Expedited Articles

Conformational Studies and Electronic Structures of Tamoxifen and Toremifene and Their Allylic Carbocations Proposed as Reactive Intermediates Leading to DNA Adduct Formation

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Toremifene, a compound which differs from tamoxifen by the substitution of a chlorine atom for a hydrogen atom in the ethyl group, is significantly less potent than tamoxifen in causing DNA adduct formation in rats. To examine the relationship of the DNA adduct-forming ability of these compounds with their physicochemical properties such as stable conformation and chemical reactivity, we carried out molecular mechanics, molecular dynamics, and quantum mechanics calculations for the two compounds. For tamoxifen, six stable conformers were identified by conformational search with CFF91 force field. Molecular dynamics simulations showed that these were often interconverted within 1.0 ns. On the other hand, although the conformation of stable conformers and dynamical behavior of toremifene were almost the same as those of tamoxifen, a few conformations were slightly different from those of tamoxifen owing to the effect of the chlorine atom at chloroethyl group. In addition, the stability of the allylic carbocation, which had been proposed as the reactive intermediate leading to DNA adduct formation, was calculated with both semiempirical and density functional methods. Results showed that the carbocation intermediate of toremifene was less stable than that of tamoxifen by 4−5 kcal/mol, suggesting that toremifene was less frequently activated to the intermediate than tamoxifen. Furthermore, the carbocation intermediates of two other tamoxifen derivatives, 4-iodotamoxifen and droxifene, which show no DNA adduct-forming ability, were also less stable compared with that of tamoxifen. These calculated results suggest a close relation between the stability of the proposed carbocation intermediate and DNA adduct-forming ability.

Introduction

Tamoxifen, a triphenylethylene class of compound, is widely used in the treatment of breast cancer as an antiestrogenic agent.[1] Toremifene, a structurally related derivative chlorinated in the ethyl side chain, has also been proven to be clinically effective.[2] Toremifene competes with estrogen for binding sites with almost the same binding constant as tamoxifen and exerts a qualitatively and quantitatively similar antiestrogen effect to tamoxifen on estrogen-dependent breast cancer cell lines *in vitro* and *in vivo*. However, the two compounds have shown a remarkable difference in hepatocarcinogenicity in rats.[3−6] Whereas tamoxifen has been shown to cause liver carcinogenesis in rats on long-term administration, toremifene has not exhibited such carcinogenicity.

Furthermore, DNA adducts were found in tamoxifen-exposed rat liver,[7] suggesting the association of DNA damage with this carcinogenesis by tamoxifen. This claim is further supported by the fact that, in contrast to tamoxifene, toremifene produces little DNA adducts in rat liver.[5,6]

In addition, it has been reported that tamoxifen forms DNA adducts via metabolic activation by cytochrome P450 enzymes.[8,9] Although the reactive intermediate generated by the enzymes has not been identified experimentally, Potter et al. have recently proposed a noteworthy hypothesis for the mechanism of DNA adduct formation (Figure 1),[10] in which tamoxifen is activated by oxidation at the α-position of the ethyl group, resulting in the formation of a reactive carbocation intermediate that would react with nucleophiles on DNA. They have presented experimental evidence to support this mechanism: the deuterated compound [*ethyl*-D_5]tamoxifen produces fewer DNA adducts than the nondeuterated compound, which can be reasonably explained by a kinetic isotope effect,[11] while α-hydroxytamoxifen, identified as a metabolite of tamoxifen, shows high DNA-binding activity.[12] Furthermore, an adduct of tamoxifen and deoxyguanosine in which the α-position of tamoxifen is linked covalently to the exocyclic amino group of deoxyguanosine has been identified as the major product of the reaction of α-acetoxytamoxifen with DNA.[13]

Recently it has been proposed that tamoxifen may be useful in long-term prophylactic therapy in healthy women at high risk of developing breast cancer, and in fact a number of clinical trials are currently underway.[14] It is therefore a matter of urgency to determine the mechanism of the hepatocarcinogenesis of tamoxifen in rats and to estimate the possibility of carcinogenesis in humans.[15,16] The large difference in both hepatic carcinogenic activity and DNA adduct-forming ability between tamoxifen and toremifene despite the small structural alteration from a hydrogen atom to a chlorine atom at the ethyl group may provide us an important clue in clarifying the mechanism of DNA adduct formation leading to carcinogenesis. Thus, we investigated

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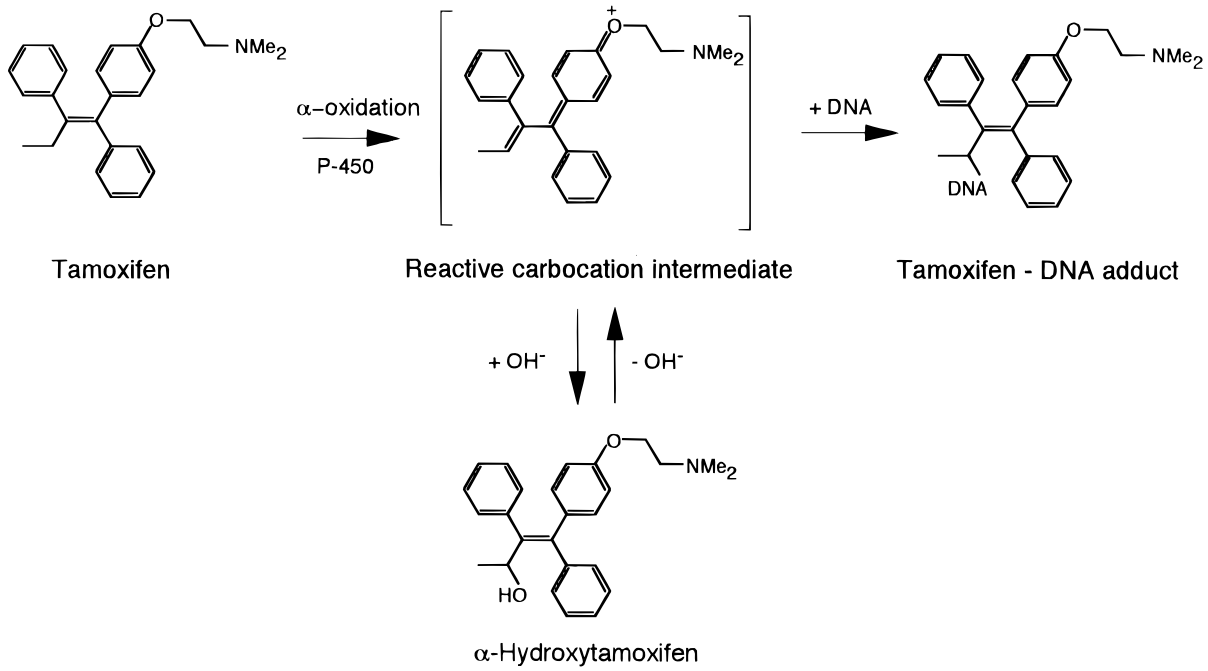
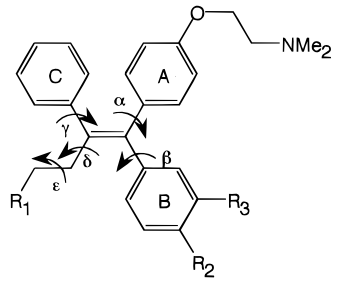


