Investigation of the metabolism and reductive activation of carcinogenic aristolochic acids in rats

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Running title: Metabolism and reductive activation of carcinogenic AAs.

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Abbreviations used are: AAs, aristolochic acids; AAIa, aristolochic acid Ia; AAN, aristolochic acid nephropathy; SPE, solid-phase extraction; HPLC, high performance liquid chromatography; ESI-MS, electrospray ionization-mass spectrometry; LC-MS-MS, liquid chromatography-tandem mass spectrometry; Qq-TOF, hybrid quadrupole-time of flight mass spectrometry; HR-MS, high resolution-mass spectrometry.
Abstract

The metabolic activation of aristolochic acids (AAs) that have been demonstrated to be mutagenic and carcinogenic was investigated. In vitro metabolism study indicated that AAs were metabolized to N-hydroxyaristolactam which could be either reduced to aristolactams or rearranged to 7-hydroxyaristolactams via Bamberger rearrangement. In vivo metabolism study is important because the intermediates (aristolactam-nitriumion) of the nitroreduction process are thought to be responsible for the carcinogenicity of AAs. LC-MS and LC-MS-MS were applied to the analyses of a series of positional isomers of hydroxyaristolactams in rat urine samples after the in vivo study of AAs. Three hydroxylated metabolites of aristolactam II and two hydroxylated metabolites of aristolactam I were identified. The structures of the positional isomers were elucidated from the interpretation of MS-MS spectra and the theoretical calculations of molecular augmentation. In addition, several new metabolites were detected in the rat urine by HR-MS and MS-MS, including those from the decarboxylation of AAs and the conjugations of acetylation, glucuronidation and sulfation of aristolochic acid Ia (AAIa).
Aristolochic acids (AAs) are a mixture of nitrophenanthrene carboxylic acid derivatives found primarily in the genus *Aristolochia* (Ong et al., 2000). Major components of AAs include aristolochic acid I (8-methoxy-6-nitro-phenanthro (3,4-\textit{d})-1,3-dioxolo-5-carboxylic acid, AAI) and aristolochic acid II (6-nitro-phenanthro (3,4-\textit{d})-1,3-dioxolo-5-carboxylic acid, AAII) that differ by a methoxy group (Fig. 1). Herbal products containing AAs have long been used for the treatment of tumour and snake bites (Kupchan and Doskotch, 1962; Rocker and Chung, 1975). Their anti-inflammatory properties were of great interest for pharmaceutical companies in Germany for 20 years until they were observed to be carcinogenic in rats (Mengs et al., 1982). Since then, extensive studies have been conducted in rodents for the probable human carcinogenicity (Bieler et al., 1997; Mengs, 1988; Pfau et al., 1990b, 1990c, 1991; Stiborova et al., 1994, 2001, 2002, 2003). The metabolic activation of AAs is a unique example of intramolecular acetylation, which leads to the ultimate carcinogen. It was postulated that an intermediate generated from nitroreduction of AAs, namely aristolactam-nitriumion with a delocalized positive charge, is the ultimate carcinogen. The aristolactam-nitriumion was found to bind to the exocyclic amino group of purine nucleotides in DNA. The DNA-AA adducts were detected in internal organs of patients suffered from the aristolochic acid nephropathy (AAN) (Bieler et al., 1997; Stiborova et al., 2002).

It is known that carcinogenic nitroaromatics (R-NO\textsubscript{2}) may be metabolized to \textit{N}-hydroxylamine (R-NHOH) before being further reduced to aromatic amines (R-NH\textsubscript{2}) by drug metabolism (Kato et al., 1969; Mitchelmore et al., 1998; Purohit and Basu, 2000; Sugimura et al., 1966; Tatsumi et al., 1986) and microbial activities (Hasegawa et al., 2000; Hughes et al., 1999). Previous studies have shown that the major metabolites of
AAs in rats were produced from nitroreduction, O-demethylation and denitration. O-demethylation and hydroxylation were also observed in aristolactam I and aristolactam II, respectively, producing aristolactam Ia as the common end-stage reductive metabolite (Chan et al., 2006; Krumbiegel et al., 1987). The formation of hydroxylamine upon metabolic activation is believed to be the key step for the carcinogenicity. The nitroreduction plays a key role on biotransformation to the ultimate carcinogen of AAs (Pfau et al., 1990b, 1991; Schmeiser et al., 1986). However, little is known about the mechanism underlying the bioactivation of AAs. The identification of 7-hydroxyaristolactam I after incubating AAI with xanthine oxidase led to the postulation of a reduction mechanism for AAI (Pfau et al., 1990b). It was also proposed that the carcinogenic activity of AAs arose from their hydroxylamine (Schmeiser et al., 1986). To the best of our knowledge, no in vivo study regarding the metabolic activation of AAs is available in the literature.

