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Metabolism of the carcinogen alpha-asarone in liver microsomes

Alexander T. Cartus, Dieter Schrenk*

University of Kaiserslautern, Food Chemistry and Toxicology, Erwin-Schroedinger-Strasse 52, 67663 Kaiserslautern, Germany

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ABSTRACT

Alpha-asarone (**1**) is a naturally occurring phenylpropene found in several plants, e.g. *Acorus calamus*. **1**-containing plant materials and essential oils thereof are used for flavoring foods and in many phytopharmaceuticals. **1** has been claimed to have positive pharmacological effects, however, it is carcinogenic in male mice (liver) and probably genotoxic. Since the metabolic pathways of **1** have not been investigated and its carcinogenic mode of action is unknown, we investigated the metabolism of **1** in liver microsomes of rat, bovine, porcine, and human origin using HPLC-DAD and LC-ESI-MS/MS and derived kinetic data on the metabolite formation.

The main metabolic pathway was the side-chain hydroxylation leading to (*E*)-3′-hydroxyasarone (**2**). Epoxidation of **1** presumably led to (*E*)-asarone-1′,2′-epoxide (**4**) which instantly hydrolyzed to form *erythro*- and *threo*-configured diols (**5b**+**5a**). As a minor reaction *O*-demethylation of **1** was observed. The metabolite formation showed little species-specific differences with the exception of porcine liver microsomes for which the formation of diols **5b**+**5a** exceeded the formation of alcohol **2**. The kinetic parameters imply a dependence of the pattern of metabolite formation from substrate concentration. On the basis of our results and earlier findings we hypothesize the genotoxic epoxide **4** being the ultimate carcinogen metabolically formed from **1**.

1. Introduction

α-asarone (**1**, *trans*-2,4,5-trimethoxy-1-propenylbenzene, Fig. 2) belongs to the group of phenylpropenes (alkenylbenzenes) like eugenol, anethole or safrol and is a known hepatocarcinogen in male mice. **1** is found in plants, e.g. of the genus *Acorus* (e.g. *Acorus calamus* L.; 'Sweet flag', *Acorus gramineus* Sol.), *Asarum* (e.g. *Acorus europaeum* L.), and some peppers (e.g. *Piper sarmentosum* Roxb.). Depending on karyotype and growth conditions of the plant, the essential oil of the rhizome of *A. calamus* consists of up to 17% of **1**, typically of 5–10% (Rana et al., 2013; Satyal et al., 2013; Qin et al., 2010; Della Greca et al., 1989) besides higher concentrations (up to 94%) of β-asarone (the *cis*-isomer of **1**) and small amounts of γ-asarone (the allylic isomer of **1**). In general, the content of asarone isomers decreases with decreasing polyploidy of the plants, i.e. the asarone content of tetraploidic Indian > tetraploidic European > diploidic American *A. calamus* (Krahulcova, 2003; Rana et al., 2013). Concentrations in dried rhizome of tetraploid Indian *A. calamus* were found to be in the range of 4.4–8.3% for β-asarone and up to 0.7% for **1** (Zuba and Byrska, 2012; Rana et al., 2013).

A. calamus is used in traditional phytomedicine of many ethnicities. Its dried rhizome is used to flavor alcoholic beverages like bitters, and is consumed as tea (Rajput et al., 2014). **1** and **1**-containing plant extracts are claimed to have multiple beneficial pharmacological actions, e.g., to act as a sedative, CNS depressant, anticonvulsant, antispasmodic, antidiarrheal, antidiabetic and anticholinergic drug (Mukherjee et al., 2007a, b; Rajput et al., 2014). Furthermore, several (beneficial) neuronal effects, e.g. on memory disorder or learning performance are described in the literature (Kim et al., 2015; Shin et al., 2014).

Pharmacokinetic studies of **1** in rats showed, that orally administered **1** is rapidly absorbed in the gastrointestinal tract (Meng et al., 2013). The acute toxicity of **1** in mice is comparatively low with LD_{50} values of 418 mg/kg body wt (oral), and 245–310 mg/kg body wt (intraperitoneal, i.p.) (Belova et al., 1985; Morales-Ramírez et al., 1992). Teratogenic effects of **1** (repeated dose, gavage) were reported in mice and rats (Salazar et al., 1992; Chamorro et al., 1996, 1998). Chronically, **1** significantly induced hepatocellular adenomas and carcinomas in male C57BL/6J × C3H/HeJ F1 mice in different dosage designs (single dose of 52, 104, and 156 mg/kg body wt, as well as repeated dose of in sum 1 mg/animal at day 1, 8, 15 and 22 in a ratio of 1:2:4:12) when administered i.p.

