On the role of copper and iron in DNA cleavage by ochratoxin A. Structure–activity relationships in metal binding and copper-mediated DNA cleavage

Jason A. Ardus, Ivan G. Gillman, and Richard A. Manderville

Abstract: Ochratoxin A (OTA, **1**: X = Cl) is a fungal carcinogen that facilitates single-strand DNA cleavage and DNA adduction when metabolically activated. To determine if redox-active transition metals induce OTA-mediated DNA damage, we have examined the toxin's ability to bind Cu(II) and Fe(III) in aqueous media and facilitate DNA cleavage in their presence using agarose gel electrophoresis and supercoiled plasmid DNA. Using fluorescence spectroscopy, **1** was found to bind Cu(II) readily at physiological pH, while acidic conditions (pH 2.6) were employed to study Fe(III) binding due to the formation of Fe-oxide precipitates at higher pH values. Structure–activity relationships employing synthetic derivatives of **1** implied that **1** binds both Cu(II) and Fe(III) by its phenolic oxygen, while the carboxylic acid of its phenylalanine moiety binds Cu(II), but does not appear to play a role in Fe(III) coordination at pH 2.6. In terms of metal-mediated DNA cleavage, no role for **1** could be detected in Fe-induced DNA strand scission. With Cu(II), DNA cleavage by the 1:1 copper-bound complex of **1** could only be initiated by addition of a suitable reducing agent (sodium ascorbate). However, **1** was found to facilitate DNA cleavage by the Cu(II) complex of 1,10-phenanthroline ($Cu(OP)_2$); a prototypical Cu-mediated nuclease system that cleaves DNA upon activation by an external reducing agent. Structure–activity relationships employing analogs lacking the chlorine atom, ochratoxin B (**2**: X = H), and the lactone (**12**), indicated that the chlorine atom is essential for activity of the OTA in potentiating DNA cleavage by $Cu(OP)_2$. The implications of our findings to the genotoxic properties of **1** are discussed.

Key words: ochratoxin, DNA cleavage, copper, iron, 1,10-phenanthroline.

Résumé : L'ochratoxine A (OTA, **1**; X = Cl) est un cancérigène de champignon qui, lorsqu'il est métaboliquement activé, facilite le clivage de l'ADN monobrin et l'adduction de l'ADN. Dans le but de déterminer si les métaux de transition actifs en oxydoréduction induisent des dommages à l'ADN sous l'influence de l'OTA, on a utilisé l'électrophorèse sur gel d'agarose et un plasmide d'ADN surtorsadé pour examiner l'habilité de la toxine à se lier au Cu(II) et au Fe(III) en milieu aqueux et à faciliter le clivage de l'ADN en leur présence. En utilisant la spectroscopie de fluorescence, on a trouvé que **1** se lie facilement au Cu(II) à pH physiologique alors que, à cause de la formation de précipités d'oxyde de Fe à des valeurs de pH plus élevées, des conditions acides sont nécessaires pour étudier la liaison avec le Fe(III). Des relations structure–activité développées à l'aide de dérivés synthétiques de **1** impliquent que **1** se lie au Cu(II) ainsi qu'au Fe(III) par son oxygène phénolique; par ailleurs, l'acide carboxylique de sa partie phénylalanine se lie au Cu(II), mais ne semble pas jouer de rôle dans la coordination du Fe(III) à un pH de 2,6. En termes de clivage de l'ADN sous l'influence des métaux, on n'a détecté aucun rôle pour **1** dans la scission de l'ADN catalysé par le Fe(III). Avec le Cu(II), le clivage de l'ADN par le complexe 1:1 de cuivre lié à **1** ne peut être initié que par l'addition d'un agent réducteur approprié (ascorbate de sodium). Toutefois, on a trouvé que **1** facilite le clivage de l'ADN par le complexe du Cu(II) avec la 1,10-phénanthroline, ($Cu(OP)_2$), un prototype de système de nucléase catalysée par le Cu qui clive l'ADN lors d'une activation par un agent réducteur externe. Les relations structure–activité utilisant des analogues ne comportant pas d'atome de chlore, ochratoxine B (**2**, X = H) et la lactone (**12**), indiquent que l'atome de chlore est essentiel pour l'activité de l'OTA pour potentialiser le clivage de l'ADN par le $Cu(OP)_2$. On discute des implications de nos observations en relation avec les propriétés génotoxiques de **1**.

Mots clés : ochratoxine, clivage d'ADN, cuivre, fer, 1,10-phénanthroline.

[Traduit par la rédaction]

This paper is dedicated to Professor Erwin Buncel, my mentor, who was the first to stimulate my interest in physical organic chemistry.

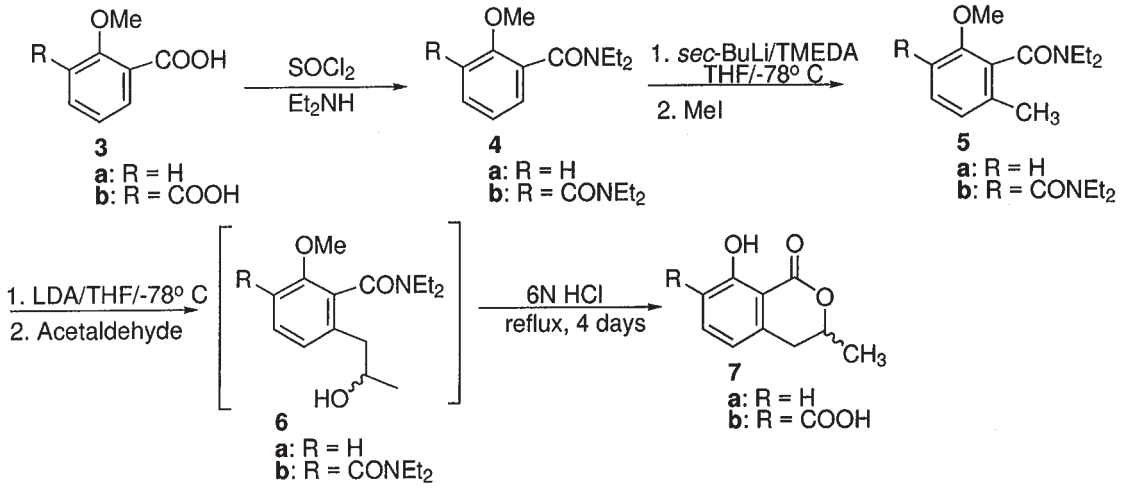
Received November 17, 1997.

J.A. Ardus, I.G. Gillman, and R.A. Manderville.[1] Department of Chemistry, Wake Forest University, Winston-Salem, NC 27109–7486, U.S.A.

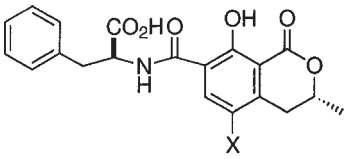
[1] Author to whom correspondence may be addressed. Telephone: (336)-758–5513. Fax: (336)-758–4698. E-mail: manderra@wfu.edu

© 1998 NRC Canada

Scheme 1. Synthetic route used for the preparation of the dihydroisocoumarine.



Ochratoxin A (OTA, **1**: X = Cl) is a fungal carcinogen produced by species of *Aspergillus* and *Penicillium* (1). Its occurrence in feed is widespread (2) and ingestion has been linked to the disease Balkan endemic nephropathy in which patients suffer from urinary tract tumors (3). The toxin consists of a phenolic dihydroisocoumarin, amide linked to L-phenylalanine, and has been shown to promote DNA adduction (4) and single-strand DNA cleavage (5); properties that establish a basis for its genotoxic action.



OTA, **1**: X = Cl
OTB, **2**: X = H

Although the mechanisms underlying the genotoxicity of OTA are uncertain, in vivo studies have established that the chlorine atom (6) and lactone (7) are essential and that activation may be mediated by cytochrome P-450 (8) with subsequent formation of activated oxygen species, such as superoxide (9) and (or) hydroxyl radicals (10, 11). The ability of **1** to bind Fe(III) has also been implicated as a possible source of reactive oxygen species that promote lipid peroxidation (12), and may facilitate DNA cleavage (10). Reports by Hecht et al. (13) and Li and Trush (14) that certain phenols and hydroquinones damage DNA by a copper-redox mechanism implies that Cu(II) ions may also play a role in OTA carcinogenicity.

Since OTA activation appears to be oxidative (3–11) and may involve participation of redox-active transition metals (9–14), we examined the toxin's ability to bind Cu(II) and Fe(III) in aqueous solutions and facilitate DNA cleavage in their presence using supercoiled plasmid DNA and agarose gel electrophoresis.

In the present article, we report that, although no role for **1** in Fe-mediated DNA cleavage could be detected, the toxin was found to bind Cu(II) to form a stable 1:1 complex that initiates DNA strand scission upon activation by a suitable reducing agent (sodium ascorbate). However, more importantly to the possible mechanism of OTA genotoxicity, the toxin was found to facilitate DNA cleavage by the Cu(II) complex of 1,10-phenanthroline ($Cu(OP)_2$), a well-known footprinting reagent (15) that requires an external reducing agent to convert Cu(II) → Cu(I). Structure–activity relationships employing synthetic derivatives of **1** that lack the chlorine atom, OTB (**2**, X = H), and the lactone functionality indicated that these components (especially the chlorine atom) facilitate the ability of **1** to potentiate DNA cleavage by $Cu(OP)_2$. This contrasting behavior for **1** in Cu-mediated DNA cleavage is discussed through consideration of the toxin's chelating versus redox properties.

Results

Synthesis of structural analogs

To derive structure–activity relationships for metal binding and DNA cleavage by ochratoxin A (OTA, **1**: X = Cl), we prepared analogs that lack the following: the chlorine atom, OTB, **2**: X = H; the dihydroisocoumarine (lactone), (**12**); and the phenylalanine moiety, (±)-mellein (**7a**) (16). Derivatives of **2** containing an altered phenylalanine side-chain, i.e., the methyl ester (**9a**) and the amide (**9b**), were also prepared.

Compounds containing the lactone ring system of **1** were synthesized according to methods outlined in Scheme 1. Using procedures described by Snieckus and co-workers (17), *o*-anisic acid (2-methoxybenzoic acid, **3a**) was converted into (±)-mellein (**7a**) (16), while 2-methoxyisophthalic acid (**3b**) yielded the carboxylic acid derivative **7b** (17).

