Reaction Mechanism of Uracil Bromination by HBrO: A New Way To Generate the Enol−Keto Form of 5-Bromouracil

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Knowledge on the uracil bromination reaction is helpful for understanding the origin of the mutagenicity of 5-bromouracil (BrU). To get more details about this reaction, we explore the corresponding reaction mechanism by theoretical method. A total of seven pathways were studied for this purpose. The diketo form of BrU is observed as the main product in these pathways, which agrees well with experimental results. The most energy-favorable reaction pathway is found to be that Br and OH attacked the opposite sides of uracil. The reaction intermediate reported in the experiment is predicted to be reasonably stable. In the following step, a dehydration process occurs between H11 and O13−H14 when there are no explicit H_2O taking part. However, when there are explicit water molecules in the environment, explicit H_2O will lower the reaction barrier in the formation of reaction intermediates and the final product BrU. A proton-transfer process from C5 to O10 is facilitated by explicit H_2O, which results in enol−keto form intermediate of this modified base (defined as BrU*). These results indicate a new way to generate the enol−keto form of BrU.

Introduction

5-Bromouracil (BrU) is an analogue of thymine and uracil and a powerful mutagen causing A-T to G-C transitions. It has been widely accepted that the mutagenic action of BrU is based on its enolization or ionization.[1−4]

Many researches, including model hypothesis,[2] experimental,[3−15] and theoretical[16] studies, have revealed that BrU does induce base−base mismatch. In earlier papers, we have shown that the explicit water molecules affect stabilization and mutagenicity on uracil simultaneously.[17,18] The bromine substitute at position 5 of uracil will lead to the loss of water protection for BrU, and BrU was indicated to be mutagenic.[16]

However, as we know, the canonical nucleic acid bases (adenine, guanine, thymine, uracil, and cytosine) should exist as the main form in the double helix. Where does BrU come from? Early studies have shown that Br_2/H_2O or hypobromous acid (HOBr) can oxidize uracil into BrU.[19,20] Furthermore, eosinophils could use eosinophil peroxidase, hydrogen peroxide (H_2O_2), and bromide ion (Br^-) to generate HBrO.[21,22] HOBr generated by eosinophil peroxidase might brominate uracil to BrU.[23−25] A similar phenomenon was also observed when myeloperoxidase, hydrogen peroxide, and bromine system coexisted with uracil.[26,27]

Though the experimental results have revealed that HOBr generated by eosinophil might brominate uracil to BrU, the reaction mechanism remains ambiguous. More details about this reaction can help us understand the origin of the mutagenicity of BrU. As we know, quantum mechanical theoretical methods have become a powerful tool to study the structures of biological molecules and corresponding biochemical process.[28−36] Especially, it has obvious advantages in explaining the mechanism of biochemical processes.[37−40] As we known, reaction intermediates are usually quite hard to detect by general experimental methods. However, the reactive intermediate and reaction transition state can be predicted by theoretical methods. Thus, in the present work, all the possible reaction pathways of uracil bromination reaction are studied and the most energy-favorable one is found out. Though the diketo form of 5-bromouracil has been observed as the main product in the reaction between HBrO and uracil,[23−25] based on our theoretical study, we found that there are enol−keto forms of 5-bromouracil appearing as reaction intermediates. Some theoretic researches have indicated that the transition from the diketo form of BrU to the enol−keto form of BrU was very hard.[16,41,42] Hence, the appearance of these enol−keto form reaction intermediates is helpful for us to understand the origin of the mutagenicity of BrU.

