SHORT COMMUNICATION

Differentiation of 2′-*O*- and 3′-*O*-Methylated Ribonucleosides by Tandem Mass Spectrometry

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Recent studies revealed that the 3′-terminal nucleotides in plant microRNAs were methylated on the ribose at the 2′ or 3′ hydroxyl groups. Here we examined the fragmentation of the electrospray-produced $[M + H]^+$ and $[M - H]^-$ ions of 2′- and 3′-*O*-methylated ribonucleosides. It turned out that the predominant fragmentation pathway for the $[M + H]^+$ ions of ribose-methylated nucleosides was the neutral loss of the methylated ribose, which made it impossible to distinguish 2′-*O*-methylation from 3′-*O*-methylation by positive-ion MS/MS. However, characteristic fragment ions, resulting from the cleavage through the ribose rings, were produced for the $[M - H]^-$ ions of each pair of ribose-methylated nucleosides. In this respect, the neutral loss of a 90-Da fragment ($C_3H_6O_3$) was observed for 2′-*O*-methylated cytidine, guanosine and adenosine, but not for their 3′-*O*-methylated counterparts. On the other hand, the neutral loss of a 60-Da fragment ($C_2H_4O_2$) was found for 3′-*O*-methyluridine, but not for 2′-*O*-methyluridine. (J Am Soc Mass Spectrom 2006, 17, 1096–1099) © 2006 American Society for Mass Spectrometry

MicroRNAs (miRNAs), a family of small noncoding RNAs 20 to 24 nucleotides long, have been identified in many plant and animal species [1]. These small RNA molecules are involved in various biological processes, including cell proliferation and cell death during development, stress resistance, and fat metabolism [1].

Many cellular RNAs are susceptible to covalent modifications such as methylation, deamination, and thiolation, providing a means to expand the chemical repertoire of the four nucleobases [2, 3]. Post-transcriptional methylation on the nucleobase or ribose is one of the most common and conserved types of RNA modifications [3, 4]. Very recently, it was revealed that the last nucleotide in miRNAs could be methylated by methyltransferase HEN1, which contributes to the accumulation of endogenous miRNA and the production of transgene siRNA involved in post-transcriptional gene silencing [5]. In addition, the ribose methylation was a crucial step in plant miRNA biogenesis [6, 7].

Because the 3′ terminal nucleotides of miRNAs have free hydroxyl groups on both C_2′ and C_3′, the methylation could potentially occur at either hydroxyl group. In this context, the terminal nucleotides of small RNAs in tobacco were suggested to be methylated on the 2′-hydroxyl group on the grounds that these small RNAs were competent substrates for T4 RNA ligase [8], which was suggested not to be able to ligate RNA blocked at the terminal 3′ hydroxyl group [9–11]. It, however, has not been experimentally verified whether the terminal 3′-*O*-methylation would prohibit the T4 RNA ligase-mediated ligation via the 2′-OH group [9, 11]. Thus, there is a need for a rapid and unambiguous method to determine the nature of ribose *O*-methylation in small RNAs.

Mass spectrometry has long provided an important means for the characterization of covalent modification of nucleosides. Previous studies have shown that 2′-*O*-methyl-ribonucleosides could be differentiated from their 3′-*O*-methylated analogs by electron impact (EI) mass spectrometry [12–14]. The diagnostic fragment ion for 2′-*O*-methylribonucleosides is the ion of *m/z* 146, which corresponds to the methylated ribose moiety minus one hydrogen atom [12–14]. In addition, 2′-*O*-methyluridine and 2′-*O*-methylguanosine have been examined by positive-ion fast-atom bombardment-tandem mass spectrometry (FAB-MS/MS) [15] and negative-ion laser desorption/Fourier-transform mass spectrometry [16].

Due to the compatibility of electrospray ionization (ESI)-MS to HPLC and the high sensitivity offered by modern ESI-MS instrumentation, we decided to examine the fragmentation of the ESI-produced $[M + H]^+$ and $[M - H]^-$ ions of 2′- and 3′-*O*-methylribonucleosides, with the goal to establish a sensitive method for the differentiation of the isomeric ribonucleosides.

Experimental

2′- and 3′-*O*-methyl-ribonucleosides were purchased from RI Chemicals (Orange, CA). Electrospray ionization (ESI) MS and MS/MS experiments were carried out

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on an LCQ Deca XP ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). An equal-volume solvent mixture of acetonitrile and water was used for electrospray, and a 2 μL aliquot of 10 μM sample solution was injected in each run. The spray voltage was 3.4 kV, and the capillary temperature was maintained at 225 °C. The mass width for the precursor ion selection in MS/MS was 3 *m/z* units, and the normalized collision energy was 30% with an activation time of 30 ms. Experiments were also carried out at other normalized collisional energies.

