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Mass Spectrometric Analysis of Aminoglycosidic Antibiotics as N-Salicylidene Schiff Bases

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Mass spectra of the N-salicylidene derivatives of thirteen members of aminoglycosidic antibiotics and the partially degraded compounds containing 2-deoxystreptamine as a common component were measured. The spectra provided easily identifiable molecular ions, and prominent fragment ions that allowed the sequential determination of the constituent units.

Aminoglycosidic antibiotics such as kanamycin, neomycin and paromomycin belong to compounds of extremely low volatility, and they are not amenable as such for mass spectrometric analysis. In 1967, DeJongh, *et al.*[2] recorded first mass spectra of paromomycins as their N-acetyl-O-trimethylsilyl ethers, demonstrating that this class of the derivative is suitable to mass spectrometric investigation. Since then, little have been reported on the mass spectra of aminoglycosidic antibiotics, except for N-acetyl-O-trimethylsilyl hybrimycins[3], N-acetyl-O-trimethylsilyl butirocins[4] and gentamicins.[5]

In the preceding paper,[6] we have shown that the N-salicylidene derivatives are suitable compounds for mass spectrometry of aminosugars, giving rise to intense molecular ions and simplified fragmentation pattern. The purpose of the present paper is to examine how the N-salicylidene Schiff bases work for the mass spectrometry of aminoglycosidic antibiotics of complex structure.

Molecular Ions

It was mentioned[6] that the M^+ of N-salicylidene aminosugars were very strong, and became the base peak in a number of monosaccharides. The relative intensities of the M^+ in the pseudooligosaccharides under investigation were considerably decreased as compared to those of monosaccharide series, but still well recognized up to pseudotrisaccharides.

In the pseudodisaccharide series, the M^+ of N-salicylidene paromamine (**1**) and 3-amino-3-deoxy-α-D-glucosyl 2-deoxystreptamine (**2**) appeared at *m/e* 635, with 5% and 3% inten-

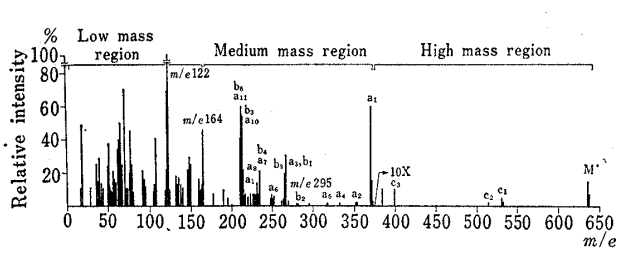


Fig. 1. Mass Spectrum of N-Salicylidene Paromamine (**1**) at 220°

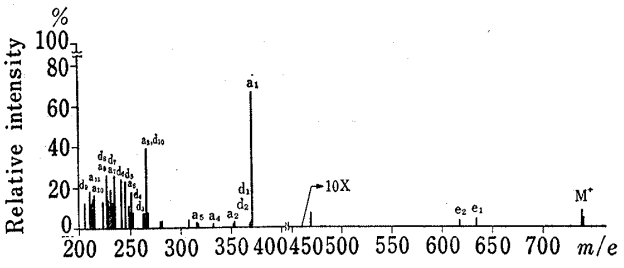


Fig. 2. Mass Spectrum of N-Salicylidene Neamine (**3**) at 220°

1) Location: *Morooka, Kohoku-ku, Yokohama.*
2) D.C. DeJongh, J.D. Hribar, S. Hanessian, and P.W.K. Woo, *J. Am. Chem. Soc.*, **89**, 3364 (1967).
3) W.T. Shier, K.L. Rinehart, Jr., and D.G. Gottlieb, *Proc. Natl. Acad. Sci., U.S.*, **63**, 198 (1969).
4) P.W.K. Woo, *Tetrahedron Letters*, **1971**, 2621.
5) D.J. Cooper, M.D. Yudis, H.M. Marigliano, and T. Traubel, *J. Chem. Soc.* (*C*), **1971**, 2876.
6) S. Inouye, *Chem. Pharm. Bull.* (Tokyo), **20**, 2320 (1972).

sities relative to the base peak at *m/e* 122. N-Salicylidene neamine (**3**) and 3,6-diamino-3,6-dideoxy-α-D-glucosyl 2-deoxystreptamine (**4**) showed the M^+ at *m/e* 738 with 0.8% and 1% intensities, respectively. The relative abundance of the M^+ (*m/e* 532) of N-salicylidene methyl 2,6-diamino-2,6-dideoxy-α-L-idosyl β-D-riboside (methyl neobiosaminide B) (**5**) amounted to 5%. These peaks were easily recognizable, since no prominent peak appeared in the neighborhood of the M^+, as seen in Fig. 1 and 2.

In comparison, the M^+ of the free bases of pseudodisaccharides such as paromamine (**1a**) (Fig. 3) and neamine (**3a**) were far more weaker, and often adhered with more stronger $(M \text{ plus } 1)^+$ peak at high temperature. For example, compound **1a** showed the M^+ at *m/e* 323 with 0.06% intensity relative to the *m/e* 59 peak at 200°. When temperature was raised to 230°, the M^+ was increased to 0.2% with concomitant increase of the $(M \text{ plus } 1)^+$ ion (0.15%). In compound **3a**, the $(M \text{ plus } 1)^+$ was stronger than the M^+, showing 0.1% and 0.02% at 240°. The mass spectrum of tetra-N-acetyl neamine (**3b**) was recorded for comparison, but neither the M^+ nor the $(M \text{ plus } 1)^+$ could not be detected. The dehydration peaks at *m/e* 472 (0.1%) and 454 (0.2%) were the highest ions observed. Dehydrated peaks were prominent in case of the free bases too, but not significant in the spectra of the Schiff bases.

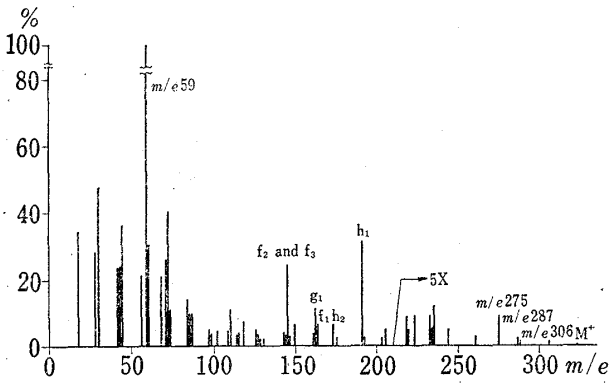


Fig. 3. Mass Spectrum of Paromamine Free Base (**1a**) at 200°

Kanamycin A and B belong to pseudotrisaccharides, and their N-salicylidene derivatives (**6** and **8**) showed the M^+ at *m/e* 900 (0.1% relative to the *m/e* 213 peak), and *m/e* 1,003 (0.2% relative to the *m/e* 245 peak). Likewise, the M^+ of N-salicylidene 2″-manno-kanamycin (**7**) and 6-amino-kanamycin (**9**) were observed at *m/e* 900 and *m/e* 1003, respectively. It was surprising that the M^+ of N-salicylidene aminosugars (**6**—**9**) having 11 hydroxyl groups in the molecules were recognizable. In comparison, the M^+ of the parent aminoglycosides (**6a** and **8a**) were not observed so far examined, or any fragment ions that allow the estimation of molecular weights.

In case of pseudotetrasaccharides such as N-salicylidene paromomycin I and II (**10** and **11**) and neomycin B and C (**12** and **13**), observation of the M^+ was unsuccessful so far, but it became possible when the Schiff bases were converted to O-trimethylsilyl derivatives. For instance, hexa-N-salicylidene trideca-O-trimethylsilyl neomycin C showed the M^+ at *m/e* 2174, with stronger intensity than that of the (M minus 15) peak. The same was true for N-salicylidene O-trimethylsilyl neamine (**3c**), whose M^+ at *m/e* 1314 was most strong in the mass region above *m/e* 1243 (see Chart 6). This is quite interesting, since the M^+ of N-acetyl-O-trimethylsilyl aminoglycosides were reported to be much weaker than the (M minus 15) peaks.[2,3] Therefore, for the compounds whose N-salicylidene derivatives fail to show the M^+, N-salicylidene-O-trimethylsilyl derivatives are alternative derivatives for the observation of the M^+.

Fragmentation of N-Salicylidene Schiff Bases

N-Salicylidene Paromamine (1)——Figure 1 presents the mass spectrum of N-salicylidene paromamine (**1**). The spectrum was divided into three parts for convenience of describing fragmentation. In the low mass region below *m/e* 164, main peaks appeared at *m/e* 164, 134, 122, 121, 107, 91 and 77. These peaks were found in all the spectra of N-salicylidene Schiff bases, and assigned to the fragment ions of the salicylidene group.[6] The relative intensities of these peaks were closely related to the positions of N-salicylidene amino groups in the sugar chains, and provided useful structural information in the case of mono-N-salicylidene

aminosugars.[6]

In poly-N-salicylidene aminosugars, however, intensities of each of these peaks are summation of those of all the salicylidene groups substituted at various positions, and therefore the correlation of the relative intensities with the structure becomes difficult, as the number of salicylidene groups increases. For this reason, fragment ions appeared in the low mass region below *m/e* 164 were not discussed in this paper.