Computational Methods

It had been shown in detail that B3LYP and MP2 gave similar results when the geometrical and vibrational features of nucleic acid bases were concerned.[43] Density functional theory (DFT) is an excellent compromise between computational cost and reasonable results. Therefore, in this paper the hybrid functional B3LYP with the basis set 6-311+G* was used for all of the calculations. Specially, the relativistic effect for Br atom was also taken into consideration by using a hybrid basis set (LANL2DZ basis set for Br atom and 6-311+G* basis set for other atoms). All the energy values reported in this work were corrected by ZPE. All calculations have been performed with the Gaussian 98 suite of packages.[44]

The computed stationary points have been characterized as minima or transition states by diagonalizing the Hessian matrix and analyzing the vibrational normal modes. In this way, the stationary points can be classified as minima if no imaginary frequencies are shown or as transition states if only one imaginary frequency is obtained.

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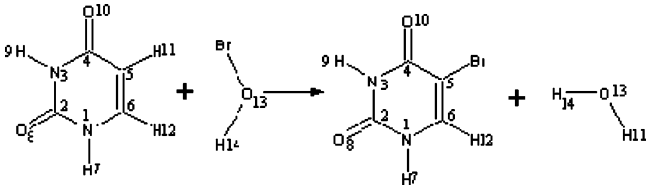

Figure 1. Bromination of uracil by HOBr.

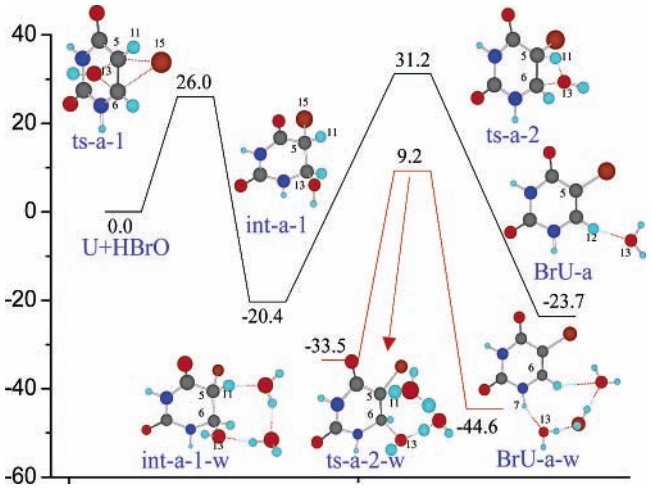

Figure 2. Reaction pathway A (Br and OH of HBrO attack C5 and C6 from opposite sides of uracil). The red line represents the reaction process assisted by explicit water molecules. The vertical axes in Figures 2−5 represent energies given in kilocalories per mole. The corresponding structure parameters are listed in Supporting Information.

The rate constant k is estimated by using transition-state theory:

$$k = \frac{k_B T}{h} \exp(-\Delta^{\ddagger} G/RT)$$

The equilibrium constant K_{eq} is provided by a statistical thermodynamic treatment with

$$K_{eq} = \exp(-\Delta_r G/RT)$$

where G is the sum of electronic and thermal free energies taken from the Gaussian output file.

Results and Discussion

The atomic labeling is presented in Figure 1. Three possible attacking methods of HBrO were considered in this work: (1) Br and OH of HBrO attack C5 and C6 from opposite sides of uracil, (2) Br and OH of HBrO attack C5 and C6 from the same side of uracil, or (3) HBrO forms a halogen bond with O10 and attack C5 in the plane of uracil. In all of these reaction pathways, the diketo form of 5-bromouracil has been obtained as the main product, which agrees well with the experimental results.[23] However, it is worth noticing that in some of the reaction pathways the enol−keto forms of 5-bromouracil appear as the reaction intermediates.

1. Br and OH of HBrO Attack C5 and C6 from Opposing Sides of Uracil. The relative energies of the different transition states or intermediates in this attacking method are presented in Figures 2−4. Figure 3 reveals another reaction pathway of the intermediate int-a-1, and Figure 4 shows another reaction pathway of the intermediate int-b-2.

Among the three possible attacking methods mentioned above, Br and OH of HBrO attacking C5 and C6 from opposing sides of uracil is the most energy-favorable one. This reaction process is via a transition state, ts-a-1, first. The barrier height of ts-a-1 is 26.0 kcal/mol, which is much lower than the other two attacking methods (45.7 kcal/mol for method 2 and 72.3 kcal/mol for method 3).

