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ESI-MS/MS for the Differentiation of Diastereomeric Pyrimidine Glycols in Mononucleosides

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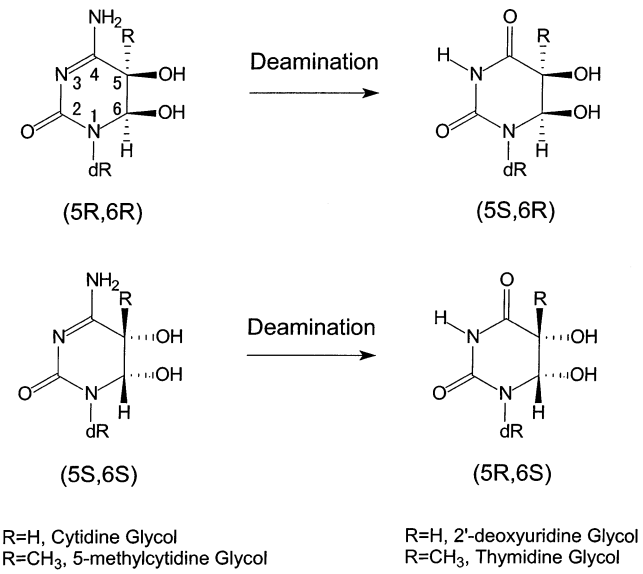
Pyrimidine glycols, or 5,6-dihydroxy-5,6-dihydropyrimidines, are primary lesions in DNA induced by reactive oxygen species. In this article, we report the preparation and tandem mass spectrometry (MS/MS) characterization of the two cis diastereomers of the glycol lesions of 2′-deoxyuridine, 5-methyl-2′-deoxycytidine, and thymidine. Our results show that collisional activation of the $[M + Na]^+$ ions of all the three pairs of cis isomers and that of the $[M + H]^+$ ions of the 2′-deoxyuridine glycols and 5-methyl-2′-deoxycytidine glycols give a facile loss of a water molecule. Interestingly, the water loss occurs more readily for the 6*S* isomer than for the 6*R* isomer. Likewise, product ion spectra of the $[M - H]^-$ ions of the two cis isomers of the 2′-deoxyuridine glycols and thymidine glycols show more facile loss of water for the 6*S* isomer than for the 6*R* isomer. MS/MS acquired at different collisional energies gave similar results, which establishes the reproducibility of spectra.

Reactive oxygen species (ROS), i.e., hydroxyl radical and superoxide anion, are produced by both endogenous aerobic metabolism and exogenous ionizing radiation and UV irradiation.[1] ROS can damage biomolecules, and the resultant nucleic acid damage has been implicated in numerous human diseases including cancer.[1−3] The 5,6-dihydroxy-5,6-dihydropyrimidines (or pyrimidine glycols, mostly their cis isomers) are primary oxidative lesions of pyrimidines (Scheme 1).[3]

Cytosines at CpG dinucleotides in mammalian DNA are methylated at the C5 position, and 5-methylcytosine (mC) plays an important role in gene expression and cancer.[4] For example, C-to-T transition mutation of cytosines at CpG sites is the most common mutation observed in human *p53* tumor suppressor gene in a number of cancers.[5] Formation and subsequent deamination (Scheme 1) of cytosine and 5-methylcytosine glycols (Cg and mCg) were proposed to be involved in the C-to-T transition mutation.[5] In line with that hypothesis, it was recently found that pol η, a human lesion bypass DNA polymerase, can synthesize

Scheme 1



past thymidine glycol (Tg) and incorporate an adenine opposite the lesion.[6] Interestingly, efficiencies of the translesion synthesis past the two cis diastereomers are different.[6] Our goal is to understand, at the molecular level, the role of Cg and mCg in the C-to-T transition mutation. One important step toward that goal is to obtain mononucleosides and oligodeoxynucleotides containing Cg, mCg, and their deaminated products, 2′-deoxyuridine glycol (Ug) and Tg, with defined stereochemistry.

Since the introduction of the electrospray ionization (ESI)[7] and matrix-assisted laser desorption/ionization (MALDI),[8] mass spectrometry (MS) has played an important role in biomolecule analysis because it is highly sensitive and tandem mass spectrometry (MS/MS) provides structure information. In the latter respect, it has been demonstrated that MS/MS can differentiate molecules with subtle differences, i.e., stereoisomers,[9−12] by giving characteristic fragment ions. Although both positive- and negative-

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ion MS/MS of Tg have been reported, it is not clear which isomer or a mixture of isomers was studied.[13] Herein we report the preparation and MS/MS characterization of two cis isomers of the glycol lesions in mononucleosides dT, dU, and dmC; the focus of our study is to use MS/MS to distinguish the two diastereomers.

EXPERIMENTAL SECTION

All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO). We prepared the glycol lesions using a procedure developed by Cadet and Teoule.[14] Briefly, 1 mg of nucleoside was dissolved in 1 mL of doubly distilled water and the resultant mixture was chilled on ice. Approximately 5 μL of bromine was then added to the chilled solution, and the mixture was vortexed and kept on ice for 15 min. The solution was light brown. Excess bromine was removed by purging air through the solution, after which the solution turned light yellow. The reaction mixture was then injected directly for HPLC analysis.

The HPLC separation was carried out on a Surveyor system with a photodiode array detector (ThermoFinnigan, San Jose, CA), and a 4.6-mm-i.d. reversed-phase C18 column (25 cm in length, 5 μm in particle size, and 300 Å in pore size; Varian, Walnut Creek, CA) was used. The flow rate was 1.0 mL/min, and a 35-min gradient of 6−12% methanol in water was used. The HPLC fractions were then dried by using a Savant Speed vacuum (Savant Instruments Inc., Holbrook, NY). ^{1}H NMR spectra were acquired on a Varian Inova 300-MHz instrument (Varian) with D_2O as solvent.

