CYTOCHROME P-450 AND PEROXIDASE OXIDIZE DETOXICATION PRODUCTS OF CARCINOGENIC ARISTOLOCHIC ACIDS (ARISTOLACTAMS) TO REACTIVE METABOLITES BINDING TO DNA IN VITRO

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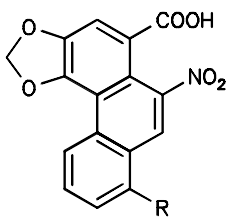
We report the analysis of DNA adducts formed from aristolactams I and II, which are the final metabolites derived from carcinogenic aristolochic acids in vivo, after their oxidation by microsomal cytochrome P-450 and horseradish peroxidase in vitro. DNA adducts were detected and quantified using the nuclease P1-enhanced variation of the ^{32}P-postlabeling assay. Quantitative analysis revelead that the extent of modification of DNA by aristolactams activated by peroxidase was more than one order of magnitude higher than for activation by microsomal cytochrome P-450. Peroxidase catalyzes the formation of active oxygen in the presence of NADH, H_2O_2 and aristolactams. Aristolactams are also oxidized by mammalian peroxidase prostaglandin H synthase. The possible role of aristolactams in carcinogenesis induced by aristolochic acid is discussed.

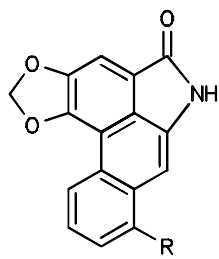
Aristolochic acids are derivatives of 3,4-methylenedioxy-10-nitro-1-phenanthrenecarboxylic acid. They are found in the roots and leaves of *Aristolochia* species[1]. These plants have been used since antiquity in obstetrics and to enhance the body's own defence mechanisms[2]. Until 1981 aristolochic acids were marketed as pharmaceutical specialities consisting of plant extracts from such species as *Aristolochia clematitis* or in the form of a mixture of aristolochic acids I and II. Besides aristolochic acid I and II the extracts of *Aristolochia* species also contain several derivatives of these substances, their aristolactams being the major components[3,4]. In 1982 Mengs and coworkers[5,6] reported aristolochic acids to be a potent carcinogen in rats, producing tumors in the forestomach and also in the kidney and lower urinary tract. Further carcinogenicity studies with aristolochic acid I alone demonstrated a high incidence of forestomach and ear-duct carcinomas in rats[7]; corresponding studies with aristolochic acid II have not yet been carried out. Because of these findings in experimental animals, pharmaceuticals containing aristolochic acids were withdrawn from the market.

Reductive metabolism of aristolochic acid I and II yielding aristolactams as final products was proposed to be the main activating pathway of these carcinogens in animals in vivo[8,9]. Recently, we were able to identify the main adducts formed in the target organ (forestomach) in rats in vivo as 7-(deoxyguanosin-N^2-yl)aristolactam I (dG-AA I), 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI) from aristolochic acid I and corresponding adducts (dG-AA II and dA-AA II) from aristolochic acid II (refs[8–10]). A cyclic *N*-acylnitrenium ion with delocalized positive charge formed during the reduction of aristolochic acid was suggested as the ultimate carcinogenic species, binding preferentially to the exocyclic amino group of purine nucleotides in DNA (ref.[9]).

Aristolactams (formed preferentially from aristolochic acid I) are the principal metabolites, which were detected unconjugated (in a minority) or conjugated (in a majority) in urine and in faeces and excreted by several animals including humans[11]. They are also stored conjugated as *N*-glucosides or bound to other cellular compounds in plants[3]. There is, however, little information in the literature regarding either the metabolism of aristolactams in animals and in plants, or their potential mutagenic or carcinogenic risks. The question hence arises whether these aristolochic acid metabolites could not be the substrates of enzyme systems that would activate these excretory products to ultimate mutagens or carcinogens. Besides microsomal cytochrome P-450 which both detoxicates and activates carcinogens in organisms, peroxidases are also believed to be implicated in these processes in tissues rich in these enzymes[12–14]. Both enzymes could also metabolize these compounds in plants, being responsible for their toxic effects in the plant itself or for their potential risks for organisms in the trophic chain[15,16]. Rat liver microsomes containing cytochrome P-450 and peroxidase (horseradish peroxidase as a model) are therefore studied from the point of view of their ability to convert aristolactams yielding active intermediates that bind to DNA. The mechanisms of reac-



R = OCH_3 aristolochic acid I
R = H aristocholic acid II



R = OCH_3 aristolactam I
R = H aristolactam II

tions catalyzed by the two enzymes are also studied. In addition, prostaglandin H synthase (PHS) is studied as another peroxidase converting both aristolactams. Here we report on the detection of DNA adducts formed by aristolactams I and II after oxidation by cytochrome P-450 and horseradish peroxidase. The ^{32}P-postlabeling assay[17] is used for both detection and quantitation of adducts.