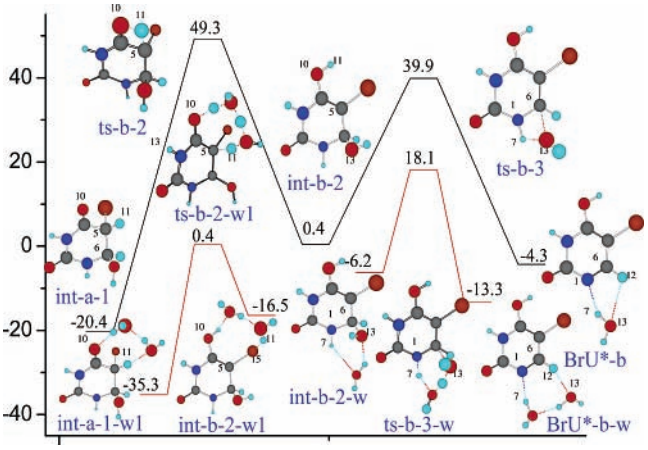

Figure 3. Reaction pathway B (another reaction pathway of int-a-1 in pathway A). The red line represents the reaction process assisted by explicit water molecules.

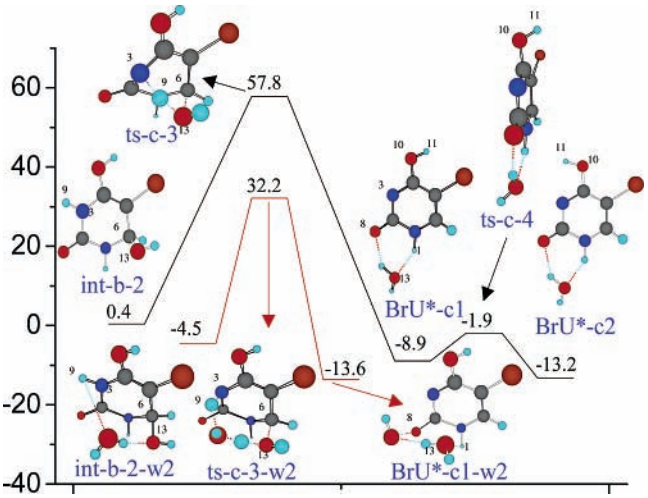

Figure 4. Reaction pathway C (another reaction pathway of int-b-2 in pathways B and E). The red line represents the reaction process assisted by explicit water molecules.

Previous kinetic analyses had suggested that the uracil bromination reaction involved an initial formation of a bromized intermediate.[19,23] However, through the experiment, it is hard to judge whether the intermediate is the trans form (int-a-1) or the cis form (int-d-1). Our calculated results reveal that the most energy-favorable pathway will generate the trans form bromized intermediate int-a-1. Int-a-1 is reasonably stable because the transition process from U + HBrO to int-a-1 is exothermic (20.4 kcal/mol) and a quite high reaction barrier follows it. Hence, it is possible to observe int-a-1 in the experiment.[19,23]

For int-a-1, there are two pathways for H11 to transfer: (a) transfer to O13 of int-a-1 (Figure 2) or (b) transfer to O10 of int-a-1 (Figure 3). As a classical pathway suggested by previous works,[19,23] H11 transfers toward O13 by intramolecular proton transfer and the 5-bromouracil is generated by a dehydration reaction. Such a pathway is described as pathway A in this work (Figure 2). It is clear that such a dehydration process must surmount a much higher barrier (51.6 kcal/mol for ts-a-2), which is 25.6 kcal/mol higher than the brominating process. It agrees well with early experimental results that the bromination of