Major peaks in the medium mass region between *m/e* 164 and 370 consisted of fragment ions involving the 2-deoxystreptamine and the 2-aminoglucose moieties, and they were characteristic fingerprint of this compound (**1**). A strong peak at *m/e* 370 had an elemental composition $C_{20}H_{22}O_5N_2$, and assigned to the di-N-salicylidene 2-deoxystreptamine cation (a_1). The formation of this ion was rationalized by the cleavage of a glycosidic bond (ii) accompanied by a hydrogen transfer (Chart 1). An alternative glycosidic cleavage (i) and/or splitting off of a hydroxyl radical from a_1 would yield a fragment ion at *m/e* 353 (a_2) with a composition $C_{20}H_{21}O_4N_2$. Comparison of the relative intensities of the *m/e* 370 and 353 peaks sug-

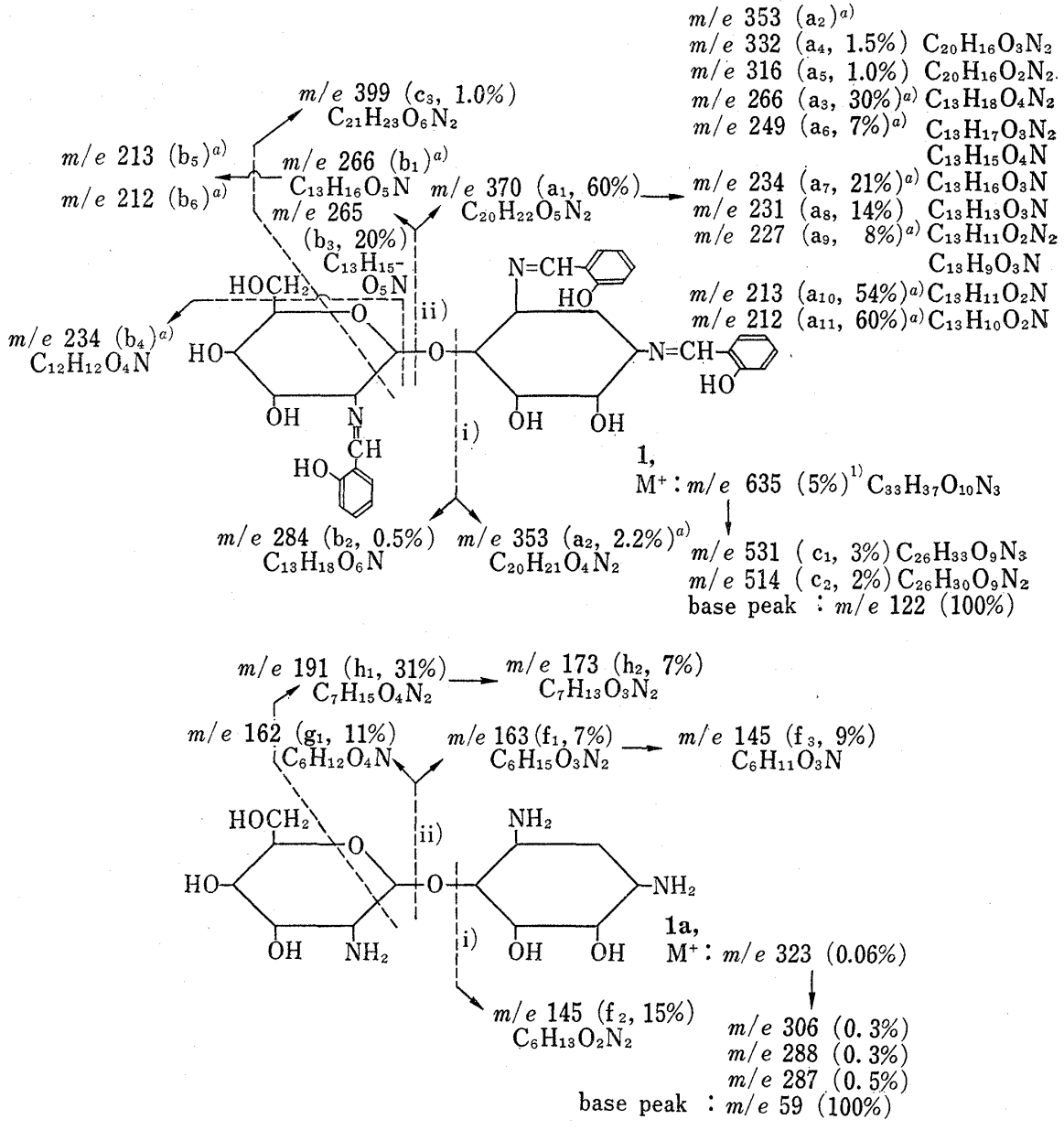


Chart 1

1) Relative abundances of the peaks below *m/e* 399 and the peaks above *m/e* 400 were obtained at 220° and 240°, respectively.
a) Accumulated relative intensities of two or more fragment ions.

gested the predominant cleavage between the ether oxygen and C-1 of the sugar moiety. A minor ion appeared at *m/e* 353 with $C_{16}H_{19}O_8N$ or $C_{19}H_{17}O_5N_2$. A strong peak at *m/e* 266 was again a doublet, of which a higher mass ion with $C_{13}H_{18}O_4N_2$ was assigned to the mono-N-salicylidene 2-deoxystreptamine cation (a_3). Another fragment ions involving the 2-deoxystreptamine portion were observed at *m/e* 332 (a_4), 316 (a_5) (di-N-salicylidene C_6 fragment ions), 249 (a_6), 234 (a_7), 231 (a_8), 227 (a_9), 213 (a_{10}) and 212 (a_{11}) (N-salicylidene C_6 fragment ions). These peaks were consistently observed in the spectra of the glycosides of 2-deoxystreptamine.

Fragment ions arising from the 2-aminoglucose portion were difficult to determine, but the strong intensities of the *m/e* 266 (b_1) with $C_{13}H_{16}O_5N$, 265 (b_3), 234 (b_4), 213 (b_5) and 212 (b_6) peaks as well as the weak *m/e* 284 peak (b_2) suggested a main contribution from the 2-aminoglucose ions. The b_1 and b_2 ions were formed directly from the rupture of the glycosidic bonds at i and ii, respectively. The assignment of the b_1 and b_3 ions was supported by the spectrum of N-salicylidene 3′-deoxyparomamine,[7] which showed the strong *m/e* 250 (266 minus 16) and 249 (265 minus 16) peaks, and the weak *m/e* 266 and 265 peaks.

There were observed a few fragment ions in the high mass region above *m/e* 370. Besides the M^+ at *m/e* 635, there appeared pseudodisaccharide ions at *m/e* 531, 514, and 399. A peak at *m/e* 531 (M minus 104) had an elemental composition $C_{26}H_{33}O_9N_3$, and assigned to the di-N-salicylidene paromamine ion (c_1), which was formed by the loss of one of three salicylidene groups from the M^+ accompanied with two hydrogens transfer. The (M minus 104) peak appeared in all the N-salicylidene Schiff bases in this investigation, and therefore a complementary peak for the confirmation of the M^+. Removal of a salicylideneimino group from the M^+ yielded a peak at *m/e* 514 (c_2). Formation of the remaining *m/e* 399 peak (c_3) was explained by the cleavage of a pyranose ring as shown in Chart 1.

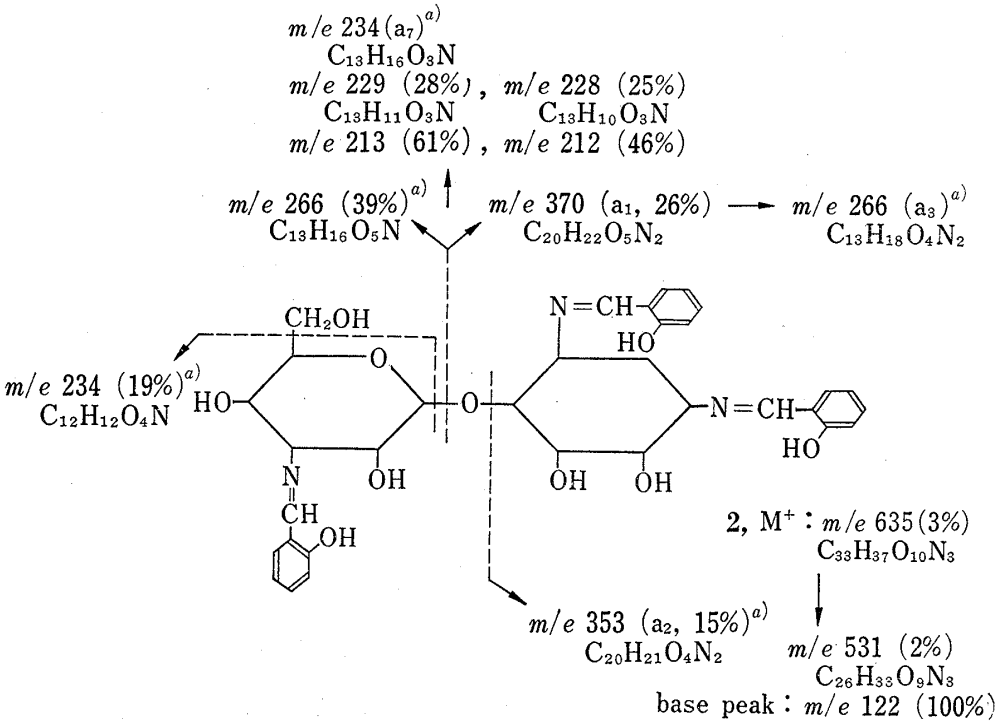


Chart 2

a) Accumulated relative intensities of two or more fragment ions.

7) S. Inouye, Y. Ogawa, S. Omoto, T. Tsuruoka, and T. Niida, *Sci. Reports, Meiji Seika Kaisha*, **12**, 65 (1972).