To attach the L-β-phenylalanine side-chain moiety, the phenolic groups were initially protected as the acetate and then reacted with the requisite phenylalanine using a dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBT) peptide coupling procedure (18), as outlined in Scheme 2. The coupled products, **9a**, **9b**, and **11**, were isolated as the free phenols as the acetate protecting groups were lost in the work-up. Hydrolysis of the methyl ester derivatives (**9a** and **11**) into the corresponding free acids (**2** and **12**) was carried out under basic conditions. These strategies provided mixtures of stereoisomers for **2**, **9a**, and **9b** that were not separated.

© 1998 NRC Canada

Scheme 2. Synthetic route used for attachment of the phenylalanine moiety.

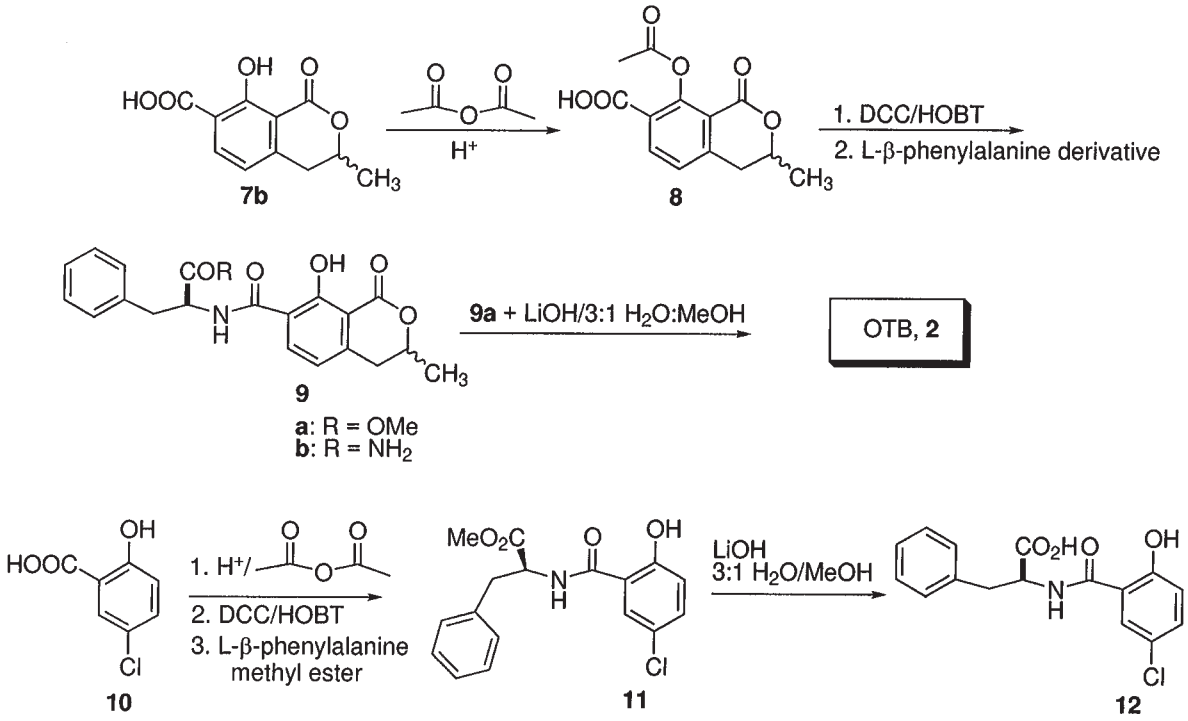
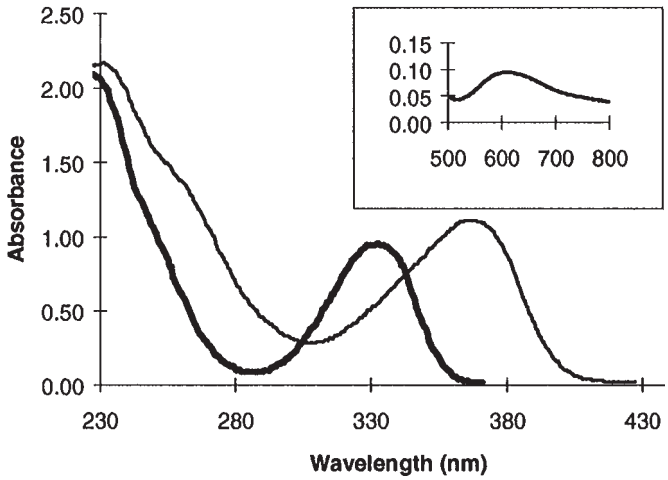


Fig. 1. The UV–absorption spectrum of **1** in ethanol (0.175 mM) in the absence (bold trace) and presence of 1 equiv. of $Cu(OAc)_2$. Inset: spectrum of Cu(II)·OTA complex at 1.75 mM highlighting the absorbance at 608 nm.



Copper and iron binding affinity

In ethanol, **1** exhibits an absorption spectrum with λ_{max} at 215 (ε = 28 000 M^{-1} cm^{-1}) and 333 nm (ε = 5500 M^{-1} cm^{-1}) (19). Addition of 1 equiv. of $Cu(OAc)_2$ yielded a pale green solution with λ_{max} at 222 (ε = 20 911 M^{-1} cm^{-1}), 258 (sh), 367 (ε = 6420), and 608 nm (ε = 55) (Fig. 1). Figure 2 shows the electrospray mass spectrum of the 1:1 mixture (in methanol) using negative ionization (ES^-). The copper complex was found to have $[M - H]^-$ = 463, which corresponds to a complex with molecular formula $CuC_{20}H_{16}NO_6Cl$ and suggests that complexation between **1** (*m*/*z* 403) and Cu(II) (*m*/*z* 63) leads to the loss of two protons. This observation strongly implies that the ligands bearing the two acidic groups of **1** (COOH and phenolic oxygen) participate in copper coordination. The OTA also forms a 1:1 complex with $FeCl_3$ that is pink in alcoholic solution (10). Generation of the complex in methanol followed by analysis by ES^- showed the presence of a single iron complex with $[M - H]^-$ = 563. This mass corresponds to a complex with molecular formula $FeC_{20}H_{18}NO_6Cl_4$ and indicates that $FeCl_3$ (*m*/*z* = 161) binding in methanol does not lead to deprotonation of **1**. Table 1 contains UV–absorption data in ethanol for the protonated and deprotonated forms of **1**, **2**, and **12**, along with their corresponding 1:1 Cu(II) and Fe(III) complexes.

The ability of **1** to bind Cu(II) and Fe(III) was determined in aqueous buffer at 25°C using fluorescence spectroscopy. Addition of Cu(II) and Fe(III) quenched the fluorescence of the toxin (10), which permitted determination of apparent equilibrium binding constants (log *K**). That the observed fluorescence quenching was due to metal binding was confirmed by performing the metal titration in the presence of excess EDTA. This metal chelator inhibited the fluorescence quenching if added prior to addition of the metal, and restored the fluorescence of OTA if added after the titration.

Figure 3*a* shows fluorescence quenching of the emission spectrum (λ_{em} = 441 nm) of **1** by $Cu(OAc)_2$ in 4-morpholineethanesulfonic acid (MES) buffer (pH 6.0), while the accompanying Scatchard analysis (20) is shown in Fig. 3*b*. From the negative slope in Fig. 3*b*, an apparent equilibrium binding constant of log *K** = 5.35 M^{-1} for formation of the 1:1 Cu(II)·OTA complex was determined. For Fe(III) binding by **1** (log *K** = 3.34 M^{-1}), aqueous HCl·KCl buffer (pH 2.6) was utilized due to the formation of Fe-oxide precipitates at higher pH values.

© 1998 NRC Canada

Fig. 2. Electrospray mass spectrum of the Cu(II)·OTA complex in methanol (m/z = 462.9). Also present is free **1** (m/z = 402.1).

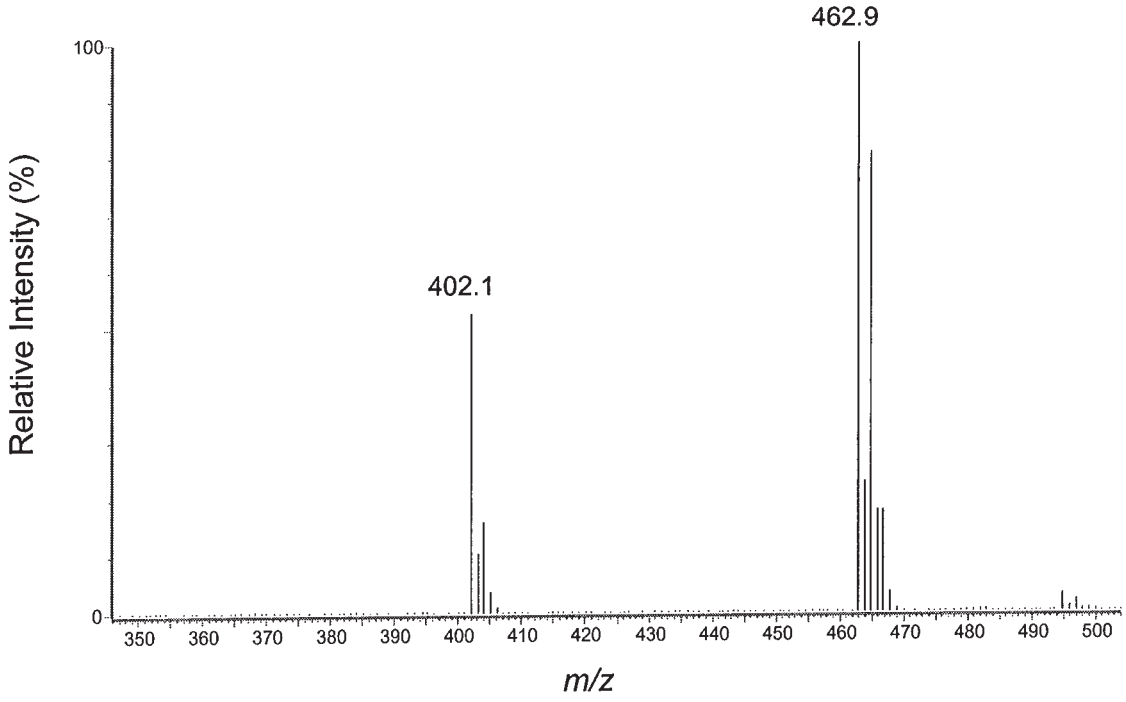


Table 1. The UV–absorption data in ethanol for ochratoxin A (OTA, **1**), OTB (**2**), and **12** in the absence (Free) and presence of base, and with 1 equiv. of Fe(III) and Cu(II).[a]

Compound	Free	+ Base[b]	+ Fe(III)[c]	+ Cu(II)[d]
OTA (**1**, X = Cl)	215 (28 000)	222 (20 300)	224, 342, 483	222 (21 000), 258 (sh),
	333 (5500)	383 (11 000)		367 (6420), 608 (55)
OTB (**2**, X = H)	218 (32 000)	230 (10 400)	241, 340, 508	235 (11 050), 331 (sh)
	320 (6900)	371 (10 200)		356 (6050), 620 (60)
12	214 (17 500)	222 (19 900)	215, 311	250 (19 600)
	312 (3600)	342 (5050)		322 (3700), 584 (130)

[a] Absorbance data are given in nm; extinction coefficients are in M^{-1} cm^{-1}.
[b] +2 equiv. of NaOH.
[c] +1 equiv. of $FeCl_3$.
[d] +1 equiv. of $Cu(OAc)_2$.