uracil is rapid whereas the dehydration process is very slow.[19,23] Some early theoretical studies have indicated that intramolecular proton-transfer processes can be accelerated by explicit water molecules.[17,29,38,45] Experimental results also showed that when these complexes containing active hydroxy group (such as water or methanol) were introduced into the reaction, the proton-transfer process would become faster.[46] It has been proven that there are at least nine water molecules in the first hydration shell of uracil, both in the plane and out of the plane of uracil.[17,47−49] The influences of water molecules on the bromination of uracil were also investigated. It is found that the transfer process of H11 also could be assisted by these explicit water molecules. Two water molecules taking part in the proton-transfer process could reduce the barrier of the dehydration process of int-a-1 by 8.9 kcal/mol (int-a-1-w → BrU-a-w).

On the basis of theoretical calculation, we found that H11 transfer to O10 of int-a-1 is also energy-possible (Figure 3). An enol−keto form intermediate int-b-2 is generated in this process. Though the barrier of the intramolecular proton transfer in the reaction of int-a-1 → int-b-2 is 18.1 kcal/mol higher than that of int-a-1 → BrU-a, when water molecules take part in the proton-transfer process, the barrier of H11 transfer to O10 is reduced by 34.0 kcal/mol. As a result, the barrier of int-a-1-w1 → int-b-2-w1 becomes lower than that of int-a-1-w → BrU-a-w. It indicates that when there are no explicit water molecules assisting, H11 transferring to O13 is more energy-favorable. However, when there are explicit water molecules assisting, it is more preferable for H11 to transfer to O10 of int-a-1 and a bromized intermediate int-b-2 is produced. For int-b-2, there is a competitive dehydration process: dehydration between O13−H14 and H7 (Figure 3) or between O13−H14 and H9 (Figure 4). Calculated results show that the dehydration between O13−H14 and H7 is always more favorable than that between O13−H14 and H9 no matter whether there are explicit water molecules or not. However, in both two dehydration processes, the enol−keto forms of 5-bromouracil tautomers are generated as the reaction intermediates. Similar enol−keto tautomers had been reported for uracil [33,50] or 5-bromouracil.[41−42] Compared with the diketo form of BrU, these enol−keto form tautomers are very unstable and they should rapidly tautomerize to the diketo form with water molecules assisting (Figure 8b).

It is interesting to find that previous experiment had revealed that for the nucleosides uridine and deoxyuridine, the reaction dehydrates to the corresponding 5-bromouridine at a much slower rate.[23] It could be indirect evidence of reaction pathways B and C. Our calculated results reveal that there is a dehydration process between H7 and O13−H14 in the minimal energy reaction pathway (Figure 3). However, for uridine, where the position of H7 is substituted by ribose, a dehydration process between H7 and O13−H14 becomes impossible. It must be by another pathway that the dehydration process happens between H9 and O13−H14 (Figure 4) or between H11 and O13−H14 directly (Figure 2), both of which possess much higher barriers. Hence, for uridine, the dehydration process is very slow.

As indicated by some pioneer works,[1−4] the mutagenic property of 5-bromouracil may come from its enol−keto forms. The conventional viewpoint about enolization believes that the presence of the bromine at position 5 significantly alters the distribution of electrons in the base, so that BrU can spend part of its existence in the rare enol form. Orozco et al.[41] had found that the diketo form of BrU was more stable than the enol tautomers in either gas phase or aqueous. Furthermore, uracil and 5-bromouracil have similar stability. Hence, they suggested that the mutagenic effect of BrU was due to its ability to lose a proton at N5 rather than its tendency to form enol tautomers.[41] Due to the same behavior of uracil and 5-bromouracil in the ground electronic state, Hanus et al.[42] suggested the electronically excited state should be taken into consideration when we discuss the mutagenicity of BrU. Two typical tautomerizing processes of uracil and 5-bromouracil are listed in Table 1. Upon comparison with uracil, there is not an obvious advantage for BrU to tautomerize to its enol−keto forms. The activation energy and Gibbs free energy change of uracil and 5-bromouracil tautomerizing are almost equal. Besides these typical tautomerizing processes, the population of the enol−keto form of 5-bromouracil may be slightly increased in some of other tautomerizing processes. For example, in our previous study, we had suggested that the bromine substitute at position 5 of uracil could lead to the loss of the protection coming from water and the possibility of tautomerism from BrU to BrU* would be higher than that from U to U*.[16,17] However, the canonical diketo form of BrU should still exist as the main form absolutely.[16] In these cases, it is disputatious to explain the mutagenic property of 5-bromouracil when BrU must tautomerize from the diketo form to its enol−keto form to induce base−base mismatch. The appearance of enol−keto forms as reaction intermediates suggests a new way to explore the mutagenic property of 5-bromouracil. In this situation, the hard tautomerizing process from diketo form to enol−keto form could be avoided and the reaction intermediate (the enol−keto form of BrU) may mismatch with guanine and adenine directly. Our research on this field is in progress.

