Reaction of the Premarin Metabolite 4-Hydroxyequilenin Semiquinone Radical with 2′-Deoxyguanosine: Formation of Unusual Cyclic Adducts

Li Shen, Shengxiang Qiu, Richard B. van Breemen, Fagen Zhang, Yumei Chen, and Judy L. Bolton*

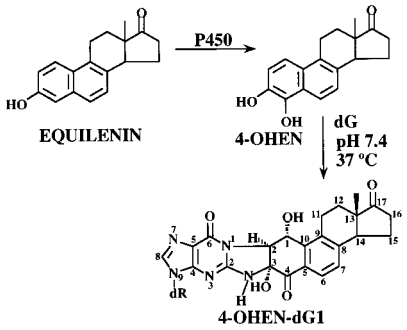
Department of Medicinal Chemistry and Pharmacognosy (M/C 781), College of Pharmacy University of Illinois at Chicago 833 S. Wood St., Chicago, Illinois 60612-7231

Received May 2, 1997

The longer women are exposed to estrogens either through early menarche and late menopause, and/or through estrogen replacement therapy, the higher is the risk of developing breast or endometrial cancer.[1] The mechanism(s) of estrogen-induced carcinogenesis could involve modification of critical cellular macromolecules by electrophilic/redox-active quinoids.[2] Aromatic hydroxylation of estrone and 17β-estradiol forming catechol metabolites represents one major metabolic pathway for endogenous estrogens. The *o*-quinones formed from oxidation of these catechols have previously been implicated as the ultimate carcinogens.[2] Redox cycling of these *o*-quinones generates reactive hydroxyl radicals which causes oxidation of the purine/pyrimidine residues of DNA.[2] Estrogen *o*-quinones are also Michael acceptors, and they could be responsible for alkylation of DNA which has been detected by ^{32}P-postlabeling methods.[3] Alternatively, we have shown that additional reactive intermediates can be produced from isomerization of the *o*-quinones to electrophilic *p*-quinone methides[4] which could be the ultimate DNA alkylating agents.

Equilenin, or its 17β-hydroxylated analog, make up 15% of the most widely prescribed estrogen replacement formulation called Premarin (Wyeth-Ayerst).[5] Although there is very little information on the human metabolism of these estrogens,[5a] it is known that treating hamsters for 9 months with either estrone, equilin + equilenin, or sulfatase-treated Premarin, resulted in 100% renal tumor incidences.[5b] In previous work, we synthesized the major catechol metabolite of equilenin, 4-hydroxyequilenin (4-OHEN, Scheme 1) and examined how aromatization of the B ring affects the formation and reactivity of the *o*-quinone.[6] 4-OHEN-*o*-quinone is much more redox-active and longer-lived than the endogenous estrone-*o*-quinones which suggests that the mechanism(s) of toxicity of the former could be quite different. It has been shown with *in vitro* models that the 4-OHEN-*o*-quinone increases the amount of oxidative damage to DNA by 50% compared to control levels.[7] In addition to DNA oxidation, an alternative mechanism for equilenin carcinogenesis may involve alkylation of DNA. We report here the synthesis and characterization of 2′-deoxyguanosine (dG) adducts of 4-OHEN quinoids.