ESI-MS and MS/MS experiments were carried out on an LCQ Deca XP ion trap mass spectrometer (ThermoFinnigan) and a Global Ultima quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, U.K.). A solution of acetonitrile and water at a volume ratio of 1:1 was used as solvent for electrospray and a 2-μL aliquot of a ~20 μM sample solution was injected in each run.

For experiments on the ion trap mass spectrometer, the capillary temperature was maintained at 220 °C, and the spray voltage was 4.6, 3.0, and 5.0 kV for acquiring MS/MS of the $[M - H]^-$, $[M + H]^+$, and $[M + Na]^+$ ions, respectively. The mass width for precursor selection was 1.5 m/z units, and the collision gas was helium. Each spectrum was obtained by averaging ~30 scans, and the time for each scan was 0.3 s.

Q-TOF experiments were done at a capillary voltage of 3.5 V and a cone voltage of 120 V. The source block and desolvation gas (N_2) temperatures were 100 and 120 °C, respectively. Collisionally activated dissociation (CAD) experiments were performed in a hexapole collision cell with Ar as collision gas and collision energies varied from 4 to 10 eV. The quadrupole mass filter before the TOF analyzer was set with LM and HM resolution of 15.0 (arbitrary units), which is equivalent to a 1.0-Da mass window for transmission of precursor ions.

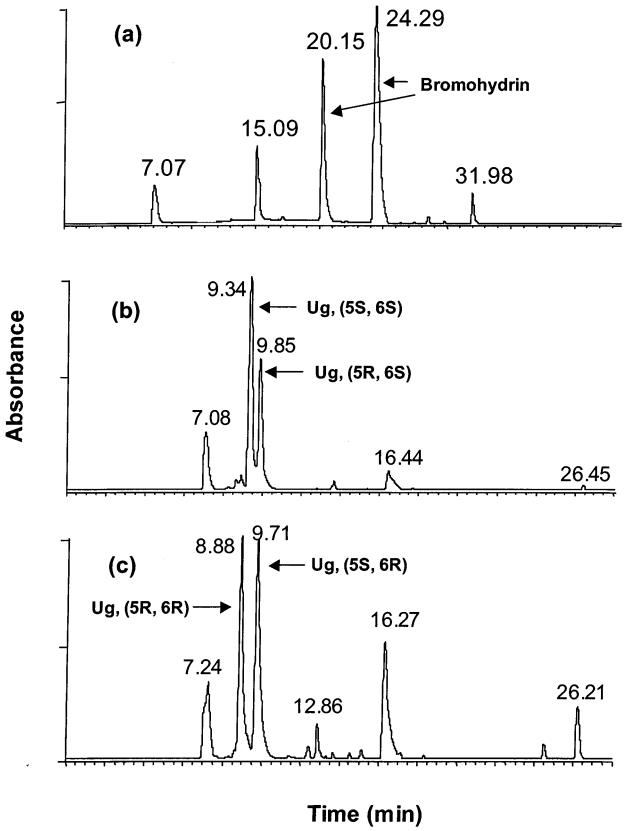

Figure 1. HPLC traces for the separation of bromohydrins (a) of 2′-deoxyuridine and the hydrolysis products of 5*S*,6*S* (b) and 5*R*,6*R* (c) bromohydrins.

Mass calibration was performed using 2′-deoxyuridine and 5-bromo-2′-deoxyuridine in positive-ion mode, and uridine monophosphate, uridine diphosphate, and adenosine monophosphate in negative-ion mode with a mass accuracy of less than 5 ppm. For MS/MS experiments, the precursor ion was used as a lock mass to measure the exact mass of the fragment ions.

RESULTS AND DISCUSSION

Preparation of Thymidine, 5-Methylcytidine, and 2′-Deoxyuridine Glycols. We prepared the Ug in a way similar to that reported by Cadet and co-workers[14−17] for the preparation of Tg, Cg, and mCg. In our hands, however, we found that the bromohydrins of 2′-deoxyuridine and 5-methyl-2′-deoxycytidine decomposed to the glycol lesions after drying. The two HPLC fractions with retention times of 20.15 and 24.29 min in Figure 1a are the 5*S*,6*S* and 5*R*,6*R* isomers of bromohydrins, and their relative mobilities are similar to those of the two isomers of the thymidine bromohydrins on a reversed-phase HPLC column.[15] After drying, we obtained both cis and trans isomers of 2′-deoxyuridine glycols by HPLC separation (Figure 1b,c). ^{1}H NMR spectra show that the latter eluted fractions are the cis isomers of Ug as compared with those of the thymidine glycols (Figure

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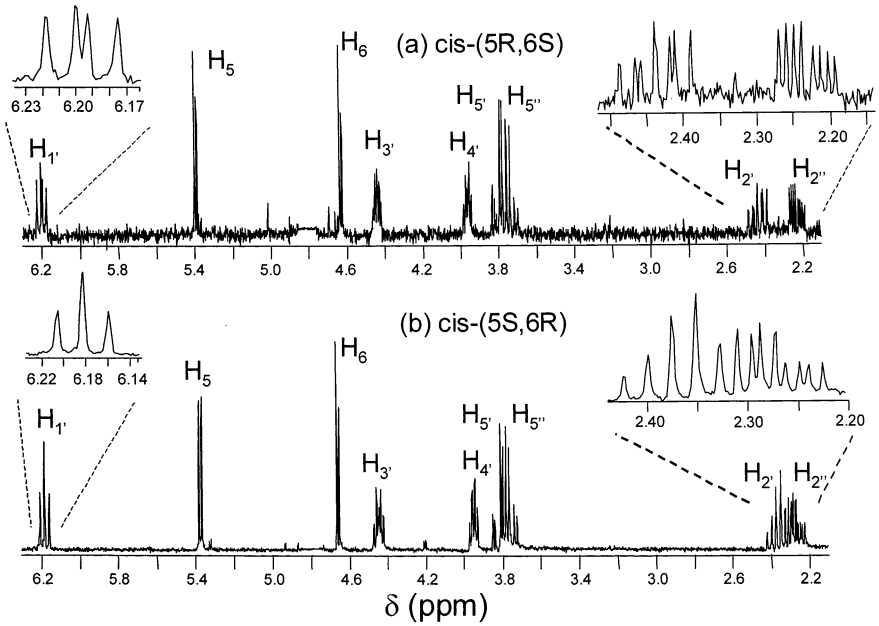

Figure 2. ^{1}H NMR spectra of the 5*R*,6*S* (a) and 5*S*,6*R* (b) isomer of 2′-deoxyuridine glycols.