* Corresponding author.
E-mail address: schrenk@rhrk.uni-kl.de (D. Schrenk).

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to preweaning animals. Pretreatment with pentachlorophenol (i.p.), a potent inhibitor of sulfotransferases, had no effect on the incidences of hepatocellular adenomas and carcinomas caused by **1** (Wiseman et al., 1987). Thus, the canonical mechanism of action of the activation of some ***allylic*** phenylpropenes (safrole, estragole, methyleugenol) *via* side-chain hydroxylation, further sulfonation and formation of a carbocation, seems not to be responsible for the carcinogenicity of **1.** Similarly, other ***propenylic*** PP, e.g. anethole or isoeugenol, are shown to be non-genotoxic although they showed carcinogenic effects (Newberne et al., 1999; NTP, 2010). Studies with pure **1** in other species or upon oral administration are not available. However, there are studies investigating different Calamus oils of Indian and European origin. Treatment of male and female rats with Indian Calamus oil (0.5% in the feed, 2-year study) consisting of about 3% **1** and 76% β-asarone (the *cis*-configured isomer of **1**) resulted in a dose-dependent higher mortality, several pathological degenerative and regenerative changes of the liver, cardiac fibrosis and atrophy as well as leiomyosarcomas of the small intestine (duodenum). In addition, hepatocellular adenomas and carcinomas (and other pathological findings) were observed in male and female rats fed with European Calamus oil (0.1, 0.5, 1.0, 2.0% in the feed, composition unknown, 2-year study) (Taylor et al., 1967; unpublished studies/abstract cited in more detail by JECFA, 1981, and SCF, 2002). The results for **1** in the Ames test were equivocal. Negative results in TA97, 98, 100, 102, 1535, 1537, and 1538 (with metabolic activation) were reported at concentrations of up to 200 μg/plate (Hsia et al., 1979; Marczewska et al., 2013). However, some positive responses in TA100 with metabolic activation were reported (Mohar et al., 1986; cited in Morales-Ramírez et al., 1992; Jin et al., 1982). **1** induced unscheduled DNA synthesis (UDS) in primary rat hepatocytes. Pre-incubation of the cells with cimetidine (a CYP450 inhibitor) markedly decreased the UDS response **1** and metabolism of **1** (measured as remaining concentration of **1**) in a concentration-dependent manner, whereas pentachlorophenol was without effect (Hasheminejad and Caldwell, 1994). Furthermore, **1** induced sister chromatid exchanges *in vitro* in human lymphocytes and *in vivo* in mice (Morales-Ramírez et al., 1992), and DNA strand breaks in the comet assay (Marczewska et al., 2013). Although **1** and β-asarone exert virtually equal effects in the mouse carcinogenicity study by Wiseman et al. (1987), β-asarone is frequently referred to be *the* 'active principle' (of toxicological concern) of *A. calamus*. However, this notion may not be due to different toxicological properties but due to the usually higher contents of β-asarone in comparison to **1** in plant material.

Because of the toxicological properties of **1** and β-asarone and the lack of sufficient data for a risk assessment, the addition of *A. calamus*, Calamus oil or Calamus extracts to food is prohibited in the U.S (21CFR189). In Europe, **1** is not regulated, but the direct use of β-asarone as a food flavoring is prohibited within the EU. Maximum levels for β-asarone in alcoholic beverages flavored with Calamus are set to a limit of 1 mg/kg in the EU (EC Regulation 1334/2008).

