Reaction of Adenine with 6-Hydroxyestrogen 6-Sulfates: Model Compounds to Demonstrate Carcinogenesis by Estrogen

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Received December 11, 1995; accepted April 16, 1996

To examine carcinogenesis by estrogens, we investigated the reactivity of 6-hydroxyestrogen 6-sulfates. Two epimeric 6-sulfates, pyridinium 3-methoxyestra-1,3,5(10)-trien-6α-yl sulfate (1) and its 6β-isomer (2), were synthesized as model compounds and reacted with adenine under mild conditions to give two common products in the ratios of approximately 3 : 1 and 5 : 1, respectively. The major product was identified as N^6-[3-methoxyestra-1,3,5(10)-trien-6β-yl] adenine (10), accompanied with its 6α-isomer (9), by comparison with synthetic specimens. These results imply that, in the metabolism of naturally occurring estrogens, hydroxylation at the C_6-position and subsequent sulfoconjugation of the benzylic hydroxyl group may produce sulfates which react with DNA to initiate carcinogenesis.

Key words 6-hydroxyestrogen 6-sulfate; carcinogen; breast cancer; pyridinium 3-methoxyestra-1,3,5(10)-trien-6α-yl sulfate; pyridinium 3-methoxyestra-1,3,5(10)-trien-6β-yl sulfate; adenine

To show whether metabolically activated estrogens, such as sulfates, act on genes to cause carcinogenesis, we synthesized two epimeric benzylic estrogen sulfates, pyridinium 3-methoxyestra-1,3,5(10)-trien-6α-yl sulfate (**1**) and 3-methoxyestra-1,3,5(10)-trien-6β-yl sulfate (**2**), as model compounds and investigated their reactivity. Both sulfates were shown to be highly reactive in water to generate C_6-carbocation.[1)]

The possibility that such estrogen sulfates could ultimately become carcinogens may be investigated by examining their reaction with DNA and the partial structure of the modified DNA produced. Confirming the partial structure requires identification of the modified nucleic acid bases (usually adenine and/or guanine). However, identification of the modified bases may be difficult, because only a few modifications of nucleic acids occur.[2)] Thus, it may be helpful to try reacting the sulfates initially not with nucleic acids, but with their bases, such as adenine and/or guanine. Modification of DNA by the sulfates may be predicted from the results of the reaction with these nucleic acid bases.

In this study, we chose adenine as the nucleophile in the reaction of estrogen sulfates **1** and **2**, and identified the products by comparison with authentic specimens.

Experimental

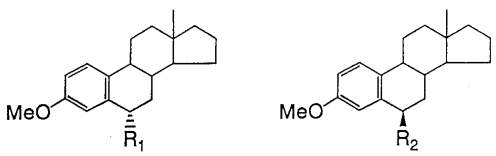
All melting points were determined on a Yanagimoto MP-500D micro melting point apparatus and are uncorrected. UV spectra, λ_{max} nm (ε), were measured in ethanol with a Hitachi 200-20 spectrometer. IR spectra (cm^{-1}) in KBr or Nujol were measured on a JASCO FT/IR-7000 spectrometer. ^{1}H-NMR spectra were recorded at 270 MHz with a JEOL JNM-GX270 spectrometer using $CDCl_3$ as a solvent. Chemical shifts are expressed in ppm relative to tetramethylsilane as an internal standard. The following abbreviations are used: s = singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublets of doublets, br s = broad singlet. EIMS (ionization voltage, 20 eV) and positive ion secondary ion mass spectrometry (SI-MS) were done with a Hitachi M-2000 mass spectrometer using a direct inlet system, and glycerol as a matrix.