Results and Discussion

Our proposed approach for identifying the nature of ribose methylation in miRNA involves digesting the miRNA by nuclease P1 and alkaline phosphatase, separating the resulting nucleoside mixture by HPLC, and monitoring the LC effluents by online MS/MS. Because the 3′ terminal nucleotide of mir173 is a cytidine, initial investigation was carried out on the differentiation of 2′-*O*-methylcytidine and 3′-*O*-methylcytidine. To distinguish these two isomeric methylated nucleosides, we first acquired the product-ion spectra of their $[M + H]^+$ ions because of the relatively high sensitivity in positive-ion mode. In sharp contrast to what were observed in the EI spectra of those *O*-methylated nucleosides [12–14], the product-ion spectra for the $[M + H]^+$ ions of the two isomeric nucleosides were identical, and both of them showed the formation of a dominant fragment ion of $[B + H]^+$ (*m/z* 112, spectra not shown), which resulted from the cleavage of the glycosidic bond and the subsequent neutral loss of the methylated ribose moiety.

Next, we acquired the product-ion spectra of the $[M - H]^-$ ions (*m/z* 256) of these two isomers and it turned out that they were distinctive from each other (Figure 1). The major product ion of *m/z* 213 for both 2′-*O*-methylcytidine and 3′-*O*-methylcytidine was induced from the loss of an HNCO moiety through the retro Diels-Alder reaction as proposed for the fragmentation of uracil [17]. The characteristic product ions of *m/z* 123 and *m/z* 153 from 2′-*O*-methylcytidine and 3′-*O*-methylcytidine, respectively, were formed from different cleavages through the ribose ring. In this regard, the fragment ion of *m/z* 123 was formed from the losses of an HNCO and a 90-Da neutral component ($C_3H_6O_3$). The consecutive neutral losses of an HNCO moiety and a $C_3H_6O_3$ fragment has been observed previously upon the collisional activation of the $[M - H]^-$ ion of 5-hydroxymethyl-2′-deoxyuridine [18]. In addition, the neutral loss of $C_3H_6O_3$ has been observed for the fragmentations of the $[M - H]^-$ ions of 2′-*O*-methylguanosine [16], 8-hydroxy-2′-deoxyguanosine, 8-hydroxy-2′-deoxyadenosine, 2-hydroxy-2′-deoxyadenosine [19], and pyrimidine glycols [20].

Rather than losing a neutral $C_3H_6O_3$ fragment, the $[M - H]^-$ ion of 3′-*O*-methylcytidine underwent a

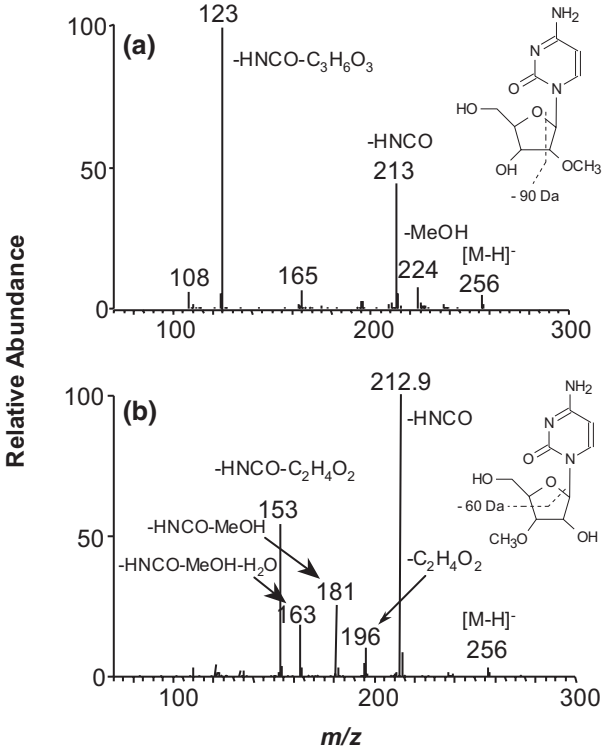

Figure 1. Product-ion spectra of the $[M - H]^-$ ions of 2′-*O*-methylcytidine (**a**) and 3′-*O*-methylcytidine (**b**).

neutral loss of a 60-Da fragment ($C_2H_4O_2$), which gave rise to the product ion of *m/z* 196 (Figure 1b). The product ion emanating from the eliminations of both $C_2H_4O_2$ and HNCO was also found (*m/z* 153) in the negative-ion MS/MS (Figure 1b). Therefore, the nature of the ribose methylation of cytidine can be readily identified from the distinctive fragmentation patterns of 2′- and 3′-*O*-methylated nucleosides in the negative-ion mode.