2);[18] the distinction of the two cis isomers lies in the spin−spin coupling patterns of the $H_{1'}$, $H_{2'}$, $H_{2''}$, and $H_{3'}$ protons on the deoxyribose ring. Similarly, the two cis isomers of mCg and Tg were prepared and structures established by ^{1}H NMR.[18,19]

During our ESI-MS measurement, there is always a small portion of mCg deaminated to Tg. Because the molecular mass of the deaminated product is 1 Da more than that of the undeaminated precursor, care was taken to avoid the selection of the deaminated product for collisional activation by using a narrow width (1−1.5 u) for parent ion isolation. The effect of isolation was demonstrated by the observation that the isotopic peak is barely detectable for the product ions (Figure 3c).

Product Ion Spectra of the $[M - H]^-$ Ions of Pyrimidine Glycols. Although the product ion spectra of the $[M - H]^-$ ions of the two cis isomers of mCg are nearly identical, those of the Ug and Tg are distinctive (Figure 3). The distinction lies in the relative propensity of water loss: The relative abundance of the water loss fragment is ∼10% for the 5*R*,6*S* isomer compared to less than 1% for the 5*S*,6*R* isomer. The water loss fragment for the two cis isomers of mCg, however, is barely detectable.

The product ion spectra also show many other fragment ions sharing common origin for the 2′-deoxyuridine glycol, 5-methyl-2′-deoxycytidine glycol, and thymidine glycol. For example, a facile cleavage occurs to form ions of *m*/*z* 115, 116, and 102 for mCg, Tg, and Ug, respectively. The structure and the proposed mechanism for the formation of this type of ion are shown in Schemes 2 and 3a by using thymidine glycol as an example. The ion of *m*/*z* 116 was also observed by Hua and co-workers.[13] However, their proposed structure was deoxyribose anion, from which we would expect to observe the ion with the same *m*/*z* for all three pairs of isomers. Our experimental results, however, clearly show that the identity of the ion is dependent on the base moiety of the three pairs of glycols. The comparison of the fragmentation of three pairs of pyrimidine glycols, therefore, supports our structure assignment.

Other fragment ions of common origin include an ion of *m*/*z* 158 and an ion corresponding to the loss of a 43 u fragment (ions of *m*/*z* 232, 218, and 231 for Tg, Ug, and mCg, Figure 3). The former is due to the loss of part of the base moiety of the glycol lesions (Schemes 2 and 3b), and the latter is probably due to the loss of HNCO.[13] Moreover, the latter fragment ion is the dominant fragment ion in the product ion spectra of the two isomers of mCg. In addition to those common fragment ions, we observe a unique ion with a loss of 28 u for the two cis isomers of Ug, which is attributed to the loss of carbon monoxide. Our structural assignments for fragment ions are further supported by high-resolution MS/MS acquired on a Q-TOF mass spectrometer (vide infra).

From above studies, we know that the product ion spectra of the $[M - H]^-$ ions of the two cis mCg are not distinctive; it is desirable to have a method that can distinguish all the three pairs of isomers. To this end, we acquired the product ion spectra of the $[M + H]^+$ and $[M + Na]^+$ ions, and it turns out they are distinctive for all three pairs of diastereomers.

Product Ion Spectra of the $[M + Na]^+$ Ions of Pyrimidine Glycols. In the positive-ion mode, the $[M + Na]^+$ ions of Ug and mCg are much more abundant than the corresponding $[M + H]^+$ ions. Moreover, the $[M + H]^+$ ions for the two cis isomers of Tg are barely detectable, preventing us from obtaining their product ion spectra. We, therefore, emphasize our discussion on the MS/MS of the $[M + Na]^+$ ions. The familiar water loss is again a facile process for the $[M + Na]^+$ ions of all three pairs of cis pyrimidine glycols, and it is more facile for the 6*S* isomer than the 6*R* isomer (Figure 4). MS/MS of the water loss fragment (MS^3) of both cis isomers gives a dominant product ion of $[Base + Na - H_2O]^+$ (Base is the nucleobase portion of the pyrimidine glycol), indicating that the water loss is likely from the modified base moiety.