Figure 1. Proposed pathway of tamoxifen−DNA adduct formation via reactive carbocation intermediate generated by α-oxidation of tamoxifen.



compound	R_1	R_2	R_3
Tamoxifen	H	H	H
Toremifene	Cl	H	H
4-Hydroxytamoxifen	H	OH	H
4-Hydroxytoremifene	Cl	OH	H
4-Iodotamoxifen	H	I	H
Droloxifene	H	H	OH

Figure 2. Structure of tamoxifen derivatives.

the difference between the two compounds in the stable conformation and the stability of the proposed reactive carbocation intermediate, with molecular mechanics (MM), molecular dynamics (MD), and quantum mechanics (QM) calculations. We also compared 4-iodotamoxifen[10] and droloxifene,[6] which exhibit no DNA adduct-forming ability.

Computational Method

A model for tamoxifen was constructed on the basis of the X-ray crystal structure determined by Precigoue et al.[17] The structure of toremifene was obtained by substitution of a chlorine atom for a hydrogen atom at the ethyl group of tamoxifen. Droloxifene and 4-iodotamoxifen were also built based on the structure of tamoxifen. The chemical structure of these tamoxifen derivatives is shown in Figure 2. Conformational search and geometry optimization of these structures were performed by MM with CFF91 force field[18] as well as a semiempirical QM method with AM1 hamiltonian.[19] The energy of intact compounds and reactive carbocation intermediates was estimated by both semiempirical and density functional theory (DFT) calculations. Molecular modeling, MM, and MD calculations were performed using Biosym software (Insight II, Discover and Search_Compare modules).

Conformational Analysis. After energy minimization of the modeled structures by Discover 2.9.5, systematic conformational search was carried out with Search_Compare as described below. The conformers were generated by varying the four or five torsion angles (α, β, γ, δ, and ϵ) shown in Figure 2 in steps of 30°, after which their energy was evaluated. A conformer was retained if its energy was less than 50 kcal/mol greater than the energy of the most stable conformer. The resulting conformers were further optimized by energy minimization, and conformers whose energy and atomic coordinates were nearly identical to a lower energy conformer were removed. Thus, stable conformers in energy local minimum were selected in above torsional space.

MD Simulation. The most stable conformation was taken as the starting point for the MD simulation of tamoxifen and toremifene. MD simulations *in vacuo* were performed with Discover 2.9.5 at a constant temperature (300 K) for 1 ns after the equilibrium step of 2 ps. The time step used in the simulation was 1 fs, and data were collected every 0.5 ps. Data analysis was carried out on the entire trajectory.

Semiempirical QM Calculation. The semiempirical calculations for tamoxifen derivatives, including 4-hydroxylated compounds and their proposed carbocation intermediates, were performed by MOPAC6[20] with AM1 hamiltonian. Energy minimization was carried out in cartesian coordinates using the eigenvector-following algorithm: the MOPAC keywords EF, GNORM=0.05, XYZ, and PRECISE.

DFT Calculation. The quantum chemical calculations were carried out using the density functional package, ADF, developed by Baerends et al.[21] Geometries were optimized at the level of Vosko−Wilk−Nusair (VWN) LSD. Bonding energies were evaluated using the VWN potential with nonlocal corrections. Becke's nonlocal exchange and Perdew's nonlocal correlation corrections were added self-consistently. The uncontracted Slater-type basis set, basis set III (double-ζ with a polarization function and frozen core approximation), was employed.

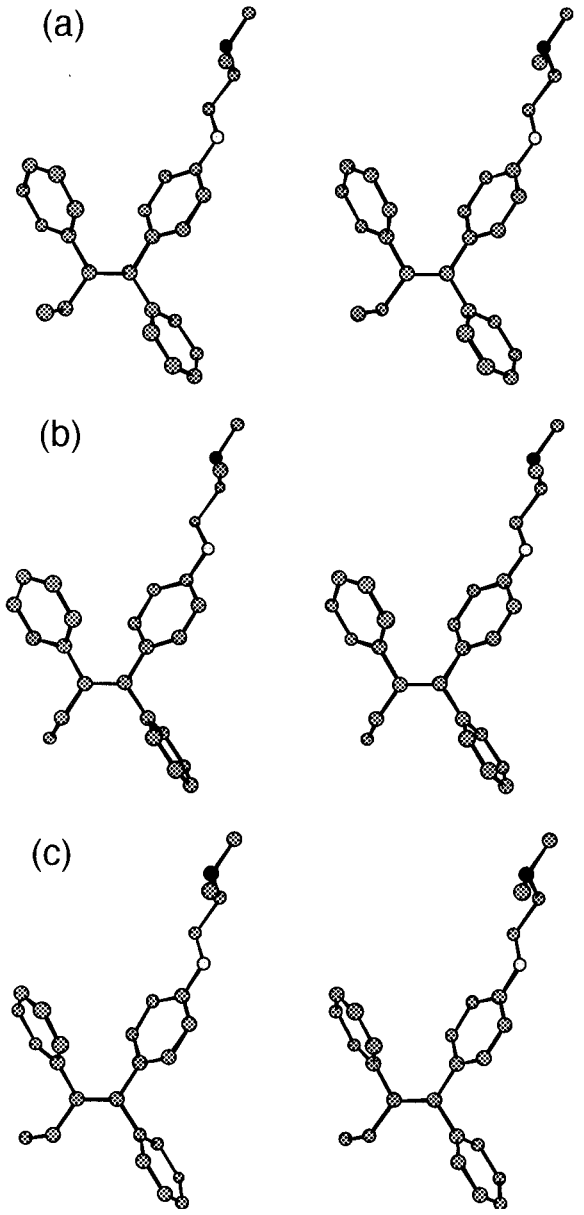


Figure 3. Stereoviews of the three low-energy conformers of tamoxifen, conformer 1(a), 2(b), and 2′(c).