Lactam formation was the major in vivo and in vitro metabolic pathway of AAs after nitroreduction (Chan et al., 2006; Krumbiegel et al., 1987). With the support of spectroscopic data from in vitro incubations, Pfau (1990b) postulated that AAs were metabolized to N-hydroxyaristolactam that might be further reduced to aristolactams or rearranged to 7-hydroxyaristolactams via Bamberger rearrangement. The 7-hydroxyaristolactams could also be produced from the 7-hydroxylation of the aristolactams. This paper reports results of the investigation of in vivo metabolic pathways by analyzing a series of positional isomers of hydroxyaristolactams. In addition to the LC-MS and MS-MS analyses, theoretical computations on the hydroxylated metabolites of aristolactams were also performed to help the isomer assignment. In
addition, several new phase II metabolites of AAs were identified in the rat urine, for the first time.
Materials and Methods

Chemicals

Aristolochic acids (mixture of AAI and AAII, 1:1 approx) were purchased from Acros Organics (NJ, USA). Aristolactam I and aristolactam II were prepared by the method described previously (Chan et al., 2006). Acetic acid and ammonium acetate were obtained from Panreac (Barcelona, Spain). Silica-bonded C<sub>18</sub> solid-phase extraction (SPE) cartridges (500 mg) were obtained from Waters (Milford, USA). HPLC-grade methanol was purchased from Tedia (OH, USA). Water was produced by a Milli-Q Ultrapure water system with the water outlet operating at 18.2 MΩ (Waters, Milford, USA).

Animal experiment and sample preparation

Male Sprague–Dawley rats (n=4) weighing 200-220 g were obtained from the Laboratory Animal Service Centre, Chinese University of Hong Kong. The animals were kept in a controlled room with constant temperature (23°C ±1) and artificial dark/light cycles. The rats were fasted for overnight but water was given ad libitum before a single oral dose of 6 mg of AAs in 0.5% NaHCO<sub>3</sub> solution. The dosed rats were then housed in metabolic cage which allowed the separated collection of urine and faces. The rat urine from 0 to 24 hour after the oral administration were collected and kept at -20°C.

Urine sample after thawing was filtered through a cellulose filter disc (25 mm, 0.2 µm) prior to the SPE. 5 mL of urine was loaded onto a pre-conditioned SPE cartridge and washed sequentially with 3 mL of water, 2 mL of water/methanol (4:1) and eluted with 3 mL of methanol. The methanolic eluent were collected and evaporated to dryness under a
stream of nitrogen. The residual was dissolved in 50 µL of methanol and then centrifuged at 13,000 rpm for 3 min prior to the LC-MS analysis. Calibration plots for the reductive metabolites of AAs were obtained from LC-MS determination of aristolactam I and aristolactam II at concentrations of 0.2, 1, 2.5, 5 and 10 µg/mL in blank urine matrix. Linear regression analysis gave calibration curves that were used to calculate the amount of aristolactam I and aristolactam II excreted in rat urine. To determine the recovery of the SPE extraction process for aristolactams, aristolactam I and aristolactam II were spiked to blank urine (2 mL) at a final concentration of 0.5 µg/mL (n=5), processed and measured in the same way as the samples from the AAs dosed rats.

**LC-MS analysis**

HPLC experiments were performed on a HP1100 HPLC system equipped with an autosampler and a micropump (Agilent Technologies, San Francisco, USA). A reverse-phase column (Phenomenex, Lunar C18, 150 mm × 2.0 mm, 5 µm) was used to separate AAs and their metabolites. The compartment of the auto-sampler was set at 4°C. The sample injection volume was 8 µL. The mobile phase system consisted of two components, with component I being 0.2% acetic acid and 5 mM ammonium acetate (A), and component II being 0.2% acetic acid in methanol (B). The solvent gradient started from 20% B and held for 5 minutes, then programmed to 80% B in 5 minutes, and held for another 15 minutes before re-conditioning, at a flow rate of 200 µL/min. The effluent of the first 5 minutes from the LC was diverted to waste.