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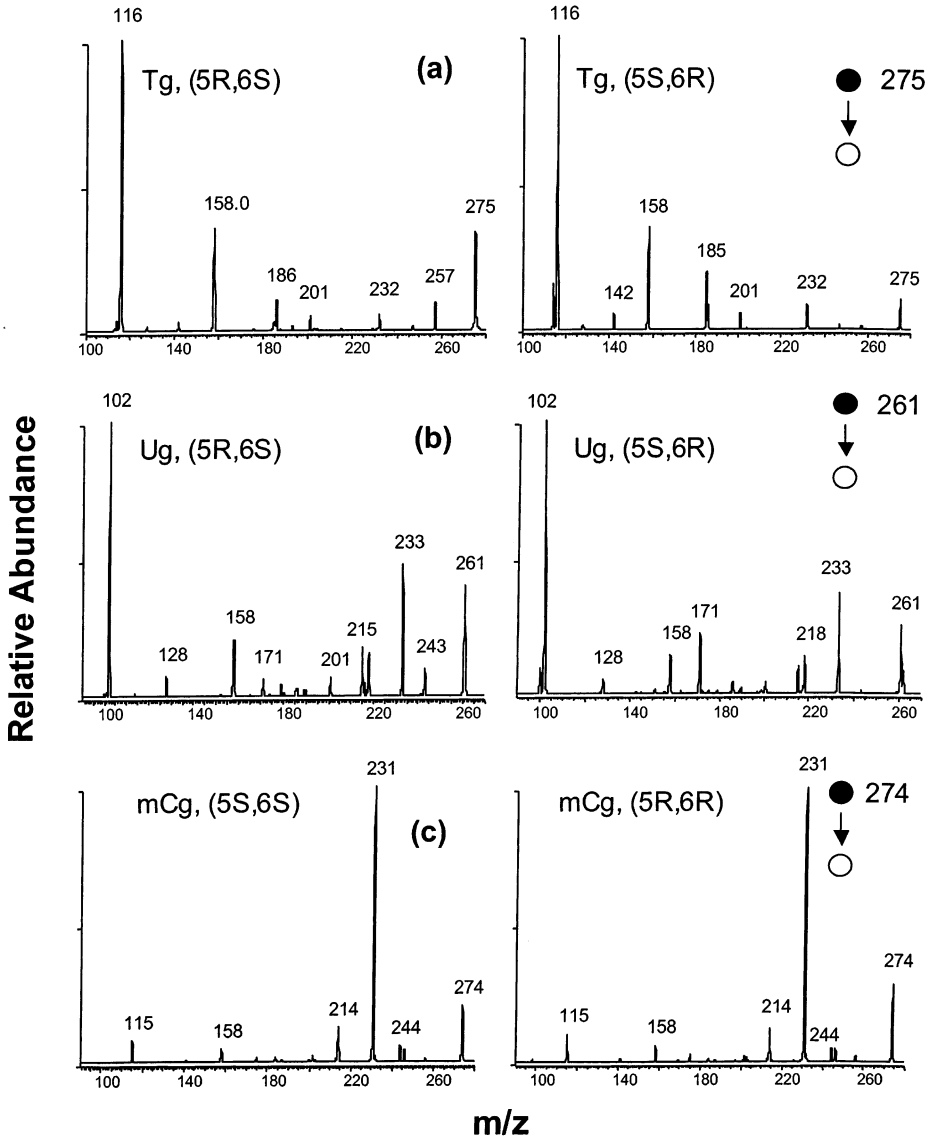

Figure 3. Product ion spectra of the $[M - H]^-$ ions of thymidine glycols (a), 2′-deoxyuridine glycols (b), and 5-methyl-2′-deoxycytidine glycols (c) acquired on an ion trap. Left, 6*S* isomers; right, 6*R* isomers. Collisional energies for (a), (b), and (c) are 23, 22, and 22%, respectively.

In addition, we observe a common fragment ion of m/z 182, which forms from a neutral loss of $C_4H_7NO_3$ from the base moiety (Scheme 4). The neutral loss was also observed in the product ion spectrum of the $[M + Na - 2H]^-$ ions of dinucleotides bearing a thymine glycol.[20] Furthermore, neutral loss of $C_5H_5NO_4$ and $C_4H_2NO_4$ (m/z 156) from the modified base moiety of Tg and Ug was observed. The neutral loss of $C_5H_5NO_4$ is the predominant cleavage in the CAD of the $[M - H]^-$ ions of dinucleotide containing a thymine glycol, and we proposed a mechanism for the cleavage.[20] It is interesting to note that this type of fragment ion is much less abundant in the product ion spectra of the $[M + Na]^+$ ions of the two cis isomers of the 5-methylcytidine glycols. The tautomerization from ketone to iminol[20] does not occur in mCg because there is no proton on N_3 of mCg. An alternative, less facile pathway may occur to form the m/z 156 ion from the $[M + Na]^+$ ion of mCg (Scheme 5b).

Fragment ions of m/z 209, 195, and 208 in Figure 4a–c, respectively, arise from the partial cleavage of the deoxyribose moiety, and they were also present in the CAD spectra of ions produced by FAB and ESI.[13,21] This type of ion was called S_1 ions,[21,22] and a mechanism involving the protonation of oxygen on the ribose ring was proposed for their formation.[22] We, however, reason that the formation of sodium ion adduct on the oxygen of the deoxyriblose ring is unlikely, and we proposed an alternative mechanism that involves a charge-remote process (Scheme 5c).

The product ion spectra of the $[M + H]^+$ ions for the two cis isomers of the Ug and mCg also show more facile water loss for the 6*S* isomer than the 6*R* isomer (Data shown in Supporting Information.). The difference in water loss is more pronounced for the two cis isomers of Ug than those of mCg

High-Resolution MS/MS Measurements on a Q-TOF Mass Spectrometer. To determine whether the distinctive water loss for the two diastereomers holds true on other types of instruments and to gain further support for the structure assignment of

