Ochratoxin A acts as a photoactivatable DNA cleaving agent

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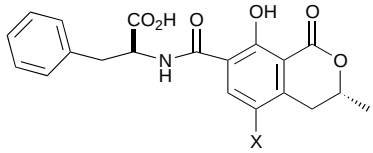
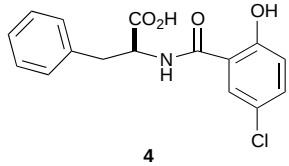
The ability of ochratoxin A to photoinduce DNA cleavage is described; in the presence of DNA the photoreaction yields the non-chlorinated derivative, ochratoxin B, while a hydroquinone derivative is produced under anaerobic conditions.

Ochratoxin A (OTA, **1**: X = Cl) is a fungal toxin produced by a species of Aspergillus and Penicillium.[1,2] It contaminates a wide range of foodstuffs and is implicated in the disease Balkan endemic nephropathy in which patients suffer from urinary tract tumors.[3] OTA induces single-strand DNA cleavage[4] and DNA adduction *in vivo*;[5] properties that establish a basis for its genotoxicity. However, the mechanism of OTA-induced DNA damage is currently not known.



OTA, **1** X = Cl
OTB, **2** X = H
OTOH, **3** X = OH



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Despite this, it is known that the chlorine atom is essential[6] and antioxidants inhibit its genotoxic activities.[7] While activation of OTA appears to be oxidative,[3–7] we have found, using fluorescence spectroscopy (λ_{ext} = 380 nm, λ_{em} = 441 nm), that the toxin is particularly susceptible to light and decomposes over time unless a suitable filter is used to suppress light intensity. Since certain halogenated compounds have been shown to efficiently photocleave DNA,[8–10] we hypothesized that **1** may photoinduce DNA damage. Such activity was also expected to provide new insight into the toxin's DNA targeting activities as photoactivatable agents have proven useful as probes of DNA structure,[11] sequence,[12] and mechanism, both in terms of DNA strand-scission[13] and DNA alkylation.[14]

Here we describe our preliminary findings on the photonuclease activity of **1**. While *in vivo* activation of **1** may be mediated by cytochrome P450[15] with subsequent formation of hydroxyl radicals,[16,17] our results show that light can also activate **1** and the products from the photoactivation provide new insight into species that may participate in its *in vivo* DNA targeting activities.

The ability of **1** to facilitate DNA photocleavage was examined using supercoiled plasmid DNA and agarose gel electrophoresis. As shown in Fig. 1, no cleavage resulted in the absence of light (lane 2), highlighting that **1** alone does not induce DNA cleavage. However, in the presence of light, DNA cleavage by **1** occurred in a concentration dependent fashion (lanes 4–7). Table 1 shows the effect of various additives on the extent of photocleavage by **1**. In an ambiently oxygenated atmosphere, inhibition was provided by the oxygen radical scavengers, DMSO, *tert*-butyl alcohol and sodium azide. However, a marked increase in the extent of photocleavage occurred in an N_2-flushed atmosphere, precluding the requirement for activated oxygen species. Inhibition of photocleavage was also provided by copper(II) ions; a metal that binds **1** effectively and quenches its fluorescence spectrum.[18] That both Cu^{II} and O_2 inhibit DNA cleavage suggests that they quench the excited state of **1**.[19]

Additional insight into the mode of photocleavage by **1** was obtained from the finding that the non-chlorinated derivative, OTB (**2**: X = H), and the derivative **4**,† which lacks the dihydroisocoumarin (lactone) ring system of **1**, failed to photoinduce DNA strand-scission (Fig. 2). These findings are remarkably similar to the *in vivo* toxicity of **1** and indicate a requirement for both the chlorine atom[6] and the lactone.[20]

Product analysis from the photoreaction of **1** in aqueous buffered media also provided information regarding the mode of photocleavage by the toxin. In an ambiently oxygenated atmosphere, no products were identified in the photoreaction of **1** alone, even though the toxin was consumed as evidenced by

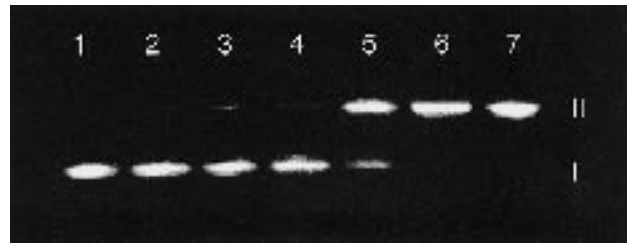


Fig. 1 Cleavage of supercoiled plasmid DNA by **1**. Reaction mixtures (20 μl total volume) contained 400 ng of plasmid DNA in 10 mM MOPS buffer, pH 7.4, and were irradiated on ice through a Pyrex filter with an ILC Technology 300 W Xenon arc lamp. After 5 min, the reaction mixtures were analyzed on a 1.2% agarose gel. Lane 1: DNA alone. Lane 2: DNA + 1000 μM **1**. Lane 3: irradiated DNA. Lane 4–7: irradiated DNA + 40, 200, 400 and 1000 μM **1**, respectively.