Electrospray ionization mass spectrometry (ESI-MS) and MS-MS analyses were conducted on a Qq-TOF tandem mass spectrometer (API Q-STAR Pulsar i, Applied
Biosystems, Foster city, USA). TurboIonspray parameters for positive ion mode ESI-MS were optimized as follow: ionspray voltage (IS) 5,300 V, declustering potential I (DPI) 20 V, declustering potential II (DPII) 15 V, focusing potential (FP) 70 V. The mass range was from m/z 200 to 800. Depending on the compound stability, the collision energy for product ion scans of AAs and their metabolites varied from 15 eV to 45 eV for the MS-MS experiments. The ion source gas I (GSI), gas II (GSII), curtain gas (CUR) and collision gas (CAD) were set at 30, 15, 30 and 3, respectively. The temperature of GSII was set at 350°C.

**Theoretical calculations**

The structure stability, vibration frequency, total energy, relative energy and dipole moment for hydroxyaristolactams were obtained from theoretical calculations using Gaussian 03 (Revision C.02, 2004, Gaussian, Inc., Wallingford CT, USA). The geometry optimization and vibrational frequency analyses were performed by using the density functional theory (DFT) at the B3LYP/6-31G(d) level in both aqueous and gas phases. The integral equation formalism-polarizable continuum solvation model (IEF-PCM), was used to treat the solvent effect in the aqueous phase. An empirical scaling factor of 0.9804 was used to correct the zero-point vibrational energies. The absence of imaginary frequencies in the theoretical calculations verified that all the structures were at their true minima at the specified computational level.
Results

Quantitative analysis of aristolactams in urine samples

The synthesized standards reported previously (Chan et al., 2006) were used for the quantitative analysis of aristolactam I and aristolactam II in the rat urine extracts. Calibration curves for aristolactam I and aristolactam II in the range of 0.2 µg/mL to 10 µg/mL were established by plotting the peak area of the [M+H]^+ ion versus the corresponding concentrations. Linear response (R^2 ≥ 0.999) was obtained for both analytes. The recoveries of aristolactam I and aristolactam II at the level of 0.5 µg/mL (n=5) were 93.5±4.0 % and 93.2±4.1 % (mean ±SD), respectively. In the rat urine samples collected after the oral administration of AAs (n = 4), 0.04±0.01µg/mL of aristolactam I and 0.10±0.03 µg/mL of aristolactam II were detected. Lower concentration of aristolactam I than aristolactam II might be due to the less availability of AAI. It has been reported that AAI could be easily converted to aristolochic acid Ia via demethylation (Fig. 1) (Chan et al., 2006; Schmeiser, 1986).

Identification of AAs metabolites

Decarboxylated metabolites of AAs

New metabolites from decarboxylation of AAs (Fig. 1) were detected in the rat urine samples, for the first time. Fig. 2 shows the extracted ion chromatogram of the decarboxylated metabolite of AAI (M1) (Fig. 2A) and its MS-MS spectrum (Fig. 2B). The MS-MS analyses of the decarboxylated metabolite of AAI resulted in the dissociate loss of –CH₃ moiety, producing the fragment ion at m/z 300 as the base peak. The characteristic loss of -NO₂ and –OCH₃ moieties were also observed from the fragment ions at m/z 268 and m/z 283, respectively.
Phase II metabolites of AAIA

Three new phase II metabolites of aristolochic acid Ia (AAIA, M2) formed by acetylation (M3), glucuronidation (M4) and sulfation (M5) (Fig. 1) were detected in the rat urine. The measured HR-MS data of the [M+NH₄]^+ ion of these metabolites (m/z 387.0802, 425.0259 and 521.1089) matched the theoretical mass of the corresponding elemental composition (m/z 387.0828, 425.0291 and 521.1044), with a mass difference of 6.8 ppm, 7.5 ppm and 8.7 ppm, respectively. Fig. 3 shows the extracted ion chromatograms of the detected phase II metabolites. The MS-MS analysis revealed the characteristic fragmentation at the conjugation linkage. The dissociate loss of 42 Da (Fig. 4A), 176 Da (Fig. 4B) and 80 Da (Fig. 4C) from the intact molecules signified the fragmentation loss of acetyl, glucuronide and sulphate groups, respectively.

