New studies on \textit{trans}-anethole oxide and \textit{trans}-asarone oxide

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The widespread use of naturally occurring alkenylbenzenes as flavoring and fragrance agents has led to a long-standing interest in their toxicity and carcinogenicity. Among them several allyl- and propenylbenzenes have been found to be mutagenic and carcinogenic. It has been shown that the carcinogenicity of several allylbenzenes can be related to the formation of electrophilic sulfuric acid esters following 1'-hydroxylation. Unlike the allylbenzenes, the mechanisms of carcinogenesis of propenylbenzenes such as anethole and asarone are not clear. It has been reported that one of the main metabolic pathways of \textit{trans}-anethole is the epoxidation of the side chain 1,2-double bond, which was responsible for cytotoxicity but not for genotoxicity. However, we report here that synthetic \textit{trans}-anethole oxide prepared from \textit{trans}-anethole and dimethyldioxirane is not only mutagenic for \textit{Salmonella} tester strains but is also carcinogenic in the induction of hepatomas in B6C3F1 mice and skin papillomas in CD-1 mice. Synthetic \textit{trans}-asarone oxide was also carcinogenic in the induction of hepatomas as well as mutagenic for \textit{Salmonella} strains. Further studies are needed on these side chain oxides of \textit{trans}-anethole and \textit{trans}-asarone as possible metabolites in the toxicity, mutagenicity and carcinogenicity of these and other propenylbenzenes.

\textbf{Introduction}

Many naturally occurring alkenylbenzene derivatives, usually relatively simple allyl- or propenylbenzenes with methoxy and/or methylenedioxy ring substituents, have been found as components of many plants or their essential oils (1,2) and have been used as important natural flavoring and fragrance chemicals. The widespread use of these alkenylbenzenes has led to a long-standing interest in their toxicity and carcinogenicity. Several allylbenzenes show carcinogenicity in experimental animals. Safrole (1-allyl-3,4-dimethoxybenzene), which is a major constituent of oil of sassafras and a component of certain other essential oils (3), has induced hepatic tumors in mice and rats (2,4–6). Estragole (1-allyl-4-methoxybenzene), which is a major constituent of oil of tarragon (estragon) and/or methylenedioxy ring substituents, has been found as active a carcinogen in the mouse liver as safrole and isosafrole (1-propenyl-3,4-methylenedioxybenzene), which induce a low incidence of hepatomas in mice (6,7). Anethole (1-propenyl-4-methoxybenzene), which is found in the essential oils of many kinds of plants, including fennel and Chinese star anise, did not seem to be carcinogenic in mice (2,15), but was a weak hepatocarcinogen in female CD rats (16). Unlike the allylbenzenes, the carcinogenic mechanism of propenylbenzenes is not clear.

\textbf{Materials and methods}

\textbf{Chemicals and animals}

Tetradecanoylphorbol 13-acetate (TPA) was obtained from ChemSyn Science Laboratory (Lenexa, KS). Triocotanol was purchased from Pfaltz and Bauer (Waterbury, CT). \textit{trans}-anethole, \textit{trans}-asarone, glyceraldehyde, activated (4 Å) molecular sieve, Oxone (monopersulfate compound), 4-(4'-nitrobenzyl)pyridine, acetone, hexane, potassium iodide (KI), starch and dimethyl sulfoxide (DMSO) were from Aldrich Chemical Co. (Milwaukee, WI). The other chemicals were obtained from Sigma Chemical Co. (St Louis, MO).

Male C3H/HeJ and female C57BL/6J mice from the Jackson Laboratory (Bar Harbor, ME) were bred in our laboratory to give B6C3F1 offspring. After weaning, the mice were fed modified AIN-76 purified diet (Teklad Diet Co., Madison, WI). The CD-1 mice from the Charles River Breeding Co. (Wilmington, MA) were fed Wayne Breeder Blox pellets (Allied Mills, Chicago, IL).