N-Salicylidene 3-Amino-3-deoxy-α-D-glucosyl 2-Deoxystreptamine (2)——The mass spectrum of compound **2** was very close to that obtained for the corresponding paromamine derivative (**1**). Thus, it showed the M^+ at *m/e* 635, the (M minus 104) peak at *m/e* 531, the N-salicylidene 2-deoxystreptamine cation (a_1) at *m/e* 370 and the N-salicylidene 3-aminoglucose cation at *m/e* 266. Other strong peaks above *m/e* 200 are shown in Chart 2. It was noted, however, that slight difference existed in the fragment ions involving the 2- and 3-aminosugar portion. The two fragment ions at *m/e* 514 (c_2) and 295 appeared in the spectrum of **1** were absent in case of **2**, while a moderate peak at *m/e* 238 appeared only in the latter. Of these, the c_2 ion was a key peak for the differentiation of **1** and **2**, since a salicylideneimino group was removed more easily from the C-2 position of the aminosugar moiety.[6]

N-Salicylidene Neamine (3)——Figure 2 shows the mass spectrum of N-salicylidene neamine (**3**) above *m/e* 200. It exhibited the M^+ at *m/e* 738, the (M minus 104) ion at *m/e* 634 (e_1) and the (M minus 121) ion (e_2) at *m/e* 617. The last ion was characteristic for the glycosides of 2-aminosugars as described above. Once again, there appeared a strong peak at *m/e* 370 due to the di-N-salicylidene 2-deoxystreptamine cation (a_1), together with its

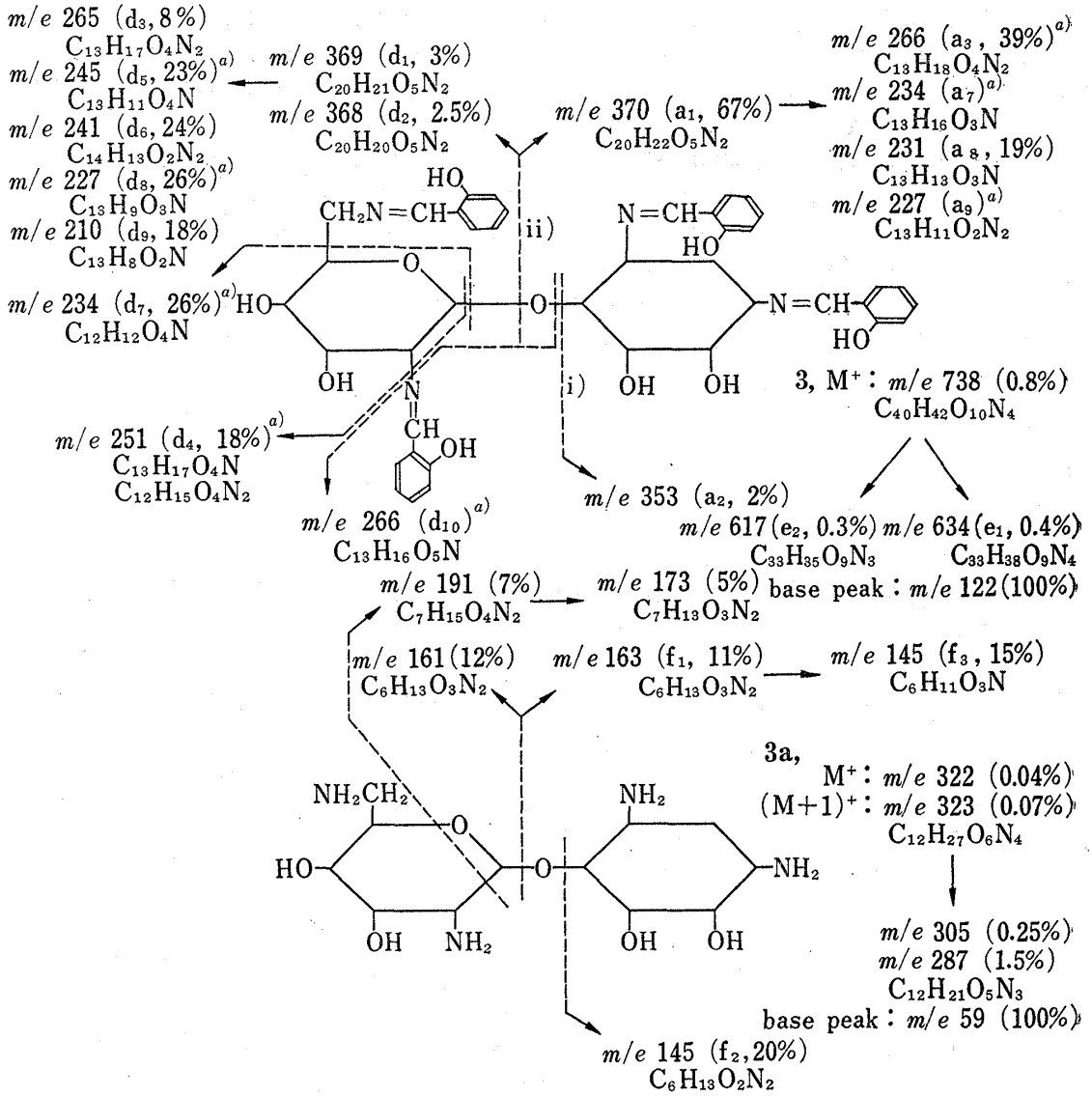


Chart 3

a) Accumulated relative intensities of two or more fragment ions.

degradation ions at *m/e* 266 (a_3), 249 (a_6), 234 (a_7), 231 (a_8), 227 (a_9), 213 (a_{10}) and 212 (a_{11}). The large intensity difference between the *m/e* 370 and 353 peaks indicated the predominant glycoside cleavage ii).

Fragment ions of the 2,6-diaminoglucose moiety (d series) were weakly observed at *m/e* 369 and 368 (d_1 and d_2), which were the products of the direct cleavage of a glycosidic bond. Release of a salicylidene or salicylideneimino group from d_1 would yield d_3 at *m/e* 265 and d_4 at *m/e* 251 (Chart 3).

The most characteristic ions of the 2,6-diaminosugar moiety were found at *m/e* 245 (d_5) and 241 (d_6). The d_5 peak was a doublet consisting of a major ion (d_{5a}) with $C_{13}H_{11}O_4N$ and a minor (d_{5b}) with $C_{13}H_{13}O_3N_2$. Of these, a diagnostic ion for the 2,6-diamino-D-glucose moiety was limited to the d_{5a}, since the d_{5b} lacked in the spectra of other neamine containing antibiotics such as **8**, **12**, and **13**, but weakly appeared in the spectrum of **4**.

Spectral comparison of **3** (Fig. 2) with **1** (Fig. 1) further revealed the intensity increase of the *m/e* 227 (d_8) and 210 (d_9) peaks, apparently compensating with the intensity decrease of the *m/e* 213 and 212 peaks in the former. This difference was reasonably ascribed to the aminosugar moieties of **1** and **3**.

The peaks at *m/e* 368, 245, 227, 251 and 241, in particular the latter two were extremely temperature-dependent, and rapidly disappeared as temperature raised over 220°. On the other hand, the *m/e* 213 and 212 peaks were intensified by raising temperature, suggesting to be the thermally decomposed ions.

N-Salicylidene 3,6-Diamino-3,6-dideoxy-α-D-glucosyl 2-Deoxystreptamine (4)——Major fragmentation of compound **4** was shown in Chart 4, and analogous to that of the neamine derivative (**3**). Difference in the fragmentation of **3** and **4** was found in the peaks at *m/e* 617 (e_2), 251 (d_4), 245 (d_5) and 241 (d_6), which appeared only in the spectrum of **3**. The fragment ions characteristic for **4** were observed at *m/e* 400 (c_4) and 227, whose intensities were more increased in **4**. The c_4 ion had a composition $C_{21}H_{24}O_6N_2$, and could be assigned

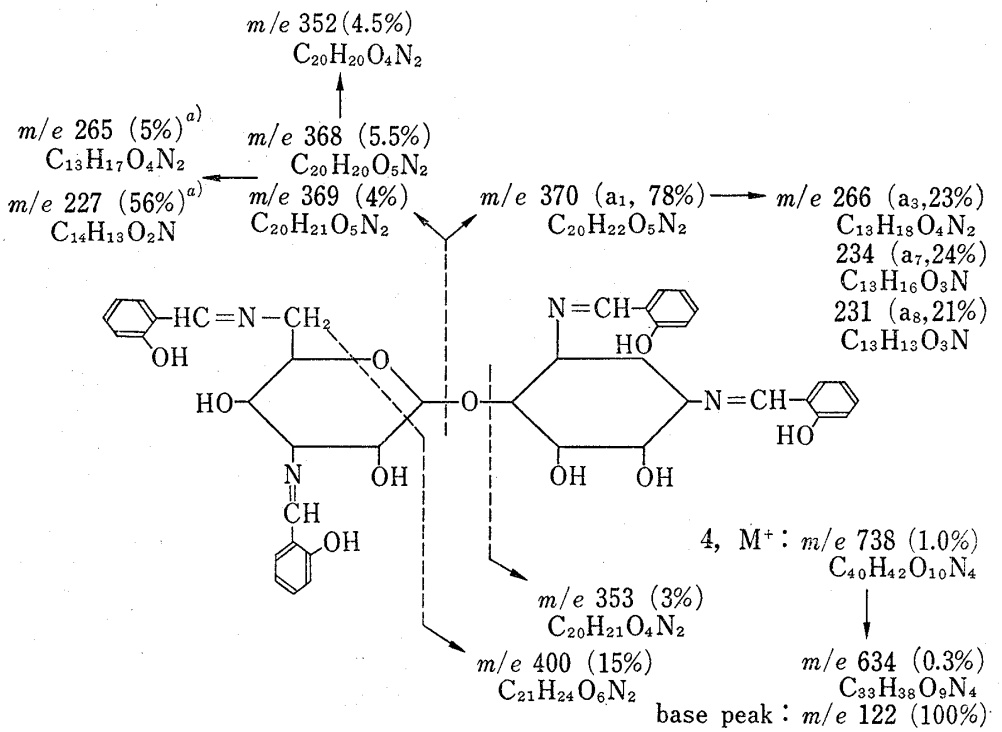


Chart 4

a) Accumulated relative intensities of two or more fragment ions.

to the diprotonated O-formyl N-salicylidene 2-deoxystreptamine. Favorable formation of the c_4 ion in **4**, in contrast to the negligible c_3 ion at *m/e* 399 that was more prominent in **1**, **7**, **8**, *etc.*, remained to be resolved.

