Characterization of methylated and unmethylated CpG oligodeoxynucleotides by electrospray ionization tandem mass spectrometry

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Methylated and unmethylated CpG oligodeoxynucleotides (ODNs) at A-rich, C-rich, G-rich and T-rich conditions were characterized by electrospray ionization tandem mass spectrometry (ESI-MS/MS). The methyalted site could be confirmed by comparison of the MS/MS spectra of methylated and unmethylated CpG-ODNs. The fragmentation patterns of the CpG-ODNs were not influenced by the presence of the methyl group but significant effects were observed for nucleobase identities and parent ion charges. The cleavage at guanine was the most facile while that at thymine was the least facile. With the increase of the parent ions charge states, the major dissociation behaviors changed from the middle to the 3′ and 5′ termini of the sequence.

Keywords: CpG-ODNs, methylation, oligonucleotides, electrospray ionization mass spectrometry

Introduction

Recently, much attention has been focused on deoxyribonucleic acid (DNA) methylation and its functional significance during normal and disease processes.[1] DNA methylation, especially the covalent modification of cytosine to 5-methylcytosine (m5C), often occurs in both prokaryotic and eukaryotic genomes. Methylation of cytosines mostly at CpG sequences, is an epigenetic phenomenon that modifies genes in a wide range of organisms.[2] The CpG-rich regions, denominated CpG islands, are largely localized in gene promoter regions or in the initial exons of genes.[3–8] Methylation of CpG islands plays a role in many cellular processes including regulating tissue and developmental-specific gene expression, X-chromosome inactivation, imprinting, suppression of parasitic DNA sequences and DNA repair.[9] Methylation of CpG sites results in silencing of the associated active genes, which is closely associated with the development of human cancers and tumors. Therefore, to better understand the effects of DNA methylation in both normal and disease processes, simple, quantitative and efficient methods for assaying DNA methylation should be developed.

A number of different methods have been developed for assaying DNA methylation, such as methylation-specific polymerase chain reaction (PCR) (MSP),[10] combined bisulfite restriction analysis (COBRA),[11] modification-sensitive single nucleotide primer extension (MsSnuPE)[12] and MethyLight.[13] Recently, Akey *et al.*[14] reported two high-throughput methods using melting curve methylation-specific PCR (McMSP) and melting curve combined bisulfite restriction analysis (McCOBRA) for DNA methylation determination. Neither of these methods, however, can provide sequence and structure information for methylated oligodeoxynucleotides (ODNs).

Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are the most widely used techniques in the field of large biopolymers. The development of ESI enabled liquid chromatography/mass spectrometry (LC/MS) to be utilized for the quantitative determination and structural characterization of DNA adduction,[15] which indicated that LC/MS has the sensitivity and selectivity to detect methylated ODNs in complex mixtures. For example, the use of LC/MS for the assessment of DNA methylation levels has been carried out by some researchers.[16,17] Humeny

et al.[18] reported enzymatic DNA methylation of oligonucleotide substrates by MALDI time-of-flight (ToF) mass spectrometry. Methylation was introduced enzymatically into the DNA by DNA methyltransferases and only canonical sites were identified by the increase in mass. More recently, Song *et al.*[19] measured the genoimic DNA methylation using LC-ESI-MS/MS and the methylation level was detected in multiple reaction monitoring (MRM) mode through measuring 5-methyl-2′-deoxycytidine levels following enzymatic hydrolysis of genomic DNA. The methods mentioned above could quantitatively determine 5-methyl-2′-deoxycytidine in genomic DNA, but the methylated sites of DNA had not yet been addressed.

