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Differentiation and sequencing of three constitutional isobaric 18-*mer* DNA oligomers using low-energy collision tandem mass spectrometry

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ABSTRACT

In this manuscript we present the differentiation and sequencing of three constitutional isobaric 18-*mer* DNA oligomers, namely: **G**ATTC**A**TA**G**CTACGAAT**C** ***1***, **A**ATTC**G**TA**G**CTACGAAT**C** ***2***, and **A**ATTC**G**TA**C**CTACGAAT**G** ***3*** using electrospray ionization-mass spectrometry with a hybrid QqToF-MS/MS instrument.

The conventional single scan ESI-QqTOF-MS analyses of the DNA oligomers afforded the same series of deprotonated molecular ions. Low-energy collision tandem mass spectrometric analyses (CID-MS/MS) were used to differentiate and to sequence the three DNA oligomers. Different CID-MS/MS analyses of the various deprotonated molecules: $[M-8H]^{8-}$ at m/z 683.9881 and $[M-9H]^{9-}$ at m/z 607.8775 were acquired for each 18-*mer* DNA oligomer. It was established that we could distinguish two different types of diagnostic product ions: (a) the product ions formed by identical cleavage sites within each precursor anion, having the same isobaric masses and (b) the product ions formed by identical cleavage sites, but having different masses. These specific diagnostic product ions facilitated the characterization of each isobaric 18-*mer* DNA oligomer. In addition, we also indicate that a series of product ions were common for at least two constitutional isobaric 18-*mer* oligomers. The Mongo Oligo Software was used exclusively to perform the characterization and the correct sequencing of each isobaric DNA oligomer.

1. Introduction

In modern molecular biotechnology, the analysis of DNA oligomers has become a topic of primary importance, since establishing the size, purity, and sequence of nucleic acids is a prerequisite to their use as molecular probes in a medicinal science. The sequence of DNA encodes the necessary information for all living organisms to survive and reproduce. Therefore, knowledge of the DNA sequences is useful in biological research [1–3]. In medicine, for example, DNA sequencing can be used to identify, diagnose and, potentially, to develop treatments for genetic diseases. Similarly, research into pathogens may lead to treatments for contagious diseases. Biotechnology is a burgeoning discipline, with the potential for many useful products and services [4].

Significant progress in the area of accurate mass determination, sequencing, and study of DNA oligomers and no covalent interactions has been made possible by the use of novel mass spectrometric techniques [4–6]. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been explored widely for DNA sequencing [7–11]. The Sanger dideoxy-procedure has been generally used for the sequencing of DNA-fragments. When compared to gel electrophoresis-based sequencing systems, mass spectrometry enables high resolution of the DNA-sequenced fragments, fast separation (on a microsecond time scale), and completely eliminates compressions that are associated with gel electrophoresis [8,9]. In addition, the use of mass spectrometry for the analysis of the monomeric constituents of nucleic acids (nucleobases, nucleosides, nucleotides and derivatives) has been widely established in the literature for the last four decades [10–12]. Before tandem mass spectrometry was used in conjunction with MALDI-MS, the gas-phase fragmentation of the DNA anions was carried out with a TOF reflectron mass spectrometer using post-source decay [13–15].

Another successful method used for the sequencing of DNAs is the ladder sequencing method [16]. It consists of the chemical cleavage of the oligonucleotide with an exonuclease (phosphodiesterase for single strands of DNA and exonuclease III for double strands) digestion that sequentially removes nucleotides from the

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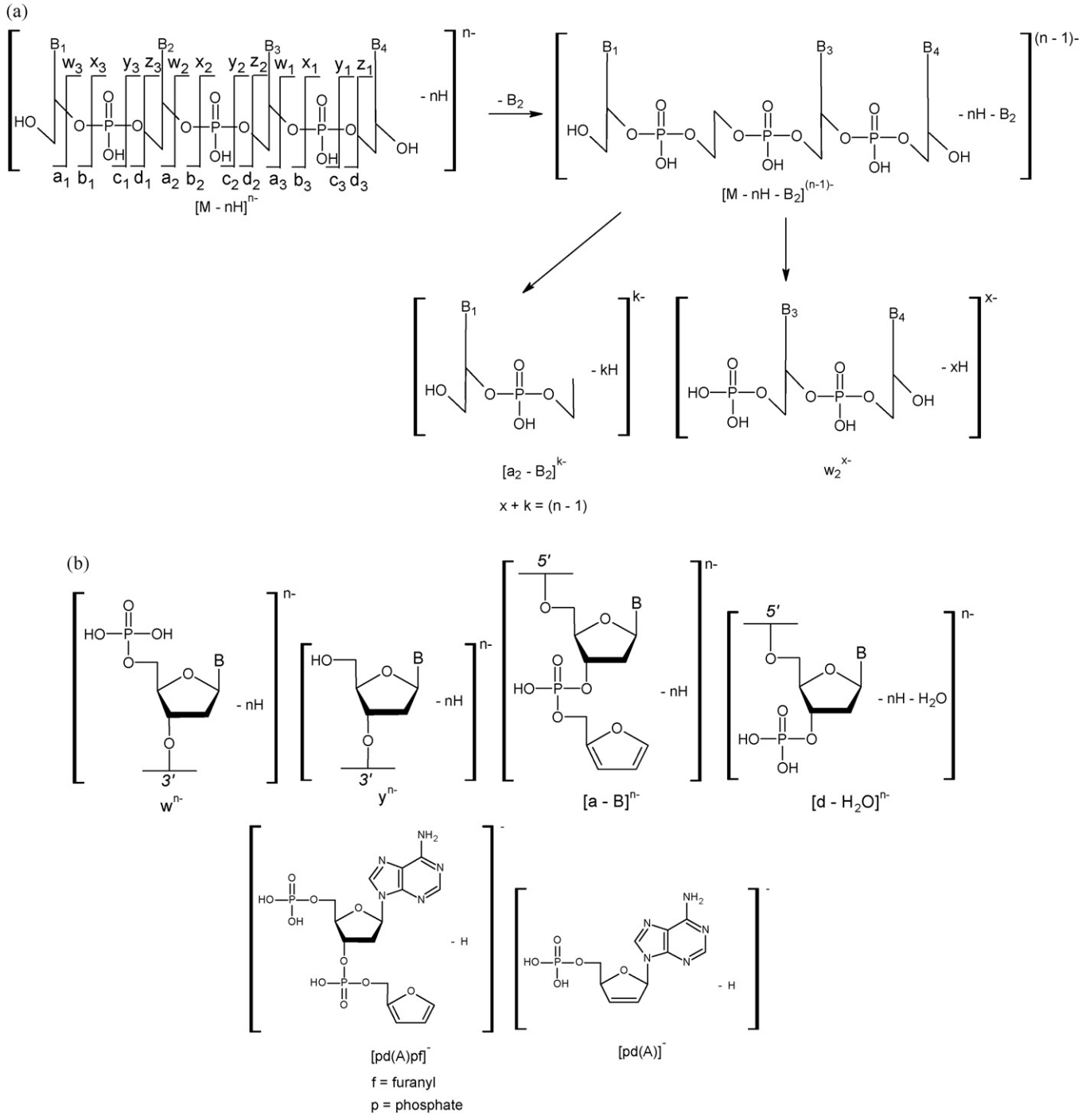

Fig. 1. Nomenclature for different oligonucleotide fragment types which includes the 3′ or 5′ terminus and complementary fragment ions formation (example: $[a_2-B_2]^{k-}$ and w_2^{x-}) (a), example of internal fragment ion $[pd(A)pf]^-$ and $[pd(A)]^-$ (b).

