New Aspects of the Fragmentation Mechanisms of Unmodified and Methylphosphonate-Modified Oligonucleotides

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A set of pentanucleotides was investigated by electrospray tandem mass spectrometry with the focus on the fragmentation mechanism. Results reveal new aspects of the fragmentation mechanism of modified and unmodified oligonucleotides and demonstrate the influence of the nucleobases on the decomposition of oligonucleotides. Adenine-rich oligonucleotides fragment easily resulting in abundant peaks corresponding to the DNA-typical a-B- and w-ions. On the other hand, thymine was found to have a stabilizing effect, which is reflected by the preferred formation of the w_4-ions and the relatively low abundance of shorter w-ions upon dissociation of pentanucleotides. Data from investigation of the formation of w_4-ions support a β-elimination mechanism. Results obtained by investigation of oligonucleotides with an abasic site confirm this mechanism, which is independent of nucleobase loss. Experiments with methylphosphonate oligonucleotides show a remarkable change in the fragmentation pattern due to the modification. It was found that charges are located on the nucleobases and initiate the fragmentation mechanism. The stability of the oligonucleotide is reduced and no a-B-fragment ions are formed wherever there is a methylphosphonate group within the backbone. This fact also demonstrates that fragmentation is locally controlled. (J Am Soc Mass Spectrom 2007, 18, 984–990) © 2007 American Society for Mass Spectrometry

Characterization of oligonucleotides by mass spectrometry has been in the focus of researchers for about three decades [1–3]. Right in the beginning it became obvious that tandem mass spectrometry of oligonucleotides is far more complex than tandem mass spectrometry of proteins and, to date, oligonucleotides still cannot be sequenced by mass spectrometry as easily and efficiently as proteins. However, sequencing oligonucleotides is a worthwhile aim, especially regarding modified oligonucleotides, which is coming up for therapeutic purposes in medicine. These modified oligonucleotides often resist sequencing by ordinary biochemical methods because of their unnatural modifications. Mass spectrometry would be an ideal tool for analyzing such molecules. Collision induced dissociation (CID) of oligodeoxynucleotides (DNA) predominantly leads to a-B- and the complementary w-ions. In 1998, Wang et al. proposed a charge remote mechanism based on experiments with thymine-rich tetra- and pentanucleotides, explaining the formation of a-B- and w-ions [4]. They demonstrated that nucleobases are protonated by the proton of the phosphate group in 5′-position and cleaved off as neutrals. The remaining zwitterionic intermediates decay into a-B- and w-fragments by an elimination reaction initiated by the deprotonated 5′-phosphate group. Hence, the intensity of the a-B-fragment ions depends on the proton affinity of the nucleobase. Also, it was assumed that there exist alternative mechanisms for the formation of w-ions. Additional information on this topic was provided by Wan et al., who presented results obtained by H/D-exchange experiments [5]. Their data give evidence for an alternative pathway of w-ion formation without protonation and subsequent cleavage of the nucleobase. Furthermore, the authors proposed a mechanism for backbone cleavage, which also results in a-B- and w-ions, but differs slightly from the one proposed previously. While the mechanism of Wang et al. assumes the nucleobases to be protonated exclusively by the proton of the adjacent 5′-phosphate group, the mechanism of Wan et al. also acknowledges protonation from the adjacent 3′-phosphate group. In a follow-up study with thymine-rich tetra-, hexa-, octa-, and decamers, the predominant cleavage site of doubly charged precursor ions was assigned to the 3′-C–O bond adjacent to the nucleobase with the highest proton affinity, thus, supporting the earlier findings [6]. It has to be taken into account that proton affinities referred to in these publications were defined by analysis of free deoxynucleosides in the gas phase [7]. The proton affinities of adenosine, guanosine, and cytidine have almost identical values, whereas thymine differs by ~9 kcal/mol. Lee and coworkers reported an additional aspect of the interaction between nucleobases and phos-

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phate groups. Their recent calculations demonstrate how charged phosphate groups influence the proton affinity of nearby nucleobases [8]. A mechanism that is independent of nucleobase loss and, thus, of the proton affinities, was introduced by Grotjahn and coworkers in the 1980s [9]. The analysis of modified oligonucleotides by tandem mass spectrometry is gaining interest, as these compounds play an increasingly important role in a wide range of biochemical and medicinal applications. Selective introduction of modifications is a versatile tool for gaining specific information on fragmentation mechanisms. Sannes-Lowery and Hofstadler investigated the behavior of oligonucleotides bearing phosphorothioate backbone and 2′-modifications [10]. They observed that modifications direct the fragmentation, resulting in preferred formation of characteristic product ions, and demonstrated the strong interaction between backbone, 2′-substituent, and the nucleobase. Although base loss and formation of a-B-ions have repeatedly shown to be dependent on the nucleobases, the influence of the nucleobases has been appointed differently in the range of publications. As pointed out by Sannes-Lowery and Hofstadler, it is dependent on the mechanism proposed [10].