Similar to the observation in the MS-MS analyses of AAIA (M2) and AAs (Chan et al., 2006), the metabolites formed by acetylation (M3) and glucuronidation (M4) of AAIA demonstrated the common neutral loss of water and carbon dioxide after the cleavage of the conjugated moiety, producing major fragment ions at m/z 310 and m/z 284, respectively. This phenomenon, however, was not observed for sulphate conjugated metabolite (M5) in the MS-MS spectrum (Fig. 4C). The tandem mass spectrometric analysis of the [M+NH₄]^+ ion (m/z 425) of the sulphate conjugated metabolite showed a major fragment ion at m/z 408. The [M-SO₃+H]^+ ion at m/z 328 from the loss of sulphate group was also observed as the base peak.

Hydroxylated metabolites of aristolactams

Three hydroxylated metabolites of aristolactam II (M6, M7 and M8) and two hydroxylated metabolites of aristolactam I (M9 and M10) were detected in the rat urine.
(Fig. 5). The identification was based on the observation of [M+H]^+ ion and [2M+H]^+ dimer ion. The MS-MS spectra of the hydroxylated metabolites of aristolactam II (M6, M7 and M8) were acquired from the protonated molecular ion at \( m/z \) 280 under the identical collision energy (40 eV). Similar fragmentation patterns were obtained (Fig. 6), except that fewer fragment ions were observed for M7. The common fragmentation included the water loss and the loss of CH\(_2\)O\(_2\), CH\(_2\)O\(_2\)+CO moieties. The common ion peak at \( m/z \) 178 represents the phenanthrene moiety from the aristolactam isomers. A fragment ion at \( m/z \) 222 was also observed for M6 and M8, resulted from the dissociative loss of both −OCH\(_2\) and −CO moieties (Fig. 6A and Fig. 6C).

Similarly, the MS-MS spectra of the hydroxylated metabolites of aristolactam I (M9 and M10) were acquired from the parent ion at \( m/z \) 310. The MS-MS analysis of M9 revealed a characteristic peak at \( m/z \) 292 corresponding to the water loss (18 Da). In contrast, no peak of water loss was observed for M10. The [M-CH\(_3\)+H]^+ ion at \( m/z \) 295 was observed as the base peak in the MS-MS spectrum of M10, along with an ion peak corresponding to the loss of -CONH moiety (data not shown). This fragmentation pattern was similar with the MS-MS fragmentation pathway of aristolactam I (Chan et al., 2006). Based on the MS-MS data and the results obtained from previous metabolism studies of AAI (Arlt et al., 2002; Pfau et al., 1990b), the peaks of the hydroxylated metabolites of aristolactam I (Fig. 5B) at 22.49 min (M9) and 23.50 min (M10) were identified as \( N \)-hydroxypyrrolidinone I and 7-hydroxyaristolactam I, respectively.

**Investigations of the isobaric hydroxylated metabolites of aristolactams by theoretical calculations**
The molecular structure, relative energy, and dipole moment of the three hydroxylated metabolites of aristolactam II (M6, M7 and M8) in aqueous and gas phases are summarized in Table 1. Among the three hydroxylated metabolites of aristolactam II postulated in previous mechanism studies of AAs (Arlt et al., 2002; Pfau et al., 1990b), aristolactam Ia (M6) had the largest dipole moment (7.5 Debye) while 7-hydroxyaristolactam had the smallest (4.6 Debye). All of them showed similar orientation of the electric dipole moment. N-hydroxyaristolactam II had the highest relative energy (38 kJ/mol) while aristolactam Ia had the smallest (0 kJ/mol). The absence of imaginary frequencies in our theoretical calculations verified that all the structures were at their true minima at the specified computational level.
Discussion

The carcinogenic and mutagenic properties of AAs have been extensively investigated. However, only limited data on \textit{in vivo} metabolism have been reported (Schmeiser, 1986; Krumbiegel, 1987). Arlt (2002) reviewed the nephrotoxic and carcinogenic mechanism of AAs in both rodents and humans, and pointed out that the phase II metabolism of AAs had not been adequately studied. Recently, we reported the identification of three new phase II metabolites of AAs, namely \textit{N}-glucuronides of aristolactam Ia and aristolactam II, and \textit{O}-glucuronide of aristolactam Ia (Chan et al., 2006). Herein we report our continuing work on the metabolic pathway of AAs after the oral administration on Sprague–Dawley rats.