MS/MS is a powerful tool for the characterization of ODNs because it can provide direct sequence and structural information.[10–12,20] For the modified ODNs, the assignment of the sites of modification within a target sequence is very important since isomeric adducts may possess different mutagenic activities due to the different biological response to the presence of the formed adducts. Although MS/MS has been widely used for identifying the DNA adduction site,[1,3,4,9] few attempts have been focused on the DNA methylation sites using ESI-MS/MS and their fragmentation mechanisms.[5,18,21]

More recently, we studied the characteristics of G- and T-rich oligonucleotides[22] and the modification of DNA involving methylation[23] and phenyl glycidyl ether (PGE)[24] by ion pair-reverse-phase high-performance liquid chromatography (IP-RP-HPLC)/ESI-MS. In our previous study of the DNA methylation,[23] T-rich ODNs were used to study the methylated site identification and fragmentation mechanism of methylated DNA using tandem mass spectrometry. As stated above, methylation of CpG sites is very important in organisms. So far, there have no studies foused on the character of the CpG-ODNs by the IP-RP-HPLC-ESI-MS/MS. So, in current research, the methylation of CpG-ODNs under A-, T-, C- and G-rich environments were investigated by ESI-MS/MS.

Experimental

Chemicals and materials

ODNs were obtained from the Dingguo Biotechnology (Beijing, China) and used as received. The sequences investigated are listed in Table 1. Triethylamine (TEA, 99.5%), glacial acetic acid (99.99%), HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was obtained from Dupont (Delaware, USA). Water was purified with an Elix-Milli-Q system (Millipore Corp., Bedford, MA, USA).

HPLC mobile phase preparation

The 400 mM hexafluoroisopropanol-16 mM triethylammonium buffer, pH 7.7, was prepared by dissolving 22 mL HFIP in 400 mL water and then slowly titrating with 1.2 mL TEA. All mobile phases were filtered through 0.45 μm film before HPLC analysis.

Table 1 Characteristics of CpG-ODNs used for IP-RP-HPLC/ESI-MS analysis.

No.	Sequence	Length	MW(Da)	
			Theoretical	Experimental
A1	**5′-AACGCGAA-3′**	8-mer	2747	2746.2
A2	5′-AA m^{5}CGCGAA-3′	8-mer	2441	2439.8
A3	5′-AACGm^{5}CGAA-3′	8-mer	2441	2439.8
A4	5′-AA m^{5}CGm^{5}CGAA-3′	8-mer	2455	2454.7
T1	5′-TTCGCGTT-3′	8-mer	2391.5	2390.6
T2	5′-TT m^{5}CGCGTT-3′	8-mer	2405.6	2403.9
T3	5′-TTCGm^{5}CGTT-3′	8-mer	2405.6	2404.2
T4	5′-TT m^{5}CG m^{5}CGTT-3′	8-mer	2419.6	2418.4
C1	5′-CCCGCGCC -3′	8-mer	2331.6	2330.2
C2	5′-CC m^{5}CGCGCC-3′	8-mer	2345.6	2344.0
C3	5′-CCCG m^{5}CGCC-3′	8-mer	2345.6	2343.9
C4	5′-CC m^{5}CGm^{5}CGCC-3′	8-mer	2359.6	2358.2
G1	5′-GGCGCGGG-3′	8-mer	2491.7	2490.6
G2	5′-GG m^{5}CGCGGG-3′	8-mer	2505.7	2504.6
G3	5′-GGCGm^{5}CGGG-3′	8-mer	2505.7	2504.4
G4	5′-GG m^{5}C G m^{5}C GGG-3′	8-mer	2519.7	2519

Instrumental analysis

All samples were analyzed using an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled with an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA). The liquid chromatograph was equipped with an on-line vacuum degassing system, a quaternary pumping system, an autosampler and a variable wavelength detector. The chromatographic separation was performed on a C_{18} column (150×3.0 mm i.d., 3.0 µm particle size, ZORBAX 300SB, Agilent Technologies, Palo Alto, CA, USA) at room temperature. The flow-rate of 300 µL min^{-1} was used in the analysis. The injection volume was 1 µL and UV detection was set at the wavelength of 260 nm. The LC effluent was introduced into a Turbon IonSpray interface without splitting. Electrospray mass data were acquired in the negative-ion mode with a spray voltage of –4.5 kV and declustering potential of –50 V. The source temperature was 400°C. Nitrogen was used as the curtain gas (setting 50), nebulizer gas (setting 20) and turbo gas (setting 20). MS/MS was performed using nitrogen as collision gas (CAD gas setting 6). The mass spectrometer was operated at unit mass resolution for both Q1 and Q3. The data were acquired with the Sciex Analyst software, version 1.3.1 (Applied Biosystems, Foster City, CA, USA).