Bartlett et al. demonstrated that fragmentation of a decamer with partly methylphosphonate-modified backbone occurs even if no protons from nearby phosphate groups are available [11]. These findings are opposed to the close relationship between the 5′-phosphate group and the nucleobase, stated by other authors within the context of backbone cleavage [4]. Although literature reflects a wide variety of results regarding backbone dissociation of oligonucleotides, generally applicable rules are still lacking.

Experimental

Oligonucleotides

Single stranded oligodeoxynucleotides were obtained from Microsynth (Balgach, Switzerland) and used without further purification. Single stranded methylphosphonate-modified oligodeoxynucleotides were obtained from Eurogentec S.A. (Seraing, Belgium) and also used without further purification. The unmodified pentamers of the sequences AAAAA, CAAAA, GAAAA, TAAAA, ACCCC, CCCCC, GCCCC, TCCCC, AGGGG, CGGGG, GGGGG, TGGGG, ATTTT, CTTTT, GTTTT, and TTTTT were used in the experiments. The set of oligodeoxynucleotides with a methylphosphonate modification was TxGxGxGxG, TxGxGxGG, TxGxGGG (x indicates the position of the methylphosphonate modification) and the set of oligodeoxynucleotides with an abasic site (dSpacer) was TdSpacerGGG and TGGdSpacerG. Solutions were prepared with concentrations of 20-40 pmol/μL oligonucleotide in water: acetonitrile (50:50 vol/vol). Acetonitrile 230 Romil SpS (purchased from Ammann-Technik AG, Obergösgen, Switzerland) was used and high purity water was purchased from Fluka (Buchs, Switzerland).

Mass Spectrometry

All experiments were performed on an Applied Biosystems/MDS SCIEX QSTAR Pulsar hybrid quadrupole time-of-flight mass spectrometer (SCIEX, Concord, Ontario, Canada), equipped with an nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark). Oligonucleotides were analyzed in the negative ion mode with a potential of 700 to 1000 V applied to the nanospray needle. Nitrogen was used as the curtain gas. Tandem mass spectrometric experiments were performed with the precursor ions selected within a window of ±1.5 *m/z* units. Collision-induced dissociation was performed with collision energies of −30 eV using nitrogen as the collision gas. Calibration of the instrument was done with cesium iodide and taurocholic acid (Fluka). The Applied Biosystems Analyst QS software package was used for data processing.

Results and Discussion

The fragmentation of single stranded oligonucleotides is being analyzed with pentamers, focusing on the molecule TGGGG and its derivatives. For having an utmost cohesive and comparable system, all tandem mass spectrometric acquisitions are based on doubly charged precursor ions, holding the collision energy fixed at −30 eV.

As illustrated by the example of the pentanucleotide TGGGG in Figure 1, the w_{n-1}-fragment ion originating from oligonucleotides of a length of *n* nucleotides appears regularly and mostly with rather high abundance. Hitherto, the mechanism leading to w_{n-1}-fragments has not been investigated in detail. The

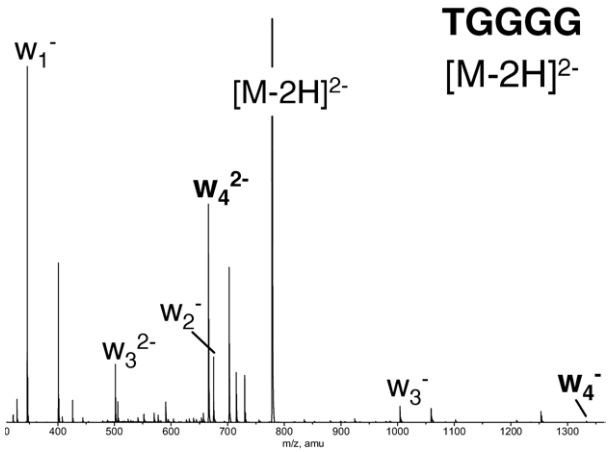


Figure 1. The product ion spectrum of the pentamer TGGGG shows peaks corresponding to the w-ion series. Among them, the peak of the doubly charged w_4-fragment ion at *m/z* 666.1 is observed with high abundance. The singly charged w_4-ion (*m/z* 1333.2) is detected as well.

generally accepted mechanism of DNA backbone cleavage with protonation of the nucleobase by the acidic proton of the adjacent 5′-phosphate group (hence termed as a-B-mechanism) is not possible in case of w_{n-1} ions because there is no phosphate group available in 5′-position that could act as a proton donator. However, the w_4-ion is readily formed and appears with high abundance despite the lack of the 5′-phosphate group.