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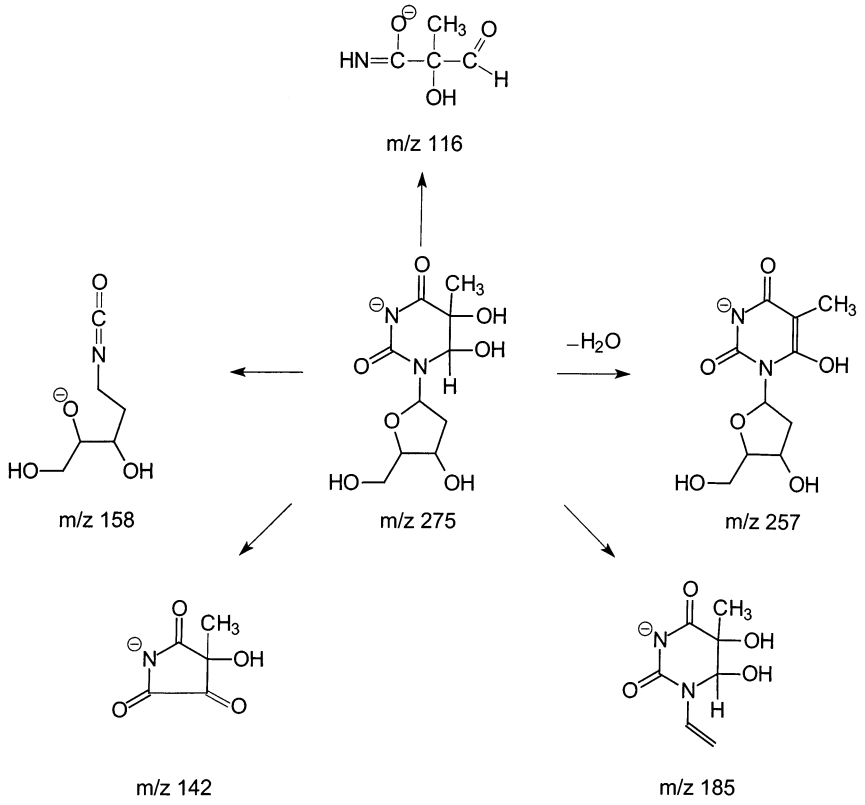
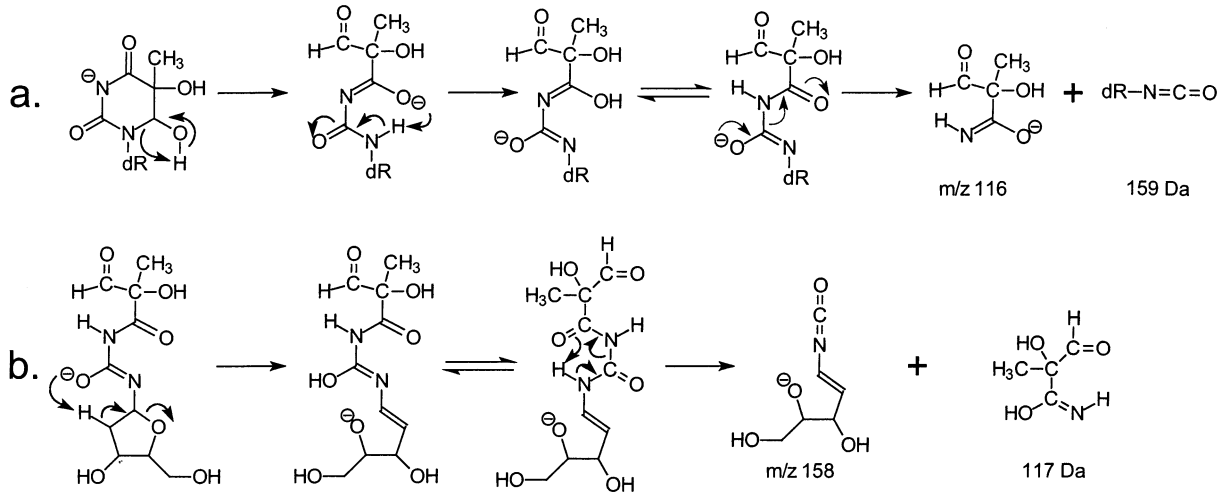
Scheme 2



Scheme 3



fragment ions, we acquired high-resolution product ion spectra on a Q-TOF mass spectrometer. Figure 6 shows the product ion spectra for the $[M - H]^-$ and $[M + Na]^+$ ions of the two cis isomers of Tg, and again the water loss is more facile for the 6*S* isomer than the 6*R* isomer. The relative abundances for some fragment ions, however, are different on Q-TOF and ion trap mass spectrometers. For example, an ion of m/z 165 is much more abundant in the Q-TOF spectra (Figure 6) than in the ion trap spectra (Figure 4). The difference is likely due to different CAD conditions on the two instruments. Similarly, MS/MS for the two diastereomers of mCg and Ug are also distinctive in that the water loss is more facile for the 6*S* than the 6*R* isomer (MS/MS for the $[M + Na]^+$ and $[M - H]^-$ ions of Ug are shown in the Supporting Information).

Results from exact mass measurement of product ions are consistent with the structures proposed in earlier sections (Table 1 shows the results for the two isomers of Tg, and the results for the two isomers of Ug are shown in the Supporting Information). The deviations between the measured masses and the calculated masses are within 20 ppm for all the ions we measured.

MS/MS at Different Collisional Energies. The product ion spectra of the $[M + Na]^+$ ions of the two cis pyrimidine glycols do not show dramatic difference in their fragmentation pattern. Rather, the difference lies in the relative abundances of the water loss fragment compared to other fragment ions. For all the three pairs of isomers, the 6*S* isomer always shows a more facile loss of water than the 6*R* isomer. To establish the reproducibility of spectra, we acquired the product ion spectra at different collisional