EXPERIMENTAL

Chemicals

Chemicals were obtained from the following sources: β-naphthoflavone from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; NADH, NADPH, DNA (from calf thymus), peroxidase (horseradish), ferricytochrome c and superoxide dismutase from Boehringer, Mannheim, Germany. The mixture of 65% aristolochic acid I and 34% aristolochic acid II was a gift from Madaus, Cologne, Germany. Compounds I and II were separated from the mixture by preparative HPLC chromatography and chemically reduced to the corresponding lactams as previously published[18]. All other chemicals were of analytical purity or better. Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained commercially from sources described previously[19].

Subcellular Preparations and Incubations

Male Sprague–Dawley rats (about 100–150 g) were injected i.p. with β-naphthoflavone dissolved in maize oil (60 mg/kg body weight) once a day for three consecutive days. The animals were starved for 16–18 h prior to being killed, and liver microsomes containing cytochrome P-450 were prepared as described by Kimura et al.[20] and stored at –70 °C.

Fresh ram seminal glands were obtained from a local slaughterhouse, trimmed of excess fat and tissue, and stored at –70 °C until use. Microsomes were prepared as described previously[21] and used as a source for PHS. PHS-cyclooxygenase activity was determined by measuring the arachidonic acid-dependent oxygen uptake in a 2.0-ml chamber equipped with a Clark-type oxygen electrode. The incubation mixture contained in 1.0 ml 0.8 mg ram seminal vesicle microsomes protein in 50 mM phosphate buffer (pH 7.4) and dimethyl sulfoxide (DMSO) as solvent, either alone or with different concentrations of aristolactams, and was preincubated for 1 min at 37 °C. The reaction was initiated by addition of arachidonic acid (0.1 mmol) dissolved in 2 μl ethanol. The initial velocity of arachidonic acid oxidation was determined from the slope of the linear portion of the O_2 uptake curve.

Incubation mixtures used for the modification of DNA by aristolactams activated by microsomal cytochrome P-450 contained in a final volume of 0.75 ml: 50 mM potassium phosphate buffer pH 7.4; 2 mM NADPH; 3.5 mg microsomal proteins; 0.3 mM aristolactam I or aristolactam II dissolved in DMSO (75 μl/0.75 ml incubation) and 1 mg DNA.

Incubation mixtures used for modification of DNA by aristolactams activated by peroxidase contained in a final volume of 0.75 ml: 50 mM Tris-HCl buffer (pH 7.4); 0.1 mg horseradish peroxidase; 1 mM hydrogen peroxide; 0.3 mM aristolactam I or aristolactam II dissolved in DMSO (75 μl/0.75 ml incubation) and 1 mg DNA. Control incubations were carried out either without activating system (DNA and aristolactams) or with activating system and aristolactams but without DNA. After incubation (37 °C, 60 min), the mixtures were extracted twice with ethyl acetate (2 × 2 ml). DNAs modified by metabolites of aristolactams formed by both activating enzyme systems were isolated from the residual water phase by the phenol/chloroform procedure as described earlier[22]. The content of DNA was measured spectrophotometrically[23].

^{32}P-Postlabeling Analysis

Materials for the ^{32}P-postlabeling assay were the same as described previously[24,25] except that T4 polynucleotide kinase was purchased from Amersham Buchler (Braunschweig, Germany) and poly(ethylenimine)cellulose (PEI-cellulose) thin layers from Macherey–Nagel (Düren, Germany). Nuclease P1 was obtained from Sigma Chemical Co.