On the basis of these observations, it seems opportune to pose the question if an alternative mechanism for formation of w-ions or even for fragmentation of oligonucleotides in general exists. It has to be assumed that alternative mechanisms are only active if fragmentation via the main pathway is hindered by some means. Aiming at elucidation of the fundamental aspects of oligonucleotide dissociation in the gas phase, the roles of the nucleobases and phosphodiester linkages have to be taken into consideration as well. Though it has been demonstrated that base loss is the key step initiating cleavage of the 3′-C–O bond of oligodeoxyribonucleotides, there is no conclusive data on how much the different types of nucleobases influence the fragmentation mechanism. Finally, to answer mechanistic questions, the influence of the phosphate group, which is the second reactive center besides the nucleobases in the fragmentation processes, has to be discussed carefully as well.

Influence of the Nucleobases

In our studies, the influence of the nucleobases on the formation of the w_4-ion was investigated. Experiments were performed with pentanucleotides of the pattern YXXXX, with a first nucleobase followed by four identical nucleobases of a different type (X, Y = A, C, G, and T). Permutation of nucleobases X and Y resulted in a set of 16 pentanucleotides totally. With these pentamers, the intensity of the w_4-ion was examined in relation to the other w-ions. Also, the abundance of the w_4^{2-}-ion was compared to the abundance of the molecular ion $[M - 2H]^{2-}$.

Repetitive analyses of one pentanucleotide demonstrated, that peak intensities can vary from one analysis to the other. Nevertheless, the product ion spectra acquired from a set of pentanucleotides reveal differences in the formation of the w_4-ions, which obviously are related to the presence of different types of nucleobases. Analyses were performed holding the settings, which affect the extent of fragmentation, such as pressure, collision energy, gas flow and interface potentials, constant.

Considering that nucleobases are involved in the fragmentation process, nucleotide composition and sequence of an oligonucleotide are likely to affect the fragmentation behavior. Data gained by dissociation of pentanucleotides give evidence for the influence of the nucleobases on the phosphate backbone and, consequently, on the fragmentation of oligonucleotides in general. However, based on the results currently available, it is not possible to set up a general scheme for assigning the effect of the nucleobases on the fragmentation, or to predict the absolute abundances of certain fragment ions. The influence of nucleobases on the fragmentation is rather observed as a trend, as illustrated by comparison of the product ion spectra of the series of pentanucleotides XAAAA to XTTTT with X = A, C, G, and T. Generally, the adenine-rich molecules show rather abundant peaks corresponding to w-ions. Consequently, the peak of the w_4-ion is less abundant than other w-ions. Results indicate that adenine-rich sequences benefit fragmentation. In contrast to XAAAA, pentanucleotides of the sequence XTTTT show the w_4-ions of higher abundance than shorter w-ions. Thus, thymines seem to stabilize the sequences, which can be assigned to their "lower" proton affinity (Figure 2).

The fact that the influence of the nucleobases on the fragmentation of oligonucleotides can hardly be predicted in detail is emphasized by a closer look at the proton affinities. The differences between adenine