TABLE 1: Activation Energies and Gibbs Free Energy Changes of Base Tautomerism[a]

	activation energy (kcal/mol)	ΔG (kcal/mol)
U → U*-c2	44.5	14.4
BrU → BrU*-c2	45.0	14.7
U-W → U*-c2-W	20.4	11.5
BrU-W → BrU*-c2-W	20.0	11.2

[a] For uracil, U*-c2 and U*-c2-W refer to similar structure like BrU*-c2 and BrU*-c2-W shown in Figure 8. The corresponding structures are listed in Supporting Information.

2. Br and OH of HBrO Attack C5 and C6 from the Same Side of Uracil. The relative energies of the different transition states or intermediates in this attacking method are given in Figures 5 and 6. Figure 6 shows another reaction pathway of the intermediate int-d-1.

Br and OH attacking C5 and C6 from the same side of uracil generate a cis form bromized intermediate int-d-1 (Figure 5). Compared with the attacking method from the opposite sides of uracil (pathway A), reaction in this attacking method must overcome a much higher barrier (19.7 kcal/mol higher than that of pathway A). It is mainly because the repulsion between Br and OH in the transition states is enhanced when they bond to the same side. However, the cis form bromized intermediate int-d-1 has similar stability compared with the trans form int-a-1. Unlike pathway A (Figure 2), the intramolecular dehydration between H11 and O13−H14 of int-d-1 is more complicated and much harder because H11 and O13−H14 are located at the opposite sides of int-d-1. Though a series of low-barrier steps follow ts-d-2, the high barrier of the rate-determining step (83.4 kcal/mol for ts-d-2) indicates that such an intramolecular dehydration process is quite unfavorable. Furthermore, it is also the most unfavorable one in all the seven reaction pathways investigated in this work.

There exists another competitive reaction pathway for int-d-1. Compared with the pathway listed in Figure 5, the proton transfer from C5 to O10 of int-d-1 is much easier, especially

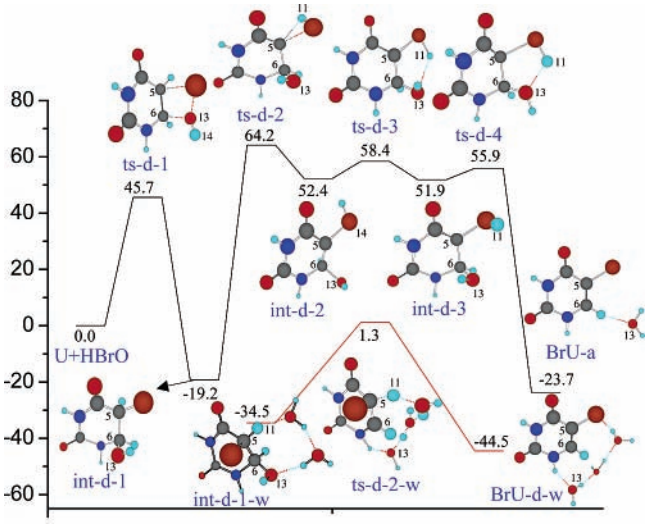

Figure 5. Reaction pathway D (Br and OH of HBrO attack C5 and C6 from the same face of uracil). The red line represents the reaction process assisted by explicit water molecules.