Scheme 1. Formation of 4-OHEN from Equilenin and Reaction with dG



4-OHEN was synthesized by treating equilin with Fremy's salt as described previously.[6] 4-OHEN (20 mg, 0.07 mmol, dissolved in 1 mL of methanol) was incubated with dG (0.14 mmol in 200 μL of DMSO) in pH 7.4 potassium phosphate buffer (25 mM, 20 mL) at 37 °C for 7 h after which the adducts were isolated and purified.[8] Four products were obtained with the same molecular weight consistent with isomers of 4-OHEN-dG (Scheme 1). What follows is the extensive characterization of the major isomer, 4-OHEN-dG1. The electrospray mass spectra (positive and negative modes) allowed the assignment of a molecular weight consistent with the addition of dG and oxygen to 4-OHEN. The molecular formula $C_{28}H_{29}N_5O_8$ was obtained from the positive ion high-resolution FAB mass spectrum, in which a protonated molecule $[M + H]^+$ was observed at *m/z* 564.2076. Negative ion electrospray MS-MS experiments were performed to obtain molecular structure information (Supporting Information). Upon collision induced dissociation, the ion at *m/z* 562 $[M - H]^-$ decomposed to form major fragment ions of *m/z* 544 $[M - H - H_2O]^-$, 516 $[M - H - H_2O - CO]^-$, 437 [M − C, D rings of 4-OHEN]$^-$, 428 $[M - H_2O -$ deoxyribose$]^-$, 410 $[M - 2H_2O -$ deoxyribose$]^-$, 400 $[M - H_2O -$ deoxyribose $- CO]^-$, 384 $[M - HCN - 2H_2O -$ deoxyribose$]^-$, 295 $[M - H_2O -$ dG without $N^2-H]^-$, 292 $[dG - H + CN]^-$, 266 $[dG - H]^-$, 176 [guanine $- H + CN]^-$, 150 [guanine $- H]^-$, and 133 [guanine $- H - NH_3]^-$. The fragment ions at *m/z* 295 and 133 (cleavage at C^2 and N^1 of dG and cleavage at dG$-C^2$ and dG$-N^2$) are consistent with reaction of 4-OHEN with the N^1 position and the N^2 exocyclic amino group of dG. In addition, pairs of ions which differ by 26 units (i.e., 410 and 384, 292 and 266, and 176 and 150) indicate loss of CN which could be formed by loss of dG-N^2-C^3 of 4-OHEN. Finally, when 4-OHEN was incubated with dG in 99% $H_2{}^{18}O$ the positive ion electrospray mass spectrum gave an apparent molecular ion at 568 $[M + H]^+$ which was 4 mass units higher than that from experiments in $H_2{}^{16}O$, although the molecular formula showed only one additional oxygen. Fragment ions corresponding to loss of the sugar (452) also were 4 mass units higher than those from $H_2{}^{16}O$ experiments. This suggests that formation of 4-OHEN-dG involved the addition of 2 molecules of water and the loss of 1 molecule of water presumably from one of the catechol hydroxy groups.

The structural characterization and stereochemical assignment of 4-OHEN-dG1 was achieved through the unambiguous rationalization of ^{1}H (Figure 1) and ^{13}C NMR resonances using a combination of DQF-COSY, ROESY, HMQC, and HMBC

(1) (a) Liehr, J. G. *Mutat. Res.* **1990**, *238*, 269−276. (b) Colditz, G. A.; Hankinson, S. E.; Hunter, D. J.; Willett, W. C.; Manson, J. E.; Stampfer, M. J.; Hennekens, C.; Rosner, B.; Speizer, F. E. *New Eng. J. of Med.* **1995**, *332*, 1589−1593.

(2) Yager, J. D.; Liehr, J. G. *Annu. Rev. Pharmacol. Toxicol.* **1996**, *36*, 203−232.

(3) Dwivedy, I.; Devanesan, P.; Cremonesi, P.; Rogan, E.; Cavalieri, E. *Chem. Res. Toxicol.* **1992**, *5*, 828−833.

(4) (a) Iverson, S. L.; Shen, L.; Anlar, N.; Bolton, J. L. *Chem. Res. Toxicol.* **1996**, *9*, 492−499. (b) Bolton, J. L.; Shen, L. *Carcinogenesis* **1996**, *17*, 925−929.

(5) (a) Purdy, R. H.; Moore, P. H.; Williams, M. C.; Goldzheher, H. W.; Paul, S. M. *FEBS Lett.* **1982**, *138*, 40−44. (b) Li, J. J.; Li, S. A.; Oberley, T. D.; Parsons, J. A. *Cancer Res.* **1995**, *55*, 4347−4351.

(6) Shen, L.; Pisha, E.; Huang, Z.; Pezzuto, J. M.; Krol, E.; Alam, Z.; van Breemen, R. B.; Bolton, J. L. *Carcinogenesis* **1997**, *18*, 1093−1101.

(7) Han, X.; Liehr, J. G. *Carcinogenesis* **1995**, *16*, 2571−2574.