The DNA samples modified by aristolactams activated by both enzyme systems (10 μg) were converted to ^{32}P-labeled deoxynucleoside-3′,5′-biphosphates by the nuclease P1 version[25] of the ^{32}P-postlabeling assay[17]. Isolated 3′-monophosphate-nucleoside adducts were labeled by incubation with 500 μCi carrier free [γ-^{32}P]ATP and T4 polynucleotide kinase under conditions as used previously[19,26]. The specific activity of [γ-^{32}P]ATP and efficiency of the kinase reaction were assayed as described[25]. Typical values for specific activity were 2 000 ± 300 Ci/mmol. ^{32}P-labeled adducts were resolved by four-directional thin-layer chromatography on PEI-cellulose according to Reddy and Randerath[25], except that the D4 direction was adjusted to pH 9.1 for better resolution[27]. Autoradiography and evaluation of relative adduct labeling (RAL) values were performed as described previously using Cerenkov counting[17,25].

Measurement of NADH Oxidation, Reduction of Ferricytochrome c and Oxygen Consumption

The reaction mixture contained in a final volume of 1 ml: 50 mM Tris-HCl buffer pH 7.4; 0.1 mM NADH; 0–0.114 mM H_2O_2; 0–5 μg horseradish peroxidase and 30–90 μM aristolactam I or aristolactam II. Reactions were started by addition of the respective aristolactams, and gradual dissappearance of NADH was followed at 340 nm using a Beckmann D-62 spectrophotometer at 20 °C.

The same reaction mixture was used for estimation of ferricytochrome c reduction except that 20 μM ferricytochrome c was added. Reduction of ferricytochrome c was monitored by the increase in absorbance at 550 nm (ref.[28]) at 20 °C. The molar extinction coefficient of cytochrome c at 550 nm (ref.[28]) was 2.9. 10^4 mol^{-1} cm^{-1}.

Oxygen consumption was measured with a Clark-type electrode at 20 °C.

RESULTS

Both microsomal cytochrome P-450 of rat liver and peroxidase in the presence of H_2O_2 are effective in supporting the activation of aristolactam I and aristolactam II to products binding to DNA. The formation of DNA adducts was analyzed using the nuclease P1-enhanced variation[25] of the ^{32}P-postlabeling assay[17].

After inspection of autoradiographs four major and two minor adduct spots were detected in DNA treated with aristolactam I and microsomes compared with two major and one minor adduct spots when treated with aristolactam II (Fig. 1). Fourteen major and six minor adduct spots were detected in DNA reacted with aristolactam I activated by the peroxidase system in vitro. The pattern of adduct spots detected in DNA treated with aristolactam II and the peroxidase system in vitro shows a lower number of adducts formed.

Quantitative analysis of the major adducts revealed that the extent of modification of DNA by aristolactams activated by peroxidase was approximately 40–70 times greater than for activation by microsomal cytochrome P-450. Highest total binding was found in DNA modified by aristolactam I activated by peroxidase, followed by DNA reacted