HPLC conditions were as follows. 1) Analytical HPLC: pump, model CCPE (Tosoh, Tokyo); detector, model UV-8011 (Tosoh) at 272 nm (for adducts) and 280 nm (for aminoestrogens); column, ODS-80T_M (250 × 4.6 mm, i.d., Tosoh) at 40 °C; mobile phase, CH_3OH–50 mM $NH_4H_2PO_4$ buffer solution (pH 2.5) (75 : 25, v/v, for adducts and 60 : 40, v/v, for aminoestrogens); flow rate, 1 ml/min. 2) Preparative HPLC: pump, model MP-301 (Tokyo Rikakikai, Tokyo), detector, model UV-8011 (Tosoh) at 272 nm (for adducts) and 280 nm (for aminoestrogens); column, μ-Bondasphere 15μ (Waters, 300 × 22 mm, i.d., joined by two columns); mobile phase, CH_3OH–H_2O (85 : 15, v/v, for adducts); CH_3OH–0.1 M $NH_4H_2PO_4$ buffer solution (pH 2.5) (70 : 30, v/v, for aminoestrogens); flow rate, 6 ml/min. 3) Three-dimensional (3D) UV-HPLC: pump, model 991J (Waters, Milford, MA, U.S.A.); detector, model photodiode array UV; column, ODS-80T_M (250 × 4.6 mm, i.d., Tosoh) at 40 °C; mobile phase, CH_3OH–50 mM $NH_4H_2PO_4$ buffer solution (pH 2.5) (75 : 25, v/v); flow rate, 1 ml/min.

Adenine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka) and 6-chloropurine was obtained from Sigma (St. Louis, MO., U.S.A.). All other reagents and solvents were reagent-grade products and were used without further purification. TLC was performed on Kieselgel 60 F_{254} (Merck, Darmstadt, Germany) with a mixture of ethyl acetate and methanol (90 : 10, v/v) as the mobile phase.

Reaction of 6α-Sulfate (1) with Adenine A mixture of dimethyl sulfoxide (DMSO, 25 ml) and 50 mM phosphate buffer solution (pH 7.0, 225 ml) containing **1**[1)] (550 mg, 1.2 mmol) and adenine (335 mg, 2.5 mmol) was stirred at 37 °C for 20 min, and then extracted with ethyl acetate (70 ml × 4). The combined organic layer was washed with water, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to give a residue, which was submitted to preparative HPLC. The eluates were divided into fractions containing adduct A and adduct B (t_R 32.0, 33.8 min, corresponding to peaks A and B in Fig. 1, respectively), both of which were concentrated *in vacuo* to give residues of 1.5 and 5 mg, respectively. Both adducts having λ_{max} at 272 nm on the 3D UV detector showed positive (red) in the Kober color reaction on the TLC plate, upon spraying 10% sulfuric acid followed by heating.

Adduct A: ^{1}H-NMR: 8.36 (1H, br s, 8′-H), 7.68 (1H, br s, 2′-H), 7.22 (1H, d, J = 8.6 Hz, 1-H), 7.14 (1H, d, J = 9.2 Hz, 6′-NH, disappeared on adding D_2O), 6.95 (1H, d, J = 2.5 Hz, 4-H), 6.76 (1H, dd, J_1 = 8.6 Hz, J_2 = 2.5 Hz, 2-H), 5.82 [1H, ddd, J_1 = 10.1 Hz, J_2 = 9.2 Hz, J_3 = 5.9 Hz (dd, J_1 = 10.1 Hz, J_2 = 5.9 Hz on adding D_2O), 6β-H], 3.67 (3H, s, OCH_3), 0.75 (3H, s, 18-H). EI-MS *m/z*: 403 (M^+), 268 [M − 135 (adenine)]$^+$, 136 [adenine + H]$^+$.



1: $R_1 = OSO_3^-$ **2**: $R_2 = OSO_3^-$
3: R_1 = OH **4**: R_2 = OH

Chart 1. **1**, **2**: as pyridinium salt

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Adduct B: ^{1}H-NMR: 8.50 (1H, br s, 8′-H), 7.67 (1H, br s, 2′-H), 7.29 (1H, d, J=8.6 Hz, 1-H), 6.90 (1H, d, J=2.6 Hz, 4-H), 6.86 (1H, dd, J_1=8.6 Hz, J_2=2.6 Hz, 2-H), 6.65 (1H, d, J=7.9 Hz, 6′-NH, disappeared on adding D_2O), 5.65 [1H, br s, $W_{1/2}$=17.6 Hz ($W_{1/2}$=11.7 Hz on adding D_2O), 6α-H], 3.73 (3H, s, OCH_3), 0.65 (3H, s, 18-H). EI-MS m/z: 403 (M^+), 268 [M−135 (adenine)]$^+$, 136 [adenine+H]$^+$.

