Identification of Tamoxifen−DNA Adducts Induced by α-Acetoxy-*N*-desmethyltamoxifen

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Received March 31, 2000

Treatment with tamoxifen increased the risk of endometrial cancers in breast cancer patients and women participating in the chemoprevention study. In our laboratory, tamoxifen−DNA adducts, including α-(N^2-deoxyguanosinyl)tamoxifen (dG-N^2-TAM), were detected in the endometrium of women taking tamoxifen [Shibutani, S., et al. (1999) *Chem. Res. Toxicol.* **12**, 646−653]. On the basis of recent animal studies, deoxyguanosinyl-*N*-desmethyltamoxifen (dG-*N*-desmethylTAM) adducts are also suspected to be formed in the liver. In the study presented here, we synthesized α-acetoxy-*N*-desmethyltamoxifen as a model activated metabolite of *N*-desmethyltamoxifen. The overall yield of α-acetoxy-*N*-desmethyltamoxifen from α-hydroxytamoxifen was approximately 42%. α-Acetoxy-*N*-desmethyltamoxifen was highly reactive to 2′-deoxyguanosine, as was similarly observed for tamoxifen α-sulfate. The two reaction products were identified as a mixture of epimers of the trans form or cis form of α-(N^2-deoxyguanosinyl)-*N*-desmethyltamoxifen (dG-N^2-*N*-desmethylTAM) by mass and proton magnetic resonance spectroscopy. In addition, the trans and cis forms of dG 3′-monophosphate-N^2-*N*-desmethylTAM were prepared as standard markers for ^{32}P-postlabeling/HPLC analysis. Using this technique, dG-N^2-*N*-desmethylTAM adducts were detected in calf thymus DNA reacted with α-acetoxy-*N*-desmethyltamoxifen.

Introduction

The Breast Cancer Prevention Trial initiated by the National Surgical Adjuvant Breast and Bowel Project recently reported that tamoxifen (TAM),[1] a chemotherapeutic agent for breast cancer, reduces the risk of development of breast cancer by 44−55% for healthy women at high risk (*1*). However, treatment with TAM increases the incidence of endometrial cancer in healthy women enrolled in the NSABP chemopreventive trial (*1*) as well as in breast cancer patients (*2*−*4*). The genotoxic effects in the human uterus (*5*, *6*) are suspected because of the observation that TAM-induced hepatocellular tumors in rats (*7*−*9*) were associated with the formation of covalent DNA adducts (*10*−*12*) by the activated TAM metabolites (*13*−*15*).

TAM is metabolically converted to α-hydroxytamoxifen (α-OHTAM), *N*-desmethyltamoxifen (*N*-desmethylTAM), tamoxifen *N*-oxide (TAM *N*-oxide), and 4-hydroxytamoxifen (4-OHTAM) (*16*−*20*) (the structures in Figure 1). These compounds are precursors to reactive species capable of damaging DNA. Among several proposed mechanisms, α-hydroxylation of these products (Figure 1), followed by sulfation or acetylation, may be a major pathway capable of forming DNA adducts (*16*−*20*). Two trans (fr-1 and fr-2) and two cis (fr-3 and fr-4) epimers of α-(N^2-deoxyguanosinyl)tamoxifen (dG-N^2-TAM) DNA adducts (*12*, *21*, *22*) can be produced via sulfation of α-OHTAM by hydroxysteroid sulfotransferases (*23*, *24*). dG-N^2-TAM adducts were miscoding and mutagenic lesions, primarily generating G → T transversions in mammalian cells (*25*, *26*). These adducts are likely to be the major DNA adducts in the liver of rats treated with TAM or α-OHTAM (*12*, *27*). dG-N^2-TAM adducts (1.5−13 adducts/10^8 nucleotides) were also detected in endometrial tissues of women taking TAM (*28*, *29*), contradicting earlier results showing that such TAM adducts were not significantly detected in other laboratories (*30*−*32*).

Using mass spectroscopy, dG-*N*-desmethylTAM was suggested to be another major adduct, in addition to dG-N^2-TAM, in the liver of rats treated with TAM (*33*). When rats were treated with *N*-desmethylTAM, one major adduct was also detected in the liver by a ^{32}P-postlabeling/HPLC analysis (*34*, *35*). Thus, another major adduct could be formed via sulfation or acetylation of α-OH-*N*-desmethylTAM.

Recently, α-OH-*N*-desmethylTAM was synthesized via desmethylation of 1-bromo-2-{4-[2-(dimethylamino)ethyl]-phenyl}-1,2-diphenylethene (*36*). α-Sulfoxy-*N*-desmethylTAM prepared from α-hydroxy-*N*-desmethylTAM was shown to be reactive to DNA in vitro, identifying a trans form of α-(deoxyguanosin-N^2-yl)-*N*-desmethyltamoxifen

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[1] Abbreviations: dG, 2′-deoxyguanosine; $dG_{3'P}$, 2′-deoxyguanosine 3′-monophosphate; TAM, tamoxifen; α-OHTAM, α-hydroxytamoxifen; dG-N^2-TAM, α-(N^2-deoxyguanosinyl)tamoxifen; TAM *N*-oxide, tamoxifen *N*-oxide; α-OHTAM *N*-oxide, α-hydroxytamoxifen *N*-oxide; dG-N^2-TAM *N*-oxide, α-(N^2-deoxyguanosinyl)tamoxifen *N*-oxide; *N*-desmethyTAM, *N*-desmethyltamoxfen; dG-N^2-*N*-desmethylTAM, α-(N^2-deoxyguanosinyl)-*N*-desmethyltamoxifen.

10.1021/tx000074o CCC: $19.00
Published on Web 07/19/2000

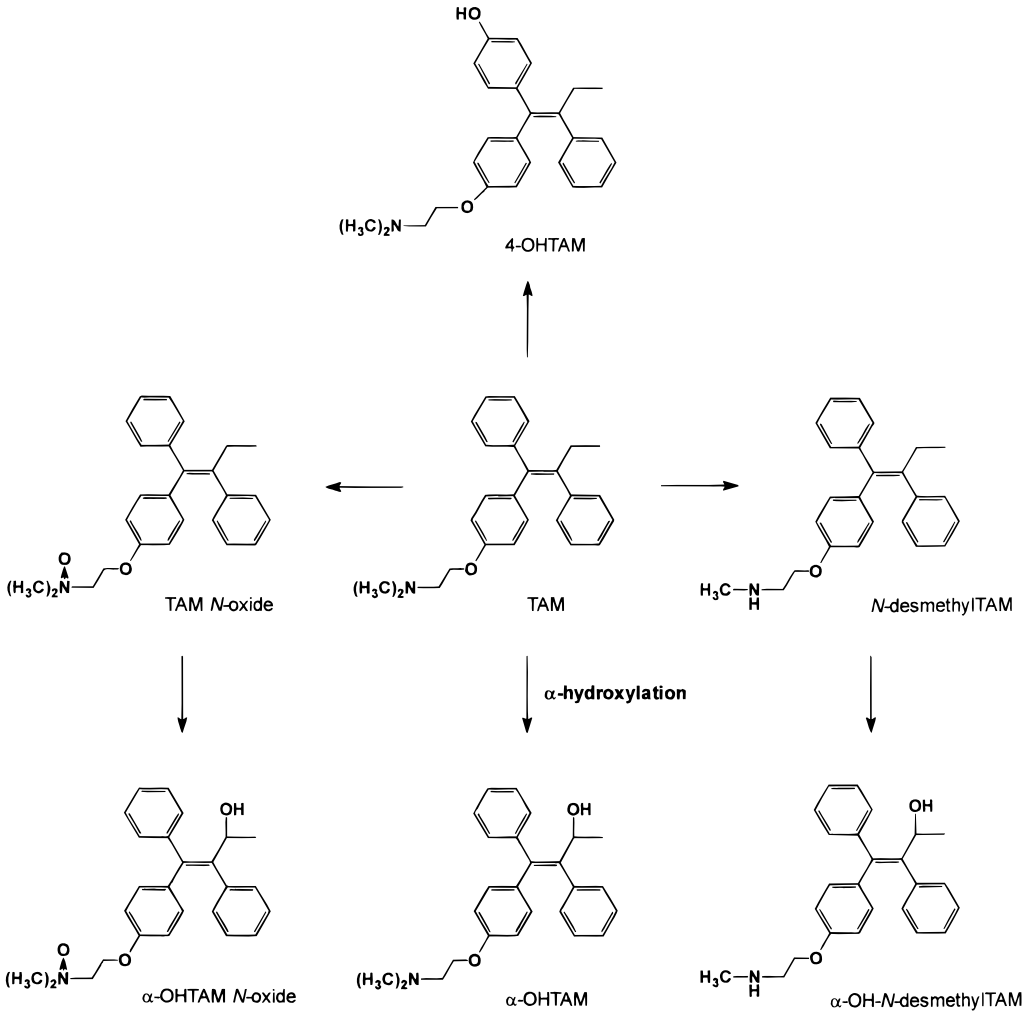


Figure 1. Metabolic pathway of tamoxifen.