N-Salicylidene Methyl 2,6-Diamino-2,6-dideoxy-α-L-idosyl β-D-Ribopyranoside (5)——Compound **5** is the only substance in this investigation that does not contain 2-deoxystreptamine. Fragmentation of **5** was studied as a model for those of paromomycin I and neomycin B as described later, since **5** is a methanolysis product of these antibiotics.

The spectrum of **5** was characterized by a number of peaks involving the N-salicylidene 2,6-diaminosugar moiety, and a few peaks of the pentose moiety (Chart 5). The fragment ions from the 2,6-diaminoidose moiety were very similar to those from the 2,6-diaminoglucose portion of **3**, including peaks at *m/e* 369, 368, 265, 245, 241, 227 and 210. However, the relatively strong *m/e* 264 and 216 peaks were characteristic for the 2,6-diaminoidose moiety, and recognized little in the spectra of **1**—**4**.

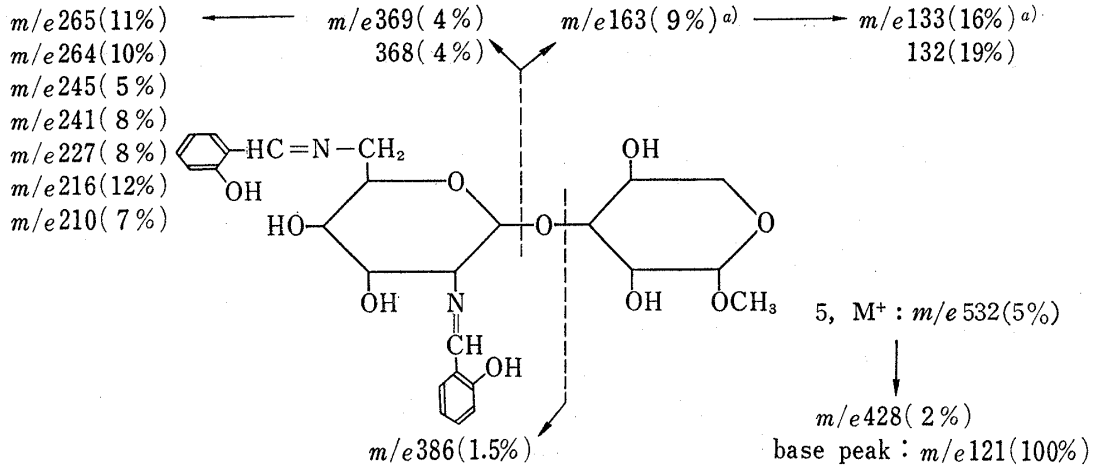


Chart 5

a) Accumulated relative intensities of two or more fragment ions.

Unique fragmentation of the N-salicylidene Schiff bases was demonstrated by comparing it with fragmentation of the parent free bases and other forms of derivatives. Fig. 3 shows a mass chart of paromamine free base (**1a**). Here, the base peak was the C_2 fragment at *m/e* 59 (C_2H_5ON). The 2-deoxystreptamine ions arising from the cleavage of a glycosidic bond were observed at *m/e* 163 and 145. The peak at *m/e* 163 had an elemental composition $C_6H_{15}O_3N_2$, and assigned to the (2-deoxystreptamine plus hydrogen) cation (f_1). Another peak at *m/e* 145 was a doublet, of which a higher mass ion with a composition $C_6H_{13}O_2N_2$ was assigned to the 2,4-dideoxysterptamine cation (f_2), and a lower mass ion with $C_6H_{11}O_3N$ to the desamino-2-deoxystreptamine cation (f_3) (Chart 1). The assignment of these ions was supported by the following fact. Both f_2 and f_3 ions appeared consistently in the spectra of glycosides of 2-deoxystreptamine, but the f_2 ion lacked in the spectrum of 2-deoxystreptamine itself. It was noted in this connection that the f_2 ion was stronger than the f_1, indicating the favorable fission of the glycosidic bond i between the ether oxygen and C-4 of 2-deoxystreptamine. The relative intensities of the f_1, f_2 and f_3 ions in the spectrum of neamine base (**3a**) were similar to those in **1a** (Chart 3).

An aminosugar fragment ion of **1a** was observed at *m/e* 162 (g_1) with a composition $C_6H_{12}O_4N$, which was shifted to an ion at *m/e* 161 with $C_6H_{13}O_3N_2$ in the spectrum of **3a**. These peaks were reasonably assigned to the 2-amino and 2,6-diaminoglucose ions, formed by the glycoside cleavage. The strong C_7 fragment ions arising from the cleavage of the C_1-C_2 bond

of the aminosugar portion were observed at *m/e* 191 (the O-formyl-2-deoxystreptamine cation, h_1) and at *m/e* 173 (h_2) (the dehydrated ion of h_1). Thus, in the spectra of the free bases, the 2-deoxystreptamine cation (f_1) corresponding to the strong a_1 ion in the N-salicylidene Schiff bases was relatively weak, and its presence was masked by other ions (f_2, f_3, g_1, h_1 and h_2) having stronger intensities.

Major fragment ions in the highest mass region of **1a** and **3a** consisted of the dehydrated peaks at *m/e* 306, 287 and 275. These dehydrated peaks were not observed in the Schiff bases.

Similar to the behavior of the free bases, the spectrum of N-acetyl neamine (**3b**) showed a favorable fission of the 2-deoxystreptamine and ether oxygen bond, as evidenced by the formation of a strong peak of the di-N-acetyl dideoxystreptamine ion at *m/e* 229 and a weak peak of the di-N-acetyl 2-deoxystreptamine ion at *m/e* 247. The di-N-acetyl 2,6-diamino-glucose ion at *m/e* 245 and the O-formyl-N-acetyl 2-deoxystreptamine ion at *m/e* 275 showed medium to weak intensity (Chart 6).

Comparative study was further extended to include the N-salicylidene-O-trimethylsilyl derivative (**3c**). Again, the glycosidic fission occurred more favorably between 2-deoxy-streptamine and the ether oxygen, supported by the strong *m/e* 641 peak and the weak *m/e* 657 peak (Chart 6).

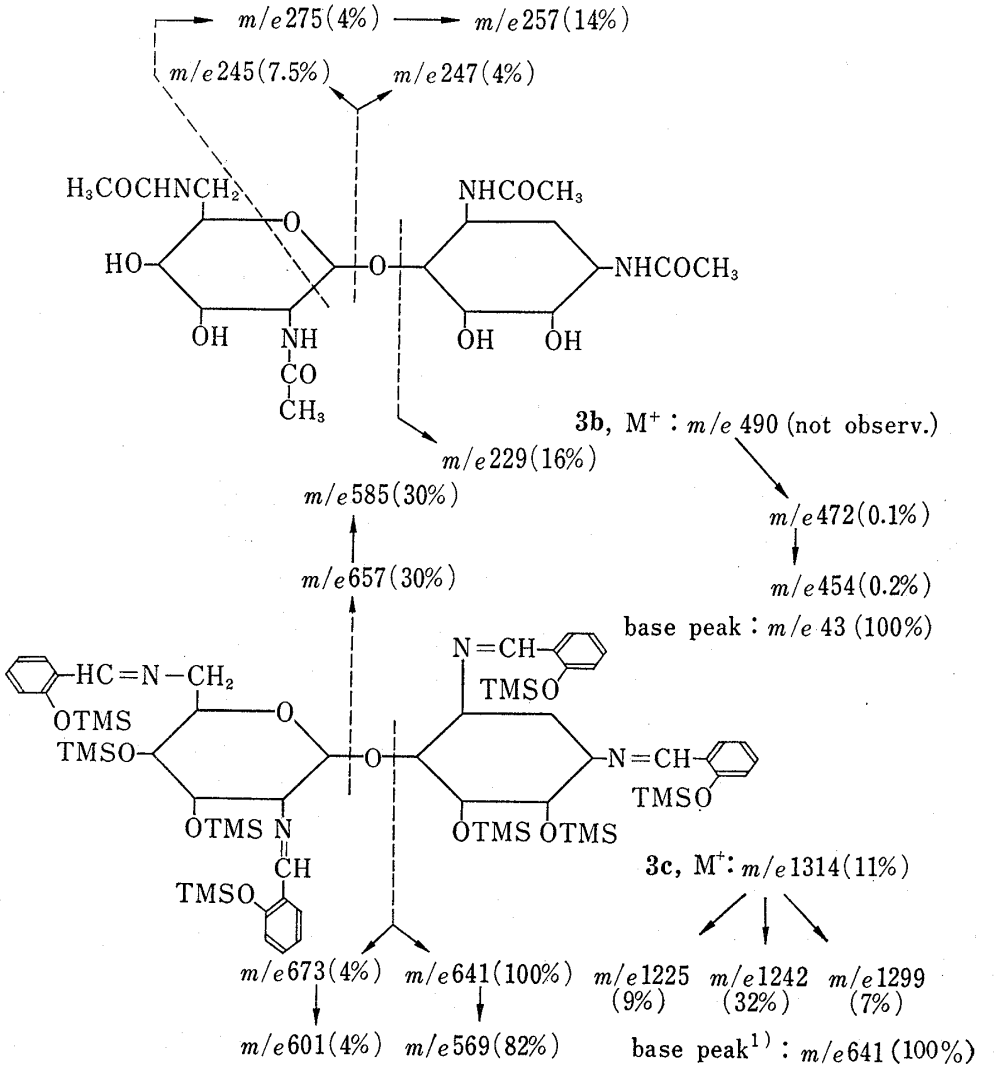


Chart 6

1) The base peak was arbitrarily selected from the peaks above *m/e* 200.