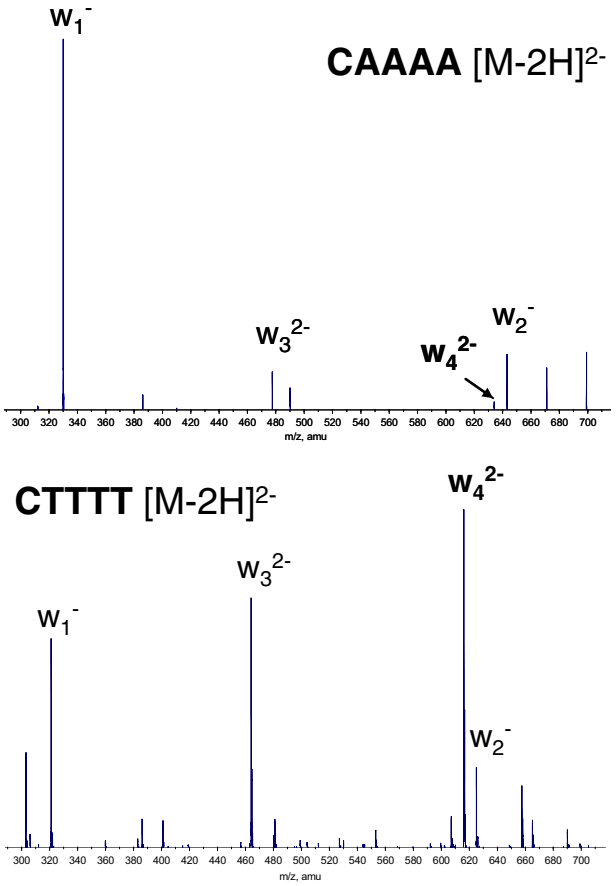
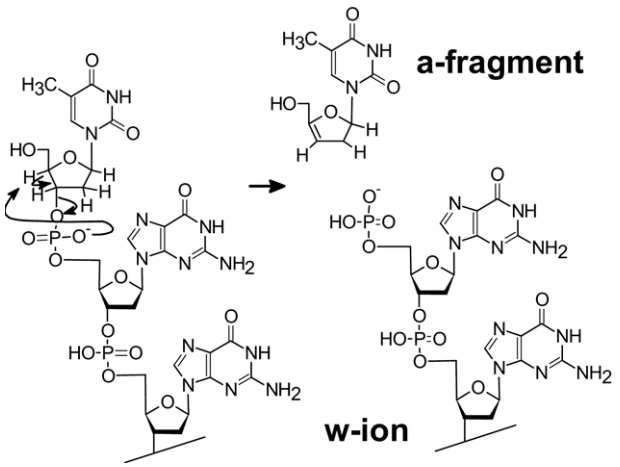


Figure 2. Comparison of the product ion spectra of the adenine-rich pentanucleotide CAAAA and the thymine-rich pentanucleotide CTTTT. The adenine-rich oligonucleotide fragments easily and the w_4^{2-}-ion (*m/z* 634.1) shows a relatively low abundance compared with the other w-ions. The thymine-rich pentanucleotide shows opposite behavior with the w_4^{2-}-ion (*m/z* 616.1) as the most abundant dissociation product.



Scheme 1

(224.2 kcal/mol), cytosine (225.9 kcal/mol), and guanine (227.4 kcal/mol) are small; only thymine (209.0 kcal/mol) differs considerably [7]. The relatively small differences between the proton affinities of adenine, guanine, and cytosine render prediction of the detailed fragmentation behavior of oligonucleotides impossible.

Mechanisms

Formation of the w_{n-1}-ion, with n indicating the number of building blocks, can be explained by a β-elimination reaction [9]. Nucleophilic attack of the deprotonated phosphate oxygen on the 4′-hydrogen results in an energetically advantageous six-membered transition state, which subsequently leads to cleavage of the 3′-C–O bond and formation of the a_1- and w_{n-1}-ions (Scheme **1**). The fact that the nucleobase is not involved in this mechanism explains the lack of a clear correlation between the type of the 5′-nucleobase and the formation of the w_{n-1}-ion. Due to the low pKa of the phosphate group, attack of the ribose by the nucleophilic site of the nucleobase is less probable. This aspect will have to be taken into account for the dissociation of the methylphosphonate-modified oligonucleotides discussed below.

Alternatively, a- and w-ions can also be formed via a basically identical β-elimination as shown in Scheme **1**, but the initial attack of the phosphate group is directed towards the 2′-hydrogen instead of the 4′-hydrogen. This β-elimination involves a six-membered cyclic intermediate as well, and is independent of the presence of an adjacent nucleobase. Experimental data do not allow ruling out one of the two mechanisms. However, it can be assumed that the 4′-hydrogen is abstracted predominantly over the 2′-hydrogen, due to the electron withdrawing effect of the neighboring oxygen atoms.

A potential third mechanism based on proton transfer from the 3′-phosphate group to the nucleobase, instead of the 5′-phosphate as in the a-B-mechanism, followed by nucleobase loss and 3′-C–O cleavage, would also result in formation of the w_{n-1}-ion [5]. However, proton transfer would be sterically hindered, rendering such mechanism rather unlikely. Thus, it is not discussed any further.

To provide supporting data for the proposed nucleobase-independent elimination reactions, experiments were performed on oligonucleotides bearing abasic sites. Such modification, also called dSpacer, is the substitution of the nucleobase at the C1′ by a hydrogen and shall reveal the role of the nucleobases in the fragmentation mechanism. Due to the presence of the abasic site, cleavage in 3′-position to the modification must occur via a nucleobase-independent mechanism, thereby excluding the existence of an a-B-mechanism at this position. Observation of the w_1-ion in the product ion spectrum of TGGdSpacerG (Figure 3) and the w_3-ion in the spectrum of TdSpacerGGG confirms that backbone dissociation does occur in the absence of an adjacent nucleobase, thus supporting the existence of a nucleobase-independent mechanism. Consequently, it can be assumed that the very same mechanism which is responsible for cleavage of the 3′-C–O bond in 3′-position to abasic sites,also applies to the formation of w_{n-1}-ions from oligonucleotides of a length of n nucleotides.