Table 1 Effect of additives on the extent of photocleavage of supercoiled DNA (Form 1) by **1**

Conditions[a]	Form I[b] (%)	Form II[c] (%)	Form III[d] (%)
Control	89	11	0
+**1**	27	73	0
+100 mM NaN_3	78	22	0
+1 μl DMSO	66	34	0
+1 μl Bu^tOH	62	38	0
+ SOD[e]	27	73	0
+ catalase[f]	27	73	0
+10 mM $Cu(OAc)_2$	74	26	0
Anaerobic[g]	0	61	39

[a] Reactions were run at pH 7.4 (10 mM MOPS buffer) using 200 μM **1** and 400 ng of supercoiled plasmid DNA in 20 μl total volume, and were irradiated in ice through a Pyrex filter with an ILC Technology 300 W Xenon arc lamp for 5 min. Densitometric quantitation of the gels was performed using a Microtek Scanmaker E_6 equipped with PhotoImpact and UTHSCSA Image Tool software. [b] Supercoiled DNA. [c] Nicked circular DNA. [d] Linear DNA. [e] 1000 units ml^{-1} super oxide dismutase. [f] 1000 units ml^{-1} catalase. [g] Reaction with **1** was purged with N_2 prior to photocleavage.

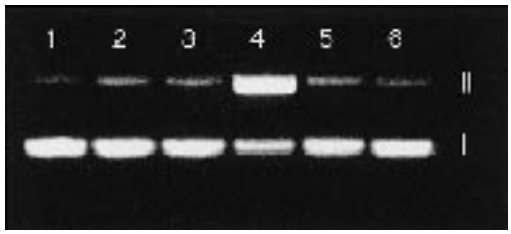
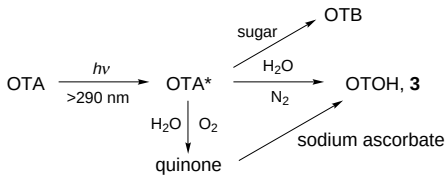


Fig. 2 Structure–activity relationships in DNA cleavage of supercoiled plasmid DNA by **1**. Reactions were carried out for 5 min as described in the caption below Fig. 1. Lane 1: DNA alone. Lane 2: DNA + 200 mM **1**. Lane 3: irradiated DNA. Lane 4: irradiated DNA + 200 μM **1**. Lane 5: irradiated DNA + 200 μM **2**. Lane 6: irradiated DNA + 200 μM 4.



Scheme 1 Summary of the photoreaction of **1**. Excitation by light (> 290 nm) to produce **1*** is accompanied by production of **2** in the presence of a sugar. Under anaerobic conditions (N_2, H_2O) substitution of chlorine by H_2O yields the hydroquinone **3**, which is also produced under reducing conditions in the presence of O_2.

HPLC. However, in the presence of calf thymus DNA or dextrose, photoirradiation of **1** yielded the non-chlorinated derivative **2**.‡ Under anaerobic conditions in the absence of a sugar, photoreaction of **1** produced the hydroquinone derivative, OTOH (**3**: X = OH),§ a finding that we attribute to $S_{RN}1$ displacement[21] of the chlorine atom by H_2O. Interestingly, **3** was also detected in the presence of O_2 when a reducing agent (sodium ascorbate) was added to the photoreaction. This observation suggests that the hydroquinone **3** may have originated from a reactive quinone precursor, in analogy to photooxidation of halogenated phenols that yield benzoquinone derivatives in the presence of O_2.[22,23] The results of these studies are summarized in Scheme 1.

In conclusion, the fungal carcinogen **1** does not facilitate DNA cleavage alone. However, we have demonstrated that light is one way to activate **1** to induce DNA damage. The finding that **2** is a product of **1** photoirradiation in the presence of DNA, suggests that strand-scission is mediated by H-atom abstraction from deoxyribose sugars through initial C–Cl bond homolysis.[8–10] In the absence of a sugar and O_2, **1** is converted in high yield to the hydroquinone derivative, **3**. This species is also formed in the presence of O_2, provided that a suitable reducing agent is added to the photoreaction. This result suggests strongly that photooxidation of **1** produces a reactive quinone derivative, a species that may be responsible for the toxin's DNA adduction properties.[5] Presently, experiments are in progress to identify the cleavage sites and the mechanistic pathways for photocleavage. We are also studying the oxidation of **1** to determine the exact nature of oxidized **1**, its chemical reactivity and lifetime in aqueous buffered media and the potential of such a species to induce DNA adduction.

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Notes and References

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† The derivative **4** was prepared in 70% yield from 5-chloro-2-hydroxybenzoic acid and L-β-phenylalanine using a DCC coupling procedure; mp 172–173 °C, δ_H[$(CD_3)_2CO$, 200 MHz] 12.3 (br, 1 H), 8.62 (d, 1 H, *J* 7.9), 7.92 (m, 1 H), 7.54–7.10 (m, 6 H), 6.94 (d, 1 H, *J* 8.9), 5.01 (m, 1 H), 3.4–3.1 (m, 2 H); δ_C[$(CD_3)_2CO$] 173.0, 169.2, 160.6, 138.3, 134.6, 130.0, 129.2, 127.7, 127.5, 120.3, 116.7, 55.0, 37.7. (Calc. for $C_{16}H_{14}ClNO_4$; C, 60.10; H, 4.41; N, 4.38. Found C, 60.02; H, 4.47; N, 4.39%.)