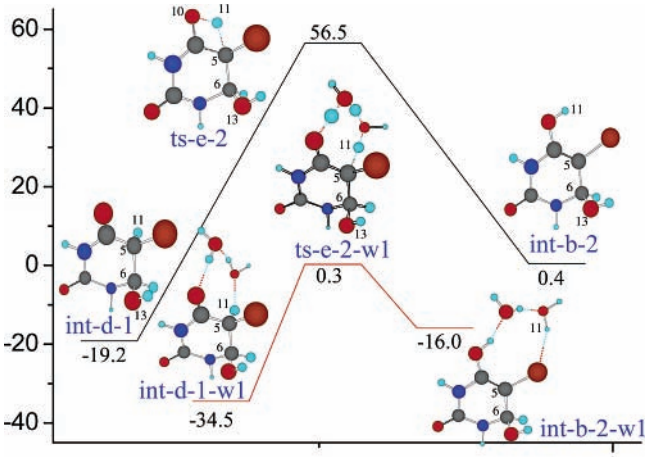

Figure 6. Reaction pathway E (another reaction pathway of int-d-1 in pathway D). The red line represents the reaction process assisted by explicit water molecules.

when water molecules participate in the transfer process (Figure 6). The barrier from int-d-1-w1 to int-b-2-w1 is 34.8 kcal/mol, which is 48.6 kcal/mol lower than that from int-d-1 to int-d-2. It indicates that if Br and OH of HBrO attack C5 and C6 from the same face of uracil, the following reaction pathway should be pathway E (Figure 6). In pathway E, an enol−keto form intermediate int-b-2 will be generated. Int-b-2 is also an intermediate in pathway B, and its reaction process has already been discussed carefully above (Figures 3 and 4). Thus, discussions about int-b-2 are omitted in this section.

3. HBrO Forms a Halogen Bond with O10 and Attacks C5 in the Plane of Uracil. The intermolecular bonding between halogen atoms and nitrogen/oxygen atoms is called a halogen bond. Recently, the halogen bond has been found to be an important interaction that can affect the structural features of biological molecules significantly.[50] Our calculated result reveals that HBrO can also form a halogen bond with O10 of uracil with a binding energy of 4.1 kcal/mol (Figure 7). This value indicates that the binding between HBrO and O10 belongs to a medium-intensity halogen bond. There also exists a halogen bond in the corresponding product of this attacking method. The binding energy of the halogen bond between H_2O and the Br atom of BrU is much weaker (1.5 kcal/mol).

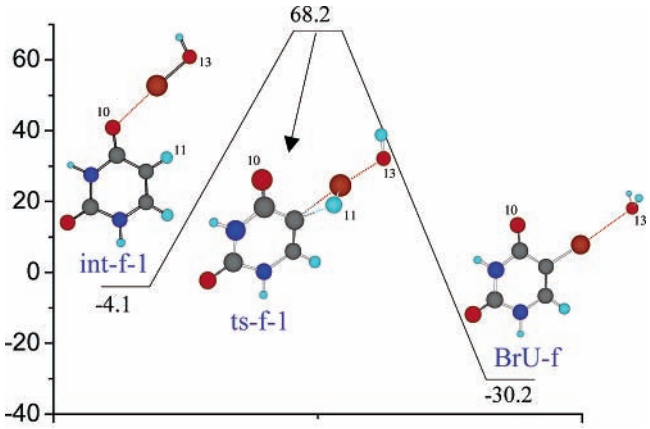

Figure 7. Reaction pathway F. Energies are corrected by ZPE.

