Mutagenic Properties of 5-Halogenuracils: Correlated Quantum Chemical ab Initio Study†

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ABSTRACT: The relative stability of all possible 5-bromouracil tautomers was studied theoretically in a gas phase, in a microhydrated environment (with one water molecule), and in bulk water. Tautomer structures were determined by gradient optimization at the correlated ab initio quantum chemical level with an extended basis set of atomic orbitals. The role of water was examined by using a self-consistent reaction field method. The relative stabilization and free energies in the gas phase, the microhydrated environment, and the bulk water clearly support the preference of the canonical keto form of 5-bromouracil in all mentioned environments. An increased abundance of enol tautomers when passing from uracil to 5-bromouracil is not supported by our calculations. Thus, the tautomeric model of the mutagenic activity of 5-bromouracil proposed previously [Hu et al. *Biochemistry* (2004) *43*, 6361] can be refuted. The validity of other mutagenic models was also discussed, and finally a new mechanism for explaining the mutagenic activity of halogenuracils based on their different behaviors in triplet excited states was suggested.

Nucleic acid (NA)[1] bases are presented in DNA mostly as the amino and keto tautomers. Despite the fact that the presence of rare forms (imino and enol forms) is only negligible (∼0.01%), their existence in NA might play a significant role in mutagenic properties of the NA. Watson and Crick (*1*) first suggested that rare forms of NA bases are responsible for these properties. Topal and Fresco (*2*) raised the hypothesis that NA bases enol tautomers are connected with point mutations. Tautomerization of NA bases, however, represents only one of several possible mutagenic mechanisms; wobble (*3*) and ionization (*4*) models were suggested as an explanation for their mutagenic activity as well. In the past, theoretical and experimental studies revealed the possible role of tautomerism of NA bases and also the possibility of proton transfer in base pairs (*5*−*7*). Tautomerizations of NA bases in the gas phase, nonpolar solvent, and water environments were also often studied theoretically (for a survey of literature, see recent reviews of Hobza and Šponer (*8*), Orozco (*9*), and Lezsczynski (*10*)). Recently, Harris (*11*) experimentally proposed the role of the equilibrium constant of tautomerization. Also the role of DNA-polymerase in incorporation of nucleic acids bases analogues was discussed (*12*).

Halogen-substituted NA bases represent interesting NA analogues with regard to the mutagenic activity. BrU is one of the well-known species with mutagenic properties and, for BrU, it was found that it can be created physiologically in inflammatory tissue (*13a*). Recently, however, the mutagenic action of BrU was questioned (*13b*,*c*). 5-Fluorouracil (FU), also known as a mutagenic agent, is also used as a common cytostatic agent in oncology and is used to treat cancer of the colon, rectum, breast, stomach, and pancreas (*14*). Clearly, the choice of halogen plays a key role, and this fact should be considered when evaluating various mutagenic mechanisms. For example, the ionization or "ionized form" mechanism that has been proposed experimentally by Sowers (*15*) would suggest the highest activity for the 5-halogenuracil XU tautomer, which exhibits the lowest PA expressed as pK_a.

It should be emphasized that experimental evidence on a preference of a specific mutagenic mechanism are ambiguous and theoretical calculations can help to explain the origin and nature of mutagenic activity of rare tautomers of NA bases (*16*). Because of the nature of interactions in nucleic acids bases, the theoretical description of these systems must be performed at highly accurate levels with the inclusion of post-HF treatments. The lack of electron correlation and the use of a small basis set can easily lead to wrong conclusions, so a possible misinterpretation of low-level theoretical data must be carefully avoided.

Recently, Hu et al. (*17*) published an article in the New Concepts section in *Biochemistry* supporting the rare tautomer mechanism of mutagenicity. This paper described a study in which far-fetched, frequently incorrect conclusions were made on the basis of wrong assumptions, misinterpreted results, and incorrectly used data from reference literature. Furthermore, they used an insufficient HF method with minimal STO-3G basis set and failed in the majority of their assumptions about the mutagenic mechanism of BrU. The

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[1] Abbreviations: NA, nucleic acid; BrU, 5-bromouracil; FU, 5-fluorouracil; XU, 5-halogenuracil; PA, proton affinity; HF, Hartree-Fock; PES, potential energy surface; FES free energy surface; SCRF, self-consistent reaction field; RI-MP2, resolution of identity; BSSE, basis set superposition error; MD/Q, molecular dynamics/quenching.

