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Genotoxicity of α-asarone analogues

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Abstract—The role of cholesterol in the formation of atherosclerotic lesions during hypercholesterolemia has been confirmed. α-Asarone is a substance of a potent hypolipidemic activity which is isolated from plants. We previously described the synthesis of several α-asarone analogues exhibiting hypolipidemic and antiplatelet activity. Genotoxic activity of four selected α-asarone analogues was theoretically evaluated based on quantum-mechanical method for calculation of enthalpy of carbocations formation (ΔH_R) according to the Testa's method. In the present paper, we evaluated the mutagenic and genotoxic activity of α-asarone isomers **2**–**5** based on the reference Ames test and micronucleus test. Results obtained in the study show that tested isomers were non-mutagenic, however, they exhibited growing cytotoxic activity. Relationship between the heat of formation of their putative metabolic intermediates and mutagenic/genotoxic activity was not confirmed.

1. Introduction

According to WHO data, diseases of cardiovascular system are a cause of 1/3 of all deaths in the world. Different plausible biological risk factors of cardiovascular heart disease include lipid abnormalities, glucose intolerance and insulin resistance, hypertension, changes in haemocoagulation or serum hyperhomocysteinemia.[1–3] In the light of these data, studies which document the influence of cholesterol and hypolipemic or hypotensive therapy on the improvement of prognosis in patients with cardiovascular diseases have become especially important.[4,5]

Hypercholesterolemia is an important cause of coronary heart disease and the role of cholesterol in the formation of atherosclerotic lesions has been confirmed in experiments.[6] Basically, cells—except for hepatic and ileal cells—do not synthesize cholesterol de novo but derive it from the blood and the cholesterol that accumulates in atherosclerotic lesion originates primarily in plasma lipoproteins (for a review, see Refs. 7 and 8). Cholesterol biosynthesis in the body is mainly regulated in the liver by the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGR).[9] Inhibition of this enzyme has proved the most efficient therapy for hyperlipidemia, since the enzymatic transformation of HMG-CoA to mevalonate represents one of the key steps in the metabolic pathway toward the biosynthesis of isoprenoids and sterols, such as cholesterol.[10]

Alpha asarone is a substance of a potent hypolipidemic activity[11–17] which is isolated from plants. It is mainly found in wild ginger (*Asarum europaeum L—Aristolochiceae*),[18] calamus (Rhizoma *Acorus Calamus—Araceae*),[19] and guatteria (*Guatteria gaumeri—Annonacea*),[20,21] the plant growing in the south-western part of Mexico. It has been found to elevate high density lipoprotein–cholesterol (HDL–cholesterol) fraction and reduce low density lipoprotein–cholesterol (LDL–cholesterol) fraction with almost unchanged total cholesterol contents in blood. The hypolipidemic action mechanism of α-asarone has recently been established in a rat model as an inhibitory effect on hepatic HMGR[14] and binding of α-asarone to HMGR active site has been theoretically examined.[22] The stimulation of bile secretion was found to be an additional mechanism reducing the serum cholesterol levels and its associated cholelitholytic activity. The results suggest that α-asarone inhibits cholesterol biosynthesis parallel to the statin mechanism.[23] However, in Ames test in the presence of S9 mix fraction

Keywords: Asarone; Genotoxicity; Mutagenicity; Ames; Micronuclei; Hypercholesterolemia.
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and *Salmonella typhimurium* TA 100 strain, mutagenic properties of α-asarone were observed.[24,25] It has previously been found that α-asarone and some of its naturally occurring isomers exhibit genotoxic activity.[26–31] In animal studies, carcinogenic[32,33] and teratogenic[34,35] activity of α-asarone derivatives was also observed. It may cause induction of sister chromatids exchange both in human lymphocytes in vitro and in mouse bone marrow cells in vivo.[36]