\textbf{Synthesis of \textit{trans}-anethole oxide and \textit{trans}-asarone oxide}

Dimethyldioxirane solution in acetone was made freshly by an improved conventional method (23). After the solution was dried with molecular sieves (4 Å, activated powder), it was titrated iodometrically with KI and starch. The titrated dimethyldioxirane solution (~0.08 M solution in acetone, 1.2 equiv.) was added to \textit{trans}-anethole or \textit{trans}-asarone in dry acetone (1 ml for 1 mmol) at 0°C and the reaction mixture was stirred for 30 min at room temperature. The reaction solution was evaporated under reduced pressure to afford the epoxides. \textit{trans}-anethole oxide was a colorless oil. The yellow oil of \textit{trans}-asarone oxide after vacuum application was dissolved in dry hexane and recrystallized at ~20°C for a few days to produce a white solid product (m.p.
Direct mutagenicity of were used within a month for the experiments. The use of dimethyldioxirane in the synthesis of the oxides gave structures whose proton NMR spectra did not show evidence that each compound contained mixtures of slightly different isomers.

37–40°C, cor.). The yields of these oxides were >95%. The oxides were kept at –80°C in dry nitrogen. In this condition trans-anethole oxide was stable for 1 year and trans-asarone oxide was stable for 1 month. The oxides were used within a month for the experiments.

Ames test
Direct mutagenicity of trans-anethole oxide and trans-asarone oxide were assayed in Salmonella typhimurium strains TA1535, TA100 and TA98 following the original procedures of Maron and Ames (24) as modified by Park et al. (25). Each oxide was dissolved in 20 µl of acetone and was preincubated with bacteria in a total volume of 0.6 ml potassium phosphate buffer (0.1 M, pH 7.4) for 30 min at 37°C in a shaking water bath. After incubation, 2 ml of top agar were added and the mixture was vortexed, plated and incubated at 37°C for 2 days before the his+ revertants were scored.

Induction of hepatic tumors
Male B6C3F1 mice (12 days old) were injected once i.p. with trans-anethole, trans-anethole oxide, trans-asarone or trans-asarone oxide. After weaning, they were fed modified AIN-76 purified diet (Teklad Diet Co.) and given water ad libitum. The mice were killed at 10–11 months and the number of hepatomas >2 mm in diameter and visible on the surface were counted. For the highest dose group of trans-asarone oxide (0.5 µmol/g body wt), 100 mice were used so that the group size was 33 mice at the end of the experiment. Each of the other groups was composed of 35 mice in the beginning.

Induction of skin papillomas
Groups of 30 female CD-1 mice (6–8 weeks old) were treated on their shaven back with 2.5 µg TPA in 0.1ml acetone. After 24 h, mice were treated with doses of 5 µmol of trans-anethole, trans-anethole oxide, trans-asarone or trans-asarone oxide once weekly for 3 weeks and thereafter twice weekly with topical TPA (2.5 µg in 0.1 ml acetone). The mice were rinsed as necessary and papillomas were enumerated at bi-weekly intervals. The experiment was terminated at 20 weeks.

Stability of trans-anethole oxide and trans-asarone oxide
The half-lives of the alkylating activity of trans-anethole oxide and trans-asarone oxide were determined by the method of Park et al. (25). The oxide solution (75 mg in 10 ml acetone) was mixed with 20 ml of 0.1 M phosphate buffer (pH 7.4) and incubated at 37°C. At various times (~0.5–25 min) after incubation at 37°C, an aliquot of 1 ml was transferred to a cap tube which contained 0.3 ml of acetone and 1.7 ml of 3.3% 4-(4'-nitrobenzyl)pyridine in ethylene glycol. Each tube was incubated at 37°C for 20 min, placed in a dry ice/acetone bath for 1 min and 2.0 ml of triethylamine/acetone (1:1 v/v) was added. The absorbance at 560 nm was measured.