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HF/STO-3G level was selected on the basis of comparisons of calculated keto−enol tautomeric equilibrium of uracil (U), and BrU with literature data (*16*). Two significant errors, made by the authors, were responsible for this wrong choice. Different energy units were used for a comparison with literature data (kJ/mol, while kcal/mol are used in original paper (*16*)). Furthermore, they used values for another enol tautomer (compare Figure 3 and Table 3 in Orozco's work (*16*) to Figure 1 in Hu's work (*17*)). When the comparison is properly performed, results obtained with the DFT method, employing a B3LYP functional with a 6-31++G(d, p) basis set, are in good agreement with the reference QCISD data (*16*), while the HF method fails.

The aim of this paper is to evaluate various mutagenic mechanisms of various 5-halogenuracils with a special attention devoted to 5-bromouracil. On the basis of high level correlated ab initio calculations, we show that the tautomeric model of mutagenic activity 5-bromouracil suggested recently by Hu et al. (*17*) can be refused. Finally, a new mechanism of mutagenic activity of this system will be proposed.

STRATEGY

To obtain the correct information on the equilibrium between different tautomers, the theoretical investigation must be performed as follows:

1. To obtain reliable gas-phase geometries, relative energies, and stabilization energies, correlated ab initio methods that include medium or extended basis set should be used.

2. Since entropy can play an important role, free energy calculations, rather than potential energy calculations, should be used to evaluate relative and stabilization energies. The determination of the structure and energy of the transition state represents a critical point, and full gradient optimization should be applied to all structures.

3. When studying the physical and chemical properties of BrU, it is necessary to consider the relativistic effects of bromine.

The role of microhydration and the bulk solvent should be taken into account. The scan of PES and FES of monohydrated systems was done using the molecular MD/Q technique followed by ab initio calculations. The hydration by bulk water was covered by the SCRF method using the COSMO approach (*18*).

METHODS

Quantum Chemical Calculations. The energetical and geometrical characteristics of 13 BrU tautomers in the gas phase were investigated using the RI-MP2 procedure (*19*) with a cc-pVTZ basis set using the TURBOMOLE 5.6 package (*20*). The results were verified by reference calculations at the full MP2 level employing the same basis set using the GAUSSIAN 03 program (*21*).

A harmonic vibration analysis providing free energies (zero-point vibration energies, temperature-dependent enthalpy terms, and entropies are included), and also the character of the stationary point (transition state) was performed at the MP2/6-31G** and B3LYP/6-31G** level of theory. Thermodynamic characteristics were taken from partition function using a rigid rotor/harmonic oscillator/ideal gas approximation. Relative gas-phase free energies were determined as the sum of the relative energy changes, zero point vibration energies, temperature-dependent enthalpies and entropies.