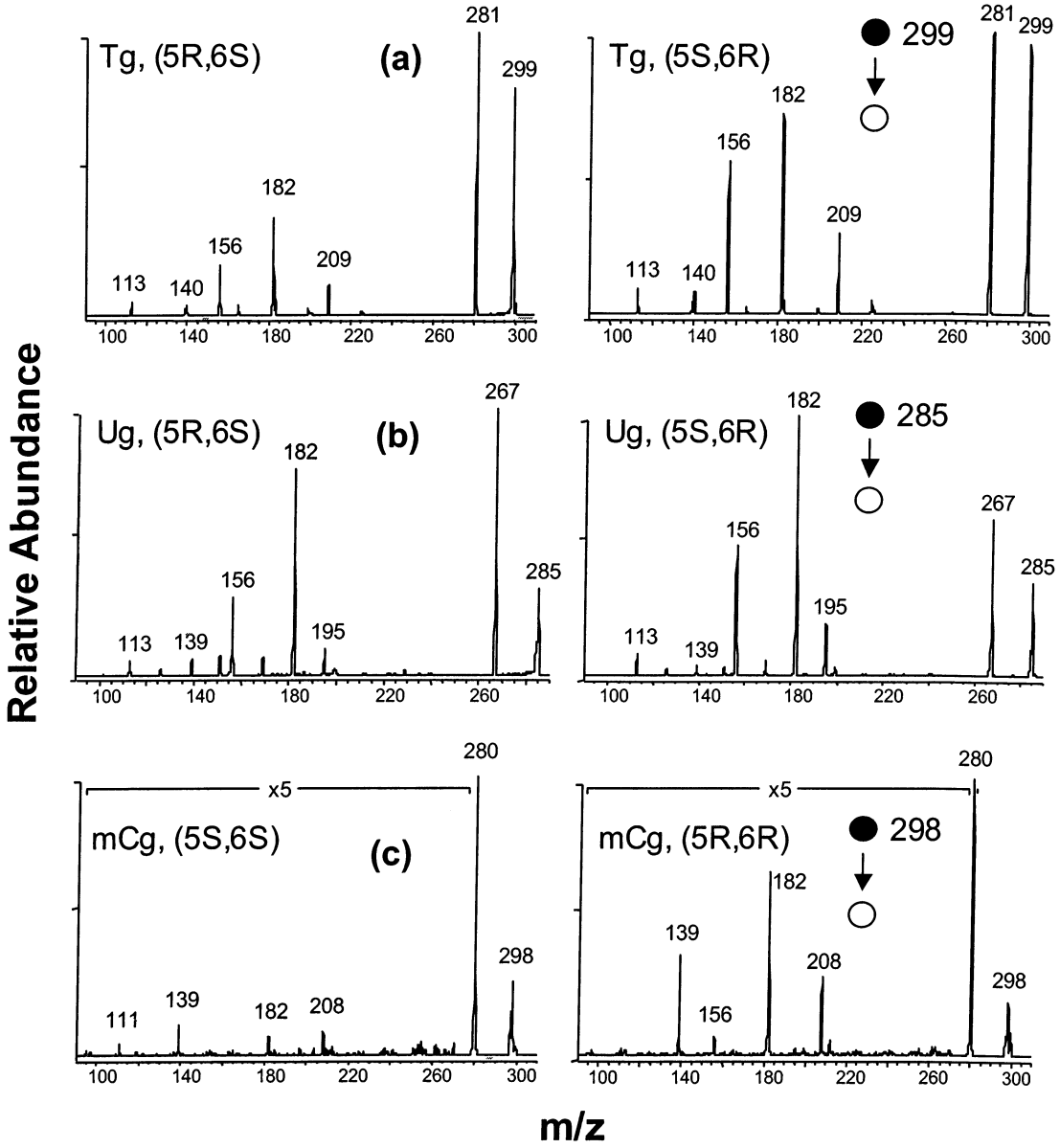


Figure 4. Product ion spectra of the $[M + Na]^+$ ions of thymidine glycols (a), 2′-deoxyuridine glycols (b), and 5-methyl-2′-deoxycytidine glycols (c) acquired on an ion trap (collisional energy, 25%). Left, 6*S* isomer; right, 6*R* isomer.

energies, a technique that is referred to as energy-resolved mass spectrometry (ERMS).[23] The results are shown in Figure 5 by plotting the intensity ratios of the water loss fragment to all other fragment ions versus the collisional energy. The data show that such a distinction is independent of the collisional energy we choose, though the ion intensity ratios vary slightly with collisional energies. Therefore, to establish the stereochemistry of an unknown cis pyrimidine glycol, we need to compare the MS/MS of the unknown with those of the two authentic cis isomers acquired at the same collisional energy.

Difference in Water Loss for the Two Cis Isomers. From the above studies of the product ion spectra of the $[M - H]^-$, $[M + H]^+$, and $[M + Na]^+$ ions, we have demonstrated that the most distinctive feature of the product ion spectra of the two cis diastereomers is the water loss fragment. It is interesting to note that postsource decay of lithiated diastereomeric benzo[*ghi*]-fluoranthene tetraols on a MALDI-TOF also gives distinctive water loss for different isomers, and water loss was not observed when the two adjacent hydroxyl groups are cis to each other.[12] Our MS^3 studies on the water loss fragment shows that the water loss, however, is likely from the base portion of the modified nucleosides, where the two hydroxyl groups are cis to each other.

Because the different propensities of water loss for the two diastereomers form the basis for distinguishing the two cis isomers, we attempted to rationalize this difference. In a previous work, we found similar results with dinucleotides bearing a thymine glycol and we attributed the difference to the water loss being a thermodynamically more favorable process for the 6*S* isomer than the 6*R* isomer.[20] We optimized the geometry for all the three pairs of cis isomers in mononucleosides with the Hartree−Fock (HF) method using a 6-31G basis set. The HF energy of the equilibrium geometry, however, does not give a

