Identification of a Reduction Product of Aristolochic Acid: Implications for the Metabolic Activation of Carcinogenic Aristolochic Acid

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Aristolochic acids are nephrotoxic and carcinogenic natural products that have been implicated both in endemic nephropathy in the Balkan region and in ailments caused by ingestion of herbal remedies. Aristolochic acids are metabolized to active intermediates that bind to DNA. In this study, reduction of aristolochic acid I with zinc in acetic acid afforded a new product that was characterized as 9-methoxy-7-methyl-2*H*-1,3-oxazolo[5′,4′-10,9]phenanthro-[3,4-*d*]-1,3-dioxolane-5-carboxylic acid, designated as aristoxazole, along with the expected aristolactam I. This new compound is a condensation product of aristolochic acid and acetic acid that may be related to the aristolochic acid−DNA adducts. The proposed mechanism of formation of aristoxazole involves nucleophilic attack of acetic acid on the nitrenium ion of aristolochic acid I. On the basis of these studies, a route to the metabolic activation of aristolochic acids and formation of adducts with DNA in in vitro systems is proposed and discussed.

Aristolochic acids are carcinogenic and nephrotoxic nitroarenes that occur in *Aristolochia* species, with aristolochic acids I and II (AAI and AAII, **1** and **2**, respectively; for numbering, see structure **1**) usually being the most abundant of these compounds. Ingestion of AAs is implicated in the type of renal fibrosis known as "Chinese herbs nephropathy" or "Balkan endemic nephropathy".[1−9] A report on the carcinogen aristolochic acid was recently issued by the U.S. Department of Health and Human Services.[10]

15   **1**   **5**

16   **17**   **18**
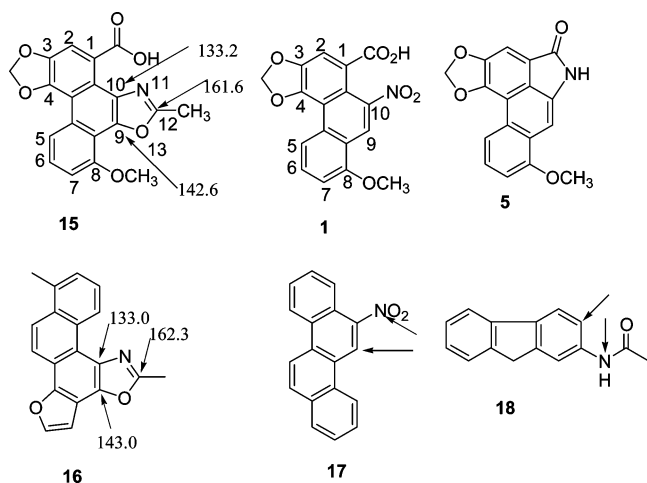
The toxicity of AAs is due to the formation of active intermediates during the detoxification process. The crucial step in the generation of DNA-reactive and mutagenic metabolites is the reduction of the nitro group of AAs. In rats and other mammals, the major metabolic pathway involves reduction of AAI (**1**) and AAII (**2**) to aristolactams [**5** and **5a** (Scheme 1)]. In addition, O-demethylation of AAI (**1**) and aristolactam I (**5**), as well as oxidation at C-8 of AAII (**2**), is observed. All of these metabolites are detected in urine and feces either in their original form or as

conjugates of glucuronic or sulfuric acids. Furthermore, covalent adducts of DNA such as (deoxyguanosin-N^2-yl)aristolactam I (dG-AAI) (**7**), (deoxyadenosin-N^6-yl)aristolactam I (dA-AAI) (**8**), (deoxyguanosin-N^2-yl)aristolactam II (dG-AAII) (**7a**), and (deoxyadenosin-N^6-yl)aristolactam II (dA-AAII) (**8a**) (Scheme 1) were identified in the renal tissue of patients that ingested herbal preparations containing AAI and AAII.[5−7] Interestingly, only adducts formed at C-9 (C-7 in an alternative numbering)[5,7,11] of the aristolochic acids are known. There are no reports of adduct formation at the C-10 nitrogen that usually occurs with nitroaromatic compounds.

The accepted mechanism for the reductive transformations of AAs into DNA adducts is shown in Scheme 1.[5,7,11] It was proposed that the aristolactam-nitrenium ion **4** is the ultimate carcinogenic species that binds to the amine group of purine nucleotides (**7**, **7a**, **8**, and **8a**) or is hydrolyzed to the corresponding 9-hydroxyaristolactam I (**6**). Note that in previous reports a different numbering system was used in which this compound was named 7-hydroxyaristolactam.[11]