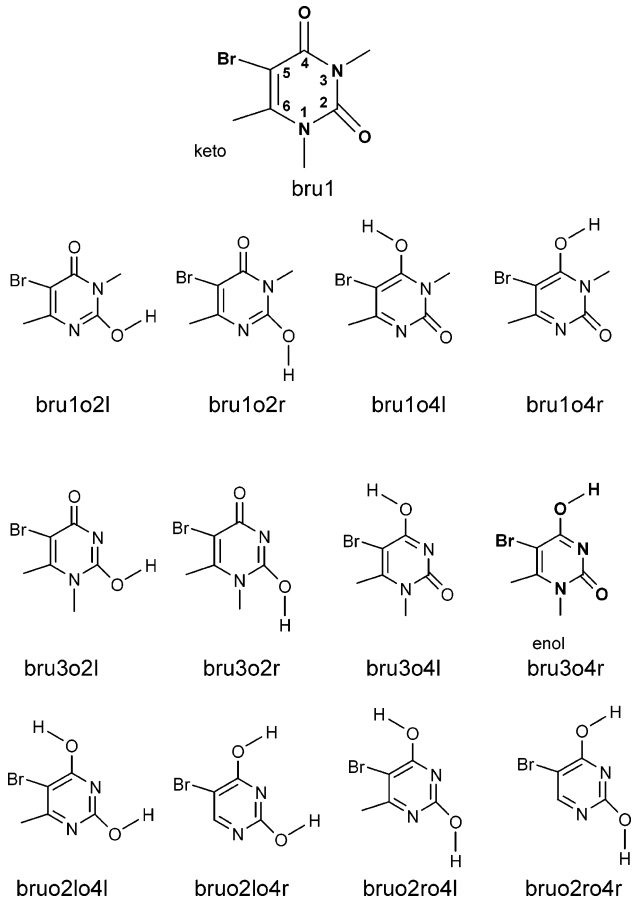

FIGURE 1: Structures of 13 tautomers of 5-bromouracil. Standard atom numbering is depicted; keto and enol forms important for the explanation of tautomeric mechanism of mutagenicity are highlighted.

The proton affinities of XUs were calculated as the difference between the total optimized energy of the neutral and the deprotonated (anionic) systems. Energies were determined at B3LYP/6-31G**, MP2/cc-pVTZ, and MP2/SDD levels. The latter one utilized pseudopotentials, which effectively covers the relativistic effects of bromine atom.

The interaction energies of BrU···water complexes were determined at the RI-MP2/cc-pVTZ level by including the BSSE (*22*) and deformation energy. The BSSE and deformation corrections were also applied systematically for other complexes mentioned below.

Base pairs stabilization energies as well as the transition states describing the proton transfer between the bases were determined at the B3LYP/6-31G** level.

MD/Q Technique. MD/Q simulations were performed in the NVE microcanonical ensemble (N,V,E means number of particles, volume, and energy) within a quaternion formalism using the modified Cornell et al. (*23*) force field.

The resulting code uses a fifth-order predictor-corrector algorithm with a 0.5 fs integration step. The MD simulations were performed at a constant total energy corresponding to the average temperature of 298 K. Every 1 ps the MD run was interrupted, the kinetic energy was removed, and the structure of the cluster of U (BrU) with one water molecule was optimized using the conjugate gradient method, the

Table 1: Relative Stability of 13 Possible Tautomers (cf. Figure 1) of 5-Bromouracil[a]

structure	dipole[b]	ΔE(RI-MP2)[c]	ΔE(MP2)[d]	ΔG(MP2)[e]	ΔG(C-PCM)[f]	ΔG_{tot}[g]
bru1	4.58	0.00	0.00	0.00	0.00	0.00
bru1o2l	4.15	16.48	16.48	17.72	−4.72	13.00
bru1o2r	4.82	8.57	8.57	9.80	1.27	11.07
bru1o4l	6.14	17.91	17.81	18.71	1.13	19.84
bru1o4r	5.32	24.03	24.01	24.87	−6.68	18.19
bru3o2l	7.48	16.36	16.36	17.71	−4.88	12.83
bru3o2r	9.87	26.53	26.53	28.07	−13.44	14.63
bru3o4l	7.39	15.38	15.24	17.31	−2.45	14.86
bru3o4r	4.16	11.55	11.54	11.95	−1.94	10.01
bruo2lo4l	2.36	10.81	10.69	13.48	4.59	18.07
bruo2lo4r	2.41	9.76	9.74	12.28	2.46	14.74
bruo2ro4l	3.37	10.77	10.64	13.53	4.26	17.79
bruo2ro4r	0.95	8.63	8.62	11.16	3.42	14.58

[a] All energies are in kcal/mol. [b] Dipole moment in Debye calculated at the HF/6-31G** level. [c] Relative interaction energy calculated at the RI-MP2/cc-pVTZ level of theory. [d] Relative interaction energy calculated at full MP2/cc-pVTZ level of theory. [e] Relative free energies calculated at full MP2/cc-pVTZ level of theory. [f] Relative hydration (COSMO) free energies calculated at the HF/6-31G*/UAHF level of theory. [g] Sum of relative free energies calculated at the MP2 level and hydration free energies (fifth and sixth column).

geometry and energy in the minimum was stored, and then the MD run was restarted from the point where it was interrupted.