Table 1. Conformational Angles and Total Energies of the Low-Energy Conformers of Tamoxifen Obtained by CFF91 Force Field Calculation

conformer	torsion angle (deg) α	β	γ	δ	total energy (kcal/mol)
1	−52	−63	−59	113	26.1
1m	52	63	59	−113	26.1
2	−52	−71	−60	−84	27.5
2m	52	71	61	84	27.6
2′	−58	−69	−74	−113	27.4
2′m	58	68	74	113	27.5
crystal structure	−48	−64	−55	114	

Results

Low-Energy Stable Conformers of Tamoxifen and the Interconversion between Them. Six local minimum conformers of tamoxifen were identified by conformational search using CFF91 force field (Table 1). The three aryl rings in all conformers exist in a propeller conformation in which the torsion angles (α, β, and γ) are in the 52−74° range. Among the conformers, conformer 1m, 2m, and 3m are the mirror images, that is, the enantiomers of conformers 1, 2, and 3, respectively. The conformer 2 and 2′ are very similar in energy and geometry, although there is slight difference in torsion angles γ and δ. Conformer 1 shows about 1.4 kcal/mol lower energy than conformer 2, with the major difference in conformation between them existing in torsion angle δ, i.e., the ethyl group is oriented in the opposite direction to the double-bond plane (Figure 3). It is noted that conformer 1 is not only the most stable conformer, but also the closest to the X-ray crystal structure with excellent agreement in structural parameters, i.e., an rms shift of 0.09 Å except for the aminoalkyl group.

Table 2. Conformational Angles and Total Energies of the Low-Energy Conformers of Toremifene Obtained by CFF91 Force Field Calculation

conformer	torsion angle (deg) α	β	γ	δ	ϵ	total energy (kcal/mol)
1-a	−52	−64	−58	114	−177	22.5
1-b	−54	−73	−63	124	−63	23.2
1-c	−52	−70	−59	104	76	24.9
1m-a	52	64	58	−114	177	22.5
1m-b	54	73	63	−124	63	23.2
1m-c	52	70	59	−104	−76	24.9
2-a	−51	−65	−57	−76	−179	24.1
2-b	−51	−62	−58	−56	−60	24.9
2m-a	51	67	57	76	179	24.1
2m-b	51	62	58	56	60	24.9
2′-a	−63	−66	−79	−117	176	24.2
2′-b	−63	−75	−80	−116	60	24.3
2′m-a	62	66	79	117	−176	24.2
2′m-b	63	75	80	117	−60	24.3

The interconversion between the six distinct conformers was further investigated using MD simulations. Figure 4 shows the distribution of torsion angles in the 1.0 ns MD simulation at 300 K. All of the distinct conformers appeared during the simulation as shown in the distribution plot of torsion angle γ and δ, indicating that the energy barrier is low enough for the conformers to interconvert within 1.0 ns. It is noted that there was correlated rotation between torsion angle α and β as well as α and γ, suggesting that the three aryl rings rotate concurrently with three-ring flip mechanism. As expected, the lowest-energy conformers 1 and 1m exhibited the highest distribution density. In contrast, the other conformers 2, 2m, 2′, and 2′m were distributed more extensively in the torsional space compared with conformers 1 and 1m, although the distribution probability was lower.

Furthermore, to examine the frequency of interconversion between the distinct conformers, the trajectories of the torsion angles γ and δ were monitored. The results are shown in Figure 5. The transition of torsion γ between −60° and −120° was observed to occur within a few picoseconds, whereas torsion δ was interconverted from −110° to 110° in the time range of 100 ps. The former corresponds to the interconversion between conformer 1 and 2m (2′m) as well as 1m and 2 (2′), while the latter reflects the transition between the 1 and 2m (2′m) conformer group and the 1m and 2 (2′) conformer group.

Low-Energy Conformers of Toremifene and the Interconversion between Them. As a result of conformational search, 14 stable conformers of toremifene were obtained (Table 2). Without taking the geometry of the chlorine atom into account, the conformers are grouped into six distinct conformers as well as tamoxi-

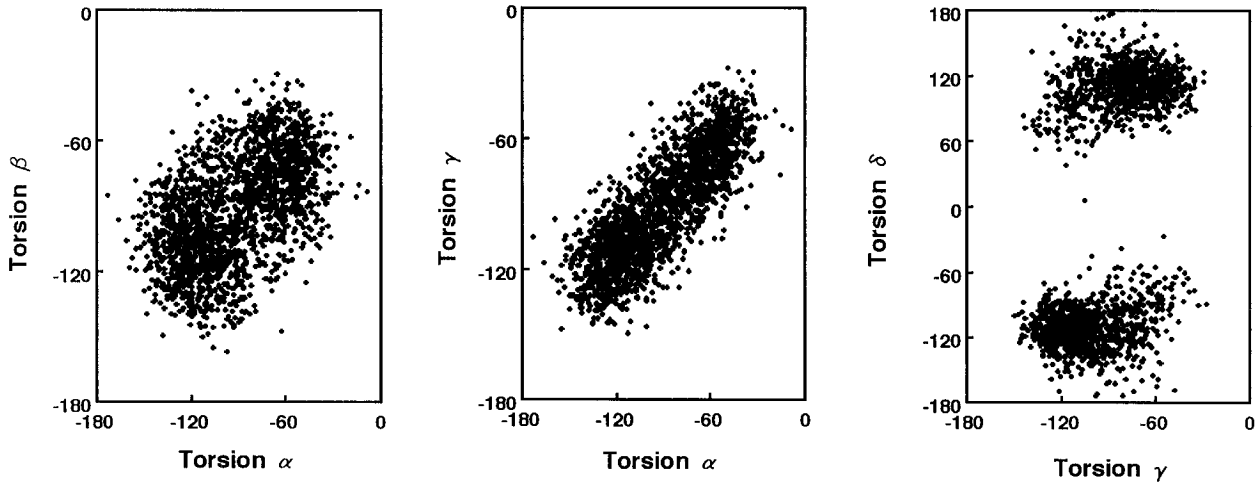


Figure 4. Distribution plots of torsion angles of tamoxifen during 1.0 ns MD.