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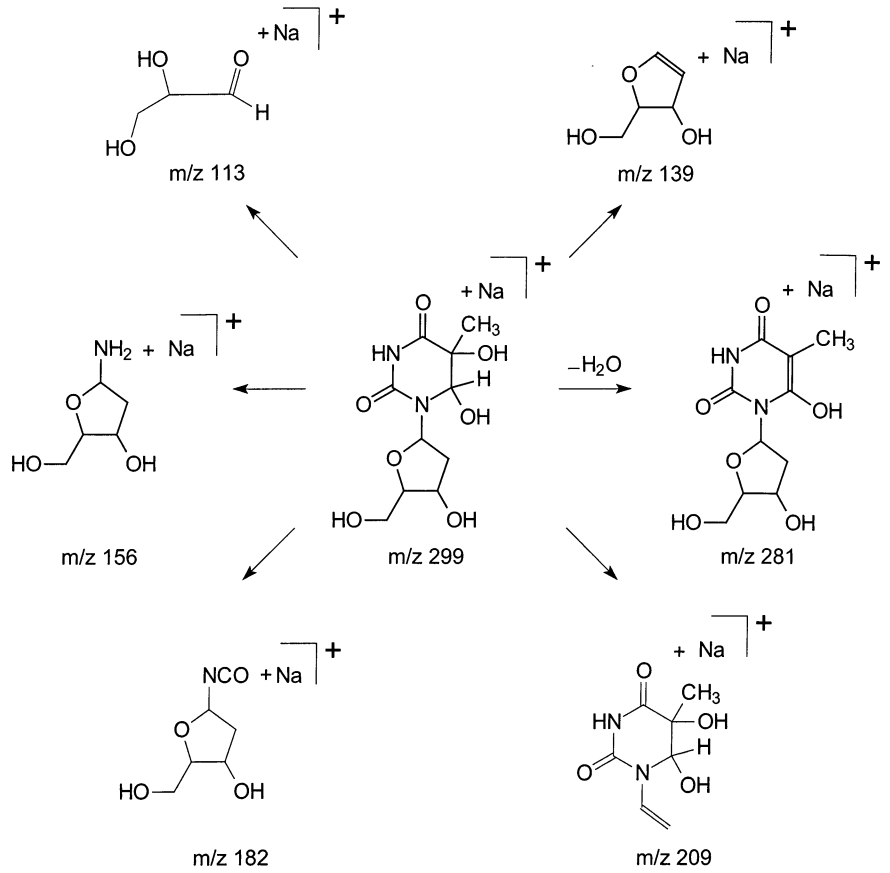
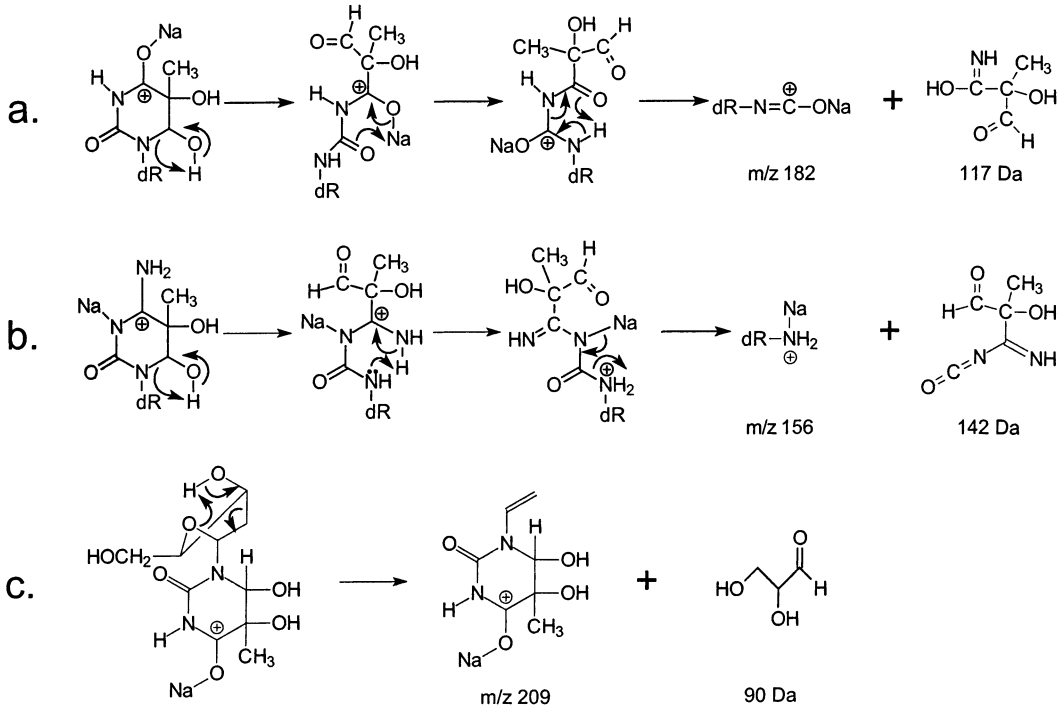
Scheme 4



Scheme 5



uniform answer about the relative ease of water loss from the two diastereomers from a thermodynamic point of view. The reason why the water loss is more facile for the 6*S* isomer than for the 6*R* isomer remains unclear at the moment.

CONCLUSIONS

We prepared two cis diastereomers of glycol lesions in mononucleosides dT, dU, and dmC via a bromohydrin intermediate, and we were able to distinguish the two isomers by MS/MS