The constants for geometrical parameters of noncanonical tautomers (not parametrized in the standard Cornell et al. force field (*23*)) were derived from quantum chemical calculations using recommended standard procedures (*24*). The atomic charges of the tautomers were generated with the electrostatic potential fitting procedure -RESP- at the HF/6-31G* level**.**

SCRF − Continuum Hydration. Bulk hydration was modeled using the continuum approach based on C-PCM (COSMO) methodology implemented in GAUSSIAN 03 (*21*) since it has been known that it adequately describes the polar solvent (*25*).

The cavity was described by United Atoms Radii optimized at the HF/6-31G* level of theory (UAHF) (*26*). In all cases, we used gas-phase RI-MP2/cc-pVTZ geometry and the GAUSSIAN03 standard parameters (UAHF, scaling 1.2, Solvent Excluded Surface as recommended for COSMO calculations (*27*, *28*).

RESULTS AND DISCUSSION

1. BrU Tautomers in the Gas Phase. Figure 1 shows all 13 possible structures of BrU tautomers. As found in our previous study (*29*) of U and thymine, only classical mesomeric forms can be derived.

Table 1 shows the dipole moments, relative energies, free energies, and C-PCM (COSMO) energies of all tautomers. The dipole moments vary from 1 to 10 D and qualitatively correspond to the values of U (*29*). Substitution of bromine in position 5 does not cause any significant changes of electronic structure compared to the unsubstituted system. Relative RI-MP2 energies (the second column of Table 1) reveal that all enol and dienol forms are significantly less stable energetically (by +9 up to +27 kcal/mol) than the canonical keto tautomer. Very similar energy penalization was detected in the case of bare U (*29*). The relative RI-MP2 and MP2 energies differ only negligibly (cf. column 2 and 3 in Table 1). Since a similar conclusion was also found for cytosine (*30*), guanine (*31*), adenine (*25*), uracil and thymine (*29*) we can confidently state that reliable relative

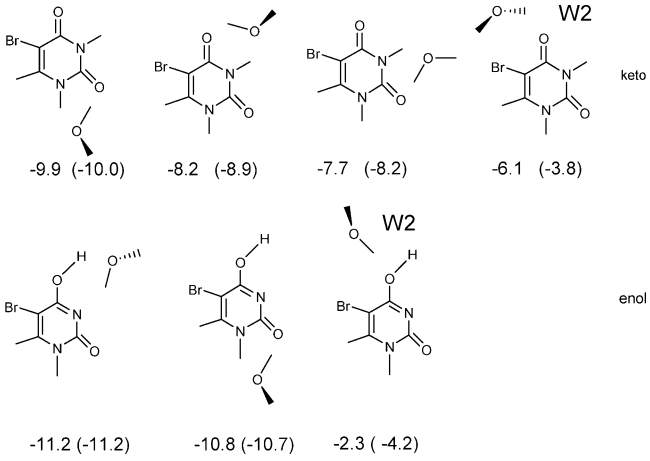

FIGURE 2: Structures of monohydrated canonical keto and enol form of 5-bromouracil considered by Hu et al. (*17*). Interaction energies (in kcal/mol) are depicted below for each structure, and numbers in parentheses indicate interaction of uracil with water in the same binding pattern.

energies of NA bases can be already obtained from the fast and efficient RI-MP2 method.

Comparisons made on relative free energies are quantitatively the same as these obtained from relative energies (see Table 1). The canonical form is even more preferable at the FES compared to the PES. Present gas-phase results are in perfect agreement with previous theoretical and experimental results on 5-substituted uracils (*16*, *32*) and also with our recent study on U (*29*). Thus, we can conclude that the canonical form of BrU can exist as the main form in the gas phase.

2. Microhydrated Tautomers. The four energetically most stable monohydrated structures of the two BrU tautomers (canonical and the enol form considered by Hu et al. (*17*)) obtained from the MD/Q simulation were optimized at the RI-MP2/cc-pVTZ level; their structures are presented in Figure 2. The stability of these structures decreases from left to right. The interaction energies of these structures are also presented in Figure 2; numbers in parentheses refer to the interaction energies of U···water complex.