Reaction of 6β-Sulfate (2) with Adenine A mixture of DMSO (20 ml) and 50 mM phosphate buffer solution (pH 7.0, 180 ml) containing **2**[1] (400 mg, 0.90 mmol) and adenine (243 mg, 1.8 mmol) was stirred at 37 °C for 20 min. The mixture was treated as for **1** to give the products having t_R 32.0 min (1 mg) and t_R 33.8 min (5 mg), the ^{1}H-NMR and EI-MS of which were completely identical with those of adduct A and B, respectively. With HPLC, a single peak was observed when they were admixed with adduct A and B, respectively.

Preparation of 6-Aminoestrogens (7a, 8a). Method A Sodium cyanoborohydride (770 mg, 12 mmol) was added in small portions to a well-dried methanolic solution (20 ml) containing 3-methoxyestra-1,3,5(10)-trien-6-one[1] (**5**, 700 mg, 2.5 mmol) and ammonium acetate (5.4 g). The mixture was refluxed for 9 h under argon, then acidified by adding 1 M HCl (15 ml), followed by neutralization with 1 M NaOH (15 ml) and extraction with ethyl acetate (100 ml × 3). The combined organic layer was washed with water (100 ml × 1), dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to give an oily residue (680 mg), which was submitted to preparative HPLC. The eluates were divided into fr. 1 (230 mg) and fr. 2 (85 mg).

3-Methoxy-6α-aminoestra-1,3,5(10)-triene (7a) Fraction 1 was recrystallized from a mixture of methanol and 25% aqueous ammonia to give needles, mp 38—42 °C. Color reaction with fluorescamine on TLC plate: positive (green). UV: 225 (8200), 279 (2100), 287 (1900). IR (KBr): 3360, 1576 (N–H). ^{1}H-NMR: 7.21 (1H, d, J=8.6 Hz, 1-H), 7.09 (1H, d, J=2.7 Hz, 4-H), 6.75 (1H, dd, J_1=8.6 Hz, J_2=2.7 Hz, 2-H), 4.01 (1H, dd, J_1=10.6 Hz, J_2=6.3 Hz, 6β-H), 3.80 (3H, s, OCH_3), 0.73 (3H, s, 18-H). SI-MS m/z: 286 [M+H]$^+$. *Anal.* Calcd for $C_{19}H_{27}NO$: C, 79.95; H, 9.54; N, 4.31. Found: C, 79.81; H, 9.39; N, 4.04.

3-Methoxy-6α-acetylaminoestra-1,3,5(10)-triene (7b) The amino compound (**7a**, 23 mg) was acetylated by the usual method to give the crude acetate (27 mg), which was recrystallized from methanol to give fine needles, mp 245—251 °C. UV: 279 (2200), 287 (2000). IR (KBr): 3274 and 1564 (N–H). ^{1}H-NMR: 7.23 (1H, d, J=8.6 Hz, 1-H), 6.84 (1H, d, J=2.4 Hz, 4-H), 6.78 (1H, dd, J_1=8.6, J_2=2.4 Hz, 2-H), 5.62 (1H, d, J=8.9 Hz, NH), 5.31 (1H, m, 6β-H), 3.77 (3H, s, OCH_3), 2.06 (3H, s, 6α-$NHCOCH_3$), 0.73 (3H, s, 18-H). SI-MS m/z: 327 [M^+].

3-Methoxy-6β-aminoestra-1,3,5(10)-triene (8a) Fraction 2 was recrystallized from a mixture of methanol and 25% aqueous ammonia to give fine needles, mp 65—68 °C. Color reaction with fluorescamine on TLC plate: positive (green). UV: 226 (8800), 281 (2100), 288 (2000). IR (KBr): 3438 and 1576 (N–H). ^{1}H-NMR: 7.23 (1H, d, J=8.6 Hz, 1-H), 6.93 (1H, d, J=2.6 Hz, 4-H), 6.78 (1H, dd, J_1=8.6 Hz, J_2=2.6 Hz, 2-H), 4.11 (1H, br s, $W_{1/2}$=7.0 Hz, 6α-H), 3.78 (3H, s, OCH_3), 0.76 (3H, s, 18-H). SI-MS m/z: 286 [M+H]$^+$. *Anal.* Calcd for $C_{19}H_{27}NO$: C, 79.95; H, 9.54; N, 4.31. Found: C, 79.74; H, 9.71; N, 4.50.