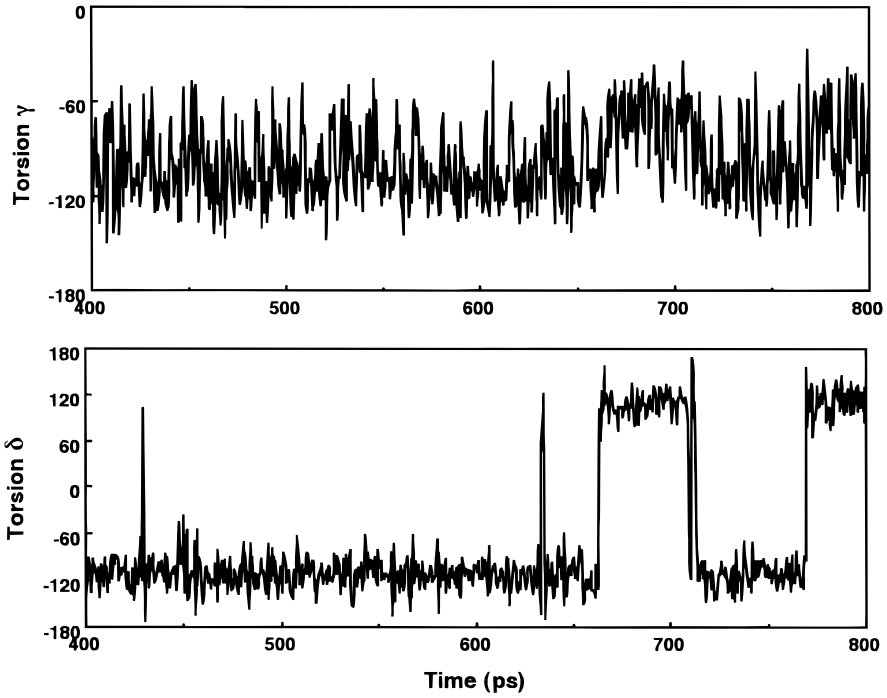


Figure 5. Dynamical motion of torsion angles γ and δ of tamoxifen during 1.0 ns MD.

fen. The energy difference between conformer 1 and 2 was about 1.6 kcal/mol, which was comparable to that of tamoxifen (1.4 kcal/mol). The geometry of the chlorine atom is most stable in the *trans* form, and the energy of the *gauche* form is 0.1 to 0.8 kcal/mol higher than the *trans* form. In the *trans* form, the four torsion angles (α, β, γ, and δ) of the low-energy conformers of toremifene are almost the same as those of tamoxifen, whereas in the *gauche* form, the torsion angles β and δ are slightly different from those of tamoxifen, due to the steric interaction between the chlorine atom and the aryl rings B and C.

As indicated in the plot of the four torsion angles (α, β, γ, and δ) in Figure 6, the distribution of toremifene conformers was basically similar to that of tamoxifen. In addition, the dynamical behavior such as the transition between the distinct conformers was almost the same as that of tamoxifen (data not shown). These calculated results indicate that the effect of the chlorine atom on the motion and molecular shape of the triaryl ethylene moiety is relatively small. Regarding the rotational isomer of the chloroethyl moiety, the *trans* form was predominant as expected. One of the two *gauche* forms also appeared with considerable frequency (Figure 6), but the frequency of the other *gauche* form was rather low, indicating that the position of the chlorine atom is confined to some extent.

Structure and Energy of Low-Energy Conformers of Tamoxifen, Toremifene, and Their 4-Hydroxylated Derivatives Obtained by AM1 Calculation. On the basis of the structure of low-energy conformers obtained by MM calculations, semiempirical calculations using the AM1 hamiltonian were performed for tamoxifen, toremifene, and their major metabolites, 4-hydroxytamoxifen and 4-hydroxytoremifene, in order to examine the electronic energy as well as the energy-minimized structure. As listed in Table 3, the energy-minimized structures corresponding to conformer 1 and 2 in MM calculation were obtained, whereas no conformer 2′ was observed. In conformer 1 of tamoxifen, the torsion angles obtained by AM1 calculation were in good agreement with those by MM calculation, although