Results and discussion

### Comparison of methylated and unmethylated ODN fragmentation patterns

ESI-MS is a gentle and sensitive method for the analysis of natural and modified nucleic acids. Determination of the molecular mass is straightforward, since ESI mass spectra for each ODN contain a distribution of charge states that can be deconvoluted. Unmethylated T-rich CpG-ODN in this study was characterized by its *m/z* 1193.9([M-2H]$^{2-}$), *m/z* 795.6 ([M−3H]$^{3-}$), *m/z* 596.7 ([M−4H]$^{4-}$), *m/z* 477.3 ([M−5H]$^{5-}$) in the total ion current (TIC) chromatograph while the ODNs with one methylated base were characterized by the *m/z* 1201.0 ([M−2H]$^{2-}$), *m/z* 800.5 ([M−3H]$^{3-}$), *m/z* 600.1 ([M−4H]$^{4-}$), *m/z* 479.8 ([M−5H]$^{5-}$) (data not shown), which corresponded to the addition of one methyl group to the original oligonucleotide.

However, to further determine the methylated sites, it is essential to obtain more sequence information from the product ion mass spectrum. Tandem mass spectrometry has proved to be very useful for DNA adducts to reveal the presence of a modified base and identify the modified sites. Many researches have studied the fragmentation mechanisms of ODNs by tandem mass spectrometry .[11,25–26] McLuckey *et al.*[25] found that several rules exist for ODN fragmentation under ion trap coalitional activation conditions. First, a base is lost either as an anion or as a neutral. Once a base is lost, the ODN shows a strong tendency to fragment at the 3′ C–O bond of sugar to which the base had been attached. The second fragmentation yields complementary fragments that are labeled a-B and w series ions, which facilitates location of the base.

This fragmentation pattern has been widely used in the explanation of the behavior of ODNs on MS/MS[26] from different types of mass spectrometers. Scheme 1 shows the fragmentation channels of T2 according to the nomenclature of McLuckey *et al*. Figure 1 gives the MS/MS spectra of the T1, T2, T3 and T4 from their parent ions of 2-charge state at the collision energy of 40 V. From Figure 1, it can be seen that the fragmentation models were similar in T1 to T4, i.e. all the dominant product ions were w2$^-$, [a3−B]$^-$, w3$^-$, [a4−B]$^-$, [M−2H]$^{2-}$, w4$^-$ and [a5–B]$^-$ ions. Furthermore, the relative intensity of these ions was similar for T1 to T4, which had the same order w2$^-$>[a3−B]$^-$>[a4−B]$^-$>w3$^-$>[a5−B]$^-$>w4$^-$. This phenomenon was also observed for other charge states, 3-, 4- and 5- (data not shown). This indicates that the presence of a methyl group has no significant influence on the fragmentation behavior of ODNs, which was also observed in our previous research.[23] Wan and Gross thought that the fragmentation mechanism involved proton transfer from the adjoining 5′- phosphate to the nucleobase.[27] They found that if ODNs were modified with methylphosphonate, the fragmentation pattern would be different, i.e. the a-B ions would not be formed. In our studies, the methyl group was located on the nucleobase, so the proton transfer and subsequent formation of [a-B]$^-$ ions would not be influenced.