These studies described above indicated that a major spectral difference between the N-salicylidene Schiff bases, and free bases and N-acetyl derivative was present in the fragment ions involving 2-deoxystreptamine, and that fission was more easily accomplished at the aminosugar and ether oxygen bond rather than the ether oxygen and 2-deoxystreptamine bond in the formers (**1**—**4**), yielding the strong a_1 ion, while the reverse was true in the latters (**1a**, **3a**, and **3b**), showing a prominent dideoxystreptamine ion. Since O-trimethylsilyl derivative of the Schiff base (**3c**) did not follow the fragmentation pattern of the Schiff bases having free hydroxyl groups, the presence of labile hydrogen atoms probably plays an important role for the stabilization of the 2-deoxystreptamine cation (a_1). It was further noted that the fragment ions from the cleavage of a pyranose ring had comparable intensity to those from the glycoside fission in the spectra of **1a**, **3a** and **3b**, thereby complicating the interpretation of the spectra.

N-Salicylidene Kanamycin A (6) and 2-Manno-kanamycin (7)——Fundamental fragmentation pattern of the N-salicylidene pseudodisaccharides could be applied to interprete fragmentation of N-salicylidene pseudooligosaccharides. Table I shows major fragment ions of N-salicylidene kanamycin A (**6**) and its stereoisomer (**7**). The mass spectra of N-salicylidene kanamycins (**6** and **7**) were very similar from each other, and characterized by the appearance of the well recognized peaks at *m/e* 635, 370 and 266. The peak at *m/e* 635 was due to the two structural subunits, N-salicylidene 3-aminoglucosyl (or 3-aminomannosyl) 2-deoxystreptamine and 6-aminoglucolys 2-deoxystreptamine, which were formed by loss of the 6- or 3-aminosugar moiety from the M^+, accompanied by a hydrogen transfer. We could not determine the contributional weight of the two subunits on this peak. The *m/e* 635 peak may be formed also from the *m/e* 739 peak, derived from the M^+ by release of a N-salicylidene C_1 fragment and H_2O. Further fragmentation of the *m/e* 635 peak would yield peaks at *m/e* 617, 531 and 514, as shown in Chart 7.

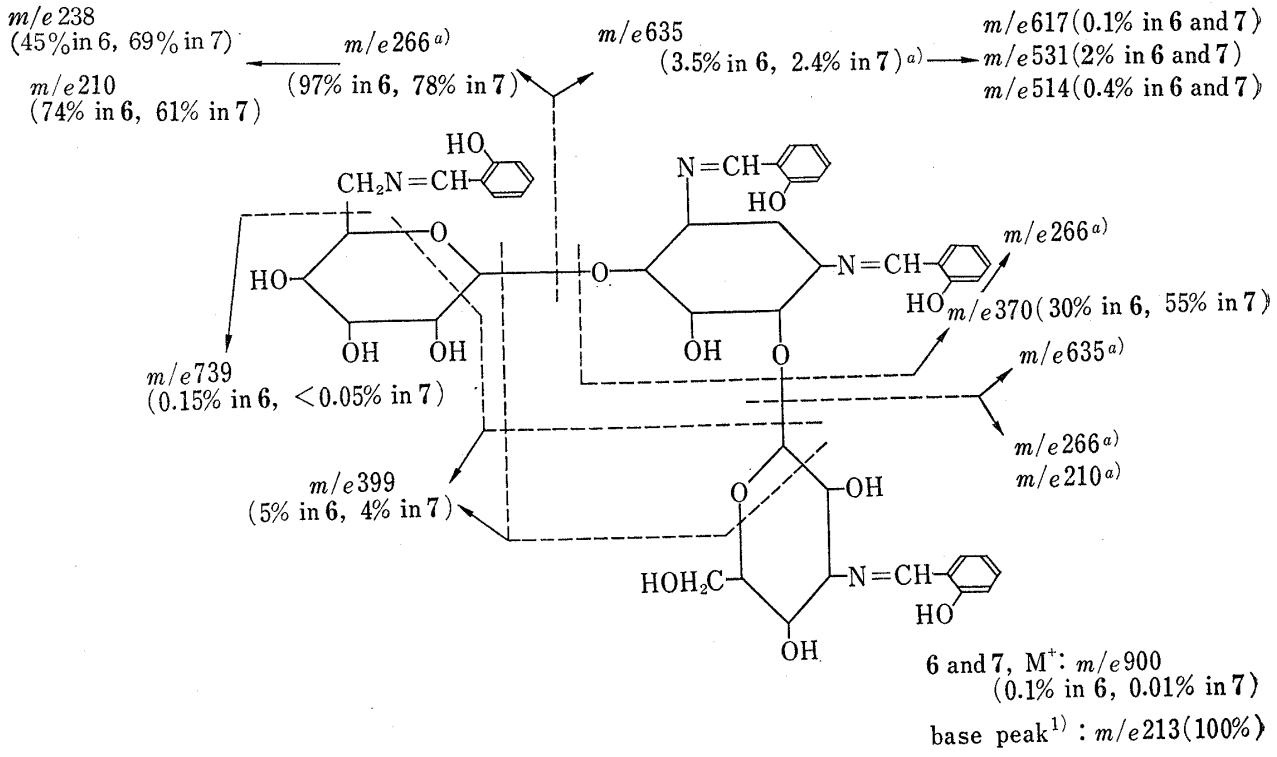


Chart 7

a) Accumulated relative intensities of two or more fragment ions.
1) The base peak was arbitrarily selected from the peaks above *m/e* 200.

The strong *m/e* 370 peak was due to the di-N-salicylidene 2-deoxystreptamine cation (a_1). The formation of this peak was accompanied by two hydrogens transfer. Other fragment ions related to the 2-deoxystreptamine moiety were observed at *m/e* 399 (c_3), 234 (a_7),231 (a_8), 213 (a_{10}), *etc.* A characteristic peak of the aminosugar moieties appeared at *m/e* 266. Its high intensity was due to double contribution from 3-amino and 6-aminohexoses, in addition to 2-deoxystreptamine. Similarly, relatively high intensities of the *m/e* 238, 213, 212 and 210 peaks suggested main contribution from the aminosugar fragments. Since the *m/e* 238 peak strongly appeared in N-salicylidene 6-aminokanamycin (**9**), but not in N-salicylidene kanamycin B (**8**) and 3′-aminokanamycin,[8] the origin of this peak could be ascribed to the 6-aminoglucose moiety, which is the common component of **6**, **7** and **9**.

N-Salicylidene Kanamycin B (8) and 6-Aminokanamycin (9)——Introduction of one amino group to C-2′ or C-6″ of the kanamycin molecule resulted in the considerable change in the mass spectra of the N-salicylidene derivatives. Main fragment ions of N-salicylidene kanamycin B (**8**) were shown in Table I and Chart 8. The spectrum of **8** showed "sequence peaks" arising from the two structural subunits, that is, the N-sali-

Table I. Relative Intensities of Major Fragment Ions of N-Salicylidene Kanamycin A (**6**), 2-Mannokanamycin (**7**), Kanamycin B (**8**) and 6-Aminokanamycin (**9**)[a]

m/e	**6** (230°) (%)	**7** (230°) (%)	**8** (240°) (%)	**9** (230°) (%)
1003			0.2	0.05
900	0.1	0.01		
792			1.0	
739	0.15	<0.05		
738			0.5	0.7
635	3.5	2.8	8.0	4.5
617	0.1	0.1	0.3	0.5
531	2.0	2.0	1.5	1.8
514	0.4	0.4		1.0
478	2.0	1.8	3.0	2.2
399	5	4	4	3.5
370	30	60	25	80
369	3	3	13	9
368	4	4	12	5
353	4	8	8	6
290	<4	<4	20	5
266	97	78	34	55
265	15	14	25	16
262	24	11	25	13
261	12	15	11	18
248	19	22	10	23
245	<4	<4	100	7
238	45	69	15	73
234	32	56	23	53
231	16	28	10	15
229	33	43	19	34
228	32	44	34	41
227	25	45	96	100
225	10	<4	60	<10
221	21	28	13	26
213	100	100	32	66
212	76	72	28	61
210	74	61	40	87

a) The base peak was arbitrarily selected from the peaks above *m/e* 200.

8) S. Inouye, *Chem. Pharm. Bull.* (Tokyo), **16**, 573 (1968).

cylidene neamine ion at *m/e* 738 and the N-salicylidene 3-aminoglucosyl 2-deoxystreptamine ion at *m/e* 635 and 531 (635 minus 104).

Except for the *m/e* 399 peak, major fragment ions above *m/e* 370 arised from the direct cleavage of the glycosidic bonds, almost exclusively by the cleavage of the ether oxygen and sugar bond. Accordingly, the spectrum became simplified, and facilitated easy correlation with the structure. The highest fragment ion at *m/e* 792 was tentatively assigned the structure as shown in Chart 8. The peak at *m/e* 399 was due to the N-salicylidene O-formyl-2-deoxystreptamine ion (c_3), which was produced from the 3-aminoglucosyl 2-deoxystreptamine moiety and/or the neamine portion.

