Investigation of the Regio- and Stereo-Selectivity of Deoxyguanosine Linkage to Deuterated 2-Hydroxyestradiol by Using Liquid Chromatography/ESI-Ion Trap Mass Spectrometry

L. Debrauwer, E. Rathahao, I. Jouanin, and A. Paris
Laboratoire des Xénobiotiques, INRA-ENSAT-ENV, Toulouse, France

G. Clodic
Laboratoire d'Enseignement des Méthodes Physico-Chimiques et Analytiques, University P. and M. Curie, Paris, France

H. Molines
Laboratoire des Chimie des Hétérocycles, University P. and M. Curie, Paris, France

O. Convert, F. Fournier, and J. C. Tabet
Laboratoire des Chimie Structurale Organique et Biologique, University P. and M. Curie, Paris, France

From previous studies on the reactivity of estradiol 2,3-quinone towards deoxyribonucleosides, it was demonstrated that several isomeric adducts were formed. Although adduction on steroid ring A or B has been evidenced using sequential MS^n experiments, in some cases attachment positions are difficult to identify unambiguously. In this work, 2-hydroxyestradiol labeled with deuterium at various positions [6β (**1**); 6α-7α (**2**); 6α-6β-7α (**3**)] have been used. Isomeric adduct differentiation could be achieved using LC-ESI-MS^n. The *m/z* shift of the quasi-molecular ions as well as the fragmentation pathways suggested that adduction could occur on both C6 and C9 sites of the steroid B ring: Nucleophilic attack of the base on the C6 position of the steroid led to major adducts and addition of the base on the activated C9 site gave minor adducts that were found to be unstable. LC-MS^n experiments carried out under deuterated medium provided information about some fragmentation processes by studying the *m/z* shift of fragment ions: (1) the loss of deoxyribose from the quasi-molecular ions took place according to a process involving a deuterium transfer from the deoxyribose alcohol function; (2) the cleavage of the steroid-base linkage involved a deuterium transfer from the hydroxy group of the catechol and likely occurred via the formation of an ion-dipole complex. The model studies conducted in this work provide new information on the fragmentation mechanisms of covalent adducts formed from estrogen quinones and deoxyguanosine, the most reactive DNA base. Besides, the first unequivocal characterization of adducts involving the steroid C9 position is shown by using deuterium labeled estrogen quinones. (J Am Soc Mass Spectrom 2003, 14, 364–372) © 2003 American Society for Mass Spectrometry

The enzymatic hydroxylation of the aromatic A ring of estrogens via cytochrome P450 enzymes leading to catechol estrogens represents one of the major metabolic pathways of estrogens [1–3]. Catechol estrogens are mainly inactivated by conversion into their *O*-methylated [4] or glucuronide and sulfate conjugated forms [5]. If those preventive pathways are not completed, catechol estrogens can undergo further metabolic activation steps to quinones, highly electrophilic species that can covalently bind to nucleophilic groups, via Michael addition reactions [2, 6–8]. In particular, the adduction of catechol estrogens to DNA bases [2, 7, 8] is known to induce mutations [9, 10] that may initiate cancer [1, 2]. The major protective pathway against this process is the reaction with glutathione [4, 11–13] yielding conjugates which can be easily excreted. The reactions of estradiol-3,4-quinone with deoxyribo-

Published online March 14, 2003

Address reprint requests to Dr. L. Debrauwer, UMR 1089 Xénobiotiques, INRA-ENSAT-ENV, 180 chemin de Tournefeuille, B.P. 3, 31931 TOULOUSE Cedex 9, France. E-mail: laurent.debrauwer@toulouse.inra.fr

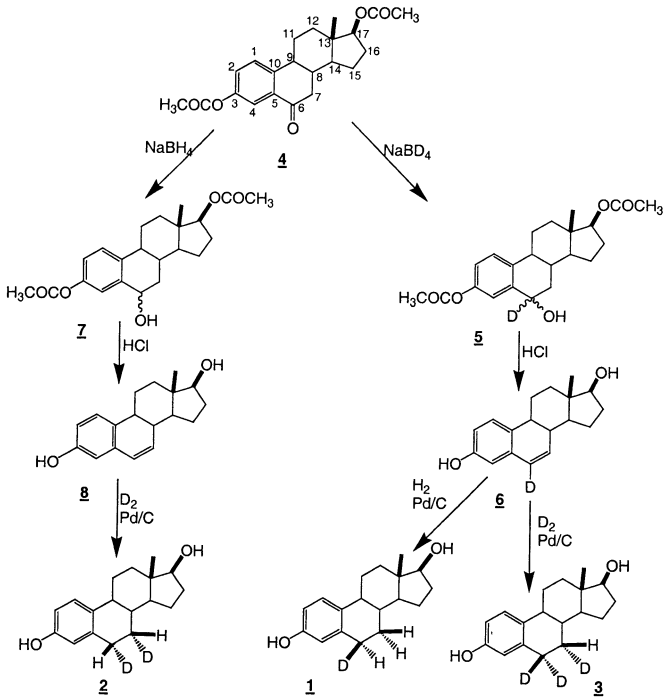
1044-0305/03/$30.00
doi:10.1016/S1044-0305(03)00066-7

Received May 16, 2002
Revised January 14, 2003
Accepted January 14, 2003

nucleosides have been extensively studied [8, 14–16] and the major covalent adduct formed from model reactions was detected *in vivo* from rats [17] or hamsters [18] treated with 4-hydroxyestradiol.

The occurrence of DNA adducts can be evidenced with great sensitivity using immunoassays and ^{32}P post-labelling [7, 19], but these methods cannot provide structural information. Because of its sensitivity and specificity, mass spectrometry appears to be a powerful tool for exploring this field of investigation [20–22]. Moreover, the possibility of on-line coupling with separation techniques, such as HPLC or CE, enhances the detection capabilities of this method [23–25]. Among different soft desorption-ionization techniques (e.g., MALDI, FAB, TSP), electrospray ionization is now commonly used for DNA adduct analysis, because of the very soft desolvation conditions which avoid fragile bond cleavages. Although negative ESI is widely used for the analysis of unmodified [26, 27] as well as modified oligonucleotides [28–30], the adducts formed between nucleosides and electrophilic reagents are generally analyzed using positive ionization [31–33], mainly for better sensitivity reasons. In previous works [34], the resulting products from the reactivity of estradiol-2,3-quinone towards desoxyribonucleosides have been investigated using LC-ESI-MS^n. Depending upon the sites of both the DNA base and the steroid involved in the Michael addition process, several isomeric compounds were formed, in particular with dG which was found to be the most reactive deoxyribonucleoside [8, 34]. Regio- and stereo-isomeric adducts have been identified using concomitant LC-ESI/MS^n and NMR methodologies [35]. Although the use of sequential LC-MS^n experiments allowed to determine the regiochemistry of adduction (e.g., at the A or B ring of the steroid), in some cases, the linkage position could not be clearly evidenced. In this context, studies using labeled compounds with deuterium atoms at various sites have been undertaken in order to highlight the differentiation of isomeric adducts by studying the mass shift and the fragmentation pattern of their corresponding quasi-molecular ions, thus providing additional information on the adduction site.

