Gas-Phase Dissociation of Oligoribonucleotides and their Analogs Studied by Electrospray Ionization Tandem Mass Spectrometry

Jan M. Tromp and Stefan Schürch
Department of Chemistry and Biochemistry, University of Bern, Bern, Switzerland

Oligoribonucleotides (RNA) and modified oligonucleotides were subjected to low-energy collision-induced dissociation in a hybrid quadrupole time-of-flight mass spectrometer to investigate their fragmentation pathways. Only very restricted data are available on gas-phase dissociation of oligoribonucleotides and their analogs and the fundamental mechanistic aspects still need to be defined to develop mass spectrometry-based protocols for sequence identification. Such methods are needed, because chemically modified oligonucleotides can not be submitted to standard sequencing protocols.

In contrast to the dissociation of DNA, dissociation of RNA was found to be independent of nucleobase loss and it is characterized by cleavage of the 5′-P—O bond, resulting in the formation of c- and their complementary y-type ions. To evaluate the influence of different 2′-substituents, several modified tetraribonucleotides were analyzed. Oligoribonucleotides incorporating a 2′-methoxy-ribose or a 2′-fluoro-ribose show fragmentation that does not exhibit any preferred dissociation pathway because all different types of fragment ions are generated with comparable abundance. To analyze the role of the nucleobases in the fragmentation of the phosphodiester backbone, an oligonucleotide lacking the nucleobase at one position has been studied. Experiments indicated that the dissociation mechanism of RNA is not influenced by the nucleobase, thus, supporting a mechanism where dissociation is initiated by formation of an intramolecular cyclic transition state with the 2′-hydroxyl proton bridged to the 5′-phosphate oxygen. (J Am Soc Mass Spectrom 2005, 16, 1262–1268) © 2005 American Society for Mass Spectrometry

Antisense oligonucleotides are nucleic acids of about 12 to 20 nucleobases, which are designed to hybridize to a complementary messenger RNA (mRNA) sequence, thus, inhibiting gene expression [1-3]. Therefore, they are of great interest in human cancer therapy and for diagnostic applications. Besides the high binding specificity of antisense oligonucleotides to their target mRNA, affinity, bioavailability, and biostability are of foremost importance. However, a major drawback of the application of unmodified phosphodiester DNA or RNA oligonucleotides is that these structures are subjected to rapid nuclease degradation under physiological conditions. To improve these factors, oligonucleotide analogs, exhibiting chemical modifications of the phosphodiester backbone, of the ribose, and, to a limited extent, also of the nucleobases, are evaluated.

Mechanisms of inhibition of gene expression by antisense oligonucleotides involve mRNA degrading enzymes such as RNase H, blockade of translation initiation, or modulation of splicing [4-7]. These mechanisms allow the application of new chemical modifications to increase the binding affinity and the nuclease resistance. Possible positions of the modifications are the phosphodiester backbone, the sugar unit and the nucleobases. Introduction of a phosphorothioate backbone increases nuclease resistance of antisense oligonucleotides and simultaneously serves as a very efficient substrate for mRNA degrading enzymes [6-9]. Unfortunately, such modification reduces the binding affinity to the complementary mRNA. Thus, further chemical modifications (e.g., derivatization of the ribose 2′-position by electronegative substituents) have been introduced to increase the affinity and to further enhance their nuclease resistance [10]. However, most of these highly modified oligonucleotide analogs do not support enzymatic activity and alternative mechanisms not relying on RNase H activity are required.

Apart from the development of appropriate synthetic methodologies, evaluation of oligonucleotide analogs is among the main focuses of antisense research. Evaluation of antisense oligonucleotides for therapeutic and diagnostic applications requires suitable analytical

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Address reprint requests to Dr. Stefan Schürch, Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, CH-3012 Bern, Switzerland. E-mail: stefan.schuerch@ioc.unibe.ch

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tools for sequence determination and detailed structural elucidation. Because of the presence of unnatural structural elements, the classical sequencing techniques [11, 12] are likely to fail and alternative approaches for rapid and accurate sequencing of chemically modified oligonucleotides have to be developed. Tandem mass spectrometry is a highly attractive candidate for this task, because it is capable of providing the high degree of structural information required.

