

Decompositions of Multiply Charged Oligonucleotide Anions

Scott A. McLuckey* and Sohrab Habibi-Goudarzi

Contribution from the Chemical and Analytical Sciences Division,
Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6365

Received April 12, 1993. Revised Manuscript Received July 12, 1993*

Abstract: Multiply charged single-strand deoxyoligonucleotide anions fragment first by loss of a nucleobase followed by cleavage at the 3' C–O bond of the sugar from which the base is lost. Both steps are proposed to proceed via 1,2-elimination involving hydrogens from the sugar and to yield a stable substituted furan as one of the products. There is a strong preference for loss of charged adenine followed by loss of charged thymine. This tendency is strongly dependent, however, upon the internal Coulombic repulsion experienced by the ion. The position of the base in the chain is not a major factor in determining which base is lost first, except in the case of the base at the 3' terminus. The loss of the base at the 3' terminus tends to be disfavored, and this tendency may result in the more abundant loss of a charged thymine, for example, than the loss of charged adenine when the only deoxyadenylate present in the sequence is at the 3' terminus. Relatively small oligomers can be fully or nearly fully sequenced via several stages of mass spectrometry. Sequencing adjacent deoxyguanylate and deoxycytidylate residues tends to be difficult due to the much lower abundances of product ions formed via reaction channels beginning with losses of cytidine and guanine. Multiple stages of mass spectrometry are facilitated by highly charged parent ions.

Introduction

The structures and reactivities of multiply charged gas-phase ions are of interest from both theoretical and applied points of view.¹ Until recently, most experimental studies on multiply charged polyatomic ions have been performed on relatively small dications,² with a much smaller number of studies focused on small dianions.³ The advent of electrospray as a means for forming gaseous ions, however, has greatly expanded the range of species that might be formed with more than one charge.⁴ The most highly charged species have been formed from polymers including polyethylene glycols⁵ and biopolymers⁶ such as peptides, proteins, carbohydrates, and oligonucleotides. Several recent reviews describe these developments with emphasis on multiply charged biopolymers.⁷

The capability of electrospray to form multiply charged biopolymers has made possible the study of the uni- and bimolecular chemistries of large molecules of biological interest, as they are affected by the internal Coulombic field created by the presence of multiple charge sites. The study of the collision-

induced dissociation of multiply charged peptides and proteins, for example, has been pioneered by the Smith group,^{7b,d,8} and several groups have described results of proton-transfer,^{7d,9} and H/D-exchange¹⁰ reactions involving multiply charged peptides and proteins. Ion-ion reaction experiments involving multiply charged proteins have also been described.¹¹

In contrast to the study of the relatively robust peptides and proteins, little work has been reported on the reactions of multiply charged oligonucleotides. In fact, gas-phase ions of oligonucleotides of any kind, be they singly or multiply charged, positive or negative, have historically been difficult to study by any technique due to their highly polar and fragile nature.¹² However, the recent successes of electrospray in forming multiply charged anions from oligonucleotides,^{7d,13} have allowed the study of the reactions of these species in the gas phase. We recently reported the results of a study of several small ($n = 4-8$) multiply charged oligonucleotide anions subjected to collisional activation in a quadrupole ion trap.¹⁴ It was noted that all of the ions studied showed structurally informative fragmentation and that only a few competitive and consecutive reaction channels dominated. The latter observation indicated that one or more stages of

* To whom correspondence should be addressed. Telephone (615) 574-2848.

† Abstract published in *Advance ACS Abstracts*, November 15, 1993.

(1) See, for example: (a) Koch, W.; Maquin, F.; Stahl, D.; Schwarz, H. *Chimia* **1985**, *39*, 376. (b) Kemp, D. L.; Cooks, R. G. In *Collision Spectroscopy*; Cooks, R. G., Ed.; Plenum Press: New York, 1978; p 257.

(2) (a) Koch, W.; Heinrich, N.; Schwarz, H.; Maquin, F.; Stahl, D. *Int. J. Mass Spectrom. Ion Proc.* **1985**, *67*, 305. (b) Appling, J. R.; Burdick, G. W.; Moran, T. F. *Org. Mass Spectrom.* **1985**, *20*, 343. (c) Ast, T. *Adv. Mass Spectrom.* **1986**, *10A*, 471.

(3) Compton, R. N. In *Negative Ions*; Esaulov, V. A., Ed.; Cambridge University Press: Cambridge, U.K., in press.

(4) (a) Yamashita, M.; Fenn, J. B. *J. Phys. Chem.* **1984**, *88*, 4451; (b) **1984**, *88*, 4671. (c) Aleksandrov, M. L.; Gall, L. N.; Krasnov, N. V.; Nikolaev, V. I.; Pavlenko, V. A.; Shkurov, V. A. *Dokl. Akad. Nauk SSSR* **1984**, *227*, 379. (d) Aleksandrov, M. L.; Baram, G. I.; Gall, L. N.; Grachev, M. A.; Knorre, V. D.; Krasnov, N. V.; Kusner, Y. S.; Nikolaev, V. A.; Pavlenko, V. A.; Shurov, V. A. *Bioorg. Khim.* **1984**, *10*, 710.

(5) Wong, S. F.; Meng, C. K.; Fenn, J. B. *J. Phys. Chem.* **1988**, *92*, 546.

(6) Meng, C. K.; Mann, M.; Fenn, J. B. *Z. Phys. D: At., Mol. Clusters* **1988**, *10*, 361.

(7) (a) Fenn, J. B.; Mann, M.; Meng, C. K.; Whitehouse, C. M. *Science* **1990**, *246*, 64. (b) Smith, R. D.; Loo, J. A.; Edmonds, C. G.; Barinaga, C. J.; Udseth, H. R. *Anal. Chem.* **1990**, *62*, 882. (c) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Mass Spectrom. Rev.* **1990**, *9*, 37. (d) Smith, R. D.; Loo, J. A.; Ogorzalek Loo, R. R.; Busman, M.; Udseth, H. R. *Mass Spectrom. Rev.* **1991**, *10*, 359. (e) Mann, M. *Org. Mass Spectrom.* **1990**, *25*, 575. (f) Huang, E. C.; Wachs, T.; Conboy, J. J.; Henion, J. D. *Anal. Chem.* **1990**, *62*, 713A.

(8) (a) Smith, R. D.; Barinaga, C. J.; Udseth, H. R. *J. Phys. Chem.* **1989**, *93*, 5019. (b) Smith, R. D.; Loo, J. A.; Barinaga, C. J.; Edmonds, C. G.; Udseth, H. R. *J. Am. Soc. Mass Spectrom.* **1990**, *1*, 53. (c) Loo, J. A.; Edmonds, C. G.; Smith, R. D. *Anal. Chem.* **1993**, *65*, 425.

(9) (a) McLuckey, S. A.; Van Berkel, G. J.; Glish, G. L. *J. Am. Chem. Soc.* **1990**, *112*, 5668. (b) McLuckey, S. A.; Glish, G. L.; Van Berkel, G. J. *Anal. Chem.* **1991**, *63*, 1971. (c) Winger, B. E.; Light-Wahl, K. J.; Smith, R. D. *J. Am. Soc. Mass Spectrom.* **1992**, *3*, 624. (d) Ogorzalek Loo, R. R.; Udseth, H. R.; Smith, R. D. *J. Phys. Chem.* **1991**, *95*, 6412. (e) Ogorzalek Loo, R. R.; Loo, J. A.; Udseth, H. R.; Smith, R. D. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 159. (f) Ikononou, M. G.; Kebarle, P. *Int. J. Mass Spectrom. Ion Proc.* **1992**, *117*, 283.

(10) (a) Suckau, D.; Shi, Y.; Quinn, J. P.; Senko, M. W.; Zhang, M.-Y.; McLafferty, F. W. Proceedings of the 40th ASMS Conference on Mass Spectrometry and Allied Topics, Washington, DC, 1992; p 477. (b) Winger, B. E.; Light-Wahl, K. J.; Smith, R. D. Proceedings of the 40th Conference on Mass Spectrometry and Allied Topics, Washington, DC, 1992; p 481.

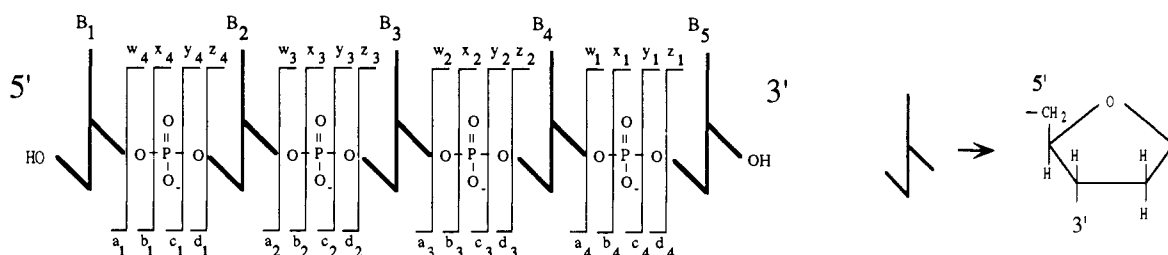
(11) (a) Ogorzalek Loo, R. R.; Udseth, H. R.; Smith, R. D. *J. Phys. Chem.* **1991**, *95*, 6412. (b) Ogorzalek Loo, R. R.; Udseth, H. R.; Smith, R. D. *J. Am. Soc. Mass Spectrom.* **1992**, *3*, 695.