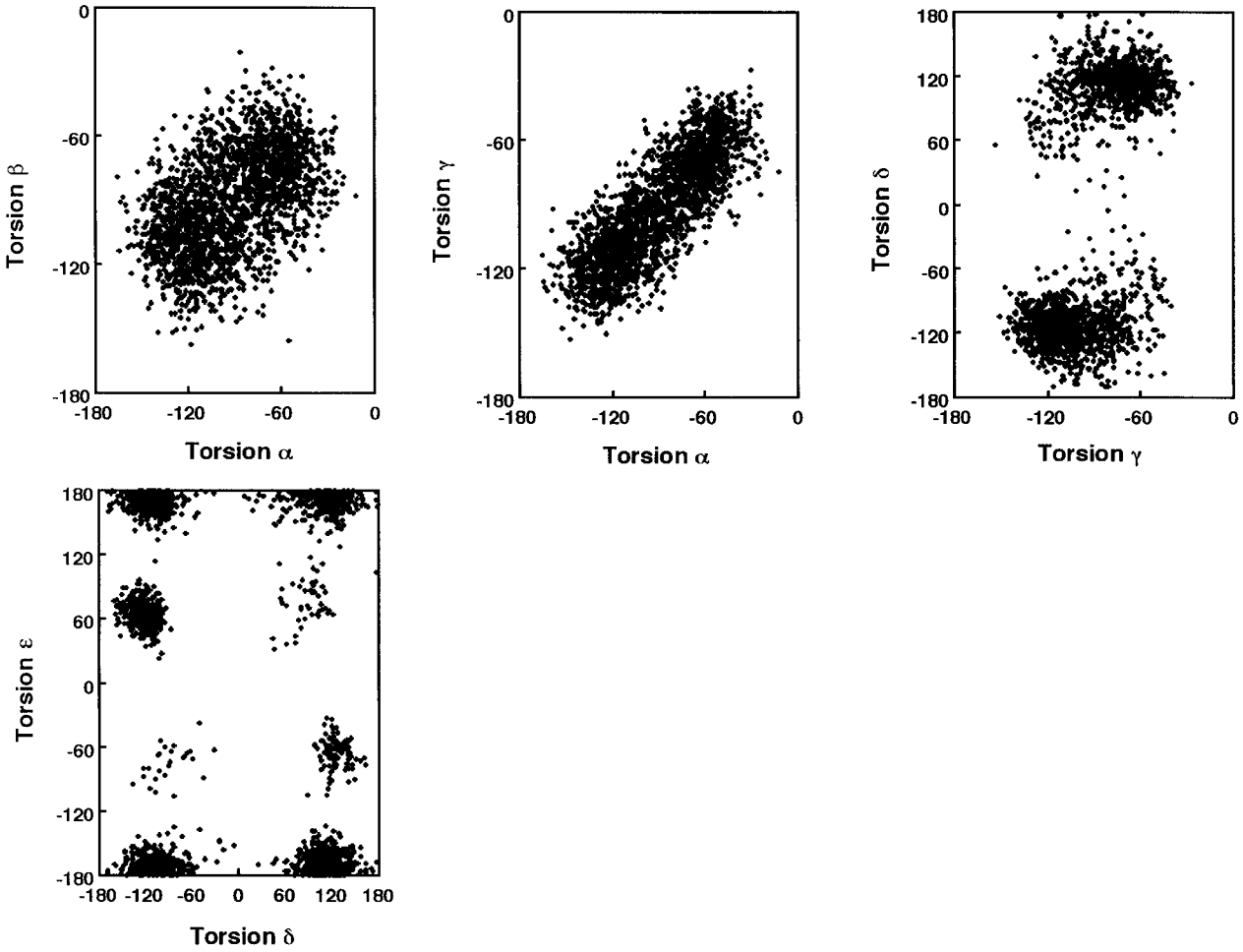


Figure 6. Distribution plots of torsion angles of toremifene during 1.0 ns MD.

Table 3. Conformational Angles and Heats of Formation of the Low-Energy Conformers of Tamoxifen, Toremifene, and Their 4-Hydroxylated Derivatives Obtained by AM1 Calculation

conformer	torsion angle (deg) α	β	γ	δ	ϵ	ΔH_f (kcal/mol)
			Tamoxifen			
1	−51	−67	−73	111		47.9
2	−51	−83	−80	−87		48.1
			4-Hydroxytamoxifen			
1	−51	−63	−72	112		3.6
2	−50	−82	−79	−86		3.9
			Toremifene			
1a	−50	−67	−70	112	176	39.4
1b	−52	−71	−75	127	−75	39.9
1c	−49	−92	−68	83	75	40.6
2a	−49	−81	−79	−84	180	39.6
2b	−55	−84	−86	−122	69	40.3
2c	−49	−75	−78	−70	−79	40.5
			4-Hydroxytoremifene			
1a	−51	−64	−69	113	176	−4.9
1b	−52	−69	−75	127	−75	−4.4
1c	−48	−91	−68	84	75	−3.6
2a	−49	−81	−79	−84	180	−4.7
2b	−55	−83	−86	−121	69	−4.1
2c	−49	−74	−77	−69	−79	−3.8

there was a slight difference of about 14° in torsion angle γ. In conformer 2, a difference of about 10−20° was observed in torsion angles β and γ. In either conformation, the aryl planes tend to be more perpendicular to the double-bond plane in AM1 calculation compared with MM calculation. Conformer 1 was more stable than conformer 2 in AM1 calculation as well as in MM calculation, but the energy difference was smaller in the former calculation than in the latter (about 0.2 vs 1.4 kcal/mol). In addition, the chloroethyl group in toremifene is most stable in the *trans* form, but in accordance with the results of MM calculation, the energy difference from the *gauche* form is small (about 0.2−0.7 kcal/mol). On the other hand, the calculated results for 4-hydroxylated derivatives revealed that the hydroxyl group has little effect on the conformation of stable conformers.

Structure and Energy of Reactive Carbocation Intermediates. The reactive carbocation intermediates for tamoxifen, toremifene, and their 4-hydroxylated derivatives were modeled on the structure proposed by Potter and then energy-minimized by the AM1 semiempirical method. As a result of the appearance of a new double-bond, both *cis* and *trans* isomers were obtained as low-energy conformers (Figure 7). In all intermediates the energy of the *trans* isomer was about 1 kcal/mol lower than that of the *cis* isomer (Table 4). In toremifene and 4-hydroxytoremifene, both *cis* and *trans* isomers were further divided into two distinct conformer groups according to the geometry of the chlorine atom.

The difference in heat of formation (ΔH_f) between the lowest energy conformer of the intact compound and that of the carbocation intermediate, $\Delta\Delta H_f$, increased in the order 4-hydroxytamoxifen < tamoxifen < 4-hydroxytoremifene < toremifene (Table 5). The difference in $\Delta\Delta H_f$ between tamoxifen and toremifene and that between 4-hydroxytamoxifen and 4-hydroxytoremifene

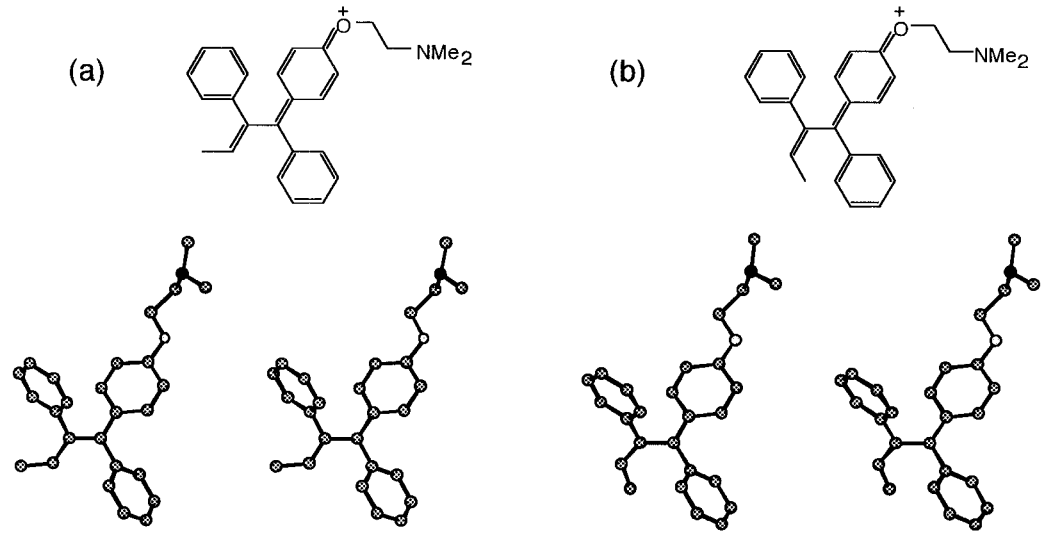


Figure 7. Stereoviews of the two low-energy conformers of reactive carbocation intermediates of tamoxifen, *trans* isomer (a) and *cis* isomer (b).