Methylphosphonate Oligonucleotides

Methylphosphonate oligonucleotides are modified oligonucleotides with one or several phosphate-hydroxyl groups substituted for methyl groups. Investigation of methylphosphonate oligonucleotides shall elucidate the role of the phosphate groups in the fragmentation as they are pivotal in all fragmentation mechanisms published.

In our experiments, we investigated the partially backbone-modified pentanucleotides TxGxGGG, TxGxGxGG,

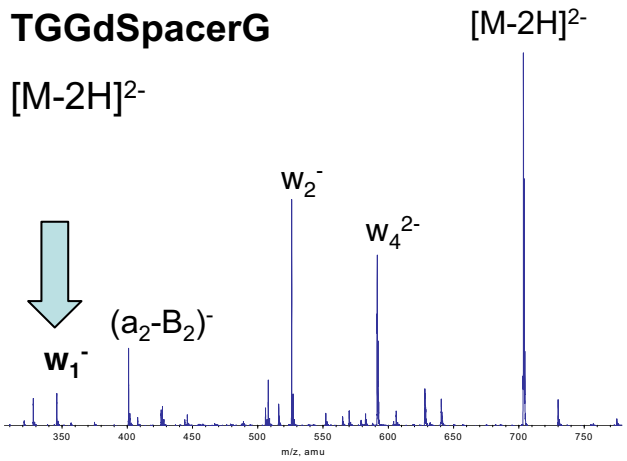


Figure 3. The presence of the w_1-ion (*m/z* 346.0) in the product ion spectrum of TGGdSpacerG proves that backbone cleavage occurs in absence of a nucleobase in 5′-position to the cleavage site.