Fragment ions of the three components were recognized in peaks at *m/e* 370, 266, 234, 231, 213 and 212 (N-salicylidene 2-deoxystreptamine ions), 369, 368, 290, 265, 245, 227 and 225 (N-salicylidene 2,6-diaminoglucose ions), and 266, 213 and 210 (N-salicylidene 3-aminoglucose ions). Of these, the *m/e* 290, 266, 227 and 210 peaks were shown to be doublets by the high resolution mass analysis, and the *m/e* 265 peak was a triplet (Chart 8).

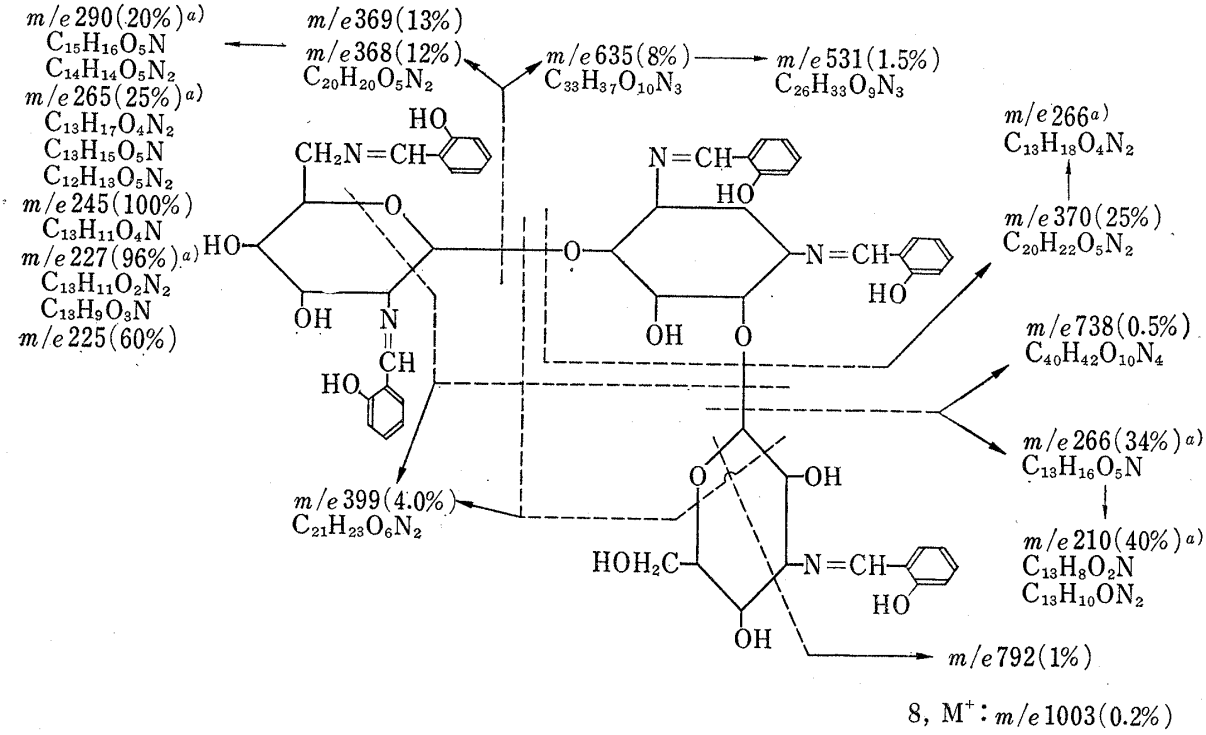


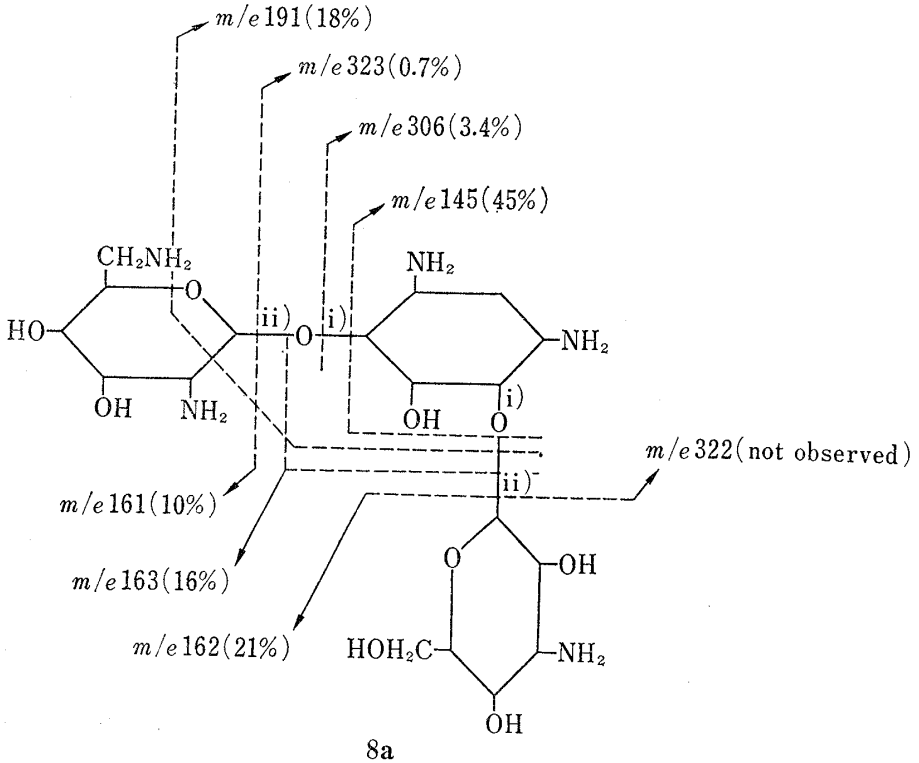
Chart 8[1)]

a) Accumulated relative intensities of two or more fragment ions.
1) The base peak was arbitrarily selected from the peaks above *m/e* 200.

Mass spectrum of N-salicylidene 6-aminokanamycin (**9**) was similar to that of **6**, **7** and **8** in the general feature, but difference existed in the fragmentation involving the amino-sugar moieties. The spectrum of **9** showed only weak peaks at *m/e* 290, 245 and 225, similar to those of **6** and **7**, which were strong in the spectrum of **8**. On the other hand, the intense *m/e* 227 peak differentiated **9** from **6** and **7**. Also strong was the *m/e* 227 peak in the spectra of **8** and N-salicylidene 3′-aminokanamycin,[8)] and therefore diagnostic for the 2,6- and 3,6-diaminoglucose moieties. Compound **9** contained **4** as a partial component, and showed the characteristic c_4 ion at *m/e* 400 (3%).

Mass spectrum of kanamycin B free base (**8a**) was examined for a comparison (Chart 8a). Fragment ions due to the three components were observed at *m/e* 163 and 145 (2-deoxystreptamine), 162 (3-aminoglucose) and 161 (2,6-diaminoglucose). The strong intensity of

the *m/e* 145 peak over the *m/e* 163 peak indicated the predominant cleavage i, as seen in the spectra of neamine (**3a**) and paromamine base (**1a**). It was noted, however, that pseudodisaccharide ions which should appear at *m/e* 323 and/or 306, and 322 and/or 305 were very weak or little observed. In this respect, the spectrum of the N-salicylidene derivative (**8**) provides more useful structural information than that of the free base (**8a**), by showing the easily recognizable pseudodisaccharide ions.



8a

M$^+$: *m/e* 484(not observed)
base peak : *m/e* 59(100%)

Chart 8a

N-Salicylidene Paromomycin I and II (10 and 11)——Mass spectra of N-salicylidene paromomycin I (**10**) and II (**11**) were similar, and showed strong peaks above *m/e* 370 at *m/e* 767, 635, 531, 502, 500 and 399 (Table II). Of these, the peaks at *m/e* 767, 635 and 531 did not appear in the neomycin counterparts, and were assigned to the N-salicylidene ribosyl paromamine, and the tri- and di-N-salicylidene paromamine ions, supported by the elemental analysis (Chart 9). The formation of the *m/e* 767 ion indicated the preferential cleavage of the glycosidic bond III rather than the bond II. In the latter case, the *m/e* 733 ion should be formed.

The peaks at *m/e* 502 and 500 were observed also in the neomycin derivatives, but not present in the kanamycin series. The *m/e* 502 ion had an elemental composition $C_{25}H_{30}O_9N_2$, and could be assigned to the N-salicylidene ribosyl 2-deoxystreptamine ion. Further fragmentation of this peak would yield an ion at *m/e* 471. The peak at *m/e* 500 with $C_{25}H_{28}O_9N_2$ was assigned to the N-salicylidene neobiosamine B or C ion, because this peak was present in the spectra of the neomycin derivatives (**12** and **13**), but not in that of N-salicylidene ribostamycin that consists of ribose and neamine.[9] The *m/e* 502 and 500 peaks appeared only

9) E. Akita, T. Tsuruoka, N. Ezaki, and T. Niida, *J. Antibiotics*, 23, 173 (1970).

