REVIEW

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Mutagenic and carcinogenic constituents of medicinal herbs used in Europe or in the USA

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Dedicated to Prof. Dr. G. Eisenbrand, Division of Food Chemistry and Environmental Toxicology, Department of Chemistry, University of Kaiserslautern, on the occasion of his 60th birthday

1. Introduction

Every year people in the developed world spend an estimated sum of $ 12.4 billion on phytomedicines – plant based remedies in the form of teas, extracts, oils and capsules [1]. In Europe, the market for herbal remedies is growing even faster than the pharmaceutical market since herbal medicines have mostly lost their "alternative" image. Indeed the new and popular programmes on preventive medicine brought plant-derived anticancer agents into the focus of interest either as preventive or as therapeutic agents [2]. Some of the most recent drugs of natural origin play a dominant role in pharmaceutical care such as paclitaxel, teniposide, and analogs of camptothecin. Regarding the sometimes severe side effects of certain herbal remedies, e.g. Chinese herbs nephropathy, an illness following the intake of a slimming regimen [3], the question arises whether a too optimistic attitude against medicinal agents of natural origin is really justified. The first step towards defusing this situation is a profound analysis of drugs of herbal origin and their ingredients; the second step comprises measures of regulation.

The most serious adverse effects produced by plant constituents are genotoxic effects. A number of herbal drugs is known to possess mutagenic and carcinogenic properties. In the last 20 to 30 years, numerous mutagenic and carcinogenic constituents from plants have been isolated, identified and tested in model systems. In some cases the mechanisms of their mutagenic and carcinogenic activities could be explained at a molecular level. With respect to preventive measures this is of great interest since the genotoxic plant constituents can cause severe toxicities and can contribute to tumor incidence in people taking these plants as herbal remedies.

In the present review, several chemical constituents of herbal medicines are critically discussed with respect to their mutagenic and carcinogenic potential. The chemical entities of these items encompass a wide spectrum of substances. They belong to the chemical classes of nitrophenanthrenes, pyrrolizidine alkaloids, propenylbenzene derivatives and anthracene-9,10-dione derivatives. These substances represent only a part of the genotoxic plant constituents but an evaluation of all substances from plants known or suspected to bear a mutagenic and/or carcinogenic risk is obviously not possible here.

2. Aristolochic acids

Aristolochic acids are constituents of *Aristolochia* species (Aristolochiaceae). *Aristolochia clematitis* L., a plant which is common in Mediterranean countries and Central Europe, was widely used in European folk medicine. From its root and upper parts aristolochic acids were isolated. Herbal teas containing Aristolochiae herba and radix were used for centuries for wound-healing, activation of the immune system and therapy of neoplasms. Pure aristolochic acid was still reported in 1982 to stimulate phagocytosis in healthy volunteers at a daily dosage of 0.9 mg per person for 10 days [4].

The tragic outcome of a slimming regimen carried out in Belgium leading to the so called "Chinese herbs nephropathy" was due to a mistake by confounding *Stephania tetrandra* S. Moore (Menispermaceae) with *Aristolochia fangchi* Y. C. Wu ex L. D. Chou et S. M. Hwang. Aristolochic acid was in fact found in the capsules of the slimming regimen [5, 6]. Up to date, at least 100 cases of extensive interstitial fibrosis of the kidneys were observed in Belgian women after using the herbal slimming pills. Seventyone patients were already registered in January 1994, 35 of those being on renal replacement therapy and 29 kidneys were transplanted [6]. Renal biopsies of 5 patients showed an extensive hypocellular interstitial fibrosis

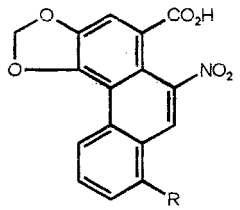
with loss of tubes, tubular atrophy, global glomerular sclerosis and fibrosis of mainly interlobular arteries. Transitional cell carcinomas were diagnosed, one of the bladder and one of the urether [5]. Similar cases were also reported in France [7], in Japan [8], and in Great Britain [9]. Although several *Aristolochia* species are officially listed in the Chinese Pharmacopoeia, including *A. contorta* Bge., *A. debilis* Sieb. et Zucc., *A. manshuriensis* Kom. and *A. fangchi*, epidemiological observations related to those in Belgium have not been reported from China, except some cases reported in Taiwan [10]. In fact, the acute nephrotoxicity of aristolochic acid was already known in the early sixties as the major dose-limiting factor in phase I clinical trial with aristolochic acid as an antineoplastic agent [11].

2.1. Pharmaceutically used drugs

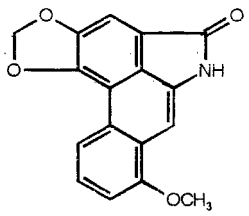
The following *Aristolochia* species served for drug use: *A. clematitis* L., *A. rotunda* L., and *A. serpentaria* L. As drugs were listed: Aristolochiae clematitis hom. HAB1 (the fresh, overground parts); A. clematitis hom. PFX (the fresh flowering overground parts); A. clematitis hom. HPUS95 (the flowering overground parts); A. rotunda rhizoma (the dried rhizome); A. rotunda hom. HAB34 (the dried rhizome); A. serpentaria root (Serpentaria USP XI, Serpentary BCP 49); A. serpen-taria hom. HAB34 (the dried root); A. serpentaria hom. HPUS89 (the dried root).

Herbal medicines containing aristolochic acids have been used in France, in Germany, in Great Britain and in the USA. In Germany, the findings of carcinogenic effects of aristolochic acids [12, 13] led the German Federal Health Office to withdraw the permission for more than 240 remedies containing aristolochic acids [14] with the exception of homeopathic dilutions of D11 and higher which are allowed for medical use.

Aristolochic acids I (**1**) and II (**2**) are derived from the phenanthrene ring system. They belong to the rare plant constituents bearing a nitro and a carboxy substituent. Reduction of the nitro group to an amino group yields corresponding aristololactams, e.g. aristololactam I (**3**) [15].



Aristolochic acid I **(1)**: R = OCH_3
Aristolochic acid II **(2)**: R = H



Aristololactam I **(3)**

2.2. Mutagenicity

Mutagenic and carcinogenic acitivities of aristolochic acids have been extensively studied and elucidated at a molecular level. Aristolochic acid I, aristolochic acid II, and the so-called aristolochic acid, a commercially available preparation consisting mainly of aristolochic acid I (65%) and aristolochic acid II (34%), have been found to be mutagenic in *Salmonella typhimurium* TA1537 and TA100 [16, 17]. In contrast, aristolochic acids exhibited no or only a very week mutagenic activity in *S. typhimurium* TA100NR, which lacks the enzyme nitroreductase [17]. Results from *S. typhimurium* YG which is highly sensitive to nitroarenes indicated the importance of the nitro group and of the reductive activation for the mutagenic activity of aristolochic acids [18]. The mutagenic activity of aristolochic acid was found to be primarily due to base substitution mutations. Frameshift mutations were found to play a marginal role in the mutagenesis of both aristolochic acid I and aristolochic acid II [19, 20].

In human lymphocytes, aristolochic acid was reported to induce chromosome aberrations and sister chromatid exchanges (SCE) [21]. The mutagenic activity of aristolochic acid was also observed in *Drosophila* [22]. In the granuloma pouch assay in rats, aristolochic acid exhibited a mutagenic activity in this test system in a dose-dependent manner [23].

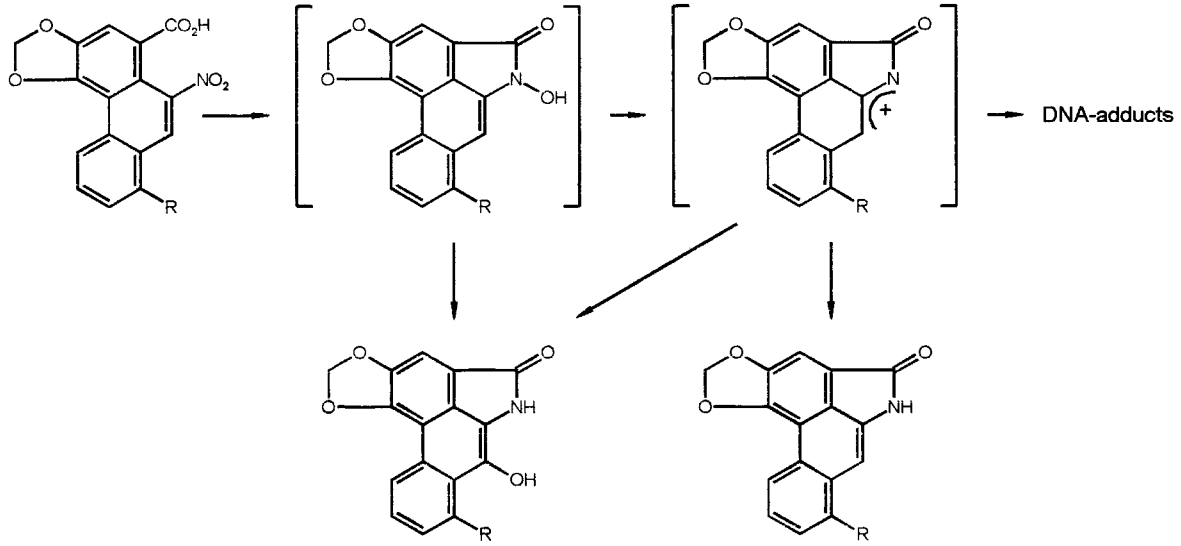
2.3. Carcinogenicity

The carcinogenic activity of aristolochic acid has been demonstrated in rats; male and female rats treated orally with aristolochic acid at daily doses of 0.1, 1.0 or 10.0 mg/kg developed dose- and time-dependently a high incidence of tumors. Rats treated for three months with aristolochic acid at daily doses of 1.0 or 10.0 mg/kg showed severe papillomatosis of the forestomach with occasional signs of malignancy. Three to six months later, the rats developed squamous cell carcinomas in the forestomach and metastases without further treatment. Anaplasia of the tubular epithelium, adenomas in the renal cortex, and hyperplasia, papillomas or carcinomas in the transitional epithelium of the renal pelvis and the urinary bladder were also observed. High doses of aristolochic acid (10.0 mg/kg) caused extensive necrosis of the forestomach epithelium, followed by regeneration and hyperplasia, papilloma formation and ultimately by invasive squamous cell carcinoma [12, 13]. Treatment of rats with aristolochic acid at a daily dose of 0.1 mg/kg did not result in tumor induction in the first 6 months, but papillomas or squamous cell carcinomas occurred in the forestomach after 12 and 16 months. In addition, hyperplasia of the transitional epithelium of the renal pelvis was observed [13]. Induction of hepatic nodules in the rat by aristolochic acid was also reported [24]. The fact that aristolochic acid is a strong forestomach carcinogen by oral administration to rats led to its use in animal experiments as a model for human stomach cancer [25, 26]. Tumor induction by aristolochic acid was also observed in mice [27, 28].

2.4. Biotransformation and mechanism of genotoxic activity

Studies on biotransformation of aristolochic acid I and aristolochic acid II *in vitro* revealed two different metabolic pathways for the two compounds under aerobic and anaerobic conditions. Whereas aristolochic acid I was extensively demethylated under aerobic conditions, only minor metabolism was detectable under anaerobic conditions. In contrast, aristolochic acid II was not metabolized anaerobically but underwent rapid reduction of the nitro group to the amino derivative and subsequent ring closure to the corresponding aristololactam II under aerobic conditions. It indicated that the reduction of the nitro group in aristolochic acid might be an activation mechanism to form cyclic *N*-acylnitrenium ion with delocalized positive charge as the ultimate carcinogenic intermediates which were able to bind to DNA *in vitro* and *in vivo* (Scheme 1) [29]. The four-electron reduction of the nitro group was the critical step in activation of aristolochic acids [30].

Scheme 1



Metabolic activation of aristolochic acid [29]

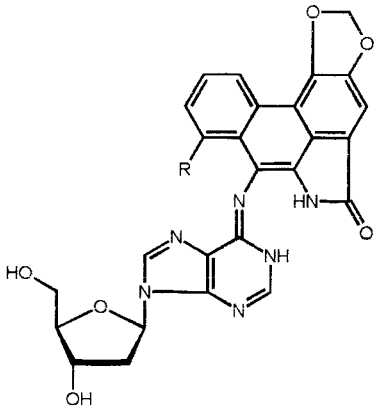
The hydroxamic acids, presumed as proximate carcinogens, could not be isolated. In order to investigate the formation of DNA adducts aristolochic acid I and aristolochic acid II were enzymatically reduced with xanthine oxidase in the presence of nucleosides, nucleotides, and calf thymus DNA. Schmeiser et al. reported that both aristolochic acid I and aristolochic acid II preferentially bind to the exocyclic amino group of purine nucleotides [31]. Simple computer calculations demonstrated that the highest positive charge in the ring system is not located at the nitrogen but at position 7 [32].