3′- or the 5′-terminus of the oligomer [17–21]. The exonuclease digestion was found to be both time and pH-dependent. Mass spectrometric analysis of the digestion products of the oligomer and the sequence of the unmodified or modified oligomer is resolved by the mass change after the loss of each nucleotide [17–21].

The ability of ESI-MS to form multiply charged ions in combination with improved mass range in novel state-of-the-art MS instruments has made possible the detection of increasingly larger oligonucleotide fragment ions. In reality, because DNA has an acidic phosphodiester backbone, oligonucleotides are usually analyzed by ESI-MS in the negative ion mode. Elucidation of the sequence of oligonucleotides by MS/MS of multiply charged DNA ions has been studied intensively over the past decade [22] and ESI-MS and CID-MS/MS protocols have been established for the characterization of intact DNA oligomers that vary in size up to 70 residues [11,23–28].

Although CID-MS/MS analyses provide an excellent basis for detecting both the length and the sequence variations of oligonucleotides [11], the presence of multiply charged ions can make interpretation of the spectra complicated. McLuckey et al. were the first to produce a nomenclature for the product ions obtained during the dissociation of multi-charged oligonucleotides (Fig. 1a) [29,30]. It was discerned, that under moderate CID-MS/MS and

MS^n conditions, further consecutive fragmentations occurred from previous charge-separation reactions. Thus, the presence of the complementary ions arising from these decompositions greatly facilitated spectral interpretation [30,31]. Furthermore, during the CID-MS/MS analysis of the studied oligonucleotides, the formation of internal product ions was also observed and these afforded valuable composition information on the sequence stretches. Needless to say, that the manual interpretation of the MS/MS analysis of oligonucleotides can be difficult, time-consuming, and prone to error because of the large number of product ions present. Consequently, efforts were made to simplify the process by relating the product ions obtained with the particular structure of a precursor oligonucleotide anion. A computer-based algorithm, to automatically derive sequence information from the MS/MS spectra for oligonucleotides of a completely unknown sequence, was developed by McCloskey's and coworkers [32].

Rozenski and McCloskey developed a novel approach for the nearest-neighbor determination, based on the analysis of fragment ions of the nucleic acid formed in the ionization region of the mass spectrometer, along with the product ions obtained from MS/MS of the precursor oligonucleotide anion [33]. The same authors wrote a software for the *ab initio* determination of unknown oligonucleotide sequences up to the *mer*-20 level and was termed Simple Oligonucleotide Sequencer (SOS) [34]. However, this method cannot be extended to longer oligonucleotide sequences because missing fragments in the series of [a−B] or *w* ions would prevent the successful passage through the whole sequence.

The Mongo Oligo Mass Calculator v2.06 software was developed by Rozenski and collaborators and is available online [35]. This software is used to determine the sequence of oligonucleotides provided by the ESI-CID-MS/MS analysis of multi-charged ions and/or enzymatic digests. It is also capable of providing the molecular mass calculation of oligonucleotides and to predict the possible CID-product ions [*a*−B], [*d*−H_2O], *y* and *w*. In addition, this software permits the determination of internal product ions (information about sequence stretches) that may obtained for the analysis of a known oligonucleotide. Please note that the formation of internal product ions [pd(A)pf]$^-$ predicted by this program correspond to the following product ions represented as $[A_2{:}A_2]^-$, $[A_6{:}A_6]^-$, $[A_8{:}A_8]^-$, $[A_{12}{:}A_{12}]^-$, $[A_{15}{:}A_{15}]^-$ and $[A_{16}{:}A_{16}]^-$ for the oligonucleotide ***1*** (Fig. 1b). The Mongo Oligo Program also calculates the possible mass of exo- and endonuclease digests ions [35].

The purpose of this work was to try to sequence, with a QqToF-MS/MS hybrid instrument, an isobaric series of three self-complementary 18-*mer* DNA oligomers which have the same base composition but different orders. The Mongo Oligo Program was used to obtain the CID-MS/MS correct sequencing. These 18-*mer* DNA oligomers are being used in our laboratory for the synthesis of model Retrosine derived-DNA adducts compounds used for the study. The retrosine is a pro-carcinogen pyrrolizidine alkaloid, and its metabolites formed during liver microsomal activation are toxics and can bind DNA, causing cancer [36,37].

2. Material and methods

2.1. Sample preparation

The three constitutional isobaric 18-*mer* DNA oligomers used in our experiments were purchased from Oswel DNA Service Laboratory (Southampton, UK). These three self-complementary 18-*mer* DNA oligomers were made up of 18 residues with the following sequences: **G**ATTC**A**TA**G**CTACGAAT**C *1***, **A**ATTC**G**TA**G**CTACGAAT**C *2***, and **A**ATTC**G**TA**C**CTACGAAT**G *3***, having the same base composition but different sequences.

2.2. Electrospray quadrupole orthogonal time-of-flight mass spectrometry

Conventional electrospray ionization-mass spectrometry for all the oligomers was acquired in the negative ion mode using an Applied Biosystems API-QSTAR XL quadrupole orthogonal time-of-flight (QqTOF)-MS/MS hybrid tandem mass spectrometer (Applied Biosystems International-MDS Sciex, Foster City, CA, USA). This instrument is capable of analyzing a mass range of *m*/*z* 5–40,000, with a resolution of 10,000 in the negative ion mode. ESI was performed with the Turbo Ionspray source operated at −4.2 kV. The ESI-MS were recorded with a cone voltage setting (declustering potential 1, DP1) of −50 V, a declustering potential 2 (DP2) of −10 V, a focusing potential (FP) of −255 V, a Curtain gas of 20 Pascal (Pa), an Ion Source Gas 1 (GS1) of 30 Pa, and an Ion Source Gas 2 (GS2) of 30 Pa, which were generally kept constant for the analyses.

A solution of each oligonucleotide at a concentration of 1 mg mL^{-1} was prepared by dissolving 1 mg of each sample in 1 mL of a mixture of acetonitrile/water (80/20) (v/v). Sample solution was then directly infused, with an integrated Harvard syringe pump (Harvard Apparatus, Hollister, MA) at a rate of 5 μL min^{-1}. One drop of triethylamine was added to the solution of oligonucleotides (500 μl) to increase the formation of the deprotonated molecular anions in the ion source of the mass spectrometer.

The TOF analyzer was calibrated for the mass range of the measured analytes using the following oligomer: TGATCA which was dissolved in a 1:1 mixture of acetonitrile (ACN or CH_3CN)-water (H_2O) and checking for the exact masses of the $[M-H]^-$ at *m*/*z* 1789.3431 and $[M-2H]^{2-}$ at *m*/*z* 894.1679, and using the TCA oligomer and checking for the exact mass of the $[M-H]^-$ at *m*/*z* 843.1870 and $[M-2H]^{2-}$ at *m*/*z* 421.0899.