In the present work, deuterium labeled 2-hydroxy-17β-estradiol (2OH$E_2\beta$) [i.e., 6β (**1**); 6α-7α (**2**); 6α-6β-7α (**3**)] have been synthesized (Scheme **1**). The corresponding labeled quinones have been formed and let to react with deoxyguanosine. The resulting crude mixtures were directly analyzed by LC-ESI-MS^n using previously developed analytical conditions [34]. Various LC-MS^2 and LC-MS^3 experiments into an ion trap have been performed via resonant excitation of the different selected ions. LC-MS^n experiments using deuterated mobile phases have also been carried out in order to clarify some fragmentation processes.



Scheme 1. Synthesis of deuterium labeled estradiol.

Experimental

Mass Spectrometry

All experiments were performed on a Finnigan LCQ ion trap mass spectrometer (Thermo Quest, Les Ulis, France) equipped with an ESI source operated in the positive mode using the following conditions: Needle voltage (5 kV); heated capillary temperature (230 °C); capillary voltage (4 V); tube lens offset (5 V). Nitrogen was used as the sheath gas at a flow rate of 80% (arb. units). All spectra were acquired using AGC (Automatic Gain Control). MS^n experiments were carried out using He as collision gas. Collision energies were adjusted in order to get maximum structural information for each experiment, i.e., typically 17–20% for MS/MS on the quasi-molecular species, and 21% for MS^3 experiments (100% collision energy corresponds to 5 V peak to peak). In all MS^n experiments, ions were isolated at q_z 0.83 with 1.5 u as ion isolation width and excited at q_z 0.25.

Liquid Chromatography

Liquid chromatography has been used to introduce the samples into the ESI source at a 0.2 ml/min flow rate. This was carried out using a Thermo Separation P4000 pump (Thermo Quest) fitted with a Rheodyne injector. The LC column used was an Ultrabase 5 μm C18 column (25 $\times$ 2 mm) from SFCC (Eragny, France). The following gradient elution was used: 100% A to 85% A from 0 to 5 min., then 85% A to 50% A from 5 to 30 min., and 50% A to 100% B from 30 to 40 min., and finally 100% B from 40 to 60 min., with A, H_2O/CH_3OH/

Table 1. Isomeric adducts obtained from reactions of deuterium labeled steroids **1**, **2**, **3** with dG.

Adducts M.W. Isomers	(**1**) + dG 554		(**2**) + dG 555		(**3**) + dG 556	
	a[a]	**b**[b]	**a**[a]	**b**[b]	**a**[a]	**b**[b]
Quasi-molecular ions *m/z*	555($M_{d1}H^+$,75%) 554($M_{d0}H^+$,25%)		556($M_{d2}H^+$,75%) 555($M_{d1}H^+$,25%)		557($M_{d3}H^+$,20%) 556($M_{d2}H^+$,80%)	

[a]retention time: 30.4 min.
[b]retention time: 31.6 min.

CH_3COOH (90:10:0.2) and B, $H_2O/CH_3OH/CH_3COOH$ (10:90:0.2).

Materials

The synthetic procedure used for the preparation of the studied adducts from the different deuterated compounds was described in the literature [8]. Briefly, the 2-hydroxy derivatives of the deuterated compounds were prepared according to the method described by Gelbke et al. [36]. The deuterated quinones were prepared by oxidation in acetonitrile at −40 °C of the corresponding catechols using activated manganese dioxide utilizing a procedure published by Abul-Hajj et al. [37]. The resulting suspension was then filtered directly into a stirred solution of dG as previously described [8] and let to react 5 h at room temperature.

Compounds **1**, **2**, **3** were prepared from the 6-keto-17β-estradiol diacetate **4** (Scheme **1**). **4** was prepared from 17β-estradiol as described by Garza and Rao [38] and reduced with sodium borohydride (deuterated or not) to give the alcohols **5** or **7**. These products were dehydrated to give the Compounds **6** or **8**, which were then hydrogenated on Pd/C as catalyst either with hydrogen or with deuterium gas. The stereochemistry of all the deuterated compounds was checked by comparison of the corresponding ^{1}H NMR spectra with that of 17β-estradiol.

*Synthesis of compounds **1** and **3**.* A solution of **4** (100 mg) in 3.3 mL of methanol-d_4-tetrahydrofuran-d_8 (10:1, vol/vol) was stirred at −15 °C with sodium borohydride-d_4 for 30 min. Acetic acid (0.15 mL) was added and the mixture stirred for 85 min. After addition of water (50 mL), extraction with ethylacetate and drying over magnesium sulfate, 6-^{2}H-6-hydroxy-17β-estradiol diacetate (**5**) was obtained (46% yield).

A methanolic solution of **5** (12 mg in 12 mL) was stirred for 8 h with 1N HCl (8 mL), then for 15 h more with 1N HCl (4 mL). After methanol evaporation, washing with water, extraction with ethylacetate, and drying over magnesium sulfate, 41mg of deuterated Compound **6** were obtained (47% yield). A solution of 30 mg of **6** in a mixture of 8 mg 10% Pd/C in ethylacetate (2 mL) was hydrogenated with D_2 at atmospheric pressure to give the Compound **3** after filtration and evaporation. A similar procedure with H_2 led to the Compound **1**.

6β-^{2}H-17β-estradiol (**1**): ^{1}H NMR (in CD_3OD), δ 0.77 (s, 3H, CH_3), 1.14 (m, 1H, $H_{14\alpha}$), 1.2-1.55 (m, 6 H, $H_{12\alpha}$, $H_{7\alpha}$, $H_{15\beta}$, $H_{8\beta}$, $H_{11\beta}$, $H_{16\beta}$), 1.65 (m, 1H, $H_{15\alpha}$), 1.84 (d, 1H, $H_{7\beta}$), 1.92 (dt, 1H, $H_{12\beta}$), 2.0 (m, 1H, $H_{16\alpha}$), 2.10 (td, 1H, H_9), 2.25 (dq, 1H, $H_{11\alpha}$), 2.75 (d, J=5.5, 1H, $H_{6\alpha}$), 3.62 (t, 1H, $H_{17\alpha}$), 6.47 (s, 1H, H_4), 6.53 (d, 1H, H_2), 7.06 (d, 1H, H_1).