The deoxyadenosine and deoxyguanosine adducts formed *in vivo* have been found to be identical to those detected *in vitro* [33]. The two major DNA adducts of aristolochic acid I, i.e. 7-(deoxyadenosin-N^6-yl)-aristololactam I (**4**) and 7-(deoxyguanosin-N^2-yl)-aristololactam I (**5**) were detected in the DNA isolated from several organs of rats treated with aristolochic acid I [34, 35]. In liver DNA of rats treated with aristolochic acid II, major DNA adducts were 7-(deoxyadenosin-N^6-yl)-aristololactam II (**6**) and 7-(deoxyguanosin-N^2-yl)-aristololactam II (**7**) [3]. The adduct levels were generally higher with aristolochic acid I than with aristolochic acid II. The highest level of aristolochic acid I adducts were found in the target organ, the forestomach. The highest level of aristolochic acid II adducts were found in the kidney [35].

Fernando et al. employed the ^{32}P-postlabelling assay to non-invasively monitor the exposure of rats to aristolochic acid I by analysis of its DNA adducts in exfoliated cells in urine [36, 37]. Detection of the DNA adducts formed by aristolochic acids in the forestomach as target organ of rats by ^{32}P-postlabelling analysis was reported [38].

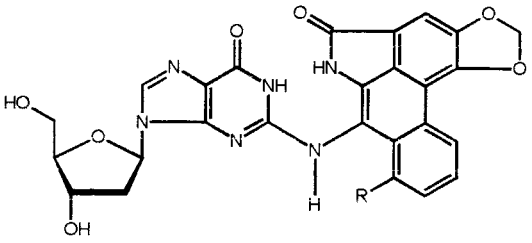
Furthermore, aristolochic acid-DNA adducts were detected in kidney [5] and ureter [3] of patients with Chinese herbs nephropathy. 7-(Deoxyadenosin-N^6-yl)-aristololactam I was found to be the major DNA adduct of aristolochic acid in the renal tissues, the minor adducts, 7-(deoxyguanosin-N^2-yl)-aristolactam I and 7-(deoxyadenosin-N^6-yl)-aristololactam II were also detected [3, 5]. Quantitative analyses of renal tissues revealed relative adduct levels from 0.7 to 5.3 adducts per 10^7 nucleotides for 7-(deoxyguanosin-N^6-yl)-aristololactam I, from 0.02 to 0.12 adducts per 10^7 nucleotides for 7-(deoxyguanosin-N^2-yl)-aristololactam I and from 0.06 to 0.24 adducts per 10^7 nucleotides for 7-(deoxyadenosin-N^6-yl)-aristololactam II [3].

Aristololactams were believed to be the principal detoxification metabolites of aristolochic acids and were detected



7-(Deoxyadenosin-N^6-yl)-aristololactam I **(4)**: R = OCH_3

7-(Deoxyadenosin-N^6-yl)-aristololactam II **(6)**: R = H



7-(Deoxyguanosin-N^2-yl)aristololactam I **(5)**: R = OCH_3

7-(Deoxyguanosin-N^2-yl)aristololactam II **(7)**: R = H

in urine and faeces of animals and humans exposed to aristolochic acids. However, Stiborova et al. have recently reported that aristololactams may also be activated by cytochrome P450 and peroxidase to form adducts with DNA. Aristololactam I activated by peroxidase-mediated one-electron oxidation led to the formation of several DNA adducts, the major two adducts were identified as 7-(deoxyguanosin-N^2-yl)aristololactam I and 7-(deoxyadenosin-N^6-yl)aristololactam I. It was therefore suggested that aristololactam I may also contribute to the potential carcinogenic effect in humans [39].

Studies on activating mutations in oncogenes detected in rat tumors induced by aristolochic acid revealed a 90%–100% high incidence of H-*Ras* mutations in forestomach and ear duct tumors [40, 41]. Analysis by sequencing and selective oligonucleotide hybridization demonstrated an AT → TA transversion mutation at the second position of codon 61 of H-*Ras* gene in all tumors that scored positive for H-*Ras* gene mutation. This apparent selectivity for mutations at adenine base in aristolochic acid I-induced tumors is consistent with the extensive formation of 7-(deoxyadenosin-N^6-yl)-aristololactam I as the major adduct in several organs as well as in the target organ, forestomach, in rats [35, 42]. Moreover, 7-(deoxyadenosin-N^6-yl)-aristololactam I was found to exhibit an apparently lifelong persistence in the forestomach DNA, whereas 7-(deoxyguanosin-N^2-yl)-aristololactam I was removed continuously over a 36 week period [37].

3. Pyrrolizidine alkaloids

More than 200 pyrrolizidine alkaloids isolated from about 6000 plant species (3% of all flowering plant species) have been structurally determined [43]. They are widely distributed in plant species belonging to the following families: Apocynaceae, Asteraceae, Boraginaceae, Celastraceae, Elaeocarpaceae, Euphorbiaceae, Fabaceae, Orchidaceae, Poaceae, Ranunculaceae, Rhizophoraceae, Santalaceae, Sapotaceae, and Scrophulariaceae, especially of the genus *Senecio* (Asteraceae) and *Crotalaria* (Fabaceae) [44]. In many countries plants containing pyrrolizidine alkaloids have been and are still used as roborants, for wound-healing and as food-items. Especially in Japan, *Petasites japonicus* Maxim. (Asteraceae), *Tussilago farfara* L. (Asteraceae) and *Symphytum officinale* L. (Boraginaceae) are known as edible plants; the young flower stalk of *P. japonicus* and buds of *T. farfara* as well as the leaf and root of *S. officinale* are used as green vegetables or tonic. Medicinally, plants containing pyrrolizidines are mainly used as remedies against rheumatism, cough, asthma bronchiale, and diarrhea. A number of plants containing pyrrolizidine alkaloids occasionally used in folk medicine in Europe were reviewed by Roeder [45].

3.1. Pharmaceutically used drugs

Some medicinal herbs containing pyrrolizidine alkaloids are officially listed in several European and in the US Pharmacopoeas. Pharmaceutically used drugs of *Borago officinalis* L. (Boraginaceae) are Boraginis flos (the dried flowers of *B. officinalis*) and Boraginis herba (the dried herb of *B. officinalis*). Both drugs are listed in the French Pharmacopoeia PFX. Moreover Borago officinalis hom. HAB34 is used (prepared from the fresh leaves).

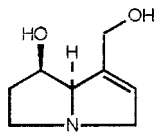
In Europe and in the USA, *Petasites hybridus* (L.) G. M. Sch. (Asteraceae) is used in herbal remedies as Petasitidis folium (the dried leaves of *P. hybridus*) and Petasitidis rhizoma (the dried root of *P. hybridus*), Petasites hybridus hom. HAB1 (from the fresh plant *P. hybridus*) and Tussilago petasites hom. HPUS88 (the whole fresh plant).

Several *Senecio* species are used in Europe and in the USA including *S. aureus* L., *S. bicolor* ssp. *cineraria* WILLD., *S. jacobaea* L., *S. ovatus* L. and *S. vulgaris* L. The following drug items are officially listed from *S. aureus*: Senecionis herba BHP83 (the dried herbs), S. aureus hom. HAB34 (the fresh, flowering plant), S. aureus hom. HPUS88 (the fresh, flowering plant). Drugs used from *S. bicolor* ssp. *cineraria* are: Cinerariae herba (the dried herbs), Cineraria maritima hom. HAB34 (fresh plant, resembled before flowering), Cineraria maritima hom. PFX (the fresh, flowering plant), Cineraria maritima hom. HPUS88 (the fresh, flowering plant). *Senecio jacobaea* delivers Senecionis jacobaea herba (the dried herbs), Senecio jacobaea hom. HAB34 (the whole, fresh plant), Senecio jacobaea hom. HPUS88 (the fresh, flowering plant).

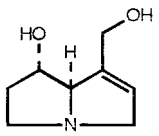
From *Tussilago farfara* L., the following drugs are used: Farfarae flos (the dried inflorescences), listed in PFVIII and in HelvVII; coltsfoot flowers Mar29, Farfarae folium (the dried leaves of *T. farfara*), listed in DAB10, ÖAB90, BHP83, Mar29; Farfara hom. HAB34 (the fresh leaves).

3.2. Constituents

The majority of pyrrolizidine alkaloids represent esters of retronecine (**8** 1-hydroxymethyl-7-hydroxy-1,2-didehydropyrrolizidine) or its 7-*cis* analog heliotridine (**9**). Pyrrolizidine alkaloids can be formed from retronecine or heliotridine in the form of monoesters, diesters as well as macrocyclic diesters, since retronecine possesses two hydroxygroups.



Retronecine **(8)**

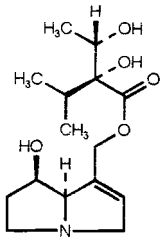


Heliotridine **(9)**

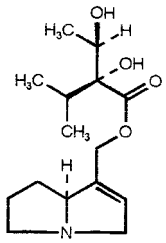
Borago officinalis was reported to contain less than 0.001% pyrrolizidine alkaloids, which were identified as amabiline, intermedine and its 7-acetyl derivative, lycopsamine and its 7-acetyl derivative, supinine and thesinine [46–48].

The root of *Petasites hybridus* was reported to contain the pyrrolizidine alkaloids integerrimine, isotussilagine, neopetasitenine, neoplatyphylline, petasitenine, senecionine, senkirkine, and tussilagine with a total content of 1 ppm to 100 ppm. The leaves of *P. hybridus* contain much less pyrrolizidine alkaloids than the root [49–51]. Pyrrolizidine alkaloids isolated from *Petasites japonicus* were identified as fukinotoxin [52], petasitenine, neopetasitenine, senkirkine and otosenine [53, 54].

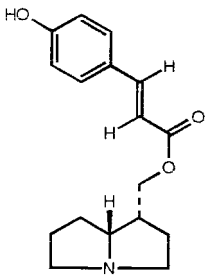
Senecio aureus was reported to contain the pyrrolizidine alkaloids floridanine, florosenine and otosenine in a total content of 0.02% [55, 56]. *Senecio jacobaea* is known to be rich in pyrrolizidine alkaloids. The total content amounts 0.2% to 0.3% of the dry flowering plants [57]. Pyrrolizidine alkaloids isolated from *S. jacobaea* were identified as (*E*)- and (*Z*)-erucifoline and acetylerucifoline, integerrimine, jacobine, jacoline, jaconine, jacozine, retror-



Intermedine **(10)**



Amabiline **(11)**



Thesinine **(12)**

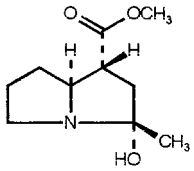
sine, riddelline, senecionine, seneciphylline, spartioidine, senecivernine, usaramine. Jacobine with a content of 0.14% was found to be the major alkaloid [58–70].

From *Tussilago farfara*, pyrrolizidine alkaloids were isolated and identified as senkirkine [71], senecionine, tussilagine, isotussilagine, tussilaginine, and isotussilaginine [72–76]. The content of senkirkine in *T. farfara* was given to be 0.15 g/kg [71].

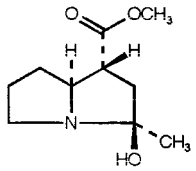
Among the pyrrolizidine alkaloids mentioned above, supinine, intermedine (**10**), and lycopsamine are monoesters of retronecine. Amabiline (**11**) is a monoester lacking the 7-hydroxy group and thesinine (**12**) is a monoester without both the 1,2-double bond and the 7-hydroxy group.

E- and *Z*-erucifoline, integerrimine (**13**), usaramine, jacobine, jacoline, jaconine, jacozine, retrorsine (**14**), senecionine (**15**), riddelline (**16**), seneciphylline (**17**), senecivernine, and spartioidine are macrocyclic diesters of retronecine, while platyphylline (**18**), and neoplatyphylline (**19**) are macrocyclic diesters of retronecine without 1,2-double bond.

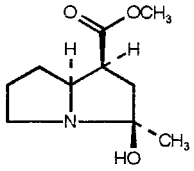
Floridanine, otosenine (**21**), florosenine, fukinotoxin, petasitenine (**22**), neopetasitenine, and senkirkine (**23**) are pyrrolizidine alkaloids derived from otonecine (**20**). Structurally otonecine is derived from a *N*-methyl-azacyclooctanone. Otonecine may act as a pyrrolizidine ring system by transannular interactions.



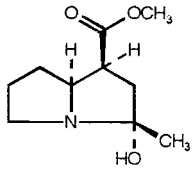
Tussilagine **(24)**



Isotussilagine **(25)**



Tussilaginine **(26)**

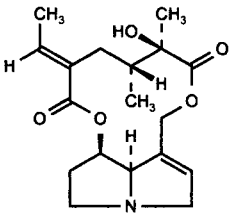


Isotussilaginine **(27)**

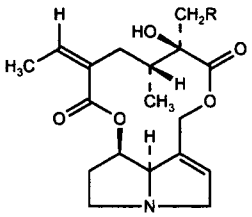
Tussilagine (**24**), isotussilagine (**25**), tussilaginine (**26**), and isotussilaginine (**27**) are derived from pyrrolizidin-7-carboxylic acid methyl ester.