From the mass spectra of T1 and T2, it can clearly be seen that both have the same [a3−B]$^-$ ion, but for the [a4−B]$^-$ ion (labeled by *) there is a mass difference of 14 Da, which would provide information about the presence of a methyl group at position 3 in T2. The mass difference between the [a5−B]$^-$ ions (labeled by #) of T1 and T2, 14 Da, and the same w2$^-$, w3$^-$ and w4$^-$ ions would further confirm that the methyl group existed at position 3. Similarly, the methylated
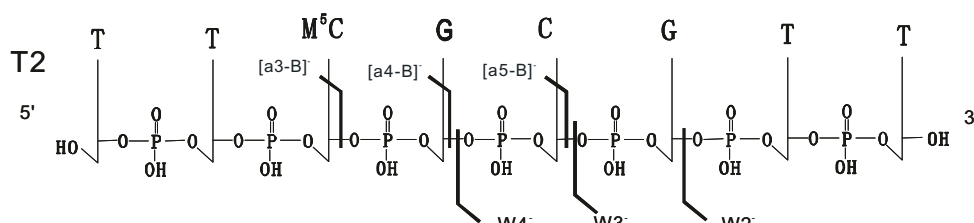
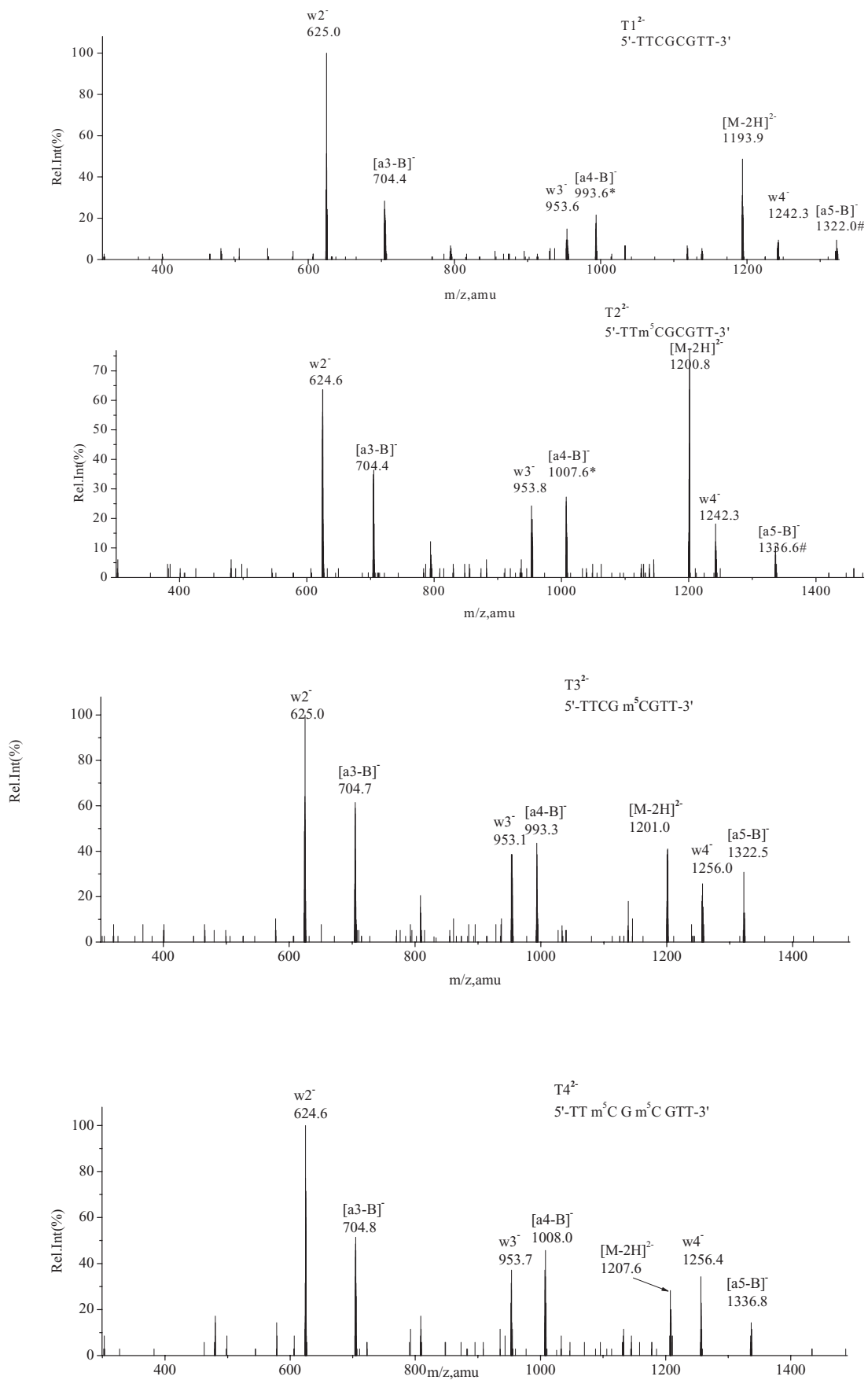**Scheme 1.** The fragmentation pattern of T2 at 2- charge state.