3-Methoxy-6β-acetylaminoestra-1,3,5(10)-triene (8b) The product (**8a**, 32 mg) was acetylated by the usual method to give the crude acetate (39 mg), which was recrystallized from methanol to give fine needles, mp 189—191 °C. UV: 279 (2100), 287 (1900). IR (KBr): 3232, 1562 (N–H). ^{1}H-NMR: 7.25 (1H, d, J=9.3 Hz, 1-H), 6.82 (1H, dd, J_1=9.3, J_2=2.7 Hz, 2-H), 6.81 (1H, d, J=2.7 Hz, 4-H), 5.82 (1H, d, J=7.9 Hz, NH), 5.17 (1H, m, 6α-H), 3.78 (3H, s, OCH_3), 2.00 (3H, s, 6β-$NHCOCH_3$), 0.76 (3H, s, 18-H). SI-MS m/z: 327 [M^+].

Method B. 3-Methoxyestra-1,3,5(10)-trien-6-one Oxime (6) A pyridine solution (100 ml) containing **5** (1.37 g, 4.8 mmol) and hydroxylamine hydrochloride (2.31 g, 33 mmol) was allowed to stand at 60 °C for 14 h. It was concentrated *in vacuo*, and then diluted with water (100 ml). The solution was weakly acidified with diluted HCl, and extracted with ethyl acetate (100 ml × 3). The combined organic layer was washed with a saturated solution of $NaHCO_3$ and water, then dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to give a residue (1.35 g). The product was recrystallized from a mixture of *n*-hexane and ethyl acetate to give needles (1.26 g, 88%), mp 193—195 °C. UV: 255 (10400), 305 (2900). IR (Nujol): 3222 (O–H), 1572 (C=N), 957 (N–O). ^{1}H-NMR: 7.44 (1H, d, J=2.6 Hz, 4-H), 7.26 (1H, d, J=8.6 Hz, 1-H), 6.91 (1H, dd, J_1=8.6 Hz, J_2=2.6 Hz, 2-H), 3.82 (3H, s, OCH_3), 0.73 (3H, s, 18-H). EI-MS m/z: 299 (M^+), 283 [M^+−16 (O)], 282 [M^+−17 (OH)]. *Anal.* Calcd for $C_{19}H_{25}NO_2$: C, 76.22; H, 8.42; N, 4.68. Found: C, 76.15; H, 8.36; N, 4.60.

3-Methoxy-6α-aminoestra-1,3,5(10)-triene (7a) An ethanolic solution (24 ml) of **6** (120 mg) containing zinc dust (0.6 g), 25% aqueous ammonia (12 ml) and ammonium acetate (1.2 g) was refluxed for 1.5 h. After cooling, the mixture was filtered, and the filtrate was diluted with water (40 ml), then extracted with ethyl acetate (40 ml × 3). The combined organic layer was washed with 10% aqueous ammonia, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to give an oily residue (119 mg), which was recrystallized from a mixture of methanol and 25% aqueous ammonia to give **7a** as needles (101 mg, 85%).

Preparation of N^6-[3-Methoxyestra-1,3,5(10)-trien-6α-yl] adenine (9) An ethanolic solution (15 ml) containing **7a** (160 mg, 0.56 mmol) and 6-chloropurine (44 mg, 0.28 mmol) was refluxed for 48 h, and the solution was concentrated *in vacuo* to give a residue, which was dissolved in chloroform (100 ml). This solution was washed with water, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to give an oil (161 mg), which was submitted to preparative HPLC. The eluates (corresponding to t_R 32.0 min) were concentrated *in vacuo* to give a crystalline residue (50 mg), which was recrystallized from a mixture of *n*-hexane and chloroform to give needles (40 mg). This product was identical with adduct A in terms of ^{1}H-NMR and EI-MS. mp 159 °C (dec.). UV: 272 (21700). IR (Nujol): 3426 and 1600 (N–H), 1243 (C–N). *Anal.* Calcd for $C_{24}H_{29}N_5O \cdot H_2O$: C, 68.38; H, 7.41; N, 16.62. Found: C, 68.70; H, 7.56; N, 14.40.