Table 4. Conformational Angles and Heats of Formation of the Low-Energy Conformers of Reactive Carbocation Intermediates Obtained by AM1 Calculation

	torsion angle (deg)					
conformer	α	β	γ	δ	ϵ	ΔH_f (kcal/mol)
			Tamoxifen			
trans	−21	−37	−57	180		223.6
cis	−14	−38	−47	−3		225.0
			4-Hydroxytamoxifen			
trans	−24	−32	−56	180		177.9
cis	−17	−32	−47	−3		179.1
			Toremifene			
trans-1	−20	−36	−55	180	−144	219.3
trans-2	−19	−37	−56	180	110	219.3
cis-1	−13	−36	−44	−2	−96	219.9
cis-2	−14	−36	−46	−4	128	220.6
			4-Hydroxytoremifene			
trans-1	−23	−31	−55	180	−142	173.3
trans-2	−22	−31	−56	180	110	173.4
cis-1	−17	−30	−43	−1	−95	174.0
cis-2	−18	−29	−45	−3	126	174.6

Table 5. Comparison of the Stability of Reactive Carbocation Intermediates by AM1 Calculation

compound	$\Delta\Delta H_f$[a] (kcal/mol)	difference (kcal/mol)
tamoxifen	208.9	0.0
toremifene	213.1	4.2
4-hydroxytamoxifen	207.5	0.0
4-hydroxytoremifene	211.5	4.0

[a] $\Delta\Delta H_f = \Delta H_f$(reactive intermediate) + $\Delta H_f(H^-) - \Delta H_f$(intact).

was 4.2 and 4.0 kcal/mol, respectively, indicating that the introduction of a chlorine atom into the β-position of the ethyl group reduced the stability of the carbocation intermediate relative to the intact compound.

Deprotonation in the Carbocation Intermediate of 4-Hydroxylated Derivatives. The dissociation of the 4-hydroxyl group of the carbocation intermediate of 4-hydroxylated derivatives should be taken into account, since the resulting deprotonated form would be stabilized by the charge dispersion resulting from the resonance effect accompanying the neutralization of a carbocation. Thus the energy-minimized structures of the deprotonated intermediates and their heats of formation were obtained using AM1 calculation. Alteration of conformation shown as the change of torsion angles α and β was observed, probably owing to the change of resonance structure accompanied by the deprotonation, i.e., the torsion angle β changed to about 0°, while the absolute α increased (Table 6). In contrast to the protonated form, the *cis* isomer in the deprotonated form was slightly more stable than the *trans* isomer.

Table 6. Conformational Angles and Heats of Formation of the Low-Energy conformers of Deprotonated Reactive Intermediates of 4-Hydroxylated Derivatives Obtained by AM1 Calculation

	torsion angle (deg)					
conformer	α	β	γ	δ	ϵ	ΔH_f (kcal/mol)
			4-Hydroxytamoxifen			
trans	−56	−2	−55	−179		44.1
cis	−56	−2	−46	1		43.7
			4-Hydroxtoremifene			
trans-1	−57	−2	−56	−179	−133	36.7
trans-2	−54	−3	−57	180	113	36.4
cis-1	−55	−3	−45	−2	−123	36.1
cis-2	−56	−2	−45	0	129	36.6

The change of heat of formation through the deprotonation process of the carbocation intermediate of 4-hydroxytoremifene was 2.5−3.2 kcal/mol lower than that of 4-hydroxytamoxifen, suggesting that the former compound is more deprotonated than the latter.

Energy of the Carbocation Intermediate of 4-Iodotamoxifen and Droloxifene. In prominent contrast to tamoxifen, two tamoxifene derivatives, 4-iodotamoxifen and droloxifene, with the substituent on the B phenyl ring of tamoxifen (Figure 1), exhibit no DNA adduct-forming ability. Thus, the effect of these substituents on the stability of the carbocation intermediates was further evaluated. The calculated energy difference between the intact compound and the carbocation intermediate for the two compounds indicated that the carbocation intermediates of 4-iodotamoxifen and droloxifene were less stable than that of tamoxifen by 2.6 and 2.0 kcal/mol, respectively, and of 4-hydroxytamoxifen by 4.0 and 3.5 kcal/mol, respectively. These results are similar to those of toremifene in the destabilization of the intermediate.

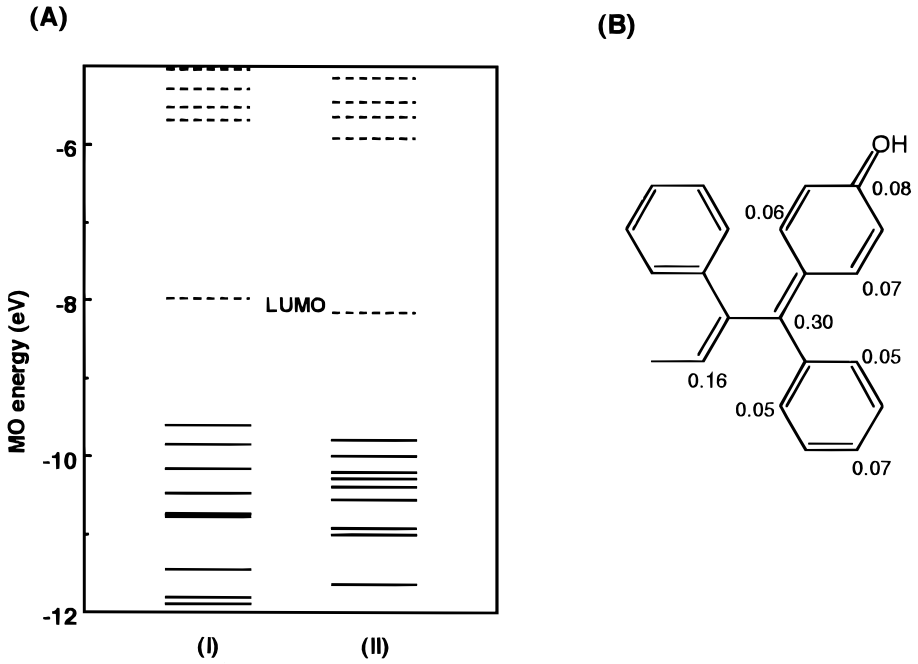


Figure 8. Electronic structures of reactive carbocation intermediates obtained by DFT calculation. (A) Energy levels of molecular orbitals of the intermediates of tamoxifen (I) and toremifene (II). (B) Electron density distribution of LUMO of the intermediate of tamoxifen. Densities of atoms without value are less than 0.05.