‡ After 5 min irradiation time, the yield of **2** from the reaction of 100 μM **1** with 100 mM dextrose was *ca.* 20% based on HPLC analysis. The isolated sample was identical to authentic **2** purchased from Sigma.

§ The hydroquinone derivative **3** was obtained in 80% from the photoreaction of (100 μM) in N_2-flushed phosphate buffer (0.1 M, pH 7.4). δ_H[$^2H_6$]DMSO) 13.08 (s, 1 H), 12.04 (s, 1 H), 9.88 (s, 1 H), 8.95 (d, 1 H, *J* 6.0), 7.72 (s, 1 H), 7.29 (m, 5 H), 4.29 (m, 2 H), 3.12 (m, 3 H), 2.66 (dd, 1 H, *J* 11.9, 11.6), 1.43 (d, 3 H, *J* 6.2). δ_C([$^2H_6$]DMSO) 172.5, 169.9, 163.1, 152.2, 145.8, 136.9, 130.2, 129.3, 128.4, 126.7, 123.4, 118.3, 109.4, 76.2, 53.9, 36.7, 28.1, 20.3. These peaks are identical to a separately prepared sample starting from 4-methoxyphenol. Full details will be published elsewhere.

1 K. J. van der Merwe, P. S. Steyn, L. Fourie, D. B. Scott and J. J. Theron, *Nature*, 1965, **205**, 1112.
2 W. van Walbeek, P. M. Scott, F. S. Thatcher, *Can. J. Microbiol.*, 1968, **14**, 131.
3 R. R. Marquardt and A. A. Frohlich, *J. Anim. Sci.*, 1992, **70**, 3968.
4 J. C. Seegers, L. H. Bohmer, M. C. Kruger, M. L. Lottering and K. M. de, *Toxicol. Appl. Pharmacol.*, 1994, **129**, 1.
5 Y. Grosse, I. Baudrimont, M. Castegnaro, A.-M. Betbeder, E. E. Creppy, G. Dirheimer and L. A. Pfohl, *Chem.-Biol. Interact.*, 1995, **95**, 175.
6 C. Malaveille, G. Brun and H. Bartsch, *Mutation Res.*, 1994, **307**, 141.
7 I. Baudrimont, A.-M. Betbeder, A. Gharbi, L. A. Pfohl, G. Dirheimer and E. E. Creppy, *Toxicology*, 1994, **89**, 101.
8 T. Matsumoto, Y. Utsumi, Y. Sakai, K. Toyooka and M. Shibuya, *Heterocycles*, 1992, **34**, 1697.
9 J. C. Quada, Jr., M. J. Levy and S. M. Hecht, *J. Am. Chem. Soc.*, 1993, **115**, 12 171.
10 Saito, T. Sakurai, T. Kurimoto and M. Takayama, *Tetrahedron Lett.*, 1994, **35**, 4797.
11 J. K. Barton, *Science*, 1986, **233**, 727.
12 P. E. Nielsen, C. Hiort, S. H. Sönnichsen, O. Buchardt, O. Dahl and B. Norden, *J. Am. Chem. Soc.*, 1992, **114**, 4967.
13 D. Ly, Y. Kan, B. Armitage and G. B. Schuster, *J. Am. Chem. Soc.*, 1996, **118**, 8747.
14 G. Büchi, K. W. Fowler and A. M. Nadzan, *J. Am. Chem. Soc.*, 1982, **104**, 544.
15 R. J. Omar, H. V. Gelboin and A. D. Rahimtula, *Biochem. Pharmacol.*, 1996, **51**, 207.
16 B. B. Hasinoff, A. D. Rahimtula and R. F. Omar, *Biochim. Biophys. Acta*, 1990, **1036**, 78.
17 D. Hoehler, R. R. Marquardt, A. R. McIntosh and H. Xiao, *J. Biol. Chem.*, 1996, **271**, 27 388.
18 J. A. Ardus, I. G. Gillman and R. A. Manderville, *Can. J. Chem.*, in the press.
19 N. J. Turro, *Modern Molecular Photochemistry*, University Science Books, Sausalito, CA, 1991, pp. 589–591.
20 D. Hoehler, R. R. Marquardt, A. R. McIntosh and G. M. Hatch, *Biochim. Biophys. Acta*, 1997, **1357**, 225.
21 J. F. Bunnett, *Acc. Chem. Res.*, 1978, **11**, 413.
22 K. David-Oudjehani and P. Boule, *New J. Chem.*, 1995, **19**, 199.
23 C. Richard and P. Boule, *New J. Chem.*, 1994, **18**, 547.

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