Within the past decade, detailed mechanistic data on the dissociation of unmodified oligodeoxyribonucleotides (DNA) in gas-phase have been provided. The loss of the nucleobase was found to be the initial dissociation step, leading to the subsequent cleavage of the 3′-C—O bond, thus, resulting in the formation of the generally abundant [a-B] and w ions. Several different mechanisms have been proposed and evaluated to explain the formation of the DNA typical [a-B] and w fragment ions [13-15] and the generally applied nomenclature of oligonucleotide fragments was proposed by McLuckey et al. in 1992 [16]. On the other hand, very limited data are available on the dissociation of RNA and oligonucleotide analogs.

Because of their use in antisense applications, methylphosphonate and phosphorothioate oligonucleotides are among the most widely studied analogs. Oligonucleotides with a methylphosphonate backbone have been investigated to study the effect of backbone charge on backbone dissociation and to trace the origin of the proton transferred to the nucleobase as the initial step of DNA dissociation [17, 18]. Oligonucleotides based on the phosphorothioate backbone are highly promising compounds for therapeutic antisense applications. The first of these antisense oligonucleotides was admitted to market in 1998 and further RNase H activating phosphorothioate oligonucleotides are currently in various stages of clinical trial [3, 19-21]. Investigation of the dissociation pattern of phosphorothioate, and phosphodiester oligodeoxyribonucleotides in gas-phase revealed that both backbone modifications yield the same types of fragment ions [22, 23].

Information on the fragmentation mechanism of RNA in gas-phase is rare. The few reports found indicate that dissociation of RNA clearly differs from the dissociation of DNA. The preferred cleavage site of RNA is the 5′-P—O bond, resulting in the formation of c- and y-type ions [24-26]. It has been shown that oligoribonucleotides possess higher gas-phase stability than oligodeoxyribonucleotides because of stabilization of the *N*-glycosidic bond by the 2′-OH substituent [10, 27]. Unlike dissociation of DNA, backbone dissociation of RNA is independent of nucleobase loss. Collision-induced dissociation (CID) of mixed sequence DNA/RNA oligonucleotides demonstrated that backbone cleavage is controlled locally and influenced by the type of adjacent ribose 2′-substituent. No evidence for any remotely located group affecting the dissociation was found and a fragmentation mechanism was proposed [26]. Furthermore, the fragmentation patterns of different 2′-ribose-modified oligonucleotides have been described without focusing on the mechanistic aspects of gas-phase dissociation [23, 28]. Reports specifically focusing on the dissociation mechanisms of 2′-modified oligonucleotides and their gas-phase stability are missing completely.

In this article we report on the dissociation of selected nucleobase- or 2′-ribose-modified oligonucleotides. To gain insight into the fragmentation mechanism of oligoribonucleotides and for better understanding of the influence of different 2′-substituents, a number of selectively modified oligoribonucleotides have been investigated by electrospray tandem mass spectrometry on a hybrid quadrupole time-of-flight mass spectrometer.

Experimental

Sample Preparation

RP-HPLC (reversed-phase high performance liquid chromatography) purified oligonucleotides were obtained from TriLink BioTechnologies (San Diego, CA) and Microsynth (Balgach, Switzerland). The lyophilized oligonucleotides were dissolved in a mixture of water/acetonitrile/triethylamine (49/49/2) resulting in a final oligonucleotide concentration of about 30 pmol/μL. Typically, a sample volume of 3 μL was used for each analysis.

Mass Spectrometry

All experiments were performed on an Applied Biosystems/MDS Sciex QStar Pulsar hybrid quadrupole time-of-flight mass spectrometer (Sciex, Concord, Canada), equipped with a nanoelectrospray ion source (Protana, Odense, Denmark). Oligonucleotides were analyzed in the negative-ion mode with a potential of −900 V applied to the nanospray needle. Nitrogen was used as the curtain gas.

Tandem mass spectrometric experiments were performed with precursor ions selected within a window of ±1.5 *m/z* units. CID was performed with collision energies in the range of 10-35 eV using nitrogen as the collision gas. The time-of-flight analyzer was tuned for an average mass resolving power of 10,000 (full width at half maximum) and calibrated externally using a mixture of cesium iodide and taurocholic acid. Calibration was performed before each series of experiments or at least once a day. The Applied Biosystems Analyst QS software package was used for data processing. Product ion spectra of tetranucleotides are based on the doubly deprotonated precursor ions.