The water-binding motifs of the most stable gas-phase tautomer (canonical keto form) and enol form used in Hu's paper (cf. Figure 2) agree with patterns found in U (*29*, *33*,

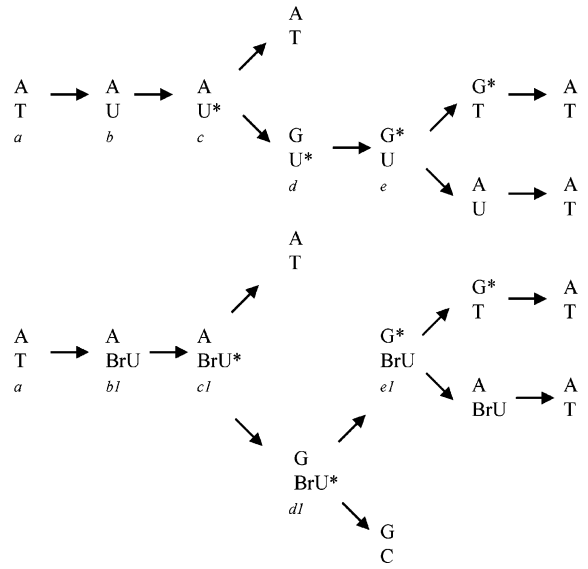

FIGURE 3: Proposed scheme of tautomeric mechanism of mutagenicity of 5-bromouracil (cf. Hu et al. work, ref *17*).

34). However, it must be stressed that these structures are not the global minima at PES.

It is thus evident that bromine substitution in position 5 has only a minor effect on the stability of monohydrated structures. Comparison of BrU and U does not show almost any difference. The exceptions are the least stable structures, where water interacts with the O4 oxygen. For these structures, we found a significant decrease of stability (by approximately 2 kcal/mol) of complexes for both tautomers of 5-bromoracil in comparison with U similar to what was found by Hu (*17*). Also the position of water molecules differs in BrU and U.

We would like to comment on an important point of Hu's et al. work (*17*) showing the importance of water W2 in region S2 (cf. Figure 2) and its "protective" features leading to the decrease of population of the enol form. This is a crucial step for further explanation of the mutagenic activity of BrU. On the basis of our data for BrU*, it is apparent that the energetic penalty of this hydration site is very high (+9 kcal/mol; cf. Figure 2, first and third structures of enol···water), which makes any conclusion about the protection/unprotection ability of water molecule in this site irrelevant. Generally, water in the most stable position has a slightly stabilizing effect on the energy difference between the keto and enol tautomers, reducing it by only about 1 kcal/mol. This is insufficient to influence the equilibrium between tautomers, and, thus, the energetic penalty remains still large enough to prevent population of the enol form.

We would like to stress further that Hu et al. (*17*) did not use any quantitative arguments to support the existence of the rare tautomer in water and the hypothesis of authors about of the protective action of water in this region is speculative.

3. COSMO Results. The results given in the last column of Table 1 show that the presence of the water environment is not decisive. Relative free energies are similar to those obtained in the gas phase. We can thus conclude that tautomeric equilibria in the gas phase, microhydrated environment, and bulk water are strongly shifted in favor of the canonical form. The probability of the existence of the enol