Figure 1. MS/MS spectra of T-rich CpG-ODNs from 2- charge states at collision energies of 40 V.

site can be determined at position 5 in the T3. For the ODNs with two methylated bases (T4), comparing the mass spectrum of T1 with that of T4, $[a_3-B]^-$ and $[a_4-B]^-$ ions can be used to determine the methyl group at position 3 in the T4 whereas $[a5-B]^-$, $w2^-$, $w3^-$ and $w4^-$ ions would confirm the presence of methyl group at position 5. So, MS/MS was very useful to confirm the methylated sites directly.

Characterization of fragmentation patterns of methylated CpG surrounded by different nucleobase

Different nucleobase composition conditions would show different behavior on the mass spectrum. Null and his co-workers found that the ESI signal decreased with the increase of G content by direct injection of the electrospray solution containing acetonitrile and 2-propanol, ammonium acetate, piperidine and imidazole.[28] Our previous researches showed that the total ion current (TIC) intensity of T-rich oligonucleotides was much higher than that of G-rich oligonucleotides.[22] Wan and Cross found that the propensity to form a-B ions in the fragmentation of twelve 8-mers fell in the order of G>C≈A>>T.[27] However, few researches have been carried out to learn the characterization of fragmentation patterns of methylated CpG surrounded by different nucleobases.

In the present study, the precursor ion of 2-charge state was used to investigate the characterization of fragmentation patterns of A-, T-, C- and G-rich methylated CpG sequences. As shown in Figure 2 (T4 see Figure 1), it can be found that $w2^-$, $w4^-$, $w3^-$, $[a4-B]^-$, $[a3-B]^-$ and $[a5-B]^-$ ions are the main product ions in these samples. However, in A4, the fragment ions also included $w7^-$, $[a2-B]^-$ and $[a7-B]^-$, in G4 are $w6^-$ and $w7^-$ and in C4 are $w6^-$, $w7^-$, $[a2-B]^-$ and $[a7-B]^-$. This showed that there were the least fragment ions in T4 compared with the other three samples and the dissociation behavior was mainly involved in the middle of the sequence, i.e. at cytosines and guanines. Therefore, it can be concluded that the formation of sequence fragments from the loss of thymine is the least facile among the four nucleobases, which agreed with the results of Wan[27] and ours.[23] Furthermore, the fragment ions $[M-A]^{2-}$, $[M-C-H]^{2-}$ and $[M-G-H]^{2-}$ were presented in A4, C4 and G4, respectively, whereas no similar ions $[M-B-nH]^{n-}$ were found in T4. This would further confirm that thymine is the most difficult to be lost to form sequence fragmentations.

The other phenomena we noticed was that $w2^-$ ions were always most abundant among the product ions of $[M-2H]^{2-}$, which implied that backbone cleavage at position 6 tended to be favored. From Figure 2, we also clearly see that the relative intensities of the main fragment ions of G4 followed the order $w2^->[a4-B]^->w4^-\approx[a3-B]^->w3^->[a4-B]^-\approx w7^->w6>[a5-B]^-$, which suggested that backbone cleavage at guanine which existed in the middle of the sequence (positions 4 and 6) tended to be favored. However, for C4 and A4, $w3^-$ ions were more abundant than $[a4-B]^-$ ions and in T4 $[a3-B]^-$ ions were more abundant than a4-B ions. These results illustrated that for A-, T- and

C-rich methylated CpG-ODNs backbone cleavage happened more easily at guanine and cytosine (positions 3 or 5 and 6). In addition, $[a6-B]^{n-}$ ions, the complementation ions of $w2^-$, were not observed in these four samples. Similar results were also observed by Wan and Gross.[27] They thought that in the 6-mers and greater ODNs the flexible structure of gas-phase oligonucleotides could offer other phosphates or deoxyribose groups to serve as the proton source. The other reason is that base loss upon CID in triple quadrupoles is much less dependent on the identity of the base than in quadrupole ion traps.[26] So the strong preference for loss of specific bases to form fragment ions was not the only way to form product ions.[29,30]

