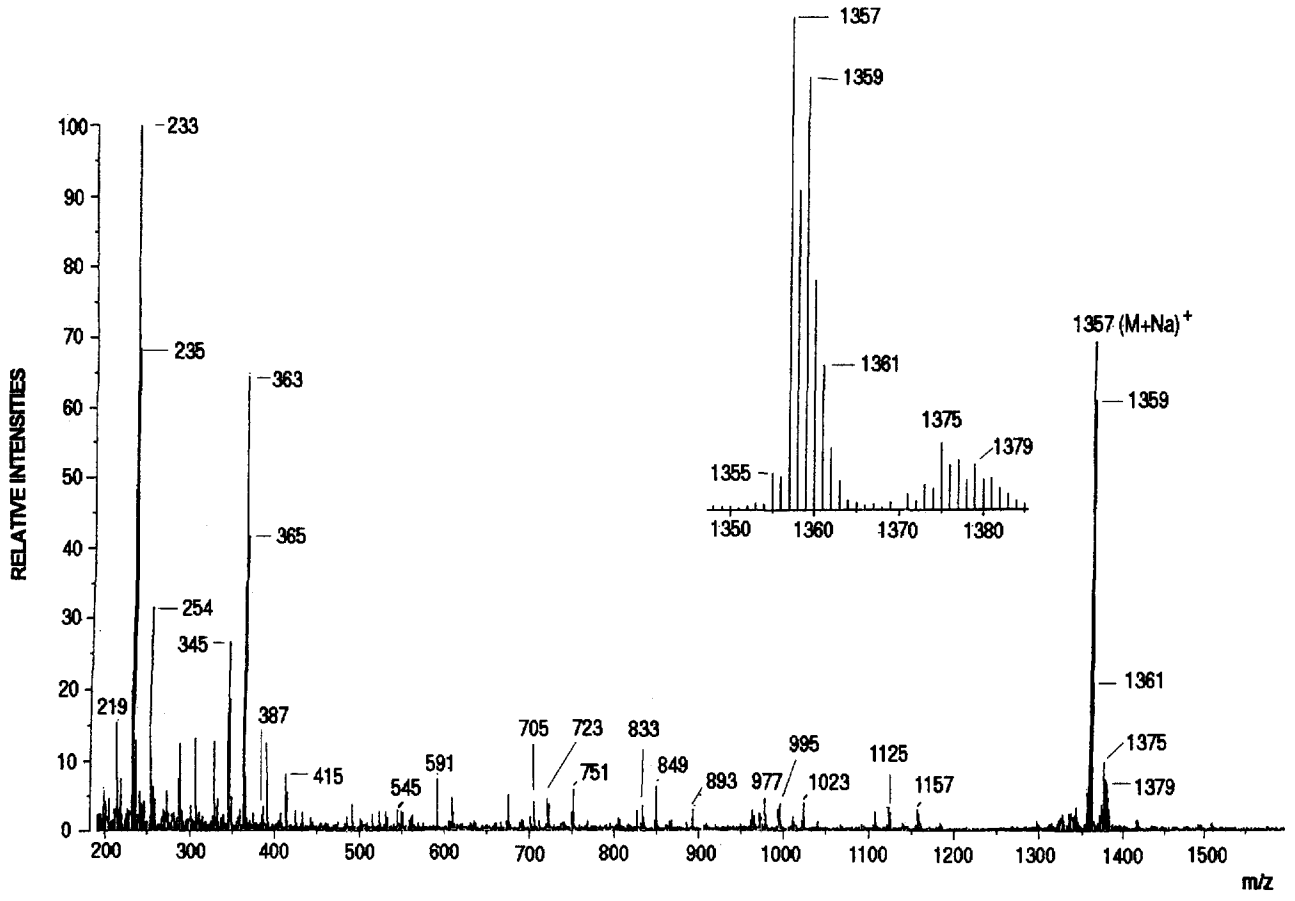

Figure 2. NaCl enhanced positive ion FAB-MS of EV-6 using 3NBA as the matrix: insert illustrates expanded molecular ion region.

of EV-6 and involve cleavages at the ether linkages. The low-abundance ion at m/z 219 is formed by cleavage of the ortho-ester joining ring h and j and is also observed with EV-D. The presence of the di-Cl aromatic moiety, marked by the two-chlorine isotope pattern, defines the left portion of EV-6. A series of fragment ions is observed containing a two-chlorine isotope pattern, starting with the sodiated lactone fragment at m/z 531 and includes the m/z 363, 345, and 233 fragment ions.