Electronic Structure and Stability of the Carbocation Intermediate Obtained by DFT Method. The more precise quantum mechanics calculations on the intact compound and carbocation intermediate of tamoxifen and toremifene were performed using the DFT method. To reduce computational time, the (dimethylamino)ethoxy group of tamoxifen, toremifene, and the corresponding carbocation intermediates was replaced by a hydroxyl group. The compounds were geometry-optimized by the LSD potential (VWN) using the AM1-minimized structure as a starting structure. The energies of LSD-optimized structures were calculated using the VWN potential with nonlocal corrections. The results showed that the carbocation intermediate of toremifene was 5.4 kcal/mol less stable than that of tamoxifen. This result is consistent with that by AM1 calculation.

The energy level of molecular orbitals (MO's) in the carbocation intermediate of tamoxifen and toremifene is illustrated in Figure 8A. Since the lowest unoccupied MO (LUMO) of the two intermediates was greater than 2 eV lower in energy than other unoccupied MO's, it is conceivable that only the LUMO plays an important role in the electrophilic reaction with DNA. In addition, the frontier electron density in the LUMO of the carbocation intermediate of tamoxifen, which is associated with the electrophilic reactivity of each atom in the molecule, is highest in one of the central double-bond carbons and second highest in the α-carbon (Figure 8B), suggesting that the central carbon rather than the α-carbon is most reactive toward nucleophilic agents such as nucleobases. The carbocation intermediate of toremifene showed similar frontier electron density (data not shown), but the LUMO energy, an index of the electrophilic reactivity of the molecule, was about 0.17 eV lower than that of tamoxifen. It is therefore possible that the reactivity of the intermediate of toremifene is higher than that of tamoxifen, corresponding to the instability of the intermediate.

Discussion

It has been often noted that the biological activity of a drug is closely associated with the topology of its molecule. X-ray crystallographic and molecular mechanics studies on synthetic estrogens and antiestrogens, for example, have shown that in some cases the biological activity of a compound is significantly dependent upon its conformation.[22,23] Among the several conformation analyses of tamoxifen and its related compounds,[23−26] Edwards et al. performed an extended conformational search for tamoxifen with MMP2 force field and identified four distinct conformers.[26] From the comparison of four torsion angles of toremifen, α, β, γ, and δ, we found that these conformers correspond to the conformers 1, 1m, 2, and 2m identified using CFF91 force field in our study. We also found another distinct conformer, 2′ (2′m), near conformer 2 (2m); these conformers form a more extended stable area with slightly higher energy than the neighboring area of conformer 1 (1m) as displayed in MD simulation (Figure 4). Furthermore, analysis of MD trajectories showed that interconversion between the distinct conformers occurs frequently within 1.0 ns, indicating that tamoxifen was not restrained to a particular conformation in the free state (Figure 5).

Although toremifene has slightly different β and γ torsion angles from tamoxifen when the chlorine atom is in a *gauche* position, it should be noted that there is no large difference between the two compounds in the overall conformation of low-energy conformers, that is, a propeller conformation, or in interconversion among the conformers. It has been proposed that the key structural elements of tamoxifen in its ability to bind to the estrogen receptor are the B ring or 4-hydroxylated

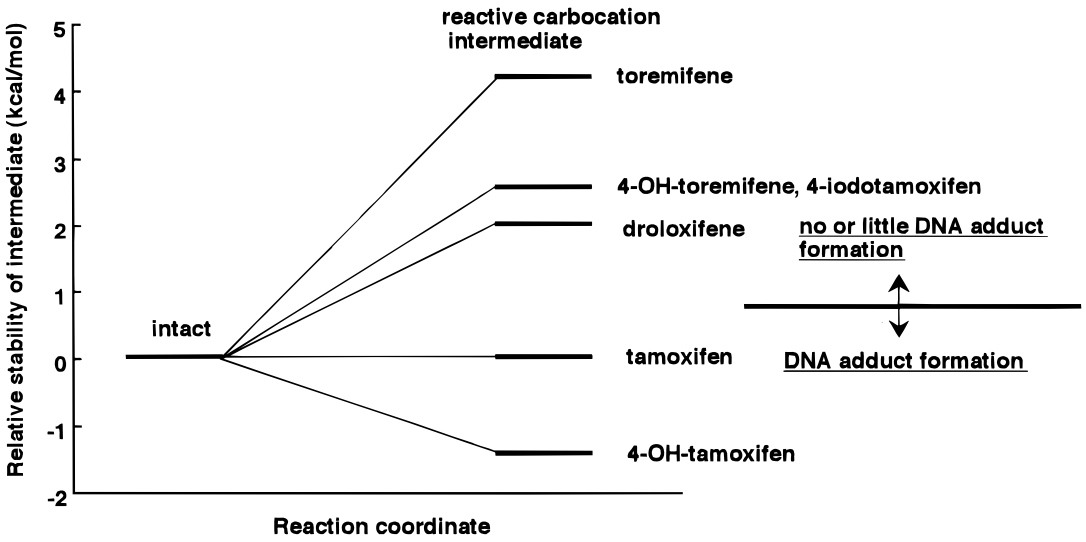


Figure 9. Relationship of the stability of reactive carbocation intermediates of tamoxifen derivatives with their DNA adduct-forming activity.

B ring and the propeller arrangement of the triaryls and that the (dimethylamino)ethoxy side chain interferes with subsequent estrogen receptor functions essential for activity.[22,27,28] It is noteworthy that these requirements are satisfied for toremifene as well. This would explain why toremifene has estrogen-binding affinity, antiesterogenecity, and antiproliferative activity comparable to those of tamoxifen despite the introduction of a sizeable chlorine atom into the ethyl group.