2.3. Low-energy collision CID-MS/MS analyses

Product ion scan experiments were recorded with the same instrument. The product ion spectra were obtained from fragmentation in the QqTOF-MS/MS hybrid instrument. In the product ion mode, the first quadrupole (Q1) selected the corresponding precursor ion. The precursor ion was fragmented into product ions by collision with nitrogen in the radio-frequency (RF), linear acceleration pulsar high pressure (LINAC) equipped, quadrupole collision cell. Nitrogen collision gas was added to the enclosed chamber of the quadrupole (Q2) to give an indicated pressure of 1×10^{-5} torr for collisional activation of the sample ions. The collision energy (CE) and the CID gas conditions ensured that the precursor ion remained abundant. The product ions were scanned and sorted in the orthogonal mass resolving time-of-flight analyzer which measured the occurrence of particular product ions, previously formed in the Q2. We have found the collision energies for the CID-MS/MS of protonated molecules of these oligomers did not exceed 40 eV while the declustering potential was kept at the same value (DP1 = −50 V) as that of the electrospray full scan ESI-MS experiments.

3. Results and discussion

Ni and Chang and Oberacher et al. have studied the sequence verification of different length oligonucleotides using ESI-QTOF-MS [38,39]. Their results were also compared to those obtained with an ESI triple quadrupole MS and an ESI Ion Trap MS. They found that the resolving power of the QTOF and its sensitivity allowed better interpretation of the spectra and also facilitated the sequencing of the oligonucleotides. It is important to mention, that the resolving power of the QqTOF-MS instrument can only reach 20,000 FWHM [39,40], which is a moderate resolution of lower orders of magni-

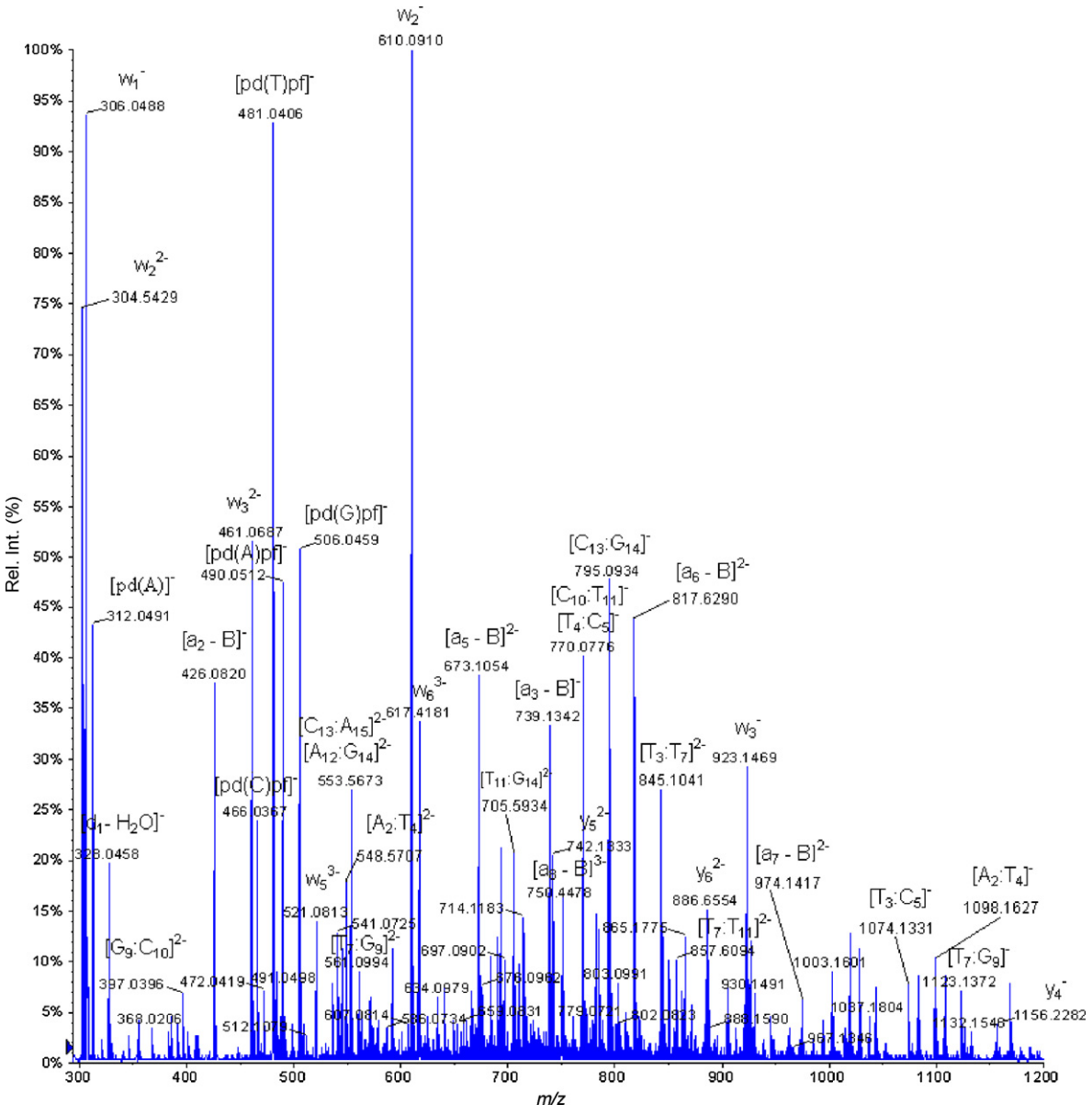

Fig. 2. Low-energy CID tandem mass spectrum of the $[M-8H]^{8-}$ anion at m/z 683.9881 of the oligomer ***1***.

tude when compared to high resolution-mass spectrometry such as Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS or FT-MS) or the recent LTQ Orbitrap XL Hybrid FT which can reach up to 1,000,000 FWHM [41,42].

In this rationale, the ESI-QqTOF-MS and CID-MS/MS analyses of this series of isobaric 18-*mer* oligonucleotides were performed using a QqTOF-MS/MS hybrid instrument. This latter instrument allowed us to identify and separate the isotopic deprotonated molecules without ambiguity.

In addition, we have opted to use reliable software Mongo Oligo Mass Calculator for the identification and characterization of the CID-product ions obtained from the product ion scan analyses of the, well resolved, diagnostic isotopic deprotonated molecules, selected of our isobaric 18-*mer* oligomers. The Mongo Oligo Mass Calculator program was developed by Rozenski and coworkers and is a clever tool capable of a variety of functions. The Mongo Oligo Mass Calculator uses the sequence of a know oligonucleotide as input for the calculations. It can be used for calculating masses of oligonucleotides, electrospray series, fragment product ions obtained by collision-induced dissociation (CID), and fragments of enzymatic digests by endonuclease and exonuclease [35]. The method consists on comparing the product ion spectra masses generated by CID of multiply charged oligonucleotide ions to the predicted m/z values, employing established fragmentation pathways obtained from a known reference sequence. The program can also calculate the masses of modified residues. An accompanying tool is Oligo Composition Calculator which finds oligonucleotide composition when the user inputs a mass. Mongo Oligo Mass Calculator is by far the most comprehensive program for the analysis of genetic mass spectrometric data with multiple functionalities [35,43].