6,6,7α-^{2}H-17β-estradiol (**3**): ^{1}H NMR (in CD_3OD), δ 0.77 (s, 3H, CH_3), 1.14 (m, 1H, $H_{14\alpha}$), 1.2-1.55 (m, 6 H, $H_{12\alpha}$, $H_{7\alpha}$, $H_{15\beta}$, $H_{8\beta}$, $H_{11\beta}$, $H_{16\beta}$), 1.65 (m, 1H, $H_{15\alpha}$), 1.84 (s, 1H, $H_{7\beta}$), 1.92 (dt, 1H, $H_{12\beta}$), 2.0 (m, 1H, $H_{16\alpha}$), 2.10 (td, 1H, H_9), 2.25 (dq, 1H, $H_{11\alpha}$), 3.62 (t, 1H, $H_{17\alpha}$), 6.47 (s, 1H, H_4), 6.53 (d, 1H, H_2), 7.06 (d, 1H, H_1).

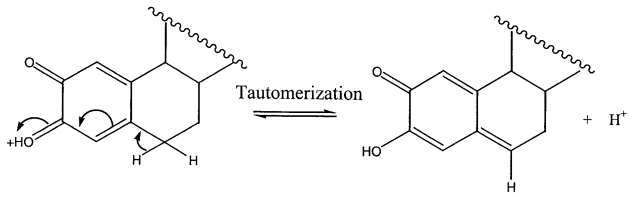
*Synthesis of compound **2**.* 100 mg of **4** in methanol (4 mL) and tetrahydrofuran (1 mL) were slowly added to a solution of sodium borohydride (70 mg in 0.1 mL water) at 0 °C. This mixture was stirred at 0 °C for 30 min and 1 h more after addition of acetic acid (0.1 mL). After extraction, washing and drying, the crude product was purified on silica plates: 65mg of solid 6-hydroxy-17β-estradiol diacetate **7** were obtained (65% yield). A mixture of 38 mg of **7** in methanol (5 mL) and 1N HCl (0.7 mL) was stirred at 80 °C for 3 h. Then water (20 mL) was added. After extraction, neutralization, drying, and purification on silica plates, 14 mg of the unsaturated Compound **8** were obtained (51% yield). As described above, catalytic hydrogenation with D_2 on **8** led to the 6,7-^{2}H-17β-estradiol **2** with a 45% yield. The H_2 or D_2 addition proceeds on the α face.

6α,7α-^{2}H-17β-estradiol (**2**): ^{1}H NMR (in CD_3OD), δ 0.77 (s, 3H, CH_3), 1.14 (m, 1H, $H_{14\alpha}$), 1.2-1.55 (m, 6 H, $H_{12\alpha}$, $H_{7\alpha}$, $H_{15\beta}$, $H_{8\beta}$, $H_{11\beta}$, $H_{16\beta}$),.1.65 (m, 1H, $H_{15\alpha}$), 1.84 (dd, 1H, $H_{7\beta}$), 1.92 (dt, 1H, $H_{12\beta}$), 2.0 (m, 1H, $H_{16\alpha}$), 2.10 (td, 1H, H_9), 2.25 (dq, 1H, $H_{11\alpha}$), 2.75 (d, J=5.0, 1H, $H_{6\beta}$), 3.62 (t, 1H, $H_{17\alpha}$), 6.47 (s, 1H, H_4), 6.53 (d, 1H, H_2), 7.06 (d, 1H, H_1).

Results and Discussion

Labeled Covalent Adducts Molecular Weights

LC-ESI-MS analyses of the different crude reaction mixtures revealed several isomeric adducts eluted at two different retention times, i.e., 30.4 and 31.6 min, and reported as **a** and **b** isomers in Table 1, together with their respective molecular weight and the *m/z* of their labeled protonated molecular species. As shown in Table 1, each chromatographic peak consisted of a mixture of isotopomers as indicated by the isotopic



Scheme 2. Proposed mechanism for the isomerization of the *o*-quinone to the *p*-quinone methide.

patterns of the quasi-molecular species. It must be noted that unstable adducts (without deoxyribose moiety) were not observed in our work in contrast with previously published results [34]. This was very likely due to the low yield of the reactions conducted in our study.

First, the direct molecular weight measurement of the various labeled covalent adducts provided information on the attachment position of the nucleoside and steroid partners (Table 1). As previously emphasized [8, 34], almost all adducts were formed by a nucleophilic attack of the deoxyguanosine at the C6 position of the steroid substrate. This suggested that o-quinones isomerized into more electrophilic p-quinone methide forms, which underwent Michael addition reactions with deoxyguanosine, thus resulting in benzylic adducts (Scheme **2**). In a similar way, the formation of adducts between quinones and glutathione was also shown to involve such isomerization processes [39–42]. Thus, by analogy with a previously reported base-catalyzed isomerization process [41], a mechanism involving acidic catalysis can be proposed for the formation of covalent adducts between estradiol-2,3-quinone and deoxyguanosine in the acidic medium used in this work (Scheme **2**).

The reaction of deuterium labeled steroids led to covalent adducts displaying shifted *m/z* of the quasi-molecular species. As indicated in Table 1, major adducts were detected at *m/z* 556 from both **2** and **3** isotopomers and at *m/z* 555 from **1**. This indicated one deuterium atom (C6α/β or C7α position) was lost from **3** whereas the deuterium atoms from **1** and **2** were preserved. This behavior suggested that the formation of these adducts may involve the elimination of one H or D atom from the C6 position of the steroid B ring. Indeed, the *m/z* of the observed quasi-molecular ions depended upon the nature of the atom lost during the oxidation step of the catechol estrogen into its corresponding quinone methide form as described in Scheme **2**. The loss of H at position C6 from **1** and **2**, led to the major isotopomeric covalent adduct ions at *m/z* 555 and 556, respectively. Conversely, the elimination of one D at position C6β for **1** and C6α for **2** observed as a minor process led to the occurrence of ions at *m/z* 554 and 555. This result indicated that an isotope effect may occur in the formation of the p-quinone methides for **1** and **2**, in which the hydrogen elimination is favored compared to that of the deuterium atom. A similar isotope effect has also been reported for the isomerization of 4-propyl-o-quinone into its tautomeric p-quinone methide form [41] and was in the range of $k_H/k_D = 5.5 \pm 0.6$ at 37 °C.