Since the 3′ terminal nucleoside of miRNAs can also be uridine, guanosine, or adenosine, we further examined the fragmentation of these three pairs of *O*-methylated nucleosides. The product-ion spectra of the $[M + H]^+$ ions of the two isomeric nucleosides in each pair were again indistinguishable, and the most abundant product ions were the protonated ions of the nucleobases ($[B + H]^+$, spectra not shown).

The product-ion spectra of the $[M - H]^-$ ions of these three pairs of *O*-methylated nucleosides are, however, distinctive. In this regard, the $[M - H]^-$ ion of 3′-*O*-methyluridine (Figure 2b) underwent similar cleavages as that of the corresponding cytidine derivative (Figure 1b). In particular, we observed the losses of HNCO (*m/z* 214), HNCO + H_2O (*m/z* 196), and $C_2H_4O_2$+HNCO (*m/z* 154). In contrast to what was found for 2′-*O*-methylcytidine, the product-ion spectrum of the $[M- H]^-$ ion of 2′-*O*-methyluridine (Figure 2a) did not show the formation of a product ion arising from the loss of the 90-Da ($C_3H_6O_3$) fragment. Instead, the most abundant product ion for 2′-*O*-methyluridine

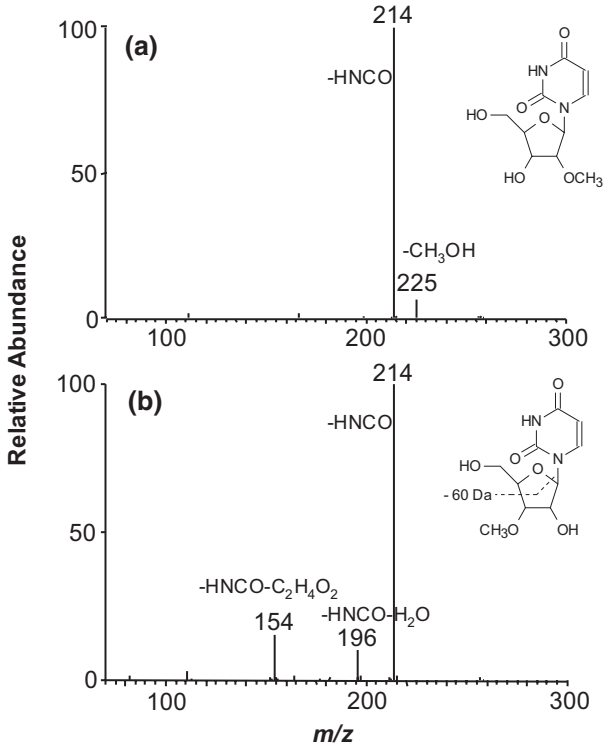

Figure 2. Product-ion spectra of the $[M - H]^-$ ions of 2′-*O*-methyluridine (**a**) and 3′-*O*-methyluridine (**b**).

was the ion of *m/z* 214, which is ascribed to the loss of an HNCO component. Although the cleavage within the ribose moiety was not observed for 2′-*O*-methyluridine, the diagnostic fragment ions of *m/z* 196 and 154 for 3′-*O*-methyluridine can be used to distinguish these two isomeric ribose-methylated nucleosides. Moreover, we observed a product ion resulting from the neutral loss of a methanol molecule for 2′-*O*-methyluridine (*m/z* 225), but not for 3′-*O*-methyluridine.

The product-ion spectra of the $[M - H]^-$ ions of the corresponding purine nucleosides showed that the most abundant ions were the deprotonated ions of the nucleobases (i.e., the ions of *m/z* 150 and 134, Figure 3). The product ions allowing for differentiating 2′-*O*-methyladenosine and 2′-*O*-methylguanosine from their corresponding 3′-*O*-methylated analogs were the ions of *m/z* 190 and 206 for 2′-*O*-methyladenosine and 2′-*O*-methylguanosine, respectively. These two ions formed from the familiar cross-ring cleavage of the ribose moiety (i.e., loss of a $C_3H_6O_3$ fragment).