(8) The adducts were isolated from the aqueous phase on C-18 extraction cartridges and eluted with methanol. The eluates were concentrated and subjected to semipreparative HPLC with an Ultrasphere ODS column (10 × 250 mm, Beckman) with a flow rate of 3.5 mL/min. The mobile phase consisted of 10% CH_3OH in water for 2 min, increased to 35% CH_3OH over 3 min, increased to 43% CH_3OH over 25 min, and increased to 90% CH_3OH over the last 5 min of the run. Using this method, the retention times of the isomers were 16, 20, 27, and 30 min. The product ratios were 2.8:1.3:1.4:1 in order of increasing elution time.

S0002-7863(97)01396-6 CCC: $14.00

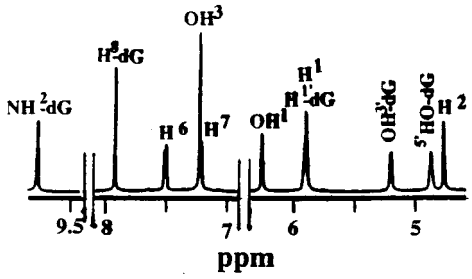


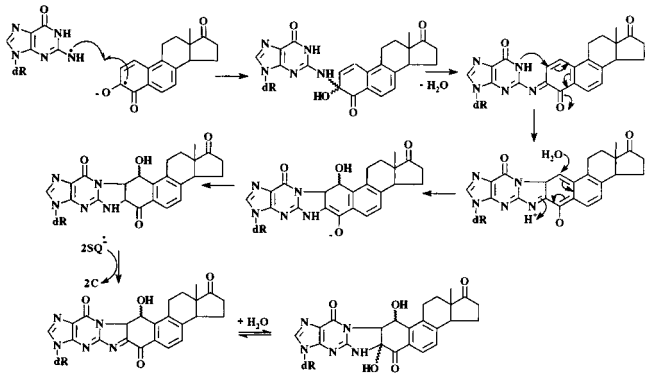
Figure 1. Partial proton NMR of 4-OHEN-dG[1] in DMSO-d_6.

techniques. The B, C, and D rings of 4-OHEN remained intact based on interpretation of the data derived from the NMR experiments which suggests that dG alkylated the A ring of 4-OHEN, resulting in saturation of the aromatic ring. The proton at δ 5.92, split by the vicinal hydroxyl proton (δ 6.28), and further coupled with the adjacent H^2 proton at δ 4.78 as shown in the DQF-COSY spectrum, was assigned to H^1 because of the significant cross-peaks between the proton pairs of $H^1/H^{11\alpha}$ and $H^1/H^{11\beta}$ in the ROESY spectrum. A typical conjugated carbonyl signal was observed in the ^{13}C NMR spectrum at δ 191.3, which was assigned to the C^4 position. The exchangeable NH proton at 9.29 ppm integrates to 1 proton suggesting reaction had occurred at the exocyclic amino group. In addition, it was clearly demonstrated that dG was attached at the C^3 position of 4-OHEN through N^2 by the observation of the informative cross-correlation of N^2H/C^2 and N^2H/C^3 in the HMBC spectrum. Similarly, alkylation of N^1-dG at C^2 of 4-OHEN was substantiated by cross-correlation of H^2/dG-C^2 in the HMBC spectra as well as the downfield chemical shift of the aliphatic H^2 proton (δ 4.78) and C^2 carbon (δ 67.2). Finally, the exchangeable proton at δ 7.25 was assigned to the hydroxyl group at C^3 from cross-correlations between $^3HO/C^2$, $^3HO/C^3$, $^3HO/C^4$, and the downfield resonances of the C^3 (δ 84.3) carbon.

The almost planar conformation of the A ring prevented the use of coupling constants to assign stereochemistry. Nevertheless, this difficulty could be overcome by the use of the ROESY spectrum and spatial relation rationalization. The significant NOE correlation between $H^1/H^{11\alpha}$ and $H^1/H^{11\beta}$ and the lack of any NOE effects between OH^1 and the two H^{11} protons favored the C^1 hydroxyl group in the α-orientation. It was notable that the C^{18} methyl group shifted to a much higher field resonance upon dG adduction; i.e., from δ 0.69 in 4-OHEN to δ 0.04 in 4-OHEN-dG1, which may only be expected from the steric repulsion and anisotropic effects resulting from a β-oriented dG substituent. Therefore, the structure of 4-OHEN-dG1 was established as depicted in Scheme 1. Similarities between the proton and ^{13}C NMR spectra of the other three dG adducts suggest that they are the corresponding diastereoisomers of 4-OHEN-dG1; however, the possibility of structural isomers cannot be ruled out at this time.