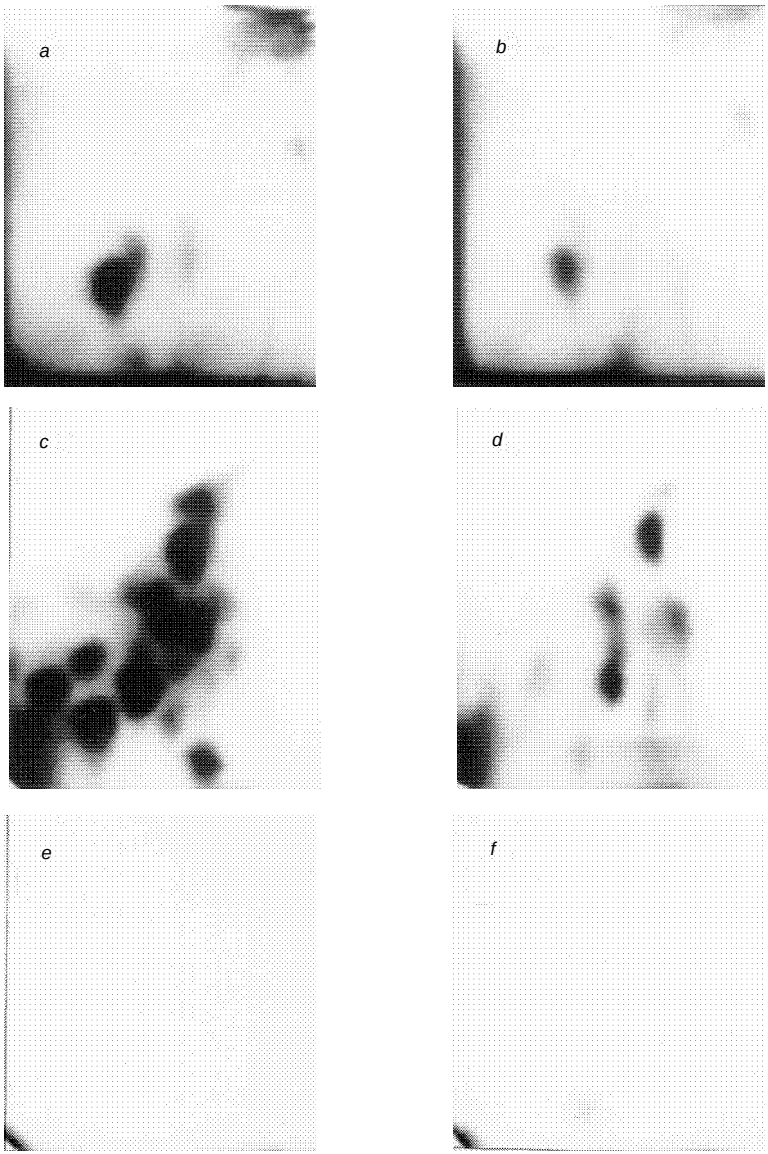


FIG. 1

Autoradiographs of PEI-cellulose TLC maps of ^{32}P-labeled digest of DNA treated with aristolactams activated by microsomes or peroxidase. *a* DNA treated with aristolactam I and microsomes, *b* DNA treated with aristolactam II and microsomes, *c* DNA treated with aristolactam I and peroxidase, *d* DNA treated with aristolactam II and peroxidase, *e* and *f* show control samples containing DNA and aristolactam I without activating enzymes (*e*) or samples containing peroxidase and aristolactam I without DNA (*f*). Analysis was performed by the nuclease P1 version of the assay. Autoradiography was done at –70 °C for 2 h (*a*, *b*, *e*, *f*) and for 30 min (*c*, *d*). The origins are located at the bottom left corners, D3 direction from bottom to top and D4 direction on four directional TLC on PEI-cellulose from left to right (see Experimental)

with aristolactam II activated by the same enzyme, and DNA modified with both lactams activated by microsomal cytochrome P-450 (Table I).

Since the chromatographic conditions of TLC used in the ^{32}P-postlabeling assay are suitable for the separation of very lipophilic adducts formed from aromatic carcinogens with DNA (ref.[25]), the adducts found with aristolactams and DNA must be strongly lipophilic. Although the exact nature of the adducts has not yet been elucidated, their pronounced lipophilicity indicates that the whole hydrophobic polycyclic aromatic system of the molecules of aristolactams is covalently linked to deoxynucleotides in DNA. As different levels of aristolactams binding to DNA after their activation by peroxidase and cytochrome P-450 were detected, the mechanisms of reactions catalyzed by the two enzyme systems should be different.

During the peroxidase-catalyzed reaction aristolactam I and II act as catalysts for oxidation of NADH by hydrogen peroxide and peroxidase. Hence, peroxidase and H_2O_2 possess NADH-oxidase activity in the presence of these compounds. We found a low oxidation of NADH with peroxidase and H_2O_2 without lactams (0.04 nmol NADH/min per 1 μg protein) or in the system with this compound but without H_2O_2 (0.06 nmol NADH/min per 1 μg protein). However, in the complete oxidizing system (peroxidase, H_2O_2, aristolactams) NADH was oxidized (Table II). An effective oxygen uptake during the reaction in the above system was also observed (Table II). The above described abilities of metabolites of aristolactams formed by peroxidase were, however, not found in the system in which both compounds were activated by microsomal cytochrome P-450.