Results and Discussion

A partial section of the product ion spectra of the tetranucleotides d(TATG) and UAUU is shown in Figure 1. It shows the unique fragment ion pattern of RNA, which clearly differs from the pattern of DNA. The

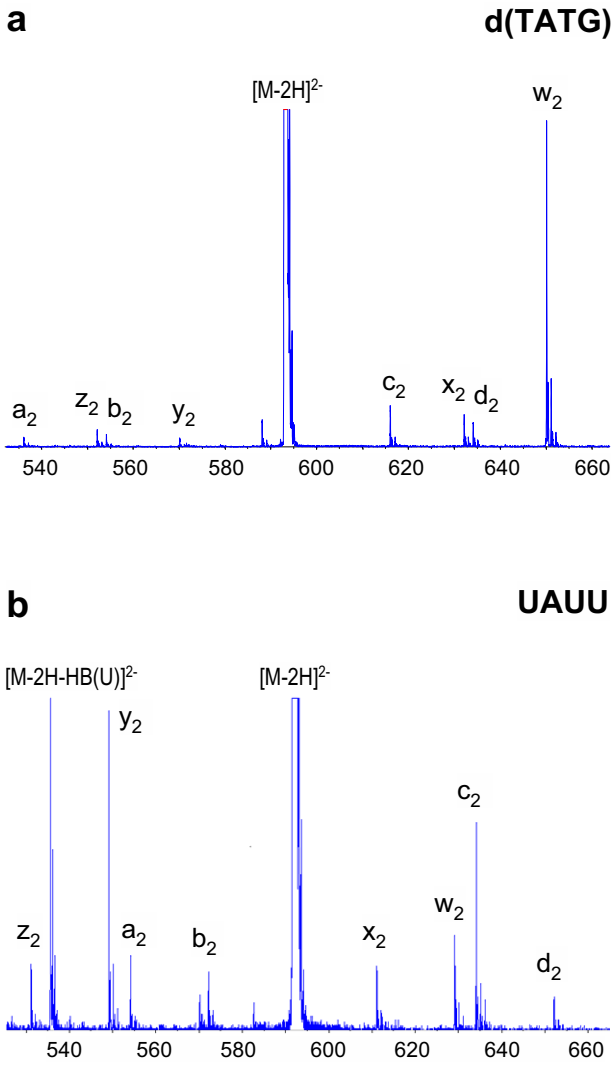


Figure 1. Comparison of the product ion spectra of DNA and RNA. (**a**) Product ion spectrum of the deoxyribonucleotide d(TATG), obtained by dissociation of the $[M\text{-}2H]^{2-}$ precursor ion with *m/z* 593.13. The abundant w_2 ion (*m/z* 650.13) is a result of the cleavage of the 3′-C—O bond, which is initiated by nucleobase loss. (**b**) The product ion spectrum of the $[M\text{-}2H]^{2-}$ precursor ion of the tetraribonucleotide UAUU (*m/z* 591.63) predominantly shows the RNA characteristic y_2 and c_2 ions with *m/z* 549.13 and 634.12, respectively. Other fragments, because of backbone cleavage, are of significantly lower intensity.