3.1. ESI-QqTOF-MS analyses

The ESI-QqTof-MS (negative ionization mode) of the three isobaric 18-*mer* DNA oligomers showed identical series of multi-charged deprotonated molecular anions (Table 1 in the supplementary material). Seeing that the identical molecular weight of the isobaric 18-*mer* DNA oligomers is high, the intensity of the monoisotopic mass of the deprotonated molecules tends to

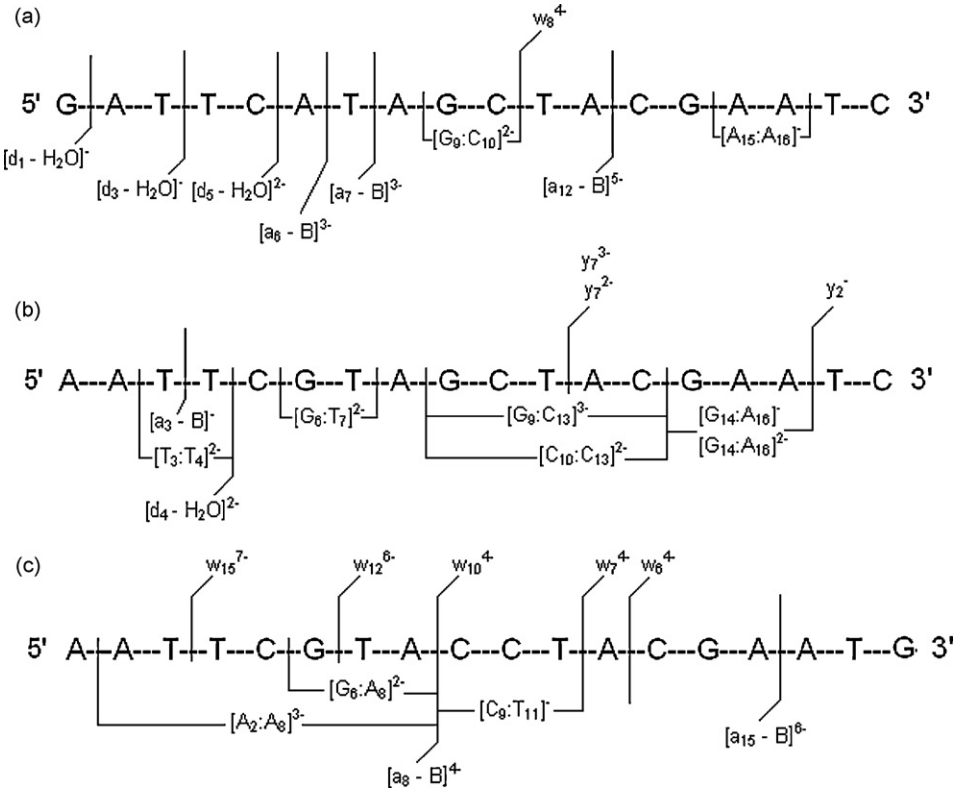

Fig. 3. Different specific product ions obtained during the CID-MS/MS analysis of the $[M-8H]^{8-}$ ion at *m/z* 683.9881, for the following (a) oligomer ***1***, (b) oligomer ***2*** and (c) oligomer ***3***.

be lower than the nominal and average masses. As expected, the ESI-QqTOF-MS analysis of the three constitutional isobaric 18-*mer* DNA oligomers gave the equivalent series of highly charged deprotonated molecular anions with the same *m/z* values. It is also well known that the DNA molecular anions, when highly charged, are extremely easy to fragment [30,31,33–35,43]. In addition the three analyzed isobaric 18-*mer* oligomers, formed in the gas-phase the same series of sodiated adduct anions. The theoretical monoisotopic *m/z* values of the deprotonated molecules and their Δ deviations (ppm) are presented in Table 1 in the supplementary material.

3.2. Low-energy collision CID-MS/MS analyses

In general, during all the tandem analyses presented herein, we performed on the extracted isotopic precursor ions. The CID-MS/MS experiments of the deprotonated molecules $[M-8H]^{8-}$ at *m/z* 683.9881 and $[M-9H]^{9-}$ at *m/z* 607.8775, were acquired for each isobaric 18-*mer* DNA oligomer. Initial attempts to generate product-ion scans from the multiply charged oligonucleotide anion precursors relied on maximizing the abundances of the product ions of high masses to obtain as much information as possible on each different sequence. Thus, the best collision energy was chosen for each molecular anion, which was about 30 eV. As already reported in the literature, we have found that when the analyzed anion was highly charged, it needed less collision energy to fragment [44].

3.3. CID-MS/MS analysis of the $[M-8H]^{8-}$ deprotonated molecules at m/z 683.9881 extracted from the isobaric 18-mer DNA oligomers ***1***–***3***

We have noticed that each individual product ion scan, created a series of unique diagnostic ions, in addition to their common product ions (Fig. 2 for oligomer ***1***). The specific product ions obtained by CID-MS/MS analysis of the $[M-8H]^{8-}$ ion at *m/z* 683.9881 of the 18-*mer* DNA oligomers ***1***–***3*** are shown in Tables 2–4 presented in the supplementary material. It is important to point out, that the examination of each diagnostic product ions indicated that their formations were not localized within the same sequential region of the constitutional 18-*mer* oligomers. Therefore, the formations of these diagnostic product ions are unique and constitute a fingerprint of the analyzed isobaric 18-*mer* oligomers. On the other hand, the CID-MS/MS fragmentations of the deprotonated molecules, yielded identical mini-sequences, occurring at the same cleavage positions; however the product ions formed had different charged states. This latter observation may be due to the existence of different deprotonation sites of the respective analyzed oligomers. In addition, it is well known that the final CID-MS/MS fragmentation pattern obtained can be influenced by the nucleobases present in each of the analyzed 18-*mer* DNA oligomers [12]. It is logical to expect that isobaric oligonucleotides with different sequences basically have different physical-chemical properties, which can be the reason for the differences observed during their gas-phase fragmentations.

Additionally, we observed the formation complementary product ions, which helped in the product ion identification. McLuckey et al. found that when a base is eliminated from the deprotonated molecule $[M-nH]^{n-}$ ($n>1$), it produces the anion $[M-nH-B]^{(n-1)-}$ (B = lost base) which fragments further to afford the complementary pair of product ions w_a^{x-} and $[a_m-B]^{k-}$ in which x, $k>1$ and $(x+k)=(n-1)$ (Fig. 1a) [30,31]. Another fragmentation pathway leading to the formation of complementary product ions involves the loss of a neutral base (A, T, C or G) to produce the following anion: $[M-nH-BH]^{n-}$ (BH = neutral base loss) which also fragments, leading to the formation of the following complementary product ions: w_a^{x-} and $[a_m-B]^{k-}$ (x, $k>1$ and $(x+k)=n$).