Human exposure to **1** resulting from foodstuffs seems to be relatively low compared to other phenylpropenes like estragole or methyleugenol but definitive data are lacking. Highest intakes are assumed to arise from the consumption of alcoholic beverages flavored with *A. calamus* rhizome and preparations thereof. For β-asarone, the total dietary intake *via* foods and alcoholic beverages was estimated by the Council of Europe to be in the range of 8.5–49 μg/day for mean and high users (COE, 2005). Based on the usually lower contents of **1** in plants, its daily intake may be in the range of one-tenth of that calculated for β-asarone, i.e. 0.85–4.9 μg/day. However, due to the possible positive pharmacological effects mentioned above, Calamus preparations are used as ingredients in plant food supplements (PFS) or herbal medicinal products sold over-the-counter and/or *via* the internet. Zuba and Byrska (2012) investigated the α- and β-asarone contents of several herbal medicinal products. In their study **1** was present in all investigated products with contents of 0.49–51.4 μg/pellet or tablet (i.e. up to 0.5 mg/g). β-asarone contents were usually higher (up to 1.53 mg/tablet or 5.1 mg/g), but not detectable in all products. The β-asarone contents in plant food supplements were found to be even higher with concentrations of 0.12–44.3 mg/g (van den Berg, 2011).

The Committee on Herbal Medicinal Products (HMPC) of the European Medicines Agency (EMEA) concluded in 2005 that the concentrations of **1** and β-asarone in herbal medicinal products should be reduced to a minimum, and diploid varieties should always be preferred. In addition to the food regulation, a limit of exposure from herbal medicinal products of approximately 115 mg/day, i.e. about 2 mg/kg body weight per day could be accepted temporarily until a full benefit/risk assessment has been carried out (HMPC, 2005). This dose level would be exceeded by most of the investigated PFS or herbal medicinal products with just one pellet or tablet per day but is substantially higher than the estimated dietary intake.

Although many pharmacological effects and suggestions for mode of actions of **1** are depicted in the literature, no data on the metabolism of **1** are yet available and thus nothing is known about its genotoxic/carcinogenic mechanism of action. To get first insights into the toxicological mechanism of action, we investigated the metabolism of **1** using rat, pig, bovine and human liver microsomes and determined kinetic parameters of metabolite formation.

2. Materials and methods

2.1. Chemicals and materials

Standard chemicals and solvents for the synthesis of metabolites were obtained in analytical grade or appropriate grade for the use intended by commercial suppliers and were used without further purification unless otherwise noted. Chemicals and enzymes for microsomal incubations were purchased from the following manufacturers: Acetone, acetonitrile, ethyl acetate, and all salts were purchased from Merck (Darmstadt, Germany). α-asarone (**1**), β-NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase, were obtained from Sigma–Aldrich/Fluka (Taufenkirchen, Germany). Methanol was obtained in HPLC gradient grade from Promochem (Wesel, Germany). Water was used double distilled and degassed (HPLC). Aroclor 1254 was originally purchased from Monsanto (St. Louis, MO, USA). The compounds **2**–**10** were synthesized and characterized as previously described (Cartus et al., 2011, 2015). Abbreviations of chemicals: **1**, α-asarone ((*E*)-2,4,5-trimethoxy-1-propenylbenzene, CAS No. 2883-98-9); **2**, (*E*)-3′-hydroxyasarone (CAS No. 1392497-89-0); **3**, (*E*)-3′-oxoasarone (CAS No. 99217-06-8); **4**, (*E*)-asarone-1′,2′-epoxide (CAS No. 124878-08-6) and (*Z*)-asarone-1′,2′-epoxide (CAS No. 321365-66-6); 5**a**, *threo*-1′,2′-dihydrodihydroxy-asarone (CAS No. 146830-05-9): mixture of (1*R*,2*R*)- and (1*S*,2*S*)-1-(2,4,5-trimethoxyphenyl)-propane-1,2-diol (CAS No. 321365-67-7 relative configuration, 853021-83-7 absolute configuration, and 137361-00-3 relative configuration, 853021-82-6 absolute configuration, respectively); **5b**, *erythro*-1′,2′-dihydrodihydroxy-asarone (CAS No. 146830-05-9): mixture of (1*S*,2*R*)- and (1*R*,2*S*)-1-(2,4,5-trimethoxyphenyl)-propane-1,2-diol (CAS No. 137361-02-5 relative configuration, 853021-85-9 absolute configuration, and 321365-68-8 relative configuration, 853021-84-8 absolute configuration); **6**, 2,4,5-trimethoxyphenylacetone (CAS No. 2020-90-8); **7**, (*E*)-6-hydroxyasarone (CAS No. 65720-05-0); **8**, (*E*)-4-hydroxyasarone (no CAS No. assigned); **9**, (*E*)-3-hydroxyasarone (no CAS No.