In contrast, there is a noticeable difference in DNA adduct-forming ability between tamoxifen and toremifene, i.e., toremifene is about 150 times less potent than tamoxifen in causing DNA adduct formation in rat liver.[6] We will discuss the cause of this difference on the basis of the proposed mechanism, in which the first step of reaction leading to DNA adduct formation is the generation of the reactive carbocation intermediate via α-oxidation, or in other words hydride abstraction from the α-position of the ethyl group by P450 enzyme (Figure 1).[10] Since a carbocation type of intermediate would be expected to react rapidly with nucleophiles, the formation step of the intermediate is considered as the rate-determining step in the reaction. Thus, to account for the difference in DNA adduct-forming ability between tamoxifen and toremifene, the steric and electronic effect of the chlorine atom of toremifene on intermediate formation was investigated. It is possible that the chlorine atom at the β-position in toremifene interferes with the hydride abstraction at the α-position owing to steric hindrance. When the chloroethyl group of toremifene is in the *trans* form, the chlorine atom is on the same side as the electrophile, that is, the activated group of P450 enzyme, whereas in the *gauche* form the electrophile should approach the α-hydrogen from the side opposite to the chlorine atom with less steric hindrance. Since the energy of the *gauche* forms is only 0.1−0.8 kcal/mol higher than that of the *trans* forms, the *gauche* forms appear with high frequency, as illustrated in Figure 6. Thus, although the steric effect of the chlorine atom would partially contribute to the decrease in the carbocation intermediate formation, it is unlikely that this effect is sufficient to account for the large difference between tamoxifen and toremifene in DNA adduct-forming ability.

A chlorine atom has not only a large steric factor, but also a remarkable electron-withdrawing property. It is possible that this electronic property affects the chemical reactivity of the drug, that is, the metabolic activation of toremifene by P450 enzyme when compared with that of tamoxifen. In fact, owing to the electronic effect of the chlorine atom on the resonance system of the molecule, the stability of the proposed reactive carbocation intermediate relative to the intact compound is 4.2 (AM1) or 5.4 (DFT) kcal/mol less in toremifene than in tamoxifen. Although the structure of the transition state in the tamoxifen−P450 enzyme reaction is not known, it is conceivable that the electronic structure of the reactive carbocation intermediate is close to that of the transition state, since P450 is mainly involved in the stabilization of hydrides rather than of carbocation intermediates. Thus, given that the stability of the transition state approximately parallels that of the carbocation intermediate, an energy difference of 4−5 kcal/mol in the intermediate would correspond to the above 1000-fold difference in reaction rate. This difference in reactivity may explain the ratio of the amount of liver DNA adducts in rats administered toremifene to that in those given tamoxifen (0.85:116).[6]

Since tamoxifen and toremifene are metabolized to their 4-hydroxylated derivatives in rat liver, it is conceivable that these metabolites also undergo α-oxidation of the ethyl group which generates the carbocation intermediate leading to DNA adduct formation. Indeed, the carbocation intermediates of 4-hydroxytamoxifen and 4-hydroxytoremifene are more stable than those of their intact compounds by 1.4 and 1.6 kcal/mol, respectively, suggesting that 4-hydroxylation might facilitate the generation of the intermediate. It should be noted, however, that in this pathway of activation via 4-hydroxylation, as well as in the direct pathway, toremifene demands about 4 kcal/mol more energy for activation than tamoxifen (Table 5). In addition, the carbocation intermediate generated from 4-hydroxylated

metabolites is expected to be deprotonated, resulting in the neutral deprotonated form with rearranged geometry. The energy difference of the deprotonated form relative to the protonated form in the intermediate is about 3 kcal/mol less in 4-hydroxytoremifene than in 4-hydroxytamoxifen, suggesting that the ratio of the deprotonated form to the protonated form in the intermediate of 4-hydroxytoremifene is about 100 times greater than that in 4-hydroxytamoxifen.

However, the dissociation of the 4-hydroxyl group in the intermediate neutralizes the carbocation, an effect which is thought to lower the electrophilic reactivity. Indeed, an increase of about 4.3 eV at the LUMO energy level was observed with dissociation in both intermediates of 4-hydroxytamoxifen and 4-hydroxytoremifene, which predicts that the reactivity of the deprotonated intermediate toward nucleophiles is less than that of the protonated intermediate (date not shown). Furthermore, as it is predicted that the rate of the deprotonated form to the protonated form in the intermediate of 4-hydroxytoremifene is considerably higher than that of 4-hydroxytamoxifen (Table 6), the reactivity of the former intermediate might be much lower than that of the latter.

Recently the major product of the reaction of α-acetoxytamoxifen with DNA has been identified as (*E*)-α-(N^2-deoxyguanosinyl)tamoxifen, which is chromatographically identical to one of the major adducts formed in tamoxifen-treated rats and in rat hepatocytes *in vitro*.[13] Interestingly, the major reaction site of the carbocation intermediate predicted from the electron density of LUMO by the frontier-electron theory of reactivity is the central double-bond carbon followed by the α-carbon, which has the second greatest density (Figure 8B). The fact that DNA forms a covalent bond with the α-carbon rather than the central carbon could be, however, explained by the possibility that the steric hindrance would be greater for the DNA attack on the central carbon than on the α-carbon, or that the central carbon adducts would be rearranged to the α-carbon adducts which is more stable.

The carbocation intermediates of two other tamoxifen derivatives, 4-iodotamoxifen and droloxifene, which show no DNA adduct-forming ability, are also less stable than that of tamoxifen by 2.6 and 2.0 kcal/mol, respectively. Thus, it is likely that the stability of the proposed carbocation intermediate is closely related with DNA adduct-forming ability in these derivatives as well as in toremifene. As shown in Figure 9, the DNA adduct-forming ability of tamoxifen derivatives is clearly distinguished by the stability of the carbocation intermediate, that is, the energy difference between the intact compound and the intermediate. This correspondence seems to support the hypothesis that the tamoxifen reacts with DNA via a carbocation intermediate. It remains to be determined if a reactive carbocation is actually involved in the covalent reaction between tamoxifen and DNA. If so, then this structure−activity relationship could be exploited to design less carcinogenic synthetic tamoxifen derivatives.

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