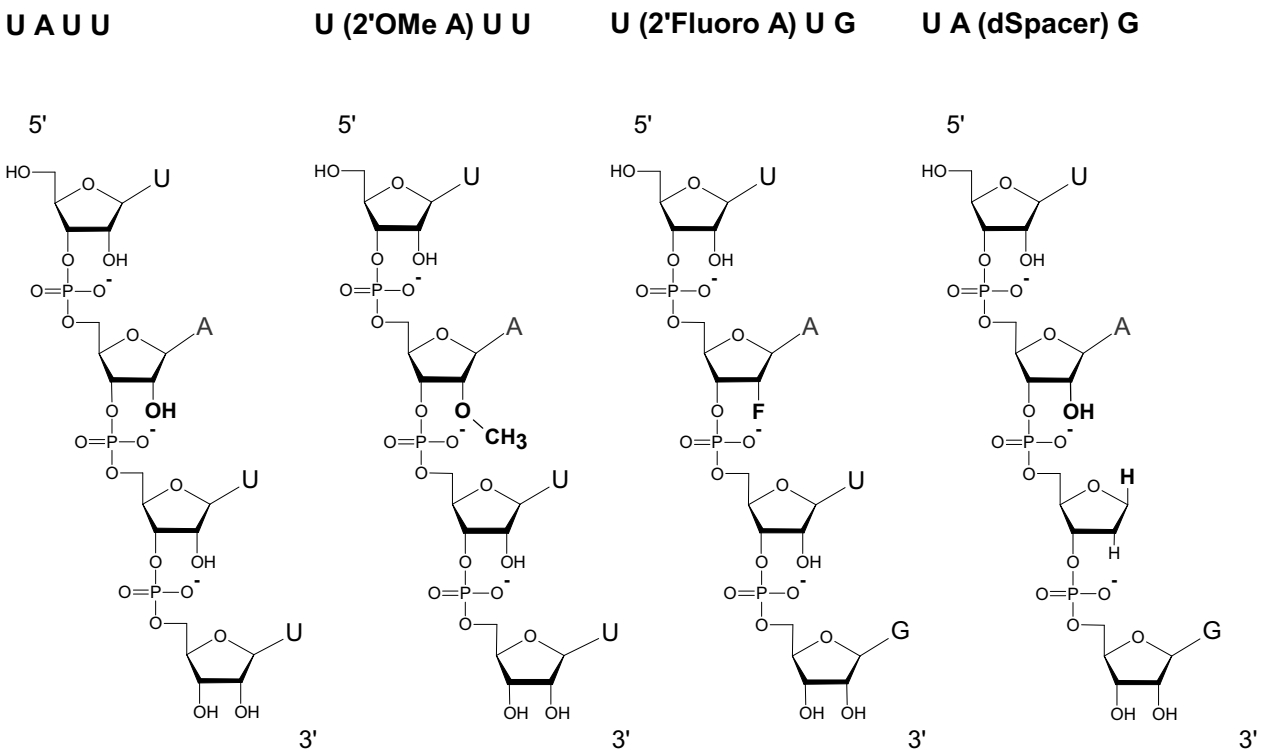
product ion spectrum of the doubly deprotonated tetradeoxyribonucleotide d(TATG) with *m/z* 593.13 is shown in Figure 1a. The spectrum is characterized by the abundant DNA typical w_1, w_2, and w_3 fragment ions with *m/z* 346.07, 650.13, and 963.20, respectively, generated by cleavage of the 3′-C—O bonds. The complementary [a-B]-type ions and additional fragments due to backbone cleavage at alternative positions and nucleobase loss are observed with reduced abundance. On the other hand, the product ion spectrum of the tetraribonucleotide UAUU ($[M\text{-}2H]^{2-}$, *m/z* 591.63) shows abundant y- and c-type ions (Figure 1b). Most of the theoretically possible fragment ions originating from backbone cleavage can be found in the spectrum. The y_2 ion (*m/z* 549.13) exhibits about a fourfold higher abundance than the other 3′-terminal ions (w_2, x_2, and z_2). Likewise, the abundance of the c_2 ion (*m/z* 634.12) is about three times higher, compared with the alternative 5′-terminal fragment ions (a_2, b_2, and d_2). The observed formation of c- and their complementary y-type ions confirms the 5′-P—O bond as the preferred cleavage site within the RNA backbone. RNA typical behavior was also found for the first and third phosphodiester group of the tetraribonucleotide (data not shown).

The different fragment ion patterns observed for DNA and RNA must be related to the presence of the altered 2′-substituent. The electronegative hydroxyl group of RNA plays a key role in the dissociation mechanism, because it effectively stabilizes the *N*-glycosidic bond. The inductive effect of such a strong electron-withdrawing group impedes formation of the carbocation at the 1′-position [10], thus, rendering nucleobase loss less prominent. Furthermore, the availability of the additional proton in the vicinity of the phosphodiester group provides alternative pathways for backbone cleavage.