Preparation of N^6-[3-Methoxyestra-1,3,5(10)-trien-6β-yl] adenine (10) Using the same procedure as described for **9**, a crude product (32 mg, corresponding to t_R 33.8 min) was obtained from **8a** (94 mg, 0.33 mmol) and 6-chloropurine (26 mg, 0.17 mmol). The product was recrystallized from a mixture of *n*-hexane and chloroform to give needles (24 mg, 80%). It was identical with adduct B in terms of ^{1}H-NMR and EI-MS. mp 179 °C (dec.). UV: 272 (20000). IR (Nujol): 3424 and 1570 (N–H), 1257 (C–N). *Anal.* Calcd for $C_{24}H_{29}N_5O$: C, 71.43; H, 7.24; N, 17.36. Found: C, 71.50; H, 7.20; N, 17.40.

Results and Discussion

Structure of the Adducts Both sulfates (**1**, **2**) are extremely hydrolyzable in water and are fairly stable in DMSO, although they contain some water.[1] The 6α-sulfate (**1**) was thus reacted in 10% aqueous DMSO with adenine (twice the molar ratio to **1**). Figure 1 shows the HPLC of the reaction mixture at 20 min after the start of the reaction; peaks 1, 2 and 3 correspond to adenine, 3-methoxyestra-1,3,5(10)-trien-6α-ol (**3**) and its 6β-isomer (**4**), respectively. Two peaks having retention times (t_R) of

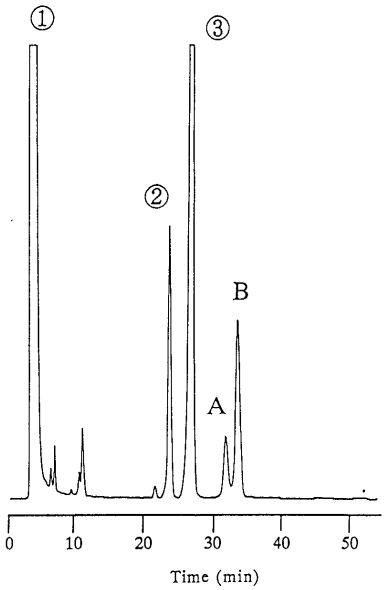


Fig. 1. HPLC of the Reaction Mixture of Pyridinium 3-Methoxyestra-1,3,5(10)-trien-6α-yl Sulfate (**1**) with Adenine in 10% Aqueous DMSO at 37 °C

Peak 1, adenine; peak 2, **3**; peak 3, **4**.

32.0 and 33.8 min (A and B in Fig. 1, respectively) were estimated to be the products, because they appeared only when both the sulfate and adenine were used.

This was supported by the following evidence: 1) characteristic UV spectra of adenine were observed with both peaks (λ_{max} 272 nm) on 3D UV-HPLC, and 2) both products separated by TLC showed a positive Kober color reaction (red) characteristic of estrogen. Thus, the mixture was submitted to preparative HPLC to give two products having t_R 32.0 and 33.8 min, which were named adducts A and B, respectively.

By MS, adduct A showed the ion peak at m/z 403, which was considered to be the molecular ion, because the mass corresponds to that of the molecular species produced by condensation between adenine and the estrogenic C_6-carbocation derived from **1**. The spectrum showed fragment ion peaks at m/z 268 and at m/z 136 due to the loss of 135 mass units (adenine) and due to the protonated adenine, respectively. The MS of adduct A was not distinguishable from that of adduct B, indicating the adducts are isomeric.

The structures of these adducts were confirmed by ^{1}H-NMR as follows: the spectra of both adducts showed common signals due to the steroidal and adenyl structures, and the two protons of the amino group in the adenine molecule decreased to one in the adduct molecules. These results suggest that the adducts might have been produced by condensation between steroid and adenine through the amino group of the adenyl molecule. The only distinct difference of the spectra between adducts A and B was the splitting pattern of the C_6-proton of the steroidal residue, which suggests that the difference between the adducts may be attributed to a steric difference at the C_6-position of 3-methoxyestra-1,3,5(10)-triene.