Figure 4 presents the mass spectral data of EV-D

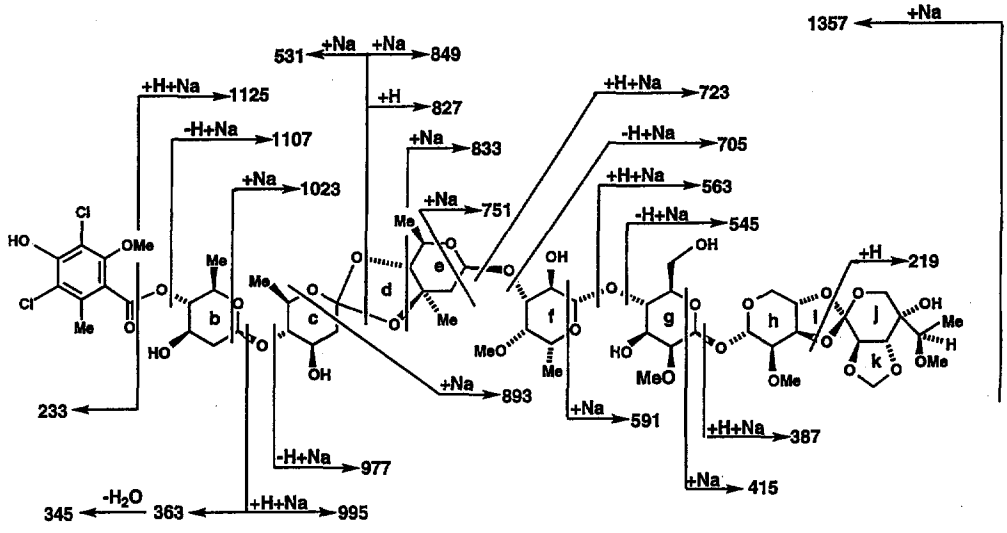

Figure 3. Structure of EV-6 demonstrating structurally informative ions found in spectrum shown in Figure 2.