In view of the importance of the reduction of AAs with respect to genotoxicity, we reinvestigated the reduction of AAI using zinc in acetic acid. As a result, we report the identification of a new reduction product from AAI, namely 9-methoxy-7-methyl-2*H*-1,3-oxazolo[5′,4′-10,9]phenanthro[3,4-*d*]-1,3-dioxolane-5-carboxylic acid, which is named aristoxazole (**15**). We discuss its mechanism of formation and the possibility that there is a nitrenium ion-based pathway leading to adduct formation, which is complementary to that proposed by Pfau et al.[11]
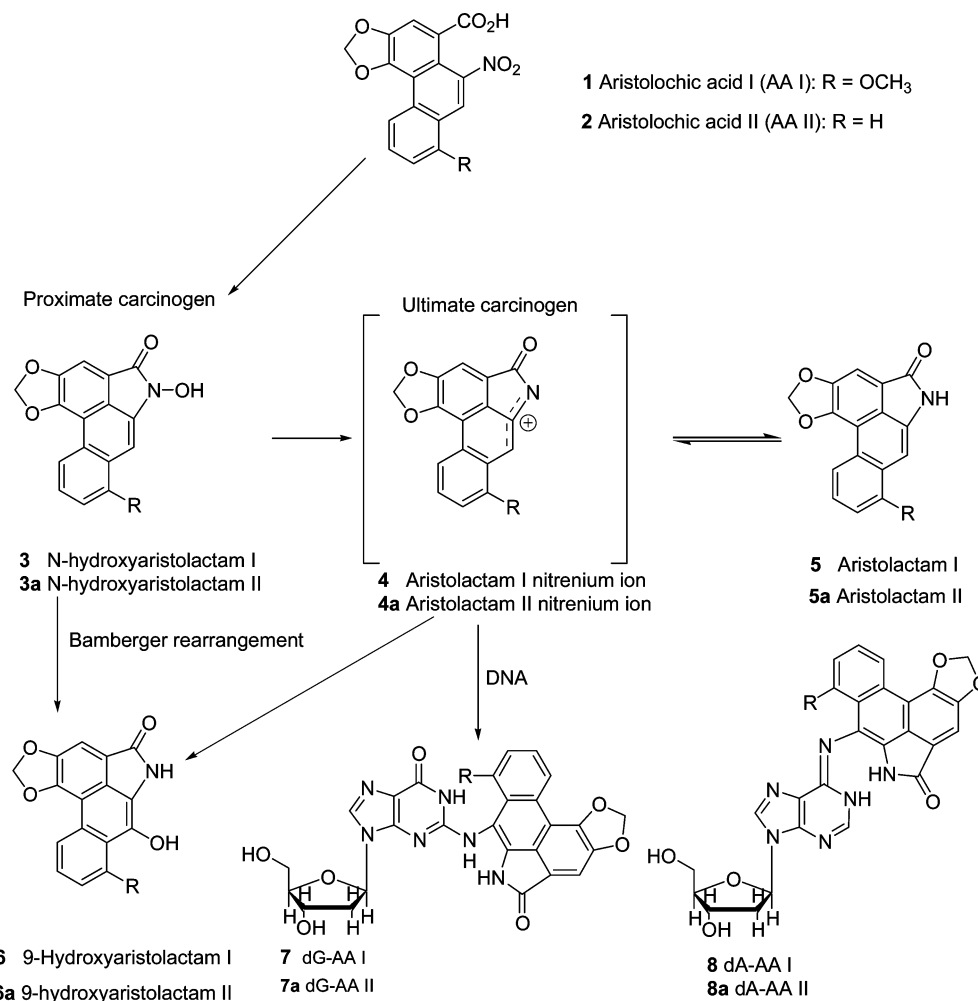
Results and Discussion

Identification of Aristoxazole. Previous studies have established that AAs underwent reduction to either the corresponding aristolactam or a complex mixture of uncharacterized products.[12] In this study, AAI was reduced with zinc powder because this is a well-established route for the reduction of nitro groups. The zinc reduction of AAI (**1**) in boiling acetic acid yielded the expected aristolactam I (**5**) as the major product. However, high-performance liquid chromatography (HPLC) analyses revealed the presence of a minor reduction product, subsequently identified as aristoxazole (**15**) [for the sake of simplicity, the numbering of the atoms matches that of AAI (**1**)]. When AAI was reduced with Zn in acetic acid between 60 and 118 °C, aristolactam I (**5**) and aristoxazole (**15**)

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Scheme 1. Metabolic Activation and DNA Adduct Formation of AAI (**1**) and AAII (**2**)[a]

1 Aristolochic acid I (AA I): R = OCH_3

2 Aristolochic acid II (AA II): R = H

Proximate carcinogen

Ultimate carcinogen

3 N-hydroxyaristolactam I
3a N-hydroxyaristolactam II

4 Aristolactam I nitrenium ion
4a Aristolactam II nitrenium ion

5 Aristolactam I
5a Aristolactam II

Bamberger rearrangement

DNA

6 9-Hydroxyaristolactam I
6a 9-hydroxyaristolactam II

7 dG-AA I
7a dG-AA II

8 dA-AA I
8a dA-AA II

[a] For **1** and **3**−**8**, R = OCH_3; for **2** and **3a**−**8a**, R = H.

were obtained in a ca. 3:1 ratio. The reaction proceeds at room temperature (25 °C), but the aristolactam I:aristoxazole ratio changes to 6:1. Aristoxazole (**15**, MW 351, $C_{19}H_{13}NO_6$) is a stable compound. It cannot be reduced further when treated under the same conditions.

The molecular formula indicates that two carbon atoms were added to AAI during the formation of aristoxazole. These were supplied by acetic acid because the reduction was conducted in this solvent. The UV spectrum shows a strong absorption at 251 nm typical of the phenanthrene nucleus. The possibility that aristoxazole was an *N*-acetyl derivative related to aristolactam I was discounted for several reasons. Evidence of an intact carboxylic acid group was provided by chemical behavior, the detection of a fragment ion at *m/z* 306 (M − 44) under electron impact, the IR spectrum (carbonyl stretch at 1668 cm^{-1}), and formation of a methyl ester with diazomethane. The ^{1}H NMR spectrum (Table 1) provided limited data because the compound contains only a small number of hydrogens. The presence of a broad singlet at ca. δ_H 13, which exchanged with D_2O, gave further confirmation of the carboxylic acid moiety. The doublet at δ_H 8.74, the triplet at δ_H 7.62, the singlet at δ_H 7.49, and the doublet at δ_H 7.34 were assigned as the aryl hydrogens at C-5, C-6, C-2, and C-7, respectively, on the basis of the splitting patterns and comparison of the chemical shifts with those of AAI.[13] The expected singlet for H-9 (ca. δ_H 7.20) was absent, as well as the signal for a probable NH lactam proton (ca. δ_H 10.70). The singlets at δ_H 6.38 and 4.04 were assigned to the methylenedioxy and methoxy groups, respectively. An extra methyl group was observed at δ_H 2.69. These results and structural

Table 1. ^{1}H NMR and ^{13}C NMR Data for Aristoxazole (**15**) and Aristoxazole Methyl Ester in DMSO-d_6 (δ in parts per million, *J* in parentheses in hertz)

position	aristoxazole (**15**)		**15** methyl ester
	δ_H	$\delta_C{}^a$	δ_H
1		127.7	
2	7.50 s (6.5)	109.1	7.57 s
3		144.4	
4		144.3	
4a		114.1	
4b		125.0	
5	8.74 d (8.4)	119.4	8.80 d (8.4)
6	7.62 t (8.4)	127.1	7.70 t (8.4)
7	7.34 d (8.0)	108.9	7.42 d (8.0)
8		153.5	
8a		111.8	
9		142.6	
10		133.2	
10a		117.3	
12		161.6	
1-COOH	13.00 s (broad)	170.1	
3,4-CH_2O_2	6.38 s	101.8	6.43 s
8-OMe	4.04 s	56.0	4.08 s
12-Me	2.69 s	14.3	2.73 s
1-COOMe			3.94 s

[a] Assignments of signals with similar chemical shifts may be reversed.

considerations suggested the phenanthroxazole structure **15** for the unknown compound, the methyl group being attributed to the C-12 methyl substituent.