(12) (a) Crain, P. F. *Mass Spectrom. Rev.* **1990**, *9*, 505. (b) McCloskey, J. A. In *Methods in Enzymology*; McCloskey, J. A., Ed.; Academic Press: New York, 1990; Vol. 193, Chapter 41.

(13) (a) Covey, T. R.; Bonner, R. F.; Shushan, B. I.; Henion, J. D. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 249. (b) Stults, J. T.; Marsters, J. C. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 359.

(14) McLuckey, S. A.; Van Berkel, G. J.; Glish, G. L. *J. Am. Soc. Mass Spectrom.* **1992**, *3*, 60.

Chart I



collision-induced dissociation could provide extensive, if not complete, sequence information. We have since studied a much larger suite of oligonucleotides and have characterized the phenomenology associated with their activation and dissociation under quadrupole ion trap conditions. The results have indicated that the fragmentation of multiply charged oligonucleotide anions follows a few simple rules which lead to extensive sequence information. This paper describes these rules and their dependence on charge state and oligonucleotide sequence. While the sequencing of large oligomers by mass spectrometry remains a challenging problem, the ability to sequence small oligomers expands the utility of mass spectrometry in some areas of nucleic acid research, such as in the study of modified oligonucleotides.

Experimental Section

Samples and Apparatus. All deoxyoligonucleotides and mononucleotides used in this study were obtained commercially as either the sodium salt or the free acid. All mononucleotides were obtained from Sigma Chemical Co., St. Louis, MO, while the oligonucleotides 5'-d(TG-CATCGT)-3', 5'-d(TCGGG)-3', 5'-d(CGTTCC)-3', and 5'-d(CCCAp)-3' were obtained from American Synthesis, Pleasanton, CA, and the oligonucleotides 5'-d(CCCC)-3', 5'-d(CCCA)-3', 5'-d(ACCT)-3', and 5'-d(TCCA)-3' were obtained from Genosys Biotechnologies, The Woodlands, TX. Solutions were prepared by dissolving the sample in a drop of HPLC-grade water and diluting with HPLC-grade methanol to give a concentration of 1–20 μM in at least 9:1 methanol:water (vol/vol). All solutions were infused at a rate of 1–3 $\mu\text{L}/\text{min}$ through a 120- μm -i.d. needle held at a potential of –3000 to –3500 V. All experiments were carried out with a home-made electrospray source coupled with a Finnigan-MAT (San Jose, CA) ion trap mass spectrometer modified for injection of ions formed external to the ion trap.¹⁵ Details of the electrospray/ion trap interface have been described.¹⁶

Ion Manipulation and Mass/Charge Analysis. Ions were injected into the ion trap for periods ranging from 0.1 to 0.5 s. The radio frequency (rf) signal amplitude applied to the ring electrode during ion injection ranged from 700 to 1000 V 0-p. In all cases, helium was admitted into the vacuum system to a total pressure of 1 mTorr, with a background pressure in the instrument of 2×10^{-5} Torr without the addition of helium.

Details of ion isolation, collisional activation, and mass analysis for multiply charged oligonucleotides have been given previously.¹⁴ Briefly, ion isolation was effected by one or more resonance ejection scans.¹⁶ A single resonance ejection scan was used for isolation of parent ions of $m/z < 650$, whereby low m/z ions were ejected by passing the ions through a q_z value of 0.908 by scanning the amplitude of the ring electrode rf signal while high-mass ions were ejected by dipolar resonance ejection using a 15-V p-p sine wave signal applied to the end caps at a frequency selected to eject ions at an m/z value slightly greater than that of the parent ion.¹⁷ For parent ions of $m/z > 650$, two resonance ejection scans were required, each using a different frequency applied to the end caps. Parent ions were typically isolated at less than unit resolution to avoid parent ion loss prior to collisional activation due either to dissociation or to ejection from off-resonance power absorption. A single stage of ion isolation was used for mass spectrometry/mass spectrometry (MS/MS) experiments, and two stages were employed for MS³ experiments. After the ions of interest were isolated, they were subjected to collisional activation by applying a supplementary rf signal to the end caps of an

amplitude of 100–500 mV p-p for 20–40 ms. Parent ions were typically subjected to collisional activation at a q_z value within a range of 0.07–0.2. This varied for each parent ion charge state because the low mass/charge cutoff during collisional activation was typically maintained at m/z 100 to allow for trapping of any charged bases should they be formed. Since parent ions vary in m/z for the various charge states, their q_z values during collisional activation were not constant for these studies.

Mass/charge analysis was effected after the completion of all ion isolation and collisional activation steps using either the mass-selective instability method in conjunction with axial modulation,¹⁸ when the nominal ion trap mass/charge range of 650 was sufficient, or resonance ejection,¹⁹ when a mass/charge range of 1300 was necessary to analyze all of the major product ions. The mass/charge scale for the MS/MS and MSⁿ spectra was calibrated using the electrospray mass spectrum of the parent compound. In this work, the mass/charge ratios of the various charge states of the parent compound were known and could be used to determine a correction for the mass scale provided by the ion trap data system. The mass accuracy associated with mass/charge assignments in the MS/MS and MSⁿ experiments is on the order of 0.1% or better. The spectra shown here were typically the result of an average of 10–30 individual scans.

Results and Discussion

Throughout this paper, the symbol for the parent deoxynucleotide, M, is intended to imply the neutral molecule with hydrogens attached to all phosphodiester linkages. The nomenclature for product ions is somewhat more complex due to the wide variety of fragments that can be formed. We have proposed a scheme¹⁴ codifying cleavages along the phosphodiester linkage, analogous to that used for peptides²⁰ to indicate cleavages along the peptide linkage, which we have found to be useful in labeling MS/MS spectra. The nomenclature is summarized in Chart I for a hypothetical 5-mer. Note that the “d” and “w” ions indicated in this scheme are not analogous to ions formed from charge-site-remote dissociations of side chains observed with peptides and proteins.

The work described previously¹⁴ applied to multiply charged deoxyoligonucleotides that contained at least one deoxyadenylate residue in the chain. It was noted that a very strong tendency is the loss of the adenine anion (m/z 134) as the first fragmentation step. The next reaction is fragmentation at the 3' C–O bond of the sugar from which the base was lost. The ion trap is particularly well-suited to identifying the lowest energy fragmentation processes, in that ion trap collisional activation²¹ can be analogous to a slow heating process. Under gentle collisional activation conditions, resulting from the use of low-amplitude supplementary rf signals, fragmentation can be largely limited to base loss. Under moderate conditions, consecutive fragmentations occur. The observations made for deoxyadenylate-containing oligomers are illustrated in Figure 1, which shows the MS/MS spectrum of the (M – 7H)⁷⁻ parent ion from the 8-mer 5'-d(TGCATCGT)-3' acquired under moderate collisional activation conditions. Note that the major route to decomposition passes through loss of the

(18) Stafford, G. C., Jr.; Kelley, P. E.; Syka, J. E. P.; Reynolds, W. E.; Todd, J. F. *J. Int. J. Mass Spectrom. Ion Proc.* **1984**, *60*, 85.

(19) (a) Kaiser, R. E., Jr.; Cooks, R. G.; Moss, J.; Hemberger, P. H. *Rapid Commun. Mass Spectrom.* **1989**, *3*, 50. (b) Kaiser, R. E., Jr.; Louris, J. N.; Amy, J. W.; Cooks, R. G. *Rapid Commun. Mass Spectrom.* **1990**, *3*, 225.

(20) Roepstorff, P.; Fohlman, J. *Biomed. Mass Spectrom.* **1984**, *11*, 601.

(21) Louris, J. N.; Cooks, R. G.; Syka, J. E. P.; Kelley, P. E.; Stafford, G. C., Jr.; Todd, J. F. *J. Anal. Chem.* **1987**, *59*, 1677.

(15) McLucky, S. A.; Glish, G. L.; Asano, K. G. *Anal. Chim. Acta* **1989**, *225*, 25.

(16) (a) Van Berkel, G. J.; McLucky, S. A.; Glish, G. L. *Anal. Chem.* **1990**, *62*, 1284. (b) McLucky, S. A.; Van Berkel, G. J.; Glish, G. L.; Huang, E. C.; Henion, J. D. *Anal. Chem.* **1991**, *63*, 375.

(17) McLucky, S. A.; Goeringer, D. E.; Glish, G. L. *J. Am. Soc. Mass Spectrom.* **1991**, *2*, 11.