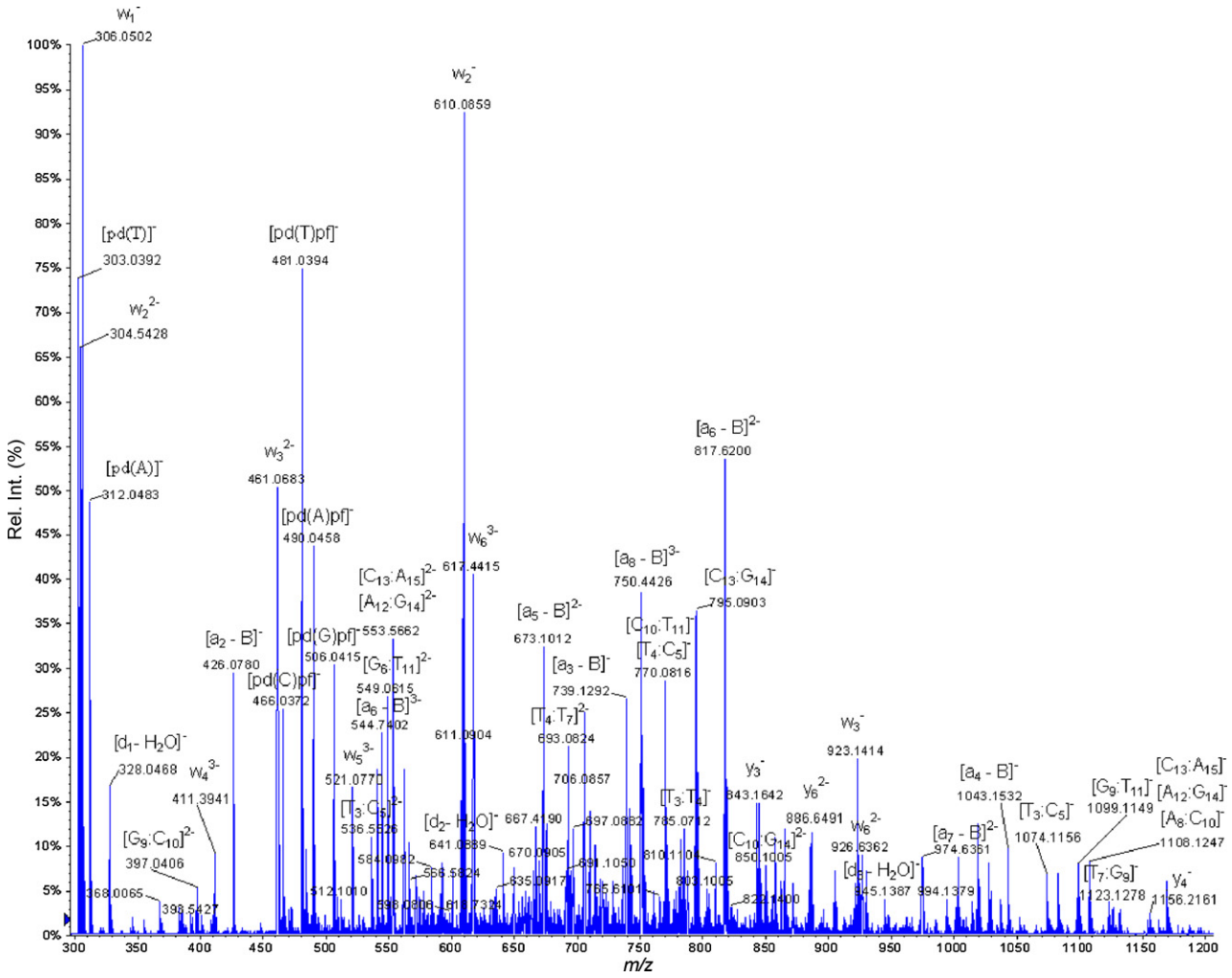

Fig. 4. Low-energy CID tandem mass spectrum of the $[M-9H]^{9-}$ anion at *m*/*z* 607.8775 of the oligomer ***1***.

Thus, in the CID-MS/MS analysis of the multi-charged precursor $[M-8H]^{8-}$ ion, the loss of a single base (A, T, C or G) can produce the hepta-charged product anion: $[M-8H-B]^{7-}$ which probably fragments spontaneously and therefore is not detected. However, this $[M-8H-B]^{7-}$ product ion is able to afford the following complementary product ions. For the oligomer ***1*** (Figs. 3a and 6a), we identified the hepta-charged anion: $[a_{16}-B(A)]^{6-}$ at *m*/*z* 787.9272 and w_2^- at *m*/*z* 610.0910 and $[a_{12}-B(A)]^{4-}$ at *m*/*z* 871.3856 and w_6^{3-} at *m*/*z* 617.4181. However, no complementary product ions were observed for the constitutional isobaric DNA oligomer ***2*** (Figs. 3b and 6b). For the DNA oligomer ***3***, we identified the complementary product ions $[a_8-B(A)]^{3-}$ at *m*/*z* 750.4395 and w_{10}^{4-} at *m*/*z* 771.6299; $[a_{12}-B(A)]^{4-}$ at *m*/*z* 861.3868 and w_6^{3-} at *m*/*z* 630.7709 and $[a_{16}-B(A)]^{6-}$ at *m*/*z* 781.2867 and w_2^- at *m*/*z* 650.0968 (Figs. 3c and 6c). Furthermore, a neutral base loss during the CID-MS/MS analysis of the precursor ion $[M-8H]^{8-}$ is able to form the octa-charged product ion $[M-8H-B]^{8-}$ (not detected in the spectra), which is an intermediate ion for the production of the following complementary product ions: $[a_{16}-B(A)]^{6-}$ at *m*/*z* 787.9272 and w_2^{2-} at *m*/*z* 304.5429 and $[a_{12}-B(A)]^{5-}$ at *m*/*z* 696.9452 and w_6^{3-} at *m*/*z* 617.4181 for the oligonucleotide ***1*** (Figs. 3a and 6a). Also, we were not able to identify any complementary product ions for the oligonucleotide ***2*** (Figs. 3b and 6b). Finally, the complementary product ions $[a_{16}-B(A)]^{6-}$ at *m*/*z* 781.2867 and w_2^{2-} at *m*/*z* 324.5523; $[a_{15}-B(A)]^{6-}$ at *m*/*z* 729.0918 and w_3^{2-} at *m*/*z* 481.0689; $[a_{12}-B(A)]^{4-}$ at *m*/*z* 861.3868 and w_6^{4-} at *m*/*z* 472.8377 and $[a_8-B(A)]^{4-}$ at *m*/*z* 562.5952 and w_{10}^{4-} at *m*/*z* 771.6299 were detected for the oligonucleotide ***3*** (Figs. 3c and 6c).

The CID-MS/MS analyses confirm without doubt, the known fact, that these three 18-*mer* DNA oligomers, while being isobaric, have indeed different sequences.

*3.4. CID-MS/MS analysis of the $[M-9H]^{9-}$ deprotonated molecules at m/z 607.8775 extracted from the isobaric 18-mer DNA oligomers **1**–**3***

The CID-MS/MS analysis of the $[M-9H]^{9-}$ ion at *m*/*z* 607.8775 for the oligonucleotide ***1*** (Fig. 4), afforded different diagnostic product ions (see Tables 2–4 in the supplementary material). The cleavage sites within each analyzed respective precursor ion are shown in Fig. 5a–c. As expected, we have identified a series of diagnostic complementary product ions that were tentatively assigned as being created from the non-detected $[M-9H-B]^{8-}$ intermediate product ion. Accordingly, we have identified for oligomer ***1*** the following complementary product ions: $[a_{12}-B(A)]^{5-}$ at *m*/*z* 696.8982 and w_6^{3-} at *m*/*z* 617.4415; $[a_{12}-B(A)]^{4-}$ at *m*/*z* 871.3750 and w_6^{4-} at *m*/*z* 462.8181; and $[a_{16}-B(A)]^{6-}$ at *m*/*z* 787.9570 and w_2^{2-} at *m*/*z* 304.5428. It is interesting to note that for the CID-MS/MS analyses of the $[M-9H]^{9-}$ ion at *m*/*z* 607.8775 extracted form the 18-*mer* oligomers ***2*** and ***3*** we did not observe the formation of any complementary product ions. Unfortunately, we were not able to identify the pairs of complementary product ions arising from the fragmen-