3.3. Toxicity

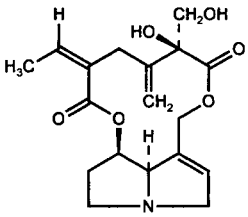
The hepatotoxicity of pyrrolizidine alkaloids in humans has long been known. Retrorsine and seneciophylline as two major alkaloids in *Senecio* species have been identified as contaminants of grain causing "bread poisoning" of humans in South Africa. Over 80 cases of *Senecio* poisoning, mainly in the young and mostly fatal, have been observed in the George and Mossel Bay districts 80 to 90



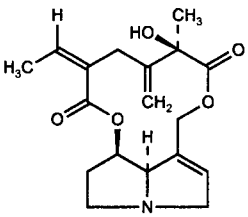
Integerrimine **(13)**



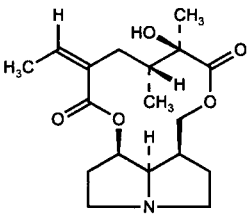
Retrosine **(14)**: R = OH
Senecionine **(15)**: R = H



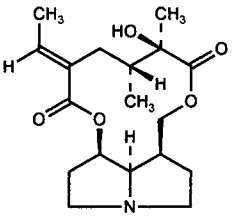
Riddelline **(16)**



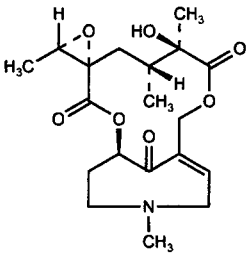
Seneciophylline **(17)**



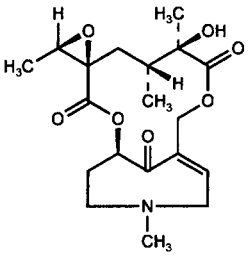
Platyphylline **(18)**



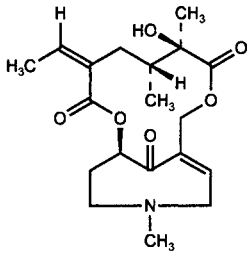
Neoplatyphylline **(19)**



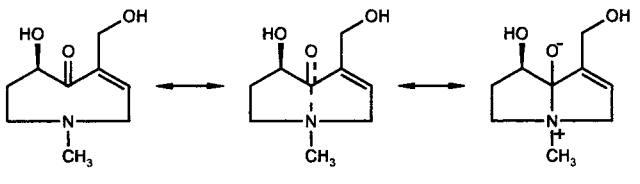
Otosenine **(21)**



Petasitenine **(22)**



Senkirkine **(23)**



Otonecine **(20)**

years ago; 12 cases were hospitalized in one area in 1931–1941. The most common symptoms were severe abdominal pain, rapidly developing ascites and hepatomegaly [77, 78].
The primary toxic effect of retrorsine in rats was reported to be on liver parenchyma and on the central and hepatic veins, with induction of centrilobular haemorrhagic necrosis followed by an apparent proliferation of the endothelium of the central and sublobular veins, leading to partial or complete occlusion of the lumen [79, 80]. Death of rats caused by a fatal dose of retrorsine is accompanied by haemorrhagic centrilobular necrosis of the liver [81]. It was further reported that retrorsine exerted a strong and persistent cell cycle block on hepatocyte proliferation. The selective proliferation of transplanted hepatocytes in retrorsine-treated animals is dependent, at least in part, on the persistent cell cycle block imposed by the alkaloid on endogenous parenchymal cells [82]. The hepatotoxic effects of petasitenine in rats were confirmed by electron microscopic examination. Biochemical tests for the mitochondrial function revealed an inhibitory effect of petasitenine on the respiratory system [83].

3.4. Mutagenicity

Pyrrolizidine alkaloids, especially those of diester and macrocyclic diester types, are known to be mutagenic. Different results in tests with *Salmonella typhimurium* have been reported. Retrorsine, seneciphylline and the extract of several *Senecio* species including *S. fuchsii* showed a weak mutagenic activity in *S. typhimurium* in the presence of an activating system [84]. An acetone extract of *S. jacobaea* did not produce mutagenic response in *S. typhimurium* TA1535 and TA100 without mammalian liver microsomes, but showed mutagenic activity in *S. typhimurium* TA1535, TA100, TA1537 and TA98 with an activating system. However, the major pyrrolizidine alkaloid of *S. jacobaea*, jacobine, produced a negative response without and with mammalian liver microsomes in test with *S. typhimurium* [85]. Genotoxic effects in the *Escherichia coli* SOS chromotest were reported only with retrorsine, but not with senecionine and senkirkine either in the absence or in the presence of rat liver S9 mix [86].
Retrorsine was found to possess a weak clastogenic potential and to induce chromosome aberrations *in vitro* in V79 cells at high concentrations without a metabolizing system. However, a strong and concentration-dependent increase in chromosome aberrations for retrorsine was observed in the presence of rat liver S9 mix [87]. Seneciphylline and senkirkine significantly induced SCE in V79 cells in the presence of primary chick embryo hepatocytes [88]. Petasitenine and senkirkine were also reported to induce chromosome aberrations in V79 cells. Petasitenine induced interchromosomal exchanges, while senkirkine caused chromatid gaps. Petasitenine and senkirkine also induced an 8-azaguanine-resistant mutation in V79 cells by direct treatment for 48 h [89]. In an *in vivo* study, a single dose of integerrimine caused chromosomal damage in bone marrow cells of mice in a dose-dependent manner. The greatest frequency of chromosome aberrations was detected 12 h after treatment [90]. Besides senecionine and seneciphylline, riddelline was also reported to be mutagenic in V79 cells in the presence of S9 mix [91].
Seventeen pyrrolizidine alkaloids were studied with the hepatocyte primary culture-DNA repair test using rat hepatocytes. DNA repair synthesis was elicited by 15 alkaloids, including senecionine, seneciphylline, jacobine. Seneciphylline and senkirkine were also positive in the DNA repair test with hamster or mouse hepatocytes [92]. Retrorsine was reported to cause increased frequencies of micronucleated rat hepatocytes *in vitro* in a concentration-dependent manner [93, 94]. The frequency of micronucleated erythrocytes in peripheral blood of male mice was increased after a single dose of riddelline. Increases in unscheduled DNA syntheses (UDS) were detected in primary hepatocytes from rats and mice treated with riddelline at doses up to 25 mg/kg for 5 or 30 days [95–97].
Structure activity relationships of some pyrrolizidine alkaloids inhibiting mitosis and inducing megalocyte formation in cultured bovine kidney epithelial cells revealed that pyrrolizidine alkaloids of macrocyclic diester type with a double bond in their acid moiety such as seneciphylline, senecionine, riddelline, retrorsine produced a dose-dependent inhibition of colony formation at 50, 100, and 300 μM and induction of megalocytosis at 500 μM. Pyrrolizidine alkaloids of the macrocyclic diester type without a double bond in their acid moiety and pyrrolizidine alkaloids of open diester type elicited only a slight inhibition of colony formation and had no effect on cellular morphology at 500 μM. Retronecine had no effect on either colony formation or cell morphology. Pyrrolic pyrrolizidine alkaloids such as dehydrosenecionine and dehydroretronecine were more active in inhibiting colony formation than their parent compounds and were potent inducers of abnormal cellular morphology at 500 μM [98]. In an assay for the induction of somatic mutation and recombination in wing cells of *Drosophila melanogaster*, pyrrolizidine alkaloids of macrocyclic diester type showed the highest mutagenicity, whereas pyrrolizidine alkaloids of the monoester type esterified at the O^9 position of retronecine showed the lowest mutagenicity. Monoester type alkaloids lacking the 7-hydroxy group were not mutagenic [99, 100].
Jacobine was found to induce the production of endogenous avian RNA tumor virus particles in cultured chick embryo fibroblasts [101].

3.5. Carcinogenicity

The carcinogenic effects of pyrrolizidine alkaloids were already reported in the fifties. They were found to strongly depend on their structure; alkaloids of the macrocyclic diester type and of the diester type were found to be carcinogenic such as retrosine, riddelline, seneciophylline [78], senkirkine [78, 102], petasitenine [102]. Studies on structure-activity-relationships revealed that the pyrrolizidine alkaloids derived from 1,2-didehydropyrrolizidine, esterified at O^7 or O^9 position with a branchy carboxylic acid having more than 5 carbon atoms, are toxic, mutagenic and carcinogenic [103, 104]. About 100 pyrrolizidine alkaloids known up to date possess these structural characteristics [105].
The carcinogenicity of retrorsine was observed in rats. Ten male and 4 female rats were treated with retrorsine in drinking water at a concentration of 0.03 mg/ml, 3 days a week until death at 10–24 months. Six male rats showed nodular hyperplasia in the liver, 4 of these were confirmed as hepatomas. Hemorrhagic liver tumor has been observed in 1 male rat; and regenerative liver changes and papillary adenoma in the lung in 1 female rat [106]. A single dose of retrorsine at 30 mg/kg to 95 rats caused 5 hepatomas and 1 case each of carcinoma of breast, lung, and uterus, hemangioendothelioma of the spleen, as well as retroperi-

toneal sarcoma and squamous cell carcinoma of the jaw in the 29 surviving rats [107].

The carcinogenicity of riddelline has also been studied in rats. Five female and 5 male rats were treated with riddelline in drinking water at a concentration of 0.02 mg/ml twice weekly for 6 months followed by 3 i.p. injections of 25 mg/kg during the 7th month. All 4 surviving males developed liver nodules in 6–16 months after the last injection [108]. In a more recent report, Chan et al. described the toxicity and carcinogenicity of riddelline following 13 weeks of oral administration to rats at doses up to 10 mg/kg and mice at doses up to 25 mg/kg, five times a week. At the end of treatment, significant findings included dose-related hepatopathy and intravascular macrophage accumulation in rats and hepatocytomegaly in mice. Fourteen weeks after the last application of riddelline, the lesions persisted and hepatic foci of cellular alteration in male rats and bile duct proliferation in female rats and in male and female mice increased in severity [95].

Senkirkine was reported to be carcinogenic in rats after i.p. administration of 22 mg/kg twice a week for 4 weeks and then once a week for 52 weeks. All 20 rats in the experimental group survived for more than 290 days after the start of treatment and 9 of them developed liver cell adenomas [109].

Petasitenine was shown to be carcinogenic in rats given drinking water containing 0.01% petasitenine. Haemangioendothelial sarcomas of the liver and liver cell adenomas developed each in five of ten treated rats [110].

In addition to the pure alkaloids, the total alkaloid fraction isolated from *Senecio jacobaea* was found to be carcinogenic in rats and chicken [111]. Carcinogenicity of the dried flowers of *Tussilago farfara* has been studied in rats. The flowers of *T. farfara* were applied in the diet at 16–32%, 8% and 4%. In the high dose group, 8 of 12 rats developed hemangioendothelial sarcomas in the liver, and 3 of them developed additional tumors including 1 hepatocellular carcinoma, 1 hepatocellular adenoma and 1 papilloma of the urinary bladder. In the middle dose group, 1 rat developed hemangioendothelial sarcoma in the liver. No tumors were observed in the low dose group [112]. Similar effects in rats were observed with *Petasites japonicus* [113].

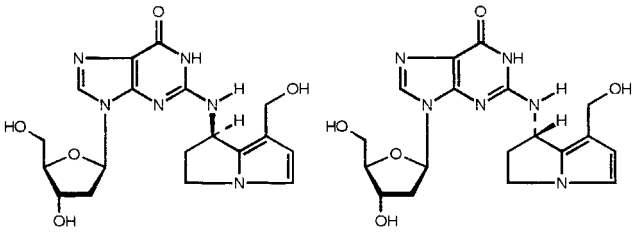
Dehydroretronecine, the main pyrrolic metabolite of pyrrolizidine alkaloids, has been shown to be carcinogenic in rats by s.c. administration at a biweekly dose of 20 mg/kg for 4 months then 10 mg/kg for 8 months. Local rhabdomyosarcomas developed in 31 of 60 treated animals and metastasis in 5 animals [114].

3.6. Biotransformation and mechanism of genotoxicity

Pyrrolizidine alkaloids derived from 1,2-didehydropyrrolizidine were found to be mainly activated by cytochrome P450 isoenzymes [115], including CYPIIIA4 [116], to 1,2,3,8-tetradehydropyrrolizidine metabolites consisting of a pyrrole and a pyrrolidine ring, which are much more electrophilic than the parent compounds and represent the ultimate reactive species. They are able to react with alcohol, amine, and thiol groups of biological macromolecules [117, 118]. Formation of N-oxides [119, 120], 3-hydroxy or 8-hydroxy derivatives as intermediates has been discussed. The conversion of the retrorsine to its pyrrolic derivative *in vivo* and *in vitro* was reported [121]. The pyrrolic metabolite of retrorsine also exerted hepatotoxic effects in rats [122]. Pyrrolizidine alkaloids with a methyl group at the nitrogen and a keto function at position 8 such as petasitenine, fukinotoxin, senkirkine, and otosenine can be activated in a similar way after cationic elimination of the methyl group (Scheme 2) [123, 124]. Pyrrolizidine alkaloids may also be converted to dehydroretronecine metabolites by human microsomal preparations. Incubation of otonecine type pyrrolizidine alkaloids with both rat and human microsomal preparations yielded both dehydroretronecine and the parent alkaloid [125].

Pyrrolizidine alkaloids of diester type can act as bifunctional alkylating agents, which may cause DNA alkylation and DNA-DNA, or DNA-protein crosslinking (Scheme 3) [126, 127]. DNA binding of pyrrolizidine alkaloids has been analysed using ^{14}C- or ^{3}H-labelled compounds, or using alkaline elution [126, 128, 129]. The reactivity of the positions 7 and 9 was found to depend on steric hindrance by the ester function.