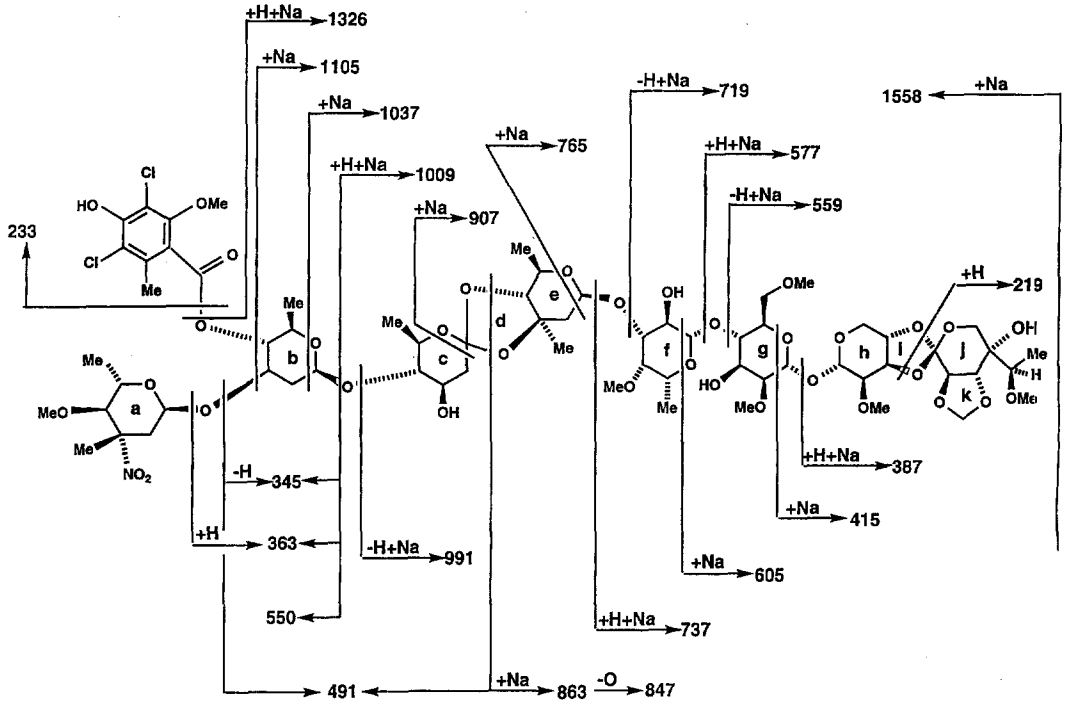

Figure 4. Structure of EV-D with structurally informative ions obtained by NaCl enhanced positive ion FAB-MS, presented for comparison to EV-6.

obtained on the JEOL JMS-HX110A mass spectrometer with NaCl-doped 3NBA as the matrix. The table below compares the above three fragmentation series involving the right portion of EV-6 with the corresponding series for EV-D; it is important to note that ions of m/z 415, 387, and 219 are observed for both compounds, indicating no structural differences for this portion of the molecule.

EV-6	1023, 893, 751, 591, 415
EV-D	1037, 907, 765, 605, 415
EV-6	995, 849, 723, 563, 387, 219
EV-D	1009, 863, 737, 577, 387, 219
EV-6	977, 833, 705, 545
EV-D	991, 847, 719, 559

A difference of 14 u is observed in fragments containing ring g; this 14-u mass difference is maintained throughout the remainder of the fragment series. This mass difference is consistent with the replacement of the $-CH_2OCH_3$ group in the g ring of EV-D with a $-CH_2OH$ group in EV-6. Thus, EV-6 has the same sugar sequence as EV-D, allowing for the 14 u difference resulting from the different g ring substituent. In addition, because rings b–k are identical for both everninomicins, except for the 14 u difference in ring g, and because the aromatic ester group is also shown to be identical (ions of m/z 233, 345, and 363), it follows that the remaining difference in mass is due to the absence of the nitro sugar; the accurate mass measurements are consistent with this assignment.

The standard chemical and mass-spectral procedure for confirming the presence of the phenol ester attached to sugar b of all everninomicins involved methylation of this phenol group. Compound **2**, formed by methylation of EV-6 **1** with diazomethane, produced a species that gave an $[M + Na]^+$ at m/z 1371. The NaCl-doped matrix gave a FAB-MS spectrum (Figure 5) of this compound that contained the same cleavage sequences as EV-6, with the exception that the ions of m/z 531, 363, 345, and 233 have been shifted 14 u higher to m/z 545, 377, 359, and 247, respectively, thus being consistent with methylation of the aromatic hydroxy group.

We have also investigated the use of negative ion FAB-MS for the structural analysis of everninomicins, and this technique appears to be an extremely powerful tool for the characterization of these oligosaccharide antibiotics [18]. The results of our negative-ion FAB study, covering all known everninomicins, will be reported as a separate article. A brief discription of the results for EV-6 are mentioned here.

Negative-ion FAB-MS analysis of EV-6 was performed in the DMSO–3NBA matrix without the addition of NaCl. It produced an abundant molecular ion $[M - H]^-$, at m/z 1333 and a series of ions of m/z 987, 811, 651, 507, and 377, resulting from the successive cleavage, from right to left (h–c), of the ether linkages between the sugar groups. In addition to this series, there was an ion at m/z 1133 due to the loss of sugar j and ring k with a transfer of two hydrogens to the observed ion. The presence of ion at m/z 249 corresponded to the aromatic ester function containing two chlorine ions. In general, negative-ion FAB-MS pro-