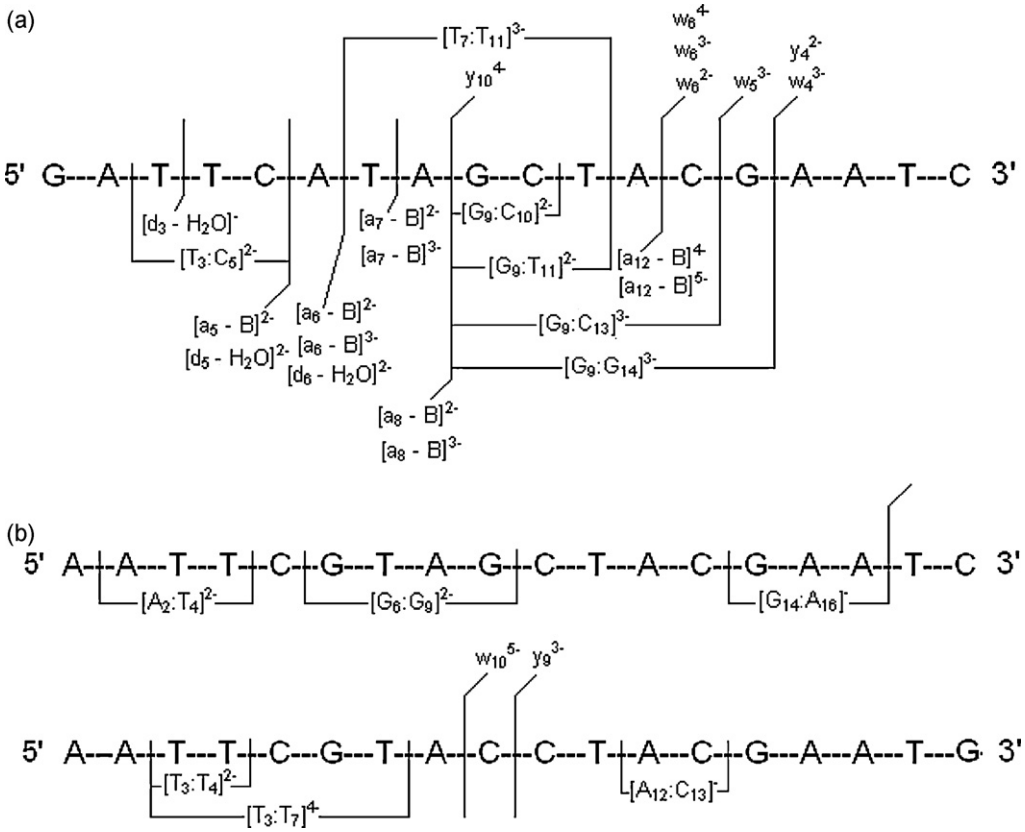

Fig. 5. Different specific product ions obtained during the CID-MS/MS analysis of the $[M-9H]^{9-}$ ion at m/z 607.8775, for the following (a) oligomer ***1***, (b) oligomer ***2*** and (c) oligomer ***3***.

tation of the intermediary nona-charged product ion $[M-9H-B]^{9-}$ (not detected), which should be produced during the CID-MS/MS analysis of the precursor ion $[M-9H]^{9-}$ via the loss of a neutral base. It is evident that the CID-MS/MS of the precursor $[M-9H]^{9-}$ ion at m/z 607.8775 affords less complementary product ions than the analysis of the $[M-8H]^{8-}$ precursor ion.

3.5. Common product ions present in the product ion scans of the oligomers *1*–*3*

During each single CID-MS/MS analysis conducted with the different series of precursor ions selected from the oligomers ***1***–***3***, we have observed a common series of either identical or similar product ions. These are shown as Tables 5 and 6 in the supplementary material. We have tried to classify them into two types: (a) the product ions formed by identical cleavage sites within each precursor anion, having the same masses which corresponded to product ions with the same bases composition and (b) the product ions formed by identical cleavage sites, however having different masses, corresponding to product ions with a different bases composition. Accordingly, depending on the cleavage site, some product ions may have identical masses.

Thus, the CID-MS/MS of the precursor ions $[M-8H]^{8-}$ at m/z 683.9881 and $[M-9H]^{9-}$ at m/z 607.8775 extracted from GATTCA**TAGCTACGAATC** ***1*** (Figs. 6a and 7a) and AATTCG**TAGCTACGAATC** ***2*** (Figs. 6b and 7b) have the following common product ions with the same masses (the common sequence from each termini of the two oligonucleotides is displayed in bold): w_n and y_n ($n \leq 12$), $[a_m-B]$ ($m \geq 7$) and $[d_p-H_2O]$ ($p \geq 6$). Indeed, during the CID-MS/MS analysis of the $[M-8H]^{8-}$ at m/z 683.9881 the common product ions, with the same masses of these two oligomers, are: w_2^{2-} at m/z 304.5429, w_1^- at m/z 306.0488, w_3^{2-} at m/z 461.0687, w_5^{3-} at m/z 521.0813, y_4^{2-} at m/z 577.6099, w_2^- at m/z 610.0910, w_6^{3-} at m/z 617.4181, y_5^{2-} at m/z 742.1333, $[a_8-B]^{3-}$ at m/z 750.4478, w_5^{2-} at m/z 782.1231, y_3^- at m/z 843.1699, y_6^{2-} at m/z 886.6554, w_3^- at m/z 923.1469, w_6^{2-} at m/z 926.6393 and $[a_7-B]^{2-}$ at m/z 974.1417.

The product ion scan of the $[M-9H]^{9-}$ at m/z 607.8775 gives us the following common product ions having the same masses of the two oligonucleotides: w_2^{2-} at m/z 304.5428, w_1^- at m/z 306.0502, w_3^{2-} at m/z 461.0683, w_2^- at m/z 610.0859, w_8^{4-} at m/z 617.0860, y_5^{2-} at m/z 742.1295, w_5^{2-} at m/z 782.1193, y_3^- at m/z 843.1642, y_6^{2-} at m/z 886.6485, w_3^- at m/z 923.1414 and y_4^- at m/z 1156.2161.

But, if we compare the CID-MS/MS of the precursor ion $[M-8H]^{8-}$ at m/z 683.9881 extracted from the **G**ATTC**A**TA**G**CTACGAAT**C** ***1*** and the **A**ATTC**G**TA**C**CTACGAAT**G** ***3*** (differences in the bases location between the two oligomers are displayed in bold) (Figs. 6c and 7c) we notice the formation of one common product ion $[a_8-B]^{3-}$ at m/z 750.4478 having the same mass (see Table 5 presented in the supplementary material). However, please note, that this product ion has the same base composition, but with a different sequence for the two other oligonucleotides. This is not surprising, as in the CID-MS/MS analyses of the deprotonated molecules $[M-8H]^{8-}$ at m/z 683.9881 and also $[M-9H]^{9-}$ at m/z 607.8775 obtained from oligomers ***1*** and ***3***, the extracted precursor ions have different termini and therefore cannot form the common product ions w_n, y_n, $[a_m-B]$ and $[d_p-H_2O]$ with the same sequence. Please note that, in the CID-MS/MS of the precursor ion $[M-9H]^{9-}$ extracted from **G**ATTC**A**TA**G**CTACGAAT**C** ***1*** and the **A**ATTC**G**TA**C**CTACGAAT**G** ***3*** no common product ion having the same mass were observed.