Table II. Relative Intensities of Major Fragment Ions of N-Salicylidene Paromomycin I (**10**), II (**11**), Neomycin B (**12**) and C (**13**) at Various Temperatures[a)]

m/e	**10**		**11**		**12**		**13**	
	220° (%)	240° (%)	220° (%)	240° (%)	230° (%)	250° (%)	220° (%)	240° (%)
870						0.05	0.04	0.1
767	0.05	0.2		0.1				
738					0.1	0.4	0.2	0.6
635	0.6	2.0		1.0				
634					0.05	0.2	0.1	0.3
617	0.1	0.2			0.05	0.2		
563		0.2		0.3		0.07		0.4
531	0.6	1.0		1.0				
514		0.5		0.1				
502	0.6	2.0		2.0	0.1	8.0	2.0	7.1
500	2.0	1.0	0.3	0.3	1.0	0.8	1.5	1.0
471	0.4	1.0	0.05	0.1	0.3	0.3	1.0	2.8
399		4.0		3.0		8.0		7.0
370	11	13	4	12	10	50	27	38
369	7	5	2	2.5	5	7.5	2.5	6
368	22	9	4	2.5	17	10	5	6
353	8	5	4	4	10	10	9	6
340	11	3	6		10	8	14	5
332	28	13	12	8	13	15	16	14
266	44	35	15	28	24	65	30	39
265	22	17	15	14	10	20	9	17
262	17	9	13	8	14	30	11	12
261	13	7	10	8	10	25	8	10
248	17	13	9	12	13	20	19	19
245			69		62	28	43	29
241			85		9.5		100	14
238	56	35	23	34	29	45	32	38
234	83	48	85	36	67	65	39	62
231	9	13	7	9	8.5	30	11	24
228	56	35	46	28	43	75	75	62
227	56	39	100	36	100	55	57	50
225	16	9	23	8	21	10	9	12
221	33	22	25	16	19	30	16	60
216	67	39	10	6	38	38	9	14
213	100	100	62	100	43	80	55	88
212	72	76	46	78	43	65	55	100
210	78	52	58	40	64	100	66	52

a) The base peak was arbitrarily selected from the peaks above *m/e* 200.

in the spectra of the ribose containing antibiotics, and hence a diagnostic value for the differentiation of the paromomycin-neomycin series from the kanamycin series that contains no pentose.

Simultaneous occurrance of the four fragment ions, N-salicylidene paromamine, ribosyl 2-deoxystreptamine, neobiosamines and ribosyl paromamine was enough to suggest the framework of paromomycins, and therefore, the most important diagnostic ions.

The peaks at *m/e* 399, 370 and 234 were assigned to the fragment ions related to the N-salicylidene 2-deoxystreptamine portion. Fragment ions involved the two 2,6-diaminohexoses were found at *m/e* 369, 368, 265, 245, 241, 227 and 216. The peaks at *m/e* 266, 213 and 212 were ascribed mainly to the 2-aminoglucose ions. The ribose ion was weakly observed at *m/e* 132.

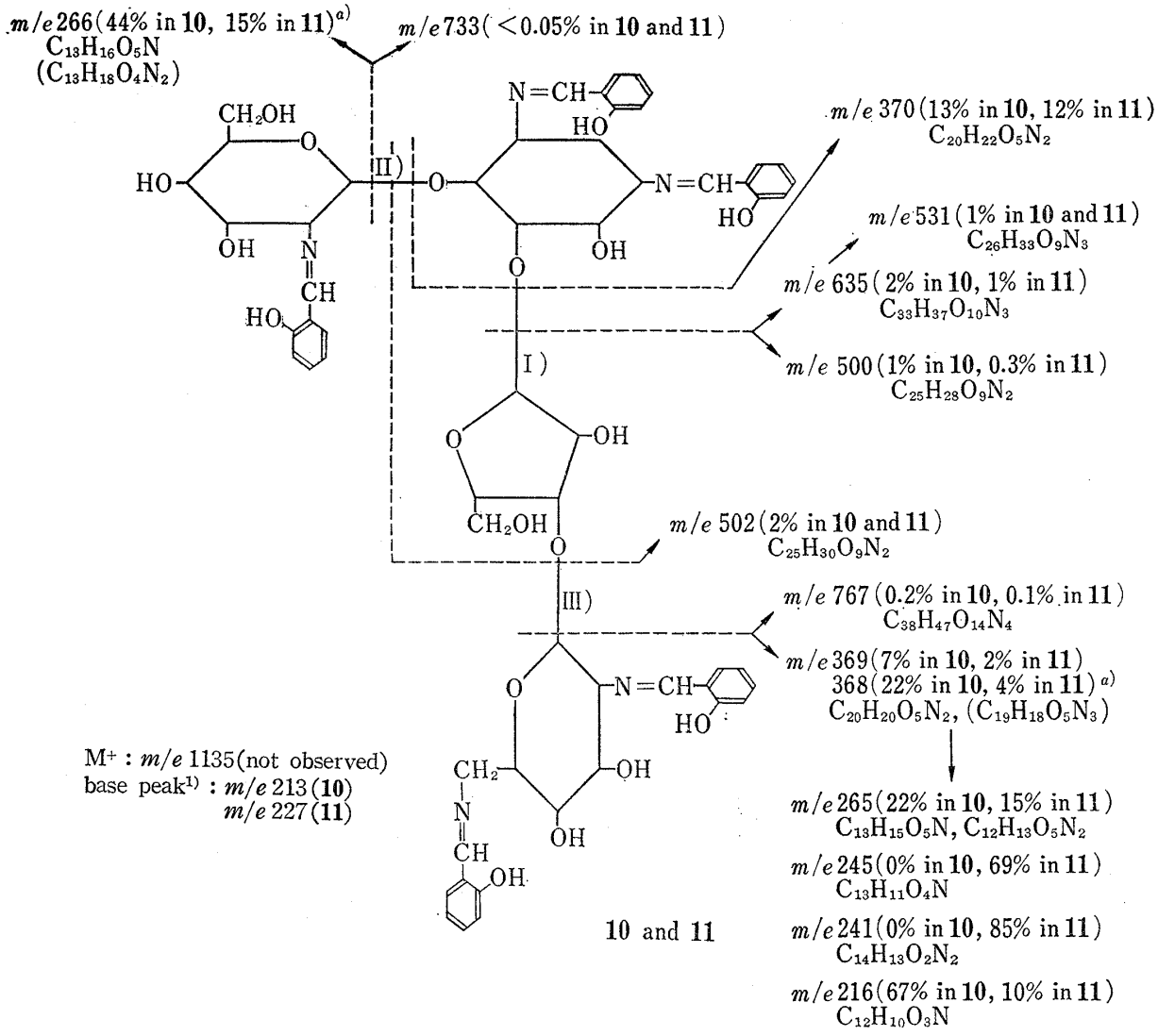


Chart 9[2]

a) Accumulated relative intensities of two or more fragment ions.
1) The base peak was arbitrarily selected from the peaks above *m/e* 200.
2) Relative abundances of the peaks below *m/e* 369 and the peaks above *m/e* 370 were obtained at 220° and 240°, respectively.

N-Salicylidene Neomycin B and C (12 and 13)——Structural difference between paromomycins and neomycins is the replacement of a terminal hydroxyl group in the former by an amino group in the latter. This small change caused a considerable change in the mass spectra of the N-salicylidene derivatives, as already seen in the kanamycin series. Table II shows main fragment ions of **12** and **13**.

The spectra of **12** and **13** showed a peak at *m/e* 870, which could be formed by the cleavage III), yielding the N-salicylidene ribosyl neamine (ribostamycin[9]) ion, and/or by the cleavage II) giving rise to the N-salicylidene neobiosaminyl 2-deoxystreptamine ion (Chart 10). However, the formation of the latter was less probable, since the cleavage III) was predominated in the fragmentation of **10** and **11**.

N-Salicylidene disaccharide ions and pseudodisaccharide ions were found at *m/e* 738, 502 and 500, and they were assigned to the tri-N-salicylidene neamine, ribosyl 2-deoxystreptamine and neobiosamine B or C ions, respectively. A complementary peak of the *m/e* 738 ion was observed at *m/e* 634 (738 minus 104). A combination of the neamine, ribosyl 2-deoxy-

