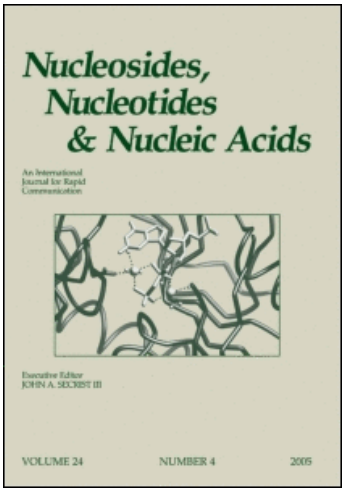

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MASS SPECTROMETRY OF OLIGONUCLEOTIDES

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□ *Expanding research in the field of modified oligonucleotides demands suitable analytical tools for size and purity verification of known compounds and accurate structure elucidation of unknowns. There is a need for characterization of the types and sites of modifications in oligonucleotides and to identify and sequence selected candidates originating from synthesis. The potential of electrospray tandem mass spectrometry (ESI-MS/MS) for structural characterization and sequencing of oligonucleotides is demonstrated. The fundamental behavior of DNA, RNA, and selected modified oligonucleotides in gas-phase is shown. Since gas-phase dissociation does not demand specific structural prerequisites, the method bears a great potential for rapid and most accurate characterization of modified oligonucleotides, e.g. from combinatorial libraries.*

Keywords Oligonucleotide sequencing; gas-phase dissociation; electrospray; tandem mass spectrometry

INTRODUCTION

Oligonucleotide-based therapeutics bear a great potential for successful human cancer therapy. A variety of compounds is evaluated for therapeutic applications and great effort is put into the development of chemically modified oligonucleotides with the goal to direct the mechanism of action, to increase their affinity towards target sequences, and to enhance their binding specificity and biostability. Apart from the development of appropriate synthetic methodologies, evaluation of nucleic acid-based drug candidates is among the main focuses of current research activities. Within this context, fast, reliable, and accurate tools are needed for verification of the structural integrity and for detailed structural characterization of selected compounds.

Tandem mass spectrometry (MS/MS) is a powerful tool for rapid structure confirmation and elucidation. The technique is based on selection of precursor ions in a first stage of mass spectrometry, followed by activation of the precursor ions by collisions with an inert gas. Collision-induced dissociation (CID) results in sequence- or structure-defining fragment ions, which

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are finally separated and detected in a second stage of mass spectrometry. In combination with soft ionization techniques, such as electrospray ionization (ESI), tandem mass spectrometry has demonstrated its potential for routine peptide sequencing and protein identification. Despite its many advantages, tandem mass spectrometry is hardly employed for sequence elucidation of oligonucleotides. This is mostly due to the fact that the resulting product ion spectra are very complex and that the fundamental aspects of oligonucleotide dissociation in gas-phase are not understood completely.

SEQUENCING OF UNMODIFIED DNA AND RNA BY TANDEM MASS SPECTROMETRY

Unlike chemical or enzymatic degradation, gas-phase dissociation of oligonucleotides in the collision cell of a tandem mass spectrometer does not require specific structural prerequisites. The nucleotide sequence is read from the product ion spectrum by assigning one or more series of fragment ions generated by cleavage of the phosphodiester bond at various positions along the backbone. The main mechanism of backbone cleavage of DNA in gas-phase was solved by Gross and co-workers almost a decade ago.[1] It was demonstrated that backbone cleavage is initiated by protonation and subsequent loss of the nucleobase. Bond rearrangement finally results in cleavage of the 3′-C-O bond, generating the [a-Base]- and w-ions as the main dissociation products. Consequently, sequence elucidation of DNA is primarily based on assignment of these two ion series. The nomenclature of oligonucleotide fragment ions shown in Figure 1, is based on the initial work of McLuckey et al.[2]

The fragment ion pattern generated by dissociation of RNA clearly differs from the one of DNA. RNA predominantly dissociates by cleavage of the 5′-P-O bond, resulting in c- and their complementary y-ions as the main products.[3–5] The mechanism responsible for formation of the RNA-typical fragment ions has recently been elucidated by Tromp et al.[6,7] by studying the influence of various 1′- and 2′-substituents on backbone cleavage. It was demonstrated that, in contrast to the dissociation of DNA, the nucleobase adjacent to the cleavage site is not involved in the cleavage mechanism of RNA. The mechanism assumes that backbone cleavage is initiated by formation of an intramolecular cyclic transition state with the 2′-hydroxyl proton bridged to the 5′-oxygen. Abstraction of the 2′-hydroxyl proton by the 5′-phosphate oxygen leads to scission of the 5′-P-O bond and finally, results in the release of a y-fragment with intact 5′-terminus and a stabilized negatively charged c-fragment ion. The independence of RNA backbone cleavage from nucleobase loss has further been demonstrated by experiments on 1′-biphenyl-substituted oligonucleotides.[8] Furthermore, experiments on chimeric oligonucleotides containing deoxyribo- and ribonucleotides