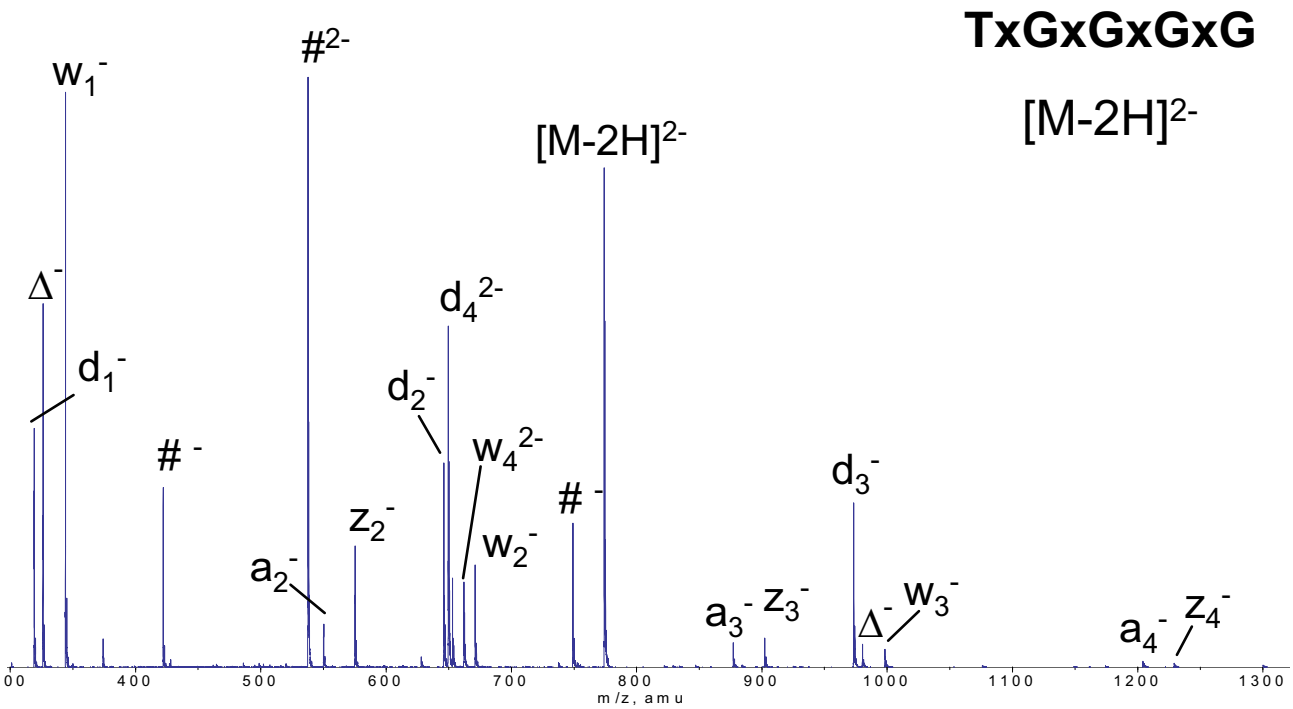


Figure 4. Product ion spectrum of the fully methylphosphonate-substituted pentanucleotide TxGxGxGxG; a-B- and M-B-ions are not detected. Besides the w- and a-ions, abundant fragments of the d/z-series are observed. Further peaks indicate the presence of the internal fragment ions, generated by repetitive backbone cleavage, e.g., [w-d]- (#) and [z-d]/[a-w]-ions (Δ).

and the entirely modified sequence TxGxGxGxG, with x indicating the position of the methylphosphonate modification. Localization of the charge is a central question in the discussion of methylphosphonate oligonucleotides. For unmodified oligonucleotides, the negative charge is most likely located on the phosphate groups, due to their low pKa. For methylphosphonate-modified oligonucleotides, however, deprotonation of the backbone is impossible wherever the phosphate group is inactivated by the methyl substituent. As proven by the spectrum of the fully methylphosphonate-substituted oligonucleotide TxGxGxGxG, ionization occurs. We have therefore evidence for charge location on the nucleobases (Figure 4). Generally, methylphosphonate-modified oligonucleotides exhibit reduced stability compared with their natural counterparts. CID with fixed collision energy of −30 eV showed that the ratios of fragment ions to the undissociated precursor ion are higher for methylphosphonate oligonucleotides than for unmodified oligonucleotides. The exchange of natural phosphate groups for methylphosphonate groups within the oligonucleotide backbone results in a significantly altered fragment ion pattern. The spectrum of TxGxGxGxG completely lacks the a-B-ions, while the complete w-fragment ion series appears with nearly identical peak abundance in the spectra of natural and modified oligonucleotides. Thus, backbone cleavage of methylphosphonate oligonucleotides is characterized by the w-/a- and d-/z-ion series and additionally, internal fragment ions such as [w-d]-ions and [a-w]-/[d-z]-ions (Figure 4 and Figure 5).

These results demonstrate that the phosphate group plays a central role in the formation of a-B-ions. The acidic proton of the neutral phosphate group and the negative charge of the deprotonated phosphate group both participate in the mechanism of nucleobase cleavage. This mechanism is now blocked by the methylphosphonate modifications. Experiments with partially substituted phosphate backbone further show that fragmentation is locally controlled: the (a_4-B_4)-ion with *m/z* 1055.2 appears in the product ion spectrum of TxGxGGG (Figure 5) but not in the spectrum of TxGxGxGG (spectrum not shown). The two sequences differ by the type of backbone linkage between the third and fourth building blocks, which is in 5′-position to the a_4-cleavage site. TxGxGGG bears an unmodified phosphate group, which acts as a proton donor. TxGxGxGG, however, bears a methylphosphonate group at this position, which is not able to perform protonation of the nucleobase and initiation of the cleavage reaction. The absence of the (a_4-B_4)-ion in the product ion spectrum of TxGxGxGG supports the DNA-cleavage mechanism proposed by Wang et al. for unmodified oligonucleotides [4]. Our results give additional evidence for the necessity of a proton donor in 5′-position to the cleavage site and emphasize local control of the a-B-mechanism [12]. The spectrum in Figure 5 also shows relatively abundant peaks corresponding to the w_3^{2-}- and w_4^{2-}-fragment ions (both of them incorporate two unmodified phosphate groups) indicating that the charges, according to their low pKa, are preferably located on the phosphate groups.