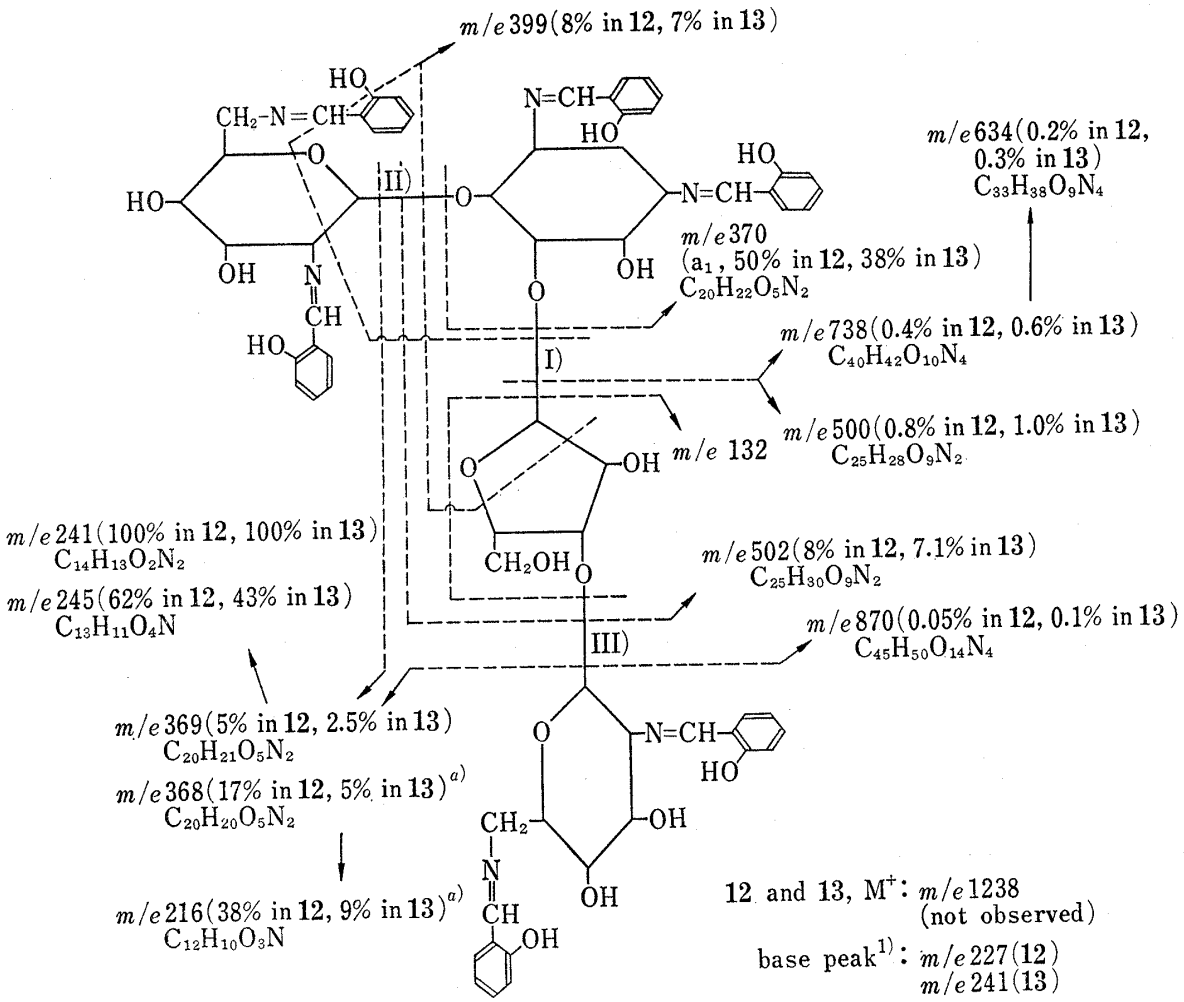


Chart 10[2)]

a) Accumulated relative intensities of two or more fragment ions.
1) The base peak was arbitarily selected from the peaks above *m/e* 200.
2) Relative abundances of the peaks below *m/e* 369 and the peaks above *m/e* 370 were obtained at 230° or 220° and 250° or 240°, respectively.

streptamine, neobiosamines and ribosyl neamine fragments easily led to construct the gross structure of neomycins, though the spectra did not give rise to the M^+.

Monosaccharide ions arising from the 2-deoxystreptamine, 2,6-diaminosugars and ribose moieties were recognized respectively as shown in Table II and Chart 10.

Although it is generally known that mass spectra are reflected little stereochemistry, some noticeable steric influence was observed in the paromomycins and neomycins series. The peaks at *m/e* 245 and 241 were relatively strong in N-salicylidene paromomycin II (**11**), and neomycins B (**12**) and C (**13**) as well as **3** and ribostamycin,[9)] but lacked in the spectrum of paromomycin I (**10**). Main contributor to these peaks, therefore, seemed to be the 2,6-diamino-D-glucose fragment, which is the common moiety in these antibiotics. Definite structures of both ions were unknown, but the low hydrogen contents suggested the extensive dehydrogenation of a pyranose ring. Since they were not formed from the 2-amino- and 3-aminoglucose, 3,6-diaminoglucose and 2,6-diaminoidose moieties, the formation by the selective removal of a salicylidene group at C-2 position of the 2,6-diaminoglucose was implied. The *m/e* 241 peak had one more carbon than the *m/e* 245 peak. The extra carbon may be derivable from an azomethine carbon or C-4 of the 2-deoxystreptamine portion.

On the other hand, the peaks at *m/e* 368 and 216 appeared strongly in N-salicylidene paromomycin I (**10**) and neomycin B (**12**) as well as **5**, indicating that the 2,6-diamino-L-idose moiety was a main contributor. The *m/e* 216 peak was a doublet, a major ion with $C_{12}H_{10}O_3N$ and a minor with $C_{12}H_{12}O_2N_2$, of which the major ion was characteristic for the 2,6-diamino-idose moiety. Since this ion was not formed from 3,6-diamino- or 2,6-diamino-D-glucose, its formation by the selective removal of the C-6 salicylideneiminomethyl group was most probable.

The mass spectra of salicylidene Schiff bases of monosaccharides were little affected by the temperature of an ion chamber, but the spectra of N-salicylidene pseudooligosaccharides, in particular, paromomycins and neomycins were considerably temperature dependent as exemplified in Table II. The remarkable temperature effect in Table II indicated that thermal decomposition plays an important role in the fragmentation of the pseudotetrasaccharides (**10**—**13**). As the temperature raised from 210° to 230° (or from 230° to 250°), fragment ions at *m/e* 500, 368, 340, 245, 241 and 227 decreased, while fragment ions at *m/e* 502, 399, 370, 266, 238, 213 and 212 increased. The temperature effect on the *m/e* 502, 500, 370, 368 and 266 peaks may be explained by assuming that the glycosidic linkages between ribose and 2,6-diamino-L-idose (or 2,6-diamino-D-glucose) (III), and between ribose and 2-deoxystreptamine (I) were initially cleaved at lower temperature, followed by the rupture of the linkage between 2-deoxystreptamine and 2-aminoglucose (or 2,6-diaminoglucose) (II) at higher temperature.

In conclusion, the mass spectra of N-salicylidene aminoglycosidic antibiotics provided the following structural information.

1) The M^+ were recognizable up to pseudotrisaccharides. The M^+ of pseudotetrasaccharides became observable in the form of O-trimethylsilyl ethers of N-salicylidene Schiff bases. The (M minus 104) peak appeared as a complementary peak of the M^+.

2) Fragment ions arising from the cleavage of glycosidic bonds became principal peaks, thereby facilitating the recognition of component sugars, and disclosing the binding sequence of components. The formation of these ions was always accompanied with transfer of one or two hydrogens.

3) The spectra of the N-salicylidene derivatives were affected more by the number of salicylideneimino groups rather than the substituted positions. Addition of one amino group in place of a hydroxyl group caused the increase of 104 mass units for fragment ions.

4) The positional and stereochemical effects of the salicylideneimino function on the fragmentation pattern were generally small, but the differentiation was possible between the 2-amino and 3-aminosugar moieties, between the 2,6-diamino and 3,6-diaminosugar moieties, and between the 2,6-diamino-D-glucose and -L-idose moieties.

The results of the present study coupled with the ease of preparation in a micro scale makes the N-salicylidene Schiff bases attractive derivatives for mass spectrometry of aminosugars, particularly aminoglycosidic antibiotics. In fact, this was demonstrated in the structural elucidation of a new paromomycin member, lividomycin A (SF-767 A[7]) and SS-56 substance [10] belonged to the destomycin family.

Experimental

Materials——The N-salicylidene derivatives employed in this work were prepared according to the procedures reported in the following literatures.

Tri-N-salicylidene paromamine (**1**),[11] tri-N-salicylidene 3-amino-3-deoxy-α-D-glucosyl 2-deoxystreptamine (**2**),[8] tetra-N-salicylidene neamine (**3**),[12] tetra-N-salicylidene 3,6-diamino-3,6-dideoxy-α-D-glucosyl

10) S. Inouye, K. Totsugawa, T. Shomura, and T. Niida, to be published.
11) S. Inouye, *Chem. Pharm. Bull.* (Tokyo), **15**, 1609 (1967).
12) T. Ito, M. Nishio, and H. Ogawa, *J. Antibiotics, Ser. A*, **17**, 189 (1964).

2-deoxystreptamine (**4**),[8] di-N-salicylidene methyl 2,6-diamino-2,6-dideoxy-α-L-idosyl-β-D-ribopyranoside (**5**),[11] tetra-N-salicylidene kanamycin A (**6**),[11] tetra-N-salicylidene 2-mannokanamycin (**7**),[13] penta-N-salicylidene kanamycin B (**8**),[11] penta-N-salicylidene 6-aminokanamycin (**9**),[14] penta-N-salicylidene paromomycin I (**10**) and II (**11**),[11] hexa-N-salicylidene neomycin B (**12**) and C (**13**).[11]

The free bases of paromamine (**1a**), neamine (**3a**) and N-acetyl neamine (**3b**) were prepared from paromomycin and neomycin in this laboratory. The O-trimethylsilyl ethers of the N-salicylidene derivatives (**3c** and **13b**) were prepared by treating **3** or **13** with the TMS-PZ reagent purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, at room temperature for 30 min. The reaction mixture was introduced directly in an ion chambr to avoid the decomposition of the TMS derivatives.

Mass Spectra——The low resolution mass spectra were recorded on a JMS-01SG double focussing mass spectrometer at 75 eV using the direct inlet system. To get a maximum gain, a low resolution of 700 (10% valley), a maximum accelerating voltage (8 kV), and a maximum ionizing current of 350—450 μA were used.

High resolution measurements were determined under the same condition, but with a resolution of ca 15000 (10% valley). The exact mass data were obtained by the photographic recording with PFK as an internal standard. The plates used were highly sensitive plates manufactured by TECH/OPS, U.S.A. All values were within ±2 milli mass units below *m/e* 400, and ±9 milli mass units above *m/e* 400 of the theoretical values calculated for elemental compositions.

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13) S. Inouye, *Sci. Reports, Meiji Seika Kaisha*, **11**, 84 (1970).
14) S. Inouye, *J. Antibiotics, Ser. A*, **20**, 6 (1967).