Increases in the rate of adduct formation were observed in basic solution, in the presence of reducing agents, and with reductive enzymes (Supporting Information). In contrast, adduct formation could be completely abolished under anaerobic conditions, in acidic solution, in methanol, and in the presence of a high concentration of chelator. These data strongly suggest that dG reacts with the semiquinone radical of 4-OHEN and not the *o*-quinone or quinone methides. These data along with unequivocal assignment of the structure of 4-OHEN-dG1 allow us to formulate a hypothetical mechanism for reaction of the 4-OHEN semiquinone radical with dG (Scheme 2). The 4-OHEN semiquinone radical generated by auto-oxidation of 4-OHEN[6] abstracts a hydrogen atom from the exocyclic amino group of dG generating dG$-N^2H^{\bullet}$. Alternatively, dG$-N^2H^{\bullet}$ could be formed by abstraction of a hydrogen atom by an

Scheme 2. Hypothetical Mechanism for Formation of 4-OHEN-dG



hydroxyl radical or a superoxide anion radical. Under physiological conditions it has been shown that radical generation at N^2 is favored over the formation of carbon-centered radicals at C^8.[9] This radical combines with another 4-OHEN semiquinone radical at the C^3 position. Loss of water generates the *o*-quinone imine which undergoes intramolecular cyclization with N^1-dG. Addition of water returns the B ring to aromaticity, and ketonization of the C^4 carbonyl group gives the monohydroxylated cyclic adduct. Two-electron oxidation of the dG$-N^2$ amine of this compound gives an imine which upon hydrolysis gives the final structure of 4-OHEN-dG1.

In conclusion, we have shown that 4-OHEN semiquinone radical forms highly unusual adducts with dG. The type of adducts formed with estrogen quinoids likely reflects the reactivity of the quinoids as well as the environment in which they are formed. For example, a recent report has shown that the 4-hydroxyestrone−semiquinone radical modifies adenine at the C^8 position on the purine ring in DMF under reductive conditions.[10] In contrast, in an acidic environment the *o*-quinone of 4-hydroxyestrone is the alkylating species which only reacts with dG and not deoxyadenosine to give an N-7 substituted adduct at the C^1 position on the A ring.[11] Under the same conditions, 2-hydroxyestrone-*o*-quinone rapidly isomerizes to a quinone methide which then alkylates the exocyclic amino group of either deoxyadenosine or dG. With 4-OHEN, we have shown that the quinoid responsible for alkylation of dG under physiological conditions is the semiquinone radical ultimately generating unusual adducts. Finally, the implications of these adducts to the biological effects of Premarin is not known; however, given the direct link between long-term estrogen replacement therapy and the enhanced risk of breast cancer, the potential for formation of redox-active/electrophilic metabolites from all of the estrogens in estrogen replacement formulations needs to be explored.

Acknowledgment. This research was supported by NIH grant CA73638 and the University of Illinois at Chicago. We thank Mr. Dejan Nikolic for the electrospray mass spectrometry analysis, Mr. Mark Bohlke for experiments obtained on the 500 MHz NMR, and Dr. Karol Bruzik for helpful discussions.

Supporting Information Available: Characterization details of the 4-OHEN-dG adducts as well as information on the mechanism of adduct formation (15 pages). See any current masthead page for ordering and Internet access instructions.

JA971396Q

(9) Zady, M. F.; Wong, J. L. *J. Am. Chem. Soc.* **1980**, *45*, 2373−2377.
(10) Abul-Hajj, Y. J.; Tabakovic, K.; Tabakovic, I. *J. Am. Chem. Soc.* **1995**, *117*, 6144−6145.
(11) Stack, D. E.; Byun, J.; Gross, M. L.; Rogan, E. G.; Cavalieri, E. L. *Chem. Res. Toxicol.* **1996**, *9*, 851−859.