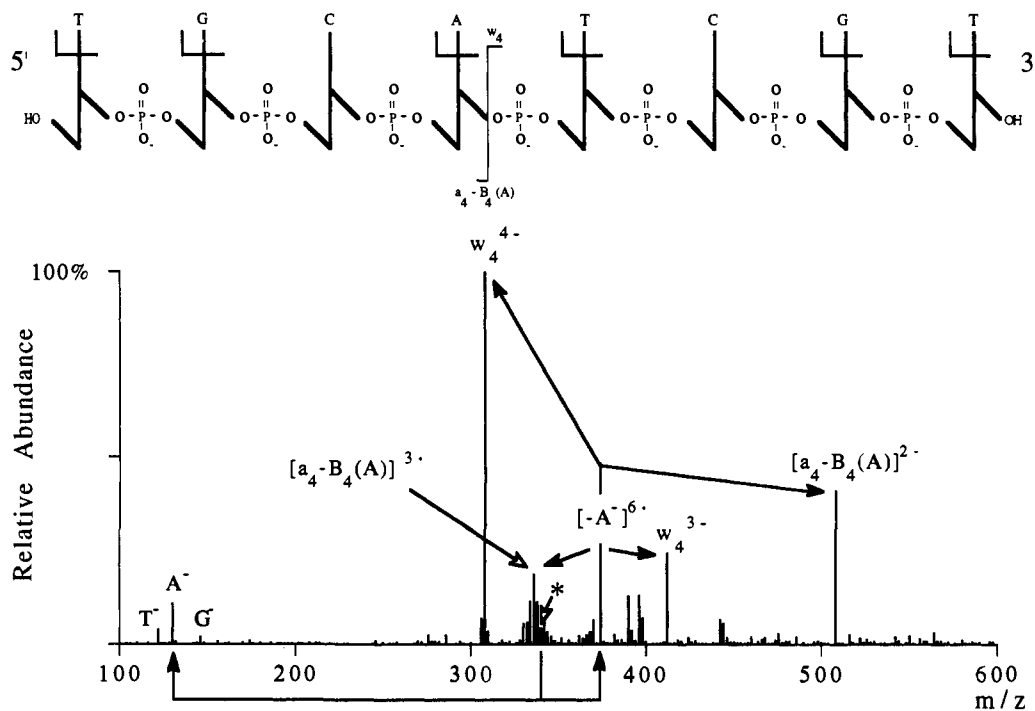
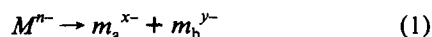


Figure 1. MS/MS spectrum of the $(M - 7H^+)^{7-}$ ion, where $M = 5'$ -d(TGCATCGT)- $3'$. The asterisk indicates the mass/charge location of the parent ion. Open arrowheads with a label at the tail are used to identify some peaks. Closed arrowheads are used to indicate the genealogy of the fragments.

adenine anion, A^- . This ion and its complement are both observed in the spectrum. Small signals arising from losses of the thymine anion, T^- , and the guanine anion, G^- , are also observed. Spectral interpretation is greatly facilitated by the fact that both complementary ions arising from decompositions involving charge separation reactions are usually observed in the spectrum. Provided that the parent ion charge is known, charge and mass conservation can be used to identify complementary ions, their charge states, and their masses. For the generic reaction



where M is the parent ion mass and m_a and m_b are the product ion masses and where n , x , and y are the respective charges, conservation of mass and charge requires that

$$((M/n) - (m_a/x)) / ((M/n) - (m_b/y)) = y/x. \quad (2)$$

Therefore, complementary ions from a particular parent ion can be identified by locating the ions in the spectrum that fall at the appropriate ratios of mass/charge differences from the parent ion. For a quadruply charged parent ion, for example, products formed from charge separation fragmentations must fall on the mass/charge scale equidistant from the parent ion if both products carry two charges each or at a ratio of 3:1 if the decomposition results in triply charged and singly charged fragments. Ions formed from consecutive fragmentations bear no relationship with the original parent ion mass/charge but, obviously, must fall at the appropriate ratios relative to the ion from which they are formed directly. Recognizing this situation facilitates the identification of ions in the MS/MS spectrum formed directly from the parent ion and those formed from consecutive decompositions. In the case illustrated in Figure 1, the most abundant ions in the spectrum arise from further decomposition of the $(M - 7H^+ - A^-)^{6-}$ anion at the $3'$ C-O bond from the sugar that once held the adenine. These consecutive fragmentation products are the complementary pairs $w_4^{4-}/(a_4 - B_4(A))^{2-}$ and $w_3^{3-}/(a_4 - B_4(A))^{3-}$. Note that these ions can be identified as complementary without prior knowledge of the sequence once it is recognized that they are not formed directly from the parent ion (they do not fall at expected mass/charge difference ratios) but follow after loss of A^- from the parent ion. For parent ions with high charge states such as this, essentially no loss of a neutral base of

any kind is observed. All decompositions involve charge separation. As illustrated below, losses of neutral bases occur for lower charge states and, in particular, for cytidine. It is also noteworthy, although not shown here, that below the 6- charge state for this oligomer, A^- is the only charged base observed to be lost.

Figure 2 shows MS^3 spectra resulting from the experimental sequences $(M - 6H^+)^{6-} \rightarrow w_4^{3-} \rightarrow ?$ (Figure 2a) and $(M - 5H^+)^{5-} \rightarrow a_4 - B_4(A)^{2-} \rightarrow ?$ (Figure 2b) for $M = 5'$ -d(TGCATCGT)- $3'$. Open arrowheads are used to point to peaks difficult to label directly, whereas closed arrowheads are used to indicate the genealogy of the peaks. The w_4^{3-} fragment contains the sequence TCGT with a phosphate group on the $5'$ end. Loss of the $5'$ PO_3^- group (the PO_3^- group itself was not trapped) competes with the major fragmentation channel, viz., the loss of T^- and its consecutive decompositions to give the w_3^{2-} ion and its neutral counterpart and the w_3^- fragment and its charged complement (m/z 176). Note that the loss of PO_3^- directly from the parent ion is commonly observed for oligomers phosphorylated on the $5'$ sugar. This observation is useful in differentiating w-type ions from $(a - B)$ -type ions in MS^3 experiments involving ions of unknown sequence. Relatively small fractions of the total product ion charge are partitioned into the loss of neutral C, followed by cleavage at its sugar and the loss of G^- , followed by cleavage at its sugar. The full sequence of the w_4 fragment can be deduced from this spectrum by using the following logic. The loss of PO_3^- suggests that the ion is a w-type ion, and loss of T^- from the ion formed by loss of PO_3^- clearly indicates the presence of at least one thymidine. This is also reflected in the intense loss of T^- in the spectrum. The location of at least one deoxythymidylate at the $5'$ end of this fragment is clearly indicated by the loss of a sugar plus phosphate indicated as "ps" in the figure. Loss of this fragment as both a neutral and as a singly charged ion is apparent. A relatively small signal due to loss of G^- is observed in the spectrum, and a search for product ions equidistant in mass/charge from this ion yields the products labeled w_1^- and $[a_7 - B_7(G)]^-$. The m/z 321 ion, labeled as w_1^- , is noteworthy in that it is the mass/charge of $5'$ phosphorylated deoxythymidine. This strongly suggests that a deoxythymidylate is also located at the $3'$ end of the ion with a deoxyguanylate adjacent to it. The loss of a neutral C yields two sets of complementary fragments comprised of $w_2^{2-}/$