In a model study, two diastereomers of N^2-(dehydroretronecin-7-yl)-deoxyguanosine (**28**) were isolated by reaction of dehydroretronecine with deoxyguanosine. The adducts were formed by nucleophilic attack of the exocyclic amino group of deoxyguanosine at the electrophilic center at C-7 position of dehydroretronecine [130].



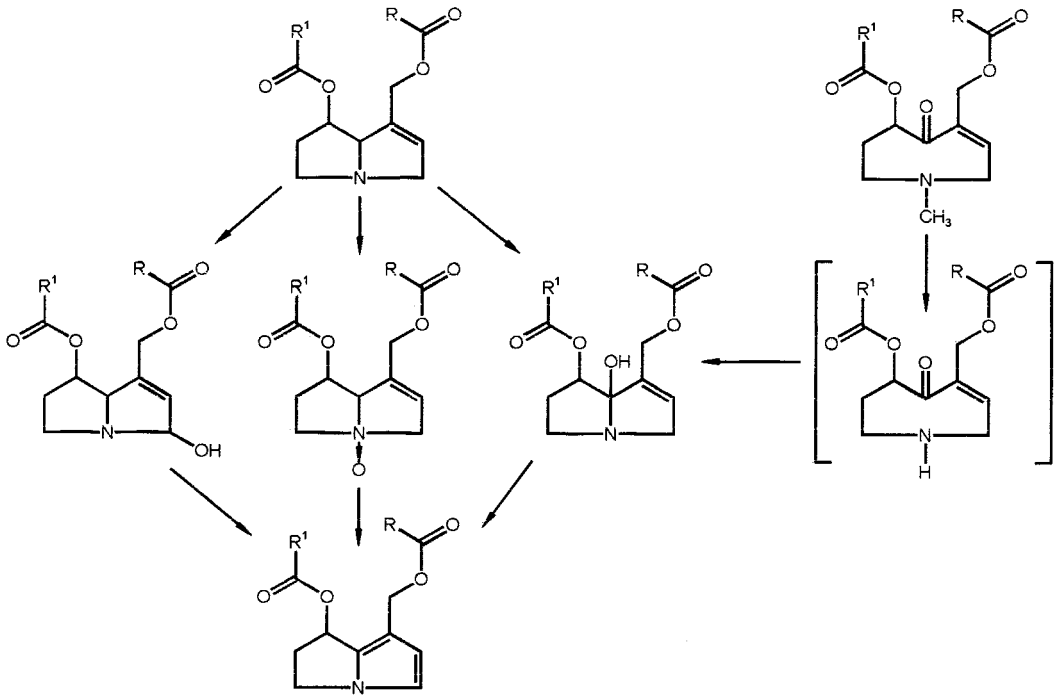
Diastereomers of N^2-(dehydroretronecin-7-yl)-deoxyguanosine (**28**)

Reaction of dehydroretronecine with adenosine, guanosine, thymidine, uridine, deoxyadenosine and the nucleotides deoxyguanosine 5′-monophosphate, deoxyadenosine 5′-monophosphate, thymidine 5′-monophosphate and deoxyuridine 5′-monophosphate also resulted in the formation of monoalkylated adducts. The reactive C-7 position of dehydroretronecine was shown to alkylate N^6 of adenosine, O^2 of thymidine and thymidine 5′-monophosphate and N^2 of deoxyguanosine 5′-monophosphate to yield N^6-(dehydroretronecin-7-yl)-adenosine (**29**), O^2-(dehydroretronecin-7-yl)-thymidine (**30**) and its 5′-monophosphate, and N^2-(dehydroretronecin-7-yl)-deoxyguanosine 5′-monophosphate. By analogy it was deduced that alkylation occurs at O^2 of uridine and its 5′-monophosphate [127].

Isolation of dehydroretronecin-7-yl-adducts as well as dehydroretronecin-9-yl-adducts by the reaction of deoxyguanosine, deoxyadenosine, thymidine, and deoxycytidine with a dehydropyrrolizidine alkaloid has been also reported [131]. Jacobine was found to significantly induce dose-dependent DNA-DNA interstrand cross-linking in hepatic DNA of male rats treated i.p. with jacobine at dose of 5–60 mg/kg. Significant DNA-protein cross-linking was also induced by doses of 15–60 mg/kg, however, no DNA single-strand breaks were detected [132].

Model experiments with a series of synthetic pyrrolic derivatives of pyrrolizidine alkaloids to alkylate 4-(*p*-nitrobenzyl)pyridine have been reported by Cooper and Huxtable [133]. The rank order of reactivity was the same for the macrocyclic and open diester pyrrolic derivatives. Pyrrolic metabolites of the primarily hepatotoxic alkaloids, retrorsine and seneciphylline, were more reactive than those of some other related pyrrolizidine alkaloids and produced additionally pneumo- or neurotoxicity. This suggests that

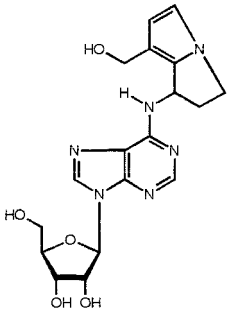
Scheme 2



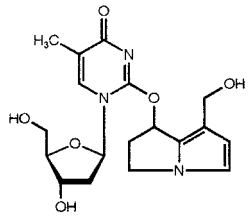
Metabolic activation of pyrrolizidine alkaloids derived from 1,2-didehydropyrrolizidine [123, 124]

pyrrolic metabolites with greater stability are able to survive long enough to reach target organs downstream from the liver.

A general binding profile of senecionine to macromolecules was reported by Candrian et al. [126]. DNA and proteins from rat liver, lung and kidney of both sexes treated with the [^{3}H]senecionine and [^{3}H]seneciphylline showed covalent binding of the alkaloids to DNA. A covalent binding index (CBI), expressed as μmol bound alkaloid/mol nucleotides per dose in mmol/kg, of 210 ± 12 was found in the liver of female animals treated with [^{3}H]senecionine, whereas binding to liver DNA of males was fourfold lower. The DNA damage 6 h after treatment persisted during

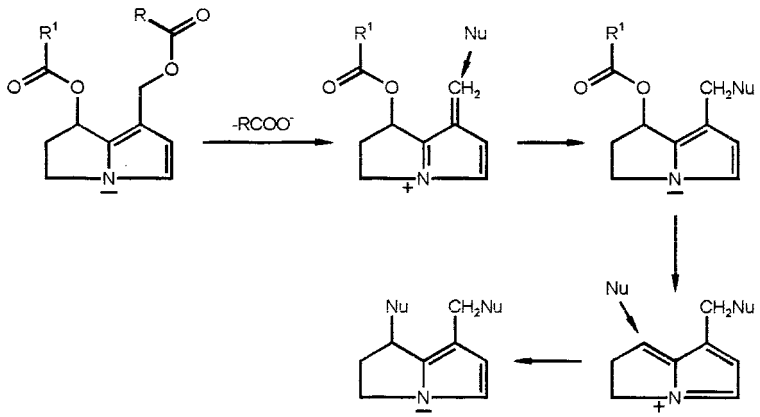


N^6-(Dehydroretronecin-7-yl)-adenosine **(29)**



O^2-(Dehydroretronecin-7-yl)-thymidine **(30)**

Scheme 3



Alkylation and crosslinking reactions by activated pyrrolizidine alkaloids [72]

the following 4 days. Administration of [^{3}H]seneciphylline to female and male rats resulted in a CBI of 69 ± 7 and 73–92 for the liver DNA. The binding of both alkaloids to DNA of lungs and kidneys in male and female rats was also observed.

Additional studies on crosslinking of DNA including intra- and intermolecular crosslinking were reported with pBR322 plasmid DNA and M13 viral DNA [134], and with synthetic oligonucleotides [135]. The desoxyguanosine of 5′-d(CG) has been found to be the predominant site for crosslinking by activated pyrrolizidine diesters [135].

A study on the structure-activity relationship of the DNA cross-linking by pyrrolizidine alkaloids in mammalian cells revealed that two structural determinants for biological activity appear to be the presence of both a macrocyclic diester and an unsaturated ester function since the cross-linking ability of seneciphylline, riddelline, retrorsine, and senecionine by far exceeded that of macrocyclic type pyrrolizidine alkaloids without an unsaturated ester function and retronecine. In addition, the stereochemical orientation of the ester linkage was found to have no effect on biological activity [136].

According to the activating mechanism, the parent compound retronecine practically does not possess any alkylating potential. The hydrolysis of pyrrolizidine alkaloids by either base catalyzed or enzymatic hydrolysis of the ester linkages should be considered as a detoxification process [137, 138].

A further detoxification mechanism is the conjugation of the active pyrrolic metabolites with glutathione to form 7-glutathionyl-1-hydroxymethyl-1,2,3,8-tetradehydropyrrolizidine. Isolated rat livers perfused with retrorsine at 0.5 mM for 1 h resulted in a removal of 93% of retrorsine by the liver. Glutathione conjugation accounted for 880 nmol per gramm liver. Release of the less toxic hydrolytic product of the dehydroalkaloid, 1-hydroxymethyl-7-hydroxy-1,2,3,8-tetradehydropyrrolizidine, was also detected [139]. Senecionine was metabolized primarily to putative pyrrolic metabolites and to its N-oxide by rat liver microsomal monooxygenases. The pyrrolic metabolites were highly reactive and either bound covalently to nucleophiles or were hydrolysed to the more stable 1-hydroxymethyl-7-hydroxy-1,2,3,8-tetradehydropyrrolizidine and the corresponding necic acid. Addition of glutathione to incubation mixtures containing rat liver microsomes and senecionine, resulted in the formation of a glutathione conjugate with 1-hydroxymethyl-7-hydroxy-1,2,3,8-tetradehydropyrrolizidine, however, only negligible amounts of conjugate were formed without rat liver microsomes, indicating the microsomal conversion of senecionine to the highly reactive metabolite followed by conjugation with glutathione [140].

Sulphur-bound pyrrolic metabolites were identified in blood and liver tissue from rats after a single i.p. injection of retrorsine, senecionine and some related pyrrolizidine alkaloids [141]. An N-acetylcysteine conjugate with pyrrolic metabolites of senecionine has been identified in rat urine following administration of senecionine [142]. A glutathione conjugate was found to be formed by pyrrolizidine alkaloids of otonecine type with rat microsomes in the presence of glutathione [143].

The acute toxicity of retrorsine in rats was found to be decreased by i.p. pretreatment with cysteine at a dose of 200 mg/kg and the glutathione level was increased to about double that of the controls. In contrast, the acute toxicity of retrorsine in rats was increased and the glutathione levels decreased by i.p. pretreatment with 2-chloroethanol at a dose 30 mg/kg [81]. The levels of pyrrolic metabolites in the livers of rats pretreated with cysteine or chloroethanol are about 60% and 200%, respectively, those of control animals 2 h after administration of retrorsine. In addition, treatment of rats with retrorsine caused a fall in the cytochrome P450 levels in the liver, 24 h after dosing. This loss of cytochrome P450 was increased in rats pretreated with chloroethanol [81].

An oral dose of retrorsine to rats was found to be metabolized and excreted in urine as isatinecic acid, pyrrolic metabolite, N-oxide and retronecine accounting for 31%, 10%, 11% and 0.4%, respectively, of the administered dose. Pretreatment of rats with phenobarbital increased the excretion of both pyrrolic metabolites and isatinecic acid with a corresponding decrease in the excretion of retrorsine and its N-oxide [144]. The relative concentration of pyrrolic metabolites after administration of retrorsine to bile duct-cannulated rats, was greatest in the bile collected in the first hour and was negligibly small 7 h after dosing [145]. Following an i.v. injection of [^{14}C]senecionine at a dose of 60 mg/kg to rats, 44% and 43% of the total administered radioactivity were excreted in the bile and urine. Senecionine N-oxide was identified as the major metabolite. Less than 5% in bile and 18% in urine was excreted as parent alkaloid. The plasma concentration of senecionine-equivalents/g decreased from 107 to 12 nmol over a 7 h periode, while red blood cell concentrations declined from 109 to 26 nmol/g. Biliary pyrrolic metabolites were estimated to be 1.4 mg [146]. [^{14}C]Senecionine an [^{14}C]seneciphylline administered to mice resulted in a rapid excretion of the radioactivity in the urine and feces accounting for 84% of the dose injected within 16 h. The liver contained over 1.5% of the dose at 16 h and a small amount, 0.04%, of the dose was transferred into the milk in 16 h [129].

An interspecies difference of the urinary retrorsine metabolites has been observed. Guinea pigs are generally resistant to the toxicity of pyrrolizidine alkaloids. The urinary excretion of the metabolites, isatinecic acid and pyrrolic metabolites from retrorsine was lower in the resistant species such as guinea pigs, than in the susceptible species including mice, hamsters and rats. In contrast, the urinary N-oxide levels were higher in guinea pigs relative to mice, hamsters and rats. It can be suggested that the resistance of guinea pigs to pyrrolizidine alkaloids is attributed to the high metabolism of pyrrolizidine alkaloids to N-oxides combined with a corresponding low conversion to pyrrolic metabolites [147]. Flavin-containing monooxygenase was found to be a major detoxifying enzyme for the pyrrolizidine alkaloid senecionine in guinea pig tissues. In guinea pig lung and kidney microsomes, N-oxide was the major metabolite formed from senecionine with little or no production of pyrrolic metabolites. The high rate of detoxication coupled with the low level of activation of senecionine in liver, lung, and kidney may help to explain the apparent resistance of the guinea pig to intoxication by senecionine and other pyrrolizidine alkaloids [148]. Carboxylesterase GPH1 from guinea pig liver microsomes was found to be able to hydrolyse seneciphylline, senecionine, integerrimine and related pyrrolizidine alkaloids, whereas carboxylesterase GPL1 was much less active [149].