Results
Purity of trans-anethole oxide and trans-asarone oxide
The structures of trans-anethole oxide and trans-asarone oxide are shown in Figure 1. trans-anethole oxide and trans-asarone oxide were found to have very pure NMR spectra with little or no impurity peaks. The yields of oxides from this method were >95%, which are much better than those of Mohan and Whalen (27) and Greca et al. (39), whose methods used m-chloroperoxybenzoic acid to oxidize anethole or asarone and gave yields of 38 and 52%, respectively. Our trans-anethole oxide had an EIMS spectrum (70 eV) of m/z (rel. int. %) 164 [M]+ (82), 135 (23), 121 (44), 120 (100) and a 1H NMR 300 MHz (CDCl3, TMS, δ p.p.m.) spectrum of δ 1.45 (d, J = 5.1 Hz, 3H), δ 3.02 (dq, J = 5.1, 2.2 Hz, 1H), δ 3.82 (s, 3H), δ 3.84 (s, 3H), δ 3.89 (s, 3H), δ 3.90 (d, J = 2.2 Hz, 1H), δ 3.51 (d, J = 2.2 Hz, 1H), δ 3.78 (s, 3H), δ 6.8–7.3 (m, 4H). This NMR spectrum agrees well with that reported (27). Likewise, our trans-asarone oxide had an EIMS spectrum (70 eV) of m/z (rel. int. %) 224 [M]+ (91), 209 (90), 208 (26), 195 (26), 193 (60), 181 (71), 165 (100) and a 1H NMR 300 MHz (CDCl3, TMS, δ p.p.m.) spectrum of δ 1.45 (d, J = 5.2 Hz, 3H), δ 2.95 (dq, J = 5.2, 2.2 Hz, 1H), δ 3.82 (s, 3H), δ 3.84 (s, 3H), δ 3.89 (s, 3H), δ 3.90 (d, J = 2.2 Hz, 1H), δ 6.52 (s, 1H), δ 6.66 (s, 1 H). The NMR spectrum reported by Greca et al. (39), who used α-asarone extracted from Acorus gramineus for synthesis, agreed with ours except that their 1° proton had an upper chemical shift. He also claimed that the oxyranylpropene was obtained from the plant, but this is doubtful because his work-up and separation methods included an aqueous environment that would destroy the oxirane structure.

Stability of trans-anethole oxide and trans-asarone oxide
Both of the oxides were stable in acetone or DMSO for 1 h at 0 or 37°C (data not shown). In aqueous environments, however, the amount of these epoxides declined, presumably because of their hydration to diols. The half-life of trans-anethole oxide was 7.6 min in 0.1 M potassium phosphate buffer (pH 7.4) at 37°C. The presence of 154 mM KCl lowered the half-life to 4.2 min. Trans-asarone oxide showed shorter half-lives; 4.0 min without or 2.4 min with 154 mM KCl.

Mutagenicity for Salmonella typhimurium
Mutagenicity studies of trans-anethole oxide and trans-asarone oxide were carried out as described in Materials and methods. Both of the oxides showed dose-dependent mutagenicity in point mutation models (TA1535 and TA100) and in a frameshift mutation model (TA98). Trans-anethole oxide was more mutagenic than trans-asarone oxide in all strains (Figures 2–4).
male B6C3F1 mice, but only the highest dose of trans-anethole oxide was statistically significant \( (P < 0.01) \). Treatment with trans-asarone induced hepatomas up to ~90% with both doses (0.25 and 0.5 \( \mu \)mol/g body wt). With the low doses of trans-asarone oxide (0.125 and 0.25 \( \mu \)mol/g body wt), hepatoma induction was not significantly different from the trioctanoin treatment. However, the dose of 0.5 \( \mu \)mol/g body wt trans-asarone oxide exhibited detectable carcinogenic activity, with 50% of hepatomas in the surviving animals, in which the average number of hepatomas increased significantly \( (P < 0.01) \).

**Induction of skin papillomas in female CD-1 mice**

Unlike the parent propenylbenzenes, topical application of \( \geq 10 \mu \)mol of the reactive oxides produced sores on mouse skin (data not shown). Therefore, a topical dose of 5 \( \mu \)mol was introduced for this experiment. Topical application of trans-anethole oxide produced a 30% incidence of skin papillomas, in which the average number of papillomas per mouse was significantly different from that of acetone application \( (P < 0.01; \text{ Table II}) \). However, treatment with trans-anelthole, trans-asarone and trans-asarone oxide did not induce papillomas significantly.