Finally, the comparison between the CID-MS/MS of the precursor ions $[M-8H]^{8-}$ at m/z 683.9881 (Fig. 6 and Table 5 pre-

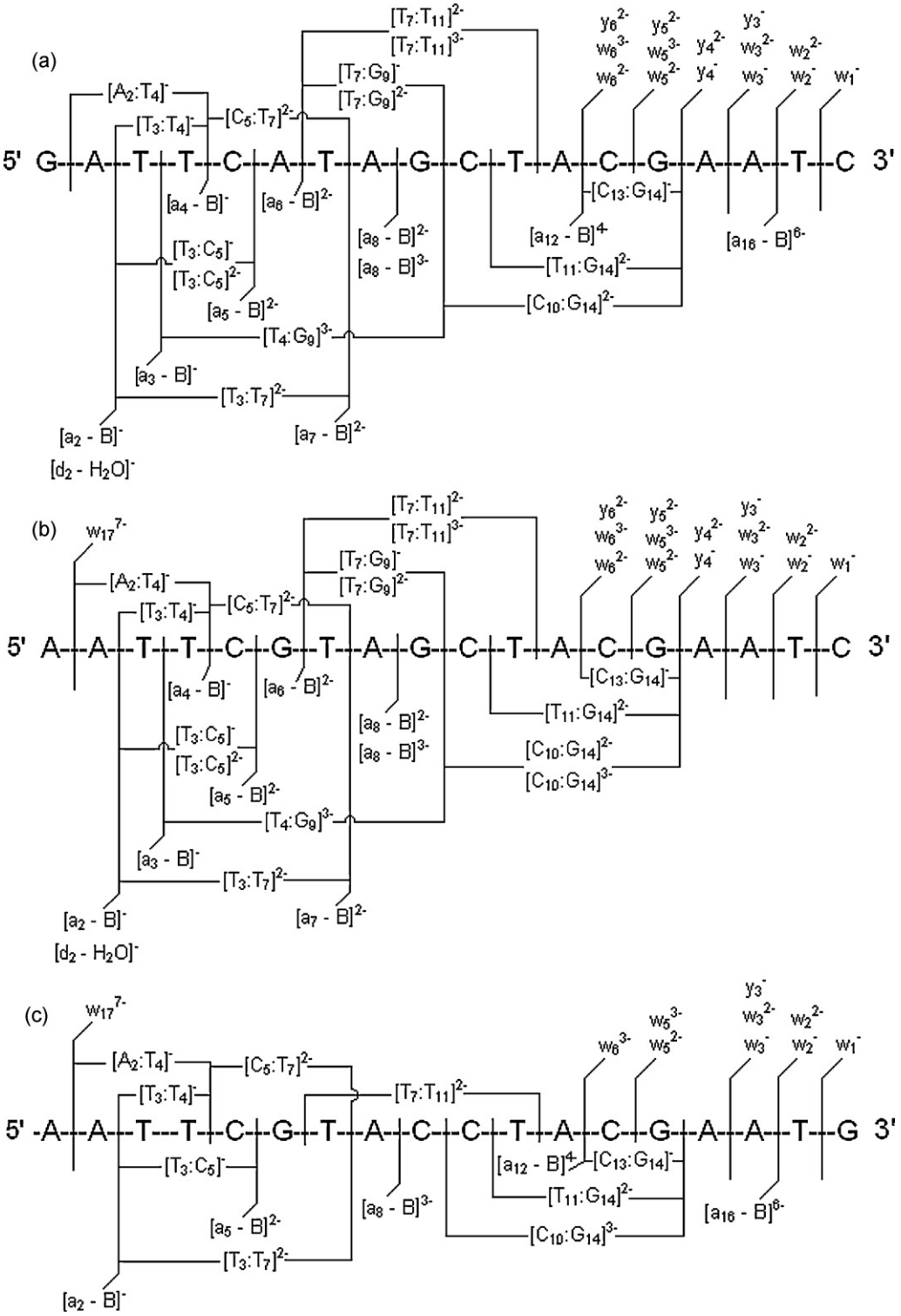

Fig. 6. Common product ions (isobaric and non-isobaric) to at least two oligonucleotides obtained during the CID-MS/MS analysis of the $[M-8H]^{8-}$ ion at m/z 683.9881, for the following (a) oligomer ***1***, (b) oligomer ***2*** and (c) oligomer ***3***.

sented in the supplementary material) and $[M-9H]^{9-}$ at m/z 607.8775 (Fig. 7 and Table 6 presented in the supplementary material) extracted from **AATTCGTA**GCTACGAATC ***2*** and **AATTCGTA**CCTACGAATG ***3*** produced the following common product ions having the same masses: w_n and y_n ($n \geq 10$), $[a_m-B]$ ($m \leq 9$) and $[d_p-H_2O]$ ($p \leq 8$). Indeed, during the CID-MS/MS of the precursor ions $[M-8H]^{8-}$ at m/z 683.9881 we observed the following common product ions having the same mass: $[a_2-B]^-$ at m/z 410.0818, $[a_5-B]^{2-}$ at m/z 665.1079, w_{17}^{7-} at m/z 748.5498 and $[a_8-B]^{3-}$ at m/z 750.4530. From these observations, it was construed that, although these common product ions contain the same bases, they do not necessarily have the same sequence.

We also observed the formation of common internal product ions with the same masses which correspond to the same sequence. For example, the internal product ion $[T_{11}{:}G_{14}]^{2-}$ at m/z 705.5935 of the CID-MS/MS analysis of the $[M-8H]^{8-}$ molecular ion at m/z