Apparent equilibrium binding constants (log K^*) for formation of 1:1 Cu(II) and Fe(III) complexes of **1** and our synthesized derivatives are listed in Table 2. Values in parentheses for Cu(II) binding represent the copper binding affinity of the anionic forms of the various derivatives (log $K_{1:1}$). For comparison to our synthesized sample of **2**, metal binding by the natural stereoisomer of **2** (purchased from Sigma) was determined, and as anticipated, the binding constants of the purchased **2** and our synthesized sample were nearly identical. The binding affinity of **1** for Zn(II) in 4-morpholinepropanesulfonic acid (MOPS) buffer (pH 7.4) was also measured to provide insight into Cu(I) binding, as both Zn(II) and Cu(I) are diamagnetic d^{10}-electron metals that favor a tetrahedral binding arrangement (21). Also presented are pK_a values for **1**, **2**, **7a**, and **12**. Values for **1** (7, 22) and **2** (7, 23) are from the literature, while pK_as for **7a** and **12** were measured at 25°C in 0.1 M aqueous buffer solutions using the recommended spectrophotometric procedure (24).

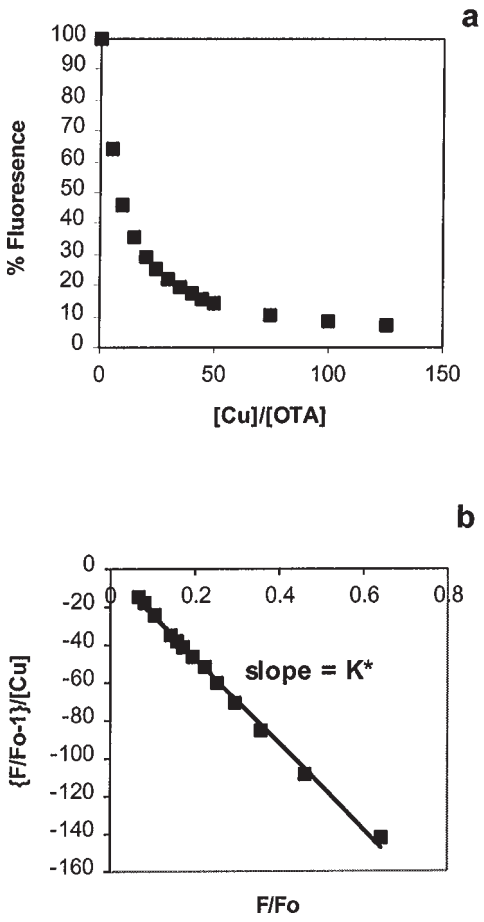
DNA cleavage

The ability of **1** to effect DNA cleavage in the presence of Fe and Cu was determined using supercoiled plasmid DNA and agarose gel electrophoresis. In our initial experiments, attempts were made to establish the toxin's ability to facilitate DNA cleavage in the presence of Fe. However, utilizing both Fe(III) and Fe(II) in the presence of excess **1** under various conditions (sodium ascorbate and HOOH as additives), no definitive role for **1** was determined, as control lanes in the gels for free Fe were equally efficient in promoting single-strand DNA cleavage (data not shown).

For DNA cleavage by Cu(II) ($Cu(OAc)_2$, 20 μM) admixture of **1** had very little effect on the extent of single-strand DNA cleavage (over a 15 h incubation period at 37°C in MOPS buffer, pH 7.4). Thus, the ability of the Cu(II)·OTA complex to facilitate DNA cleavage in the presence of sodium ascorbate was examined. For these experiments, 20 μM Cu(II) was premixed with 60 μM **1** prior to addition of plasmid DNA

© 1998 NRC Canada

Fig. 3. (*a*) Emission spectrum (λ_{ex} = 380 nm, λ_{em} = 440 nm) of fluorescence quenching of **1** (10 μM) at increasing $Cu(II)(OAc)_2$ concentrations in 10 mM MES (pH 6.0) and 100 mM $NaClO_4$ buffer solution at 25°C, and (*b*) Scatchard plot of the fluorescence quenching of part *a*.



and sodium ascorbate. Under these conditions, approximately 99% of Cu(II) is bound by the toxin, determined from log *K** = 6.26 M^{-1} for formation of the 1:1 Cu(II)·OTA complex at pH 7.4. Figure 4 shows a representative gel highlighting that the Cu(II)·OTA complex is capable of redox cycling and thus inducing DNA cleavage in the presence of sodium ascorbate. Additional experiments showed the cleavage to be completely inhibited by the enzyme catalase (1000 units/mL) and sodium azide (100 mM), while the hydroxyl radical scavengers, dimethyl sulfoxide (1 M) and *tert*-butanol (1 M), provided only partial protection (data not shown). These results indicate a requirement for HOOH and favor the involvement of a copper-oxo species over a freely diffusible hydroxyl radical (15).

To further explore Cu-mediated DNA cleavage by **1**, its ability to induce DNA cleavage by Cu(II) prebound to 2 equiv. of 1,10-phenanthroline (OP) was examined. The OP ligand binds Cu(II) to form a $Cu(OP)_2$ complex that is a prototypical copper nuclease system and facilitates DNA cleavage in the presence of an external reducing agent (15). As reported by Sigman (15), reduction of $Cu(II)(OP)_2$ to $Cu(I)(OP)_2$ followed by HOOH oxidation produces a copper-oxo species that cleaves DNA through H1′-atom abstraction of the deoxyribose sugar. The utility of $Cu(OP)_2$ here was to test if **1** could facilitate the production of $Cu(I)(OP)_2$, the species necessary for DNA cleavage (15). Figure 5 shows that DNA cleavage by $Cu(OP)_2$ was potentiated by OTA in a concentration dependent fashion. Additional experiments showed that the reaction was completely inhibited by the enzyme catalase, which mimicked the findings observed by Sigman and co-workers (15) for $Cu(OP)_2$ cleavage in the presence of thiol. However, unlike thiol-potentiation, the cleavage reaction was also partially inhibited by superoxide dismutase (SOD) (ca. 70%, data not shown), which suggests that the reduction of $Cu(II)(OP)_2$ is also mediated by O_2^- (15).

Since in vivo studies on OTA genotoxicity suggest a role for the chlorine atom and lactone (6, 7, 11), we also examined $Cu(OP)_2$-mediated DNA cleavage in the presence of **2** and **12** (Scheme 2). Figure 6 shows that under conditions where **1** effectively potentiates DNA cleavage by $Cu(OP)_2$ (lane 4), the derivative **12** (lane 5) was only slightly less efficient, while **2** (lane 6) failed to induce DNA cleavage above background (lane 2). To test if differences in the phenolic pK_as for these species (Table 2) could account for these results, the experiment was repeated in 2-(cyclohexylamino)-ethanesulfonic acid (CHES) buffer at pH 8.6. Interestingly, no change in the extent of cleavage was detected for all species (data not shown), indicating that the presence of the phenoxy anion does not explain the differences noted in the gel.

The ability of **1** to induce DNA cleavage in the presence of Fe(III) prebound to the metal chelator EDTA (25) was also examined. At 20 μM Fe(III)·EDTA, additions of **1** up to 250 μM, followed by 30 min incubation time at 37°C, failed to induce DNA cleavage (data not shown). This result was contrasted by our findings with $Cu(OP)_2$ and by the reaction of Fe(III)·EDTA (20 μM) with sodium ascorbate (50 μM), which almost facilitated the complete conversion of Form I into Form II DNA after the 30 min incubation time.

Discussion

One of the major goals in the field of toxicology is to elucidate the mechanisms by which xenobiotics produce detrimental biological responses in an organism (26). This knowledge can be used to identify chemical structures that predispose a molecule to a sequence of events that leads to toxicity. In the present study, we have evaluated the role played by Fe(III) and Cu(II) ions in DNA cleavage by the fungal carcinogen ochratoxin A (OTA, **1**). Since it had been shown that an Fe(III) complex of **1** produces hydroxyl radicals to an extent greater than free iron (10), it was anticipated that **1** would facilitate Fe-mediated DNA cleavage. That **1** possesses a *para*-chlorophenolic group also suggested that copper ions may promote OTA-induced DNA damage, based on analogy to Cu-mediated DNA cleavage by hydroquinones (13, 14).

Initial studies focused on the ability of the toxin and several structural analogs (Schemes 1 and 2) to bind Fe(III) and Cu(II) using fluorescence spectroscopy (Table 2). Both metals were found to quench the fluorescence of the toxin, which permitted determination of apparent equilibrium binding constants (log *K**) for formation of the 1:1 complexes. Of particular relevance to the biological properties of these compounds was the ability to monitor Cu(II) binding at physiological pH, while acidic conditions (pH 2.6) were employed for Fe(III) binding due to the formation of Fe-oxide precipitates at higher

© 1998 NRC Canada

Table 2. Equilibrium binding data for ochratoxin A (OTA, **1**) and structural analogs in aqueous buffered solutions at 25°C.