(dG-N^2-*N*-desmethylTAM) (*36*). However, the synthetic step from 1-bromo-2-{4-[2-(methylamino)ethyl]phenyl}-1,2-diphenylethene to α-OH-*N*-desmethylTAM was critical, and the yield was only 12%. In addition, the cis form of the dG-N^2-*N*-desmethylTAM adduct has not yet been characterized.

In this paper, α-acetoxy-*N*-desmethylTAM was prepared from α-OHTAM as a model activated form of *N*-desmethylTAM (Scheme 1). The overall yield was approximately 42%. α-Acetoxy-*N*-desmethylTAM was highly reactive to dG, resulting in two reaction products identified as a mixture of two epimers of the trans or cis form of dG-N^2-*N*-desmethylTAM. These trans and cis isomers of dG 3′-monophosphate-N^2-*N*-desmethylTAM (dG$_{3'P}$-N^2-*N*-desmethylTAM) were also prepared and used as standard markers for quantification of *N*-desmethylTAM−DNA adducts by ^{32}P-postlabeling/HPLC analysis.

Materials and Methods

Caution: *TAM α-sulfate and α-acetoxy-N-desmethylTAM are potentially genotoxic and should be handled with proper care.*

Chemicals. Organic chemicals used for synthesis were supplied by Aldrich Chemical (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA). [γ-^{32}P]ATP (specific activity of 6000 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Polyethyleneimine (PEI)-cellulose plates were purchased from Machery-Nagel (Duren, Germany). Calf thymus DNA, proteinase K, and potato apyrase were purchased from Sigma (St. Louis, MO) and nuclease P1 and alkaline phosphatase (type III) from Boehringer Mannheim (Indianapolis, IN). RNase A, RNase T1, micrococcal nuclease, and spleen phosphodiesterase were obtained from Worthington Biochemical Co. (Freehold, NJ). T4 polynucleotide kinase was purchased from Stratagene (La Jolla, CA).

Synthesis of Chemical Materials. A trans form of α-hydroxytamoxifen (α-OHTAM) was synthesized by the established protocol (*37*). TAM α-sulfate and α-acetoxyTAM were synthesized by the established method (*12*, *21*).

Synthesis of α-Acetoxy-*N*-desmethylTAM. (*E*)-4-{4-[2-(*N*-Ethoxycarbonyl-*N*-methylamino)ethoxy]phenyl}-3,4-diphenyl-3-buten-2-ol (2). To a solution of α-OHTAM (**1**, 99 mg, 0.26 mmol) in benzene (5 mL) was added ethylchloroformate (0.05 mL), and the mixture was refluxed for 1 h. After the solvent was removed by evaporation, the residue was chromatographed on a column of silica gel with (a) 1:3 and (b) 1:2 ethyl acetate/hexane. Eluant b gave **2** (87 mg, 0.20 mmol) as a syrup. The yield of **2** was 79%. ^{1}H NMR for compound **2** ($CDCl_3$): δ 1.15−1.29 (6H, m), 2.95 (3H, s), 3.52 (2H, m), 3.94 (2H, m), 4.05 (2H, q, J = 7.1 Hz), 4.83 (1H, q, J = 6.5 Hz), 6.52 (2H, m), 6.81 (2H, m), 7.15−7.42 (10H, m).

(*E*)-4-{4-[2-(*N*-Benzyloxycarbonyl-*N*-methylamino)-ethoxy]phenyl}-3,4-diphenyl-3-buten-2-ol (4). A mixture of **2** (87 mg, 0.20 mmol), ethylene glycol (3 mL), hydrazine hydrate (0.3 mL), and potassium hydroxide (500 mg) was stirred at 140 °C for 2 h. The reaction mixture was poured into ice/water and extracted with diisopropyl ether. The extract was washed with brine and dried using anhydrous sodium sulfate, and then the solvent was evaporated. The residual syrup was dissolved in methanol (5 mL) (added at 0 °C), triethylamine (109 μL), and

benzylchloroformate (56 μL). The mixture was stirred at 0 °C for 1 h, and then concentrated. The residue was extracted with ethyl acetate, washed with brine, and evaporated. The residue was chromatographed on a column of silica gel with toluene/ethyl acetate (3:1) to give **4** (95 mg, 0.19 mmol) as a syrup. The yield of **4** from compound **2** was 93%. ^{1}H NMR for compound **4** ($CDCl_3$): δ 1.20 (3H, d, J = 6.5 Hz), 2.98 (3H, s), 3.56 (2H, m), 3.95 (2H, m), 4.83 (1H, q, J = 6.5 Hz), 5.10 (2H, s), 6.49 (2H, m), 6.80 (2H, m), 7.12−7.43 (15H, m).

(*E*)-2-Acetoxy-4-{4-[2-(*N*-benzyloxycarbonyl-*N*-methylamino)ethoxy]phenyl}-3,4-diphenyl-3-butene (5). To a solution of **4** (95 mg, 0.19 mmol) in pyridine (4 mL) was added acetic anhydride (1 mL), and the mixture was stirred at room temperature for 15 h and then evaporated to a syrup. The product was purified by chromatography on a column of silica gel with toluene/ethyl acetate (4:1) to afford **5** (75 mg, 0.14 mmol) as a syrup. The yield of **5** from compound **4** was 73%. ^{1}H NMR for compound **5** ($CDCl_3$): δ 1.27 (3H, d, J = 6.6 Hz), 1.91 (3H, s), 2.98 (3H, s), 3.56 (2H, m), 3.95 (2H, m), 5.10 (2H, s), 5.76 (1H, q, J = 6.6 Hz), 6.47 (2H, m), 6.78 (2H, m), 7.06−7.45 (15H, m). ^{1}H NMR for compound **5** (CD_3OD): δ 1.25 (3H, d, J = 6.6 Hz), 1.88 (3H, s), 2.94 (3H, s), 3.56 (2H, t, J = 5.5 Hz), 3.95 (2H, m), 5.05 (2H, brs), 5.74 (1H, q, J = 6.6 Hz), 6.49 (2H, m), 6.77 (2H, m), 7.10−7.44 (15H, m).

α-Acetoxy-*N*-desmethylTAM (6) ((*E*)-2-Acetoxy-4-{4-[2-(methylamino)ethoxy]phenyl}-3,4-diphenyl-3-butene). To a solution of **5** (75 mg, 0.14 mmol) in ethyl acetate (10 mL) was added 5% Pd-C catalyst (50 wt %, 10 mg), and the mixture was stirred for 2 h at room temperature in a hydrogen atmosphere. Filtration of the catalyst and evaporation of the solvent gave α-acetoxy-*N*-desmethylTAM (**6**, 56 mg, 0.13 mmol) as a crude syrup. The purity of **6** according to NMR data was ∼80%. The yield of **6** from compound **5** was 79%. MS (FAB, positive): m/z 416 $[M + H]^+$. ^{1}H NMR for compound **6** (CD_3OD): δ 1.25 (3H, d, J = 6.6 Hz), 1.88 (3H, s), 2.41 (3H, s), 2.88 (2H, t, J = 5.0 Hz), 3.94 (2H, t, J = 5.0 Hz), 5.74 (1H, q, J = 6.6 Hz), 6.58 (2H, m), 6.77 (2H, m), 6.95−7.43 (10H, m).