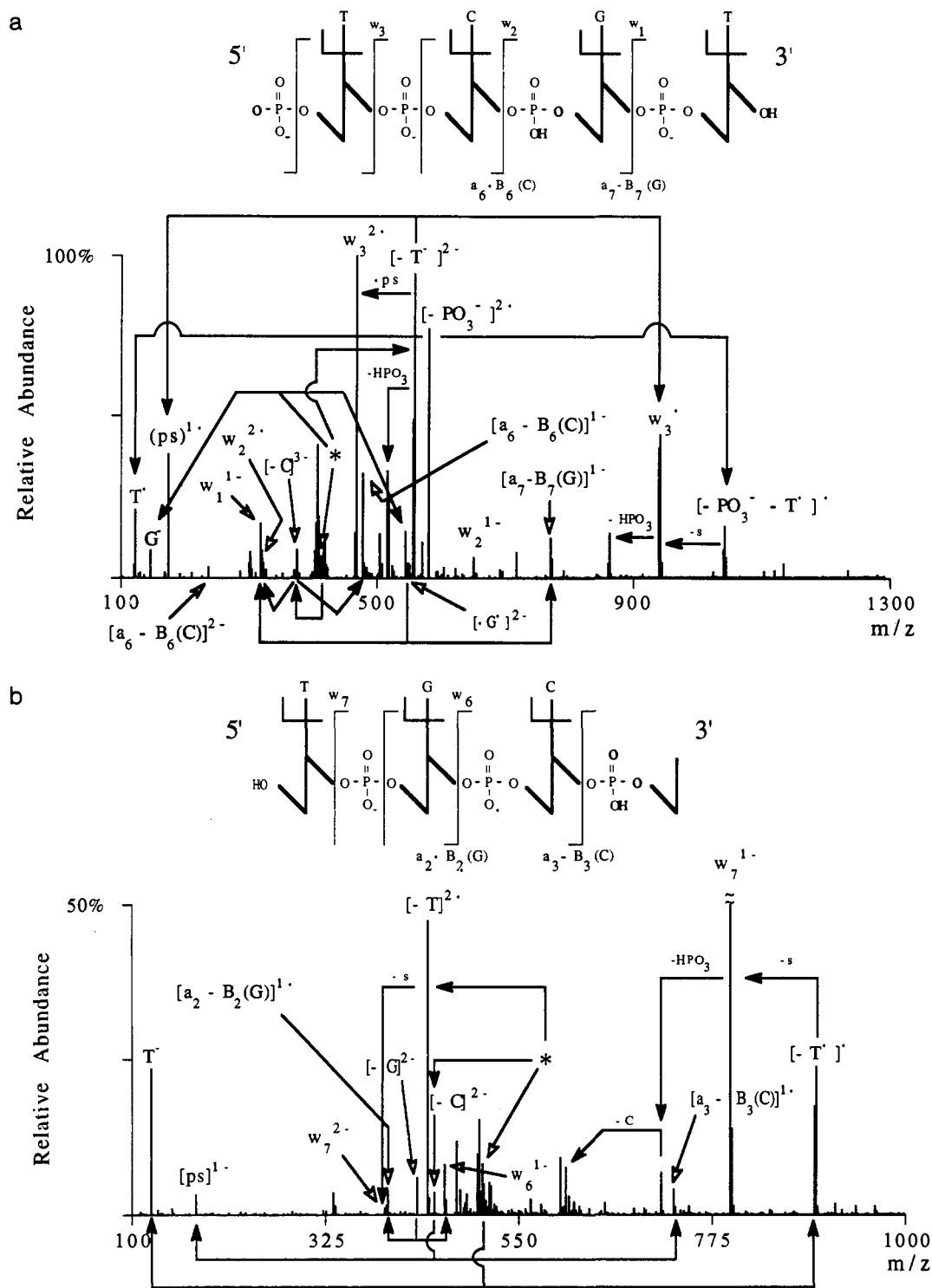


Figure 2. (a) MS^3 spectrum resulting from the sequence $(M - 6H^+)^{6-} \rightarrow w_4^{3-} \rightarrow ?$, where $M = 5'$ -d(TGCATCGT)-3'. (b) MS^3 spectrum resulting from the sequence $(M - 5H^+)^{5-} \rightarrow [a_4 - B_4(A)]^{2-} \rightarrow ?$, where $M = 5'$ -d(TGCATCGT)-3'. Open arrowheads with a label at the tail are used to identify some peaks. Closed arrowheads are used to indicate the genealogy of the fragments. The asterisk indicates the mass/charge location of the parent ion.

$[a_6 - B_6(C)]^-$ and $w_2^-/[a_6 - B_6(C)]^{2-}$ (arrows indicating the origin of the latter pair are omitted from the figure). The masses of these fragments alone indicate that the deoxycytidylate is in the middle of the sequence. Further confidence that the pTCGT sequence of the ion is correctly identified can be gained by comparing the masses of the indicated fragments with tabulated masses of w -type and $(a - B)$ -type ions with various compositions. We have not resorted to such a comparison here, aside from identifying fragments corresponding in mass/charge to those of the mononucleotides, but comparisons of product ion masses with such a tabulation would facilitate automated spectral interpretation.

The $a_4 - B_4(A)$ dianion (Figure 2b) includes the TGC sequence and shows that the loss of T^- and loss of neutral T are the dominant decompositions. The fact that no direct loss of HPO_3 or PO_3^- is observed from the parent ion indicates that this ion is likely to be the $(a - B)$ ion of the complementary pair indicated in Figure 1. The loss of the neutral sugar moiety in both channels involving loss of T indicates that a T is present on the sugar of the 5' terminus. There are also relatively small signals due to losses of neutral guanine and cytosine and further fragmentation therefrom that indicate their relative positions in the sequence. The complementary ions formed from loss of neutral cytosine, the pair labeled $[ps]^-$ and $[a_3 - B_3(C)]^-$, indicate that deoxycytidylate

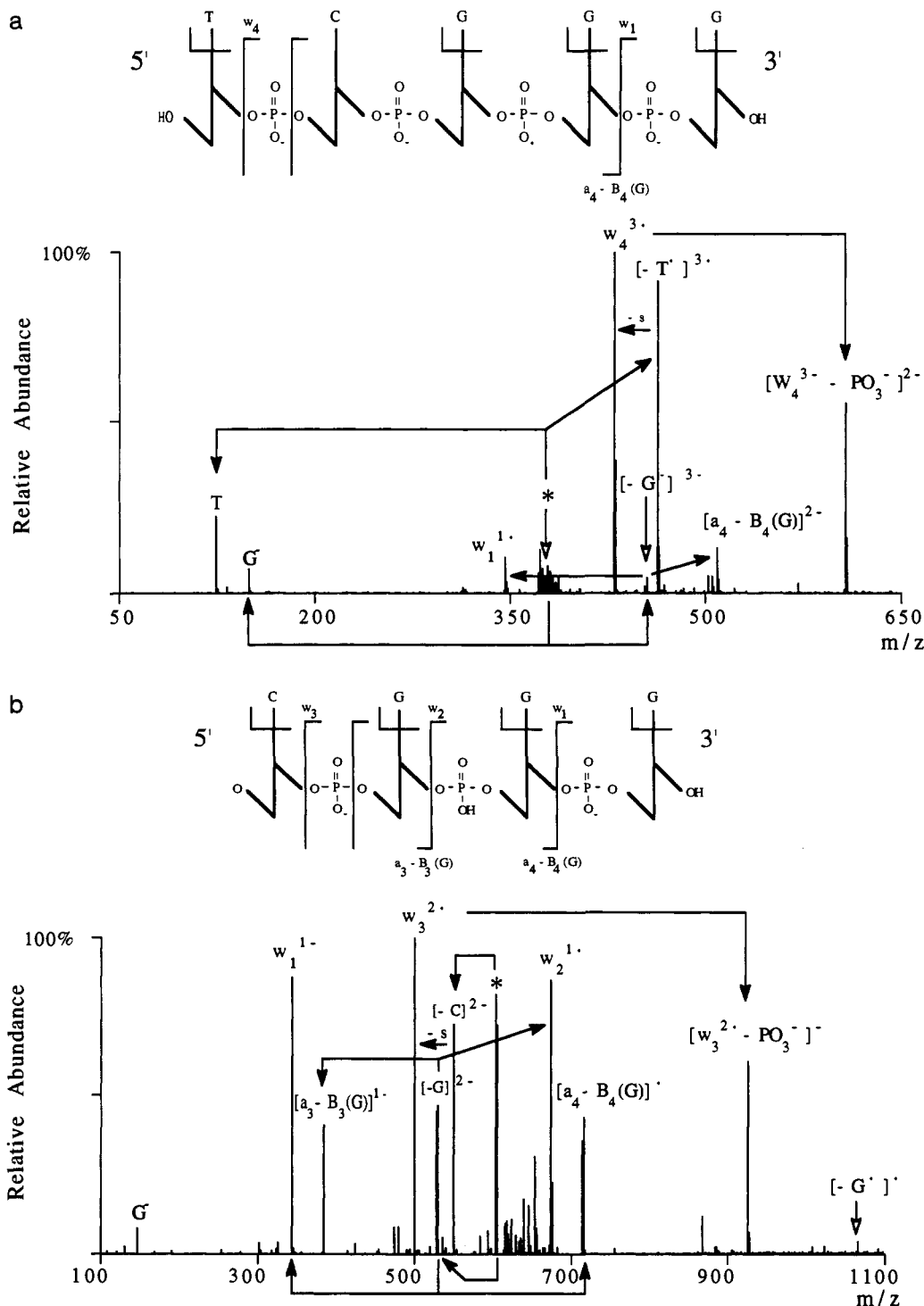


Figure 3. (a) MS/MS spectrum of the $(M - 4H^+)^{4-}$ ion, where $M = 5\text{'-d(TCGGG)-3\text{'}}$. (b) MS³ spectrum resulting from the sequence $(M - 4H^+)^{4-} \rightarrow [w_4^{3-} - PO_3]^{2-} \rightarrow ?$. Open arrowheads with a label at the tail are used to identify some peaks. Closed arrowheads are used to indicate the genealogy of the fragments. The asterisk indicates the mass/charge location of the parent ion.

is nearest to the 3' terminus, and the complementary ions formed from loss of neutral guanine, labeled as $[a_2 - B_2(G)]^-$ and w_6^- , clearly show that deoxyguanylate is located in between the thymidine and cytidine residues. In the case of this 8-mer, the MS/MS spectrum and the two MS³ spectra combine to give the complete sequence. However, dissociations involving the guanidine- and cytosine-containing residues did not make major contributions to the total product ion signal. This is a general observation when these residues are interspersed with those of deoxyadenylate and deoxythymidylate.