**Fig. 3.** Mutagenicity of trans-anethole oxide and trans-asarone oxide for *S.typhimurium TA100*. The indicated amount of each oxide dissolved in 20 \( \mu \)l of acetone was preincubated with bacteria in a total volume of 0.6 ml potassium phosphate buffer (0.1 M, pH 7.4) at 37°C for 30 min before plating. The his\(^+\) revertants were scored 2 days later.

**Fig. 4.** Mutagenicity of trans-anethole oxide and trans-asarone oxide for *S.typhimurium TA98*. The indicated amount of each oxide dissolved in 20 \( \mu \)l of acetone was preincubated with bacteria in a total volume of 0.6 ml potassium phosphate buffer (0.1 M, pH 7.4) at 37°C for 30 min before plating. The his\(^+\) revertants were scored 2 days later.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (( \mu )mol \times 3)</th>
<th>Mice with papillomas (%)</th>
<th>Papillomas per mouse at 20 weeks (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>trans-anethole</td>
<td>5 ( \mu )mol\times 3</td>
<td>6</td>
<td>0.06 ± 0.5</td>
</tr>
<tr>
<td>trans-anethole oxide</td>
<td>5 ( \mu )mol\times 3</td>
<td>30( ^b )</td>
<td>0.4 ± 0.8( ^t )</td>
</tr>
<tr>
<td>trans-asarone</td>
<td>5 ( \mu )mol\times 3</td>
<td>11</td>
<td>0.1 ± 0.4</td>
</tr>
<tr>
<td>trans-asarone oxide</td>
<td>5 ( \mu )mol\times 3</td>
<td>3</td>
<td>0.03 ± 0.2</td>
</tr>
<tr>
<td>acetone</td>
<td>4</td>
<td>2</td>
<td>0.04 ± 0.2</td>
</tr>
</tbody>
</table>

Groups of 30 mice were treated topically on their shaven backs with 2.5 \( \mu \)g TPA in 0.1 ml acetone 18–24 h before each topical dose of the test compounds. Three applications of test compounds were given at 1 week intervals followed by twice weekly doses of 2.5 \( \mu \)g TPA in 0.1 ml acetone. The test compounds were dissolved in 0.2 ml acetone. Treatment with 10 or 50 \( \mu \)mol of the oxides produced raw skin sores. However, trans-anethole and trans-asarone did not produce skin sores at these levels.

\( ^t \)Significant; \( P < 0.05 \), Fisher’s test.

\( ^b \)Significant; \( P < 0.01 \), Student’s \( t \)-test.

**Table II.** Initiation of skin papilloma formation in 6–8-week-old female CD-1 mice by multiple topical doses of trans-anethole, trans-anethole oxide, trans-asarone or trans-asarone oxide

**Table I.** Induction of hepatomas by a single i.p. injection of trans-anethole, trans-anethole oxide, trans-asarone or trans-asarone oxide in 12-day-old B6C3F1 mice

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (( \mu )mol/g body wt)</th>
<th>Survival to weaning (%)</th>
<th>Mice with hepatomas(^a) (%)</th>
<th>Hepatomas per surviving mouse (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>trans-anethole</td>
<td>0.50</td>
<td>100</td>
<td>20</td>
<td>0.2 ± 0.5</td>
</tr>
<tr>
<td>trans-anethole oxide</td>
<td>0.25</td>
<td>100</td>
<td>19</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>trans-anethole oxide</td>
<td>0.50</td>
<td>100</td>
<td>29( ^c )</td>
<td>0.3 ± 0.5( ^t )</td>
</tr>
<tr>
<td>trans-asarone</td>
<td>0.25</td>
<td>100</td>
<td>91( ^d )</td>
<td>2.2 ± 1.2( ^t )</td>
</tr>
<tr>
<td>trans-asarone oxide</td>
<td>0.50</td>
<td>100</td>
<td>91( ^d )</td>
<td>2.7 ± 1.5( ^t )</td>
</tr>
<tr>
<td>trans-asarone oxide</td>
<td>0.125</td>
<td>97</td>
<td>12</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>trans-asarone oxide</td>
<td>0.25</td>
<td>100</td>
<td>17</td>
<td>0.2 ± 0.5</td>
</tr>
<tr>
<td>trans-asarone oxide</td>
<td>0.50</td>
<td>33( ^b )</td>
<td>50( ^c )</td>
<td>1.0 ± 1.5( ^t )</td>
</tr>
<tr>
<td>10% DMSO in trioctanoin</td>
<td>100</td>
<td>6</td>
<td>0.1 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