To obtain mechanistic data on the gas-phase dissociation of RNA and to evaluate the influence of different 2′-substituents on the dissociation and stability of the *N*-glycosidic bond, a number of tetraribonucleotides bearing a single modified nucleotide have been studied. Modifications include 2′-methoxy and 2′-fluoro substituents at the 2′-position of the second nucleotide, and a deoxynucleotide lacking the nucleobase (dSpacer) as the third building block within the sequence (Scheme **1**). Thus, the influence of the modifications on the second phosphodiester group can be observed.

Tetramers were chosen for this study because they can be dissociated efficiently under low-energy CID conditions, and the length of the nucleotide sequences is still sufficient to study the effect of selected local modifications on backbone dissociation. Hydrogen/deuterium exchange experiments, which are often used for localizing the origin of protons transferred on dissociation reactions, do not provide conclusive information on the dissociation of RNA, because 2′-hydroxyl protons, remaining phosphate protons on the backbone, and amino protons of the nucleobases would be exchanged.

Compared with unmodified RNA, oligoribonucleotides with a 2′-methoxy-adenosine as the second nucleotide exhibits a different fragmentation behavior, as shown by the product ion spectrum of the doubly deprotonated U(2′OMeA)UU (*m/z* 598.60) in Figure 2a. Although the first and third phosphodiester groups dissociate into RNA typical y- and c-type ions, no evidence for a preferred dissociation pathway of the second phosphodiester group was found, and all of the theoretically possible fragment ions were observed with similar abundance. This experiment shows that the fragmentation mechanism of RNA is not exclusively influenced by the stabilization of the *N*-glycosidic bond



Scheme 1. Structures of the tetraribonucleotides investigated. All oligonucleotides are shown in completely deprotonated form. Unmodified RNA is shown on the left. Modifications are located between the second and the third building block and include 2′-methoxy and 2′-fluoro substituents and a deoxynucleotide lacking the nucleobase (dSpacer).

by the 2′-hydroxyl oxygen. The 2′-hydroxyl proton most likely contributes to the formation of y- and c-type ions as well. Otherwise, backbone dissociation next to 2′-methoxy-modified nucleotides would result in fragment ion patterns identical to those of unmodified nucleotides.

Figure 2b further shows the influence of an electronegative 2′-substituent on backbone dissociation. The product ion spectrum obtained by dissociation of doubly deprotonated U(2′FluoroA)UG (*m/z* 612.11), which bears a 2′-fluoro modification at the second nucleotide, is shown in Figure 2b. The stabilizing effect of the electronegative 2′-substiuent on the *N*-glycosidic bond reduces the rate of nucleobase loss and, consequently, the abundance of the corresponding fragment ions is decreased. Formation of the w_2 ion (*m/z* 668.10) even becomes less likely than the formation of the alternative x_2, y_2, and z_2 ions. Also, the complementary [a_2-B_2] ion (*m/z* 444.09) is of significantly reduced abundance, when compared with the dissociation pattern of an unmodified DNA. The 2′-fluoro nucleotide does not exhibit any preferred dissociation pathway, and the various types of fragment ions are of comparable abundance. The resulting fragment ion pattern is quite similar to the one observed for 2′-methoxy derivatives (Figure 2a). Although 2′-hydroxyl, 2′-methoxy, and 2′-fluoro substituents all stabilize the *N*-glycosidic bond, the fragmentation patterns of 2′-methoxy- and 2′-fluoro-modified tetranucleotides, which lack the exchangeable 2′-proton, clearly differ from the one of RNA. These experiments on oligonucleotides incorporating building blocks with modified 2′-substituents show the important role the 2′-hydroxyl group is playing in the dissociation of RNA. The electronegative 2′-hydroxyl group stabilizes the *N*-glycosidic bond and simultaneously provides a mobile proton.

A mechanism explaining backbone dissociation of RNA, which involves the 2′-hydroxyl proton, has been proposed previously [26]. It proposes initiation of backbone dissociation by formation of an intramolecular cyclic transition-state with the 2′-hydroxyl proton bridged to the 5′-phosphate oxygen. Abstraction of the 2′-hydroxyl proton by the 5′-oxygen leads to dissociation of the 5′-P—O bond and, finally, complementary c- and y-type fragment ions are released. Subsequent bond rearrangement leads to stable c-type fragment ions (Scheme **2a**). The aforementioned results obtained from experiments with 2′-modified tetraribonucleotides are consistent with this mechanism.