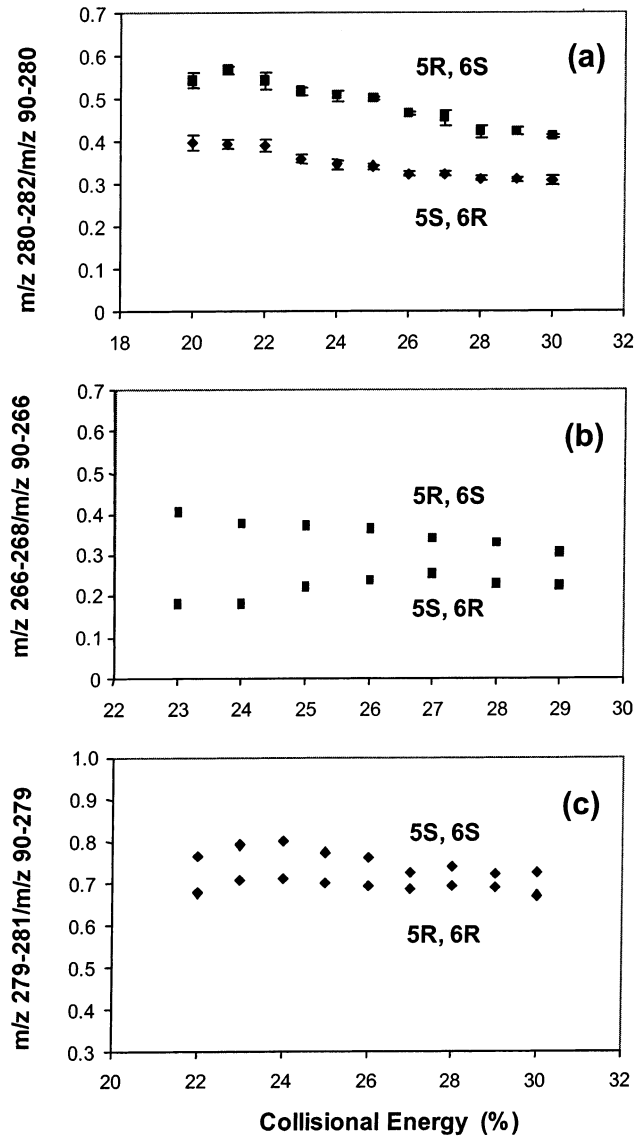


Figure 5. Ion intensity ratio of water loss fragment versus other fragment ions as a function of collisional energy for the product ion spectra of the $[M + Na]^+$ ions of (a) thymidine glycol, (b) 2′-deoxyuridine glycol, and (c) 5-methyl-2′-deoxycytidine glycol acquired on an ion trap. The error bars in (a) are standard deviations based on three independent measurements.

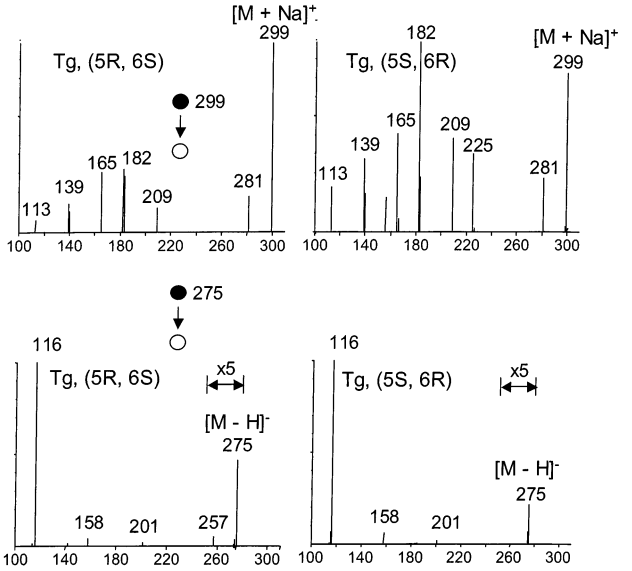


Figure 6. Product ion spectra of the $[M + Na]^+$ (top) and $[M - H]^-$ (bottom) ions of isomeric thymidine glycols: 5*R*,6*S* (left) and 5*S*,6*R* (right). The spectra were acquired on a Q-TOF mass spectrometer.

Table 1. High-Resolution Data for Fragment Ions in the MS/MS of the (A) $[M - H]^-$ and (B) $[M + Na]^+$ Ions of Isomeric *cis*-2′-Thymidine Glycols

			5*R*,6*S*		5*S*,6*R*	
ion (*m*/*z*)	elemental composition	calcd mass	measd mass	ppm	measd mass	ppm
			(a) $[M - H]^-$			
275	$C_{10}H_{15}N_2O_7$	275.0879	275.0887	2.9	275.0883	−1.5
257	$C_{10}H_{13}N_2O_6$	257.0774	257.0785	4.3	257.0771	−1.2
232	$C_9H_{14}NO_6$	232.0821	232.0841	8.6	232.0821	−0.0
201	$C_7H_9N_2O_5$	201.0511	201.0513	1.0	201.0535	11.9
185	$C_7H_9N_2O_4$	185.0562	185.0574	6.5	185.0576	7.6
158	$C_6H_8NO_4$	158.0453	158.0462	5.7	158.0466	8.2
142	$C_5H_4NO_4$	142.0140	142.0136	−2.8	142.0151	7.7
116	$C_4H_6NO_3$	116.0348	116.0352	3.4	116.0352	3.7
			4(b) $[M + Na]^+$			
299	$C_{10}H_{16}N_2O_7Na$	299.0851	299.0844	−3.7	299.0844	−3.7
281	$C_{10}H_{14}N_2O_6Na$	281.0750	281.0747	−1.1	281.0735	−5.3
209	$C_7H_{10}N_2O_4Na$	209.0538	209.0549	5.3	209.0541	1.4
182	$C_6H_9NO_4Na$	182.0429	182.0440	6.0	182.0444	−8.2
156	$C_5H_{11}NO_3Na$	156.0637	156.0663	16.7	156.0632	−3.2
139	$C_5H_8O_3Na$	139.0371	139.0380	6.5	139.0362	−6.5
113	$C_3H_6O_3Na$	113.0215	113.0214	−0.9	113.0218	2.7

of the $[M - H]^-$, $[M + H]^+$, and $[M + Na]^+$ ions on both a quadrupole ion trap and a quadrupole time-of-flight mass spectrometer. Those glycol lesions fragment in a similar way upon collisional activation: water loss occurs readily for all the three pairs of pyrimidine glycols. The relative propensities of water loss form the basis for distinguishing the two isomers: The 6*S* isomer always shows more facile loss of water than the 6*R* isomer in all the product ion spectra we studied except those of the $[M - H]^-$ ions of the mCg. MS/MS of the latter does not show water loss. High-resolution MS/MS measurement on a Q-TOF instrument supports our structural assignment of fragment ions.

Because the difference of product ion spectra of the two cis isomers lies only in the relative abundances of fragment ions, we established the reproducibility of the spectra by acquiring MS/MS at various collisional energies.

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SUPPORTING INFORMATION AVAILABLE

Additional data as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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