TABLE I

Quantitative analysis od adducts formed in DNA reacting with aristolactams I and II activated by microsomes and peroxidase. The numbers are averages and standard deviations of triplicate analyses, relative adduct labeling (RAL) represents the adducts in normal nucleotides, total adduct content was determined by summing up of RAL of individual adducts. For other conditions see Experimental

Activation system	Total DNA adduct content	
	RAL . 10^7	μmol/kg
Aristolactam I/cytochrome P-450	1.30 ± 0.21	0.39 ± 0.06
Aristolactam II/cytochrome P-450	0.46 ± 0.10	0.13 ± 0.03
Aristolactam I/peroxidase	93.40 ± 8.31	28.08 ± 2.50
Aristolactam II/peroxidase	19.45 ± 1.32	5.85 ± 0.40

As shown in Table III aristolactams enhanced the initial reduction of ferricytochrome c in the peroxidase–H_2O_2–NADH system. No reduction of ferricytochrome c occurred with aristolactams alone in the absence of H_2O_2. The enhancement by aristolactams of the reduction rate was completely prevented by superoxide dismutase (Table III) suggesting that the superoxide radical was formed, being responsible for the reduction of ferricytochrome c. Ferricytochrome c decreased the oxygen consumption due to the peroxidase–H_2O_2–NADH–aristolactams system. Addition of 0.1, 0.15 and 0.2 mM ferricytochrome c resulted in 78%, 93% and 100% inhibition of oxygen consumption, respectively. However, no effect of ferricytochrome c on NADH oxidation by the system

TABLE II
Oxidation of NADH and oxygen uptake per protein content catalyzed by the peroxidase–H_2O_2–aristolactams system. Values shown are averages and standard deviations of three parallel experiments. For experimental conditions see the text

Compound	Converted NADH mol/min kg	Oxygen uptake mol/min kg
None	0.04 ± 0.006	0.008 ± 0.001
Aristolactam I	5.71 ± 0.200	4.570 ± 0.075
Aristolactam II	5.60 ± 0.200	4.480 ± 0.080

TABLE III
Reduction rate (v) of ferricytochrome c during aristolactam-mediated NADH oxidation by the peroxidase–H_2O_2 system at various aristolactam concentrations (c). Values in the table are averages and standard deviations of three parallel experiments

c, μmol/l	v, nmol/min	
	aristolactam I	aristolactam II
0	0.033 ± 0.010	0.033 ± 0.010
20	0.769 ± 0.100	1.003 ± 0.134
30	1.137 ± 0.134	1.338 ± 0.200
60	2.040 ± 0.167	3.010 ± 0.200
90	3.344 ± 0.301	4.682 ± 0.301
90[a]	0	0

[a] 0.2 μM superoxide dismutase added.

was observed. This indicates that superoxide radicals are not responsible for NADH oxidation and that the oxidation of NADH is mediated by the reaction of peroxidase with lactams (see above). These findings suggest that radicals are formed by a one-electron oxidation of aristolactams catalyzed by peroxidase, which is effective in the oxidation of NADH.

Although horseradish peroxidase has served as a suitable model peroxidase for mammalian peroxidases in the present and in other studies[12], it was of interest to investigate the metabolism of aristolactams by prostaglandin H synthase, another enzyme with peroxidative activity present in several mammalian tissues, including the tissues of the lower urinary tract. These tissues are the target organs for aristolochic acid. We determined that ram seminal vesicle microsomes containing PHS, a key enzyme for prostaglandin biosynthesis, oxidizes aristolactams during the conversion of arachidonic acid to prostaglandins. Conversion of arachidonic acid requires the incorporation of molecular oxygen catalyzed by PHS cyclooxygenase and the presence of a reducing cofactor for the reduction of endogeneous substrates (prostaglandins) catalyzed by the hydroperoxidase activity of the enzyme[21]. Oxygen uptake thus serves as a measn of measuring cyclooxygenase activity of PHS (refs[12,21]). We investigated aristolactams as cofactors for PHS by measuring oxygen incorporation into arachidonic acid catalyzed by ram seminal microsomes containing PHS. Table IV shows the concentration-dependent stimulation of oxygen uptake produced by aristolactams, which is completely inhibited by indomethacin, a well-known PHS cyclooxygenase inhibitor. This indicates that both lactams serve as cosubstrates or "cofactors" for PHS, radicals being formed during the reaction[21]. Lactam II is a better cosubstrate of PHS. It follows from the finding that aristolactam II has a more stimulating effect on the arachidonic acid-dependent oxygen

TABLE IV
Stimulation of PHS-cyclooxygenase activity (measured as oxygen uptake rate per protein content, *v*) by aristolactams various concentration (*c*)

c, μmol/l	*v*, mol/min kg	
	aristolactam I	aristolactam II
0	110.0 ± 9.8	110.0 ± 9.8
10	169.2 ± 11.0	163.6 ± 10.5
50	125.5 ± 10.2	211.5 ± 15.1
100	98.7 ± 6.8	218.6 ± 15.8
10[a]	9.1 ± 0.9	8.2 ± 0.9

[a] 100 μM indomethacin added.

uptake rates than lactam I. Moreover, at high concentrations of aristolactam I, the stimulatory effect of this compound on PHS activity was reversed; this lactam I inhibited the oxygen uptake at higher concentrations.