However, the pyrrolizidine alkaloid jacobine is unexpectedly toxic to this animal species. Studies on the metabolism of jacobine with isolated guinea pig liver enzymes revealed that the combination of high pyrrolic metabolism and low N-oxide formation, together with low hydrolysis,

are the major factors contributing to the susceptibility of guinea pigs to jacobine [150]. The reaction of glutathione with jacobine, containing an epoxide ring was found to be catalyzed by hepatic glutathione-S-transferase enzymes of guinea pig *in vitro* [151].

The Commission D and Commission E of the German Federal Health Office prepared monographies on several pyrrolizidine alkaloids containing plants as e.g. *Tussilago farfara, Petasites hybridus*. On the basis of the proven genotoxicity of pyrrolizidine alkaloids, the German Federal Health Office ordered in 1992 a maximal pyrrolizidine content for remedies containing pyrrolizidine alkaloids with a 1,2-unsaturated necine structure. This restriction comprises about 550 medicinal preparations containing herbal materials from e.g. *Anchusa officinalis*, *Borago officinale*, *Cineraria maritima*, *Cynoglossum officinale*, *Eupatorium cannabinum*, *Lithospermun officinale*, *Petasites hybridus*, *Senecio aureus*, *Symphytum officinale*, and *Tussilago farfara*. The maximal daily intake of pyrrolizidine alkaloids should not be more than 1 μg, in topical application not more than 100 μg/day. For preparations containing more than 0.1 μg pyrrolizidine alkaloids in the daily dose of the medicinal preparations, a maximal application period of 6 weeks per year is further prescribed [152].

In Austria remedies containing the plant *Tussilago farfara* or parts of it were withdrawn from the market in 1992.

The variability of the content of plant constituents is a known problem. Taxonomic and ecologic methods are described which enable a rational interpretation of the content of pyrrolizidine alkaloids, exemplified with *Symphytum officinale* [153]. Several methods exist for the quantitative determination of pyrrolizidine alkaloids. For *Tussilaginis folium* an exact description for GC-analysis with special emphasis on the extraction procedure is given [154].

4. Safrole and related propenylbenzenes

Very diverse plant families possess propenylbenzenes as constituents, e.g. the Lauraceae with *Sassafras albidum* (Nutt.) Nees and *Cinnamomum* species; the Araceae with *Acorus calamus* L. and *Acorus gramineus* Soland.; the Aristolochiaceae with *Asaraum europaeum* L. and *Asarum canadense* L.; the Myristicaceae with *Myristica fragrans* Houtt and other *Myristica* species. Their volatile oils possess an aromatic smell and are therefore used as aromatics. Moreover, the drugs containing propenylbenzenes are also used in many pharmaceutical preparations for medical purpose.

4.1. Pharmaceutically used drugs

The following pharmaceutical preparations are known for:

Sassafras albidum: Sassafras aetheroleum, Sassafras cortex, Sassafras lignum, Sassafras hom. HAB34, Sassafras officinale hom. HPUS88. Examples for use in folk medicine are rheumatism, dysmenorrhoe and diseases of the urogenital tract. However, the use of preparations of sassafras is not recommended due to their toxic ingredients. A very popular beverage in the USA was "root beer", which was brewed from Sassafras cortex. Since it contained up to 27 ppm safrole, its use was forbidden in 1960.

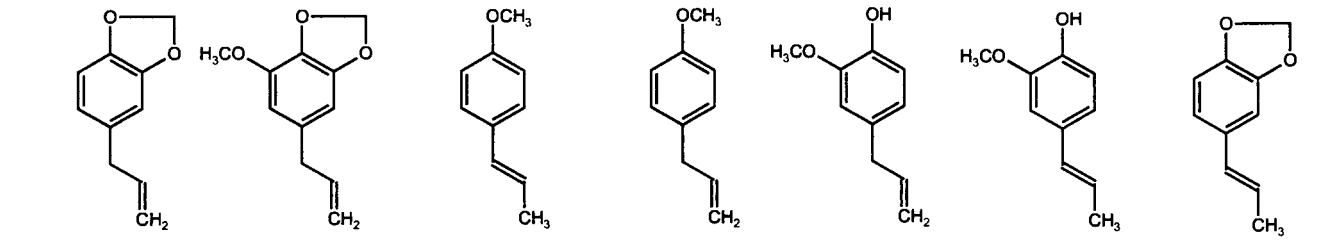
Acorus calamus: Calami aetheroleum, C. rhizoma, Acorus calamus hom. HAB1. *Acorus gramineus*: Acorum graminеus rhizoma. *A. calamus* is used in the treatment of ingestions.

Asarum canadense: A. canadense rhizoma, Asarum canadense hom. HAB34, Asarum canadense hom. HPUS88; *Asarum europaeum*: Asari rhizoma (Radix asari EB 6), Asarum europaeum hom. HAB1, Asarum europaeum hom. HPUS78. The pharmaceutical preparations of *A. europaeum* are used for the treatment of asthma bronchiale, acute and chronic bronchitis. Fields of use of the homeopathic drugs are mental exhaustion, diarrhoea, irritation of mucous membranes. A negative monography of the Commission D of the Federal Health Office exists for *"Asarum canadense"* [155].

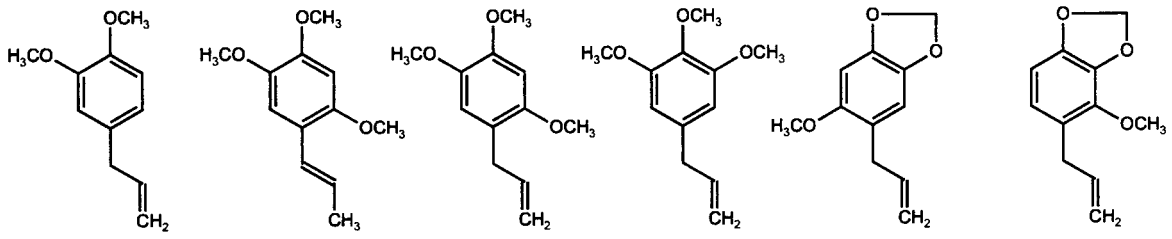
Cinnamomum aromaticum Nees (*C. cassia* Presl): Cassiae flos, Cinnamomi cassiae aetheroleum; *Cinnamomum camphora* (L.) Sieb.: Cinnamomi camphorae aetheroleum; *Cinnamomum culilawan*: Culibabani cortex*; Cinnamomum loureirii* Nees: Cinnamomi loureirii cortex; *Cinnamomum ceylanicum* Blume: Cinnamomi aetheroleum, Cinnamomi cortex, Cinnamomum ceylanicum hom. HAB1, Cinnamomum hom. HPUS78. These preparations are mainly used for the treatment of indigestion. The stem bark of *C. ceylanicum* and *C. aromaticum* is an old and widely used spice. A negative monography of the Commission D of the Federal Health Office exists for "Cinnamomi flos" [156].

Myristica argentea: arillus and seed; *Myristica dactyloides*: leaves, cortex and seed; *Myristica fragrans*: Myristicae aetheroleum, arillus (Macis) and seed, Myristica fragrans hom. HAB1; nux moschata hom. PFX, nux moschata hom. HPUS88); *Myristica malabarica*: arillus and seed. Medical use of myristica preparations are in dental and mouth care. In folk medicine *Myristica arillus* is used for the treatment of indigestion, diarrhoea, and flatulence.

Naturally occurring propenylbenzenes comprise a group of compounds that contain a 2-propenyl or 1-propenyl group attached to a substituted benzene ring. The most extensively studied 2-propenylbenzene of plant origin is safrole (**31**), which is contained in sassafras oil up to 93% [157–159] and is also present in less quantity in essential oils of nutmeg, the seeds of *Myristica fragrans* and other *Myristica* species [160, 161]. The principal 2-propenylbenzene in the essential oil of nutmeg was found to be myristicin (**32**) [162]. Cinnamon and cinnamon oil are known to contain cinnamaldehyde as the major constituent, however, safrole and related propenylbenzenes including anethole (**33**), estragole (**34**), eugenol (**35**), isoeugenol (**36**), isosafrole (**37**) and methyleugenol (**38**) are also detected as minor constituents [163]. Safrole was found to be one of the major constituents in the essential oil from *Cinnamomum camphora*, especially in young trees [164]. The principal propenylbenzenes in the essential oil from *Acorus calamus* [165–167] and *Asarum* species [166] are reported to be α-asarone (*trans*-asarone, **39**), β-asarone (*cis*-asarone) and γ-asarone (**40**). α-Asarone and β-asarone are present in the essential oil of *Acorus calamus* in about 6 and 100 μg/ml and 500 and 25 μg/ml in the essential oil of *Asarum europaeum* [166]. In addition, safrole, methyleugenol, elemicin (**41**), asaricin (**42**), myristicin, and croweacin (**43**) are detected in some *Asarum* species [168–170]. Among the propenylbenzenes mentioned above, anethole, isoeugenol, α-asarone represent 1-propenylbenzenes, whereas all others are 2-propenylbenzenes.



Safrole (31) Myristicin (32) Anethole (33) Estragole (34) Eugenol (35) Isoeugenol (36) Isosafrole (37)



Methyleugenol (38) α-Asarone (39) γ-Asarone (40) Elemicin (41) Asaricin (42) Croweacin (43)

4.2. Mutagenicity

The mutagenic, genotoxic and carcinogenic activities of the propenylbenzenes especially of safrole are of great importance since they are distributed in many spices and essential oils and were studied extensively both *in vitro* and *in vivo*. Safrole has been tested in a variety of mutagenicity tests. In bacterial tests like *S. typhimurium* and in *E. coli* safrole presented itself as negative [171–175], but modifications of the routine conditions led to mutagenic effects [176]. However, safrole was reported to be mutagenic in the *Bacillus subtilis* DNA-repair test [163, 177]. When investigated in *Saccharomyces cerevisiae* safrole was mostly negative with the following endpoints: gene mutations, recombinant effects, mitotic aneuploidy and DNA repair synthesis [178–180].

Results from gene mutation tests in mammalian cells were equivocal or negative possibly due to the metabolic systems used. Positive results were gathered with methodogically or statistically unsufficient tools. In Chinese hamster ovary (CHO) cells safrole showed mutagenic activity inducing SCE and micronuclei but only in the presence of human hepatoma (Hep G2) S9 microsomal fractions [181, 182]. Safrole did not induce UDS in HeLa cells [183] and did not show mutagenic effects in the initiator tRNA acceptance assay [184]. In *Drosophila melanogaster* safrole was found to be not mutagenic [185–188]. Daimon et al. [189] reported that safrole is able to induce chromosome aberrations and SCE, and to form DNA adducts in Chinese hamster lung cells in culture in the presence of rat liver S9 fraction. They also reported that both a single dose and repeated doses of safrole induced SCE; chromosome aberrations and the formation of DNA adducts in hepatocytes of rats exposed *in vivo* to safrole [190]. Cytogenetic effects of safrole were also observed in CHO-K1 cells [191]. Galli and Schiestl [192] as well as Carls and Schiestl [180] could show that safrole induced intrachromosomal recombination in strain RS112 of *Saccharomyces cerevisiae* preferentially in G2-arrested cells which might be explained by preferential induction of unequal sister chromatid recombination leading to deletions. On the other hand, safrole was more recombinagenic in G1-arrested than in growing cells.

4.3. Carcinogenicity

The carcinogenicity of safrole has been studied in mice and rats. Induction of tumors, especially hepatocarcinomas in mice and rats fed diets containing safrole was reported by different groups [193–200]. Safrole produced liver tumors after oral treatment; liver and lung tumors appeared after s.c. injection in male infant mice. Intraperitoneal injection of safrole to mice resulted in an increase of lung tumor incidence [201]. Infant mice which had received safrole s.c. developed hepatomas and at higher dosage pulmonary adenomas and adenocarcinomas [199]. Hepatic tumors also developed in the male offspring of safrole-treated mothers; whereas in female offspring of safrole treated mothers only a low incidence of renal carcinomas but no hepatic tumors were seen [202]. Administration of safrole in the diet to rats induced hepatocellular carcinomas [193, 198].