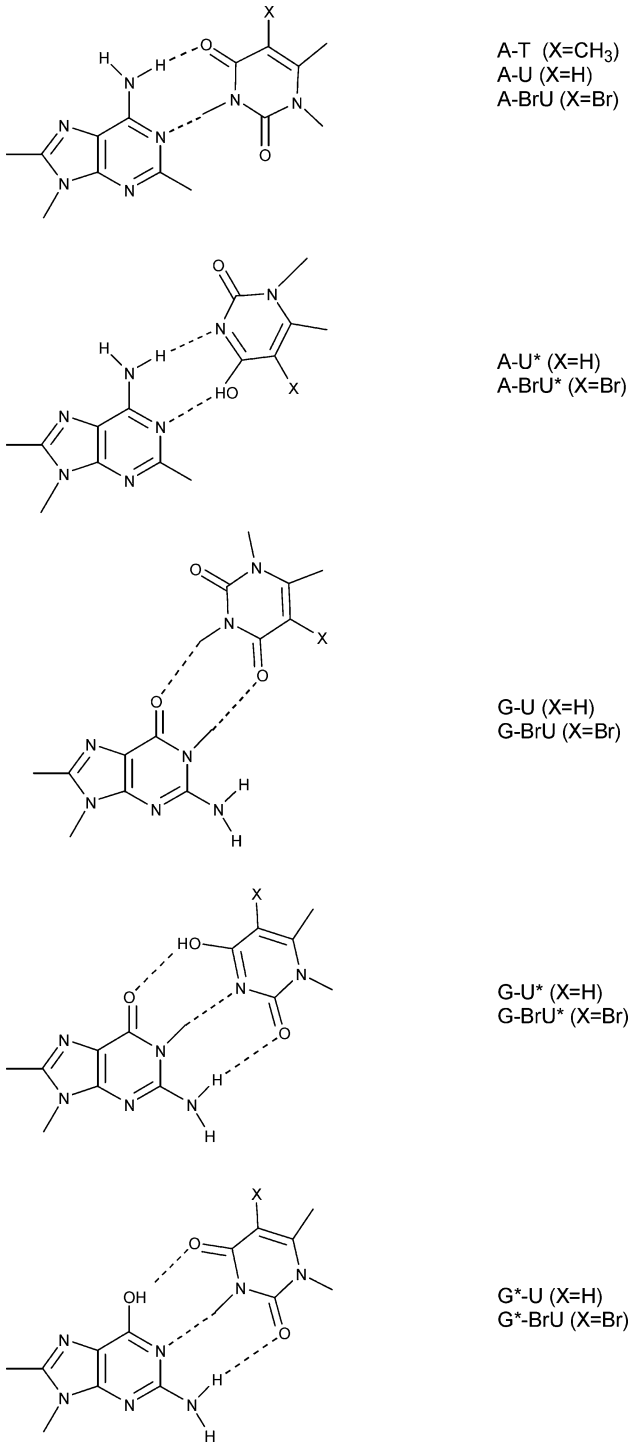

FIGURE 4: Structures of base pairs participating in tautomeric mechanism (cf. Figure 3).

form (cf. Figure 1) is very low, of the order 10^{-7}, which is too low to explain mutagenic activity of BrU.

4. Mutagenic Model of BrU. The mutagenic mechanism of the transition from A−T to G−C induced by BrU (cf. Figures 3 and 4) is based on the fact that BrU is an analogue of thymine and it can interact both with adenine or guanine. The latter base is preferred. If bare U is incorporated during DNA synthesis and replaces thymine, no mutagenic activity is observed. In the following paragraphs, we will discuss the probability of each step as suggested by Hu et al. (*17*) (cf. Figure 3). For this purpose, we compare our results with those obtained by Hu et al.

Table 2: Energy (ΔE) and Free Energy (ΔG) Changes (in kcal/mol) of Base and Base Pair Tautomerism[a]

reaction	U ↔ U*	BrU ↔ BrU*	AU ↔ AU*	ABrU ↔ ABrU*
ΔE	10.8	11.6	8.4	8.5
ΔG	12.5	12.0	8.1	8.0
ΔG/water	10.1	9.5	7.3	8.3

[a] All data were obtained at the B3LYP/6-31G** level. An asterisk means an enol form of base. If not stated otherwise, values in vacuo are presented.

We expect that steps leading to the incorporation of U and BrU into DNA (a → b and a → b1) are possible and can occur.

b → c and b1 → c1 Steps. This is the critical step of the whole process. Tautomeric equilibria between U → U* and BrU → BrU* (* denotes the enol forms discussed; cf. Figures 1 and 4) are clearly shifted to the keto forms and the probability of the enolization process is negligible in vacuo, in a microhydrated environment, and in bulk water (see above). The ΔE, ΔG/gas-phase values as well as ΔG/water values are highly positive (see Table 2), which indicates a very low probability of the processes considered. Adding bromine affects the equilibrium only marginally. Contrary to the results reported by Hu et al. (*17*), our results do not support the statement that tautomeric reaction from BrU to BrU* is more likely than from U to U*. On the other hand, our results fully agree with Orozco's results (*16*).