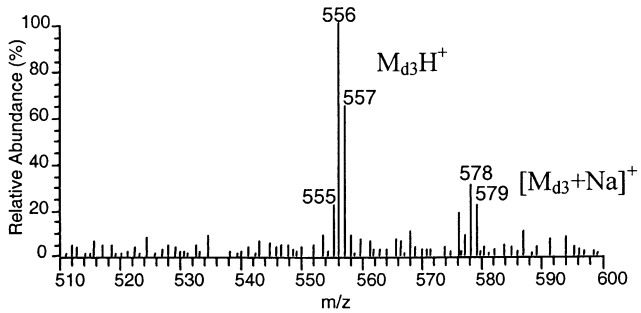


Figure 1. Positive ESI mass spectrum of the **b** isomer of (**3** + dG).

On the other hand, the presence of some adducts detected as the $M_{d3}H^+$ ions at *m/z* 557 (and $[M_{d3} + Na]^+$ ions at *m/z* 579) from **3** indicated that the initial labeling could also be preserved on the trideuterated compound (Figure 1). Hence, in this case, a base addition occurring at a site other than the C6 position (i.e., at the C9 position or the A ring) had to be considered.

MS^n Experiments on the m/z 555 and 556 Ions from 1, 2, and 3

As indicated on Figure 2 a, b, and c, all the ionic adduct species (**a** isomers as well as **b** isomers) at *m/z* 555 and 556 ion decomposed according to the same fragmentation pathway. This fragmentation mainly involved the loss of the deoxyribose moiety from the quasi-molecular ions in the MS^2 step, whereas the resulting fragment ion underwent the cleavage of the steroid-base bond leading to the formation of the protonated base (*m/z* 152) and $[MH\text{-}dG]^+$ (*m/z* 288 or 289) as a pair of complementary ions. However, slight differences can be observed on the CID spectrum of **1** (Figure 2a) when compared to the CID spectra of **2** and **3** (Figure 2b and c), concerning in particular the stability of the molecular species. Indeed, when submitted to the same isolation and excitation conditions in the ion trap cell, the $\mathbf{1}H^+$ species seems to be more stable than its $\mathbf{2}H^+$ and $\mathbf{3}H^+$ counterparts. This difference may be due to isotope effects, which seem difficult to interpret. However, the subsequent MS^3 spectra acquired from the *m/z* 440 (or 439) daughter ions are completely identical for **1** (Figure 2a), **2** (Figure 2b), and **3** (Figure 2c). This shows that these *m/z* 440 (or 439) selected product ions are the same ionic species. These results were consistent with the attachment of the base at the steroid B ring and indicated that all the adduct ions (**a** and **b** isotopomers), possessed similar structures. As already reported [8], nucleophilic attack at the prochiral C6 atom of the steroid resulted in the formation of two diastereoisomers (**a** and **b** isomers) which corresponded to the α and β forms as illustrated in Scheme **3** on the basis of Compound **3**. The formation of the quinone methide

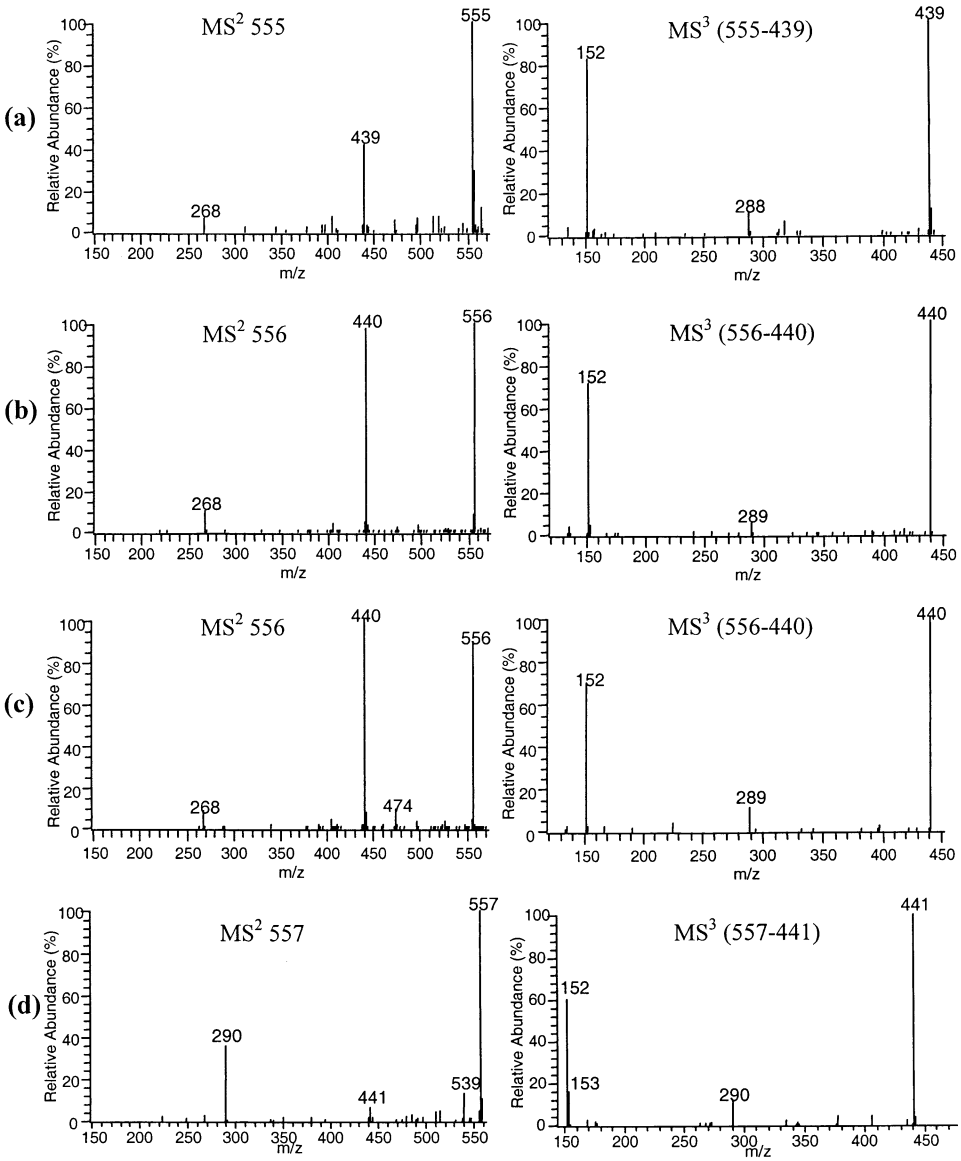
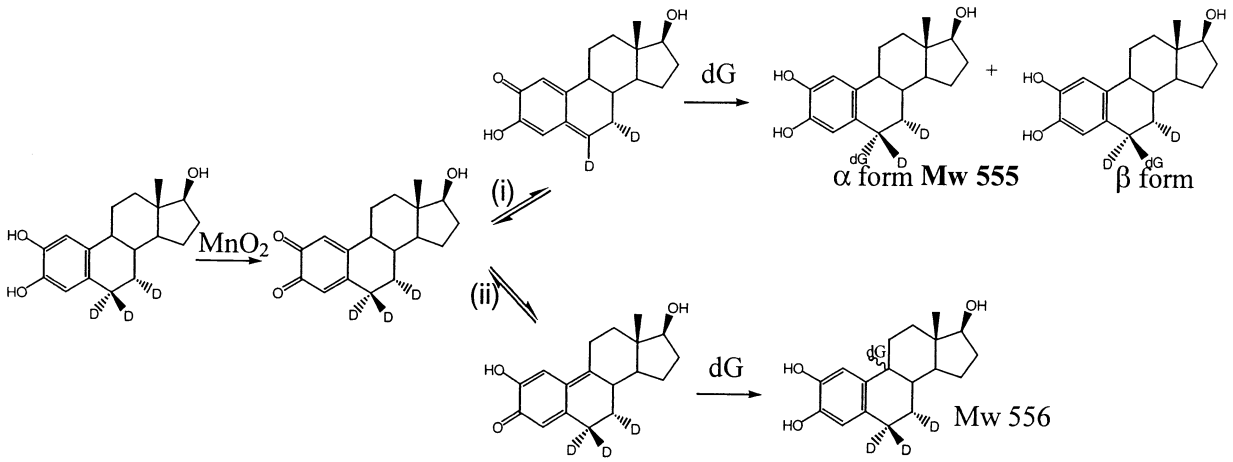