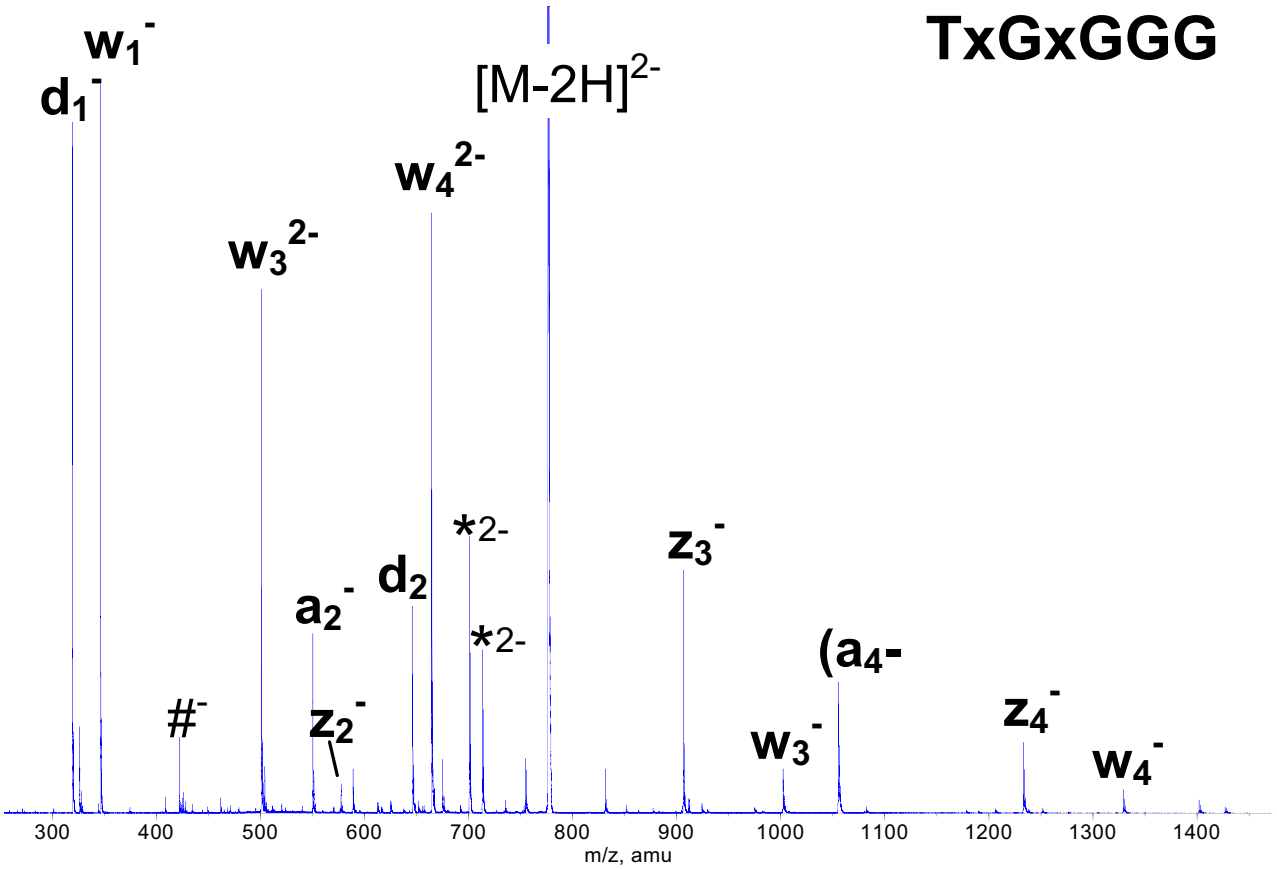
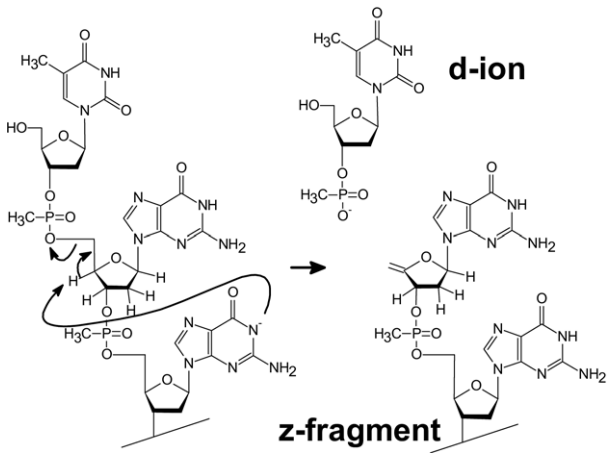


Figure 5. Product ion spectrum of TxGxGGG. Abundant peaks of w_3^{2-}- and w_4^{2-}-ions indicate that the charge is preferably located on unmodified phosphate groups. # indicates the [w-d]-ion; * indicates M-B-ions.