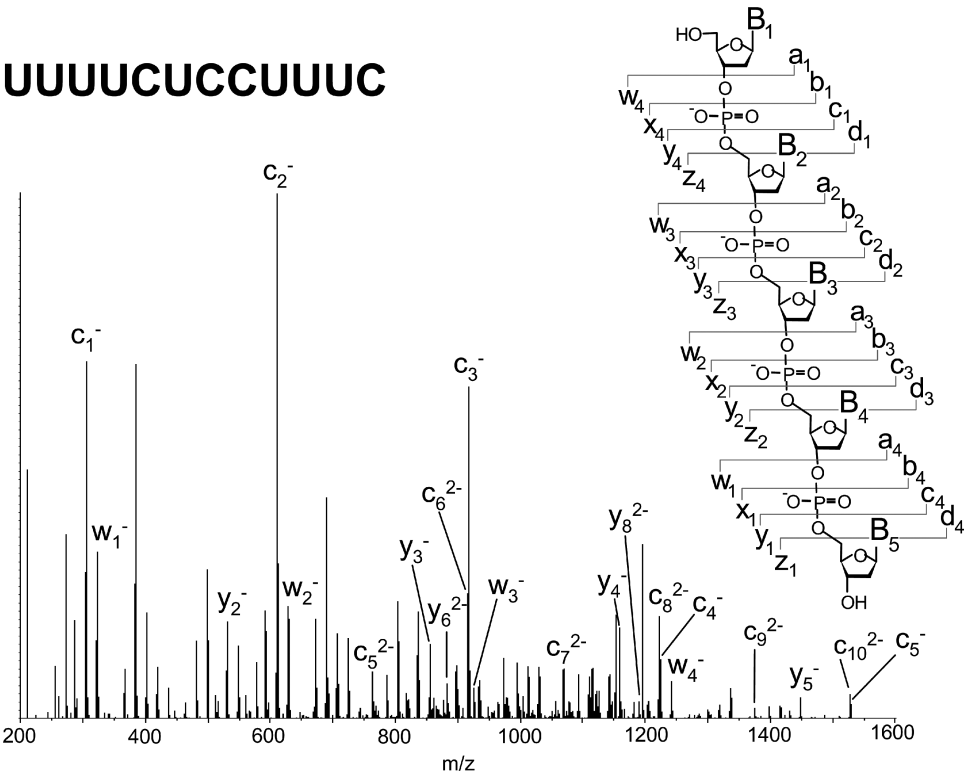

FIGURE 1 Product ion spectrum of the dodecaribonucleotide UUUUCUCCUUUC (3606 Da). Sequence-defining c- and y-ions are generated by cleavage of the 5′-P-O bond and provide full sequence coverage. Right: General nomenclature of ions generated by gas-phase dissociation of oligonucleotides.

demonstrated that backbone cleavage is locally controlled, influenced by the adjacent functional groups only.[5] An example of RNA sequencing by tandem mass spectrometry is given by Figure 1, which shows the product ion spectrum of the dodecaribonucleotide UUUUCUCCUUUC (3606 Da). The triply charged molecular ion with m/z 1201.1 was selected as the precursor ion and subjected to collision-induced dissociation. The spectrum shows the two series of oppositely directed c- and y-type fragment ions as the main dissociation products. Additional sequence information is obtained from the w-ion series. Besides these main series of sequence-defining fragment ions, numerous fragments generated by alternative backbone cleavage are observed as well, though with less abundance.

SEQUENCING OF MODIFIED OLIGONUCLEOTIDES

Due to the unnatural structural elements present in modified oligonucleotides, they often resist degradation by conventional methods. Since gas-phase dissociation does not depend on the activity of a specific cleavage