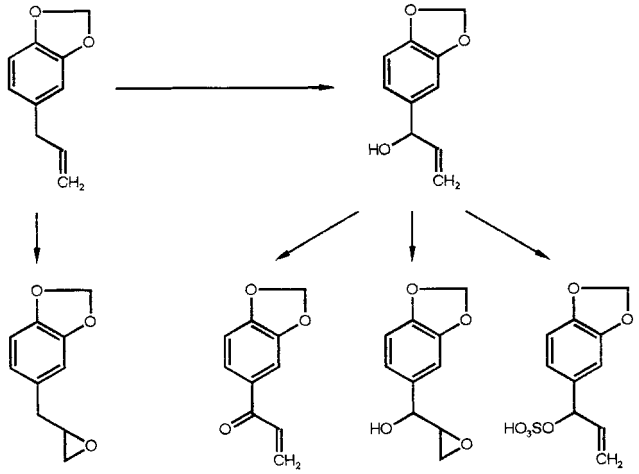
4.4. Biotransformation

Safrole is metabolized by rat and mouse liver microsomes to 1′-hydroxysafrole [173, 202, 203], which can undergo further hepatic metabolism to electrophilic compounds by different ways, such as sulfonation to sulfuric acid ester catalyzed by sulfotransferase or oxidation of the side chain double bond to give 1′-hydroxysafrole-2′,3′-epoxide [204, 205]. Epoxidation of the 2-propenyl side chain of safrole and 1′-hydroxysafrole occurred also with NADPH-fortified hepatic microsomes [206–209] (Scheme 4).

The sulfuric acid ester of 1′-hydroxysafrole was proposed to be the major electrophilic metabolite reacting with the hepatic DNA *in vivo* [210, 211]. This assumption was strengthened by the fact that pretreatment of rats with pentachlorophenol as sulfotransferase inhibitor significantly decreased the chromosomal aberrations, SCE, replicative DNA synthesis, and the formation of DNA-adducts in the liver of rats induced by safrole [210]. The formation of 1′-hydroxysafrole-2′,3′-epoxide *in vitro* and *in vivo* did not contribute significantly to the formation of hepatic DNA adducts *in vivo* [212].

Rats, guinea pigs, and hamsters excreted 1%–3.5% of an i.p. injected dose of safrole as 1′-hydroxysafrole; male

Scheme 4



Biotransformation of safrole [208]

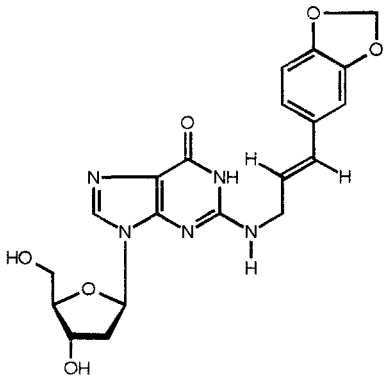
mice excreted 33% as 1′-hydroxysafrole and female mice 19% [193]. The major metabolite of safrole in the urine of rats was identified as 1,2-dihydroxy-4-(2-propenyl)-benzene formed *via* demethylenation of the methylenedioxy moiety [213, 214]. Formation of *o*-quinone 4-(2-propenyl)-3,5-cyclohexadien-1,2-dione, and isomerization to the more electrophilic *p*-quinone methide 2-hydroxy-4-propenylidene-2,5-cyclohexadien-1-one was described [215]. The glutathione adducts of the *o*-quinone and *p*-quinone methide were characterized [216].

Administration of a single i.p. dose of [2′,3′-^{3}H]-1′-oxosafrole to male Sprague-Dawley rats or female CD-1 mice with cannulated bile ducts resulted in the excretion of 2 major biliary metabolites identified as 3′-(glutathion-*S*-yl)-1′-oxo-2′,3′-dihydrosafrole and 3′-(*N*-acetylcystein-*S*-yl)-1′-oxo-2′,3′-dihydrosafrole. The latter conjugate was also found in the urine. The major biliary and urinary metabolite in rats after a single i.p. dose of [2′,3′-^{3}H]-1′-hydroxysafrole, was its glucuronide. Although the sulfuric acid ester of 1′-hydroxysafrole was believed to be the major metabolite leading to the formation of DNA adducts in the liver, it was, at most, of minor importance in the formation of glutathione adducts. Only a very small percentage of a dose of 1′-hydroxysafrole was excreted in the bile of rats or mice as glutathione conjugate [217].

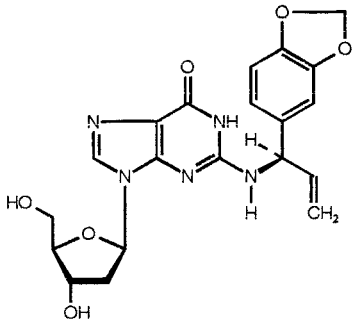
1′-Hydroxysafrole induced hepatic tumors by oral administration to female mice. 1′-Acetoxysafrole showed activities similar to those of 1′-hydroxysafrole [218]. However, 1′-oxosafrole did not show carcinogenic activity under similar conditions [196]. Safrole-2′,3′-epoxide and 1′-hydroxysafrole, when administered topically to female mice at relatively high doses, initiated benign skin tumors that were promoted by croton oil.

Safrole-2′,3′-epoxide, 1′-hydroxysafrole, 1′-hydroxysafrole-2′,3′-epoxide and 1′-acetoxysafrole were all found to be mutagenic in *Salmonella typhimurium* TA1535 and TA100 without metabolic activation [173, 196, 203, 219]. No mutagenic activity was detected for the corresponding 1′-oxosafrole [196, 204].

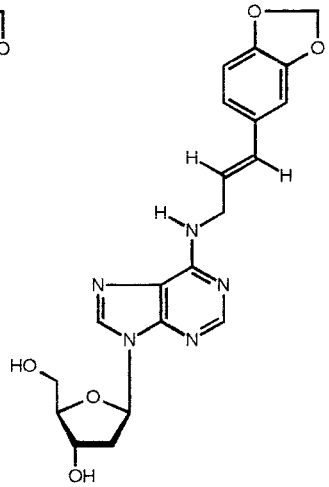
1′-Acetoxysafrole has been used as model compound to study the interactions of metabolically activated safrole with DNA. The major adduct of the reaction with guanosine involved covalent binding of the 3′ position of the propenylbenzene with the exocyclic amino group to N^2-(*trans*-isosafrol-3′-yl)-deoxyguanosine (**44**), indicating an allylic shift of the reactive intermediates prior to reaction. Minor guanosine adducts were identified as the enantiomers of N^2-(sa-



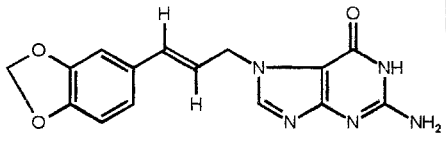
N^2-(trans-Isosafrol-3'-yl)-deoxyguanosin **(44)**



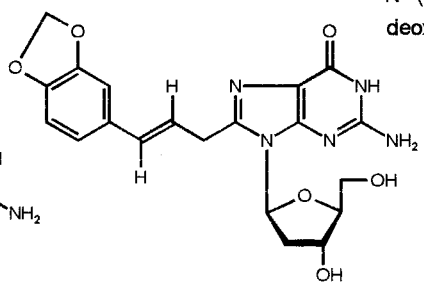
N^2-(Safrol-1'-yl)-deoxyguanosine **(45)**



N^6-(trans-Isosafrol-3'-yl)-deoxyguanosine **(46)**



7-(trans-Isosafrol-3'-yl)-guanine **(47)**



8-(trans-Isosafrol-3'-yl)-deoxyguanosine **(48)**

frol-1′-yl)-deoxyguanosine (**45**), 7-(*trans*-isosafrol-3′-yl)-guanine (**46**), and 8-(*trans*-isosafrol-3′-yl)-deoxyguanosine (**47**). The major adduct with adenosine has been found to be N^6-(*trans*-isosafrol-3′-yl)-deoxyadinosine (**48**) [208, 220, 221].

4.5. Mechanism of genotoxic activation

The DNA adducts of safrole and its metabolites formed *in vivo* have been reported. The characterization of the DNA adducts was carried out using either radiolabelled substances in earlier studies or using ^{32}P-postlabelling technique in more recent studies. DNA adducts formed in mouse liver by 1′-hydroxysafrole were identified as N^2-(*trans*-isosafrol-3′-yl)-deoxyguanosine, N^2-(safrol-1′-yl)-deoxyguanosine, 7-(*trans*-isosafrol-3′-yl)-guanine, 8-(*trans*-isosafrol-3′-yl)-deoxyguanosine, and N^6-(*trans*-isosafrol-3′-yl)-deoxyadenosine. N^2-(Safrol-1′-yl)-deoxyguanosine was reported to be the major adduct with a relative amount of about 60% [204, 212, 222]. A similar adduct pattern was found in mouse liver treated with 1′-acetoxysafrole [221]. Daimon et al. [189] have detected two major and two minor DNA adducts in hepatocytes of rats after a single dose or repeated doses of safrole. The maximal amount of total DNA-adducts was calculated to be about 90 adducts per 10^7 nucleotides [189].

The chromatographic properties of two of the DNA adducts caused by safrole were identical with the adducts formed by the reaction of 1′-acetoxysafrole with deoxyguanosine monophosphate, and two with adducts with deoxyadenosine monophosphate [223]. It was estimated that most of these adducts were still present after 43 days. Significant levels of DNA binding of elemicin were also found, but the adducts were less persistent [223–225].

Administration of safrole to pregnant mice on day 18 of gestation resulted in the formation of adducts in different maternal and fetal tissues, including skin, kidney, heart, lung, intestine, uterus, and brain. Adduct levels were higher in maternal organs than in the corresponding fetal organs, except in the case of brain [226]. This result was a further evidence for the transplacental carcinogenic activity of safrole [202]. Oral administration of safrole caused higher levels of DNA adducts in the livers of pregnant mice than of non-pregnant mice [227]. The adduct levels have been found to be much lower in DNA of white blood cells compared to those in liver [228].

Alkali-labile sites were detected in DNA of human cells treated with 1′-acetoxysafrole [229–231]. Reaction of electrophilic propenylbenzenes, including 1′-acetoxysafrole and 1′-acetoxyelemicin, with supercoiled SV40 DNA resulted in production of 20-fold range in apurinic or apyrimidinic sites. However, it was directed that no correlation is present between the abilities of the reactive derivatives to induce apurinic or apyrimidinic sites and their mutagenic activity in *S. typhimurium* TA100 [232, 233].

Ireland et al. [234] reported that modification of plasmids containing the human H-*RAS* proto-oncongene by 1′-hydroxysafrole and transfection into mouse NIH3T3 cells resulted in transformed foci containing the activated oncogene. Sequence analysis indicated that the activating mutation in two cases was a GC → TA transversion: at the first base of codon 12 in one case and at the first base of codon 61 in the other. The third transformant was apparently not mutated in codon 12, 61 or 117 [234]. Mutations of a shuttle vector, *oriP-tk*, with 1′-acetoxysafrole and introduction of the vector into human lymphoblastoid cells resulted in 5 of the 6 possible base substitution mutations in the thymidine kinase gene. The most frequent mutation was GC → AT transition, followed by AT → GC transition, GC → TA, GC → CG, and AT → TA transversions and frameshift mutations at GC base pairs [235].

High frequency of DNA adducts with safrole were found in oral squamous cell carcinomas associated with betel quid, containing safrole (77%, 23/30) using a ^{32}P-postlabelling method, but not in oral squamous cell carcinomas, which are not associated with betel quid chewing. Six of seven oral submucous fibrosis also exhibited the same safrole-like DNA adduct. Using co-chromatography and re-chromatography techniques, it was further demonstrated that these adducts were identical to synthetic safrole-dGMP adducts as well as DNA adducts from 1′-hydroxysafrole-treated HepG2 cells [236]. An epidemiological study revealed that safrole in betel quid may be a risk factor for hepatocellular carcinoma [237].

The Food and Drug Adminsitration has withdrawn the registration of sassafras root extracts for aromatising of food items. The German Cosmetic Act restricted the highest concentration of safrol in cosmetic products to 100 ppm, in prescriptions for teeth to 50 ppm, safrol is not allowed in prescriptions for children [238].

Like safrole, estragole, methyleugenol, its proximate 1′-hydroxy metabolites, were reported to be hepatocarcinogenic in mice [239]. Estragole and 1′-hydroxy-estragole induced hepatic tumors after administration for 12 months in the diet of female mice. Methyleugenol and its 1′-hydroxy metabolite showed activities similar to estragole and 1′-hydroxy-estragole for the induction of hepatic tumors in male mice treated prior to weaning [200, 212]. 1′-Acetoxy-estragole had an activity similar to those of its respective 1′-hydroxy derivative. 1′-Oxoestragole, 1′-hydroxyelemicin and its acetic acid ester each exerted a very weak, but statistically significant hepatocarcinogenic activity. Species different susceptibility was described [213]. Eugenol was not carcinogenic in female mice when administered for 12 months in the diet. Myristicin and elemicin did not induce tumors in male mice [200, 213, 240].

1′-Hydroxyestragole showed mutagenic and estragole very weak mutagenic effect in *S. typhimurium* TA100 in the absence of NADPH-fortified rat liver microsomes [173]. Estragole and basil oil containing about 88% estragole were reported to markedly induce UDS in rat hepatocytes *in vitro* and in rat liver in an *ex vivo* test [241]. Estragole, methyleugenol [242, 243] and elimicin but not myristicin [176] were found to show genotoxicity as determined by the UDS assay in cultured rat hepatocytes. Eugenol induced chromosomal aberrations in CHO cells *in vitro* [244]. In a battery of *in vivo* assays with end points including the frequency of both micronucleated polychromatic erythrocytes in the bone marrow and micronucleated hepatocytes after partial hepatectomy; the *in vivo-in vitro* induction of DNA fragmentation, and of UDS in hepatocyte primary cultures, eugenol never produced effects indicative of genotoxic activity [245, 246].