In this study, two major metabolites from the nitroreduction of AAs (aristolactam I and aristolactam II) were quantified in the rat urine. The quantitative analysis of aristolactams is of great interest not only because the lactam formation was the major \textit{in vivo} and \textit{in vitro} metabolic pathway of AAs after the nitroreduction (Chan et al., 2006; Krumbiegel et al., 1987) but also because the intermediates (aristolactam-nitriumion) of the reduction process plays a key role on biotransformation to the ultimate carcinogen of AAs (Pfau et al., 1990b, 1991; Schmeiser et al., 1986). The preliminary quantitative analysis indicated that sub µg/mL level of aristolactam I and aristolactam II existed in the rat urine samples collected after the oral administration of AAs. The concentration of aristolactam II was around 2 times higher than that of aristolactam I, probably because the parent compound of aristolactam I (i.e., AAI) could be easily demethylated to aristolochic acid Ia (Fig. 1). Further study would focus on the determination of mass balance of AAs and the lactam metabolites after the \textit{in vivo} experiments. The quantitative analysis might provide
important information of carcinogenesis of AAs because the aristolactams are associated
with the formation of DNA adducts (Arlt et al., 2002).

Several \textit{in vivo} metabolites were identified in the rat urine samples for the first time. In
addition to the observation of decarboxylative metabolite of AAs, three new conjugated
metabolites formed by acetylation, glucuronidation and sulfation of AAIa were identified.
A series of positional isomers were detected as hydroxyaristolactam I and
hydroxyaristolactam II. All metabolites were characterized from the HR-MS and MS-MS
analyses.

Being \textit{peri}-substituted nitrophenanthrene carboxylic acids, AAs are weak mutagens in \textit{S.
typhimurium} strain TA100 when compared with other nitroaromatic compounds (Purohit
and Basu, 2000). It has been reported that steric repulsion between the carboxyl and nitro
group rendered the nitro group to deviate from co-planarity with reference to the aromatic
rings (Pfau et al., 1990a). Nitroaromatics with perpendicularly orientated nitro group has
been proved to be poor substrates for nitroreductases in mutagenicity assay (Fu et al.,
1985), which might be the reason for the observed low mutagenicity of AAs. However,
the decarboxylation of AAs (M1) alleviates the steric repulsion experienced by the nitro
group, making it less deviated from co-planarity with reference to the aromatic rings,
which might enhance their mutagenicity.

Conjugation is one of the most important routes for the elimination of xenobiotics from
biological systems. The conventional methodology for the analysis of conjugated
metabolites comprises a chemical or enzymatic hydrolysis step. Combining the high
separation efficiency of HPLC and the soft ionization technology of ESI-MS, LC-MS
provides a sensitive and efficient way for direct analysis of phase II metabolites in the
complex sample matrix. In our previous study, three glucuronide conjugates of arylactams were detected and characterized (Chan et al., 2006).

An extensive O-demethylation has been observed for AAI in both in vitro and in vivo studies, producing AAIA as the metabolite (Chan et al., 2006; Krumbiegel et al., 1987; Schmeiser et al., 1986). However, the metabolic fate of AAIA is not well understood apart from the lactam formation, which produces arylactam IA. Herein, we report the first identification of three conjugated metabolites formed by acetylation (M3), glucuronidation (M4) and sulfation (M5) of AAIA in rat urine after the oral administration of AAs. The phase II metabolites showed very characteristic fragmentation at the conjugation linkage in MS-MS experiments. The loss of 42 Da (Fig. 4A), 176 Da (Fig. 4B) and 80 Da (Fig. 4C) from the intact molecules signified the neutral loss of acetyl, glucuronide and sulphate groups, respectively. The assignments made for the conjugates were further supported by the HR-MS data. The phenomenon of water and carbon dioxide loss after the cleavage of the conjugated moieties in LC-MS-MS analysis was in consistent with the data obtained when AAs and AAIA were analyzed (Chan et al., 2006).