It is worth noting that the above differences in product-ion spectra are observed for a wide range of normalized collisional energies (20–40%) for all four pairs of isomers except the isomeric *O*-methylated adenosines. For the latter pair of isomers, the small difference observed in Figure 3a and b is only obvious while the normalized collisional energy lies between 35 and 40%. It is also important to note that the most abundant fragment ions resulting from the collisional activation of the $[M - H]^-$ ions of the unmodified uridine, cytidine, adenosine, and

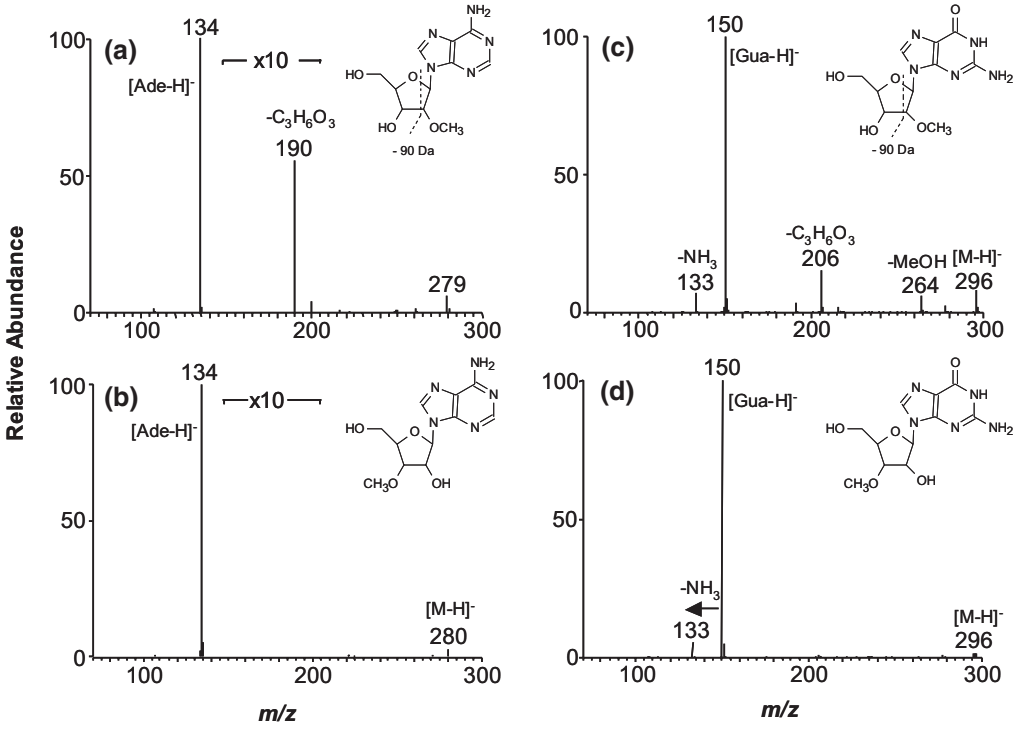

Figure 3. Product-ion spectra of the $[M - H]^-$ ions of 2′-*O*-methyladenosine (**a**), 3′-*O*-methyladenosine (**b**), 2′-*O*-methylguanosine (**c**), and 3′-*O*-methylguanosine (**d**).

guanosine are the deprotonated ions of the nucleobases [21, 22].

Conclusions

Here we showed that negative-ion ESI-MS/MS allows for the differentiation of 2′-*O*-methylribonucleosides from their 3′-*O*-methylated counterparts. Several characteristics are common in the fragmentation chemistry of ribose-methylated nucleosides. First, the two isomeric *O*-methylated nucleosides in each pair cannot be distinguished by positive-ion ESI-MS/MS, where the collisional activation of the $[M + H]^+$ ions of both modified nucleosides leads to facile cleavage of the glycosidic bond to give protonated nucleobase as the dominant fragment ion. In negative-ion detection mode, however, all four pairs of ribose-methylated nucleosides can be differentiated from the characteristic cleavages within the ribose rings. Second, the neutral loss of a 90-Da fragment is observed for 2′-*O*-methylated cytidine, adenosine, and guanosine, but not for the corresponding 3′-*O*-methylated nucleosides. Third, both 2′- and 3′-*O*-methylated uridine and cytidine undergo a common retro Diels-Alder type dissociation in the pyrimidine moiety, resulting in the neutral loss of an HNCO moiety (43 Da). Moreover, the unique neutral loss of a 60-Da $C_2H_4O_2$ fragment from 3′-*O*-methylated cytidine and uridine can facilitate their differentiation from the 2′-*O*-methylated counterparts. The tandem mass spectrometric method reported here can be potentially applied for the determination of the nature of ribose methylation in endogenous miRNAs as well as small interference RNAs (siRNA), which could improve our understanding of the functions of these small but important RNAs.

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