Reaction of α-Acetoxy-*N*-desmthylTAM with dG. 2′-Deoxyguanosine (70 mg) was reacted at 37 °C for 18 h with 56 mg of α-acetoxy-*N*-desmethylTAM (∼80% pure) in 35 mL of 100 mM Tris-HCl buffer (pH 8.0) and 1.75 mL of acetonitrile. The reaction mixture was extracted twice with 30 mL of butanol, and the pooled butanol extracts were evaporated to dryness. To isolate *N*-desmethylTAM-modified dG, the pooled butanol extracts were subjected to a reverse-phase μBondapak C_{18} column (0.78 cm × 30 cm, Waters), eluted over 45 min at a flow rate of 2.0 mL/min with a linear gradient of 0.05 M triethylammonium acetate (pH 7.0) containing 10 to 70% acetonitrile (*21*, *23*). An isocratic HPLC condition consisting of 0.05 M triethylammonium acetate (pH 7.0, 65%) and acetonitrile (35%) was also used for the purification. HPLC analysis was performed on a Waters 990 HPLC instrument, equipped with a photodiode array detector. Mass spectrometry was performed on a Platform liquid chromatograph (Micromass). The dG-*N*-desmethylTAM fraction was chromatographed using a HP1100 system (Hewlett-Packard, Palo Alto, CA), equipped with a photodiode array detector. A PLRP-S column (1.0 mm × 50 mm, 8 μm; Microm Bioresources) was employed at a flow rate of 50 μL/min with a linear gradient of 0.05 M triethylammonium acetate (pH 7.0) containing 5 to 50% acetonitrile over the course of 35 min and subsequently 50 to 95% acetonitrile over the course of 15 min. The effluent was directed to the source of the mass spectrometer operated in the positive or negative ion mode. The mass rage of m/z 250−900 was scanned in 2 s. NMR spectroscopy was performed on a Bruker 600 MHz NMR instrument. ^{1}H NMR data of dG reaction products are described in Table 1.

Reaction of α-Acetoxy-*N*-desmethylTAM with dG 3′-Monophosphate. 2′-Deoxyguanosine 3′-monophosphate ($dG_{3'P}$, 1.0 mg) was reacted at 37 °C for 18 h with 0.8 mg of α-acetoxy-*N*-desmethylTAM (∼80% pure) in 500 μL of 100 mM Tris-HCl buffer (pH 8.0) and 25 μL of acetonitrile. The reaction mixture was extracted twice with 500 μL of butanol. To isolate *N*-desmethylTAM-modified $dG_{3'P}$, the pooled butanol extracts were concentrated at reduced pressure and subjected to HPLC, as similarly described for isolation of dG-N²-*N*-desmethylTAM. A part of the purified *N*-desmethylTAM-modified $dG_{3'P}$ was evaporated to dryness and incubated at 37 °C for 1 h with alkaline phosphatase (3 units) in 100 μL of 100 mM Tris-HCl buffer (pH 7.0). The reaction mixture was extracted twice with 100 μL of butanol. The butanol extract was evaporated at reduced pressure and subjected to a reverse-phase column using the same HPLC condition described for dG-N²-*N*-desmethylTAM. The retention time and UV spectra of the products were compared with that of diastereoisomers of dG-N²-*N*-desmethylTAM. The molecular weight of *N*-desmethylTAM-modified $dG_{3'P}$ was also measured by LC/mass spectrometry.

Reaction of DNA with Activated Forms of TAM Analogues. Aliquots of DNA (10 μg) were incubated at 37 °C for 1 h with 400 μg of TAM α-sulfate or α-acetoxy-*N*-desmethylTAM in 100 μL of 100 mM Tris-HCl buffer (pH 8.0). The samples were evaporated to dryness in a vacuum desiccator, and extracted three times with 1.0 mL of ethanol. The recovered DNA (0.4 μg) was digested at 37 °C for 2 h in 30 μL of 17 mM sodium succinate buffer (pH 6.0) containing 8 mM $CaCl_2$, using 1.5 units of micrococcal nuclease and 0.15 unit of spleen phosphodiesterase. Afterward, 1.0 unit of nuclease P1 was added and the reaction mixture was incubated at 37 °C for 1 h. Samples were dissolved in 100 μL of distilled water and extracted twice with 200 μL of butanol. The butanol fraction was back-extracted with 50 μL of distilled water, dried, and then used for analysis of TAM−DNA adducts. Approximately 95% of the TAM adducts were recovered by butanol extraction. The concentration of the DNA was estimated to be 50 μg per 1 OD_{260} unit.

Quantitation of ^{32}P-Labeled DNA Adducts by HPLC. The digests of pooled extracts were incubated with 40 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) and 3 μL of T4 polynucleotide kinase (10 units/μL) (*38*) and developed for 16 h on a 10 cm × 10 cm PEI-cellulose thin-layer plate using 1.7 M sodium phosphate buffer (pH 6.0) with a paper wick. The position of adducts was established by autoradiography, using Kodak Xomat XAR film. ^{32}P-labeled products remaining on the TLC plate were recovered, using 4 M pyridinium formate (pH 4.3), and evaporated to dryness. The recovery of ^{32}P-labeled products was approximately 84%. Using a minor modification of a method established by Martin et al. (*27*), the ^{32}P-labeled products were subjected to a Hypersil BDS C_{18} analytical column (0.46 cm × 25 cm, 5 μm; Shandon), eluted at a flow rate of 1.0 mL/min with an isocratic condition of 2.0 M ammonium formate (pH 4.0) containing 20% acetonitrile/methanol (6:1, v/v) over the course of 40 min followed by a linear gradient from 20 to 45% over the course of 25 min. The radioactivity was monitored by a radioisotope detector (Berthold LB506 C-1, ICON Scientific Inc.) lined to a Waters 990 HPLC instrument. Standard stereoisomers of $dG_{3'p}$-N²-TAM (*23*) and $dG_{3'p}$-N²-*N*-desmethylTAM were also labeled with ^{32}P (*38*).

The relative adduct levels were calculated according to Levay et al. (*39*), using disintegrations per minute instead of counts per minute [(total disintegrations per minute in adducts)/1.15 × 10^{11} dpm, assuming that 5 μg of DNA represented 1.52 × 10^4 pmol of $dN_{3'P}$ and the specific activity of the [γ-^{32}P]ATP was 5.06 × 10^7 dpm/pmol]. The detection limit was approximately 2.5 × 10^{-10} adduct. The specific activity of [γ-^{32}P]ATP was corrected by calculating the extent of decay.

Results and Discussion

Synthesis of α-Acetoxy-*N*-desmethylTAM. To prepare α-acetoxy-*N*-desmethylTAM, we initially attempted to synthesize α-OH-*N*-desmthylTAM via desmethylation of 1-bromo-2-{4-[2-(dimethylamino)ethyl]phenyl}-1,2-diphenylethene using the modified procedures of Foster et al. (*37*) and Olofson et al. (*40*). Although the reaction was performed at −70 °C, the 2-methylaminoethoxy

Scheme 1. Synthesis of α-Acetoxy-*N*-desmethyltamoxifen

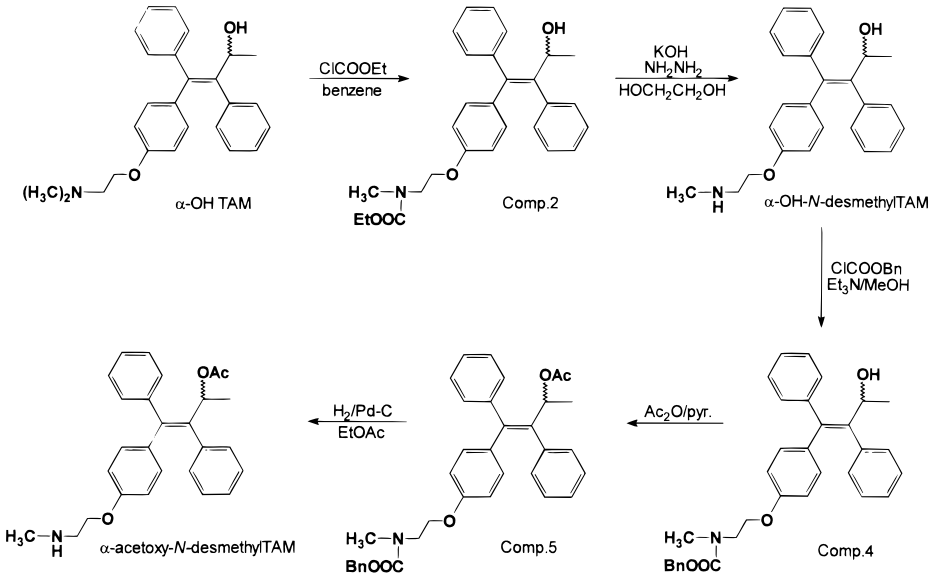
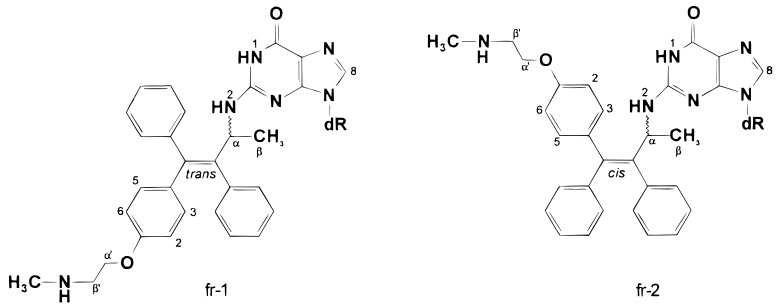