The mechanism underlying N-hydroxylation of arylamines and the nitroreduction of nitropolycyclic hydrocarbons to aryl hydroxyamines has been considered common. Similar to other nitroaromatics, nitroreduction is the crucial step in metabolic activation of AAs to their ultimate carcinogen. Being the first example of intra-molecular acetylation upon metabolic activation, the mechanism for the in vitro reductive conversion of AAI to arylactam I was postulated (Pfau et al., 1990b). It was proposed that AAI was first metabolized to its N-hydroxyaristolactam I, which was either reduced to arylactam I or underwent Bamberger rearrangement to give 7-hydroxyaristolactam I.
However, the intermediate N-hydroxyaristolactam I had not been identified probably because of the lack of analytical sensitivity.

In this study, three hydroxylated metabolites of aristolactam II (M6-M8) (Fig. 5A) and two hydroxylated metabolites of aristolactam I (M9, M10) (Fig. 5B) were detected in the rat urine. Based on the previous metabolism study of AAs (Arlt et al., 2002; Pfau et al., 1990b), the hydroxylated metabolites from aristolactam II (MW 279) were identified as aristolactam Ia, N-hydroxyaristolactam II and 7-hydroxyaristolactam II, while those from aristolactam I (MW 309) as N-hydroxyaristolactam I and 7-hydroxyaristolactam I. However, distinguishing the positional isomers was challenge because the corresponding authentic standards were not available. Theoretical computation was therefore performed to help the assignment of the hydroxyaristolactams in the LC-MS chromatograms.

The reverse-phase liquid chromatographic retention time generally depends on the polarity and structural parameters (e.g., size/shape) of the eluted compounds (Kaliszan et al., 1986). Of a congeneric series of analytes, the more polar compounds generally have less retention under the identical chromatographic conditions. The chemical polarity can be expressed in terms of electronic properties such as dipole moment and polar surface area. Compounds with larger dipole moment tend to have shorter retention time (Kaliszan et al., 1986; Niessen, 1999). For the three hydroxylated metabolites of aristolactam II (M6-M8), the calculated dipole moments of aristolactam Ia, N-hydroxyaristolactam II and 7-hydroxyaristolactam II in the aqueous phase were 7.5, 6.1 and 4.6 Debye, respectively, with a similar orientation (Table 1), indicating that aristolactam Ia might have the highest polarity. Accordingly, the reversed-phase liquid chromatographic peaks
at retention time of 21.15 min, 22.77 min and 24.02 min were assigned to be aristolactam Ia (M6), N-hydroxyaristolactam II (M7) and 7-hydroxyaristolactam II (M8), respectively. The isomer identification of the three hydroxylated metabolites of aristolactam II (M6-M8) was further confirmed by the comparison of the MS-MS results (Fig. 6) together with the relative energy values obtained from the computation calculation (Table 1). The MS-MS data under the identical collision energy were in agreement with the molecular stability represented by the calculated relative energy. The most stable aristolactam Ia with the lowest relative energy had the most intensive parent ion peak at m/z 280 (Fig. 6A), while the least stable N-hydroxyaristolactam II showed intensive fragmentation (Fig. 6B). Moreover, the MS-MS fragmentations of aristolactam Ia (M6) (Fig. 6A) and 7-hydroxyaristolactam II (M8) (Fig. 6C) were similar but far more intensive than that of N-hydroxyaristolactam II (M7) (Fig. 6B), which agreed well with the proposal that M6 and M8 were the same type of (phenolic) compounds, while M7 was different (N-hydroxy).