Figure 3 shows MS/MS and MS³ spectra of the $(M - 4H^+)^{4-}$ parent ion from 5'-d(TCGGG)-3', a molecular species that lacks deoxyadenylate. In the absence of adenine as a base, and for

highly charged species, loss of T⁻ followed by cleavage at the 3' C-O bond of the relevant sugar is the dominant decomposition pathway (Figure 3a). Note that there is also a relatively small signal associated with loss of G⁻ and some consecutive fragmentation from this route. For a T present at the 5' end of the molecule, a loss of the sugar moiety accompanies formation of the w-type ion. The MS/MS spectrum also illustrates another commonly observed tendency: the loss of PO₃⁻ when it is present at the 5' position of the chain as it is for the w_4^{3-} anion, in this case and for the case described above for the 8-mer. Figure 3b shows the spectrum that results from the collisional activation of the doubly charged $[w_4^{3-} - PO_3]^{2-}$ anion. This spectrum shows relatively rich fragmentation and reflects the losses of neutral cytosine and

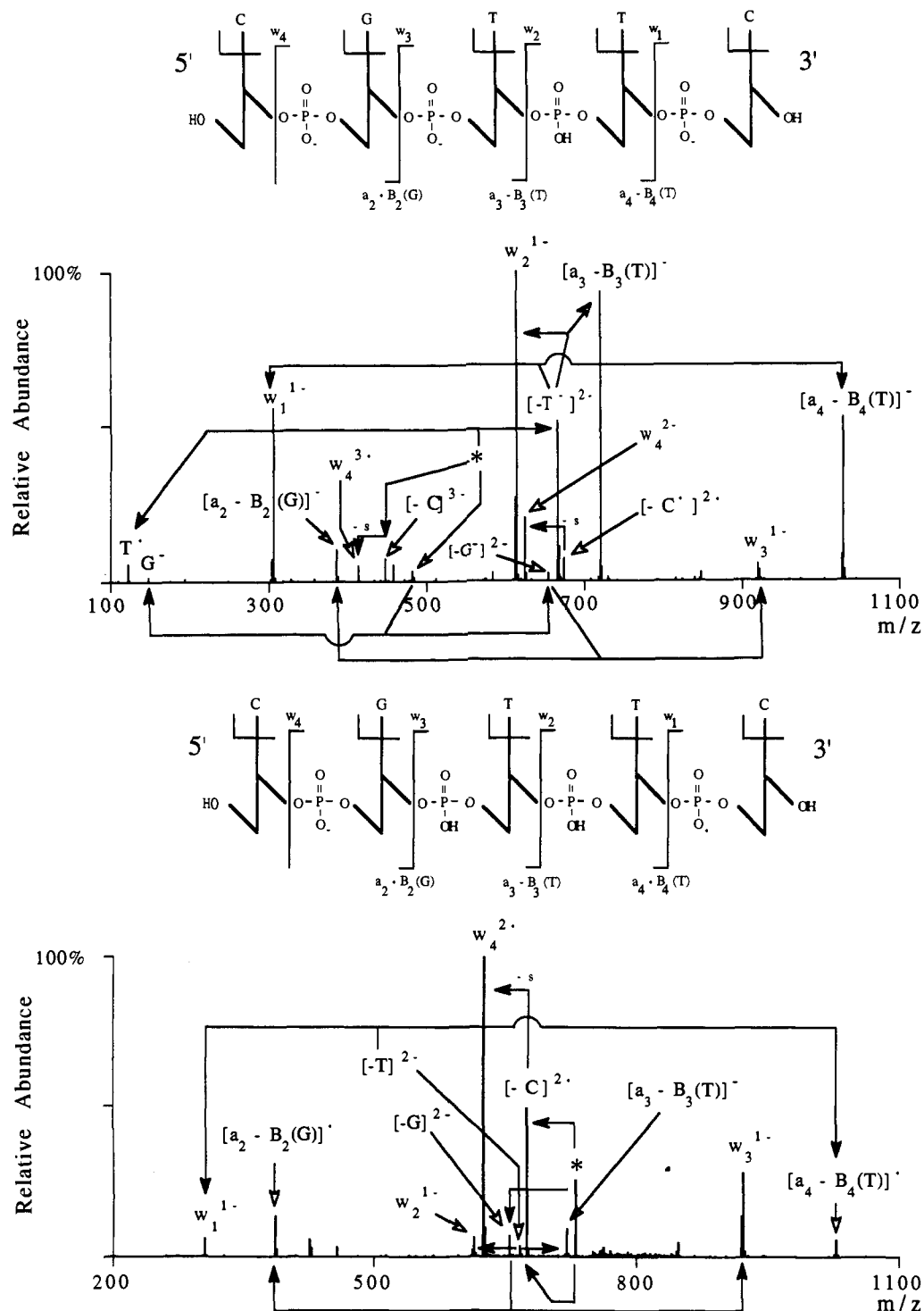


Figure 4. MS/MS spectra of the $(M - 3H^+)^{3-}$ ion (a) and the $(M - 2H^+)^{2-}$ ion (b), where $M = 5'$ -d(CGTTTC)-3'. Open arrowheads with a label at the tail are used to identify some peaks. Closed arrowheads are used to indicate the genealogy of the fragments. The asterisk indicates the mass/charge location of the parent ion.

guanine followed by consecutive fragmentation at the respective 3' C-O bonds of the sugars. As illustrated above, the location of the cytidine residue at the 5' end is indicated by sugar loss. Two sets of complementary ions are observed from the ions resulting from loss of guanine, labeled $[a_3 - B_3(G)]^-/w_2^-$ and $[a_4 - B_4(G)]^-/w_1^-$. The ion at m/z 346 (w_4) corresponds to the mass/charge of phosphorylated deoxyguanosine, indicating its location on the 3' terminus. In this example, which is illustrative of those in which the molecule is not highly charged, losses of the neutral bases dominate. In this case, the loss of G dominates over the loss of G^- , whereas the opposite was observed in the decomposition of $(M - 4H^+)^{4-}$. It is also noteworthy that

the complete sequence of the molecule can be derived from the two spectra in Figure 3.

As a rule, loss of a charged base, as opposed to loss of neutral base, increases with the ratio of the charge to the number of phosphate groups in the parent ion. This behavior is demonstrated qualitatively by comparison of the MS/MS spectra of the $(M - 3H^+)^{3-}$ and $(M - 2H^+)^{2-}$ ions derived from 5'-d(CGTTTC)-3', as shown in Figure 4. There are four phosphodiester linkages in this molecule, and if it is assumed that the number of such groups constitutes the maximum possible negative charge for the system, the 3- and 2- ions are 75% and 50% charged, respectively. In the case of $(M - 3H^+)^{3-}$ (Figure 4a), the two major competing