Characterization of fragmentation patterns of methylated CpG sequence at different parent ions charge states

Although the multiply charged nature of the parent ion introduces complexity in the mass spectrum of the product ions,[30] more sequence information could be obtained from the product ion spectra of ODNs with different charge states to determine their structure and sequence. The charge-state distribution of ODNs in our experiments typically covers a range from 2- to 5-charge states and Figure 3 shows the mass spectra of A2 at 2-, 3-, 4- and 5-charge states.

Oberacher *et al.*[31] found that collision energy had a major impact on the ability to interpret MS/MS spectra. The optimum collision energy should be high enough to generate fragments covering the sequence as completely as possible by a-B and w ions, but low enough to suppress secondary and/or alternative fragmentation pathways. In our study, the optimum collision energies for 2-, 3-, 4- and 5-parent ions of A2 were 40, 30, 18 and 13 V, respectively. This showed that higher energies were required for lower charge states, which was consisted with the results obtained in our previous study. This may be that the energy for break-up of proton-bound intermediates is higher with lower charge state oligonucleotides.[22]

It is well known that the total charge content is an important factor in interpreting the fragmentation mechanisms of ODNs.[32] If one takes an ODN with all the protons deprotonated from the phosphodiester linkages as being 100% charged, then those ODNs in our experiments at 2-, 3-, 4-, and 5-charge states would correspond to 29%, 43%, 57% and 71% charged, respectively. From Figure 3, it can be seen that the main product ions from 2-charge state of A2, $w2^-$, $w3^-$, $w4^-$, $w5^-$, $[a3-B]^-$ and $[a5-B]^-$ ions were indicative of the cleavage in the middle of the sequence (cytosine and guanine). For 3-charge state, $w2^-$, $w4^{2-}$, $w5^{2-}$, $[a3-B]^-$, $[a4-B]^-$, $[a5-B]^{2-}$ and $[a6-B]^{2-}$ suggested the cleavage of the middle of the sequence. For 4-charge states, only two product ions, $w3^{2-}$ and $w5^{2-}$, were indicative of the cleavage in the middle of the sequence, but more product ions, $w1^-$, $w7^{3-}$, $[a2-B]^-$, $w2^-$ and $[a7-B]^{3-}$ ions, suggested cleavage at the 5′ and 3′ terminal of the sequence. No product ions indicative of cleavage in the middle of the sequence were observed for 5-charge state, but only $w7^{4-}$ and $w1^-$ ions suggested cleavage at the 5′ and 3′
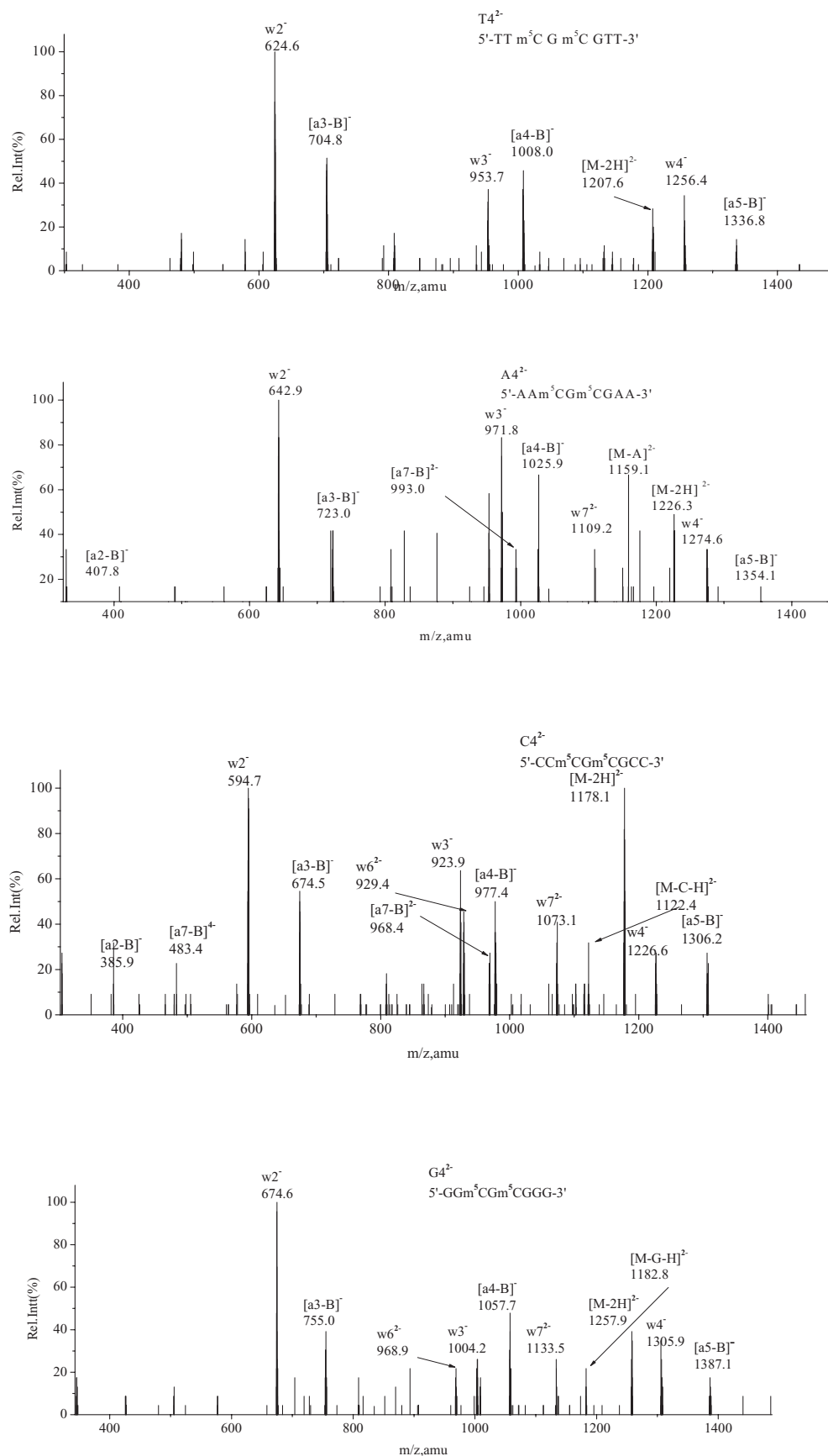
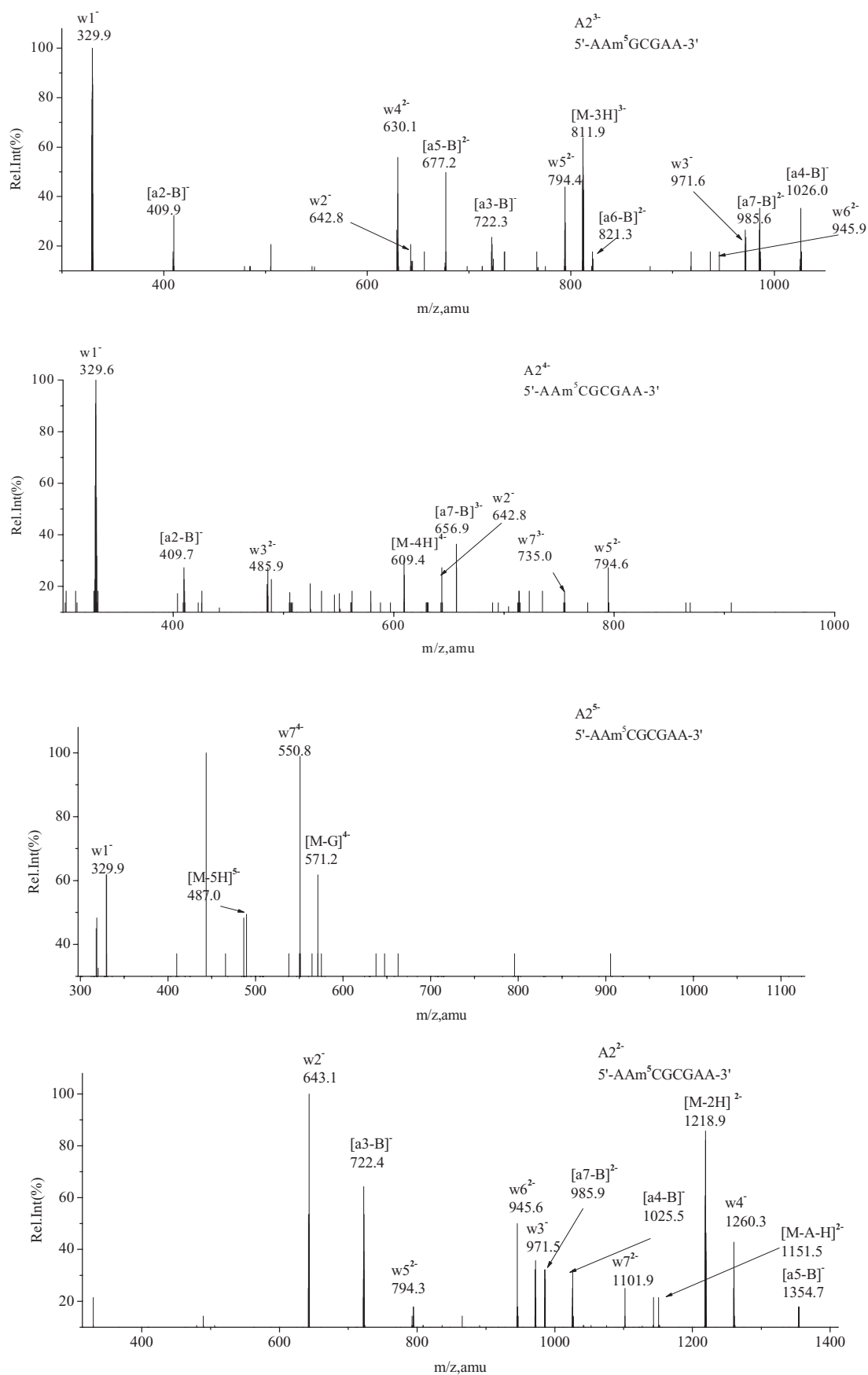**Figure 2. MS/MS spectra of T4, A4, C4 and G4 from 2- charge states at collision energies of 40 V.**