Figure 1. Calculated ball-and-wire structures of aristolic acid and aristoxazole.
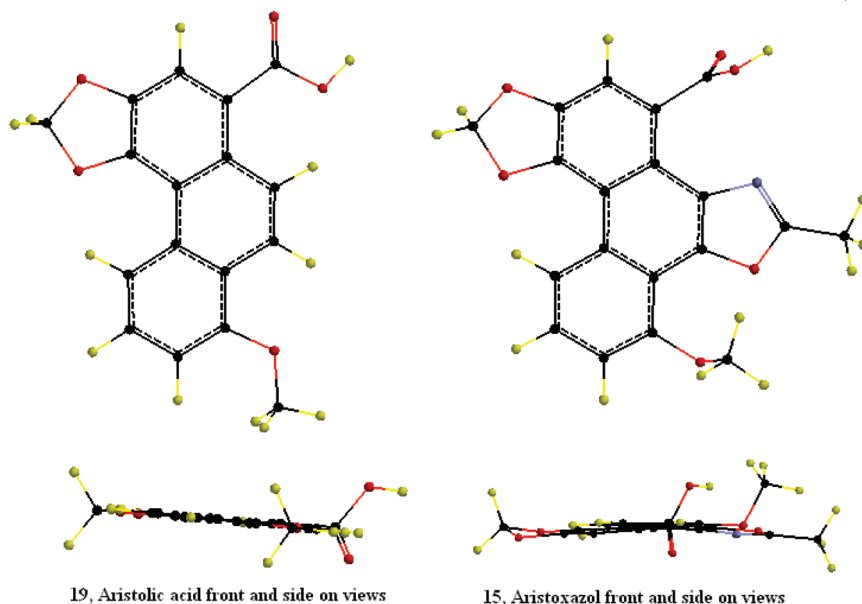
19, Aristolic acid front and side on views   15, Aristoxazol front and side on views

The proton-decoupled ^{13}C NMR spectrum (Table 1) supported structure **15**. The spectrum was composed of 19 signals, seven of which appeared above δ_C 130. Four of these signals were assigned by comparison with those of AAI[14] as follows: δ_C 170.1 (carboxyl group), δ_C 153.4 (C-8), and δ_C 144.3 and 144.2 (C-3 and C-4 or vice versa). The remaining three downfield signals at δ_C 161.6, 142.6, and 133.2 were assigned as C-12, C-9, and C-10 of the benzoxazole unit, respectively, based on a comparison with the corresponding carbons of isosalviamine C (**16**) that have chemical shifts of δ_C 162.3, 143.0, and 133.0, respectively.[15] Further support for the oxazole ring is provided by the signal at δ_C 14.3, whose chemical shift matches very well with that of the corresponding methyl substituent in 2-methylbenzoxazole (δ_C 14.4) and natural products containing this moiety (δ_C 14.9–15.1).[15] Equally important, this signal is much further upfield than the methyl carbons of acetyl groups on aryl substrates such as acetanilide (δ_C 24.1), phenyl acetate (δ_C 21.1), and acetophenone (δ_C 25.7).

The UV spectrum of aristoxazole shows a remarkable resemblance to that of aristolic acid [**19** (Figure 1)] (λ_{max} values of 254.5, 296.2, 319sh, 327.1, 355.0, and 374.5 nm), which indicates that these compounds have similar chromophores. The two minor bands of aristoxazole at 363 and 382 nm are ~8 nm bathochromically shifted from those of the desnitro AAI, a shift that can be attributed, in part, to the presence of the oxazole ring at C-9 and C-10. On the basis of the comparison of the λ_{max} values for benzene (254 nm) and 2-methylbenzoxazole (271 and 277 nm),[16] a bathochromic shift of ~20 nm would have been expected. The smaller bathochromic shift observed with aristoxazole is attributed to steric compression by crowding of the substituents at the *peri* 8, 9, 10, and 1 positions.[17] Molecular modeling of the aristoxazole revealed distortion within the phenanthrene ring (Figure 1), presumably as a means of reducing steric compression. Support for this proposal was obtained from the modeling of aristolic acid, which lacks the oxazole ring. The data revealed that the phenanthrene ring of aristolic acid was essentially planar (Figure 1). In summary, chemical and spectroscopic data support the proposed structure **15** for aristoxazole in which the oxazole unit is fused to the C-9–C-10 bond of the phenanthrene moiety.

Considerations about the Formation of Aristoxazole. Reduction of AAI with Zn in acetic acid produces aristolactam I (**5**) accompanied by smaller amounts of aristoxazole. Overall, the formation of aristoxazole from AAI involves the reduction of the nitro group and the insertion of an acetoxy group at C-9, which will generate the oxazole unit after several steps. Thus, aristoxazole is a derivative of an intermediate reduction product of AAI. In contrast, aristolactam I (**5**) represents the final product from the complete reduction of the nitro group of AAI.

Reduction of nitro arenes to amines using Zn occurs via the nitroso and hydroxylamine intermediates. Each reduction step occurs by a series of single-electron transfers (SETs) in which an electron from the Zn and a proton are transferred to the species being reduced.[18] Thus, the formation of the *N*-hydroxyarylamine **9**, and the aryl amine **10**, which are probable intermediates in the formation of aristoxazole (**15**) and aristolactam I (**5**), is thought to occur via SET reactions (Scheme 2). Both amines **9** and **10** can readily cyclize to form the more stable aristolactam, **5**, either directly or via the postulated *N*-hydroxylactam, **3**. Thus, formation of aristolactam I from the reduction process is entirely expected. Furthermore, the stability of the lactam makes it an unlikely precursor to aristoxazole, indicating that the pathway must diverge prior to the reduction that leads to aristolactam.