Figure 2. MS^2 and MS^3 spectra of ESI-produced protonated molecular ions from the **b** isomer of (**a**) (**1** + dG), (**b**) (**2** + dG), and (**c** and **d**) (**3** + dG).

intermediate implies a loss of stereochemistry at the C6 position of the steroid as reported in Scheme **2**, accompanied by an isotope effect concerning the H or D elimination. This explains the occurrence of isotopomers at M and M + 1 on both diastereoisomers formed according to Michael addition processes. Such isomeric adduct species have already been evidenced by LC-ESI-MS/MS and NMR and it has been shown that both diastereoisomers could be separated by HPLC [34, 35].

MS^n Experiments on the m/z 557 Ions from 3: Evidence for a Reaction at the Steroid C9 Position

The *m/z* 557 ions from the labeled Compound **3** possessed a significant relative abundance (65% of the peak *m/z* 556, see Figure 1). In this case, the contribution of the ^{13}C natural isotopic abundance of the *m/z* 556 ions, and the occurrence of covalent adducts which preserved the three initial D atoms should be considered. Taking into account that the natural isotopic contributions at M + 1 for a $C_{28}H_{35}O_7N_5$ species, the relative abundance of the peak at *m/z* 557 should correspond to ca. 33% of the protonated ions at *m/z* 556. The actual relative abundance observed for the *m/z* 557 ion is 65%. The same trend is observed for the MNa^+ species, with the *m/z* 579 ion relative abundance corresponding to 65% of the peak at *m/z* 578 (Figure 1). This indicated that the *m/z* 557 ion also contained a molecular species in which the three initial D atoms were preserved, suggesting that the adduction process may also occur on a site other than the C6 position. Moreover, the CID spectrum presented in Figure 2d also showed that the behavior of the *m/z* 557 ions from **3** towards collisional



Scheme 3. Proposed reaction pathway for adduct formations in the reaction between **3** and dG.

activation was specific. In this case, the main fragmentation pathways consisted of a water elimination and steroid-base linkage cleavage to yield the *m/z* 539 and 290 ions, respectively, whereas the loss of deoxyribose was observed as a weak process. This particular pattern has never been observed on estrogen quinone-deoxyribonucleoside covalent adducts until this work, indicating that this adduct should have a different structure. From previously published results [8], the adduction process at the steroid A ring could be ruled out since the nucleobase and steroid are strongly bonded in such covalent adducts, which mainly dissociate according to charge-remote fragmentations [8, 34, 35]. Moreover, in this case, the presence of both complementary ions at *m/z* 152 and 290 in the MS^3 spectrum of the $[MH\text{-deoxyribose}]^+$ *m/z* 441 ions (resulting from the decomposition of the parent *m/z* 557 ions) was characteristic of the cleavage of the steroid-base linkage, consistent with a linkage at the steroid B ring. The presence of a weak signal at *m/z* 153 was very likely due to the ^{13}C contribution of the *m/z* 556 ions also selected at *m/z* 557 and could be attributed to the ^{13}C contribution of the protonated base. Thus, it can be concluded that the *m/z* 557 ions probably correspond to a covalent adduct resulting from the addition of the base on the activated C9 site as reported in Scheme **3**. As the C9 site was also a prochiral center, two diastereoisomers could also be expected. However, these C9 attachment adducts detected at *m/z* 557 appeared to be eluted at retention times similar to those of the **a** and **b** isomers of the C6 attachment adducts, respectively. The greater abundance of the *m/z* 290 ions relative to the *m/z* 441 ions in the MS^2 spectrum of the *m/z* 557 ions (Figure 2d) could be expected from a C9 adduct since the elimination of the base moiety can be favored because of steric decompression effects and the tertiary character of the produced carbonium site.

The formation of some adduct ions at *m/z* 555 (d_1) and 556 (d_2) could also be expected from **1** and **2**, respectively, considering the same nucleophilic attack at the C9 position of the steroid resulting in the preservation of the initial number of deuterium atoms, but the occurrence of these species could not be evidenced in our experiments.