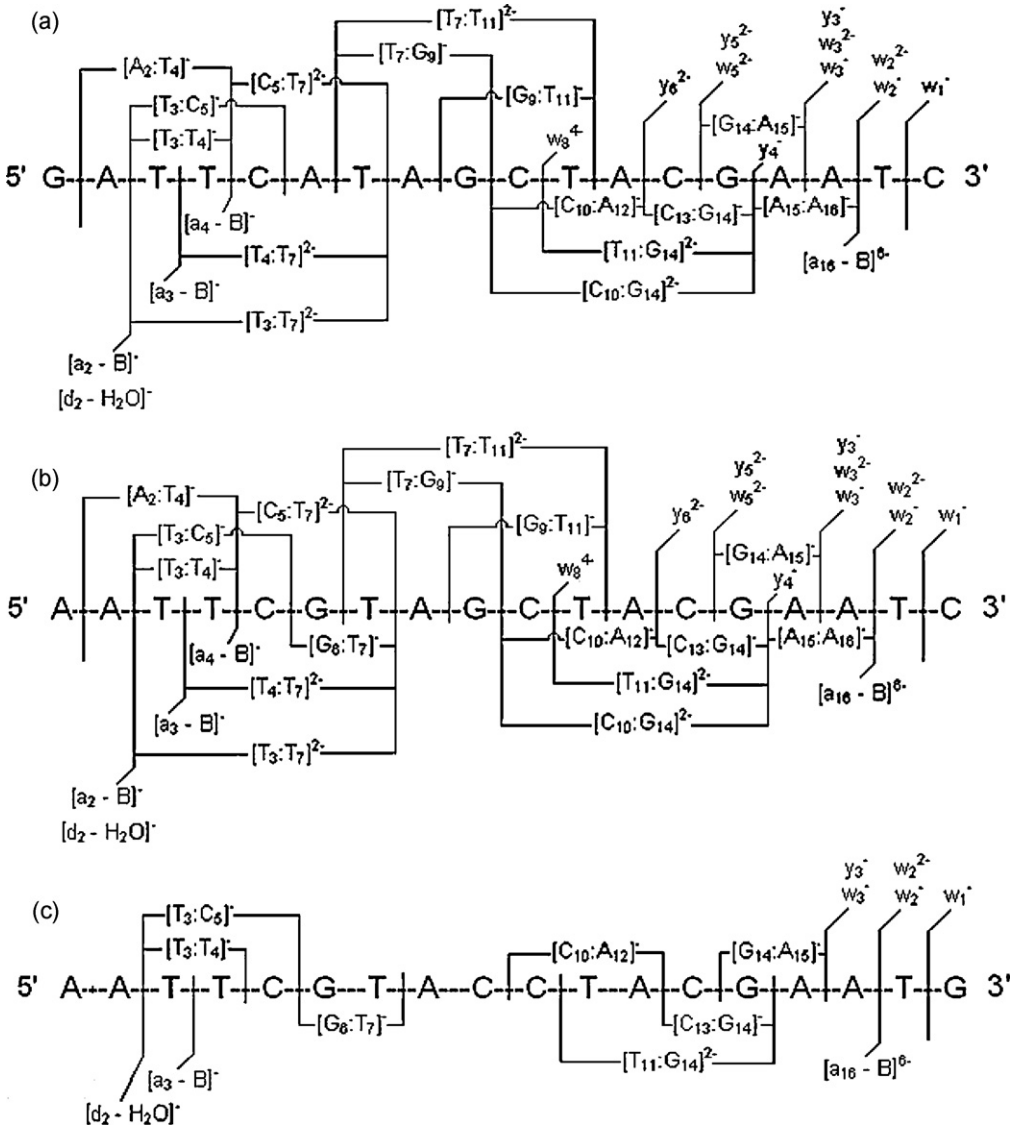

Fig. 7. Common product ions (isobaric and non-isobaric) obtained during the CID-MS/MS analysis of the $[M-9H]^{9-}$ ion at m/z 607.8775, for the following (a) oligomer ***1***, (b) oligomer ***2*** and (c) oligomer ***3***.

683.9881 (and $[M-9H]^{9-}$ at m/z 607.8775) correspond to the following sequence $[pd(TACG)pf]^{2-}$ for the oligomers ***1***–***3*** (Fig. 6 and Tables 5 and 6 presented in the supplementary material). We can also say that the product ions w_n and y_n ($n \leq 12$) correspond to part of a same sequence for the GATTCA**TAGCTACGAATC** ***1*** and AATTCG**TAGCTACGAATC** ***2*** oligomers (the common sequence from each termini of the two oligonucleotides is displayed in bold), as well as the product ions $[a_m-B]$ ($m \leq 9$) and $[d_p-H_2O]$ ($p \leq 8$) which have the same sequence for the **AATTCGTA**GCTACGAATC ***2*** and **AATTCGTA**CCTACGAATG ***3***. However in the case of the CID-MS/MS of $[M-8H]^{8-}$ at m/z 683.9881 and $[M-9H]^{9-}$ at m/z 607.8775 extracted from **G**ATTC**A**TA**G**CTACGAAT**C** ***1*** and **A**ATTC**G**TA**C**CTACGAAT**G** ***3*** oligomers do not have common product ions. This is explained by the fact that these two oligonucleotides ***1*** and ***3*** have different bases at their 3′- and 5′-termini. In addition, these constitutional isobaric 18-*mer* DNA oligomers can break down from there 3′- and 5′-termini.

Furthermore, we also have observed the formation of internal product ions, having the same masses, but different sequences. For example, the internal product ion $[C_5{:}T_7]^{2-}$ at m/z 541.07 correspond to the ion $[pd(CAT)pf]^{2-}$ for the oligonucleotide ***1***. Unfortunately, this product ion at m/z 541.0725, can also correspond to the following ions: $[pd(TAC)pf]^{2-}$, $[pd(ATC)pf]^{2-}$ and $[pd(CAT)pf]^{2-}$ for the oligonucleotide ***1***; $[pd(TAC)pf]^{2-}$ and $[pd(ATC)pf]^{2-}$ for the oligonucleotide ***2*** and $[pd(TAC)pf]^{2-}$ and $[pd(CTA)pf]^{2-}$ for the oligonucleotide ***3*** (see Tables 8 and 9 presented in the supplementary material). In addition, the internal product ion $[C_5{:}T_7]^{2-}$ represents the sequence $[pd(CGT)pf]^{2-}$ at m/z 549.0629 for the oligomers ***2*** and ***3***. We can conclude from this example, that the internal product ions, can give limited information about the sequence stretches of the oligonucleotides.

In the CID-MS/MS analyses of the precursor ions $[M-8H]^{8-}$ at m/z 683.9881 and $[M-9H]^{9-}$ at m/z 607.8775 extracted form the 18-*mer* DNA oligomers ***1***–***3***, we noticed the presence of the product ions arising from the same specific site cleavages but which have different masses. Thus, the **G**ATTC**A**TAGCTACGAATC ***1*** and **A**ATTC**G**TAGCTACGAATC ***2*** give the following common product ions with different masses: w_n and y_n ($n \geq 13$), $[a_m-B]$ ($2 \leq m \leq 6$) and $[d_p-H_2O]$ ($p \leq 5$). The **G**ATTC**A**TA**G**CTACGAAT**C** ***1*** and **A**ATTC**G**TA**C**CTACGAAT**G** ***3*** have the following common product ions which differ in their masses: w_n and y_n ($n \leq 9$ and $13 \leq n \leq 17$), $[a_m-B]$ ($2 \leq m \leq 6$ and $m \geq 10$) and $[d_p-H_2O]$ ($p \leq 5$ and $9 \leq p \leq 17$).

Finally, for the pair AATTCGTA**G**CTACGAAT**C** ***2*** and AATTCGTA**C**CTACGAAT**G** ***3***, we identified the common prod-

uct ions, which have not the same masses: w_n and y_n ($n \leq 9$), [a_m−B] ($m \geq 10$) and [d_p−H_2O] ($9 \leq p \leq 17$).

4. Conclusion

The constitutional isobaric 18-*mer* DNA oligomers **G**ATTC**A**TA**G**CTACGAAT**C**, **A**ATTC**G**TA**G**CTACGAAT**C** and **A**ATTC**G**TA**C**CTACGAAT**G** ***1–3*** (molecular mass = 5479.9560 Da) were analyzed by negative electrospray mass spectrometry. The resolution of the QqToF mass spectrometer allowed us to observe the isotopic distribution of each ion, which is helpful for the identification of the molecular ions and the fragments ions.

The conventional ESI-QqTOF-MS, these 18-*mer* DNA oligomers exhibited identical series of multi-charged deprotonated molecular ions. Low-energy collision-induced dissociation tandem mass spectrometric analysis of the multi-charged oligonucleotide anions $[M-8H]^{8-}$ at m/z 683.9881 and $[M-9H]^{9-}$ at m/z 607.8775 provided characteristic and distinct fingerprint patterns which permitted discrimination amongst the individual oligomers and allowed complete bi-directional sequence verification. On the other hand, the *ab initio* sequencing was not possible for the 18-*mer* oligonucleotides because complicated CID-MS/MS spectra derived form highly charged precursor ions were obtained.

Finally, we would like to indicate that the use of the Mongo Oligo Mass Calculator v2.06 software, developed by Rozenski is an excellent program which facilitates the characterization and sequencing of DNA oligomers and help to decipher the complex CID-MS/MS analyses obtained in this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2010.06.006.

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