However, the possibility of an alternative gas-phase dissociation mechanism, similar to the one known for backbone cleavage of oligoribonucleotides in solution, still could not be excluded. The mechanism shown in Scheme **2b** involves the 2′-hydroxyl proton and leads to the same RNA characteristic y- and c-type fragment ions as the mechanism shown in Scheme **2a**. Although in gas-phase there is no free base present, which could act as a proton acceptor,

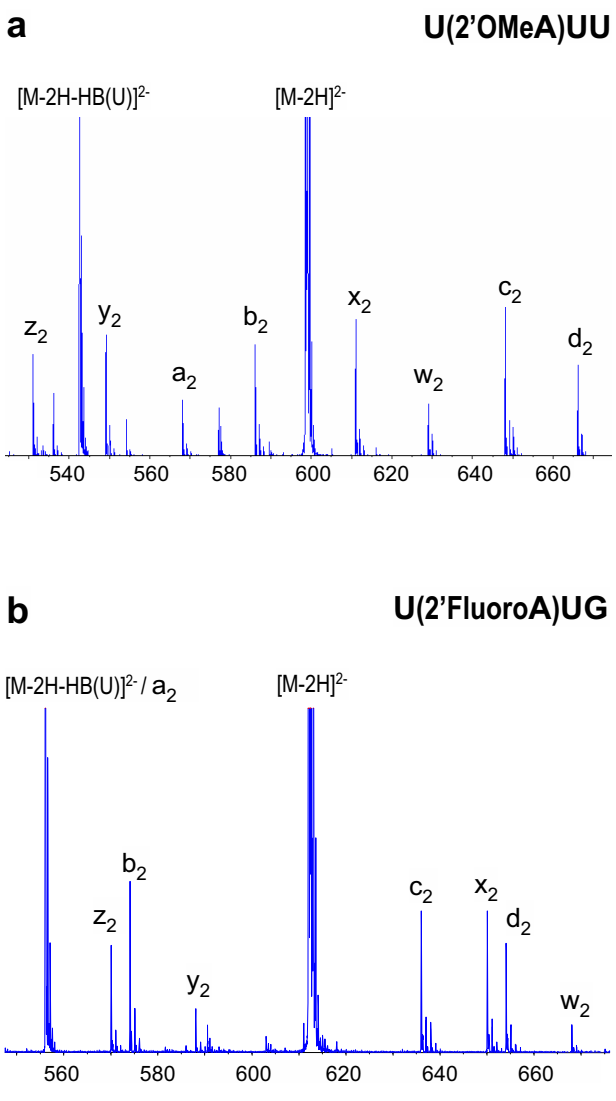


Figure 2. (**a**) Product ion spectrum of the doubly deprotonated modified tetraribonucleotide U(2′-OMeA)UU with *m/z* 598.60. The spectrum gives no evidence of a preferred dissociation pathway. (**b**) Product ion spectrum of doubly deprotonated U(2′-Fluoro)UG with *m/z* 612.11. The a_2 fragment ion (*m/z* 556.10) is overlapped by the very abundant $[M\text{-}2H\text{-}HB(U)]^{2-}$ ion.