TABLE 2: Rate Constants (*k*) and Equilibrium Constants (K_{eq}) of Base Tautomerism

	k (s^{-1})		K_{eq}	
	isolated[a]	H_2O[b]	isolated[a]	H_2O[b]
BrU*-b2 → BrU*-b3	7.0×10^{-12}	4.6×10^{3}	5.0×10^{5}	1.8×10^{2}
BrU*-b4 → BrU*-c2	3.6×10^{-14}	8.6×10^{4}	6.4×10^{1}	5.3×10^{3}
BrU*-c2 → BrU	4.4×10^{-10}	2.0×10^{6}	2.8×10^{10}	1.5×10^{8}

[a] Isolated tautomerizing processes. [b] Tautomerizing processes with water assisting.

Such an attacking method must surmount a much higher barrier (72.3 kcal/mol), and a corresponding water-assisted proton-transfer process was not found. Compared with the other two attacking methods, HBrO attacking C5 of uracil directly is the most energy-unfavorable one.

4. Tautomerizing Processes of BrU*. As indicated by the experimental results, the diketo form of BrU was generated as the main product.[19,23] However, our calculated results indicate that there are other reaction pathways producing enol−keto form bromized intermediates BrU*. Why was BrU* not observed in the experiments? We believe it is mainly because that there exists a rapid tautomerizing process from the enol−keto form BrU* to the diketo form of BrU (Table 2).

As we can see in Figure 8, the diketo form BrU is the most stable structure among all the tautomers of 5-bromouracil. The enol−keto form intermediates generated in pathway B (BrU*-b) and pathway C (BrU*-c1, BrU*-c2) should spontaneously tautomerize toward the diketo form by proton transfer or proton rotation processes. The barrier of proton rotation is very low (7.5 kcal/mol for ts-BrU*-b2 and 7.8 kcal/mol for ts-BrU*-b4). Though the barriers of intramolecular proton transfer process are much higher (32.7, 35.8, and 30.2 kcal/mol for ts-BrU*-b3, ts-BrU*-c2, and ts-BrU, respectively; Figure 8a), when there are explicit water taking part in the transfer process, the barriers are reduced to 12.5, 10.7, and 8.9 kcal/mol for ts-BrU*-b3-w, ts-BrU*-c2-w1, and ts-BrU-w, respectively (Figure 8b). The barriers of the tautomerizing processes are about 30.0 kcal/mol lower than that of the rate-limiting step listed in pathways A and D. Hence, compared with the bromination reaction of uracil, the tautomerizing processes of the enol−keto tautomers assisted by water molecules are very rapid.

Furthermore, the Gibbs energy changes from the enol−keto form to the diketo form are all larger than 11.2 kcal/mol, which indicates that the equilibrium constants from the enol−keto form to the diketo form are all larger than 10^9. That is, the amount of the enol−keto form tautomers is very small.

The rapid tautomerizing process from the enol−keto form to the diketo form as well as the tiny quantity of the enol−keto