More than 100 years ago, Bamberger reported that *N*-phenyl-hydroxylamine rearranged in a sulfuric acid solution to give 4-aminophenol.[19] The key features of this reaction are the O-protonation of the NOH group followed by N–O bond cleavage and loss of water to form an intermediate nitrenium ion.[20,21] The studies mentioned above suggest that formation of aristoxazole (**15**) during reduction of AAI with Zn in acetic acid involves formation of the corresponding *N*-hydroxylamine and a nitrenium ion as the reactive intermediate species.

It was important to consider whether the aristoxazole was formed by a variation of the reactions of the nitrenium ion, **4**, of Scheme 1, which was proposed for the activation of AAs in biological systems. However, we were unable to account for the formation of aristoxazole via this pathway. The stumbling block is that hydrolysis of the lactam structure, **3**, in Scheme 1, which is required to generate the *N*-hydroxylamine **9**, the expected initial precursor of aristoxazole, would not be possible under the reaction conditions. This topic is discussed further in Probable Routes of Aristoxazole Formation.

In light of these difficulties, it was necessary to propose an alternative pathway, which is shown in Scheme 2. Before discussing the most reasonable pathway to aristoxazole, we needed to examine the possibility that aristoxazole formation occurred by direct heterolysis of the N–O bond of the *N*-hydroxylamine **9** to give the nitrenium ion **12** prior to the formation of the lactam **3** or

Scheme 2. Proposed Mechanism for the Formation of Aristoxazole (**15**)[a]
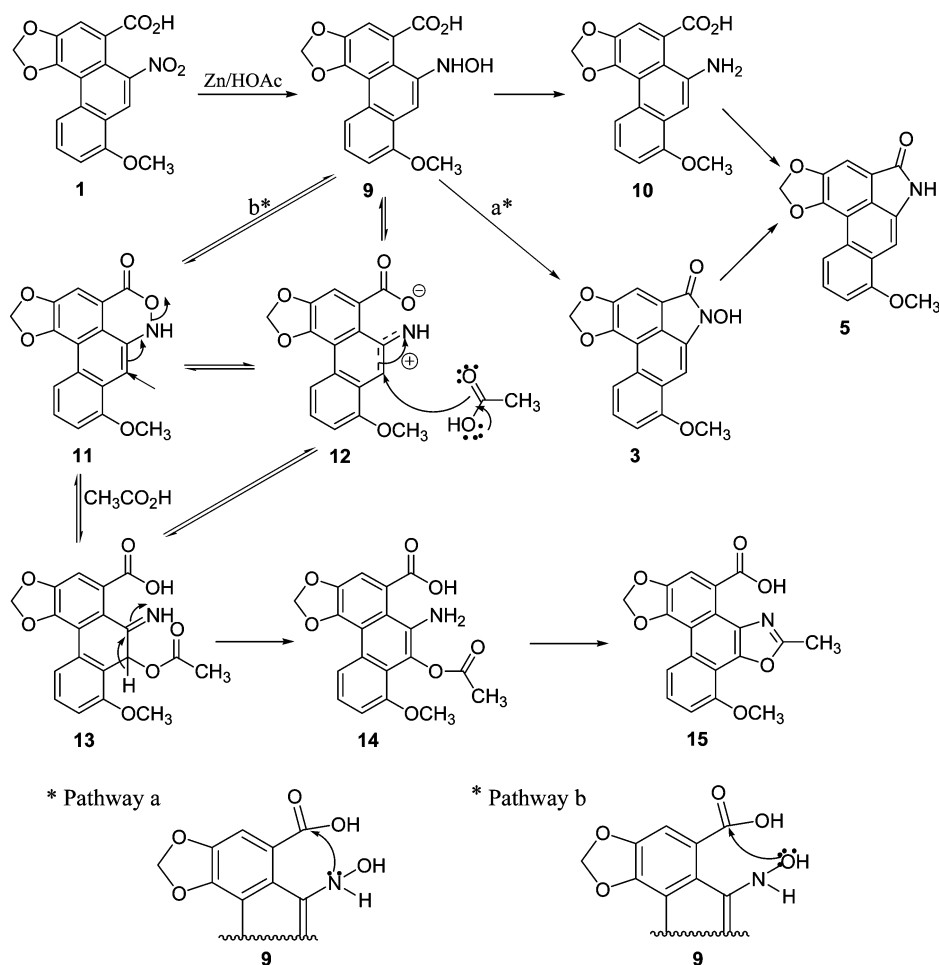
[a] With the exception of structures **1**, **5**, and **15**, none of the structures in this scheme have been isolated. Thus, it is not possible to determine their bioactivities. The bioactivities of structures **1** and **5** are well-established.[5,7,11,25]

reduction (Scheme 2). This conversion of *N*-hydroxylamine **9** into nitrenium ion **12** seems unlikely because the acetic acid medium is insufficiently strong to drive the transformation of **9** into **12**. Even if it occurs, it is probable that it would be very slow, which would mean that spontaneous formation of lactam **3** or reduction to **10** would be by far the predominant process. Although the **9**-to-**12** conversion cannot be ruled out, an alternative and more reasonable mechanism is shown in Scheme 2. In this mechanism, formation of aristoxazole is proposed to occur via the formation of an oxazinone **11**.