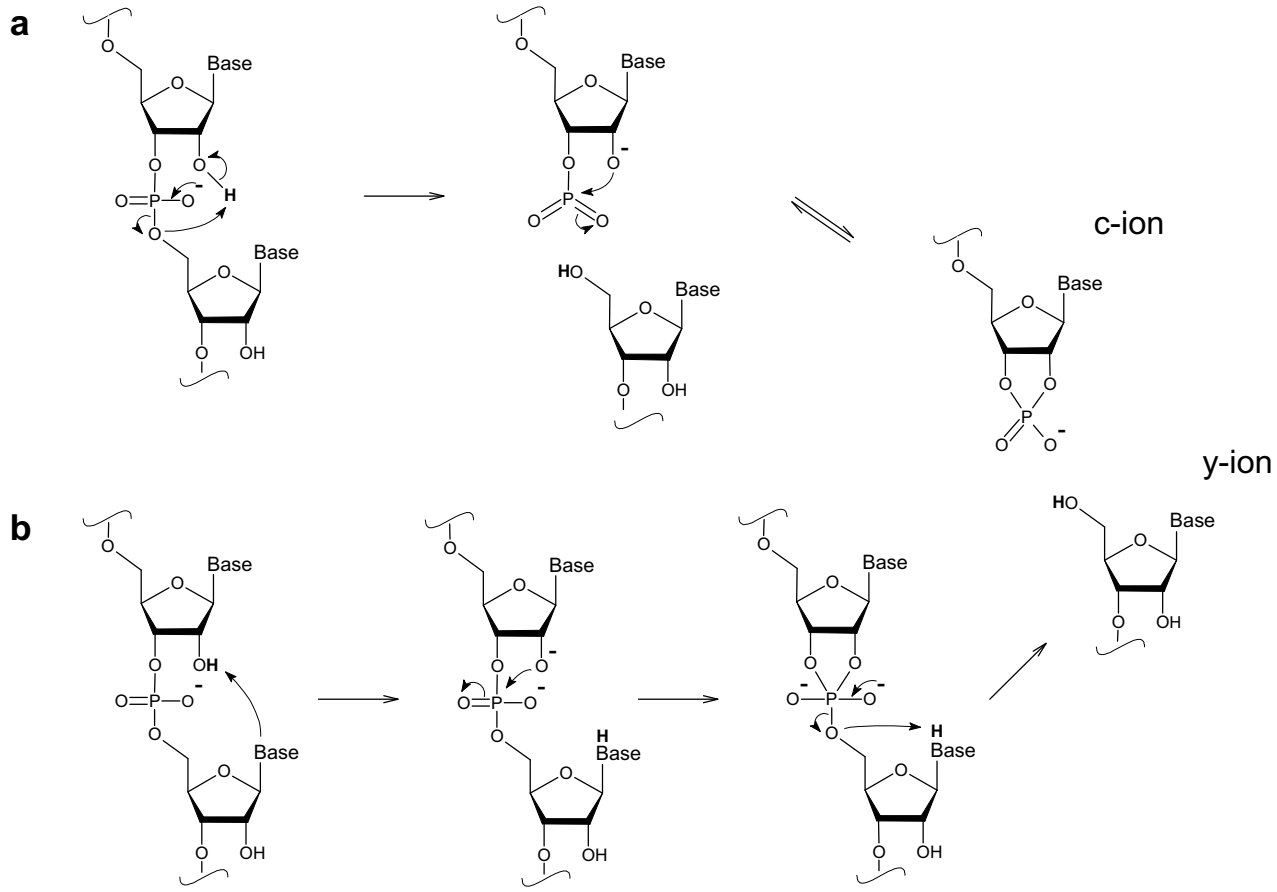
deprotonation of the 2′-hydroxyl group could still be accomplished by intramolecular proton transfer to the adjacent 3′-nucleobase. Deprotonation of the 2′-hydroxyl group would be followed by transesterification, in which the 2′-oxygen participates as a nucleophile, generating a cyclic transition-state. Bond rearrangement and protonation of the leaving group (y ion) by the protonated nucleobase would finally result in cleavage of the phosphodiester backbone and in the formation of the c- and its complementary y-type fragment ions (Scheme **2b**).

To verify such an alternative mechanism the tetranucleotide UA(dSpacer)G, incorporating a deoxy building block lacking the nucleobase (Scheme **1**), has been subjected to CID. Incorporation of the dSpacer into the sequence enables evaluation of the role of the nucleobase in the dissociation of the phosphodiester backbone, because the dSpacer does not provide any nucleobase, which could potentially act as an acceptor for the 2′-hydroxyl proton. If the nucleobases were involved in the dissociation of RNA (e.g., as protonation sites), the presence of a building block lacking the nucleobase would block such mechanism. The altered fragmentation pathway should result in a product ion pattern that differs from the one observed for RNA. If, on the other hand, the nucleobase was not involved in the mechanism, dissociation of the phosphodiester backbone would not be affected by the dSpacer and c- and y-type ions would be formed predominantly.