The configuration was decided on the basis of data for estra-1,3,5(10)-triene-3,6,17β-triol triacetate by Wintersteiner *et al.*[3] According to their analytical results on ^{1}H-NMR of various C_6-substituted estrogen derivatives, the signal of the 6β-proton of 6α-derivatives appears as a double doublet in an ABX-system, having two coupling constants of about 8.5 and 5.5 Hz, because of the magnetically unequal relationship to the C_7-protons. In contrast, the signal of the 6α-proton of 6β-derivatives appears as a doublet, having only one coupling constant of about 3.0 Hz, in the AB-system between the 6α- and C_7-proton.

Figure 2 shows a partial ^{1}H-NMR of both adducts in our study which explains the difference in the splitting patterns between adducts A and B. The signal at 5.83 ppm of the C_6-proton of adduct A was observed as a triple doublet ($J_1 = 10.1$ Hz, $J_2 = 9.2$ Hz, $J_3 = 5.9$ Hz). When deuterated water (D_2O) was added to the sample, the splitting pattern was converted to a double doublet ($J_1 = 10.1$ Hz, $J_2 = 5.9$ Hz), because the spin–spin coupling constant between C_6- and the imino proton ($J = 9.2$ Hz) disappeared. On the other hand, the signal at 5.63 ppm of the C_6-proton of adduct B appeared as a broad singlet ($W_{1/2} = 17.6$ Hz), which was converted to a slightly narrower broad singlet ($W_{1/2} = 11.7$ Hz) when D_2O was added. Judging from these splitting patterns of the C_6-protons, we propose that adducts A and B have the

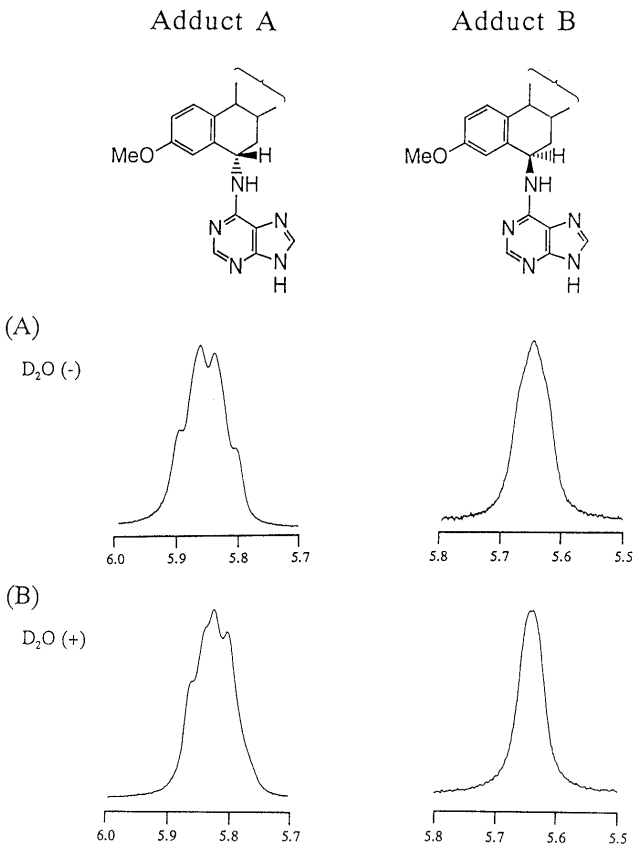


Fig. 2. Partial ^{1}H-NMR Spectra of Adduct A and Adduct B Obtained by Coupling of Estrogen-6-sulfate (**1**) with Adenine

Spectra were determined in $CDCl_3$ (A) without D_2O and (B) with D_2O.

structures with the adenyl group at the 6α- and 6β-positions, respectively.