Compound	pK_a COOH/PhOH	Cu(II)[e] log K^* (log $K_{1:1}$)[f]	Fe(III)[i] log K^*	Zn(II)[j] log K^*
OTA (**1**, X = Cl)	4.2/7.0[a]	5.35 (6.40)	3.34	3.92
OTB (**2**, X = H)	4.2/7.8[b]	4.42 (6.24)	3.49	
9a (R = OMe)	—	3.73 (5.52)[g]	3.24	
9b (R = NH_2)	—	3.65 (5.45)	3.28	
12	7.5[c]	5.17 (6.69)[h]	3.71	
(±)-Mellein (**7a**)	10.4[d]	2.55 (6.95)	3.93	

[a] Taken from refs. 7 and 22.
[b] Taken from refs. 7 and 23.
[c] Determined by UV-vis spectroscopy in 0.1 M phosphate buffers.
[d] Determined by UV-vis spectroscopy in 0.1 M carbonate buffers containing 20 vol.% 1,4-dioxane.
[e] Determined by fluorescence spectroscopy in 10 mM MES buffer (pH 6.0) containing 100 mM $NaClO_4$.
[f] log $K_{1:1}$ = [Cu(II)·OTA]/[Cu(II)][OTA^{2-}]; determined from log K^* by accounting for the percent anionic forms at pH 6.0.
[g] Phenolic pK_a of **9a** and **9b** has been taken as 7.8 to estimate log $K_{1:1}$.
[h] pK_a of COOH has been taken as 4.2 to estimate log $K_{1:1}$.
[i] Determined by fluorescence spectroscopy in 10 mM KCl·HCl (pH 2.6), 100 mM $NaClO_4$.
[j] Determined by fluorescence spectroscopy in 10 mM MOPS buffer (pH 7.4), 100 mM $NaClO_4$.

Fig. 4. The DNA cleavage by Cu(II)·OTA in the presence of sodium ascorbate. Cleavage was carried out at 37°C for 30 min in 10 mM MOPS (pH 7.4), 50 mM NaCl buffer solution. Lane 1: DNA alone; lane 2: 20 μM $Cu(OAc)_2$; lane 3: 20 Cu(II)·OTA; lane 4–7: 20 μM Cu(II)·OTA + 10, 20, 50, and 250 μM sodium ascorbate, respectively.

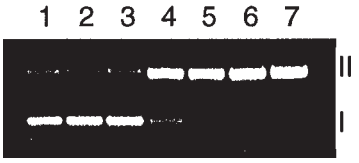
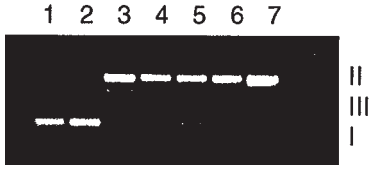


Fig. 5. The OTA–potentiation of Form I DNA cleavage by Cu(II)$(OP)_2$. Cleavage was carried out at 37°C for 30 min in 10 mM MOPS (pH 7.4), 50 mM NaCl buffer solution. Lane 1: DNA alone; lane 2: 20 μM Cu(II)$(OP)_2$; lane 3: 20 μM Cu(II)$(OP)_2$ + 10 μM sodium ascorbate; lane 4–7: 20 μM Cu(II)$(OP)_2$ + 50, 100, 150, and 250 μM **1**, respectively.



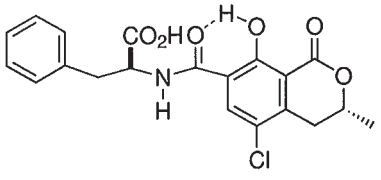
pH values. This finding indicates that **1** would be more efficient at binding Cu(II) than Fe(III) ions in biological systems.

Although we have not unequivocally determined the exact Cu(II) binding mode for **1**, that formation of the Cu(II)·OTA complex in methanol (Fig. 2) was accompanied by the loss of two protons suggests strongly that the two acidic groups of **1** (COOH and phenol) participate in Cu(II) coordination. Support for participation of the phenolic group stems from the fact that at pH 6, log $K^* = 5.35$ M^{-1} for **1**, while for **2** log $K^* = 4.42$ M^{-1} (Table 2). Clearly the binding affinity is sensitive to ionization of the phenolic group. Participation of the COOH group was supported by the finding that the OTB derivatives **9a** and **9b** (Scheme 2), which possess the methyl ester and amide

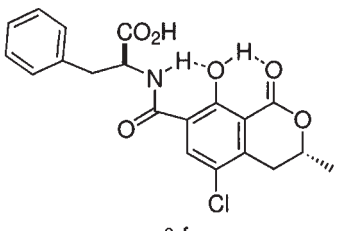
As demonstrated by Bredenkamp et al. (27), **1** adopts a β phenylalanine group respectively, showed diminished Cu(II) binding affinity when compared to the free acid **2**. The results presented in Table 2 also indicate that the dihydroisocoumarin moiety (lactone) of **1** is not important for formation of the 1:1 Cu·OTA complex. This hypothesis stems for the fact that the derivative **12**, which lacks the lactone moiety, has a greater affinity for Cu(II) than **1** (compare log $K_{1:1}$ values in Table 2).

For both the phenolic oxygen and the carboxylic acid of the phenylalanine moiety of **1** to participate in Cu(II) binding, it also appears that the amide nitrogen coordinates Cu(II). This hypothesis was derived from inspection of molecular models and from consideration of the conformation of the free toxin derived from single-crystal X-ray structural studies (27).

form (crystals obtained from benzene) conformation that



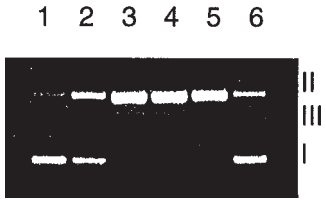
α form



β form

© 1998 NRC Canada

Fig. 6. Structure–activity relationships in OTA–potentiation of Form I DNA cleavage by $Cu(II)(OP)_2$. Cleavage was carried out at 37°C for 30 min in 10 mM MOPS (pH 7.4), 50 mM NaCl buffer solution. Lane 1: DNA alone; lane 2: 20 μM $Cu(II)(OP)_2$; lane 3: 20 μM $Cu(II)(OP)_2$ + 10 μM sodium ascorbate; lane 4: 20 μM $Cu(II)(OP)_2$ + 50 μM OTA; lane 5: 20 μM $Cu(II)(OP)_2$ + 50 μM **12**; lane 6: 20 μM $Cu(II)(OP)_2$ + 50 μM **2**.



maximizes hydrogen bonding interactions between the amide, phenolic group, and lactone carbonyl. The alternative α form was not detected. Although the fully protonated form of **1** is not important for Cu(II) coordination, the orientation of the phenylalanine moiety in the β form appears to facilitate coordination to Cu(II) by the phenolic oxygen and carboxylic acid of the phenylalanine. This conformation also predicts that the amide nitrogen participates in Cu(II) binding, which is consistent with previous studies on Cu(II) binding by the amide group of peptides (28), and Cu(II)·phenylalanine complexes (29).

For Fe(III) binding the data in Table 2 indicates that all derivatives possess similar binding affinity at pH 2.6. These results are consistent with the mass spectral data obtained in methanol, which indicated that $FeCl_3$ binding by **1** is not accompanied by proton removal from the toxin. This suggests that $FeCl_3$ forms a coordinate bond with the phenolic oxygen. Consistent with this hypothesis, **7a**, which lacks both the electron-withdrawing chlorine atom and amide group ($pK_a = 10.4$), possesses the greatest affinity for $FeCl_3$ at pH 2.6 (Table 2). In contrast to Cu(II) binding, the derivatives **9a** and **9b** do not show significant differences in binding from **2**. This result appears to indicate that the carboxylic acid does not participate in Fe(III) binding at pH 2.6 and may suggest that these compounds bind Fe(III) by the phenolic oxygen (7, 10) and an *ortho*-carbonyl, either from the lactone (10) or the amide group (7, 30).

To define the role played by Fe(III) and Cu(II) in OTA-induced DNA cleavage, we examined the ability of **1** to facilitate DNA strand-scission in the presence of free iron and copper ions. Utilizing both Fe(III) and Fe(II) ions, no definitive role for the toxin could be identified. This result was surprising, given reports that **1** coordinates Fe(III), facilitates lipid peroxidation (12), and produces the hydroxyl radical via the Fenton reaction (10). However, results by Marquardt and co-workers (11*b*) on production of the hydroxyl radical in a microsomal-NADPH preparation, showed conclusively that addition of **1** and Fe(III) produces the hydroxyl radical in a lower yield than NADPH and Fe(III) alone. Our in vitro results are in accord with this finding and reaffirm that coordination of **1** to Fe(III) is not responsible for OTA genotoxicity.

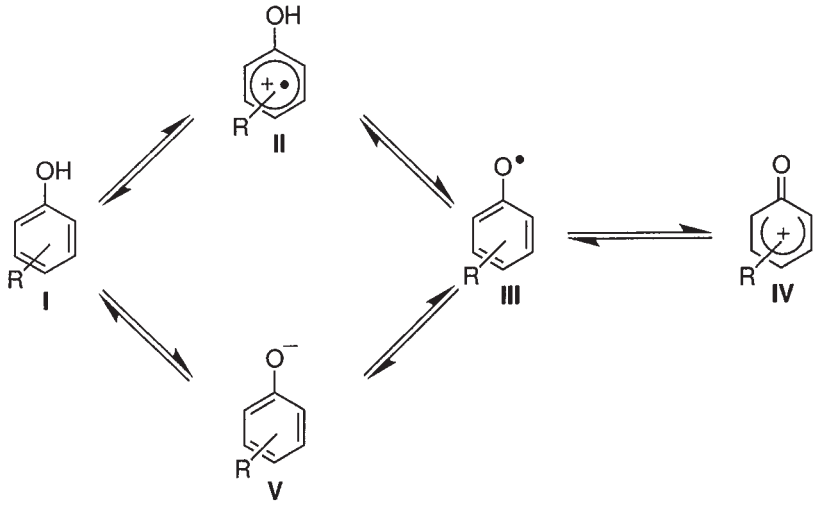
For Cu-mediated DNA cleavage, **1** was found to form a 1:1 Cu(II)·OTA complex that induced DNA strand-scission only upon activation by a suitable reducing agent (Fig. 4). This result is in sharp contrast to the reports by Hecht and co-workers (13) and Li and Trush (14) on the ability of hydroquinones to induce copper-mediated DNA cleavage. They found that 30 min incubation time at 37°C was more than sufficient to cleave the DNA through reduction of Cu(II) to Cu(I) with concomitant oxidation of the hydroquinone to the respective benzoquinone (13, 14). The inability of **1** to induce Cu-mediated DNA cleavage in the absence of a reducing agent, however, can be rationalized by consideration of the toxin's chelating properties. Unlike *ortho*- or *para*-hydroquinone, which are not expected to coordinate Cu(II) with high binding affinity, **1** binds Cu(II) very efficiently (Table 2). If binding affinity of the OTA for Zn(II) ($\log K = 3.92\ M^{-1}$ at pH 7.4) is representative of the toxin's affinity for Cu(I) (both are d^{10}-electron metals that favor a tetrahedral binding arrangement (21)) then the Cu(II) complex is significantly more stable than the putative Cu(I) complex ($\log (K_{Cu}/K_{Zn})$ at pH 7.4 = 6.26/3.92 = 1.6). This finding would explain the inability of **1** to facilitate Cu-mediated DNA damage in the absence of a reducing agent, and is consistent with the fact that Cu(II) is much less efficient in reduction when it is bound to protein ligands than when it is free in solution (31). Studies by Marquardt and co-workers (11*a*) on hydroxyl radical production by **1** in bacteria also show that additions of divalent cations such as Fe(II) and Cu(II) ions fail to enhance hydroxyl radical production, which appears consistent with the findings presented here on the inability of **1** to cleave DNA in the presence of these metal ions free in solution. Furthermore, Cu(II)·OTA mediated DNA cleavage in the presence of sodium ascorbate is probably irrelevant to OTA genotoxicity, since antioxidants inhibit the toxicity of **1** (9).