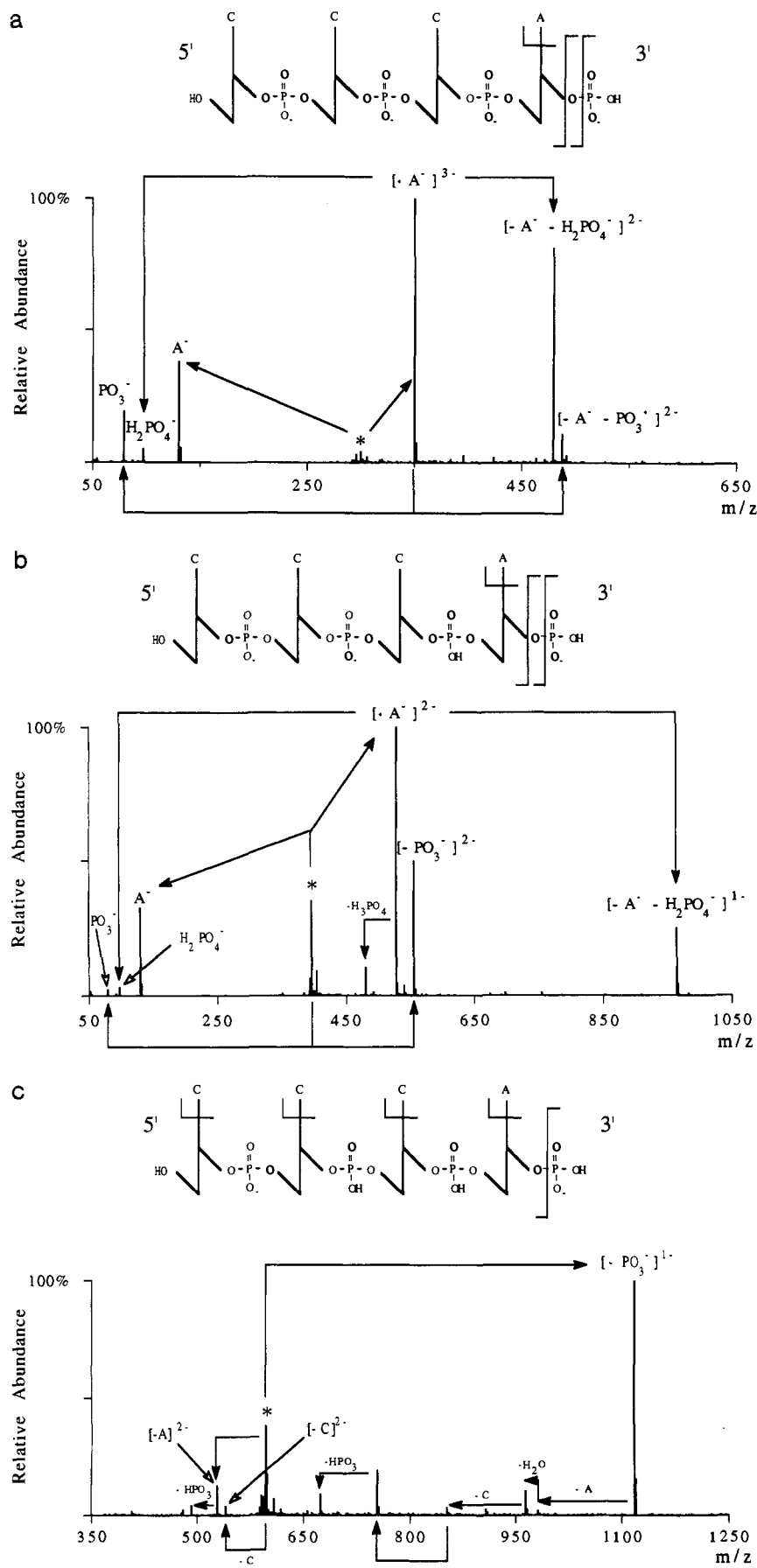
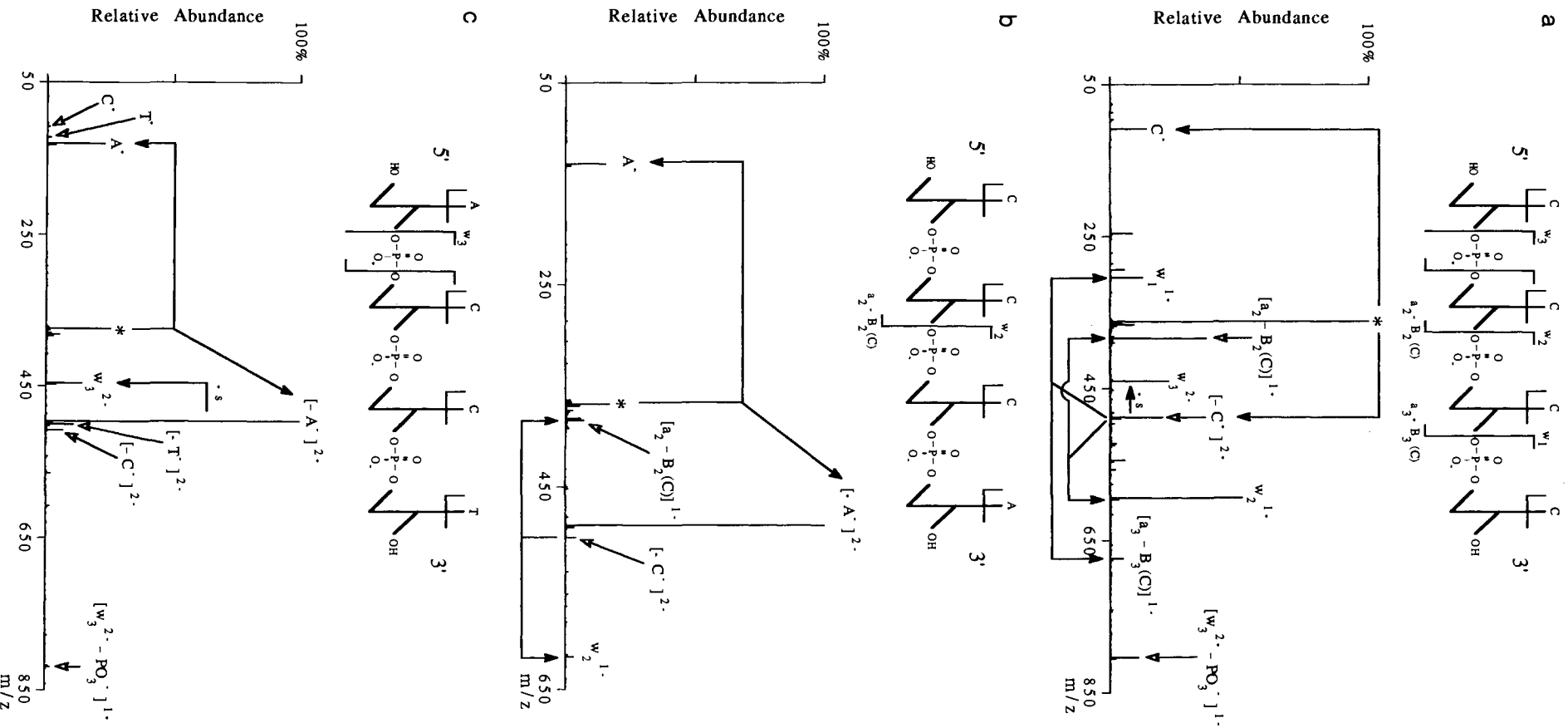


Figure 5. MS/MS spectra of the $(M - 4H^+)^4+$ ion (a), the $(M - 3H^+)^3+$ ion (b), and the $(M - 2H^+)^2+$ ion (c) derived from 5'-d(CCCAp)-3'. The asterisk indicates the mass/charge location of the parent ion.

decomposition channels involve the losses of the thymidines as anions and cleavages at their respective sugars. Less competitive channels involve losses of charged guanidine and cytosine, the

latter being a relatively rare observation. A relatively small extent of loss of neutral cytidine, a common observation, also appears. It is noteworthy that, although the losses of T⁻ dominate, the



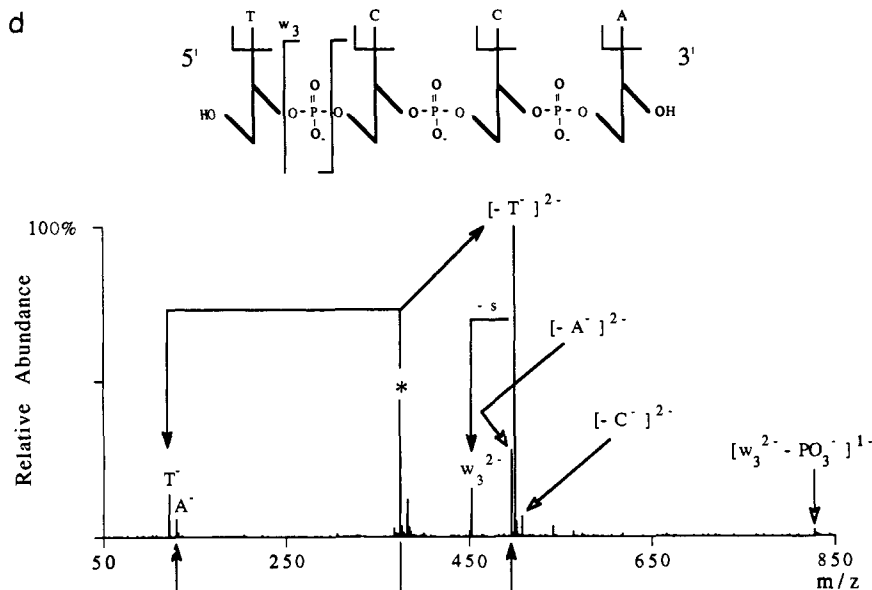


Figure 6. MS/MS spectra of the $(M - 3H^+)^{3-}$ anions derived from (a) 5'-d(CCCC)-3', (b) 5'-d(CCCA)-3', (c) 5'-d(ACCT)-3', and (d) 5'-d(TCCA)-3'. The asterisk indicates the mass/charge location of the parent ion.