The mice were injected once i.p. with the test compounds. The compounds were dissolved in 10% DMSO in trioctanoin. The number of hepatomas >2 mm in diameter and visible on the surface were counted.

\( ^a \)Mice were killed at 10–11 months when each group had ~35 mice.

\( ^b \)Adhesion at autopsy.

\( ^c \)Significant; \( P < 0.05 \), Fisher’s test.

\( ^d \)Significant; \( P < 0.01 \), Fisher’s test.

\( ^e \)Significant; \( P < 0.01 \), Student’s \( t \)-test.
Discussion

The culinary use of naturally occurring alkenylbenzene derivatives, which are found in many spicy plants or their essential oils, has led to an interest in their toxicity and carcinogenicity. In the experiments presented here, the epoxides of propenylbenzenes were positive in the Salmonella mutation assay and induced tumors in mice. These results suggest that epoxidation of the side chain in vivo might be a possible pathway of carcinogenic metabolism of propenylbenzenes.

Studies on the metabolism of trans-anethole have revealed three major metabolic pathways: O-demethylation, ω-hydroxylation and side chain epoxidation (17–19). By virtue of its high chemical reactivity, epoxidation of the 1,2-double bond of trans-anethole is the pathway of greatest toxicological interest. The preferred route of metabolism in the rat was via epoxidation to diols. At a dose of 1500 mg/kg, 15% of the dose was found as diols in urine (18). In the mouse, 4.5% of the dose was excreted in the urine as diols. The cytotoxicity of trans-anethole in rat hepatocytes due to its metabolism to epoxide has been reported (20,21). inhibition of cytosolic epoxide hydrolases by 4-fluorochalcone oxide increased the cytotoxicity of anethole in cultured hepatocytes (21) and anethole epoxide itself also showed strong cytotoxicity (22).

In these reports, however, the unscheduled DNA synthesis (UDS) of hepatocytes was not altered by anethole and an epoxide hydrolase inhibitor (21) nor by anethole epoxide itself (22). These results led Marshall and Caldwell to conclude that anethole epoxide was not genotoxic but was cytotoxic (21,22).

Indeed, we have found that trans-anethole epoxide is more toxic to animals than trans-anethole. Application of 10 μmol trans-anethole epoxide to CD-1 mouse skin produced skin sores although no skin sores were found with anethole up to a dose of 50 μmol (Table II). Intraperitoneal injection of trans-anethole epoxide at a dose of 5 μmol/g body wt killed all 12-day-old male B6C3F1 mice in the group, whereas trans-anethole did not (data not shown). However, we also found that trans-anethole epoxide was mutagenic both in point mutation (Figures 2 and 3) and in frameshift mutation (Figure 4) models of the Ames test. The oxide produced revertant colonies in a dose-dependent manner. Bacterial cells are probably more resistant to the cytotoxicity of trans-anethole epoxide than hepatocytes because the oxide did not show toxicity in Salmonella strains up to a level of 90 μg/plate. It is reported that concentrations >0.5 mM trans-anethole epoxide caused a slightly increased UDS ratio to fall below 1.0 in rat hepatocytes because of cytotoxicity (22) and that the GSH level was reduced to ~60% of the control value by 1 mM trans-anethole epoxide (22). Therefore, trans-anethole epoxide might be too toxic to hepatocytes to reveal genotoxic effects in the UDS assay. Isosafrole (1-propenyl-3,4-methylenedioxybenzene), which is weakly carcinogenic in mice (6,7), did not induce UDS in hepatocytes (35). Thus, the UDS assay does not seem to properly extrapolate the mutagenicity or carcinogenicity of propenylbenzenes, although it is a well-known method to assess genetic damage.

