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Ochratoxin A Forms a Carbon-Bonded C8-Deoxyguanosine Nucleoside Adduct: Implications for C8 Reactivity by a Phenolic Radical

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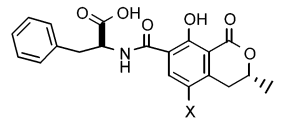
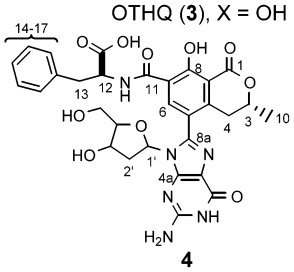
Ochratoxin A (OTA, **1**) is a *para*-chlorophenolic mycotoxin produced by strains of *Aspergillus* and *Penicillium*[1] that is widely found as a contaminant of improperly stored food products.[2] The toxin is a potent renal carcinogen in rodents[3] and is implicated in human kidney[4] and testicular carcinogenesis.[5]



OTA (**1**), X = Cl
OTB (**2**), X = H
OTHQ (**3**), X = OH



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In cells, OTA induces oxidative stress[6] and oxidative DNA damage[6b,7] through production of reactive oxygen species (ROS).[8] From the ^{32}P-postlabeling assay, it appears that OTA facilitates guanine-specific DNA adducts,[9] properties that establish a basis for its mutagenicity.[10] However, recent studies[11] suggest that the spots observed in the ^{32}P-postlabeling assay may be due to OTA cytotoxicity rather than to direct adduct formation.

Our laboratory has utilized a number of chemical approaches to assess OTA oxidation.[12−14] These studies point to the intermediacy of the OTA phenoxyl radical and benzoquinone formed from oxidative dechlorination of OTA. Recently, we presented evidence that the benzoquinone reacts covalently with reduced glutathione (GSH).[15] Here, the photooxidative properties of the hydroquinone derivative (OTHQ, **3**)[13] were utilized to generate an OTA-GSH conjugate that acted as an analytical standard for biochemical analyses.[15] These results confirmed that OTA undergoes oxidative dechlorination by liver enzymes, horseradish peroxidase (HRP)/H_2O_2, and free Fe(II), which supported our chemical findings.

Presently, we use the photochemistry of OTA[13,16] to assess its reactivity toward deoxyguanosine (dG), the base implicated in OTA-mediated DNA adduction.[9] These studies have led to the isolation and identification of the carbon-bonded C8-OTA-dG adduct **4** that is also formed upon oxidation of OTA with HRP/H_2O_2, free Fe(II), and Cu(II) ions. These results represent the first definitive evidence that OTA can react with a DNA-derived nucleophile and suggest involvement of the OTA phenoxyl radical in reaction with dG.

The photoreactivity of OTA (100 μM) in the presence of 50 mol equiv of dG was initially investigated using electrospray mass spectrometry (ES$^-$). Photoirradiation of the OTA/dG mixture for 2 min in 100 mM phosphate buffer (pH 7.4) generated three major products eluting at 19.4, 23.7, and 24.6 min (see Supporting Information and Figure S1 for experimental details). Products at 23.7 and 24.6 min had parent ions at *m*/*z* 384 and 368 and were assigned to OTHQ, **3** ([M − H]$^-$ = 384), and OTB, **2** ([M − H]$^-$ = 368).[13] The product at 19.4 min also lacked the chlorine isotope pattern and had a parent ion at [M − H]$^-$ = 633. Its MS/MS spectrum exhibited a prominent ion at *m*/*z* 517 (−deoxyribose (dR)), which was accompanied by ions at *m*/*z* 473 ([M − dR − CO_2 − H]$^-$) and 429 ([M − dR − 2CO_2 − H]$^-$), suggesting covalent attachment of dG to OTA with loss of HCl (i.e., OTA(403) − Cl(35) + dG(267) − 1H = 634).

Semipreparative scale photoreactions yielded the OTA-dG adduct as an off-white solid; its ^{1}H NMR spectrum in DMSO-d_6 is shown in Figure 1a. Surprisingly, all NH and OH protons of the dG moiety could be accounted for.[17] The sharp singlet at δ 8.09 ppm was either H6 of the OTA moiety or H8 of dG. However, the ^{1}H−^{13}C long-range heteronuclear correlation NMR spectrum (HMBC) shown in Figure 1b established the structure as **4**. The sugar H1′ proton showed a correlation with C8a at 143.3 ppm and C4a at 152.3 ppm, while the singlet at 8.09 ppm showed correlations with C8a and the phenolic C8 and amide carbonyl C11. These correlations confirmed the identity of H6 (8.09 ppm) and that it was the H8a proton of dG that was lost to yield **4** (NMR assignments of **4** are given in Figure S2, Supporting Information). It is also worthy to note that in the NOESY spectrum the sugar H1′ proton showed a strong cross-peak to the aromatic H6 proton (Figure S3, Supporting Information), not to the H4 protons, suggesting that the sugar and the phenylalanine moiety were in close proximity, as depicted for **4**.