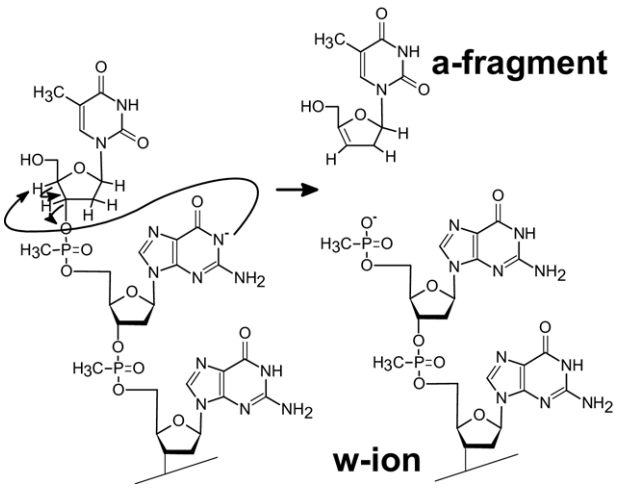
The product ion spectra of both, fully and partially methylphosphonate-modified oligonucleotides show the d- and their complementary z-ions with high abundance (Figures 4 and 5). For their formation, we propose the mechanism shown in Scheme **2**. The negatively charged nucleobase abstracts the proton from the 4′-position, which is then followed by a β-elimination leading to subsequent bond rearrangement and cleavage of the 5′-C–O bond, resulting in formation of the d- and z-ion. After cleavage, the charge is located on the methylphosphonate group of the d-ion. Abstraction of the 4′-hydrogen by the charged nucleobase in 5′-position is very unlikely, due to steric hindrance.



Scheme 2

In analogy, we propose a mechanism for the formation of a- and w-ions from methylphosphonate oligonucleotides, which again involves the charged 3′-nucleobase as the key element. Attack of the 4′-hydrogen, or alternatively the 2′-hydrogen, by the negative charge on nitrogen N1 induces β-elimination (Scheme **3**). Bond cleavage between C3′ and the oxygen atom of the methylphosphonate group results in formation of the corresponding a- and w-ion. The charge is located on the 5′-terminal methylphosphonate group of the w-ion, as shown in Scheme **3**. This mechanism resembles the β-elimination proposed for formation of the w_{n-1}-ion (see Scheme **1**), with the difference that the charge, which is responsible for the initial attack of the ribose hydrogen, is located on the nucleobase and not on the phosphate group.

Based on tandem mass spectrometric investigation of a modified dinucleoside with all exchangeable protons removed, Sindona and coworkers [13] showed that the 5′-terminal nucleobase can be involved in scission



Scheme 3

of the phosphodiester group as well. Calculations on the structure of the analyzed dimer revealed an energetically favorable six-membered ring intermediate, which induces abstraction of the 2′-hydrogen by the 5′-nucleobase and subsequently, leads to cleavage of the 3′-C–O bond. The reactive position is the 2-O of the thymine in deprotonated enol form at the 5′-position.

Our experiments on pentanucleotides with adenine in 5′-terminal position and an adjacent methylphosphonate group demonstrated that such mechanism is not restricted to pyrimidine bases only, as formation of a w_4-ion from Ax(dSpacer)GGG, with a purine in 5′-terminal position, still occurred. The abasic site (dSpacer) adjacent to the adenine excluded participation of the nucleobase in 3′-position.

Conclusions

Decomposition of oligonucleotides results in fragment ions, the formation of which cannot be explained by the generally accepted mechanism responsible for generation of the main fragment ions. Studies show that the influence of the nucleobases on the fragmentation of oligonucleotides is limited to a general trend. Adenine-rich oligonucleotides fragment more easily, while thymine-rich sequences show less abundant dissociation. Results demonstrate that it is currently not possible to predict the abundances of fragment ions based on the sequence of the nucleobases.

Investigation of oligonucleotides incorporating abasic sites proved backbone cleavage in absence of a nucleobase. Results support a nucleobase-independent β-elimination mechanism whereby the deprotonated phosphate group, as the nucleophile attacks either the 4′-hydrogen or the 2′-hydrogen, initiating the backbone cleavage process. This mechanism can also be applied to the formation of w_{n-1}-fragment ions originating from oligonucleotides of a length of n nucleotides.

The locations of charges, which often act as reactive centers within the fragmentation process, were investigated by CID of methylphosphonate-modified oligonucleotides. Our studies showed that charges are preferably located on the phosphate groups. If phosphate groups are inactivated by methylphosphonate-modifications, charges can alternatively be located on the nucleobases. In such cases, the nucleobases become the reactive centers and initiate the fragmentation process. Structural modification of oligonucleotides leads to a change of the fragmentation mechanism and, consequently, to a considerably altered fragment ion pattern. Since methylphosphonate oligonucleotides cannot form a-B-ions, a-, d-, and z-ions as well as the internal fragment ions ([w-d]-ions and the isobaric [z-d]- and [a-w]-ions) are formed alternatively, in addition to the generally observed w-ions.

Acknowledgments

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