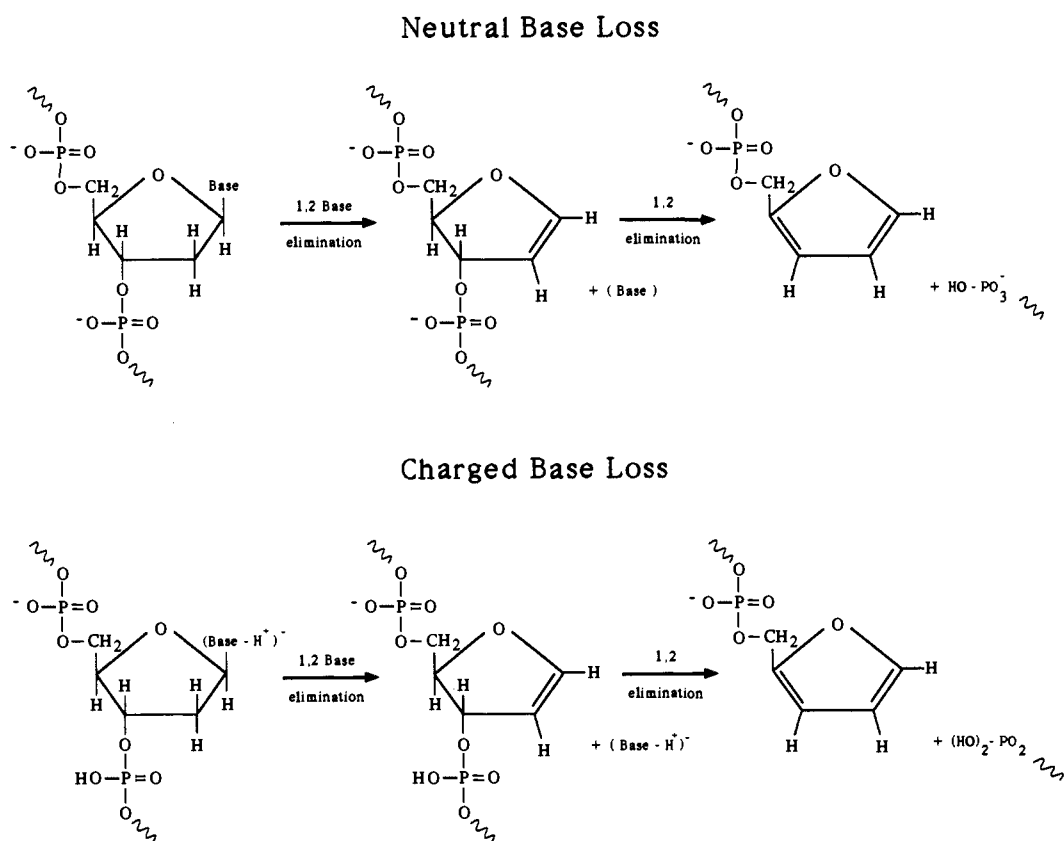
entire sequence of this molecule can be determined from the MS/MS spectrum using the reasoning illustrated above for the 8-mer and for 5'-d(TCGGG)-3'. This can also be said of the MS/MS spectrum of the $(M - 2H^+)^{2-}$ parent ion (Figure 4b), but the fragmentation behavior of this ion differs markedly from that of the triply charged ion. For one, the major dissociation pathway goes through the loss of neutral cytidine followed by the loss of $C_5H_6O_2$ (elements of the 5' sugar), indicating the location of C at the 5' end of the molecule. Another difference is that essentially no loss of any of the bases as charged species is observed. The other structurally diagnostic fragmentations proceed first through the loss of neutral guanine or thymine. As already indicated, the loss of a charged cytidine is not commonly observed, unless the molecule is highly charged. The loss of a neutral cytidine is a facile reaction, however, if Coulombic repulsion does not promote the loss of some other base as a charged species. This particular comparison was chosen to illustrate how dramatically the number of charges on the ion can affect the MS/MS spectrum. The change in Coulombic repulsion for a 5-mer going from 3- to 2- is relatively large. However, the same qualitative trend is observed for all species, although the change in Coulombic repulsion in going from one charge state to the next depends both upon the number of charges and upon their relative locations.

Phosphorylation on the 3' terminus results in the loss of PO_3^- in competition with base loss. The importance of this reaction channel is highly dependent, however, upon the charge of the parent ion, as illustrated in Figure 5. This figure shows the MS/MS spectra of the $(M - 4H^+)^{4-}$, $(M - 3H^+)^{3-}$, and $(M - 2H^+)^{2-}$ ions derived from 5'-d(CCCAp)-3, respectively. Note that the $(M - 4H^+)^{4-}$ ion (Figure 5a) fragments almost exclusively through the channel beginning with the loss of A^- . The first step of this channel results in the complementary A^- and $(M - 4H^+ - A^-)^{3-}$ anions, while a second step leads to the loss of $H_2PO_4^-$ resulting from cleavage at the 3' C-O bond of the 3' terminus sugar. These two sets of complementary ions account for over 90% of the product ion signal. Relatively weak signals corresponding to the complementary ions arising from the loss of PO_3^- from the $(M - 4H^+ - A^-)^{3-}$ ion also appear in the spectrum. Essentially no loss of PO_3^- directly from the $(M - 4H^+)^{4-}$ parent ion is observed. Similar product ions also appear in the MS/MS spectrum of the $(M - 3H^+)^{3-}$ ion (Figure 5b), with some differences in charge states. However, there is a significant contribution from the loss of PO_3^- directly from the parent ion in this case. The complementary ions are, of course, produced in equal abundances, but the trapping efficiency for the PO_3^- fragment under the collisional activation conditions used to acquire the spectrum is relatively poor. In the

case of the $(M - 2H^+)^{2-}$ parent ion (Figure 5c), however, the relative tendency for loss of PO_3^- is dramatically increased. (Note that conditions used to dissociate this ion did not allow for the storage of the complementary PO_3^- ion.) In analogy with the comparison of Figure 4, which illustrated the effect of charge state on the relative propensities for loss of neutral vs charged bases, the base losses associated with the $(M - 2H^+)^{2-}$ ion are neutral A and C, whereas only A^- loss was observed with the more highly charged parent ions of this molecule. Note that at least one further stage of collision-induced dissociation is required for each parent ion charge state to observe significant signals from losses of C and cleavage at the respective sugars.

The data presented above as well as results from most other oligonucleotides that we have examined point to an order of preference for charged base loss of $A^- > T^- > G^- > C^-$. The only notable exceptions to this rule arise for the 3' base. The fragmentation at the 3' C-O bond for the 3' terminus residue following base loss would lead to water loss. We rarely observe this reaction. The reasons why this reaction sequence is rarely observed could be that the energetics for water loss (step 2) following base loss (step 1) are unfavorable, that loss of the 3' base is disfavored, or both. We have no quantitative basis from which to draw conclusions about the relative propensities for loss of water versus formation of a w-type ion, but the evidence presented below suggests that the loss of the 3' base is disfavored. Figure 6 compares the MS/MS spectra of the $(M - 3H^+)^{3-}$ parent ions derived from 5'-d(CCCC)-3', 5'-d(CCCA)-3', 5'-d(ACCT)-3', and 5'-d(TCCA)-3'. In all cases, relatively low-amplitude collisional activation was employed to promote observation of the lowest energy decompositions. The $(M - 3H^+)^{3-}$ parent ion from CCCC is shown to illustrate a case in which base location *only* should affect the likelihood for base loss. In this case, as with all of the parent ions in Figure 6, the number of charges equals the number of phosphodiester linkages, resulting in a relatively high charge density. It is in such a situation that the loss of C- is observed as illustrated in Figure 6a. Note that the 5' residue is indicated by sugar loss to give the w_3^{2-} product and that the second and third residues are indicated by the appearance of the complementary pairs $w_2^-/[a_2 - B_2(C)]^-$ and $w_1^-/[a_3 - B_3(C)]^-$, respectively. Very little loss of water is observed from the $(M - 3H^+ - C^-)^{2-}$ ion which might be expected from the 3' residue. It might be argued that the remaining $(M - 3H^+ - C^-)^{2-}$ ions are enriched in the ion resulting from the loss of the 3' base, but further collisional activation of this ion does not result in enhanced water loss.

Scheme I



The results of Figure 6a suggest that, in the absence of a preference for base identity, the least likely base to be lost is that of the 3' residue. The relative importance of base location versus base identity is illustrated in Figures 6b-d. For triply-charged CCCA, A⁻ is lost despite its location on the 3' residue and very little C⁻ loss is observed. In this case, the tendency for A⁻ loss apparently overcomes the tendency against loss of the 3' base. In the case of triply charged ACCT (Figure 6c), A⁻ is preferentially lost, as expected. Relatively small signals due to loss of T⁻ and loss of C⁻ are also observed, with the former channel appearing to be slightly more important. Perhaps most interesting, the MS/MS spectrum of triply charged TCCA shows a strong preference for loss of T⁻, with significant competition from loss of A⁻. Hence, this ion constitutes an exception to the usually observed order of charged base loss. Apparently the preference for loss of A⁻ over loss of T⁻ does not overcome the tendency against base loss from the 3' residue. These results are qualitatively consistent with those of Cerny et al. in a study describing the behavior of anions derived from various deoxydinucleotides formed by fast atom bombardment and studied by conventional tandem mass spectrometry.²² The results showed a clear preference for base loss from the 5' residue over that from the 3' residue. Ion trap MS/MS results for the same species show an even greater preference for loss of the 5' base.²³ The propensity for base loss has also been reported to be higher for the 3' phosphorylated mononucleotides than for the corresponding 5' phosphorylated species.^{22,24}

The internal Coulombic fields experienced by the parent ions comprising the comparison of Figure 6 are presumably very similar. All ions carry three charges and all are of very similar size. The experiments were designed in this way to remove the Coulombic field as a variable. We have already shown that charge state can have a dramatic effect on the appearance of the MS/

MS spectrum (see Figures 4 and 5, for example). It might, therefore, be expected that charge state can also affect the likelihood for loss of a base at the 3' terminus relative to that for loss of a base from elsewhere in the oligonucleotide. We have not studied systematically the effect of charge on the likelihood for loss of the base on the 3' terminus, but the results that we have acquired for the doubly charged parent ions of the compounds of Figure 6 certainly show an effect. For example, the MS/MS spectrum of doubly charged CCCA (not shown) shows both loss of charged adenine and loss of charged cytosine along with the losses of both nucleobases as neutrals.