To explore the role played by Cu(II) ions in OTA-induced DNA cleavage in more detail, we also examined the ability of **1** to potentiate DNA cleavage by the Cu(II) complex of 1,10-phenanthroline ($Cu(OP)_2$). As described by Sigman (15), the nuclease activity of $Cu(OP)_2$ proceeds by an obligatory, ordered mechanism, whereby the cupric complex $Cu(II)(OP)_2$ is reduced to the cuprous complex, $Cu(I)(OP)_2$, which is then oxidized by HOOH to generate the copper-oxo species directly responsible for strand-scission. Since the reaction is independent of the reductant used to potentiate it (15), we used the $Cu(OP)_2$ nuclease system to gain insight into redox properties of the OTA in DNA cleavage. Interestingly, **1** was found to potentiate DNA cleavage by $Cu(OP)_2$ (Fig. 5), presumably by reducing $Cu(II)(OP)_2 \rightarrow Cu(I)(OP)_2$ with the concomitant oxidation of **1**. Although the exact nature of OTA oxidation is uncertain, it is informative that the nonchlorinated derivative **2** failed to induce DNA cleavage by $Cu(OP)_2$. The derivative **12**, which lacks the lactone of **1**, showed only slightly diminished activity (Fig. 6), which indicates that the chlorine atom is more important than the lactone for OTA potentiation of $Cu(OP)_2$-mediated DNA cleavage.

To provide a rationale for the ability of **1** to induce DNA cleavage by $Cu(OP)_2$, it is informative to consider oxidation of phenols, where the oxidation takes place according to the routes presented in Scheme 3 (32). Under aprotic conditions, oxidation of phenol (**I**) produces the cation radical (**II**), which deprotonates to the neutral radical (**III**). For solutions containing the phenolate ion (**V**), oxidation to the phenoxyl radical (**III**) proceeds readily and the resulting phenoxyl radical (**III**) may be further oxidized to the phenoxonium ion (**IV**), or couple to yield a variety of products (32).

© 1998 NRC Canada

Scheme 3. Oxidation pathway for common phenols.



Insight from Scheme 3 suggests that single electron transfer from **1** to $Cu(II)(OP)_2$ would yield the OTA phenoxyl radical ($OTA^{\cdot}$) and $Cu(I)(OP)_2$ (eq. [1*a*]). For $Cu(I)(OP)_2$ to initiate DNA strand-scission, it must combine with hydrogen peroxide (HOOH) to generate a copper-oxo species (15). The finding that SOD inhibits the reaction (70%) suggests that the reduction of $Cu(II)(OP)_2$ is also mediated by $O_2^{\cdot-}$. Although oxidation of $Cu(I)(OP)_2$ by O_2 can yield $O_2^{\cdot-}$ (15), another possible source of superoxide is the oxidation of $OTA^{\cdot}$ by O_2 to yield the phenoxonium ion of OTA (OTA^+), eq. [1*b*]. The liberated $O_2^{\cdot-}$ could assist OTA in the reduction of $Cu(II)(OP)_2$ (eq. [1*c*]) and yield HOOH after disproportionation (eq. [1*d*]). Oxidation of $Cu(I)(OP)_2$ by HOOH would yield a copper-oxo species (eq. [1*e*]), that initiates DNA cleavage (eq. [1*f*]).

[1*a*] $OTA + Cu(II)(OP)_2 \rightarrow OTA^{\cdot} + Cu(I)(OP)_2$

[1*b*] $OTA^{\cdot} + O_2 \rightarrow OTA^+ + O_2^{\cdot-}$

[1*c*] $Cu(II)(OP)_2 + O_2^{\cdot-} \rightarrow Cu(I)(OP)_2 + O_2$

[1*d*] $2O_2^{\cdot-} + 2H^+ \rightarrow HOOH + O_2$

[1*e*] $Cu(I)(OP)_2 + HOOH \rightarrow$ copper-oxo species

[1*f*] Copper-oxo species + DNA $\rightarrow$ DNA cleavage

While we have yet to identify the products resulting from OTA oxidation, it is informative that the intermediacy of the OTA phenoxyl radical in the in vivo genotoxicity of **1** has been proposed by Malaveille et al. (6). Oxidation to the putative phenoxonium ion (OTA^+) may also provide a rationale for the toxin's ability to facilitate DNA adduction (4). A DNA-derived nucleophile could react directly with OTA^+, or reaction of OTA^+ with H_2O to furnish a quinone intermediate after loss of HCl could precede DNA adduction. In this regard, chlorinated phenols have been converted to the respective *para*-quinones with $CuCl_2$ and O_2 (33) and by enzymatic oxidative pathways (34), and quinones are Michael acceptors that have been implicated in DNA adduct formation (35).

Conclusions

The present study implies that the genotoxic properties of **1** may be attributable to the toxin's redox properties, instead of its ability to bind redox-active transition metals. The toxin does not facilitate DNA cleavage in the presence of free Fe, or Fe(III) bound to EDTA. With Cu(II), **1** forms a stable 1:1 Cu(II)·OTA complex that facilitates DNA cleavage upon activation by sodium ascorbate. However, since OTA toxicity is inhibited by antioxidants, this pathway is unlikely to play a role in the mechanism(s) of OTA genotoxicity. A more significant finding was the demonstrated ability of **1** to initiate DNA cleavage by $Cu(II)(OP)_2$. Here, **1** provides the reducing equivalents to convert $Cu(II)(OP)_2 \rightarrow Cu(I)(OP)_2$. The chlorine atom was found to be critical for this activity, which is consistent with in vivo studies on OTA genotoxicity (6). Currently, we are studying oxidation of **1** by electrochemical and chemical means in an effort to determine the nature of the oxidized OTA species and the potential of such a species to induce DNA adduction.

Experimental section

Materials

Ochratoxin A (OTA, **1**: X = Cl) was purchased from Dr. Ronald Marquardt, Department of Animal Sciences, University of Manitoba, and was used without further purification. Supercoiled plasmid (Form I) DNA was a gift from Dr. Fred W. Perrino, Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University. The plasmid was a derivative of pOXO4 (36) containing the dnaQ gene. The following enzymes and reagents were obtained commercially and used without further purification: 1,10-phenanthroline (OP), copper acetate ($Cu(OAc)_2$), ferric chloride ($FeCl_3$), sodium perchlorate ($NaClO_4$), 4-morpholinepropanesulfonic acid (MOPS), 4-morpholineethanesulfonic acid (MES), 2-(cyclohexylamino)ethanesulfonic acid (CHES), ethylenediaminetetraacetic acid (EDTA), 1,3-dicyclohexylcarbo-diimide (DCC), 1-hydroxybenzotriazole hydrate (HOBT), L-phenylalanine methyl ester hydrochloride, 5-chlorosalicylic acid, *sec*-butyllithium (*sec*-BuLi), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA), methyl iodide (MeI), lithium diisopropyamide (LDA), acetaldehyde, and acetic anhydride

© 1998 NRC Canada

(Aldrich); ochratoxin B (**2**: X = H), catalase, superoxide dismutase (SOD), and L-β-phenylalanine amide (Sigma).

Methods

Elemental analyses were carried out by Atlantic Microlab, Inc. High-resolution mass spectral analyses were carried out by the Nebraska Center for Mass Spectrometry. Melting points were taken on a Mel-Temp apparatus and are not corrected. NMR spectra were recorded on a Varian VXR-200 spectrometer (^{1}H, 200 MHz; ^{13}C, 50 MHz). Chemical shifts are given in parts per million (ppm) relative to tetramethylsilane (TMS), and coupling constants (*J*) are reported in hertz (Hz). The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Absorption measurements were made on a Hitachi U-2001 UV-vis spectrophotometer, and fluorescence spectra were acquired with a Hitachi F-2000; both instruments were equipped with a thermostated cell compartment. Distilled, deionized water from a Milli-Q system was used for all aqueous solutions and manipulations. Other solvents were purified and dried according to standard procedures. Agarose gel electrophoresis was carried out in 40 mM Tris-acetate buffer (pH 8.0), containing 5 mM EDTA. Agarose gel loading buffer: 40 mM Tris-OAc (pH 8.0), 5 mM EDTA, 40% glycerol, 0.3% bromophenol blue.

Mass spectra were acquired using a Micromass Quattro II operating in the negative ion spray mode (ES^-). The system acquired signal over a *m*/*z* range of 200–1000 at 8 s/scan with a 0.06 step. Samples of **1** and Cu(II)·OTA (0.1mg/mL) were prepared in methanol and injected via syringe into a fixed loop injector port (100 μL volume) interfaced to the ion spray source. The Cu(II)·OTA sample was also prepared in 10 mM NH_4OAc (pH 7.0) buffer and subjected to reverse-phase high-pressure liquid chromatography (HPLC, Hewlett Packard (HP) 1100 series with photodiode array detection, 190–600 nm, using a gradient of MeOH in 2 mM ammonium acetate, pH 7.0), prior to analysis by ESI/MS. The sample of Fe(III)·OTA (0.1mg/mL) was prepared in methanol and was analyzed by ESI/MS without prior separation by HPLC.