According to the Testa's method,[37] the relative stability of carbonium ions formed during the metabolic pathway (Fig. 1) may be one of the key factors in the genotoxicity of some allyl benzenes (asarones) and propenyl benzenes, the non-genotoxicity being due to a greater difficulty of the carbonium ions formation. A high value of energy ΔH_{carb} (above 231 kcal/mol) may be indicative of lack of genotoxic activity, whereas low value of this parameter (below 231 kcal/mol with the mean value of 227.7 ± 2.2 kcal/mol) qualifies the compound to the group of genotoxic compounds.[32,37]

We have previously described the synthesis of several α-asarone analogues. All the compounds were examined for their hypolipidemic activity on Wistar male rats. Some of the obtained analogues exhibited hypolipidemic and antiplatelet activity.[15,16,38] Genotoxic activity of four selected α-asarone analogues **2**–**5** (Fig. 2) was theoretically evaluated[38,15] based on quantum-mechanical method for calculation of enthalpy of carbocations formation according to the Testa's method.[37]

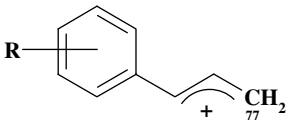


Figure 1. Carbocationic product of allyl benzenes metabolic activation.[30]

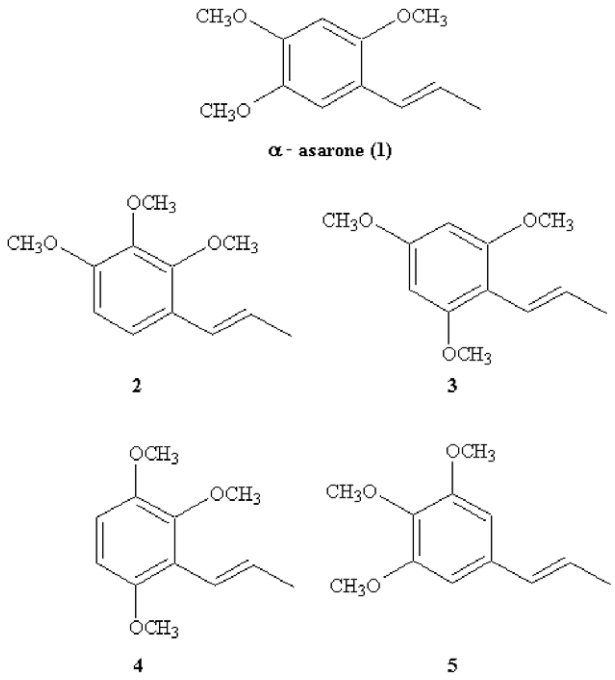


Figure 2. Structures of α-asarone and its isomers **2**–**5**.

Table 1. Heat of formations of metabolic intermediates (Fig. 1) for α-asarone and its isomers[15,38]

Compound	$\Delta H_{R(carbocat.)}$ [kcal/mol]
α-Asarone (**1**)	227.5
2	229.3
3	217.3
4	227.9
5	235.9

It was found that for α-asarone analogues **2**–**5** (Fig. 2) the value of the heat of carbocation formation falls within the range of 217.3–235.9 kcal/mol (Table 1). One of these compounds, 5, has a value higher than 231 kcal/mol and the remaining three compounds show the average value of 224.8 kcal/mol. Based on the above, it might be concluded that the compounds **2**–**4** possess genotoxic activity whereas compound **5** does not.

In the present paper, we have evaluated the mutagenic and genotoxic activity of α-asarone isomers **2**–**5** based on the reference Ames test and micronucleus test.[39–42]

2. Results and discussion

2.1. Ames test

Mutagenic activity of four α-asarone isomers **2**–**5** was studied with the use of Ames test.[39] Four *Salmonella typhimurium* strains (TA97, TA98, TA100, and TA102) were used in the experiments. The strains differed with respect to the type of mutation in the histidine gene, which results in different susceptibility of these strains to the action of chemical mutagenesis and enables the determination of a compound mutagenic action evaluated as an induction of specific bacterial DNA changes. In the test α-asarone has been found to exhibit potent mutagenic activity.[43] The results of Ames test for α-asarone isomers **2**–**5** (Tables 2–5) indicated that none of the tested compounds caused the increase in his^+ revertants number in any of the four studied bacterial strains, both in experiments with and without metabolic fraction. It strongly suggests that the compounds do not exhibit any mutagenic activity.