The high energy and free energy penalty of both enolization processes of the isolated species still does not mean that b → c and b1 → c1 steps cannot proceed. It is necessary to consider a more realistic situation in DNA molecule. Thus, we have studied the enolization process of U and BrU not only for an isolated system but also in a complex with adenine. This study has been performed because the possibility that the complexation of U* (BrU*) with A will compensate for the positive ΔG of the enolization process. From the Table 2 it is, however, apparent that ΔG/gas-phase values as well as ΔG/water values for AU → AU* and ABrU → ABrU* (for structures of base pairs see Figure 4) are highly unfavorable (positive), and they are practically identical for U and BrU. The equilibrium constant of both processes (water included) is equal with the value 6.8×10^6, which gives the probability of both processes of 1.5×10^{-7}. These findings put doubts on any speculation that the tautomeric model can explain the mutagenic activity of BrU. In addition, we can exclude a direct tautomerization process from AU to AU* within the strand (see Figure 4). The only possibility of changing the binding pattern is during the DNA replication.

c → d and c1 → d1 Steps. U* as well as BrU* prefer binding to guanine over adenine. From Table 3 it follows that these steps proceed with high probability. Values reported by Hu et al. (cf. Table 3) are qualitatively correct but quantitatively different. In addition, the results presented in Table 3 indicate that bromation of U in the keto form does not affect its binding to A or G.

d → e and d1 → e1 Steps. The calculated energetical profiles of both processes are collected in Table 4. The results obtained at the B3LYP and MP2 levels slightly differ from each other, and the more reliable MP2 method favors complexes with guanine in enol form. In addition, the relative free energy values differ from the energy ones, and the inclusion of entropy affects the relative values substantially. On the other hand, a water environment affects both equilibria only marginally. From Table 4, it follows that, if we consider all effects, G*U and G*BrU forms (cf. Figure 4) are preferred. Consequently, the transition from A−T to G−C will not happen either in the case of bare U or BrU. In addition to the energy penalty for the step b → c discussed above, the barrier of proton transfer is 5.3 and 2.4 kcal/mol for U and BrU, respectively. Thus, the energy difference between these two barriers is not significant enough to prefer this step (d → e, d1 → e1) for bromated species under physiological conditions. Table 4 further shows that the proton transfer from GBrU* to G*BrU and the reverse process are definitively not barrier-free processes (as mentioned by Hu et al. (*17*)), but they are characterized by barriers of 2.4 and 3.0 kcal/mol, respectively. We would like to comment that we did not consider hydrogen tunneling, which would make the process more likely to proceed. However, this would not change the overall picture described in this paragraph.

Evaluation of the Tautomeric Model for the Explanation of Mutagenic Activity of the BrU. Putting all steps together, we can conclude that the probability that BrU induces mutation from A−T to G−C is very low, at the order of 10^{-8}, similar to that of U. Such a low probability cannot explain the known mutagenic activity of BrU and some other mechanism is probably taking place.

Can the Ionization Model Explain Mutagenic Activity of BrU? The ionization model proposed by Sowers (*15*) supposes that at physiological pH the canonical keto form of U exists in equilibrium with anionic form, and it is an ionized form that makes a mispair with guanine. However, only a negligible amount of the anionic form is produced (0.2%). XUs possess a pK_a value 2 orders of magnitude lower than U and a higher abundance of the anionic form and, consequently, a higher probability of mutations in organisms can thus be expected.

If the ionization reaction represents a key process among various XUs, that with the lowest PA will be the most potent. Since bromine exhibits relativistic effects which can be important for some properties, the calculations were performed using relativistic pseudopotential as well (cf. Table

Table 3: Energy (ΔE) and Free Energy (ΔG) Changes (in kcal/mol) of Complexation of U* and BrU* with A and G in Vacuo[a]

reaction	A+U* → AU*	A+BrU* → ABrU*	G+U* → GU*[b]	G+BrU* → GBrU*[c]
ΔE	−21.1 (−15.2)	−22.7 (−15.6)	−34.7 (−26.7)	−35.9 (−31.9)
ΔG	−1.6	−8.8	−22.2	−23.5

[a] All data were obtained at the B3LYP/6-31G** level. Numbers in parentheses were taken from the paper of Hu et al. (*17*). [b] ΔE and ΔG values for the GU complexation (G+U → GU) (cf. Figure 4) amount to −17.6 and −6.8 (B3LYP) and −16.3 and −7.1 (MP2) kcal/mol. [c] ΔE and ΔG values for the GBrU complexation (G+BrU → GBrU) (cf. Figure 4) amount to −17.7 and −7.0 (B3LYP) and −16.5 and −7.5 (MP2) kcal/mol.