Table 1. ^{1}H NMR Spectrum of α-(Deoxyguanosin-N^2-yl)-*N*-desmethyltamoxifen Adducts



	δ (ppm)[a,b]	
	fr-1	fr-2
tamoxifen fragment		
C**H$_3$**CH	1.26, d, J = 6.8 Hz	1.28, d, J = 6.0 Hz
CH$_3$N**H**	1.85,[c] bs	1.88,[c] bs
C**H$_3$**NH	2.26, s	2.34, s
C**H$_2$**NH	2.70, t, J = 5.6 Hz	2.83, t, J = 5.6 Hz
C**H$_2$**O	3.82, t, J = 5.6 Hz	4.01, t, J = 5.6 Hz
CH$_3$C**H**	4.90, m	4.95, m
H3 and **H**5	6.52, 6.54, each bd, J = 8.8 Hz	7.16, 7.18, each bd, J = 8.4 Hz
H2 and **H**6	6.76, 6.78, each bd, J = 8.8 Hz	7.33, 7.35, each bd, J = 8.4 Hz
phenyl	7.01−7.49, m	6.88−7.12, m
guanine fragment		
N^2**H**	5.96 and 6.01,[c] two d, J = 7.4 Hz	5.95 and 6.01,[c] two d, J = 7.4 Hz
H8	7.97, s	7.98, s
N1	10.08,[c] bs	10.08,[c] bs
sugar fragment		
2′	2.4−2.5, m	2.4−2.6, m
5′	3.51, q	3.50, q
4′	3.88, m	3.90, m
3′	4.39, m	4.40, m
5′-O**H**	4.78,[c] t, J = 5.2 Hz	4.75,[c] t, J = 5.2 Hz
3′-O**H**	5.42,[c] d, J = 3.3 Hz	5.34,[c] d, J = 3.3 Hz
1′	6.18 and 6.22, two t, J = 6.2 Hz	6.22 and 6.26, two t, J = 6.2 Hz

[a] Recorded in DMSO-d_6. [b] Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad. [c] Can be exchanged with D_2O.

moiety was readily cleaved from the brominated compounds. Using a similar synthetic method at a temperature of less than −100 °C, Gamboa da Costa et al. (*36*) synthesized α-OH-*N*-desmethylTAM. The lower reaction temperature may be required for synthesis of such an unstable compound. However, the yield of α-OH-*N*-desmethylTAM was only 12% (*36*). Therefore, we used the alternative procedure to synthesize α-acetoxy-*N*-desmethylTAM, as shown in Scheme 1. Treatment of α-OHTAM with ethylchloroformate in benzene gave **2** in 79% yield. Benzyloxycarbonyl compound **4** was prepared by the following two processes: hydrazine hydrate/KOH and benzylchloroformate/MeOH. Compound **4** was converted into the corresponding acetate **5** by acetic anhy-

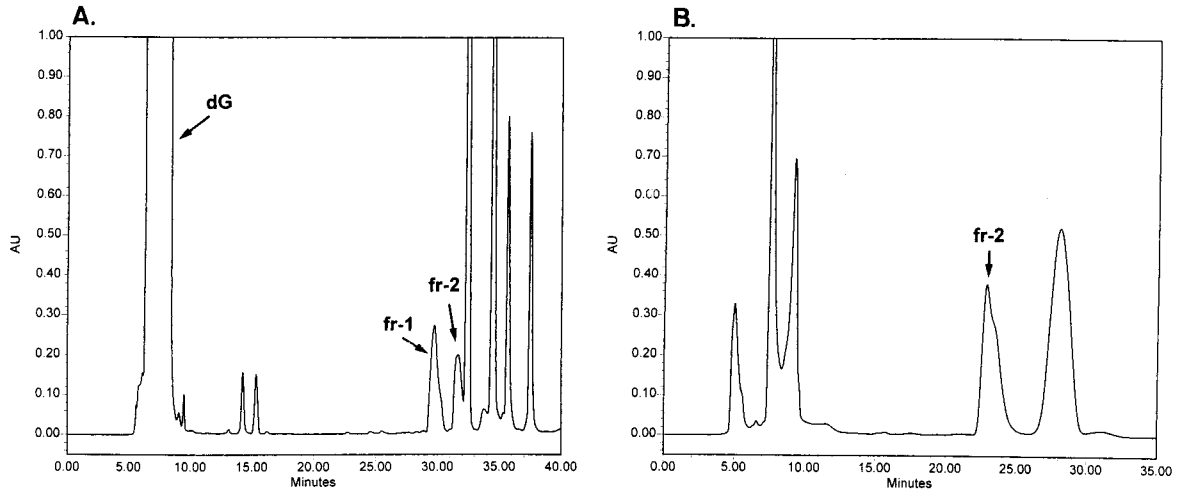


Figure 2. HPLC separation of dG-*N*-desmethyltamoxifen formed by α-acetoxy-*N*-desmethyltamoxifen. dG (70 mg) was reacted at 37 °C for 18 h with α-acetoxy-*N*-desmethylTAM (56 mg) in 100 mM Tris-HCl buffer (pH 8.0), as described in Materials and Methods. The reaction mixture was extracted twice with butanol, and the pooled butanol extracts were evaporated to dryness. (A) To isolate dG-*N*-desmethylTAM (fr-1 and fr-2), 9 mg of the pooled butanol extracts was subjected to a reverse-phase μBondapak C_{18} column (0.78 cm × 30 cm), eluted over the course of 45 min at a flow rate of 2.0 mL/min with a linear gradient of 0.05 M triethylammonium acetate (pH 7.0) containing 10 to 70% acetonitrile. (B) dG-*N*-desmethylTAM (fr-2) was further purified by an isocratic HPLC condition using 0.05 M triethylammonium acetate (pH 7.0, 65%) and acetonitrile (35%).

drite/pyridine. Finally, hydrogenolysis of **5** in the presence of a H_2/5%Pd-C/EtOAc mixture gave α-acetoxy-*N*-desmethylTAM **6** as an unsuitable syrup. The overall yield from α-OHTAM was approximately 42%. Since α-acetoxy-*N*-desmethylTAM was highly unstable upon exposure to the atmosphere as was observed for TAM α-sulfate (*21*), the purity of α-acetoxy-*N*-desmethylTAM was approximately 80%, which is based on the NMR data. Thus, this synthetic procedure can be used to prepare α-acetoxy-*N*-desmethylTAM with reasonable yield.

Characterization of dG Products Induced by α-Acetoxy-*N*-desmethylTAM. dG was reacted with α-acetoxy-*N*-desmethylTAM as described in Materials and Methods. The reaction products were extracted with butanol from the reaction mixture, and isolated by a preparative HPLC column using a linear gradient of 0.05 M triethylammonium acetate containing 10 to 70% acetonitrile (Figure 2A). The retention times of fr-1 and fr-2 were 29.7 and 31.6 min, respectively. Since fr-2 was contaminated with other compounds, this fraction was further purified by the isocratic HPLC system using 0.05 M triethylammonium acetate and acetonitrile (65:35) (Figure 2B). Fr-2 was isolated at 22.8 min. When fr-1 or fr-2 was subjected to an analytical HPLC column (panels A and C of Figure 3), both fractions have two peaks exhibiting identical UV spectra (panels B and D of Figure 3). The UV maximum (251 nm) of fr-1 was slightly different from that of fr-2 (247 nm). Using positive ion LC/mass spectroscopy, the protonated molecular ion of fr-1 and fr-2 exhibited a peak at *m/z* 623 (Figure 4), showing the molecular weight to be 622 Da. Other peaks in this spectrum were due to background from the chromatographic system. Thus, these products are suspected to be the diastereoisomers of dG-*N*-desmethylTAM, as was similarly observed for dG-N²-TAM (*21*) and dG-N²-TAM *N*-oxide (*41*).