Figure 3. MS/MS spectra of A2 from 2-, 3-, 4- and 5- charge states at collision energies of 40 V, 30 V, 18 V, 13 V, respectively.

terminals of the sequence could be found. So, these results showed that the fragmentation patterns involved at the 5′ and 3′ terminals were predominant at higher charge states (or higher charge contents). Similar results were obtained by Virkic and his co-workers.[33] They found that the 5′ and 3′ base losses were preferred over internal base loss for 2-charge states of trimer and tetramer ODNs (the total charge content was 100% and 67%, respectively). In our previous study,[23] we also found that with the increase of the parent ions, the charge states of the main fragmentation channels changed from the middle to the 5′ and 3′ terminals of T-rich ODNs. For other samples, A1, A3, A4 and T-rich, C-rich and G-rich CpG-ODNs, similar results were also obtained.

Conclusions

Methylated and unmethylated CpG-ODNs could be well characterized by ESI-MS/MS and the methylated sites can be confirmed by comparison of their MS/MS spectra. The presence of a methyl group has no significant influence on the fragmentation behavior of ODNs. The formation of sequence fragments from the loss of thymine is the most difficult among the four nucleobases. Backbone cleavage would happen more easily at guanine and cytosine for A-, T-, C-rich methylated CpG-ODNs. The parent ion charges play an important role in the fragmentation patterns. At lower charge states, the fragmentation mainly happened in the middle of sequence whereas at higher charge states the broken sites mainly happened at the 5′ and 3′ terminals.

Acknowledgements

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