It is assumed that the 9-hydroxylamine, **9**, can be stabilized by spontaneous cyclization. The carboxyl group of **9** can undergo an addition−elimination reaction with the hydroxylamine unit to form either the lactam **3** by reaction at the nitrogen or the oxazinone **11** by reaction at the oxygen (pathways a and b in Scheme 2). Also, the nucleophilic addition−elimination reaction of a hydroxylamino oxygen atom with a carboxylic acid has been documented.[22,23] It is proposed that part of the *N*-hydroxylamine **9** is converted into the oxazinone **11** instead of being transformed into either lactam **3** or amine **10** by further reduction. In contrast to *N*-hydroxylamine **9**, oxazinone **11** could readily ionize to give nitrenium ion **12**, which could be attacked by the available nucleophiles such as acetic acid and water. The 9-acetoxy adduct **13** could then undergo aromatization and dehydration to give aristoxazole (**15**). Like the nitroso derivative of AAI (not shown), *N*-hydroxyarylamine, **9**, arylamine, **10**, and oxazinone, **11**, would be too reactive to be either isolated or detected among the reaction products. Similarly, it is proposed that transformation of **13** into **15** via the more stable isomer, **14**

Table 2. Density Function Calculations[a] of Likely Intermediates to Aristoxazole and Related Compounds

compound	energy (arbitrary units)	compound	energy (arbitrary units)
1	−1235.9836	11	−1085.5324
3	−1085.5347	13	−1314.7125
5	−1010.3526	14	−1314.7325
9	−1161.9774	15	−1238.2741
10	−1086.8029		

[a] Calculations were conducted at the B3LYP 6-311+G** level.

(Table 2), may occur rapidly and synchronously because formation of the lactam by reaction of the C-1 carboxyl group and the C-10 amino group in **14** was not observed.

The *N*-hydroxyaristolactam, **3**, was not detected directly in this synthesis, which is not altogether surprising because this compound has also not been isolated as a product of other chemical or enzymatic reductions of AAI. Intriguingly, the analogous *N*-hydroxyaristolactam of aristolochic acid II is a stable compound that has been characterized in plants (see Are *N*-Hydroxyaristo-lactams Toxic?).

Support for the formation of **11** is provided by the known cyclization of the oximes of 2-acylbenzoic acids to form 2,3-benzoxazin-1-ones.[22] A similar reaction also occurs during the reduction of *o*-nitrobenzoic acid with tin or zinc, leading to the formation of 2,1-benzisoxazolin-3-one following cyclization of the reduction intermediate, *o*-hydroxylaminobenzoic acid.[23] Additionally, the final condensation between the amino and acetoxy groups in adduct **14** to give the oxazole ring resembles the reaction of

2-aminophenols with carboxylic acids, which is usually employed to prepare oxazole derivatives.[24]

Formation of aristoxazole is discussed further in connection with problems concerning the mechanisms of aristolochic acid activation and formation of adducts (see Probable Routes of Aristoxazole Formation).

Molecular Mechanics Calculations. In view of the fact that most of the compounds that are postulated in Scheme 2 were not isolated, we conducted molecular mechanics density function calculations at the B3LYP 6311+G** level. The energies of the calculated structures are summarized in Table 2. The resultant model of aristoxazole shows a distortion within the phenanthrene skeleton (Figure 1) that would explain the smaller than expected bathochromic shifts in its UV spectrum (Identification of Aristoxazole). The calculations indicated that compound **14** was more stable than its isomer, **13**, which would be expected. Calculations also showed that the *N*-hydroxyaristolactam, **3**, was only slightly more stable than the isomeric oxazinone, **11**. This result was unexpected because the 8-demethoxy analogue, **3a**, of the *N*-hydroxylactam, **3**, is a stable compound that was isolated from plants (see Are *N*-Hydroxyaristolactams Toxic?). We repeated the calculations at different levels of sophistication, but in all cases, the energies of the two compounds were similar. Nevertheless, we have included the data (Table 2) because it is the first time that such information has been reported for aristolochic acid and related compounds. The bond lengths, bond angles, and molecular dipole moments for the compounds that are listed in Table 2 are given in the Supporting Information.

Mechanism of Aristolochic Acid Activation via the *N*-Hydroxyaristolactam and Formation of DNA Adducts. The mechanism for metabolic activation of AAs and DNA adduct formation proposed by Pfau et al. is shown in Scheme 1 in which the key features are the formation of the *N*-hydroxyaristolactam **3** and the aristolactam-nitrenium ion **4**.[5,7,11] The cyclic nitrenium ion **4** with its delocalized positive charge was suggested to be the ultimate carcinogen. Formation of the AA adduct occurs by reaction of the electrophilic C-9 of the nitrenium ion with the nucleophilic exocyclic amino group of purine nucleotides in DNA. While adducts of AA reduction intermediates with DNA (**7**, **7a**, **8**, and **8a**) and lactams **5** and **5a** have been characterized, the reactive species that covalently binds to DNA and their precursors remain unknown. Attempts to isolate the *N*-hydroxyaristolactam (**3**) were unsuccessful.[11] However, support for the proposed mechanism was obtained by the generation of DNA adducts from both aristolactam I (**5**) and *N*-chloroaristolactam II.[25,26] Isolation of 9-hydroxyaristolactam I (**6**) from enzymatic reduction of AAI also provided indirect evidence of the existence of the cyclic hydroxamic acid intermediate **3**.[11]

Although the proposed mechanism explains much of what is observed, some factors in the formation of DNA adducts remain unclear. For example, in vitro experiments revealed that AAs readily form DNA adducts under conditions in which it would be impossible to activate the hydroxylamine **3** as an *O*-sulfate or *O*-acyl ester. Similarly, the regioselectivity of DNA adduct formation at C-9 is somewhat surprising because nitroarenes usually afford both N and C adducts. Another intriguing question is why medicinal and edible plants containing significant concentrations of *N*-hydroxyaristolactams are nontoxic. These issues are discussed below in relation to the reduction product aristoxazole.

Activation of Nitroarenes: Nitroreduction and O-Esterification of the *N*-Hydroxylarylamine. Polycyclic nitroaromatic compounds are known to be metabolized into potent carcinogens in laboratory animals. They undergo reduction of the nitro group to give reactive nitrenium ions that bind to DNA with *N*-hydroxyarylamine intermediates being the immediate precursors of the nitrenium ions. Mutagenicity and carcinogenicity are dependent upon two activation reactions, namely, nitroreduction and O-esterification of the *N*-hydroxylamine. Specific nitroreductases and

acyltransferases are involved in these processes.[27,28] The *N*-hydroxylamines can undergo N—O bond cleavage under mildly acidic conditions; however, for this reaction to be biologically relevant, a previous activation step involving formation of reactive esters is necessary to render the N—O bond labile enough for cleavage. There is strong evidence that the sulfuric or carboxylic acid esters of the resulting *N*-hydroxylamines are among the more important carcinogenic metabolites because in aqueous solution they undergo heterolysis of the N—O bond to yield the arylnitrenium ions.[28-32] It is likely that the *N*-hydroxylamine **3** is activated as a sulfate or acyl ester prior to the formation of the nitrenium ion **4** because it was demonstrated that expression of the human sulfotransferase SULT1A1 in bacterial and mammalian target cells enhances the mutagenicity of AAs.[33]

O-Esterification of *N*-hydroxylamine intermediates of nitroarene carcinogens is required for the exertion of biological effects. However, in the case of AAs, there is also evidence of formation of DNA adducts under reduction conditions that are not conducive to such esterification processes.