The structures of fr-1 and fr-2 were analyzed by 500 or 600 MHz ^{1}H NMR. The spectra were recorded in DMSO, and the assignment of the peaks was determined by comparison with the NMR data of the trans and cis forms of α-OHTAM (*37*), dG-N²-TAM (*21*), and dG-N²-TAM *N*-oxide (*41*), and by D_2O exchange for analyzing the exchangeable NH and OH protons (Table 1). Two doublet signals corresponding to monosubstituted amino group (N²H) were observed at δ 5.96 and 6.01 in fr-1 and at δ 5.95 and 6.01 in fr-2. Two triplet signals corresponding to sugar H1′ were detected at δ 6.18 and 6.22 in fr-1 and at δ 6.22 and 6.26 in fr-2. This indicates that both fr-1 and fr-2 contain two epimers. The integration values of these peaks authenticates the existence of two epimers in a ratio of 1:1 for fr-1 and 3:2 for fr-2. These epimers could be produced via a carbocation generated at the α-position of the *N*-desmethylTAM moiety, as observed for TAM α-sulfate (*42*). Since fr-1 contains two epimers, the two doublet signals corresponding to protons H3 and H5, and H2 and H6, of alkoxyphenyl were overlapped and observed at δ 6.53 and 6.77, respectively (Figure 5). This indicates that fr-1 is a mixture of two trans epimers of dG-N²-*N*-desmethylTAM. The NMR spectrum of fr-1 was consistent with that observed recently by Gamboa da Costa (*36*).

On the other hand, the two doublet signals in fr-2 were shifted downfield (δ 7.17 and 7.34) (Figure 5), confirming that fr-2 is a mixture of two cis epimers of dG-N²-*N*-desmethylTAM (*21*, *37*, *41*). The doublets of N²H at δ 5.96 and 6.01 for fr-1 and at δ 5.95 and 6.01 for fr-2 were coupled to the methine moiety at δ 4.90 and 4.95, respectively. The shift in the signals for proton H8 of guanine indicates that the TAM moiety is linked to the N² position of guanine (Table 1 and Figure 5). Thus, fr-1 and fr-2 were identified as the mixture of epimers of dG-*trans*-N²-*N*-desmethylTAM and dG-*cis*-N²-*N*-desmethylTAM, respectively.

Detection of *N*-DesmethylTAM−DNA Adducts. To prepare $dG_{3'P}$-N²-*N*-desmethylTAM that is used as a standard for ^{32}P-postlabeling analysis, $dG_{3'P}$ was reacted with α-acetoxy-*N*-desmethylTAM. As shown in Figure 6A, three fractions (***a***, t_R = 20.3 min; ***b***, t_R = 21.5 min; and ***c***, t_R = 26.6 min) were separated via HPLC. The UV spectra of fractions ***a*** and ***b*** were similar to that of the epimers of the trans form of dG-N²-*N*-desmethylTAM, and the UV spectrum of fraction ***c*** was similar to that of the epimers of the cis form of dG-N²-*N*-desmethylTAM

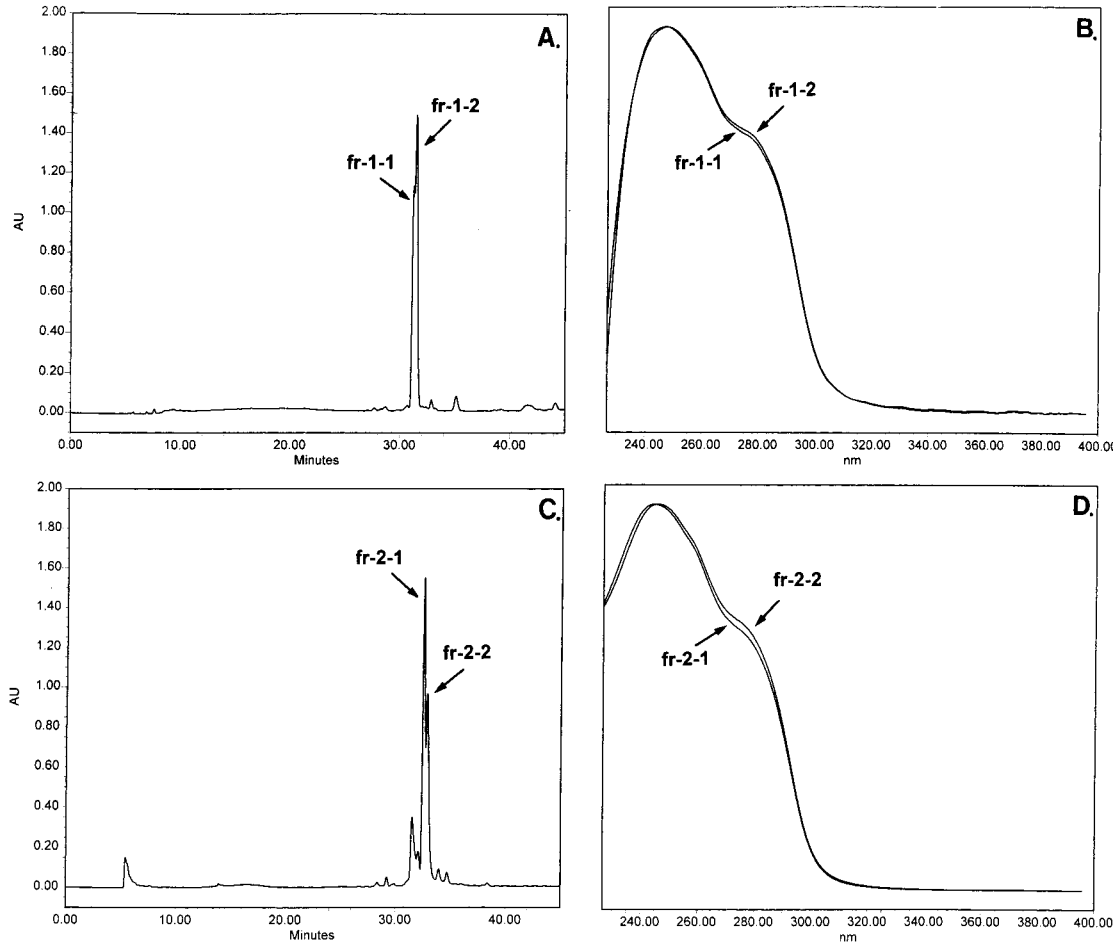


Figure 3. HPLC pattern and UV spectra of dG-N^2-*N*-desmethyltamoxifen. Partially purified fr-1 (A) and fr-2 (C) were subjected to the analytical μBondapak C_{18} column (0.39 cm × 30 cm), eluted over the course of 45 min at a flow rate of 1.0 mL/min with a linear gradient of 0.05 M triethylammonium acetate (pH 7.0) containing 10 to 70% acetonitrile. During the isolation of dG-*N*-desmethylTAM by HPLC, UV spectra (230−400 nm) of fr-1 (B) and fr-2 (D) were monitored every 2 s using a multiple-photodiode array detector (Waters).

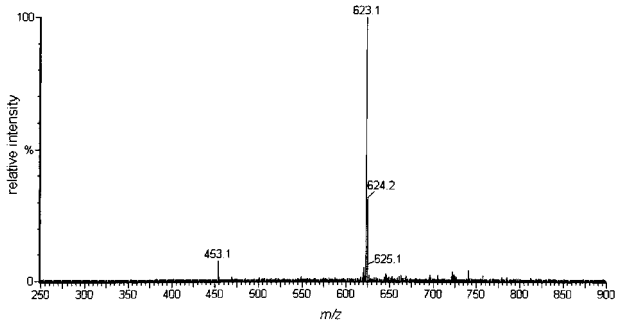


Figure 4. Mass spectrum of dG-N^2-*N*-desmethytamoxifen. Fr-1, whose spectrum is shown in Figure 2A, was subjected to positive ion FAB mass spectroscopy.

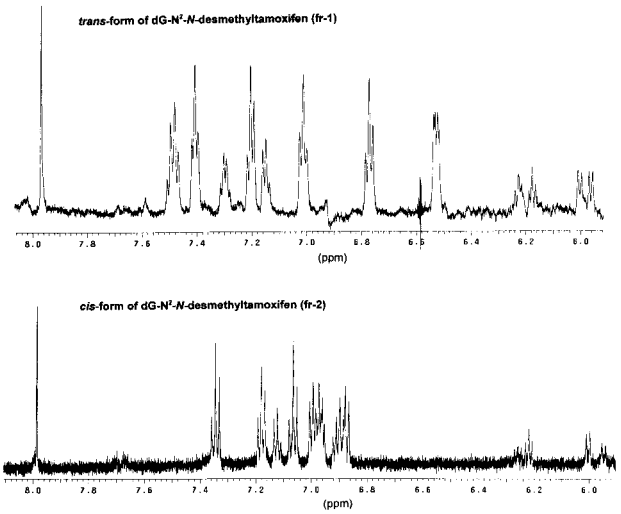


Figure 5. ^{1}H NMR spectra of trans and cis forms of dG-N^2-*N*-desmethytamoxifen. Fr-1 and fr-2, whose spectr are shown in panels A and B of Figure 2, were further purified using the same HPLC system and identified by 500 or 600 MHz NMR, as described in Table 1.