Table 4: Relative Energies and Relative Free Energies (in kcal/mol) of Base Pair Tautomerism Proceeding via Transition State (TS)

structure	GU*	TS	G*U	GBrU*	TS	G*BrU
ΔE^a	0	4.3	−0.5	0	3.4	−0.5
ΔE^b	0	4.9	−0.4 (−2.0)[f]	0	3.4	−2.2 (−2.4)[f]
ΔG^c	0	3.3	1.0	0	1.7	1.1
ΔG/water[d]	0	4.6	1.0	0	2.5	1.3
ΔG/water[e]	0	5.3	0.8 (−0.8)[f]	0	2.4	−0.4 (−0.6)[f]

[a] Relative energy in vacuo calculated at the B3LYP/6-31G** level. [b] Relative energy in vacuo calculated at the MP2/6-31G**//B3LYP/6-31G** level. [c] Relative free energy in vacuo calculated at the B3LYP/6-31G** level. [d] Relative free energy in water (SCRF/COSMO) calculated at the B3LYP/6-31G** level. [e] Relative free energy in water (SCRF/COSMO) calculated at the MP2/6-31G**//B3LYP/6-31G** level. [f] MP2/6-31G**.

Table 5: Proton Affinities (in kcal/mol) of Uracil and Various Halogenuracils

structure	U	FU	ClU	BrU
B3LYP/6-31G**	368	361	358	358
MP2/cc-pVTZ	359	352	351	350
MP2/SDD	359	350	351	351

5). On the basis of the results obtained at the MP2/cc-pVTZ and MP2/SDD levels for U, FU, and 5-chlorouracil (ClU), we can conclude that the results of pseudopotentials calculations with less accurate description of valence electrons are reliable for obtaining PA values. In addition, comparison of the results obtained by these two methods shows that the inclusion of the relativistic effect does not change the BrU's PA values. Putting the MP2 data together, we conclude that the PA of all mentioned XUs are very similar. This result is supported by experimental data showing that the pK_a values of FU and BrU are practically identical (*15*, *35*). Close PA and pK_a values of FU and BrU suggest that both systems behave very similarly within an ionization model. Unfortunately, we do not have enough experimental data to prove or exclude this conclusion. On one hand, FU is known to be a cytostatic agent (*14*) while BrU exhibits mutagenic activity (*2*). On the other hand, both systems are used as supporting chemotherapeutic agents (magnifying activity of *cis*-platinum anticancer drug (*36*)) and both exhibit similar activity. The ionization mechanism of BrU mutagenic activity cannot be thus either supported or excluded.

What Makes BrU Unique among XUs? In the previous paragraphs, we have shown very similar behavior of U, FU, and BrU, which prevents any unambiguous explanation (within tautomeric or ionization models) for the known mutagenic activity of BrU. The mutagenic schema of U and BrU depicted in Figure 3 failed because both systems behave very similarly during the reactions depicted in Figure 3. However, in the present study, we consider the system in its ground electronic state only. In its electronically excited state, the system can exhibit completely different behavior (*37*, *38*). We are certainly aware that excited singlet states are eliminated from further consideration due to their very short lifetime. In contrast, triplet states are known to live considerably longer, but they are generally not populated due to very weak S → T intersystem crossing. There are, however, ways to overcome the spin-forbidness of this transition and to increase the population of relatively long-lived triplet states. Considering the 5-XUs, the substitution of nucleic acid bases by heavy atoms such as Br seems to be promising for explaining the mechanisms of mutagenic activity of various 5-XUs. The study based on the mutagenic schema depicted in Figure 3, which considers BrU in the electronically excited *triplet* state, has been already started (*39*).

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