This subject is discussed below.

"In Vitro" Experiments. Formation of Adducts by Aristolochic Acids without Activation of the *N*-Hydroxylamine by O-Sulfation or O-Esterification. AAs can efficiently form DNA adducts under in vitro conditions where the activation of the *N*-hydroxylamine is not possible. For example, DNA adducts were formed by reduction of AAs with either zinc in potassium phosphate buffer (pH 5.8) at 37 °C or zinc in aqueous 1% acetic acid at 37 °C in the presence of DNA.[34-36] Likewise, enzymatic reduction of AAI by xanthine oxidase in the presence of hypoxanthine and calf thymus DNA also yielded DNA adducts.[11] The nitroreduction products of most nitroarenes are barely mutagenic unless they are activated by esterification;[27-32] however, a few nitroarenes seem to be mutagenic in bacteria after nitroreduction without esterification.[37-40]

As was the case in the reactions described above and the enzymatic generation of the 9-hydroxyaristolactam **6**,[11] aristoxazole was obtained under conditions that were not conducive to activation of the hydroxy group of the *N*-hydroxylamine by O-sulfation or O-acylation. Formation of the oxazinone **11** would provide an explanation for the apparent lack of activation with aristolochic acids during in vitro reductions, which will be discussed in Proposed Mechanism for the Formation of Aristoxazole via Oxazinone **11**.

Probable Routes of Aristoxazole Formation. In principle, it could be possible to account for the formation of aristoxazole by reduction of AAI with Zn in acetic acid using the mechanism of Scheme 1 in the following way. Under acidic conditions, *N*-hydroxyaristolactam **3** could ionize to nitrenium ion **4**, which would then be trapped by acetic acid. The resulting 9-acetoxy adduct could undergo hydrolysis of the lactam moiety to yield the acyclic form (10-amino-1-carboxyl derivative). Then, condensation of the 10-amino group with the 9-acetoxy group would generate aristoxazole. Thus, nitrenium ion **4** could be a precursor of both the 9-hydroxyaristolactam I (**6**) and aristoxazole (**15**). However, this possibility fails to take into account several factors.

The ionization of *N*-hydroxylamines is moderate under weakly acidic conditions at pH ∼6. Such cleavage should be even slower with the *N*-hydroxyaristolactam **3** because of "intramolecular acylation". The N—O bond heterolysis is energetically less favorable in arylamides than in the parent *N*-hydroxyarylamines. For example, studies with model esters of *N*-arylhydroxylamines established that the reaction rate of heterolysis of the N—O bond to give the nitrenium ion would be 10^6-fold slower when the NH group is replaced with the *N*-acetyl group.[41] Consistently, computational studies show that N-acylation is deactivating (as opposed to O-acylations that are activating).[42,43] Consequently, the N—O bond cleavage in the *N*-hydroxyaristolactam **3** would be strongly suppressed, and the **3**-to-**4** ionization without activation via O-esterification would be extremely slow for reactions such as the in

vitro reductions described above. The same problem is likely to occur in the formation of aristoxazole from the *N*-hydroxyaristolactam **3**. To form aristoxazole from **3**, the cleavage of the N—O bond (**3** to **4**) must be as fast as reduction (of **3** to **5** or of **9** to **10**) which does not seem to be the case. Thus, the participation of the *N*-hydroxyaristolactam **3** in the formation of aristoxazole seems unlikely.

The strongest evidence against the formation of aristoxazole from either the *N*-hydroxyaristolactam I (**3**) or the putative 9-acetoxy derivative of **5** is that these species would have to undergo hydrolysis of the stable lactam ring to form the acyclic 10-amino-1-carboxylic acid. In that way, the 9-acetoxy group can react with the C-10 amine. Ring opening of the lactam is difficult, at best. For example, the amide units of aristolactams and related compounds (e.g., benzanilide) are not cleaved when treated with boiling acetic acid. Furthermore, the opening of the lactam unit of aristolactams probably generates substantial strain from interactions of the substituents at C-9, C-10, and C-1. Clearly, opening the lactam ring is an energetically unfavorable process, which is very unlikely to occur under the reaction conditions that lead to the formation of aristoxazole. An alternative pathway for aristoxazole formation is proposed below.

Proposed Mechanism for the Formation of Aristoxazole via Oxazinone 11. The 9-hydroxyaristolactam I (**6**) and aristoxazole (**15**) are significant in vitro reduction products of AAI, accompanying the major product aristolactam I (**5**). It seems likely that these compounds were not formed by the simple dissociation of the *N*-hydroxyaristolactam **3** (without activation) for the reasons discussed above. If the reduction products 9-hydroxyaristolactam I and aristoxazole are congeners to the known AA—DNA adducts, both of them may be derived from a common nitrenium ion precursor. The high reactivity of AA intermediates during in vitro reductions suggests that aristoxazole is formed via the pathway shown in Scheme 2 in which the oxazinone **11**, which would be the activated form of the *N*-hydroxylamine **9**, forms the nitrenium ion. This pathway also avoids the need for the unfavorable ring opening of the lactam ring, which is a key step in the route based on *N*-hydroxyaristolactam **3** (Scheme 1).