Similar to AAI, a reductive mechanism for converting AAII to aristolactam II and related hydroxyaristolactams was suggested. AAII was metabolized to N-hydroxyaristolactam II and then either rearranged to 7-hydroxyaristolactam II via Bamberger rearrangement, or further reduced to aristolactam II. The in vivo metabolic pathway of AAs producing aristolactams and related hydroxylated aristolactams is summarized in Fig. 7. Because the position of carbon 8 in AAI was blocked by a methoxy group, further oxidation at this position was not possible. Therefore, only two hydroxyaristolactams were produced from AAI while three were produced from AAII, which was in consistent with the experimental results. Identification of the two hydroxylated metabolites of aristolactam I was confirmed with the MS-MS analysis (see the description in “Results”).
In summary, the *in vivo* metabolic pathway underlying the nitroreduction of AAs to the corresponding aristolactams was proposed. The animal study revealed extensive phase II metabolism of AAIA that was the demethylated metabolite of AAI. In addition to the identification of the metabolites from decarboxylation of AAs, a series of positional isomers were detected as hydroxyaristolactams. Interpretation of LC-MS and MS-MS results combined with theoretical molecule computation was used for the identification of the hydroxyaristolactam metabolites. The results from this work may represent one step forward in understanding the metabolism and bioactivation of AAs.
Reference


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Footnotes

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Figure legends

Figure 1. A schematic illustration of AAI metabolism.

Figure 2. Extracted ion chromatogram of the decarboxylated metabolite of AAI (M1) at m/z 315 (A) and its MS-MS spectrum (B).

Figure 3. Extracted ion chromatograms of the phase II metabolites of AAla from acetylation (M3, m/z 387, A), glucuronidation (M4, m/z 521, B) and sulfation (M5, m/z 408, C).

Figure 4. MS-MS spectra of the phase II metabolites of AAIa from acetylation (M3, A), glucuronidation (M4, B) and sulfation (M5, C).

Figure 5. Extracted ion chromatograms of the hydroxylated metabolites of aristolactam II and aristolactam I at m/z 280 (A) and m/z 310 (B), respectively.

Figure 6. MS-MS spectra of the hydroxylated metabolites of aristolactam II, (M6, A; M7, B; and M8, C) under the identical collision energy (40 eV).

Figure 7. Postulated pathway for the in vivo metabolic activation of AAs.
Table 1. The molecular structure, relative energy (RE), and dipole moment (DM) of the three hydroxylated metabolites of aristolactam II at the DFT B3LYP/6-31G(d) level in the aqueous and gas phases using Gaussian 03. The electric dipole moment vectors in the aqueous phase are also displayed with arrow.

<table>
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<th></th>
<th>Aristolactam Ia</th>
<th>N-hydroxyaristolactam II</th>
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Aristolochic acid I

M3

Aristolochic acid Ia (M2)

M4

M5

Acetylation
Acetyl-CoA
CoA-SH

Sulfation
PAPS
PAP

Glucuronidation
UDPGA
UDP
Figure (A) shows the chromatogram with peaks at various times and mass-to-charge ratios (m/z). The peaks correspond to different ion forms:

- \([\text{M-CH}_3^{+}\text{NH}_4]^+\)
- \([\text{M-OCH}_3^{+}\text{NH}_3]^+\)
- \([\text{M-NO}_2^{+}\text{NH}_3]^+\)

Figure (B) presents the mass spectrum with mass-to-charge ratios (m/z) and corresponding intensities. The peaks at m/z values of 123.01, 139.07, 223.06, 255.15, 268.05, 283.08, and 315.10 indicate different ion forms:

- \([\text{M-CH}_3^{+}\text{NH}_4]^+\) at 300.09 amu
- \([\text{M-CH}_3^{+}\text{NH}_4]^+\) at 22.02 amu
- \([\text{M-CH}_3^{+}\text{NH}_4]^+\) at 244.09 amu
- \([\text{M-CH}_3^{+}\text{NH}_4]^+\) at 240.06 amu
- \([\text{M-CH}_3^{+}\text{NH}_4]^+\) at 268.05 amu
- \([\text{M-CH}_3^{+}\text{NH}_4]^+\) at 283.08 amu
- \([\text{M-CH}_3^{+}\text{NH}_4]^+\) at 315.10 amu

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aristolochic acid I (AAI): R=OCH₃,
aristolochic acid II (AAII): R=H

N-hydroxyaristolactam II (M7, MW 279),
N-hydroxyaristolactam I (M9, MW 309)

7-hydroxyaristolactam II (M8, MW 279),
7-hydroxyaristolactam I (M10, MW 309)

aristolactam Ia (M6, MW 279)
aristolactam II (M11, MW 263),
aristolactam I (M12, MW 293)