N,N-Diethyl-2-methoxybenzamide (4a)

To prepare **4a** (37), 2-methoxybenzoic acid (1.00 g, 6.57 mmol) was reacted with excess thionyl chloride ($SOCl_2$) and diethylamine using standard conditions. After purification by silica-gel chromatography (1:1 EtOAc–hexane), **4a** was obtained as a colorless oil: yield 1.21 g (89%); ^{1}H NMR ($CDCl_3$), δ: 7.29 (m, 1H), 7.18 (m, 1H), 6.93 (m, 1H), 3.79 (s, 3H), 3.59–3.54 (m, 2H), 3.14 (q, 2H, *J* = 7.02), 1.24 (t, 3H, *J* = 7.02), 1.02 (t, 3H, *J* = 7.02); ^{13}C NMR ($CDCl_3$), δ: 168.3, 154.7, 129.5, 126.9, 126.5, 120.3, 110.5, 55.0, 42.4, 38.4, 13.6, 12.5.

N,N-Diethyl-2-methoxy-6-methylbenzamide (5a)

Using the procedure described by Snieckus and co-workers (17*a*), **5a** was obtained as a colorless oil: yield 0.191 g (80%); ^{1}H NMR ($CDCl_3$), δ: 7.13–6.66 (m, 3H), 3.78–3.68 (m, 1H), 3.72 (s, 3H), 3.39–3.29 (m, 1H), 3.05 (q, 2H, *J* = 7.19), 2.17 (s, 3H), 1.18 (t, 3H, *J* = 7.33), 0.95 (t, 3H, *J* = 7.19); ^{13}C NMR ($CDCl_3$), δ: 168.2, 155.1, 135.4, 128.9, 126.2, 122.3, 107.9, 55.3, 42.2, 38.3, 18.5, 13.6, 12.6.

8-Hydroxy-3-methyl-3,4-dihydroisocoumarin (Mellein) (7a)

To a stirred solution of **5a** (0.193 g, 0.873 mmol) in dry THF (75 mL) at –78°C under argon was added lithium diisopropylamide (LDA, 0.655 mL, 1.31 mmol, from 2.0 M solution in heptane–THF–ethylbenzene). After stirring for 1 h at –78°C, the resulting burgundy red mixture was quenched with acetaldehyde. The mixture was allowed to warm to room temperature, stirred for an additional 12 h, and then washed with 1 N HCl (50 mL) and extracted with EtOAc. The organic layer was dried over $MgSO_4$, filtered, and the solvent removed under reduced pressure. The resultant crude residue was then refluxed in 6 N aqueous 6 N HCl (50 mL) for 4 days. The mixture was filtered, washed with water, and dried under vacuum to afford **7a** as a light yellow solid. Recrystallization from acetone gave **7a** as a white solid: yield 0.080 g (52%); mp 38.0°C (lit. (17*b*) 38°C); ^{1}H NMR ($CDCl_3$), δ: 11.0 (s, 1H), 7.38 (m, 1H), 6.86 (m, 1H), 6.66 (m, 1H), 4.71 (m, 1H), 2.91 (m, 2H), 1.51 (d, 3H, *J* = 6.3 Hz); ^{13}C NMR ($CDCl_3$), δ: 162.9, 161.3, 142.1, 134.6, 119.3, 111.0, 74.2, 56.3, 36.2, 20.8.

N,N-Diethyl-2-methoxy-3-(diethylcarbamoyl)benzamide (4b)

The benzamide **4b** (17*c*) was prepared from 2-methoxyisophthalic acid (0.850 g, 4.33 mmol), excess $SOCl_2$ and diethylamine using standard conditions. After purification by silica-gel chromatography (elution with EtOAc), **4b** was obtained as a white solid: yield 1.13 g (85%); mp 82°C from Et_2O–hexane (lit. (17*c*) 82°C); ^{1}H NMR ($CDCl_3$), δ: 7.27 (m, 3H), 3.86 (s, 3H), 3.80–3.20 (br, 8H), 1.27 (t, 6H, *J* = 7.18 Hz), 1.06 (t, 6H, *J* = 7.14 Hz); ^{13}C NMR ($CDCl_3$), δ: 166.8, 150.6, 127.0, 42.0, 37.9, 12.8, 11.7.

N,N-Diethyl-2-methoxy-3-(diethylcarbamoyl)-6-methylbenzamide (5b)

To a stirred mixture of **4b** (0.304 g, 0.993 mmol) in dry THF (75 mL) at –78°C under argon, was added via syringe *sec*-BuLi (1.25 mL, 1.19 mmol, from 0.950 M in cyclohexanes) and TMEDA (0.138 g, 1.19 mmol). The resulting yellow solution was allowed to stir for 1 h at –78°C, and dry methyl iodide (0.423 g, 2.98 mmol) was added. The stirred mixture was allowed to warm to room temperature and stirred for an additional 12 h. The solution was then washed with 1 N HCl (100 mL) and extracted with EtOAc. The organic layer was separated, washed with three 20 mL portions of brine, then dried over $MgSO_4$. The solution was concentrated under reduced pressure to afford a crude product that was purified by silica-gel chromatography. Elution with ether (containing 1% triethylamine) afforded **5b** as a light yellow oil: yield 0.27 g (85%); ^{1}H NMR ($CDCl_3$), δ: 7.13 (d, 1H, *J* = 7.6), 7.00 (d, 1H, *J* = 7.6), 3.84 (s, 3H), 3.80–3.20 (br, 8H), 2.27 (s, 3H), 1.27 (m, 6H), 1.06 (t, 6H, *J* = 7.06 Hz); ^{13}C NMR ($CDCl_3$), δ: 167.9, 167.1, 151.3, 136.2, 127.2, 42.6, 42.3, 18.3, 13.5, 13.4, 12.3; mass spectrum (HREI), *m*/*z*: 320.209 ($C_{18}H_{28}N_2O_3$ requires 320.210).

3-Methyl-7-carboxy-8-hydroxy-2,3-dihydroisocoumarin (7b)

To a stirred solution of **5b** (1.20 g, 3.75 mmol) in dry THF (75 mL) at –78°C under argon was added LDA (2.25 mL, 4.50 mmol, from 2.0 M in heptane–THF–ethylbenzene). After

© 1998 NRC Canada

stirring for 1 h at –78°C, the resultant burgundy red solution was quenched with acetaldehyde. The mixture was allowed to warm to room temperature, stirred for an additional 12 h, washed with 1 N HCl (100 mL) and then extracted with EtOAc. The organic layer was separated, dried over $MgSO_4$, and then concentrated under reduced pressure. The crude residue was then refluxed in 6 N HCl for 4 days. The mixture was filtered, washed with water, and dried under vacuum, producing **7b** as a white solid: yield 0.528 g (63%); mp 233–235°C from acetone (lit. (17*c*) 234–236°C); ^{1}H NMR (DMSO-d_6), δ: 7.96 (d, 1H, J = 7.9), 6.88 (d, 1H, J = 7.9), 4.70 (br, 1H), 3.00 (m, 2H), 1.39 (d, 3H, J = 6.27); ^{13}C NMR (DMSO-d_6), δ: 168.9, 165.4, 161.9, 146.9, 136.6, 117.9, 115.7, 110.9, 74.8, 34.5, 20.2.

3-Methyl-7-carboxy-8-acetate-2,3-dihydroisocoumarin (8)
To a suspension of **7b** (0.200 g, 0.901 mmol) in acetic anhydride (10 mL) was added phosphoric acid (0.10 mL). The resulting solution was stirred for 24 h at 50°C. Concentration under reduced pressure, followed by purification by silica-gel chromatography (EtOAc) afforded **8** as a white solid: yield 0.233 g (98%); mp 136–137°C; ^{1}H NMR ($CDCl_3$), δ: 10.4 (br, 1H), 8.22 (d, 1H, J = 8.01), 7.24 (d, 2H, J = 8.01), 4.63 (m, 1H), 2.99 (m, 2H), 2.21 (s, 3H), 1.50 (d, 3H, J = 6.4); ^{13}C NMR ($CDCl_3$), δ: 169.3, 168.6, 161.0, 146.8, 136.7, 125.1, 123.3, 119.4, 77.2, 74.3, 35.8, 21.0, 20.6. Anal. calcd. for $C_{13}H_{12}O_6$: C 59.09; H 4.58; found: C 59.03; H 4.62.

Methyl ester of ochratoxin B (9a)
To a stirred solution of **8** (0.250 g, 0.87 mmol) in dry acetonitrile–DMF (10:3, 65 mL) at 0°C under argon was added DCC (0.217 g, 1.05 mmol) and HOBT (0.141 g, 1.05 mmol). The mixture was allowed to warm to room temperature and then stirred for an additional 1.5 h. L-Phenylalanine methyl ester (0.157 g, 0.87 mmol) in pyridine (2 mL) was then added in a dropwise fashion. After stirring for 12 h, the solvent was removed under reduced pressure. The resulting residue was redissolved in EtOAc (25 mL), washed with three 20 mL portions of brine, dried over $MgSO_4$, and concentrated under diminished pressure to yield a crude product. Purification by silica-gel column chromatography (elution with EtOAc–hexane, 80:20) afforded **9a** as a colorless residue: yield 0.276 g (83%); ^{1}H NMR ($CDCl_3$), δ: 12.7 (s, 1H), 8.53 (d, 1H, J = 6.4), 8.36 (d, 1H, J = 7.9), 7.27 (br, 5H), 6.84 (d, 1H, J = 7.9), 5.06 (m, 1H), 4.77 (m, 1H), 3.74(s, 3H), 3.24 (m, 2H), 2.99 (m, 2H), 1.56 (d, 3H, J = 6.4); ^{13}C NMR ($CDCl_3$), δ: 172.0, 170.3, 163.6, 160.5, 143.6, 139.0, 136.1, 129.3, 128.6, 127.1, 119.3, 118.4, 114.0, 108.7, 54.2, 52.3, 38.0, 34.6, 20.6; mass spectrum (HREI), *m*/*z*: 383.136 ($C_{21}H_{21}NO_6$ requires 383.137).

Ochratoxin B (2)
The hydrolysis of the methyl ester **9a** (0.383 g, 1.00 mmol) into the free acid, OTB (**2**), was achieved by reaction in a 3% LiOH solution in 3:1 H_2O–MeOH. Recrystallization from benzene afforded **2** as a white solid: yield 0.243 g (66%); mp 205–208°C (lit. (19) 220°C); ^{1}H NMR (DMSO-d_6), δ: 8.50 (d, 1H, J = 7.4), 8.06 (d, 1H, J = 7.9), 7.24 (m, 5H), 6.95 (d, 1H, J = 7.9), 4.81 (m, 2H), 3.16 (m, 2H), 1.41 (d, 3H, J = 6.1); ^{13}C NMR (DMSO-d_6), δ: 172.6, 169.1, 163.7, 159.6, 144.8, 137.0, 136.9, 129.3, 128.3, 126.7, 118.5, 118.3, 109.4, 76.1, 53.9, 36.7, 33.7, 20.2.