It can be concluded from these results that the reaction of dG at the C9 position led to unstable adducts, which could be evidenced by the specific fragmentation pathway observed from the *m/z* 557 ion of **3** from freshly prepared reaction mixtures, and which decomposed after several hours. This assumption was further supported by the change in the isotopic profile initially observed (Figure 1) from a *m/z* 557 relative intensity of 65% to a relative intensity below ca. 30% when the same sample was analyzed some days after. Furthermore, the CID spectrum of the *m/z* 557 ions selected from **3** became similar to those of the other quasi-molecular ions described in Figure 2a to c, i.e., it displayed a major fragment ion at *m/z* 441 whereas no diagnostic fragment ions at *m/z* 290 and 539 were observed (data not shown). This behavior indicated that after several hours, the C9 attachment adducts were degraded and the detected *m/z* 557 ions only corresponded to the ^{13}C isotopic contribution of the *m/z* 556 ions formed according to the C6 addition process.

Although adducts with the C9 attachment had never been clearly evidenced in previous works, a compound identified as the 11-oxo-2OHE$_2\beta$ was detected in crude reaction mixtures, and its origin was attributed to the degradation of such C9 covalent adducts [35, 43]. Alternatively, the nucleophilic attack at the C9 position was also proposed for the formation of some estrogen quinone-glutathione adducts, but the occurrence of such species could not be evidenced [39, 42]. In this case, the unstable character of such adducts was also reported and only products formed by elimination of glutathione (i.e., 9-dehydro-4OHE and 9-dehydro-2OHE from 4OHE-o-quinone and 2OHE-o-quinone, respectively) were identified as the end products of the degradation of such transient adduct species [39, 42].

Table 2. *m/z* ratios of the various ionic species obtained by LC-ESI/MSn analyses of crude reaction mixtures in deuterated medium.

Adducts	M_dD^+ (*m/z*)		$[M_dD\text{-deoxyribose}]^+$ (*m/z*)		BD_2^+ (*m/z*)
$2OHE_2\beta$ + dG	562	$\xrightarrow{MS^2}$	445	$\xrightarrow{MS^2}$	157
(**2**) + dG	564	$\xrightarrow{MS^2}$	447	$\xrightarrow{MS^2}$	157
(**3**) + dG	564	$\xrightarrow{MS^2}$	447	$\xrightarrow{MS^2}$	157

Fragmentation Processes Under MS^n Conditions as Revealed by LC-MS^n Analyses in Deuterated Medium

In order to gain complementary information on the covalent adduct fragmentation processes, LC-MS^n analyses have been performed using deuterated mobile phases. The *m/z* of the various ionic species of interest are reported in Table 2. Under these conditions, H/D exchanges occurred in solution and the *m/z* of quasi-molecular ions were shifted by 8 Th, which is consistent with the occurrence of seven exchangeable hydrogen atoms in the covalent adduct structure and the associated addition of a deuterium for ionization. MS/MS as well as MS^3 experiments were carried out on these various species (Table 2). The resulting low energy CID spectra of these species mainly displayed product ions at *m/z* 445 from ($2OHE_2\beta$ + dG) and *m/z* 447 ions from (**2** + dG) or (**3** + dG), corresponding to the loss of the deoxyribose moiety from the molecular species. Surprisingly, a specific loss of 117 u instead of the expected 118 u loss has been observed for the deoxyribose moiety elimination process. Taking into account the exchangeable hydrogen atoms on the deoxyribose moiety, the formation of these fragment ions by loss of 117 u was unexpected, since the H/D exchanges in the ion trap were minor during the MS/MS experiment period as observed from the fragmentation of protonated standard compounds in deuterated medium (data not shown). Hence, a process involving a deuterium migration from the deoxyribose to the base moiety could explain the *m/z* shift of fragment ions formed by the loss of deshydrogenated deoxyribose from the quasi-molecular ions (Scheme **4**), although other processes (e.g., formation of an ion–dipole complex followed by H/D exchange) could also rationalize the formation of such species.

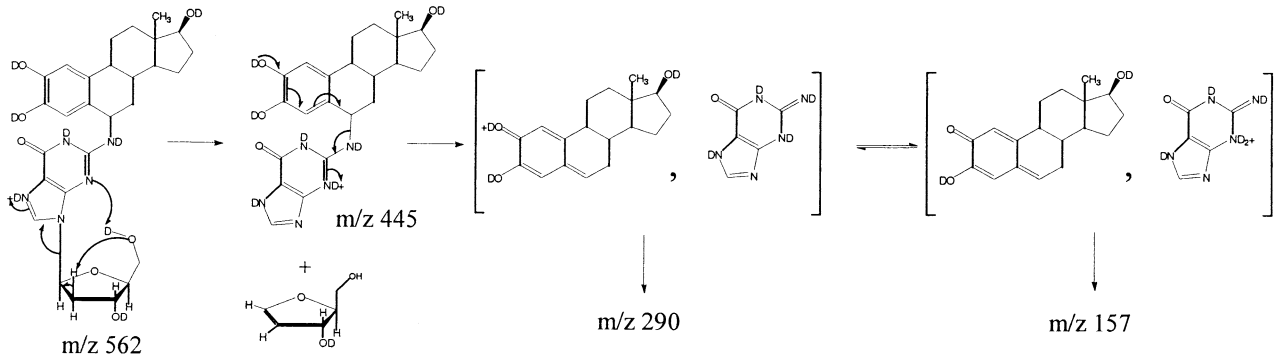
From the MS^3 experiments carried out on the *m/z* 445 or 447 ions, the *m/z* shift observed for the product protonated base (*m/z* 152 to 157) showed that an elimination mechanism involving a hydrogen on C7 position could be ruled out for the formation of such species. A mechanism involving a D transfer should preferably be considered. Hence, the cleavage of the steroid-base linkage could rather be explained via the formation of an ion–dipole complex accompanied by a deuterium transfer from the OD group of the catechol to the neutral base moiety of such a complex as presented in Scheme **4**.

Conclusion

In this study, the use of deuterated 2-hydroxyestradiol allowed the differentiation of isomeric adducts using LC-ESI-MS^n. The study of the *m/z* shift as well as the fragmentation pathways observed for the quasi-molecular ions have shown that addition of the base could occur on both the C6 and C9 sites of the steroid B ring in its quinone-methide form. Although compounds attributed to the degradation of an adduct formed by nucleophilic attack of the base on C9 position have already been identified [39, 42, 43], the corresponding adducts had never been clearly evidenced by LC-MS^n until this work. Some complementary work needs now to be developed in order (1) to achieve a complete chromatographic separation of the C6 and C9 adducts which coelute in our LC-MS experiments and (2) to improve the understanding of the particular fragmentation process of this adduct.

Acknowledgments

The authors thank Huguette Petit for his excellent technical assistance in the syntheses of deuterated compounds, and Christel Van Aerden for preliminary works on this topic.