The mutagenicity of anethole has been controversial. In Salmonella studies, anethole was negative without metabolic activation. With metabolic activation, however, it was mutagenic (31,37,38) in some studies but not in others (29). Anethole showed positive results with metabolic activation in the mouse lymphoma assay (29,30). Other mutagenic assays, including the Escherichia coli point mutation (30) and Saccharomyces cerevisiae reversion assays (32), showed negative results. These controversial results, especially with metabolic activation, might arise from the instability of anethole oxide.

As shown in Table I, i.p. injection of trans-anethole did not significantly induce hepatomas in male B6C3F1 mice. However, the highest dose of trans-anethole oxide (0.5 μmol/g body wt) increased the incidence of hepatomas significantly. Topical application to the skin of female CD-1 mice also induced skin papillomas (Table II). As mentioned earlier, we found that trans-anethole oxide is more toxic to animals than trans-anethole. Its induction of hepatomas and skin papillomas suggests that the epoxidation of anethole in vivo might be responsible for its carcinogenicity as well as toxicity. Asarone is reported to be mutagenic for Salmonella bacteria (36) and genotoxic to hepatocytes (33). It induced sister chromatid exchange in both human lymphocytes in vitro and in murine bone marrow cells in vivo (34,38). It is also teratogenic to rats (26) as well as carcinogenic in the pre-weanling mouse model (15). However, there are few studies on the metabolism of asarone. Since asarone has a similar structure to anethole, they may have the same metabolic fate. Trimethoxycinnamic acid was identified as a metabolite of asarone in cultured rat hepatocytes (33), suggesting that asarone might have been metabolized through ω-hydroxylation, like anethole. Therefore, it may also be metabolized by the other pathway of anethole, i.e. formation of the epoxide through oxidation of the 1,2-double bond. It has been reported that addition of cimetidine, a CYP2C11 inhibitor, reduced UDS induced by asarone (33). Furthermore, metabolism of asarone was decreased by cimetidine. It was suggested that the genotoxicity of asarone might be due to an oxidized metabolite, yet it was not made clear what the oxidized structure would be.

As shown in Figures 2–4, the synthetic trans-asarone oxide showed positive mutagenicity in all strains of Salmonella used in a dose-dependent manner. The effects of trans-asarone oxide were stronger than those of trans-anethole oxide. Trans-asarone oxide, of course, has a shorter half-life, suggesting a higher reactivity than trans-anethole oxide. Unfortunately, induction of skin papillomas by topical application of trans-asarone oxide was not observed (Table II). However, trans-asarone oxide induced hepatomas in up to 50% of male B6C3F1 mice with a single i.p. injection (Table I). The lower incidence than with its parent structure, trans-asarone, is probably due to its instability in physiological fluid in vivo. Our results suggest that trans-asarone oxide could be the oxidized metabolite of trans-asarone which is responsible for its mutagenicity and carcinogenicity as well as toxicity.

As shown in this study, trans-anethole oxide and trans-asarone oxide are mutagenic to bacterial cells and induce hepatomas and/or skin papillomas. Since it has been reported that one of the main metabolic fates of anethole is formation of the epoxide (17–19), anethole oxide may be responsible for the mutagenicity and the weak carcinogenicity of anethole. Likewise, trans-asarone could also be oxidized to the epoxide to be mutagenic and carcinogenic. This proposed mechanism, epoxidation of the 1,2 double bond, might distinguish the propenylbenzenes from the allylbenzenes, whose carcinogenicity is derived from formation of electrophilic sulfuric acid esters following 1'-hydroxylation.

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References


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Trans-anethole oxide and trans-anasore oxide

Trans-anethole oxide and trans-anasore oxide