The authentic sample **4** from photoexcitation of OTA may have formed via an OTA carbon-centered radical, as carbon radicals form C8 adducts[18] and photolysis of OTA yields the OTA phenolic radical and solvated electrons (e_{aq}^-);[16] the latter may initiate decomposition of the toxin to afford the carbon radical and Cl$^-$.[13,16] Thus, it was pertinent to determine whether **4** could be formed under biologically relevant oxidative conditions where the e_{aq}^- would not be present. Treatment of OTA (100 μM) with 50 equiv of dG in 100 mM phosphate buffer (pH 7.4) at 37 °C in the presence of either 100 μM $Fe(NH_4)_2(SO_4)_2$, 100 μM $Cu(OAc)_2$, or HRP (Type VI, 25 units/mL)/1 mM H_2O_2 gave the extracted ion chromatograms (EIC) in Figure 2 following 24 h of incubation time. Each oxidation system converted OTA into **4**, but clearly free Fe(II) was the most efficient (panel b), yielding **4** ca. 5 orders of magnitude greater than Cu(II) (panel c) and HRP/H_2O_2 (panel d). These results suggest that Fe may be an important cofactor in OTA-mediated mutagenicity.

The results presented in Figure 2 suggest involvement of the OTA phenoxyl radical in formation of **4**. The toxin undergoes a 1e oxidative process at ∼0.8 V versus SCE (1.04 V vs NHE) to form

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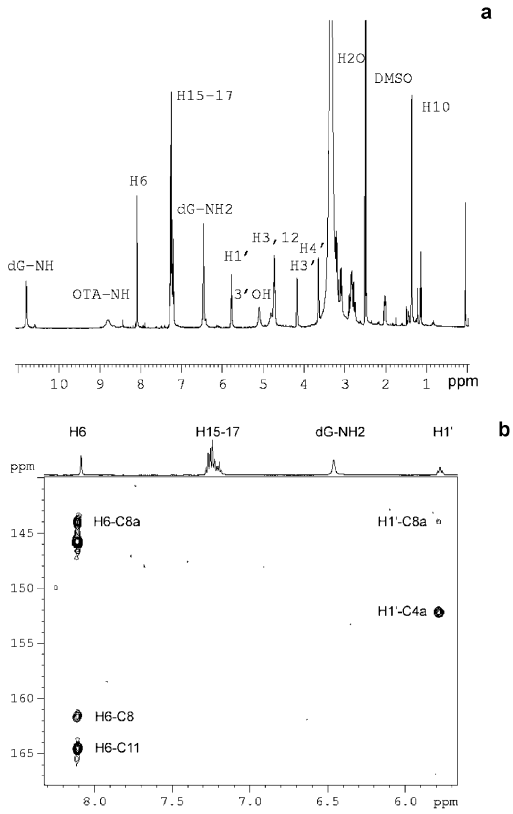

Figure 1. (a) 500 MHz ^{1}H NMR spectrum of the isolated OTA-dG adduct **4** recorded in DMSO-d_6. (b) Aromatic region of the HMBC spectrum of **4** in DMSO-d_6.

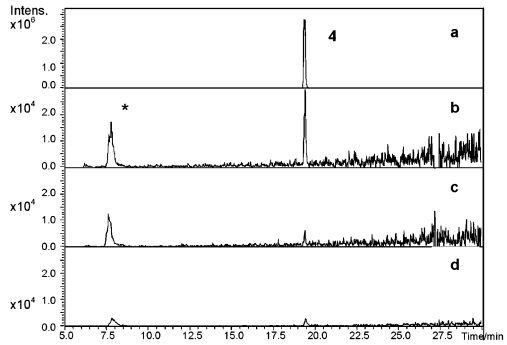

Figure 2. Extracted ion chromatograms (EIC) of *m*/*z* 633 (ES^-) for **4** from reaction of 100 μM OTA with 5 mM dG in 100 mM phosphate buffer (pH 7.4). (a) Photoirradiation (2 min); (b) incubation at 37 °C for 24 h with 1 equiv of Fe(II); (c) with 1 equiv of Cu(II); (d) with HRP (25 units/mL)/H_2O_2 (1 mM). The peak marked by the asterisk has $[M - H]^- = 595$ and $[M - 2H + K]^- = 633$; this peak does not contain an OTA moiety.

the phenoxyl radical,[14] and structure−activity relationships implicate the OTA phenoxyl radical in OTA-mediated DNA damage.[19] The susceptibility of the C8 position of dG to radical attack has been amply proven through formation of the hydroxyl radical-derived DNA lesion, 8-oxodeoxyguanosine (8-oxoG).[18] Estrogen radical anions react at the purine C8 position,[20] and most importantly Ni(salens) that form phenoxyl radicals upon oxidation are speculated to form C8-dG adducts.[21] Thus, the findings reported herein may help to define the mechanism of mutagenicity for phenolic toxins: formation of ROS with subsequent oxidative DNA damage (strand breaks and 8-oxoG) and coupling of reactive phenolic radical intermediates to the C8 position of G to form covalent DNA adducts.

In summary, the C8-deoxyguanosine nucleoside adduct **4** of the chlorinated phenolic toxin OTA (**1**) has been definitively identified by mass spectrometry and NMR. This represents the first structurally characterized DNA nucleoside adduct of a chlorophenolic toxin and is an important standard for ^{32}P-postlabeling experiments dealing with in vivo DNA adduction by OTA. The dG reactions of other chlorophenolic toxins are being examined to gain mechanistic insight into this C8 reactivity.

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Supporting Information Available: Experimental procedures and Figures S1−S3 described in the text (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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