(Figure 6B). A part of each fraction was evaporated to dryness, incubated with alkaline phosphatase to produce the deoxynucleoside, and subjected to HPLC. The retention times of products obtained from fractions ***a*** and ***b*** were consistent with fr-1-1 and fr-1-2, an epimer of the trans form of dG-N^2-*N*-desmethylTAM (data not shown). Two products (fr-2-1 and fr-2-2) were observed in fraction ***c***; the retention times were also consistent with those of the epimers of the cis form of dG-N^2-*N*-desmethylTAM. The ratio of fr-2-1 to fr-2-2 was 45:55. Using negative ion LC/mass spectroscopy, the deprotonated molecular ions of fractions ***a*** (Figure 7), ***b***, and ***c*** (data not shown) exhibited peaks at *m*/*z* 701, showing the molecular mass to be 702 Da. Background ions at *m*/*z* 338 also appeared in this spectrum. Thus, fractions ***a*** and ***b*** were the epimers of the trans form of dG$_{3'P}$-N^2-*N*-desmethylTAM, and fraction ***c*** was a mixture of epimers of the cis form of dG$_{3'P}$-N^2-*N*-desmethylTAM.

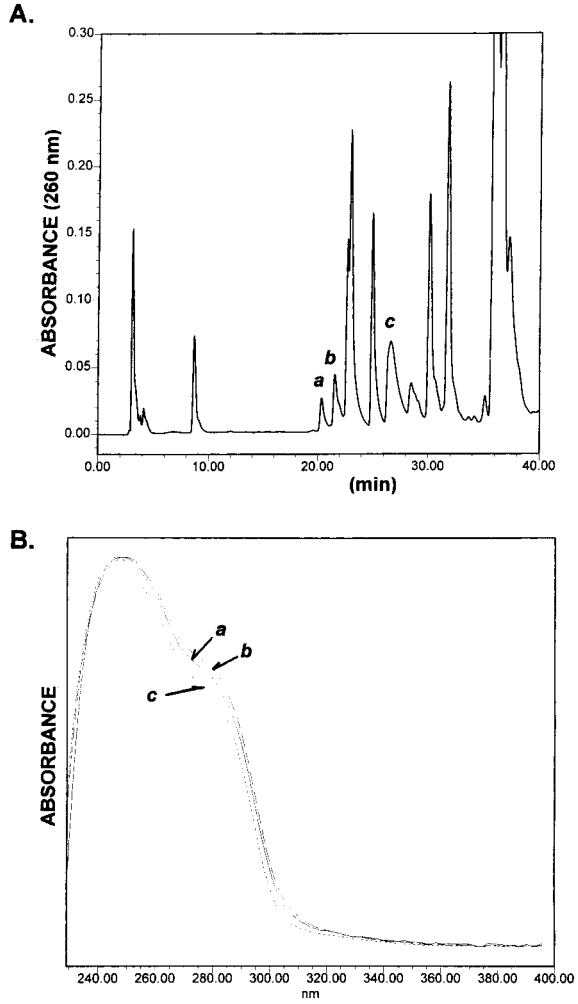


Figure 6. HPLC separation of $dG_{3'P}$-N^2-*N*-desmethyltamoxifen. $dG_{3'P}$ (1.0 mg) was reacted at 37 °C for 18 h with α-acetoxy-*N*-desmethylTAM (0.8 mg) in 100 mM Tris-HCl buffer (pH 8.0), as described in Materials and Methods. The reaction mixture was extracted twice with butanol. (A) To isolate *N*-desmethyl-TAM-modified $dG_{3'P}$, the pooled butanol extracts were concentrated at reduced pressure and subjected to HPLC, as similarly described for isolation of dG-N^2-*N*-desmethylTAM. (B) UV spectra of the of trans (***a*** and ***b***) and cis forms (***c***) of $dG_{3'P}$-N^2-*N*-desmethylTAM.

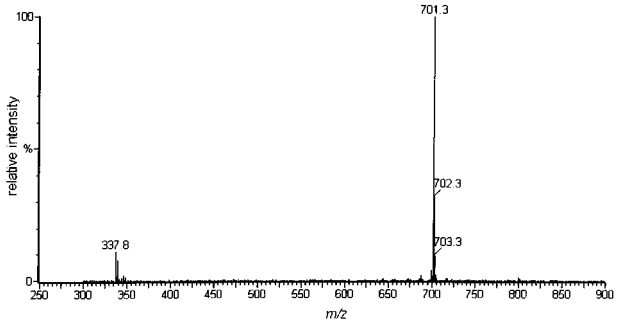


Figure 7. Mass spectrum of $dG_{3'P}$-N^2-*N*-desmethytamoxifen. Fraction ***a***, whose spectrum is shown in Figure 6A, was subjected to negative ion FAB mass spectroscopy.

When these trans and cis forms of $dG_{3'P}$-N^2-*N*-desmethylTAM were labeled with ^{32}P, and analyzed by using ^{32}P-postlabeling/HPLC on-line with a radioisotope detector, as described in Materials and Methods, the retention times of ***a***, ***b***, and ***c*** were 20.9, 24.0, and 59.5 min, respectively (Figure 8). When a calf thymus DNA was reacted with α-acetoxy-*N*-desmethylTAM, three adducts

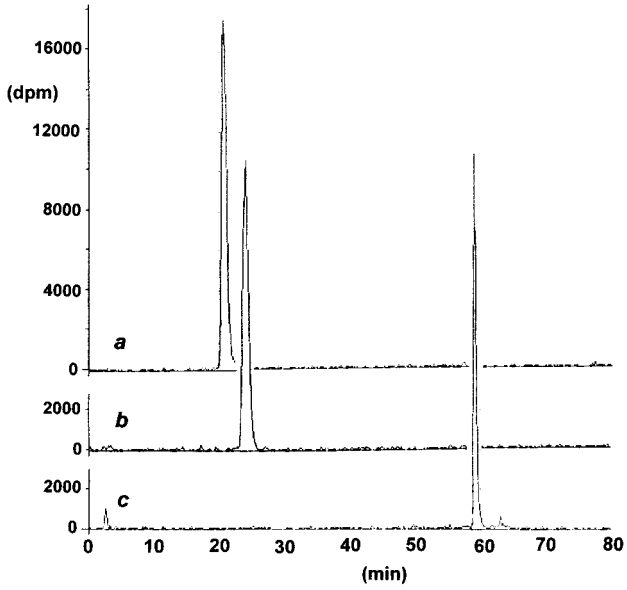


Figure 8. ^{32}P-postlabeling/HPLC analysis of $dG_{3'P}$-N^2-*N*-desmethytamoxifen. Standard stereoisomers of $dG_{3'P}$-N^2-*N*-desmethylTAM (***a***−***c***) were labeled with ^{32}P and developed for 16 h on a PEI-cellulose TLC plate using 1.7 M sodium phosphate buffer (pH 6.0) as described in Materials and Methods. ^{32}P-labeled products remaining on the TLC plate were recovered using 4 M pyridinium formate (pH 4.3) and evaporated to dryness. ^{32}P-labeled products were subjected to a Hypersil BDS C_{18} analytical column (0.46 cm × 25 cm), eluted at a flow rate of 1.0 mL/min with an isocratic condition of 2.0 M ammonium formate (pH 4.0) containing 20% acetonitrile/methanol (6:1, v/v) over the course of 40 min followed by a linear gradient from 20 to 45% over the course ofr 25 min. The radioactivity was monitored by a radioisotope detector connected to a Waters 990 HPLC instrument.

were detected (Figure 9A). Fr-2 was detected as the major adduct, and its retention time was consistent with that of the peak ***b***, an epimer of the trans form of $dG_{3'P}$-N^2-*N*-desmethylTAM. Fr-1 and fr-3 were detected as the minor adducts. The retention times of fr-1 and fr-3 were consistent with those of peaks ***a*** and ***c***, epimers of the trans and cis forms, respectively, of $dG_{3'P}$-N^2-*N*-desmethylTAM (Figure 9A). The levels of fr-1, fr-2, and fr-3 were 1.2, 8.7, and 0.9 adducts/10^5 nucleosides, respectively. When the DNA was incubated only in the buffer, no DNA adducts were detected (data not shown).