Estragole is supposed to be metabolically activated in a similar manner as safrole. 1′-Hydroxy-estragole and estragole-2′,3′-epoxide were reported to be the active metabolites. The rates of epoxidation and hydroxylation were greater for the estragole derivatives than for the safrole derivatives [206, 208]. The formation of 1′-hydroxyestragole in rat and mouse was found to be dose-dependent [247, 248]. Sulfuric acid ester of 1′-hydroxyestragole was postulated to be the ultimate carcinogen [249] whereas the

tumor incidence induced by estragole paralleled sulfotransferase activity as shown in interspecies comparisons. In contrast, glucuronidation of the proximate carcinogen [250] and conjugation with glutathione [251] should be considered as a detoxification mechanisms. Both epoxide hydrolases and glutathione S-transferases could effectively detoxify estragole epoxide and effectively prevent its cellular or genetic toxicity. Epoxide hydrolases appeared to play the major role in the detoxication of the epoxide *in vivo* [252, 253].

In human volunteers five metabolites of estragole were detected in the urine after given [methoxy-^{14}C]-labelled estragole. The two volunteers eliminated 0.2 and 0.4% of the dose respectively as 1′-hydroxy-estragole, the major routes of elimination of ^{14}C were in the urine and in the expired air as $^{14}CO_2$ [254]. The metabolites were products of side chain oxidations.

The major metabolic reactions of elemicin in rats followed both the cinnamoyl pathway to give 3-(3,4,5-trimethoxyphenyl)propionic acid and its glycine conjugate as major urinary metabolites and the epoxide-diol pathway to give 3-(3,4,5-trimethoxyphenyl)propane-1,2-diol as the most prominent metabolite. Small amounts of the epoxide of the 3-O-demethylated derivative of elemicin were identified in the urine [255]. The metabolism of myristicin resembles that of safrole. A weak DNA-binding capacity of myristicin has been demonstrated, but there are no indications that myristicin exerts carcinogenic activity in short-term assays using mice [162].

Like safrole, estragole and methyleugenol exhibited strong binding to mouse-liver DNA with 1 adduct in 10000–15000 DNA nucleotides or 200–300 pmol adduct/mg DNA after administration of a 10 mg dose, whereas myristicin, and elemicin bound to mouse liver DNA at much lower levels. Eugenol did not bind to mouse liver DNA [223, 224]. Four adduct spots were detected in the liver DNA from both adult and preweanling mice administered with methyleugenol [223, 224]. Four adducts were also detected by reaction of estragole 2′,3′-epoxide with 2′-deoxyguanosine. The major persistent adduct was 3′-N^1-deoxyguanosyl-(2′-hydroxypropyl-4-methoxy-benzene) [256].

The 1-propenylbenzenes α-asarone and β-asarone induced hepatic tumors in male mice administered in the diet prior to weaning. The hepatocarcinogenicity of the asarones was not inhibited by pretreatment with sulfotransferase inhibitors [216]. A long-term carcinogenicity study of the essential oil from *A. calamus* of Indian origin containing about 80% β-asarone resulted in induction of tumors in the duodenal region after oral administration to rats. The carcinogenic effect of the essential oil of *A. calamus* in experimental animals was ascribed to β-asarone. Induction of leiomyosarcomas of the small intestine in rats by ingestion of a high level of either β-asarone or calamus oil have also been reported [241].

In contrast, the 1-propenylbenzenes *trans*-isosafrole and *trans*-anethole were not carcinogenic in mice [216]. Truhaut et al. [257] have reported that *trans*-anethole at high doses (5% and 1% in the diet for 117–121 weeks) significantly increased the incidence of some hepatic lesions, including altered cell foci, nodular hyperplasia, benign tumors and malignant tumors in Sprague-Dawley rats. However, the authors think that the changes observed in the chronic feeding study are not of genetic origin. These effect have not been observed in studies of mice fed *trans*-anethole. Since the metabolism of *trans*-anethole in the mouse is similar to that in man, *trans*-anethole is not considered a significant carcinogenic risk to man [257, 258].

trans-Anethole was not mutagenic in the *B. subtilis* DNA-repair test (Rec assay) without S9 and in the *E. coli* WP2 uvrA reversion test [163]. *trans*-Anethole was only slightly effective in the *in vitro* UDS test using rat hepatocytes [241] and did not increase the mutant frequency in the *Salmonella*/microsome test. In contrast, a dose-related response was confirmed in the L5178Y mouse lymphoma TK+/− assay with metabolic activation. *trans*-Anethole did not induce chromosome aberrations in CHO cells. The molecular nature of the genetic change induced in mouse lymphoma cells by *trans*-anethole has not yet been identified but the available genotoxicity data are consistent with either a recombination event or a non-DNA reactive mechanism [259]. α-Asarone and β-asarone [176], but not *trans*-anethole and isosafrole [242] were genotoxic as determined by the UDS assay in cultured rat hepatocytes. α-Asarone did not produce germinal mutations in either male or female mice by oral treatment at doses of 10 and 20 mg/kg, 5 days/week, for 8 weeks. Epididymal sperm examination of male mice immediately after treatment failed to reveal any alteration in sperm count on shape. No significant alterations were observed in testicular or epididymal weights or testicular histology [260, 261]. β-Asarone showed genotoxic effects in SOS chromotest using *E. coli* [175].

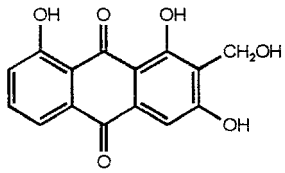
In contrast to the 2-propenylbenzenes, the metabolic activation of 1-propenylbenzenes such as *trans*-anethole and α-asarone are not clear. It has been reported that one of the main metabolic pathways of *trans*-anethole is the epoxidation of the side chain 1,2-double bond, which was responsible for cytotoxicity but not for genotoxicity [262, 263]. However, it has been recently reported that synthetic *trans*-anethole 1′,2′-epoxide is not only mutagenic for *Salmonella* but is also carcinogenic inducing hepatomas in B6C3F1 mice and skin papillomas in CD-1 mice. Synthetic α-asarone 1′,2′-epoxide was also carcinogenic inducing hepatomas and was mutagenic in *Salmonella* strains [264].

trans-Anethole was found to be hydroxylated at the 3′-carbon in male rat liver microsomes to yield about 270 nmol of *trans*-3′-hydroxy-anethole/mg protein/h [206, 208]. Eleven ^{14}C-containing urinary metabolites were identified in the rat and ten in the mouse after a single oral dose of 50 mg/kg *trans*-[methoxy-^{14}C]anethole. The metabolites arose from side-chain oxidation, side-chain cleavage and various conjugations. The major urinary metabolites were two isomers of 1-(4-methoxyphenyl)propane-1,2-diol, 2-hydroxy-1-methylthio-1-(4-methoxyphenyl)propane and 4-methoxyhippuric acid, the first three all being excreted as glucuronides. In addition to these ^{14}C-labelled metabolites, 4-hydroxypropenylbenzene, the unlabelled product of oxidative O-demethylation of *trans*-[methoxy-^{14}C]anetho-le, was excreted extensively in urine as the glucuronide [265]. Eighteen *trans*-anethole metabolites were identified in the urine of rats and mice given single doses of 250 mg/kg [1′-^{14}C]-*trans*-anethole. The metabolites were formed by three primary oxidation pathways, O-demethylation, side chain oxidation, and side chain epoxidation, followed by a variety of secondary pathways of oxidation and hydration, the products of which are extensively conjugated with sulfuric acid, glucuronic acid, glycine, and glutathione [266]. A species-specific regioselectivity in the side-chain oxidation of *trans*-anethole was observed [267]. Nine radioactive urinary metabolites were found in the urine of human volunteers after *trans*-[methoxy-^{14}C]anethole administration with 4-methoxyhippuric acid (56% of dose) as the major metabolite, accompanied by much smaller

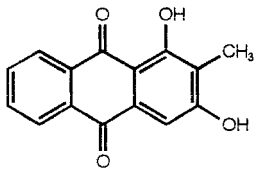
amounts of the two isomers of 1-(4-methoxyphenyl)propane-1,2-diol (together 3%) [254, 268].
trans-Anethole and isosafrole bound to mouse liver DNA only at low levels [223]. Two main adducts, which appeared to be guanine derivatives, were detected in the livers of mice treated with *trans*-anethole or isosafrole by ^{32}P-post-labelling technique [223, 224].
Structure-genotoxicity relationship studies of propenylbenzenes using the UDS assay in cultured rat hepatocytes revealed that the number and position of methoxy and methylenedioxy substituents on the benzene ring seemed to have a major effect in determing the carcinogenic activities of propenylbenzene. The stereochemical hindrance produced by such substitutions may alter the metabolism of the compounds [269]. Structure-genotoxicity relationship of propenylbenzenes was also studied using quantum chemistry. The compounds which were genotoxic or non-genotoxic in the UDS assay did not show significant differences in the relative stability of the radical species formed as intermediates in C-sp3 hydroxylation. In contrast, the carbonium ions of the genotoxic compounds were comparatively more stable than those of the inactive compounds, with the exception of eugenol. The relative stability of the carbonium ion appears to be one of the key factors in the genotoxicity of propenylbenzenes [270].

5. Drugs containing anthraquinone derivatives (anthracene-9,10-diones)

Besides madder root, the root of *Rubia tinctorum* L., Rubiaceae, medicinal plants containing anthraquinones as active principles are rhubarb, the root and rhizome of *Rheum officinale* Baillon, *R. palmatum* L. (Polygonaceae); and kreaking buckthorn bark, the stem bark of *Rhamnus frangula* L. (*Frangula alnus* Miller, Rhamnaceae). Plants belonging to the family Rubiaceae including *R. tinctorum* were reported to contain anthraquinones with substitutions in only one of the aromatic rings [281].
In contrast to madder root, rhubarb and kreaking buckthorn bark contain anthraquinones possessing a 1,8-dihydroxy group and further substituents in both aromatic rings. Aloe, the dried matter of the leaf exudate from *Aloe barbadensis* Miller (Curacao Aloe), *Aloe ferox* Miller (Cape Aloe) or other *Aloe* species (Liliaceae), contain anthraquinones and anthrone glycosides with aloin as the major component. Senna, the leaf of *Cassia acutifolia* Delile or *Cassia angustifolia* Vahl (Fabaceae), contains bianthrone glycosides like sennoside A and sennoside B as the major components.
The following pharmaceutical preparations are known for
Rubia tinctorum L.: the root, called madder.
Rheum officinale Baillon and *Rheum palmatum*: Rhei radix, Rheum hom. HAB1, Rheum officinale hom. HPUS88.
Rhamnus frangula: Frangulae cortex, Rhamnus frangula hom HAB1, Rhamnus frangula hom. PFX, Rhamnus frangula hom. HPUS88.
Rhamnus californica: Rhamnus californica hom. HPUS88.
Rhamnus catharticus: Rhamni cathartici fructus, Rhamni cathartici fructus recentes, Rhamnus cathartica hom HAB34, Rhamnus cathartica hom. HPUS88.
Rhamnus purshianus: Rhamni purshiani cortex, Cascara sagrada hom. PFX, Rhamnus purshiana hom HPUS88.
Aloe ferox and the hybrids *Aloe capensis*: Aloe hom. HAB1, Aloe hom. PFX.
Cassia angustifolia: Sennae folium, Sennae fructus angustifoliae, Senna hom. HAB34, Senna hom PFX.
Cassia acutifolia (Cassia senna): Sennae folium, Sennae fructus acutifoliae, Senna hom. HAB34, Senna hom HPUS78, Senna hom. PFX.
Madder root had a long tradition in folk medicine as remedy against uroliths. It contains lucidin (**49**), rubiadin (**50**) and a number of other related anthraquinones [271–273]. It is believed that its mechanism of action is related to the calcium ion chelating potential of anthraquinones such as alizarin (1,2-dihydroxy-anthraquinone) and purpurin (1,2,4-trihydroxy-anthraquinone) [274].
On the basis of the mutagenic and carcinogenic potentials of the chemical constituents, the German Federal Health Office announced in 1993 the countermand of the permission of 159 medicinal preparations containing the root of *R. tinctorum* [275].