Amide of ochratoxin B (9b)
To a stirred solution of **8** (0.264 g, 0.379 mmol) in dry acetonitrile–DMF (8:1, 45 mL) at 0°C under argon was added DCC (0.094 g, 0.455 mmol) and *N*-hydroxysuccinimide (0.52 g, 0.455 mmol). The resulting mixture was allowed to warm to room temperature and then stirred for an additional 1.5 h under argon. L-Phenylalanineamide (0.062 g, 0.379 mmol) in pyridine (2 mL) was then added in a dropwise fashion. The mixture was stirred for an additional 12 h, then concentrated under reduced pressure to leave a residue that was resuspended in EtOAc (25 mL). The resulting solution was washed with three 20 mL portions of brine, dried over $MgSO_4$, then concentrated under diminished pressure to afford a crude product that was purified by silica-gel chromatography. Elution with 80/20 EtOAc–hexanes produced **9b** as a white residue: yield 0.027 g (20%); ^{1}H NMR ($CDCl_3$), δ: 12.4 (br, 1H), 8.53 (d, 1H, J = 7.1), 8.29 (d, 1H, J = 8.1), 7.24 (m, 5H), 6.82 (d, 1H, J = 8.1), 6.32 (s, 1H), 5.82 (s, 1H), 4.89 (m, 1H), 4.78 (m, 1H), 3.20 (m, 2H), 2.96 (m, 2H), 1.55 (d, 3H, J = 6.2); ^{13}C NMR ($CDCl_3$), δ: 173.3, 170.2, 164.0, 160.3, 143.7, 138.7, 136.6, 129.3, 128.6, 126.9, 118.9, 118.5, 108.7, 54.9, 37.8, 34.5, 20.6; mass spectrum (HREI), *m*/*z*: 368.13734 ($C_{20}H_{20}N_2O_5$ requires 368.1372).

5-Chloro-2-hydroxy-*N*-L-phenylalaninebenzamide methyl ester (11)
To a stirred solution of the acetate of 5-chloro-2-hydroxybenzoic acid (1.00 g, 4.66 mmol) in dry acetonitrile–DMF (5:1, 55 mL) at 0°C under argon was added DCC (1.15 g, 5.59 mmol) and HOBT (0.755 g, 5.59 mmol). The mixture was allowed to warm to room temperature, stirred for an additional 1.5 h, and then L-phenylalanine methyl ester (1.55 g, 4.66 mmol) in dry pyridine (3 mL) was added in a dropwise fashion. The resulting mixture was stirred for an additional 12 h and then concentrated under diminished pressure. The resulting residue was resuspended in EtOAc (25 mL), filtered, and then washed with three 20 mL portions of brine. The organic layer was collected, dried over $MgSO_4$, and concentrated under reduced pressure to afford a crude product that was purified by silica-gel chromatography. Elution with 40/60 EtOAc–hexanes afforded **11** as a white solid: yield 0.9 g (60%); mp 83–85°C. ^{1}H NMR ($CDCl_3$), δ: 11.9 (s, 1H), 7.40–6.60 (m, 9 H), 5.07 (m, 1H), 3.80 (s, 3H), 3.26 (m, 2H); ^{13}C NMR ($CDCl_3$), δ: 171.7, 168.4, 160.1, 135.3, 134.5, 129.2, 128.8, 127.5, 125.2, 123.5, 120.1, 114.7, 114.0, 53.3, 52.7, 37.8. Anal. calcd. for $C_{17}H_{16}ClNO_4$: C 61.18; H 4.83; N 4.20; found: C 61.07; H 4.87; N 4.16.

5-Chloro-2-hydroxy-*N*-L-phenylalaninebenzamide (12)
The hydrolysis of the methyl ester **11** (1.00 g, 3.00 mmol) into the free acid **12** was achieved by reaction in a 3% LiOH solution in 3:1 H_2O–MeOH (33). Recrystallization from MeOH–H_2O afforded **12** as a white solid: yield 0.67 g (70%); mp 172–173°C. ^{1}H NMR (acetone-d_6), δ: 12.3 (br, 1H), 8.62 (d, 1H, J = 7.9), 7.92 (m, 1H), 7.54–7.10 (m, 6H), 6.94 (d, 1H, J = 8.9), 5.01 (m, 1H), 3.4–3.1 (m, 2H); ^{13}C NMR (acetone-d_6), δ: 173.0, 169.2, 160.6, 138.3, 134.6, 130.0, 129.2, 127.7, 127.5, 123.7, 120.3, 116.7, 55.0, 37.7. Anal. calcd. 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.

© 1998 NRC Canada

Metal binding

The ability of **1** and our synthesized derivatives to bind Fe(III) and Cu(II) was studied by fluorescence spectroscopy at 25°C. Stock solutions of the toxin samples were prepared in 1,4-dioxane. Concentrations were determined by UV-vis in ethanol using extinction coefficients for the phenolic absorptions, ca. 330 nm. For Cu(II) binding, a solution (2 mL) containing 10 mM MES (pH 6.0), 100 mM $NaClO_4$ buffer, or 10 mM MOPS (pH 7.4), 100 mM $NaClO_4$ buffer was utilized. Studies on Fe(III) binding were performed in buffer solutions (2 mL) containing 10 mM HCl·KCl (pH 2.6), 100 mM $NaClO_4$. In each case, 2 μL of the toxin dioxane stock solution was added for a final concentration of 10 μM. For determination of the metal binding constants, quenching of the toxin's emission spectrum (emission ca. 440 nm, excitation ca. 330–380 nm) was measured 5 min after each addition of $Cu(II)(OAc)_2$ or $Fe(III)Cl_3$ to allow binding equilibration. Scatchard analysis (20) of the quenching was performed using the following equation: $(F/F_o) - 1)/[L] = (k_{11}/k_s)K_{11} - K_{11}(F/F_o)$ where F_o is the fluorescence intensity of free toxin, [L] is the ligand concentration, and k_{11}/k_s are proportionality constants (20). From a plot of $(F/F_o) - 1/[L]$ versus F/F_o, an association constant was derived from the negative slope. For $Zn(OAc)_2$ binding at pH 7.4 (10 mM MOPS, 100 mM $NaClO_4$), emission spectra were recorded at 400 nm, and the data was analyzed by the Benesi–Hildebrand equation (20*b*, 38). Double-reciprocal plots of $1/\Delta A$ (change in emission) versus 1/[Zn] afforded straight lines from which the association constant (K_{11}) was determined from K_{11} = (*y*-intercept)/(slope) (20*b*).

pK_a determination

The pH measurements were obtained on a Fisher Scientific Accumet pH meter 910 using standard glass electrodes. Calibration was done using commercial buffers (BDH, pH 4.00, 7.00, and 10.00, all ± 0.01). The pK_a of **12** (Scheme 2) was determined using 0.1 M phosphate buffers, which were prepared by dilution of a stock (1 M K_2HPO_4) solution with deionized water. For (±)-mellein (**7a**), 0.1 M $NaHCO_3$–Na_2CO_3 buffer solutions were utilized. All UV-vis spectra were acquired at 25°C using a standard matched pair of 1 cm quartz cells. In the case of **12**, the sample and reference were filled with 1.00 mL of buffer solution, and to the sample cell was added 2 μL of a stock solution of **12** in 1,4-dioxane. For **7a**, 1 mL of carbonate buffer containing 20 vol.% 1,4-dioxane was used, and to the sample cell was added 2 μL of the stock solution of **7a** in 1,4-dioxane. The UV-visible spectra were recorded by the overlay method in the wavelength range of 250–500 nm.

Relaxation of supercoiled DNA by Fe and Cu in the presence of ochratoxin A

Reaction mixtures (20 μL total volume) contained 400 ng of Form I DNA, 10 mM MOPS (pH 7.4), 50 mM NaCl, and 20 μM each of OTA (**1**) and metal ion. The metal salts employed were $Cu(OAc)_2$, $Fe(Cl)_3$, and $Fe(NH_4)_2(SO_4)_2$. For reactions with iron, external reducing agents (200 μM sodium ascorbate) and oxidants (200 μM HOOH) were also employed. Reaction mixtures were incubated at 37°C for varying lengths of time, then quenched by the addition of 4 μL of loading buffer. Samples were loaded onto a 1% agarose gel containing ethidium bromide (1 ug/mL). The gel was run at 110 V for 2 h and visualized by UV illumination.

Densitometric quantitation was performed using a Microtek Scanmaker E_6 equipped with PhotoImpact and UTHSCSA Image Tool software. Supercoiled plasmid DNA values were corrected by a factor of 1.3, based on average literature estimates of lowering binding of ethidium to this structure (39).

Relaxation of supercoiled DNA by Cu(II)·ochratoxin A

Reaction mixtures (20 μL total volume) contained 400 ng of Form I DNA, 10 mM MOPS (pH 7.4), 50 mM NaCl, 20 μM $Cu(OAc)_2$, and 60 μM OTA. The OTA and $Cu(OAc)_2$ were premixed prior to addition of Form I DNA. Addition of sodium ascorbate 10–250 μM initiated the reaction, and mixtures were incubated at 37°C for 30 min, then quenched by the addition of 4 μL loading buffer. Samples were loaded onto a 1% agarose gel containing ethidium bromide (1 μg/mL). The gel was run at 110 V for 2 h and visualized by UV illumination. In a separate experiment, the quenching effect of catalase (1000 units/mL), sodium azide (100 mM), DMSO (1 M), and *tert*-butanol (1M) was examined.

Relaxation of supercoiled DNA by $Cu(OP)_2$ in the presence of ochratoxin A

Reaction mixtures (20 μL total volume) contained 400 ng of Form I DNA, 10 mM MOPS (pH 7.4), 50 mM NaCl, 20 μM $Cu(OAc)_2$, 40 μM OP, and either 10 μM sodium ascorbate or 50–250 μM OTA (**1**). Reaction mixtures were incubated at 37°C for 30 min, then quenched by the addition of 4 μL loading buffer. Samples were loaded onto a 1% agarose gel containing ethidium bromide (1 μg/mL). The gel was run at 110 V for 2 h and visualized by UV illumination. In a separate experiment, the quenching effect of catalase (1000 units/mL) and SOD (1000 units/mL) on the ability of 50 μM OTA to potentiate DNA cleavage by $Cu(II)(OP)_2$ was examined.