The compounds exhibited, however, moderate cytotoxic activity in the extent depending on the strain and the experimental conditions. Compound **2** at the concentration of 500 μg/plate reduced the number of his^+ revertants in four examined strains by 23–42% in the experiments without metabolic fraction (9–19% with metabolic fraction). Compounds **3** and **4** exhibited minor cytotoxic activity (28%) only against TA102 reducing the number of his^+ revertants by 28% and 43%, respectively, (in experiments with metabolic fraction by 27% and 46%, respectively). Compound **5** exhibited the

Table 2. The influence of compound **2** on the number of revertants his$^+$ of bacterial strains *Salmonella typhimurium*

Strains	Concentration of studied compound[a] (μg/plate)							
	Without S9mix				With S9mix			
	0[b]	100	500	1000	0	100	500	1000
TA97	177[a] ± 18	151 ± 7	151 ± 15	131 ± 14	200 ± 17	212 ± 4	200 ± 6	172 ± 18
TA98	31 ± 6	27 ± 2	22 ± 3	20 ± 2	30 ± 8	44 ± 2	43 ± 2	26 ± 8
TA100	130 ± 19	108 ± 7	116 ± 18	100 ± 13	145 ± 16	167 ± 8	160 ± 11	132 ± 12
TA102	277 ± 31	236 ± 27	203 ± 40	160 ± 36	298 ± 14	343 ± 48	335 ± 58	233 ± 54

[a] Mean number of colonies obtained in two experiments carried out in triplicate ± SD.
[b] Number of revertants colonies on plates without studied substance (spontaneous reversion).

Table 3. The influence of compound **3** on the number of revertants his$^+$ of bacterial strains *Salmonella typhimurium*

Strains	Concentration of studied compound (μg/plate)[a]							
	Without S9mix				With S9mix			
	0[b]	100	500	1000	0	100	500	1000
TA97	177 ± 18	163 ± 4	190 ± 30	223 ± 24	200 ± 17	175 ± 135	200 ± 23	187 ± 9
TA98	31 ± 6	32 ± 6	32 ± 7	26 ± 3	30 ± 8	24 ± 1	18 ± 1	22 ± 2
TA100	130 ± 19	138 ± 30	134 ± 16	148 ± 18	145 ± 16	153 ± 18	156 ± 18	126 ± 12
TA102	277 ± 31	275 ± 27	298 ± 43	195 ± 20	298 ± 14	333 ± 48	328 ± 49	218 ± 43

[a] Mean number of colonies obtained in two experiments carried out in triplicate ± SD.
[b] Number of revertants colonies on plates without studied substance (spontaneous reversion).

Table 4. The influence of compound **4** on the number of revertants his$^+$ of bacterial strains *Salmonella typhimurium*

Strains	Concentration of studied compound (μg/plate)[a]							
	Without S9mix				With S9mix			
	0[b]	100	500	1000	0	100	500	1000
TA97	177 ± 18	153 ± 11	137 ± 22	141 ± 9	200 ± 17	191 ± 14	126 ± 17	171 ± 7
TA98	31 ± 6	26 ± 4	26 ± 4	23 ± 4	30 ± 8	35 ± 11	27 ± 2	42 ± 11
TA100	130 ± 19	112 ± 19	104 ± 17	96 ± 14	145 ± 16	188 ± 37	186 ± 17	162 ± 12
TA102	277 ± 31	270 ± 28	159 ± 34	90 ± 22	298 ± 14	270 ± 16	162 ± 22	49 ± 21

[a] Mean number of colonies obtained in two experiments carried out in triplicate ± SD.
[b] Number of revertants colonies on plates without studied substance (spontaneous reversion).