Figure 3 shows an enlarged section of the product ion spectrum of the doubly deprotonated tetraribonucleotide UA(dSpacer)G with *m/z* 548.11. It represents the fragment ions generated by cleavage of the precursor between the second ribonucleotide (A) and the dSpacer. The RNA typical y_2 fragment ion (*m/z* 462.12) was observed with high abundance, whereas all alternative 3′-terminal fragment ions (x_2, z_2, and w_2) were of significantly decreased abundance, only reaching about 10-30% of the intensity of the y_2 ion. Likewise, c_2 (*m/z* 634.09) was the most abundant 5′-terminal fragment ion. The product ion spectrum of the unmodified oligoribonucleotide UAUU (Figure 1b) and the spectrum of the modified sequence incorporating the dSpacer (Figure 3) both show similar fragment ion patterns with the y- and c-type ions as the most abundant fragments. The fragment ion pattern is independent of the presence of the nucleobase. Consequently, the nucleobase does not have any significant influence on the dissociation mechanism of oligoribonucleotides. An alternative mechanism, in which the nucleobase plays a key role as acceptor of the 2′-hydroxyl proton, can be eliminated. These experiments clarify the role the nucleobases are playing in the fragmentation mechanism of oligoribonucleotides and confirm the previously proposed dissociation mechanism of RNA [26], which suggests backbone dissociation to be initiated by formation of an intramolecular cyclic transition-state with the 2′-hydroxyl proton bridged to the 5′-phosphate oxygen (Scheme **2a**), while the adjacent nucleobase is not involved.

Conclusions

Investigation of oligoribonucleotides and their analogs by tandem mass spectrometry establishes the importance of the 2′-hydroxyl substituent as the structural key element in the fragmentation mechanism of RNA. In contrast to DNA, which predominantly fragment into [a-base]- and w-type ions, the presence of the 2′-hydroxyl group of RNA results in formation of abundant c- and y-type ions. The electron-withdrawing 2′-substituent induces a stabilizing effect on the *N*-glycosidic bond, thus, rendering nucleobase loss less prominent. In addition, the availability of a proton in



Scheme 2. Dissociation mechanisms of RNA. (**a**) Previously proposed mechanism describing the dissociation of RNA. (**b**) Alternative dissociation mechanism similar to the one known for backbone cleavage of oligoribonucleotides in solution. Both mechanisms include the 2′-hydroxyl proton and lead to the RNA-typical fragment ions. In contrast to the mechanism (**a**), the alternative mechanism (**b**) does involve the nucleobase.

the vicinity of the phosphate group is mandatory for the RNA typical backbone cleavage.

Experiments on oligoribonucleotides incorporating the dSpacer, a nucleotide lacking the nucleobase, showed that gas-phase dissociation of the RNA backbone is not influenced by the nucleobases. Therefore, a mechanism similar to the one known for backbone cleavage of oligoribonucleotides in solution can be eliminated. The results support the mechanism in which backbone dissociation is initiated by formation of an intramolecular cyclic transition-state with the 2′-hydroxyl proton bridged to the 5′-phosphate oxygen.

Results provide fundamental information on the gas-phase dissociation of oligoribonucleotides. This information is needed to provide the basis for reliable tandem mass spectrometric sequencing methods, which will greatly facilitate the identification and verification of oligonucleotides and their analogs used for therapeutic applications, as genetic probes or as primers for amplification.

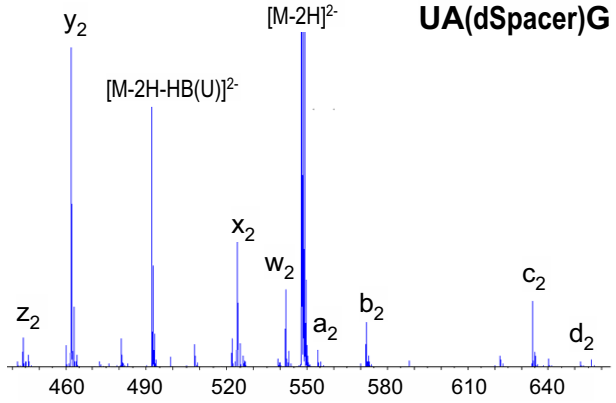


Figure 3. Product ion spectrum of the doubly deprotonated UA(dSpacer)G (*m/z* 548.11), which incorporates a building block lacking the nucleobase. The spectrum predominantly shows the RNA characteristic y_2 ion with *m/z* 462.12. Other fragments indicate backbone cleavage at alternative positions.

Acknowledgments

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