When TAM adducts in the livers of rats treated with TAM or *N*-desmethylTAM were analyzed by a similar ^{32}P-postlabeling/HPLC technique, the retention time of the second major adduct induced by TAM was consistent with that of the major adduct observed with *N*-desmethylTAM (*34*, *35*). In fact, when DNA was reacted with α-acetoxy-TAM, two epimers of the trans form of dG-N^2-TAM adducts (fr-1 and fr-2) and a mixture of two epimers of the the cis form (fr-3) were detected at 23.8, 29.6, and 60.3 min, respectively (Figure 9B). Via cochromatography of dG-N^2-TAM adducts with dG-N^2-*N*-desmethylTAM adducts, the retention time of one epimer (fr-1) of the trans form of dG-N^2-TAM was consistent with one epimer (fr-2) of the trans form of dG-N^2-*N*-desmethylTAM (Figure 9C).

Using the same ^{32}P-postlabeling/HPLC condition as in the study presented here, 16 endometrial samples collected from women taking TAM were analyzed (*29*). We detected trans and cis epimers of dG-N^2-TAM as the major adducts in eight patients treated for various periods of time with TAM. Fr-1, termed one of the trans

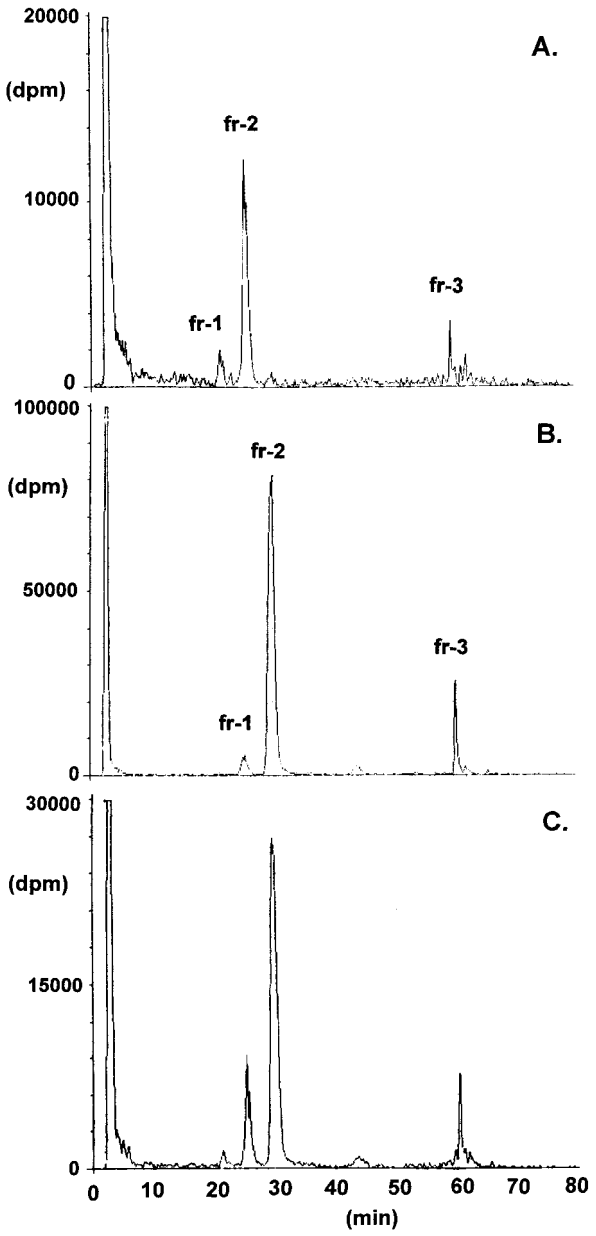


Figure 9. ^{32}P-postlabeling/HPLC analysis of DNA reacted with α-acetoxy-*N*-desmethyltamoxifen and α-acetoxytamoxifen. Aliquots of calf thymus DNA (10 μg) were incubated 37 °C for 1 h with 50 μg of α-acetoxy-*N*-desmethylTAM (A) or 400 μg of α-acetoxyTAM (B) in 100 mM Tris-HCl buffer (pH 8.0). The recovered DNA (0.4 μg) was digested using the nuclease P1 enrichment procedure and labeled with ^{32}P, as described in Materials and Methods. (C) Spectrum for a sample containing a half of that from panel A and one-third of that from panel B. These samples were analyzed using the ^{32}P-postlabeling/HPLC method for analysis of TAM−DNA adducts, as shown in the legend of Figure 8.

epimers of dG-N^2-TAM, was detected in six women taking TAM; the level of adduct was 0.5−6.0 adducts/10^8 deoxynucleotides (*29*). Since the retention time of fr-1 of dG-N^2-TAM was similar to that of fr-2 of dG-N^2-*N*-desmethylTAM, fr-1 detected in endometrial samples may contain the trans epimer of the dG-N^2-*N*-desmethylTAM−DNA adduct.

We have now prepared standards of dG$_{3'p}$-N^2-*N*-desmethylTAM for ^{32}P-postlabeling analysis, in addition to dG$_{3'p}$-N^2-TAM (*23*) and dG$_{3'p}$-N^2-TAM *N*-oxide (*41*). When an appropriate HPLC system that can resolve all the iso forms of these standards is established, TAM−DNA adducts observed in tissues of animals treated with TAM and in endometrial samples of women undergoing TAM treatment will be identified.

Acknowledgment. This research was supported by National Institute of Environmental Health Sciences Grant ES09418.

References

(1) Fischer, B., Costantino, J. P., Redmond, C. K., Fisher, E. R., Wickerham, D. L., and Cronin, W. M. (1994) Endometrial cancer in tamoxifen-treated breast cancer patients: Findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP, B-14). *J. Natl. Cancer Inst.* **86**, 527−537.

(2) Clarke, M., Cillins, R., Davies, C., Godwin, J., Gray, R., and Peto, R. (1998) Tamoxifen for early breast cancer: An overview of the randomized trials. *Lancet* **351**, 1451−1467.

(3) Barakat, R. R. (1996) Endometrial cancer and tamoxifen. In *Tamoxifen: a Guide for Clinicians and Patients* (Jordan, V. C., Ed.) pp 101−117, PRR Inc., Huntington, NY.

(4) American Cancer Society. Cancer facts and figures-1998 (1998) American Cancer Society, Atlanta.

(5) Stearns, V., and Gelman, E. P. (1998) Does tamoxifen cause cancer in human? *J. Ciln. Oncol.* **16**, 779−792.

(6) Wogan, G. N. (1997) Review of the toxicology of tamoxifen. *Semin. Oncol.* **24** (Suppl. 1), 87−97.

(7) Williams, G. M., Iatropoulos, M. J., Djordjevic, M. V., and Kaltenberg, O. P. (1993) The triphenylethylene drug tamoxifen is a strong liver carcinogen in the rat. *Carcinogenesis* **14**, 315−317.

(8) Greaves, P., Goonetilleke, R., Nunn, G., Topham, J., and Orton, T. (1993) Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. *Cancer Res.* **53**, 3919−3924.

(9) Hard, G. C., Iatropoulos, M. J., Jordan, K., Radi, L., Kaltenberg, O. P., Imondi, A. R., and Williams, G. M. (1993) Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. *Cancer Res.* **53**, 4534−4541.

(10) Han, X., and Liehr, J. G. (1992) Induction of covalent DNA adducts in rodents by tamoxifen. *Cancer Res.* **52**, 1360−1363.

(11) White, I. N. H., de Matteis, F., Davies, A., Smith, L. L., Crofton-Sleigh, C., Venitt, S., Hewer, A., and Phillips, D. H. (1992) Genotoxic potential of tamoxifen and analogues in female Fischer F344/n rats, DBA/2 and C57B1/6 mice and in human MCL-5 cells. *Carcinogenesis* **13**, 2197−2203.

(12) Osborne, M. R., Hewer, A., Hardcastle, I. R., Carmichael, P. L., and Phillips, D. H. (1996) Identification of the major tamoxifen-deoxyguanosine adduct formed in the liver DNA of rats treated with tamoxifen. *Cancer Res.* **56**, 66−71.