Relaxation of supercoiled DNA by $Cu(OP)_2$ in the presence of OTA, OTB, and 12

Reaction mixtures (20 μL total volume) contained 400 ng of Form I DNA, 10 mM MOPS (pH 7.4), 50 mM NaCl, 20 μM $Cu(OAc)_2$, 40 μM OP, and 50 μM OTA (**1**), OTB (**2**), or **12**. Reaction mixtures were incubated at 37°C for 30 min, then quenched by the addition of 4 μL loading buffer. Samples were loaded onto a 1% agarose gel containing ethidium bromide (1 μg/mL). The gel was run at 110 V for 2 h and visualized by UV illumination. In a separate experiment, the reactions described above were repeated, but in 10 mM CHES buffer, pH 8.6.

Relaxation of supercoiled DNA by Fe^{III}·EDTA in the presence of OTA

Reaction mixtures (20 μL total volume) contained 400 ng Form I DNA, 10 mM MOPS (pH 7.4), 50 mM NaCl, 20 μM $FeCl_3$, 20 μM EDTA, and either 50 μM sodium ascorbate or 50–250 μM OTA. Reaction mixtures were treated as described in the previous experiments.

Acknowledgment

We thank Dr. Fred W. Perrino (Department of Biochemistry, Wake Forest University School of Medicine) for the sample of

© 1998 NRC Canada

plasmid DNA, Dr. Gary Byrd (RJR Tobacco Co.) for acquisition of the mass spec data, and Mr. Sumit Borah, Mr. Keith Merritt, and Ms. Jennifer M. Yezek for preliminary experiments. This work was supported by grant no. IRG from the American Cancer Society and Wake Forest University.

References

1. K.J. van der Merwe, P.S. Steyn, L. Fourie, D.B. Scott, and J.J. Theron. Nature (London), **205**, 1112 (1965).
2. (*a*) T. Kuiper-Goodman and P.M. Scott. Biomed. Environ. Sci. **2**, 179 (1989); (*b*) A.E. Pohland, S. Nesheim, and L. Friedman. Pure Appl. Chem. **64**, 1029 (1992).
3. (*a*) A.M. Bendele, W.W. Carlton, P. Krogh, and E.B. Lillehoj. J. Natl. Cancer Inst. **75**, 733 (1985); (*b*) M. Castegnaro, R. Plestina, G. Dirheimer, I.N. Chernozemsky, and H. Bartsch. Mycotoxins, Endem. Nephropathy Urinary Tract Tumours. Vol. 115. IARC Sci. Publ., Oxford Univ. Press, Oxford, U.K. 1991; (*c*) R.R. Marquardt and A.A. Frohlich. J. Anim. Sci. **70**, 3968 (1992).
4. (*a*) Y. Grosse, I. Baudrimont, M. Castegnaro, A.-M. Betbeder, E.E. Creppy, G. Dirheimer, and L.A. Pfohl. Chem. Biol. Interact. **95**, 175 (1995); (*b*) P.S. Obrecht, Y. Grosse, L.A. Pfohl, and G. Dirheimer. Arch. Toxicol. **70**, 244 (1996).
5. (*a*) E.E. Creppy, A. Kane, G. Dirheimer, C. Lafarge-Frayssinet, S. Mousset, and C. Frayssinet. Toxicol. Lett. **28**, 29 (1985); (*b*) J.C. Seegers, L.H. Bohmer, M.C. Kruger, M.L. Lottering, and K.M. de A. Toxicol. Appl. Pharmacol. **129**, 1 (1994).
6. C. Malaveille, G. Brun, and H. Bartsch. Mutat. Res. **307**, 141 (1994).
7. H. Xiao, S. Madhyastha, R.R. Marquardt, S. Li, J.K. Vodela, A.A. Frohlich, and B.W. Kemppainen. Toxicol. Appl. Pharmacol. **137**, 182 (1996).
8. (*a*) G.E. de Groene, I.G. Hassing, M.J. Blom, W. Seinen, G.J. Fink, and G.J. Horbach. Cancer Res. **56**, 299 (1996); (*b*) R.F. Omar, H.V. Gelboin, and A.D. Rahimtula. Biochem. Pharmacol. **51**, 207 (1996).
9. I. Baudrimont, A.-M. Betbeder, A. Gharbi, L.A. Pfohl, G. Dirheimer, and E.E. Creppy. Toxicology, **89**, 101 (1994).
10. B.B. Hasinoff, A.D. Rahimtula, and R.F. Omar. Biochim. Biophys. Acta, **1036**, 78 (1990).
11. (*a*) D. Hoehler, R.R. Marquardt, A.R. McIntosh, and H. Xiao. J. Biol. Chem. **271**, 27 388 (1996); (*b*) D. Hoehler, R.R. Marquardt, A.R. McIntosh, and G.M. Hatch. Biochim. Biophys. Acta, **1357**, 225 (1997).
12. (*a*) A.D. Rahimtula, J.C. Bereziat, V. Bussacchini-Griot, and H. Bartsch. Biochem. Pharmacol. **37**, 4469 (1988); (*b*) R.F. Omar, A.D. Rahimtula, and H. Bartsh. J. Biochem. Toxicol. **6**, 203 (1991).
13. (*a*) R.T. Scannell, J.R. Barr, V.S. Murty, K.S. Reddy, and S.M. Hecht. J. Am. Chem. Soc. **110**, 3650 (1988); (*b*) U.S. Singh, R.T. Scannell, H. An, B.J. Carter, and S.M. Hecht. J. Am. Chem. Soc. **117**, 12 691 (1995).
14. (*a*) Y. Li and M.A. Trush. Carcinogenesis, **14**, 1303 (1993); (*b*) Cancer Res. **54**, 1895s (1994).
15. (*a*) D.S. Sigman. Acc. Chem. Res. **19**, 180 (1986); (*b*) T.B. Thederahn, M.D. Kuwabara, T.A. Larsen, and D.S. Sigman. J. Am. Chem. Soc. **111**, 4941 (1989).
16. J. Blair and G.T. Newbold. J. Chem. Soc. 2871 (1955).
17. (*a*) M. Watanabe, M. Sahara, M. Kubo, S. Furukawa, R.J. Billedeau, and V. Snieckus. J. Org. Chem. **49**, 742 (1984); (*b*) M.P. Sibi, S. Chattopadyyay, J.W. Dankwardt, and V. Snieckus. J. Am. Chem. Soc. **107**, 6312 (1985).
18. S. Terao, M. Shiraishi, K. Kato, S. Ohkawa, Y. Ashida, and Y. Maki. J. Chem. Soc. Perkin Trans. 1, 2909 (1982).
19. H. Xiao, R.R. Marquart, A.A. Frohlich, and Y.Z. Ling. J. Agric. Food Chem. **43**, 524 (1995).
20. (*a*) G. Scatchard. Ann. N.Y. Acad. Sci. **51**, 660 (1949); (*b*) K.A. Conners. Binding constants. The measurement of molecular complex stability. John Wiley & Sons, New York. 1987. pp. 339–343.
21. F.A. Cotton and G. Wilkinson. Advanced inorganic chemistry. A comprehensive text. 4th ed. John Wiley & Sons, New York. 1980.
22. F.S. Chu. Arch. Biochem. Biophys. **147**, 359 (1971).
23. F.S. Chu. CRC Crit. Rev. Toxicol. **2**, 499 (1974).
24. A. Albert and E.P. Sergeant. The determination of ionization constants. Chapman & Hall, London. 1962.
25. W. Knapp Pogozelski, T.J. McNeese, and T.D. Tullius. J. Am. Chem. Soc. **117**, 6428 (1995).
26. I.M.C.M. Rietjens, C. den Besten, R.P. Hanzlik, and P.J. van Bladeren. Chem. Res. Toxicol. **10**, 629 (1997).
27. M.W. Bredenkamp, J.L.M. Dillen, P.H. van Rooyen, and P.S. Steyn. J. Chem. Soc. Perkin Trans. 2, 1835 (1989).
28. H. Sigel and R.B. Martin. Chem. Rev. **82**, 385 (1982).
29. (*a*) J. Balla, P.V. Bernhardt, P. Buglyo, P. Comba, T.W. Hambley, R. Schmidlin, S. Stebler, and K. Varnagy. J. Chem. Soc. Dalton Trans. 277 (1991); (*b*) F.V Pamatong, C.A. Detmer III, and J.B. Bocarsly. J. Am. Chem. Soc. **118**, 5339 (1996).
30. A. Stassinopoulos, G. Schulte, G.C. Papaefthymiou, and J.P. Caradonna. J. Am. Chem. Soc. **113**, 8686 (1991).
31. (*a*) D.P. Mack, B.L. Iverson, and P.B. Dervan. J. Am. Chem. Soc. **110**, 7572 (1988); (*b*) C. Harford and B. Sarkar. Acc. Chem. Res. **30**, 123 (1997).
32. H. Lund and M.M Baizer (*Editors*). Organic electrochemistry, an introduction and a guide. 3rd ed. Revised and expanded. Marcel Dekker, Inc., New York. 1991. pp. 615–635.
33. (*a*) K. Takehira, M. Shimiza, Y. Watanabe, H. Orita, and T. Hayakawa. J. Chem. Soc. Chem. Commun. 1705 (1989); (*b*) Y. Takizawa, T. Munakata, Y. Iwasa, and T. Mitsuhashi. J. Org. Chem. **50**, 4383 (1985).
34. (*a*) T. Ohe, T. Mashino, and M. Hirobe. Tetrahedron Lett. **36**, 7681 (1995); (*b*) V.M. Samokyszyn, J.P. Freeman, K.R. Maddipati, and R. Lloyd. Chem. Res. Toxicol. **9**, 1368 (1996).
35. (*a*) Y.J. Abul-Hajj, K. Tabakovic, and I. Tabakovic. J. Am. Chem. Soc. **117**, 6144 (1995); (*b*) M.A. Lewis, D.G Yoerg, J.L. Bolton, and J.A. Thompson. Chem. Res. Toxicol. **9**, 1368 (1996).
36. D. Parsonage, H. Miller, R.P. Ross, and A. Claiborne. J. Biol. Chem. **268**, 3161 (1993).
37. S.O. de Silva, M. Watanabe, and V. Snieckus. J. Org. Chem. **44**, 4802 (1979).
38. H. Benesi and J.H. Hildebrand. J. Am. Chem. Soc. **71**, 2703 (1949).
39. C.K. Mirabelli, C.H. Huang, and S.T. Crooke. Cancer Res. **40**, 4173 (1980).

© 1998 NRC Canada