Considerations about the Pattern of Adducts Formed. Only One Type of Aristolochic Acid Adduct Is Identified, whereas Nitroaromatic Compounds Usually Generate Two or More Types of Adducts. Oxidation of aromatic amines or amides or reduction of aromatic nitro compounds produces nitrenium ions with reactive sites that are targets for nucleophilic reagents. Although nitroarenes may produce several types of adducts,[44,45] the two that are most often encountered involve the N atom or the *ortho* ring carbon that can bind to C-8 or the amino group of the purine base, respectively.[44,45] For example, 6-nitrochrysene (**17**), which has an aromatic skeleton similar to AAs, forms adducts at the N and at the adjacent carbon (see arrows in formula **17**).[45,46] The carcinogenic *N*-acetyl-2-aminofluorene (**18**) also forms two adducts after being metabolically activated by N-hydroxylation followed by O-esterification (see arrows in formula **18**). Structure **18** resembles the *N*-hydroxyaristolactam **3** in that it too is an N-acylated compound.

A priori predictions of the base or carcinogen type of adduct formed are difficult because multiple factors are involved, which include, for example, DNA repair mechanisms and the stability of the adducts during isolation.[44,47−49] Nevertheless, the relative preferences of carcinogenic aromatic nitrenium ions for adduct formation with different DNA base sites have been predicted by computational studies.[49−51] All these theoretical studies point toward the specificity of the N-site of the nitrenium ion toward C-8 of purine bases as well as the specificity of the *ortho* C-site of the nitrenium ion toward the adenine and guanine amino group.

In contrast to typical nitroaromatic compounds, only one type of adduct is commonly encountered with AAs in which C-9 is

bound to either a guanine or adenine amino group (**7**, **7a**, **8**, and **8a**). The apparently preferred formation of C-9 adducts by AAs, as opposed to N-6 adducts formed by 6-nitrochrysene, suggests that the AAI carboxyl group plays a role in controlling the regiospecificity of the reaction and the pattern of adducts formed. Possible explanations are given below.

Tentative Explanation for the Formation of C-9 Adducts by Aristolochic Acids Based on the Oxazinone Pathway. There are two possible mechanisms that account for the regiospecificity. The reaction might occur via an S_N2' mechanism in which the intermediate **11** undergoes a concerted addition−elimination reaction upon being attacked by the nucleophile (H_2O or CH_3CO_2H). Alternatively, **11** could generate the nitrenium ion **12** as an "ion pair" in which the ring carbon will be the more reactive site for nucleophilic attack because of the influence of the carboxylic acid group.

(i) S_N2' Mechanism. Concerted Addition and Elimination without Formation of a Nitrenium Ion. In this mechanism, there is direct attack of acetic acid, or another nucleophile, on C-9 of the oxazinone **11** (see arrows in structure **11**) to give the imine **13**. Subsequently, tautomerization and condensation between the substituents at C-9 and C-10 would afford aristoxazole (**15**). This pathway would seem to be the less likely of the two on the basis of studies with small model molecules, which indicate that solvolysis of N—O bond cleavage occurs through an ionic pathway (S_N1' mechanism) involving a nitrenium ion.[52]

(ii) Regiospecificic Formation of the C-9 Adducts via a Nitrenium Ion Pair in Which the Nitrogen Is Blocked from Attack. "Ion pairs" or "electrostatic complexes" are implicated in the formation of nitrenium ions by carcinogens.[43,52,53] The nitrenium ions from AAs may have the intriguing ability to form a "tight ion pair" by intramolecular interaction of the cation with the carboxylate ion. A tight ion pair consists of a cation and an anion held strongly together by electrostatic attraction, and further stabilized by hydrogen bond interaction between the ions, which prevent the solvent from forming solvent-separated ions.[43,52] Consequently, tight ion pairs are insensitive to the presence of trapping agents (H_2O, Cl^-, and reducing agents) in the medium. The nitrenium ion **12** would be an ion pair in which the cation and the anion (the carboxylate group) occur in the same molecule. The regiospecificity of DNA base attack at C-9 may be due to the occurrence of this intramolecular ion pair that fixes the anion at one side of the reacting centers. The ion pair prevents the participation of the nitrenium nitrogen atom in reactions with nucleophiles, leaving the electron-deficient C-9 position as the only site for nucleophilic attack. In summary, formation of an intramolecular nitrenium ion pair could account for the generation of the dominant C-9 adducts with DNA. In contrast, other nitroarenes lacking the carboxyl group can generate adducts at both the nitrogen and *ortho* carbon. Although computational studies are needed to test this hypothesis, the proposed oxazinone **11** intermediate provides a plausible explanation not only for the unusual pattern of adducts from AAs but also for the activation processes needed to generate nitrenium ions.

Are *N*-Hydroxyaristolactams Toxic? *N*-Hydroxyaristolactams such as **3** and **3a** seem to be implicated in nitrenium ion formation and toxicity, but there is evidence that questions the suspected toxicity of *N*-hydroxyaristolactams. *N*-Hydroxyaristolactams have not been documented in the Aristolochiaceae; however, *N*-methoxy- and *N*-hydroxyaristolactams are stable compounds that have been isolated from *Piper* spp. (Piperaceae).[54−56] Of particular interest is *Piper umbellatum*, a medicinal plant widely used in Cameroon traditional medicine.[56] It contains significant amounts of *N*-hydroxyaristolactams (~100 mg/kg of air-dried branches).[56] Leaves, stems, inflorescences, and fruits of *P. umbellatum* are also used as food in many parts of Africa.[57] There have been no reports that this plant is toxic. Because the *N*-hydroxyaristolactams (**3** and **3a**)

are the immediate precursors of the nitrenium ions (**4a** and **4b**), they are expected to be as toxic as the AAs themselves because they can be activated in vivo by sulfotransferases and acyltransferases. The lack of toxicity of *N*-hydroxyaristolactam-containing plants and a number of additional questions concerning AA activation and toxicity remain unanswered.