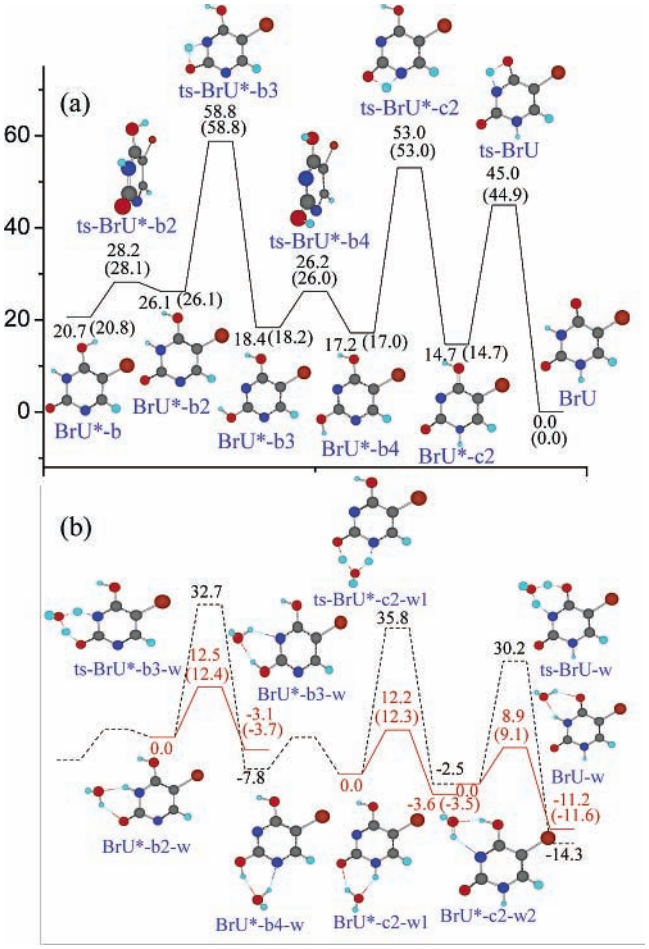

Figure 8. Tautomerizing processes from enol−keto form to diketo form; values in parentheses include the pseudopotential for Br atom. (a) Isolated tautomerizing processes; (b) tautomerizing processes with water assisting. The broken line in panel b represents the isolated tautomerizing processes.

form gives an answer to why it was very hard to detect the enol−keto forms as reaction intermediates. However, by some special experimental techniques, it is also possible to observe rapid processes and detect minor amounts of compound.[46,51]

5. Relativistic Effect for Br Atom. To investigate the relativistic effect for Br atom, we also used a pseudopotential for the Br atom. We calculated the base-tautomerizing processes of BrU* at both B3LYP/6-311+G* and B3LYP/Gen (LANL2DZ basis set for Br atom and 6-311+G* basis set for other atoms) (Figure 8). Both calculations give almost the same results, which indicates that the relativistic effect for Br atom is very small. Hence, though the results listed in Figures 2−7 do not include a relativistic effect for Br atom, we believe they are also credible.

Conclusions

In the present article, we describe a theoretical study on the reaction mechanism of uracil bromination by HBrO. All of the investigated reaction pathways indicate that the diketo form of BrU is the main product, which is well in agreement with the experiment results.[19,23] It has been found that the most energy-favorable reaction pathway is that Br and OH of HBrO attack uracil from opposite sides with a barrier of 26.0 kcal/mol. Then a dehydration process happens between H11 and O13−H14 with a barrier of 51.6 kcal/mol when there are not explicit water molecules. However, when explicit water molecules participate in the reaction, a proton transfer from C5 to O10 becomes much easier (with a barrier of 35.7 kcal/mol), which will generate the enol−keto form of 5-bromouracil intermediates. These enol−keto form tautomers will tautomerize to the most stable diketo form.

Our theoretic results can explain the following experiment phenomena: Why is the diketo form of BrU generated as the main product? Why is the dehydration process slower than the bromination process? Why could the bromized intermediate int-a-1 be observed in the experiment? Why is the dehydration process of uridine very slow? More important, our theoretical results indicate that there are enol−keto forms of BrU appearing as reaction intermediates.

Though the enol−keto form tautomer of 5-bromouracil is short lived and present in small amounts, its appearance as the reaction intermediate should have significant meaning on the mutagenicity of 5-bromouracil. It is easy to imagine that when there are guanine and adenine in the environment, the mismatch between these enol−keto form reaction intermediates and guanine/adenine should be quite competitive. Such a competitive pathway may avoid the hard tautomerizing process from the diketo form to the enol−keto form and induce base−base mismatch directly. Further research about the mismatch between the enol−keto form reaction intermediates and guanine/adenine is now in progress.

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Supporting Information Available: Imaginary frequencies of all the transition states and Z-MATRIX of all the calculated structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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