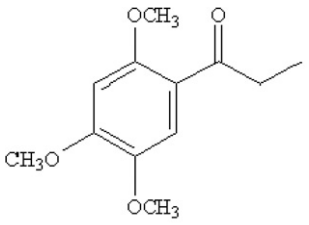
Table 5. The influence of compound **5** on the number of revertants his$^+$ of bacterial strains *Salmonella typhimurium*

Strains	Concentration of studied compound (μg/plate)[a]							
	Without S9mix				With S9mix			
	0[b]	100	500	1000	0	100	500	1000
TA97	177 ± 18	109 ± 16	88 ± 12	73 ± 9	200 ± 17	194 ± 9	164 ± 13	155 ± 11
TA98	31 ± 6	30 ± 7	24 ± 5	32 ± 1	30 ± 8	30 ± 1	18 ± 4	15 ± 4
TA100	130 ± 19	109 ± 16	88 ± 12	73 ± 9	145 ± 16	119 ± 3	108 ± 23	87 ± 2
TA102	277 ± 31	241 ± 25	155 ± 27	Toxic	298 ± 14	281 ± 30	236 ± 48	165 ± 47

[a] Mean number of colonies obtained in two experiments carried out in triplicate ± SD.
[b] Number of revertants colonies on plates without studied substance (spontaneous reversion).

highest toxicity. In the experiments without metabolic fraction, it inhibited growth of his$^+$ revertants in TA97 and TA100 strains by 59% and 44%, respectively, and caused thinning out of bacterial growth of strain TA102 (being non-toxic for strain TA100). In the experiments with metabolic fraction the compound reduced the number of his$^+$ revertants by 22–50% in all examined strains.

Thus the results obtained in the Ames test (with the use of the standard strains) showed no relationship between the heat of formation (ΔH_R) of putative metabolic intermediate and mutagenic activity of tested substances. It indicates that the formal rearrangement of substituents within α-asarone framework results in non-mutagenic compounds which, however, exhibit growing cytotoxic activity, as observed in in vitro test.



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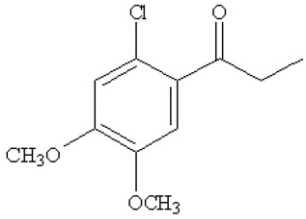

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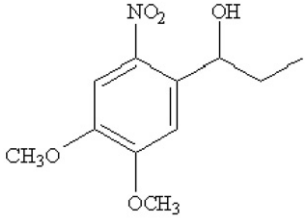

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Figure 3. Structures of α-asarone analogues **6**–**8**.

The obtained results are similar to those obtained by Cassani-Galindo et al.[43] who examined structural α-asarone analogues **6**–**8** (Fig. 3) and observed the lack of mutagenic activity with simultaneous increase in toxicity for the test strains in Ames test. They observed, however, significant increase in sister chromatid exchange (SCE) produced by compound **8** in human cultured lymphocytes. The lack of compounds **6**–**8** mutagenic activity was attributed to the lack of the unsaturated side chain (present in α-asarone) preventing the possibility of epoxide formation which was suggested to be responsible for α-asarone mutagenic activation.[44] It should be noted that compounds **2**–**5** did not show mutagenic activity in spite of the presence of unsaturated side chain and formal possibility of epoxide formation.

2.2. Micronucleus test

Genotoxic activity of α-asarone has been observed in micronucleus test in vitro.[45] In the present paper the mouse L929 cells recommended (due to their susceptibility to cytotoxic activation) for this type of experiments were used. Compounds **2**–**5** were examined in the concentration range from 0.1 to 100 μg/ml and doxorubicin used as a positive control in the study in the concentration range from 0.01 to 1 μg/ml (use of doxorubicin at higher concentration was impossible because of its cytotoxic activity).

Obtained results showed that in the positive control induction of formation of micronuclei occur already at concentration of 0.01 μg/ml. In control (untreated) cultures, the level of cells with micronuclei within binucleated cells population was of 1.44–1.80%. Statistical analysis revealed that isomers **2** used at concentration of 100 μg/ml and **3** at 50 μg/ml exhibit low, but statistically significant genotoxic activity toward L929 cells (3.79% and 3.44%, respectively). Compounds **4** and **5** did not induce the increase in the number of micronuclei within the studied concentration range (1.61% and 1.74% of micronuclei at the highest concentration) (Fig. 4).