Scheme 4. Proposed mechanism for the fragmentation of the $[M_{d7} + D]^+$ ion of 2-OHE_2-6-N^2-dG under MS^n conditions.

References

1. Cavalieri, E.; Frenkel, K.; Liehr, J. G.; Rogan, E.; Roy, D. Estrogens as Endogenous Genotoxic Agents—DNA Adducts and Mutations. *J. Natl. Cancer Inst. Monogr.* **2000,** *27,* 75–93.
2. Bolton, J. L.; Pisha, E.; Zhang, F.; Qiu, S. Role of Quinoids in Estrogen Carcinogenesis. *Chem. Res. Toxicol.* **1998,** *11,* 1113–1127.
3. Zhu, B. T.; Conney, A. H. Functional Role of Estrogen Metabolism in Target Cells: Review and Perspectives. *Carcinogenesis* **1998,** *19,* 1–27.
4. Butterworth, M.; Lau, S. S.; Monks, T. J. 17β-Estradiol Metabolism by Hamster Hepatic Microsomes: Comparison of Catechol Estrogen*O*-Methylation with Catechol Estrogen Oxidation and Glutathione Conjugation. *Chem. Res. Toxicol.* **1996,** *9,* 793–799.
5. Rathahao, E; Hillenweck, A.; Paris, A.; Debrauwer, L. Investigation of the in Vitro Metabolism of 17β-Estradiol by LC-MS/MS Using ESI and APCI. *Analysis* **2000,** *28,* 273–279.
6. Abul-Hajj, Y. J.; Tabakovic, K.; Gleason, W. B.; Ojala, W. H. Reactions of 3,4-Estrone Quinone with Mimics of Amino Acid Side Chains. *Chem. Res. Toxicol.* **1996,** *9,* 434–438.
7. Dwivedy, I.; Devanesan, P.; Cremonesi, P.; Rogan, E.; Cavalieri, E. L. Synthesis and Characterization of Estrogen 2,3- and 3,4-Quinones. Comparison of DNA Adducts Formed by the Quinones Versus Horseradish Peroxidase-Activated Catechol Estrogens. *Chem. Res. Toxicol.* **1992,** *5,* 828–833.
8. Stack, D. E.; Byun, J.; Gross, M. L.; Rogan, E. G.; Cavalieri, E. L. Molecular Characteristics of Catechol Estrogen Quinones in Reactions with Deoxyribonucleosides. *Chem. Res. Toxicol.* **1996,** *9,* 851–859.
9. Terashima, I.; Suzuki, N.; Shibutani, S. Mutagenic Properties of Estrogen Quinone-Derived DNA Adducts in Simian Kidney Cells. *Biochemistry* **2001,** *40,* 166–172.
10. Chakravarti, D.; Mailander, P. C.; Li, K. M.; Higginbotham, S.; Zhang, H. L.; Gross, M. L.; Meza, J. L.; Cavalieri, E. L.; Rogan, E. G. Evidence that a Burst of DNA Depurination in SENCAR Mouse Skin Induces Error-Prone Repair and Forms Mutations in the H-*ras* Gene. *Oncogene* **2001,** *20,* 7945–7953.
11. Cao, K.; Stack, D. E.; Ramanathan, R.; Gross, M. L.; Rogan, E. G.; Cavalieri, E. L. Synthesis and Structure Elucidation of Estrogen Quinones Conjugated with Cysteine, N-Acetylcysteine, and Glutathione. *Chem. Res. Toxicol.* **1998,** *11,* 909–916.
12. Cao, K.; Devanesan, P.; Ramanathan, R.; Gross, M. L.; Rogan, E. G.; Cavalieri, E. L. Covalent Binding of Catechol Estrogens to Glutathione Catalyzed by Horseradish Peroxidase, Lactoperoxidase, or Rat Liver Microsomes. *Chem. Res. Toxicol.* **1998,** *11,* 917–924.
13. Ramanathan, R.; Cao, K.; Cavalieri, E. L.; Gross, M. L. Mass Spectrometric Methods for Distinguishing Structural Isomers of Glutathione Conjugates of Estrone and Estradiol. *J. Am. Soc. Mass Spectrom.* **1998,** *9,* 612–619.
14. Abul-Hajj, Y. J.; Tabakovic, K.; Tabakovic, I. An Estrogen-Nucleic Acid Adduct. Electroreductive Intermolecular Coupling of 3,4-Estrone-*o*-Quinone and Adenine. *J. Am. Chem. Soc.* **1995,** *117,* 6144–6145.
15. Akanni, A.; Tabakovic, K.; Abul-Hajj, Y. J. Estrogen-Nucleic Acid Adducts: Reaction of 3,4-Estrone-*o*-Quinone with Nucleic Acid Bases. *Chem. Res. Toxicol.* **1997,** *10,* 477–481.
16. Akanni, A.; Abul-Hajj, Y. J. Estrogen-Nucleic Acid Adducts: Reaction of 3,4-Estrone-*o*-Quinone Radical Anion with Deoxyribonucleosides. *Chem. Res. Toxicol.* **1997,** *10,* 760–766.
17. Cavalieri, E. L.; Stack, D. E.; Devanesan, P. D.; Todorovic, R.; Dwivedy, I.; Higginbotham, S.; Johansson, S. L.; Patil, K. D.; Gross, M. L.; Gooden, J. K.; Ramanathan, R.; Cerny, R. L.; Rogan, E. G. Molecular Origin of Cancer: Catechol Estrogen-3,4-Quinones as Endogeneous Tumor Initiators. *Proc. Natl. Acad. Sci. U.S.A.* **1997,** *94,* 10937–10942.
18. Devanesan, P.; Todorovic, R.; Zhao, J.; Gross, M. L.; Rogan, E. G.; Cavalieri, E. L. Catechol Estrogen Conjugates and DNA Adducts in the Kidney of Male Syrian Golden Hamsters Treated with 4-Hydroxyestradiol: Potential Biomarkers for Estrogen-Initiated Cancer. *Carcinogenesis* **2001,** *22,* 489–497.
19. Zeisig, M.; Möller, L. 32P-Postlabeling High-Performance Liquid Chromatographic Improvements to Characterize DNA Adduct Stereoisomers from Benzo[a]pyrene and Benzo[c-]phenanthrene, and to Separate DNA Adducts from 7,12-Dimethylbenz[a]anthracene. *J. Chromatogr. B* **1997,** *691,* 341–350.
20. Farmer, P. B.; Sweetman, G. M. A. Mass Spectrometric Detection of Carcinogen Adducts. *J. Mass Spectrom.* **1995,** *30,* 1369–1379.
21. Chaudhary, A. J.; Nokubo, M.; Oglesby, T. D.; Marnett, L. J.; Blair, I. A. Characterization of Endogeneous DNA Adducts by Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry. *J. Mass Spectrom.* **1995,** *30,* 1157–1166.
22. Chiarelli, M. P.; Lay, J. O. Mass Spectrometry for the Analysis of Carcinogen-DNA Adducts. *Mass Spectrom. Rev.* **1992,** *11,* 447–493.
23. Esmans, E. L.; Broes, D.; Hoes, I.; Lemière, F.; Vanhoutte, K. Liquid Chromatography-Mass Spectrometry in Nucleoside, Nucleotide, and Modified Nucleotide Characterization. *J. Chromatogr. A* **1998,** *794,* 109–127.
24. Andrews, C. L.; Vouros, P.; Harsh, A. Analysis of DNA Adducts Using High-Performance Separation Techniques Coupled to Electrospray Ionization Mass Spectrometry. *J. Chromatogr. A* **1999,** *856,* 515–526.
25. Apruzzese, W. A.; Vouros, P. Analysis of DNA Adducts by Capillary Methods Coupled to Mass Spectrometry: A Perspective. *J. Chromatogr. A* **1998,** *794,* 97–108.
26. McLuckey, S. A.; Van Berkel, G. J.; Glish, G. L. Tandem Mass Spectrometry of Small, Multiply Charged Oligonucleotides. *J. Am. Soc. Mass Spectrom.* **1992,** *3,* 60–70.
27. Wang, Y.; Taylor, J.; Gross, M. L. Fragmentation of Electrospray-Produced Oligonucleotide Ions Adducted to Metal Ions. *J. Am. Soc. Mass Spectrom.* **2001,** *12,* 550–556.
28. McLuckey, S. A.; Habibi-Goudarzi, S. Ion Trap Tandem Mass Spectrometry Applied to Small Multiply Charged Oligonucleotides with a Modified Base. *J. Am. Soc. Mass Spectrom.* **1994,** *5,* 740–747.
29. Marzilli, L. A.; Wang, D.; Kobertz, W. R.; Essigmann, J. M.; Vouros, P. Mass Spectral Identification and Positional Mapping of Aflatoxin B_1-Guanine Adducts in Oligonucleotides. *J. Am. Soc. Mass Spectrom.* **1998,** *9,* 676–682.
30. Wang, Y.; Taylor, J. S.; Gross, M. L. Fragmentation of Photomodified Oligonucleotides Adducted with Metal Ions in an Electrospray Ionization Ion Trap Mass Spectrometer. *J. Am. Soc. Mass Spectrom.* **2001,** *12,* 1174–1179.
31. Ding, J.; Barlow, T.; Dipple, A.; Vouros, P. Separation and Identification of Positively Charged and Neutral Nucleoside Adducts by Capillary Electrochromatography-Microelectrospray Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **1998,** *9,* 823–829.
32. Kambouris, S. J.; Chaudhary, A. K.; Blair, I. A. Liquid Chromatography/Electrospray Ionization Tandem Mass Spectroscopy (LC/ESI/MS/MS) Analysis of 1,2-Epoxybutene Adducts of Purine Deoxynucleosides. *Toxicology* **1996,** *113,* 331–335.
33. Hoes, I.; Lemière, F.; Van Dongen, W.; Vanhoutte, F.; Esmans, E. L.; Bockstaele, D. V.; Berneman, Z.; Deforce, D. Van den; Eeckhout, E. G. Analysis of Melphalan Adducts of 2'-Deoxynucleotides in Calf Thymus DNA Hydrolysates by Capillary High-Performance Liquid Chromatography-Electrospray Tandem Mass Spectrometry. *J. Chromatogr. B* **1999,** *736,* 43–59.