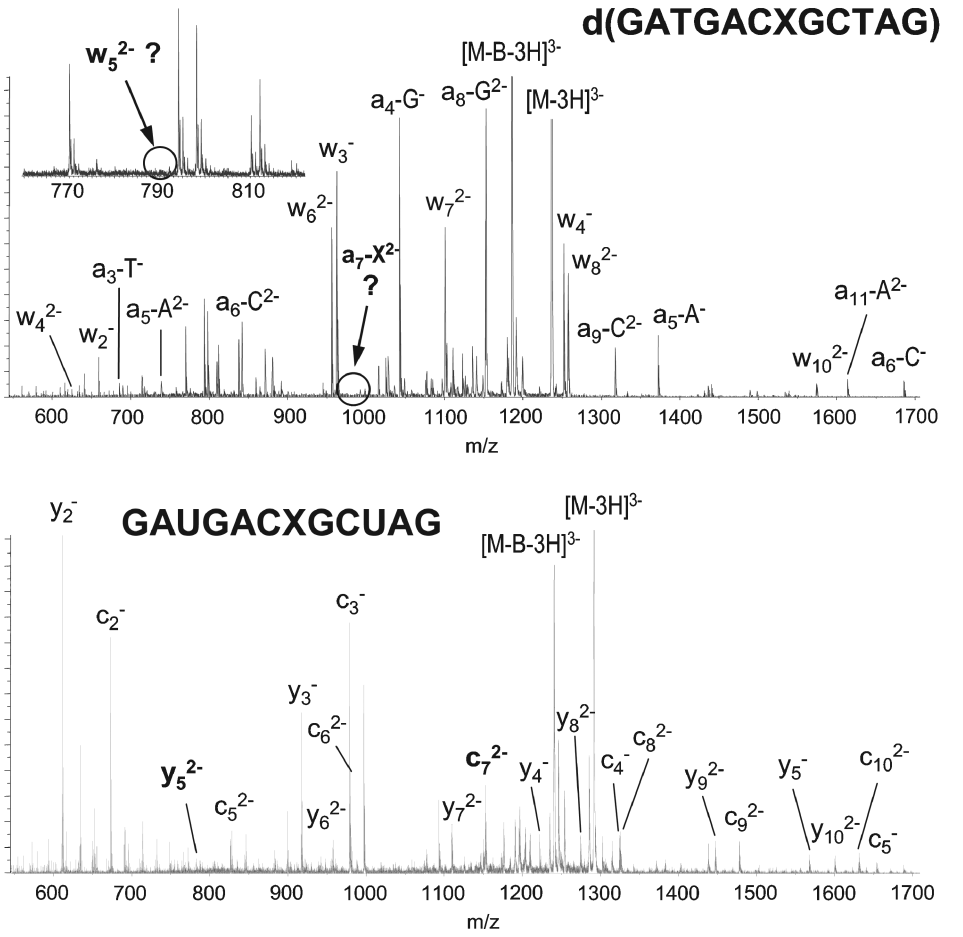

FIGURE 2 Product ion spectra of the 1′-biphenyl-modified dodecadeoxyribonucleotide d(GATGACXGCTAG) and the corresponding dodecaribonucleotide GAUGACXGCUAG, with X indicating the 1′-biphenyl modification.

reagent, tandem mass spectrometry is a promising alternative for characterization of such compounds. Product ion spectra offer rapid read-out of nucleotide sequences and simultaneously, provide information on types and positions of modifications. Product ion spectra of oligonucleotides incorporating a 1′-biphenyl modification are shown in Figure 2. Since backbone dissociation of DNA depends on loss of the nucleobase, the series of [a-B]- and w-ions show a gap at the position of the modification. In the spectrum of d(GATGACXGCTAG) the w_5^{2-} and the a_7-X^{2-} fragment ion with m/z 790.12 and m/z 986.67, respectively, are not observed. On the other hand, RNA dissociation is independent of nucleobase loss and the product ion spectrum of GAUGACXGCUAG shows the uninterrupted series of sequence-defining

c- and y-fragment ions, including the c_7^{2-} (m/z 1152.66) and the y_5^{2-} (m/z 783.12) ion.

CONCLUSIONS AND OUTLOOK

Structural characterization and sequence determination of oligonucleotides by ESI-MS/MS is an accurate and sensitive technique. Isolation of precursor ions is highly selective, thus, allowing mixture analysis. Dissociation of oligonucleotides in the collision cell of the mass spectrometer does not require specific cleavage reagents and is tolerant towards structural modifications. The high sensitivity of the method and the high degree of structural information obtained make the method highly attractive for sequence determination of modified oligonucleotides and oligonucleotides from combinatorial libraries.

In contrast to other biopolymers, oligonucleotides generate a large number of fragment ions upon CID. The presence of alternative dissociation products, whose mechanisms of formation are not known yet, results in highly complex spectra. Overlapping isotopic peaks patterns of fragments exhibiting similar m/z render peak assignment and data interpretation difficult. Further refinement of sequencing protocols are sought to direct backbone dissociation and generate simplified fragment ion patterns which enable fast and unambiguous read-out of the nucleotide sequences.

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