DISCUSSION

The results presented here demonstrate that aristolactams (the major metabolites of carcinogenic aristolochic acid) excreted or stored free or as conjugates in animals or plants represent strong mutagenic or carcinogenic risks for living organisms. Both these metabolites of aristolochic acids are activated either by microsomal enzymes containing cytochrome P-450 or by peroxidase to reactive intermediates or products binding to DNA. Furthermore, reactive species (radicals) or the superoxide radicals are produced by peroxidases. Although the mechanism in which these activation reactions are implicated into the stages of carcinogenic processes remains to be explained, it might be speculated as reported below.

It is well established that the development of cancer (carcinogenesis) requires multiple steps. In several model systems, the stages of initiation, promotion and progression can be operationally defined through the use of discrete chemical agents[29,30].

The initiation stage of carcinogenesis induced by aristolochic acids is supposed to be the formation of deoxyadenosine adduct characterizing as 7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AA I)[7,8,10]. The data obtained previously showed convincingly that the adenine base is the critical site of modification in vivo, and this in turn does tally, at least for aristolochic acid I, with expectations from the known base substitution of H-ras oncogene sequences found in cancerous forestomach tissues of rats treated with aristolochic acid I (refs[7,31]).

In the present work we observed that the metabolic products of aristolochic acid, aristolactams (which are excretory products) are converted by cytochrome P-450 and peroxidase to ultimate carcinogens which bind to DNA in vitro and may hence be implicated in the initiation of carcinogenesis induced by aristolochic acid. In particular, peroxidase is strongly effective from this point of view. The pattern of adducts formed in DNA after treating with aristolactam I activated by peroxidase is similar to the adduct pattern obtained in DNA of urothelial cells or exfoliated cells in urine of rats exposed to aristolochic acid I (ref.[26]). ^{32}P-Postlabeling of urothelium DNA of rats treated with aristolochic acid I led to autoradiograms with an abundance of adduct spots[26] in comparison with autoradiograms of liver, forestomach or urinary bladder DNA (refs[8,10,26]). Urothelial cells and bladder epithelium are rich in some peroxidases[12,32,33] and as known for several arylamines, can mediate the formation of a DNA adduct[34]. Hence it was suggested by Fernando et al.[26] that the higher aristolochic acid-DNA adduction in urothelium could arise from extrahepatic peroxidation of aristolactams directly in the urothelium by the action of urothelium peroxidases (i.e. prostaglandin H synthase). Indeed, we determined that aristolactams are oxidized by a

model peroxidase (horseradish peroxidase) and are also cosubstrates of PHS. However, only preliminary results without studies of the mechanism of the PHS-mediated reactions are shown in present paper. Further extensive studies must be carried out to confirm the above hypothesis that this oxidation leads to formation of DNA adducts in vitro and/or in vivo.

The reaction catalyzed by horseradish peroxidase as a model peroxidase was studied in detail. At present, aristolactams have been found to be oxidized by horseradish peroxidase, in contrast to cytochrome P-450, to reactive metabolites with NADH oxidation activity. In addition, they mediate an effective oxygen uptake producing active forms of molecular oxygen (superoxide radicals) under the conditions used. These findings could imply that the reactions are one-electron redox processes having free radicals as primary products, similarly as observed with several other xenobiotics oxidized by peroxidase to radicals[12,14]. The radicals of aristolactams are also formed in the oxidation reaction of these compounds catalyzed by PHS.

Several lines of evidence suggest a role of free radicals not only in the initiation of carcinogenesis (formation of adducts) but also in processes of tumor promotion[35,36] and even in progression[37]. In view of the role radicals and superoxide radicals may play in carcinogenesis, the formation of aristolactam radicals and of the superoxide radical during the peroxidase-catalyzed oxidation of aristolactams could have biological consequences in all phases of chemical carcinogenesis. The importance of the data described in this paper in an in vivo system has yet to be determined.

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