(13) Pathak, D. N., and Bodell, W. J. (1994) DNA adduct formation by tamoxifen with rat and human liver microsomal activation systems. *Carcinogenesis* **15**, 529−532.

(14) Pathak, D. N., Pongracz, K., and Bodell, W. J. (1995) Microsomal and peroxidase activation of 4-hydroxy-tamoxifen to form DNA adducts: comparison with DNA adducts formed in Sprague-Dawley rats treated with tamoxifen. *Carcinogenesis* **16**, 11−15.

(15) Hemminki, K., Widlak, P., and Hou, S. M. (1995) DNA adducts caused by tamoxifen and toremifene in human microsomal system and lymphocytes *in vitro*. *Carcinogenesis* **16**, 1661−1664.

(16) Potter, G. A., McCague, R., and Jarman, M. (1994) A mechanism hypothesis for DNA adduct formation by tamoxifen hepatic oxidative metabolism. *Carcinogenesis* **15**, 439−442.

(17) Phillips, D. H., Potter, G. A., Horton, M. N., Hewer, A., Crofton-Sleigh, C., Jarman, M., and Venitt, S. (1994) Reduced genotoxicity of [D$_5$-ethyl]-tamoxifen implicates α-hydroxylation of the ethyl group as a major pathway of tamoxifen activation to a liver carcinogen. *Carcinogenesis* **15**, 1487−1492.

(18) Phillips, D. H., Carmichael, P. L., Hewer, A., Cole, K. J., and Poon, G. K. (1994) α-Hydroxytamoxifen, a metabolite of tamoxifen with exceptionally high DNA-binding activity in rat hepatocytes. *Cancer Res.* **54**, 5518−5522.

(19) Jarman, M., Poon, G. K., Rowlands, M. G., Grimshaw, R. M., Horton, M. N., Potter, G. A., and McCague, R. (1995) The deuterium isotope effect for the α-hydroxylation of tamoxifen by rat liver microsomes accounts for the reduced genotoxicity of [D$_5$-ethtyl]tamoxifen. *Carcinogenesis* **16**, 683−688.

(20) Poon, G. K., Walter, B., Lonning, P. E., Horton, M. N., and McCague, R. (1995) Identification of tamoxifen metabolites in

human Hep G2 cell line, human liver homogenate, and patients on long-term therapy for breast cancer. *Drug Metab. Dispos.* **23**, 377−382.

(21) Dasaradhi, L., and Shibutani, S. (1997) Identification of tamoxifen-DNA adducts formed by α-sulfate tamoxifen and α-acetoxytamoxifen. *Chem. Res. Toxicol.* **10**, 189−196.

(22) Osborne, M. R., Hardcastle, I. R., and Phillips, D. H. (1997) Minor products of reaction of DNA with α-acetoxytamoxifen. *Carcinogenesis* **18**, 539−543.

(23) Shibutani, S., Dasaradhi, L., Terashima, I., Banoglu, E., and Duffel, M. W. (1998) α-Hydroxytamoxifen is a substrate of hydroxysteroid (alcohol) sulfotransferase, resulting in tamoxifen DNA adducts. *Cancer Res.* **58**, 647−653.

(24) Shibutani, S., Shaw, P., Suzuki, N., Dasaradhi, L., Duffel, M. W., and Terashima, I. (1998) Sulfation of α-hydroxytamoxifen catalyzed by human hydroxysteroid sulfotransferase results in tamoxifen DNA adducts. *Carcinogenesis* **19**, 2007−2011.

(25) Shibutani, S., and Dasaradhi, L. (1997) Miscoding potential of tamoxifen-derived DNA adducts: α-(*N*²-deoxyguanosinyl)tamoxifen. *Biochemistry* **36**, 13010−13017.

(26) Terashima, I., Suzuki, N., and Shibutani, S. (1999) Mutagenic potential of α-(*N*²-deoxyguanosinyl)tamoxifen lesions, the major DNA adducts detected in endometrial tissues of patients treated with tamoxifen. *Cancer Res.* **59**, 2091−2095.

(27) Martin, E. A., Heydon, R. T., Brown, K., Brown, J. E. B., Lim, C. K., White, I. N. H., and Smith, L. L. (1998) Evaluation of tamoxifen and α-hydroxytamoxifen ^{32}P-post-labelled DNA adducts by the development of a novel automated on-line solid-phase extraction HPLC method. *Carcinogenesis* **19**, 1061−1069.

(28) Shibutani, S., Suzuki, N., Terashima, I., Sugarman, S. M., Grollman, A. P., and Pearl, L. M. (1999) Tamoxifen-DNA adducts in the endometrium of women treated with tamoxifen. *Chem. Res. Toxicol.* **12**, 646−653.

(29) Shibutani, S., Ravindernath, A., Suzuki, N., Terashima, I., Sugarman, S. M., Grollman, A. P., and Pearl, L. M. (2000) Identification of tamoxifen-DNA adducts in the endometrium of women treated with tamoxifen. *Carcinogenesis* (in press).

(30) Carmichael, P. L., Ugwumadu, A. H. N., Neven, P., Hewer, A. J., Poon, G. K., and Phillips, D. H. (1996) Lack of genotoxicity of tamoxifen in human endometrium. *Cancer Res.* **56**, 1475−1479.

(31) Hemminki, K., Rajaniemi, H., Lindahl, B., and Moberger, B. (1996) Tamoxifen-induced DNA adducts in endometrial samples from breast cancer patients.*Cancer Res.* **56**, 4374−4377.

(32) Carmichael, P. L., Sardar, S., Crooks, N., Neven, P., Van Hoof, I., Ugwumadu, A., Bourne, T., Tomas, E., Hellberg, P., Hewer, A., and Phillips, D. H. (1999) Lack of evidence from HPLC ^{32}P-post-labeling from tamoxifen-DNA adducts in the human endometrium. *Carcinogenesis* **20**, 339−342.

(33) Rajaniemi, H., Rasanen, I., Koivisto, P., Peltonen, K., and Hemminki, K. (1999) Identification of the major tamoxifen-DNA adducts in rats liver by mass spectroscopy. *Carcinogenesis* **20**, 305−309.

(34) Phillips, D. H., Hewer, A., Horton, M. N., Cole, K. J., Carmichael, P. L., Davis, W., and Osborne, M. R. (1999) *N*-Demethylation accompanies α-hydroxylation in the metabolite activation of tamoxifen in rat liver cells. *Carcinogenesis* **20**, 2003−2009.

(35) Brown, K., Heydon, R. T., Jukes, R., White, I. N. H., and Martin, E. A. (1999) Further characterization of the DNA adducts formed in rat liver after the administration of tamoxifen, *N*-desmethyltamoxifen or *N*,*N*-didesmethyltamoxifen. *Carcinogenesis* **20**, 2011−2016.

(36) Gamboa da Costa, G., Hamilton, L. P., Beland, F. A., and Marques, M. M. (2000) Characterization of the major DNA adduct formed by α-hydroxy-N-desmethyltamoxifen in vitro and in vivo. *Chem. Res. Toxicol.* **13**, 200−207.

(37) Foster, A. B., Jarman, M., Leung, O.-T., McCague, R., Leclercq, G., and Devleeschouwer, N. (1985) Hydroxy derivatives of tamoxifen. *J. Med. Chem.* **28**, 1491−1497.

(38) Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

(39) Levay, G., Pongracz, K., and Bodell, W. J. (1991) Detection of DNA adducts in HL-60 cells treated with hydroquinone and *p*-benzoquinone by ^{32}P-postlabeling. *Carcinogenesis* **12**, 1181−1186.

(40) Olofson, R. A., Martz, J. T., Senet, J.-P., Piteau, M., and Malfroot, T. (1984) A new reagent for the selective, high-yield N-dealkylation of tertiary amines: improved syntheses of naltrexone and nalbuphine. *J. Org. Chem.* **49**, 2081−2082.

(41) Umemoto, A., Monden, Y., Komaki, K., Suwa, M., Kanno, Y., Suzuki, M., Lin, C., Ueyama, Y., Momen, M. A., Ravindernath, A., and Shibutani, S. (1999) Tamoxifen-DNA adducts formed by α-acetoxytamoxifen *N*-oxide. *Chem. Res. Toxicol.* **12**, 1083−1089.

(42) Sanchez, C., Shibutani, S., Dasaradhi, L., Bolton, J. L., Fan, P. W., and McClelland, R. A. (1998) Lifetime and reactivity of an ultimate tamoxifen carcinogen: The tamoxifen carbocation. *J. Am. Chem. Soc.* **120**, 13513−13514.

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