The major reduction product of AAI in both chemical and biological reductions is aristolactam I (**5**). The AA−DNA adducts (**7**, **7a**, **8**, and **8a**) and 9-hydroxyaristolactam I (**6**) are byproducts from reactive intermediates generated during reduction of AAI. The new reduction product, aristoxazole (**15**), may also belong to the same family of adducts. The formation of aristoxazole probably occurs via a nitrenium ion pathway in which an acetic acid molecule attacks C-9 of the nitrenium ion pair **12**. Although the formation of aristoxazole may proceed via the *N*-hydroxyaristolactam **3** (Scheme 1), it seems more likely that the compound is formed from the oxazinone **11** (Scheme 2). The oxazinone **11**, which can be considered an activated form of the *N*-hydroxylamine, could also provide a rational explanation for adduct formation in in vitro systems that lack other means of activating the *N*-hydroxylamine. In addition to the aristolochic acid−DNA adducts generated via the *N*-hydroxyaristolactam pathway (Scheme 1), the oxazinone **11** and the nitrenium ion pair **12** could also serve as important electrophiles involved in DNA adduct formation in vivo.

Experimental Section

General Experimental Procedures. UV spectra were recorded with an Agilent 8453 UV−vis spectrophotometer. IR spectra were recorded on a Perkin-Elmer Spectrum 2000 instrument with KBr pellets. NMR spectra were recorded on a Bruker 400 MHz FT-NMR spectrometer in DMSO with TMS as an internal standard. ESI and APCI mass spectra were recorded on a Thermo Scientific LCQ Deca XP MAX instrument and Thermo Scientific DSQ mass spectrometer, respectively. HPLC analysis was conducted with a Thermo-Finnigan chromatograph (Thermo Electron Corp., San Jose, CA). The chromatograph consisted of a SpectraSystem SMC1000 solvent delivery system, a vacuum membrane degasser, P4000 gradient pumps, and an AS3000 autosampler. Column effluent was monitored at 254 nm with a SpectraSystem UV6000LP variable-wavelength PDA detector and ChromQuest version 4.1. Analytical separations were performed using a C18 RP Hypersil GOLD column (RP5, 250 mm × 4.6 mm, pore size of 5 μm, Thermo Electron Corp.). HPLC solvents were employed without further purification. They were filtered through a 0.22 μm Millipore membrane. The water used was deionized and filtered through a nylon membrane (0.45 μm). The following eluting systems were used: system 1, 0.1% TFA in MeCN (A), 0.1% TFA in H_2O (B), linear gradient from 10 to 100% A over 120 min; system 2, MeCN (A), 0.1 M NH_4OAc buffer (pH 7.5) (B), linear gradient from 10 to 100% A over 120 min; system 3, 0.1% TFA in MeCN (A), 0.1% TFA in H_2O (B), linear gradient from 10 to 100% A over 30 min. The flow rate was 1.0 mL/min at room temperature. In systems 1−3, aristolactam I exhibited t_R values of 48.4, 47.6, and 19.0 min, respectively.

Molecular mechanics calculations were conducted using Spartan '06 for Windows (Wave function Inc.). The calculations were conducted at the B3LYP 6311+G** level.

Aristolochic acid I was obtained from *Aristolochia argentina* as previously described and purified by recrystallization from dioxane.[13]

9-Methoxy-7-methyl-2*H*-1,3-oxazolo[5′,4′-10,9]phenanthro[3,4-*d*]-1,3-dioxolane-5-carboxylic Acid. Aristoxazole (15). Aristolochic acid I (**1**) (15.3 mg) was refluxed for 50 min with zinc powder (70 mg) and glacial HOAc (1 mL) with magnetic stirring. The reaction mixture was treated with H_2O (5 mL) and EtOAc (5 mL), shaken, and centrifuged. The upper phase was removed, washed with H_2O, and extracted with aqueous 5% $NaHCO_3$. Evaporation to dryness of the EtOAc phase gave a yellow residue of aristolactam I (**5**) (9.1 mg). The $NaHCO_3$ solution containing aristoxazole was acidified to pH 3 with dilute HCl and extracted with EtOAc. Removal of the solvent from the organic phase under vacuum yielded a white residue of aristoxazole (3.3 mg): colorless needles (isoPrOH); HPLC t_R = 46.60 min (system 1), 24.1 min (system 2), 18.2 min (system 3); UV/PDA λ_{max} 251, 300, 326, 363, 383 nm; UV (H_2O/NaOH) λ_{max} 253.0, 299.5, 325.1, 363.8, 382.0 nm; IR (KBr) ν_{max} 1668, 1587, 1459, 1310, 1104, 1018 cm^{-1}; APCI/MS *m/z* 380.1101

[M + C_2H_5]$^+$ (calcd for $C_{21}H_{18}NO_6$, 380.1134), 352.0824 [M + H]$^+$ (calcd for $C_{19}H_{14}NO_6$, 352.0821), 334 [M + H − H_2O]$^+$, 308 [M + H − CO_2]$^+$; ESI/MS (MeOH) positive mode 352.0 [M + H]$^+$, 406.0 [M + MeOH + Na]$^+$, 703.0 [2M + H]$^+$, 725.0 [2M + Na]$^+$; EIMS *m/z* (relative intensity) 350.9 [M]$^+$ (98), 306.9 [M − CO_2]$^+$ (66), 291.9 [M − CO_2 − CH_3]$^+$ (100), 263.9 [M − CO_2 − CH_3 − CO]$^+$ (15).

Aristoxazole Methyl Ester. Aristoxazole was treated with ethereal diazomethane as usual to give aristoxazole methyl ester: t_R 57.3 min (system 1); UV/PDA λ_{max} 254, 300, 324, 363, 384 nm; APCI/MS *m/z* 394.1298 [M + C_2H_5]$^+$ (calcd for $C_{22}H_{20}NO_6$, 394.1291), 366.0952 [M + H]$^+$ (calcd for $C_{20}H_{16}NO_6$, 366.0978), 365.0872 [M]$^+$ (calcd for $C_{20}H_{15}NO_6$, 365.0899), 350.0864 [M − MeO]$^+$.

Reduction at Lower Temperatures. Aristoxazole can also be produced when the AAI/Zn/HOAc mixture is heated at lower temperatures. HPLC analyses of reaction mixtures and yields of products (**5** and **15**) revealed that the ratio of aristolactam I to aristoxazole on heating at 60 °C, at 90 °C, and at reflux (118 °C) ranged from 2.2−3 to 1. In contrast, the ratio of aristolactam I to aristoxazole was 6:1 when the reaction was conducted at room temperature (25 °C).

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