Although compound **5** with relatively high heat of cationic intermediate formation (ΔH_R = 235.9 kcal/mol) appeared to be non-genotoxic, it does not explain other observations. Compound **4**, whose heat of cationic intermediate formation was similar to the value calculated for α-asarone, did not exhibit toxic activity. Genotoxic activities of isomers **2** and **3** were also much weaker than expected.

3. Conclusions

Results obtained in the study show that there is no relationship between the heat of formation (ΔH_R) of putative metabolic intermediates and mutagenic activity of four tested α-asarone isomers. The assumption of diminishing genotoxicity with the growing heat of cationic intermediate formation (Fig. 1 and Table 1) was not confirmed.

It seems that the formal rearrangement of substituents within α-asarone framework results in non-mutagenic compounds which, however, exhibit growing cytotoxic activity. The tested α-asarone isomers did not exhibit mutagenic activity in spite of the presence of unsaturated side chain and formal possibility of epoxide formation.

Our experiments did, however, allow to identify two compounds (**3** and **4**) which possess hypolipidemic activity[38] and do not show mutagenic and genotoxic activity. The compounds should thus undergo further pharmacological evaluation.

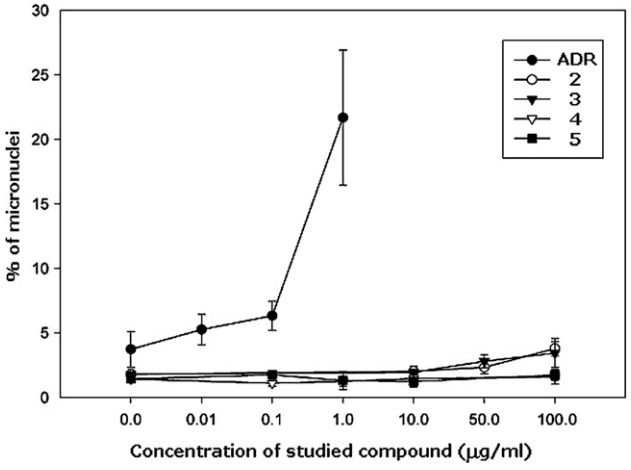

Figure 4. The ability of doxorubicin and α-asarone derivatives to induce the formation of micronuclei in L929 cells.

4. Materials and methods

4.1. Bacteria

Salmonella typhimurium strains: TA97, TA98, TA100, and TA102. These are alimentary mutants requiring presence of histidine and biotin for growth. Bacterial strains characteristics—spontaneous reversion level, ampicillin resistance (R plasmid presence), crystal violet sensitivity (*rfa* mutation), and UV sensitivity (*uvrB* mutation) were checked before the study according to the procedure of Maron and Ames.[39]

4.2. Cells

L929 line (cells of a mouse connective tissue neoplasm C3H/AN) from ATCC collection. The cells were cultured on the growth medium MEM with serum and antibiotics.

4.3. Media for bacterial cultures

Liquid broth medium (Nutrient Broth Oxoid); minimal agar medium (Bacto Agar Difco with Vogel and Bonner salt and 40% glucose); semi-liquid top agar (Bacto Agar—Difco).

4.4. Liquids for cell cultures

The MEM medium, fetal bovine serum, trypsin 0.5% + EDTA (Gibco); PBS without calcium and magnesium ions (IITD, Wrocław), antimycotic antibiotic (Sigma).

4.5. Positive control (micronucleus test)

Doxorubicin (Adriblastyna® RD), Pharmacia and Upjohn.

Compounds **2**–**5** were obtained from the Institute of Chemistry, University in Białystok.

The concentration range of the studied substances was limited by the cytotoxicity and solubility in the solvent (DMSO) used and the lack of precipitation in top agar.