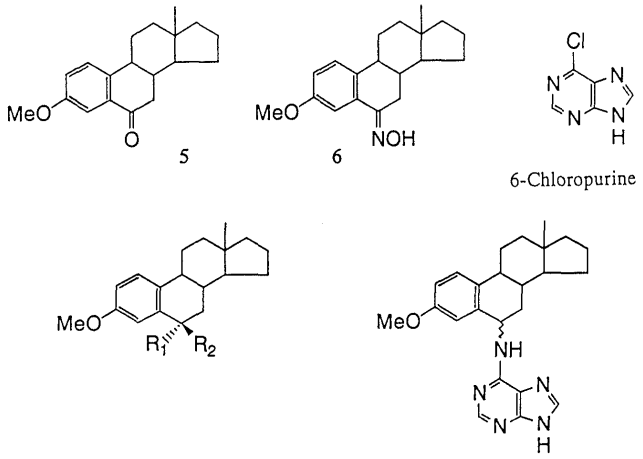
The same reaction with adenine was carried out on the 6β-sulfate (**2**) to give the same two products, which were identified by HPLC as adducts A and B.

Synthesis of the Adducts by Different Methods To confirm the structures proposed for adducts A and B, we tried to synthesize them by condensation reaction of 6α- and 6β-aminoestrogens (**7a**, **8a**) with 6-chloropurine.

Two different methods are known to obtain 6-aminoestrogens: 1) the reaction of 6-oxoestrogen oxime with zinc dust in the presence of ammonium acetate,[4] and 2) the direct conversion of the 6-ketone by using sodium borocyanohydride, also in the presence of ammonium acetate.[5] Both reactions are reported to be efficient for stereoselective preparation of 6α-aminoestrogens.

First, we selected the latter method to prepare the 6-aminoestrogen derivative from 3-methoxyestra-1,3,5(10)-trien-6-one (**5**). Upon HPLC of the reaction mixture, two product peaks were observed, with peak area ratios of 7 : 3.

The reaction mixture was submitted to preparative HPLC to give two products, each of which was a C_6-aminoestrogen isomer based on the following evidence: both products showed a positive reaction with fluorescamine and gave on SI-MS the same molecular ion peak at m/z 286. The configuration of the C_6-amino group of each product was deduced from the splitting pattern of the C_6-proton in ^{1}H-NMR. That is, the C_6-proton of the major product was observed at 4.01 ppm as a double doublet ($J_1 = 10.6$ Hz, $J_2 = 6.3$ Hz), and the C_6-proton of



7a : $R_1 = NH_2$, $R_2 = H$ 9 : 6α
7b : $R_1 = NHCOCH_3$, $R_2 = H$ 10 : 6β
8a : $R_1 = H$, $R_2 = NH_2$
8b : $R_1 = H$, $R_2 = NHCOCH_3$

Chart 2

the minor product was observed at 4.11 ppm as a broad singlet ($W_{1/2} = 7.0$ Hz), which suggests that the products were 3-methoxy-6α-aminoestra-1,3,5(10)-triene (**7a**) and 3-methoxy-6β-aminoestra-1,3,5(10)-triene (**8a**), respectively. These results for the C_6-amino group agreed with the results obtained for similar steroids by Tiefenauer *et al.*[5] Thus, stereoselective reduction of **5** did not occur with this method. However, we could obtain, in addition to **7**, the isomeric 6β-amino derivative (**8a**) at the same time.

To reconfirm the structures of the above isomeric C_6-aminoestrogens (**7a**, **8a**), another procedure reported by Hamacher and Christ[4] was applied to **5**. Treatment of **5** with hydroxylamine gave its oxime (**6**), followed by reduction to give a single product, which was identified by ^{1}H-NMR as the 6α-amine (**7a**). Thus, this method[4] is excellent for stereoselective synthesis of 6α-aminoestrogen.

We then tried the coupling reaction of the 6α-amine (**7a**) with 6-chloropurine in ethanol. Upon HPLC of the reaction mixture, a single product peak appeared. Isolation of the product by preparative HPLC gave a crystalline material, and the UV, MS and ^{1}H-NMR were identical with those of adduct A. In addition, the peak height of this product raised in proportion to the amount of adduct A added.

Similarly, the sole product obtained by a coupling reaction of the 6β-amine (**8a**) with 6-chloropurine was identical with adduct B in terms of the spectral analyses and chromatographic behavior.

In conclusion, adducts A and B produced by reacting estrogen 6-sulfates with adenine were identified as N^6-[3-methoxyestra-1,3,5(10)-trien-6α-yl] adenine (**9**) and N^6-[3-methoxyestra-1,3,5(10)-trien-6β-yl] adenine (**10**), respectively.