The decomposition chemistry of oligonucleotides is dominated by the stepwise loss of base followed by cleavage at the relevant sugar. A possible mechanism for base loss, either as a neutral or as an ion, involves a 1,2-elimination as indicated as the first step in Scheme I. If, in a multiply charged ion, charge resides on a base, then the loss of that base as an anion is favored over the loss of any of the neutral bases due to the relief of Coulombic strain. The general order of preference for charged base loss of A⁻ > T⁻ > G⁻, C⁻ is not intuitive. One possible rationale could be that intramolecular proton transfer from a nucleobase to a phosphodiester linkage could be driven by the Coulombic repulsion associated with charges on the adjacent phosphodiester groups. Why this might favor loss of adenine and thymine over guanine and cytosine for either thermodynamic or kinetic reasons is not yet clear. Neither can it be precluded that the charges are present on the nucleobases upon formation by electrospray. Scheme I indicates a mechanism with no charges on the nucleobases and a mechanism in which charge is already present on a base prior to base elimination. The phenomena underlying the observed base loss preference and the mechanism for ionization of the base are the subjects of further study. The fact that loss of the base on the 3' residue is disfavored would seem to indicate that phosphorylation on the 3' side of a sugar facilitates the loss of its base. However, 3' phosphorylation is clearly not an absolute criterion, as indicated by the fact that the triply charged parent ion of 5'-d(CCCA)-3' loses A⁻ almost exclusively (see Figure

(22) Cerny, R. L.; Gross, M. L.; Grotjahn, L. *Anal. Biochem.* **1986**, *156*, 424.

(23) Habibi-Goudarzi, S.; McLuckey, S. A. Oak Ridge National Laboratory, 1993, unpublished results.

(24) Sindona, G.; Uccella, N.; Weclawek, K. *J. Chem. Res.* **1982**, *Suppl.* 1982, 184.

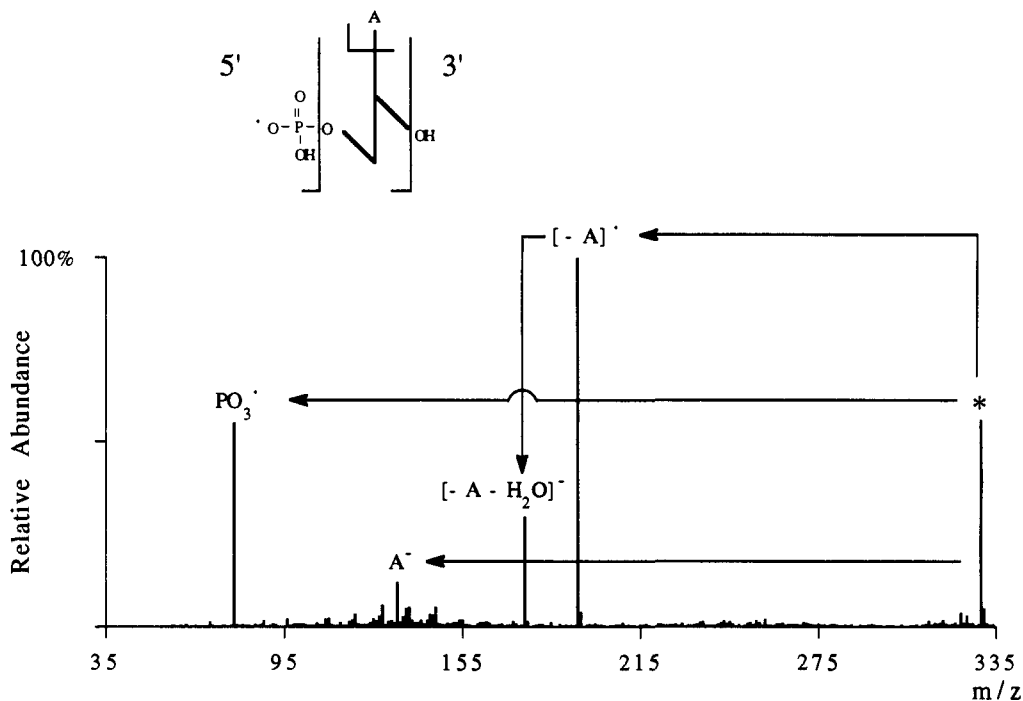


Figure 7. MS/MS spectrum of the $(M - H^+)^-$ ion of deoxyadenosine 5'-monophosphate. The asterisk indicates the mass/charge location of the parent ion.

6b). Furthermore, all of the ion trap MS/MS spectra of the singly charged 5' and 3' nucleotide anions show predominantly loss of the neutral base as the first step in dissociation,²³ although base loss does appear to be more facile from the 3' phosphorylated species.²² For example, Figure 7 shows the MS/MS spectrum of $(M - H^+)^-$ derived from deoxyadenosine 5'-monophosphate. Like the oligonucleotides, the base is lost first, primarily as a neutral in this case. The next step is a loss of water, which might occur as a 1,2-elimination involving the 3'-OH group and a hydrogen from the 4-carbon, in analogy to the mechanism for the second step of the dissociation of oligonucleotides discussed below.

The second step of dissociation, cleavage at the sugar, is particularly important in revealing structural information, in that it indicates the position of the lost base and can point to the composition of the oligomer on either side of the lost base. This fragmentation occurs regardless of the identity of the base and regardless of charge. The result of a 1,2 base elimination is a five-membered ring with a double bond between the 1 and 2 carbons of the sugar. A 1,2-elimination of the 3' phosphate group, as indicated in the second step of Scheme I, yields furan attached to the 5' carbon. The driving force for the reaction is the formation of this stable structure rather than the relief of Coulombic strain, although this may also occur. This conclusion is based on the fact that the loss of 98 mass units, corresponding to a substituted furan, is observed when the 5' base of the nucleotide is lost, even when the parent ion is a highly charged species. We have not observed consecutive loss of charged bases, for example, without cleavage of the relevant sugars to be an important process in any system studied thus far.

Conclusions

The spectra discussed above are a small subset of data acquired for a variety of deoxy oligomers, including several charge states for each, extending up to a 20-mer. They were selected to illustrate the "rules of decomposition" that these species obey with remarkable consistency. These rules and some general observations regarding the behavior of multiply charged anions of single-stranded deoxyoligonucleotides can be summarized as follows.

(i) The first decomposition step involves the loss of a base. All bases can be lost, and, at least for highly charged species, the order of preference is $A^- > T^- > G^-$, C^- . The propensity for the

loss of a charged base is dependent upon the total charge on the parent ion. At low charge states, all bases have been observed to be lost as a neutral without a particularly strong preference. There appears to be little dependence upon base location except for the base on the 3' residue. Loss of this base is disfavored. The major variables in determining if a particular base is lost first are the identity of the base, the charge state of the parent ion, and whether the base is located on the 3' residue.

(ii) The second step involves the cleavage of the 3' C-O bond of the sugar from which the base was lost. This rule is obeyed regardless of the base or its charge and typically yields complementary w-type and (a - B)-type ions. Cleavages at other locations along the phosphodiester linkage have not as yet been observed to make a significant contribution.

(iii) Loss of a PO_3^- group from phosphorylated ions can compete effectively with loss of a base. Phosphorylated parent ions and w-type product ions fall into this category.

(iv) Complementary ions are typically observed, provided that ion trapping conditions are such that both products are stored. This greatly simplifies spectral interpretation.

(v) As a result of the preference for losses of A^- and T^- from highly charged ions, it is sometimes difficult to sequence adjacent C and G residues. On the other hand, at low charge states, the channels that begin with loss of A^- or T^- do not dominate, but the parent ions are of significantly higher mass/charge ratio and are therefore more difficult to manipulate than more highly charged ions. Furthermore, the lower total charge in the system tends to minimize the number of stages of mass analysis that might be performed.

Acknowledgment. This work was supported by the National Institutes of Health under Grant No. R01 GM45372. Oak Ridge National Laboratory is operated for the U.S. Department of Energy under Contract DE-AC05-84OR21400 by Martin Marietta Energy Systems, Inc. The authors acknowledge Dr. Gary J. Van Berkel and Dr. Rose Ramsey for their helpful discussions and assistance with sample preparation and electrospray procedures. S.H.G. acknowledges support through an appointment to the Oak Ridge National Laboratory Postdoctoral Research Associates Program administered jointly by the Oak Ridge Institute for Science and Education and Oak Ridge National Laboratory.