4.6. Positive controls (without metabolic activation) (Ames test)

4-Nitro-1,2-phenyldiamine (NPD) (Merck) at the concentration of 20 μg/plate for strains—TA97 and TA98, sodium azide (Sigma) at the concentration of 1.5 μg/plate for strain—TA100, methyl methanesulfonate (MMS) at the concentration of 1.0 μg/plate for strain—TA102.

4.7. Positive controls (with metabolic activation) (Ames test)

2-Aminofluorene (2AF) (Fluka) at the concentration of 10 μg/plate for strains—TA97, TA98, TA100, 9-aminoacridine (Sigma) at the concentration of 50 μg/plate for strain—TA102.

4.8. S9 mix fraction

Mutagenic properties of many chemical compounds are revealed after metabolic activation. In the Ames test, the studied compound and bacterial cells were incubated in the presence of S9 fraction (MP Biochemicals, Inc.) containing enzymes which are able to induce biochemical metabolism of the studied substances.

4.9. Ames test

The evaluation of mutagenic action of α-asarone isomers was carried out on the basis of the reference Ames test. The test was prepared according to the procedure proposed by Maron and Ames.[39] The test consists in the evaluation of the ability of the studied compound to induce reverse mutation in auxotrophic cells of *Salmonella typhimurium* strains. The bacterial cells, in which reverse mutation occurred, show the ability to reproduce and form colonies on the minimal medium lacking growth factor (histidine).

The test strains were cultured in the liquid broth medium for 18 h in water bath at the temperature of 37 °C under agitation. After incubation 0.1 ml of bacterial culture and 0.1 ml of the studied compound were added to 2 ml of semi-liquid superficial agar containing 0.05 mM of biotin and 0.05 mM of histidine and poured on the plate with minimal agar medium. For tests with metabolic activation, 0.5 ml of S9 mix fraction was added. After 48–72 h of incubation at the temperature of 37 °C, revertant his^+ colonies were counted on the plates. To confirm the validity of the test in each experiment, the parallel positive controls were performed.

According to the generally accepted procedure regarding this test, substance in concentration which causes doubling of the number of his^+ revertants in relation to the spontaneous reversion level is defined as mutagenic.[46] Substance in concentration which causes gradual reduction or lack of a bacterial growth is defined as toxic.

4.10. Micronucleus test in vitro

The micronucleus test is a cytogenetic method used to detect chromosome breaks or damage of the cleavage spindle.[40–42,47]

Micronuclei are formed from acentric fragments of chromosomes which remain in the back during divisions of cell nucleus and are not incorporated to the descendant nuclei after the end of telophase or from the whole chromosomes remaining outside the nucleus as a result of a damage of cleavage spindle. In the test, each population of dividing cells may be used.

For the evaluation of genotoxic activity of α-asarone derivatives, micronucleus test in vitro was performed using the technique of cytokinesis blocking by cytocha-

lasin B[48] with the use of cells of the mouse connective tissue neoplasm C3H/An (line L929).

After 48 h cultures were treated with trypsin, suspended in MEM culture medium enriched with fetal bovine serum, inoculated on 4-chamber culture dishes in the number of 10^5cells/chamber and incubated for 24 h at the temperature of 37 °C. Then the cells were treated with the studied substance in various concentrations for 2 h. Afterwards, the medium with the studied substance was removed. Fresh medium containing cytochalasine B was added to the cell culture. The cells were incubated for 24 h at the temperature of 37 °C. After incubation, the cultures were washed with PBS twice, dried, fixed with ethanol for 10 min and stained for 20 min with 10% solution of Giemsa stain in 0.05 M phosphate buffer of pH 6.8. The dried preparations were evaluated microscopically.

The effect of genotoxic action is evaluated as the percentage of cells with micronuclei in the population of binuclear cells (not less than 1000 binuclear cells counted).

To check the statistical significance of the obtained results, Medistat System (microcomputer statistical system for medicinal purposes, version 2.1; 1992) was used.

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