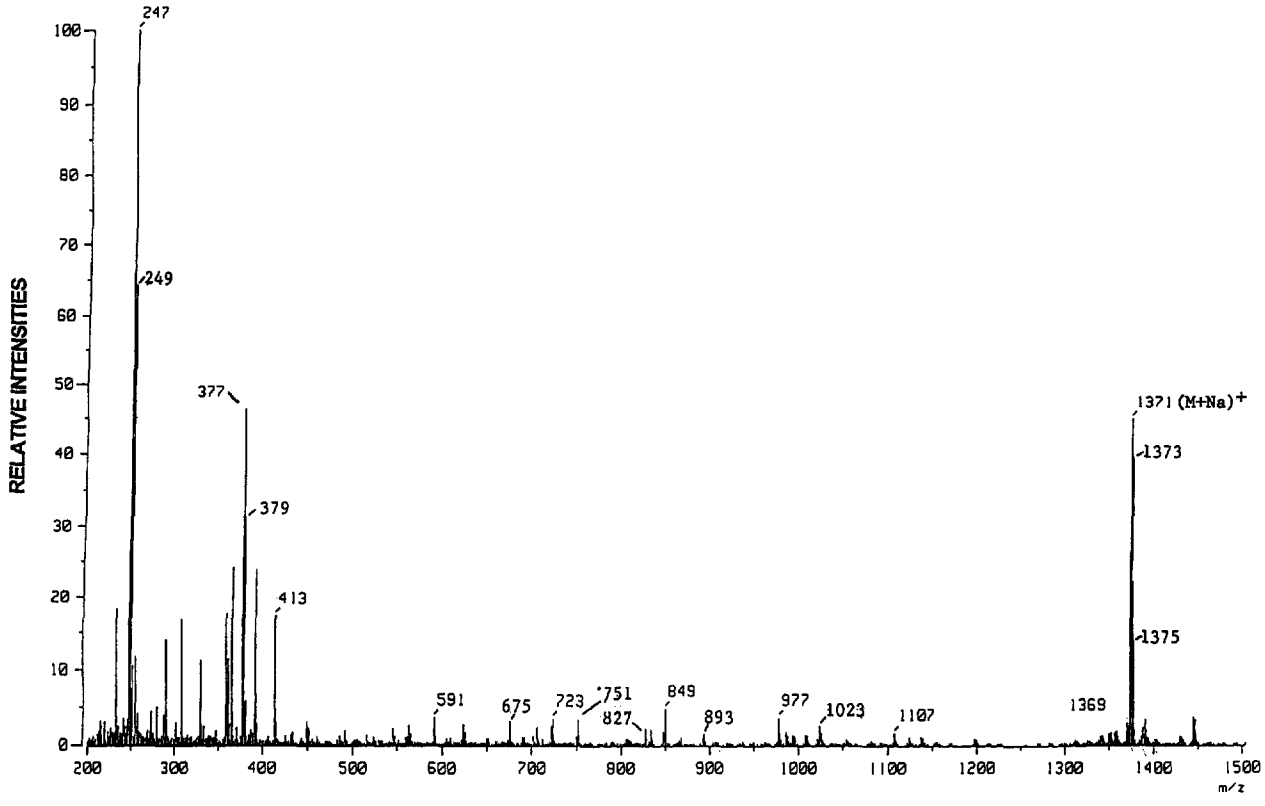

Figure 5. NaCl enhanced spectrum of methyl-EV-6 in 3NBA.

duces an abundant molecular ion and a fragment ion series that clearly defines the sugar sequence.

Conclusions

We have found that the novel everninomicin component, EV-6 **1**, closely resembles the structure to EV-D; (a) EV-6 has no nitro sugar, which is unique within the everninomicin family, (b) sugars b, c, d, e, f, h, j, and k have identical compositions and sequences as those in EV-D, and (c) sugar g is the same except that a $-CH_2OH$ group has replaced the $-CH_2OCH_3$ group of EV-D. This article again illustrates the importance of positive ion FAB (LSIMS) in the mass spectrometry analysis of complex oligosaccharides of the type represented by the everninomicin family, as well as the following points: (a) that the use of 3NBA and NaCl for FAB analysis of everninomicins produced abundant sodiated molecular ions plus fragmentation series that define the sequence of the sugar chain; and (b) that 3NBA did not produce displacement of the aromatic chlorines as observed when glycerol/thioglycerol was the matrix and that although ESI produced valuable information for EV-6, the results obtained with FAB were superior.

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