Modification of Adenine by Sulfates Two reactive estrogen sulfates were shown to react with adenine under mild conditions to give common diastereoisomeric products (**9**, **10**); the product ratios with sulfates **1** and **2** were approximately 1 : 3 and 1 : 5, respectively. The results suggest that both products were mainly produced *via* a common steroidal C_6-carbocation intermediate (S_N1 mechanism). Such a carbocation might also attack DNA.

As an example of DNA damage by an initiator, alkylation of DNA at the base residue is well-known. Such alkylation generally occurs at multiple positions in the base moiety. With the adenine residue in DNA, alkylation is possible not only at the N^6-position, but also at the N^1-, N^3- and N^7- positions. In our study, therefore, alkylation by sulfates at positions other than at N^6 of adenine was expected. The unknown minor peaks (Fig. 1) may be due to products alkylated at other positions.

Examples of sulfate metabolites acting as ultimate carcinogens are well-known in the metabolism of such foreign compounds as safrole,[6] estragole,[7] 7,12-dimethylbenz[*a*]anthracene (DMBA),[8] and other related aromatic compounds.[9] The metabolic pathways by which these substances form carcinogens involve two reactions: hydroxylation at benzylic positions (phase I reaction), followed by sulfation of the hydroxyl group (phase II reaction). The benzylic sulfates thus produced are highly hydrolyzable, producing a benzyl carbocation. The key step of carcinogenesis by these foreign substances is believed to be reaction with DNA *via* such reactive species.

It has long been recognized that the female hormone estrogen plays an important role in the occurrence of breast cancer, based on evidence that elevated enzyme activities of phase I and II reactions for estrogens are observed as a general tendency in breast cancer patients.[10] Nevertheless, no conclusive evidence for carcinogenesis by an estrogen has yet been reported.

We assumed that 6-hydroxyestrogen 6-sulfate may be an ultimate carcinogen on the basis of the analogy with carcinogenesis by metabolically activated safrole, estragole, DMBA, *etc.*, and the tendency for abnormal estrogen metabolism in breast cancer patients. Although phase I reaction of estrogen at the C_6-position is not a major metabolic pathway in humans, 6-hydroxyestrogens (especially the 6α-isomer) are always detected in the urine.[11] The present findings imply that, if sulfoconjugation then occurs at the same C_6-position, the resultant sulfate might have the ability to modify DNA.

Acknowledgement This work was supported in part by a Grant-in-Aid for Cancer Research (1993, Yoshizawa) from the Ministry of Education, Science, Sports and Culture of Japan.

References

1) Takagi H., Komatsu K., Yoshizawa I., *Steroids*, **56**, 173 (1991).
2) Watabe T., Hiratsuka A., Ogura K., *Carcinogenesis*, **8**, 445 (1987).
3) Wintersteiner O., Moore M., Cohen A. L., *J. Org. Chem.*, **29**, 1325 (1964).
4) Hamacher H., Christ E., *Arzneim-Forsch./Drug Res.*, **33**, 347 (1983).
5) Tiefenauer L. X., Bodmer D. M., Frei W., Andres R., *J. Steroid Biochem.*, **32**, 251 (1989).
6) Phillips D. H., Miller J. A., Miller E. C., Adams B., *Cancer Res.*, **41**, 2664 (1981).
7) Phillips D. H., Miller J. A., Miller E. C., Adams B., *Cancer Res.*, **41**, 176 (1981).
8) Watabe T., Ishizuka T., Isobe M., Ozawa N., *Science*, **215**, 403 (1982).
9) Okuda H., Hiratsuka A., Nojima H., Watabe T., *Biochem. Pharmacol.*, **35**, 535 (1986); Surh Y.-J., Liem A., Miller E. C., Miller J. A., *Biochem. Biophys. Res. Commun.*, **172**, 85 (1990).
10) Dao T. L., *Biochim. Biophys. Acta*, **560**, 397 (1979).
11) Knuppen R., Haupt O., Breuer H., *Biochem. J.*, **101**, 397 (1966); Cohen S. L., Ho P., Suzuki Y., Alspector F. E., *Steroids*, **32**, 279 (1978).