34. Van Aerden, C.; Debrauwer, L.; Tabet, J. C.; Paris, A. Analysis of Nucleoside-Estrogen Adducts by LC-ESI-MS-MS. *Analyst* **1998,** *123,* 2677–2680.
35. Convert, O.; Van Aerden, C.; Debrauwer, L.; Rathahao, E.; Molines, H.; Fournier, F.; Paris, A. Reactions of Estradiol-2,3-Quinones with Deoxynucleosides: Possibles Insights in the Reactivity of Estrogen Quinones with DNA. *Chem. Res. Toxicol.* **2002,** *15,* 754–764.
36. Gelbke, H. P.; Haupt, O.; Knuppen, R. A Simple Chemical Method for the Synthesis of Catechol Estrogens. *Steroids* **1976,** *21,* 205–218.
37. Abul-Hajj, Y. J. Synthesis of 3,4-Estrogen-o-Quinone. *J. Steroid Biochem.* **1984,** *21,* 621–622.
38. Garza, G. A.; Rao, P. N. Chromic Anhydride-3,5-Dimethylpyrazole Complex: An Efficient Reagent for Oxidation of Steroidal Estrogens to 6-Oxo-Derivatives. *Steroids* **1983,** *42,* 469–474.
39. Iverson, S. L.; Hu, L. Q.; Vukomanovic, V.; Bolton, J. L. The Influence of the*p*-Alkyl Substituent on the Isomerization of*o*-Quinones to*p*-Quinone Methides: Potential Bioactivation Mechanisms for Catechols. *Chem. Res. Toxicol.* **1995,** *8,* 537–544.
40. Bolton, J. L.; Shen, L. *p*-Quinone Methides are the Major Decomposition Products of Catechol Estrogen *o*-Quinones. *Carcinogenesis* **1996,** *17,* 925–929.
41. Bolton, J. L.; Wu, H. M.; Hu, L. Q. Mechanism of Isomerization of 4-Propyl-*o*-Quinone to its Tautomeric *p*-Quinone Methide. *Chem. Res. Toxicol.* **1996,** *9,* 109–113.
42. Iverson, S. L.; Shen, L.; Anlar, N.; Bolton, J. L. Bioactivation of Estrone and its Catechol Metabolites to Quinoid-Glutathione Conjugates in Rat Liver Microsomes. *Chem. Res. Toxicol.* **1996,** *9,* 492–499.
43. Van Aerden, C.; Debrauwer, L.; Fournier, F.; Rathahao, E.; Convert, O.; Molines, H.; Paris, A.; Tabet, J. C. Investigation of the Reaction of Estradiol-2,3-Quinone and Deoxyribonucleosides Using LC-ESI-MS^n. *Proceeding of the 46th ASMS Conference on Mass Spectrometry and Allied Topics*; Long Beach, CA, June 2000, p 103.