Lucidin **(49)**



Rubiadin **(50)**

5.1. Mutagenicity

The mutagenicity of an extract of madder root in *Salmonella typhimurium* TA100 and TA98 with or without metabolizing system was first reported by Yasui and Takeda [276] who have identified lucidin as the mutagenic principle. Lucidin and lucidin 3-O-primveroside were found to be mutagenic in *S. typhimurium* with or without metabolic activation, but the mutagenic activity was increased after addition of activation system [277, 278]. Rubiadin was highly mutagenic in *S. typhimurium*, however, in contrast to lucidin, it required metabolic activation. Lucidin 2-ethylether, formed from lucidin by extraction of the root with boiling ethanol, was also mutagenic in *S. typhimurium*, but only after addition of rat liver S9 mix [279] inducing mainly frameshift mutations [280].
Further mutagenic anthraquinones present in madder root were identified as 1-hydroxy-2-methylanthraquinone, 2-ethoxymethylanthraquinone, 1,3-dihydroxyanthraquinone, 7-hydroxy-2-methylanthraquinone, and 1-methoxymethylanthraquinone [273]. Studies on the structure-mutagenicity relationship of the anthraquinones revealed that the greatest activity is exhibited by 1,3-dihydroxyanthraquinones possessing a methyl or hydroxylmethyl group at position 2 [273], such as rubiadin, lucidin and lucidin 2-ethyl ether [278].
Evidence for the mutagenicity of hydroxy-substituted anthraquinones from madder root was also observed in mammalian test systems such as the V79 mutation assay at the hypoxanthine guanine phosphoribosyl transferase (HGPRT) gene locus and the DNA repair test in primary rat hepatocytes and the transformation assay in C3H/M2 mouse fibroblasts [277, 279, 282]. Lucidin induced DNA single-strand breaks and DNA-protein cross-links in V79 cells determined by the alkaline elution [279]. Lucidin 2-ethylether was weakly mutagenic to V79 cells co-cultivated with rat hepatocytes [279]. In a UDS assay in primary rat hepatocytes, rubiadin was even more potent than lucidin and

equal to the positive control 7,12-dimethylbenzo[a]anthracene (DMBA) [283]. Alizarin 2-O-primeveroside was inactive in the *Salmonella* assay, but induced UDS in primary rat hepatocytes. After oral application to rats it was metabolized to alizarin and 1-hydroxyanthraquinone. The reductive cleavage of alizarin 2-O-primeveroside was also observed after incubation with rat liver S9 mix and NADPH. 1-Hydroxyanthraquinone has been reported to induce UDS in primary rat hepatocytes [283].

5.2. Carcinogenicity

The carcinogenicity of madder root in rats has been reported. Male and female ACI rats received either a normal diet or a diet supplemented with 1% or 10% root for a total period of 780 days. Non-neoplastic lesions related to the treatment were evident in the liver and kidneys of both sexes. Moreover, dose-dependent increases in benign and malignant tumor formation were observed in the liver and kidneys of treated animals [284]. In (C57BL/6 X C3H)F1 male and female mice, the extract of madder root administered in the diet at concentrations up to 5% for 90 days did not show hyperplastic, preneoplastic nor neoplastic lesions and other pathological signs [285]. Possible explanations for the different results may be differences in the sensitivity of mice *versus* rats, in the composition of the anthraquinones of the madder root used or the time of treatment.

1-Hydroxyanthraquinone has been reported to induce gastric, intestinal and liver tumors in rats after chronic treatment [286]. 1-Hydroxyanthraquinone induced hyper-cell proliferation in rat colonic crypts with ulcerative changes, crypt abscess, severe inflammation and erosion before the occurrence of tumors, which are similar to those found in human ulcerative colitis. In addition, 1-hydroxyanthraquinone has a synergistic effect with methylazoxymethanol acetate on colon carcinogenesis. The polymerase chain reaction-single strand conformation polymorphism analysis revealed no mutations in Ki-*ras*, *p53* and in colonic neoplasms induced by 1-hydroxyanthraquinone [287]. Moreover, no mutations of APC gene were found in these tumors [288]. These findings are comparable to those found in human ulcerative colitis-associated colon cancer in contrast with sporadic colon cancers. Increased expression of TNFα and ILα was found in the induced colonic neoplasms. These cytokines may therefore act as growth factors in rat colon carcinogenesis by 1-hydroxyanthraquinone and the synergistic effect of 1-hydroxyanthraquinone with methylazoxymethanol acetate might be related to the biological effects of the cytokines expressed in the inflammatory conditions induced by 1-hydroxyanthraquinone [289].

It was demonstrated that the uptake of the anthraquinone glycosides alizarin 2-O-primeveroside and lucidin 3-O-primeveroside lead to the formation of the rodent carcinogens 1-hydroxyanthraquinone and lucidin, and to the highly genotoxic compound rubiadin. Therefore it was suggested that the therapeutic use of *R. tinctorum* may involve a carcinogenic risk [290]. Oral administration of lucidin 3-O-primeveroside to rats resulted in the excretion of lucidin and rubiadin. Treatment of lucidin 3-O-primeveroside with rat liver extract and NADPH led to the conversion to rubiadin 3-O-primeveroside. Rubiadin was formed from rubiadin 3-O-primeveroside after hydrolysis [290].

5.3. Mechanism of genotoxic activity

The mechanism of the genotoxic activity of madder root and its two major anthraquinones lucidin and rubiadin has been intensively studied. Up to five different DNA adducts in the range from about 1 to 3 adducts per 10^8 nucleotides were formed by incubation of mouse DNA with lucidin in the presence of S9 mix using the ^{32}P-postlabelling technique. A similar adduct pattern was observed by incubation of polydGpolydC with lucidin in the presence of S9 mix. DNA adduct formation was not detected after incubation of DNA with alizarin. Lucidin, a mixture containing alizarin 2-O-primeveroside and lucidin 3-O-primeveroside, and Rubia Teep, a phytotherapeutic preparation from *R. tinctorum* containing lucidin, gave rise to DNA adducts after incubation with primary rat hepatocytes for 24 h, but alizarin did not. DNA adduct formation in liver, kidney, duodenum, and colon was observed after oral administration of lucidin, the glycoside mixture, or Rubia Teep to mice. The adduct patterns were organ-specific [291]. Lucidin reacted with nucleic bases under physiological conditions in the order of the reactivity as follows: adenine $>$ guanine $\gg$ pyrimidine bases approximately 0. The isolated purine base adducts were identified as condensed reactants at the benzylic position 1 with a nitrogen atom of a purine base. This indicated a strong possibility of the formation of an exomethylenic compound as an electrophilic intermediate [292]. ^{32}P-postlabelling analysis showed an increase in the overall level of DNA adducts observed in the liver, kidney and colon of rats treated with 10% madder root in the diet for 2 weeks. HPLC analysis of ^{32}P-labelled DNA adducts revealed a peak co-migrating with an adduct obtained after *in vitro* treatment of deoxyguanosine-3′-phosphate with lucidin [283]. The DNA adduct formation by lucidin strongly suggested that the genotoxic effects of lucidin observed in *in vitro* tests resulted from covalent interaction between lucidin and the cellular DNA [291].

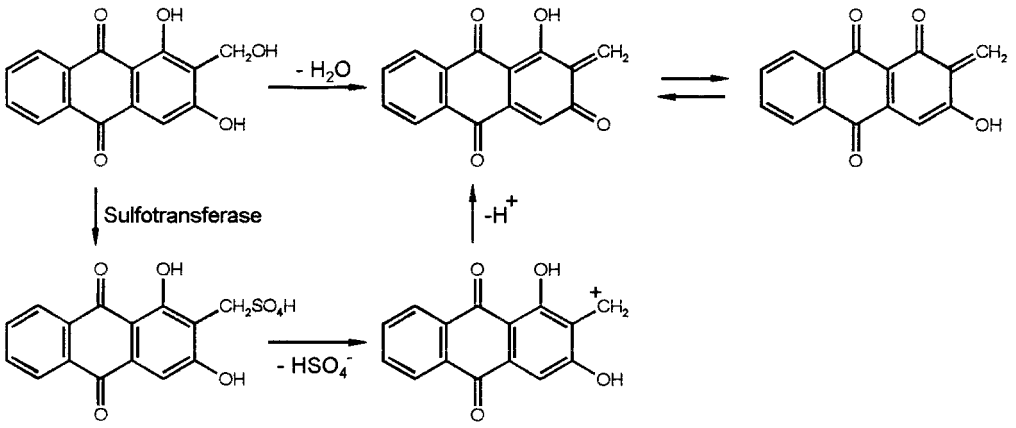
The enzymatic activation of lucidin and formation of electrophilic intermediates from benzylic alcohol was postulated to proceed *via* elimination of water or *via* sulfuric acid ester (Scheme 5) [291].

The potential mutagenicity and carcinogenicity of anthraquinone derivatives are very important because several common vegetables and useful herbal medicines contain a great number of anthraquinones and anthraquinone glycosides. Fresh peas, cabbage lettuce, and beans contain anthraquinones with a total content ranging from 0.04 to 36 mg/kg. Physcion predominated in all vegetables tested [293].

The root and rhizome of *Rheum officinale* and *R. palmatum* have a long history as laxatives with clinical use. The major anthraquinone constituents from rhubarb are rhein (**51**), aloe emodin (**52**), emodin (**53**), physcion (**54**) and chrysophanol (**55**) [294, 295]. The anthraqiunone derivatives occur in rhubarb root mainly as glycosides [296]. All the five anthraquinone derivatives are derivatives of 1,8-dihydroxyanthraquinone.

Emodin was reported to induce mutations in *S. typhimurium* TA97 [297], TA100, TA2637 [298] and especially in *S. typhimurium* TA1537 after metabolic activation, indicating that emodin represents mainly a frame-shift mutagen [299–302]. In addition, emodin monoglucoside showed mutagenic activity in *S. typhimurium* TA100 in the presence of a cell-free extract of rat cecal bacteria [279]. In contrast, emodin was also reported to be not active in inducing reversions in *S. typhimurium* TA98 and TA100 or

Scheme 5



Possible toxification pathways to reactive electrophilic intermediates of lucidin [291]

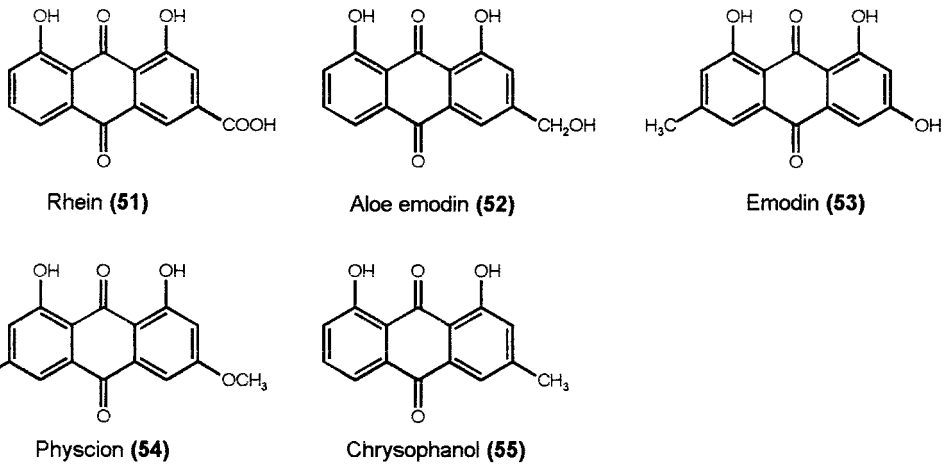
forward mutations to 8-azaguanine resistance in strain TM677 with metabolic activation [303]. In addition, emodin dose-dependently reduced the mutagenicity of benzo[a]pyrene and the food-borne mutagens IQ and Trp-P-2 [304] in *S. typhimurium* TA98, as well as the genotoxicity of 1-nitropyrene using the SOS chromotest with *E. coli* PQ37 [305]. Emodin reduced the mutagenicity of IQ by direct inhibition of the hepatic microsomal activation and not by interaction with proximate metabolites of IQ or by modification of DNA repair processes in the bacterial cell [304]. Moreover, emodin was also reported to significantly inhibit the formation of 1-nitropyrene-DNA adducts in *S. typhimurium* TA98 in the ^{32}P-postlabeling assay [305].

Emodin could be metabolized by rat hepatic S9 mix to give at least 5 quinone metabolites with 2-hydroxyemodin as a direct mutagen in test using *S. typhimurium* TA1537 [306, 307]. Other metabolites including 4-hydroxyemodin, 5-hydroxyemodin and 7-hydroxyemodin were not mutagenic in *S. typhimurium* TA1537 without metabolic activation [308]. A further metabolite of emodin was found to be ω-hydroxyemodin, formed from emodin in the presence of hepatic microsomes derived from various animal species in the following range: guinea pig and rat > mouse > rabbit [309]. Incubation of emodin with microsomes from rats that had been pretreated with inducers for different cytochrome P450 enzymes did not lead to different rates of ω-hydroxyemodin, indicating that the metabolisation by cytochrome P450 enzymes is of minor importance. The formation of 2-hydroxyemodin was increased in liver microsomes from rats pretreated with 3-methylcholanthrene and was inhibited by α-naphthoflavone, by an anti-rat CYP 1A1/2 antibody, and, to a lesser degree, by an anti-rat CYP 1A1 antibody [310].

After the administration of a single oral dose of 91 mg/kg emodin to mice, approximately 30% of the dose was excreted as unchanged emodin or its derivatives in urine, and 21% in feces within 24 h. The major free anthraquinone metabolites of emodin in 24 h urine were found to be 6-hydroxyaloe-emodin, 6-hydroxyrhein, chrysophanol, and physcion [311]. Rhein, aloe-emodin, and possibly 4-hydroxychrysophanol, 4-hydroxyemodin, 4-hydroxyrhein, 4,6-dihydroxyaloe-emodin, and 4,6-dihydroxyemodin were also detected in the urine [312].

ω-Hydroxyemodin was not mutagenic in the Ames assay in the absence of S9 mix, but exhibited mutagenicity in the presence of an activating system. The mutagenic potential in the Ames test of ω-hydroxyemodin is comparable with that of 2-hydroxyemodin [309]. Compared with emodin, 2-hydroxyemodin induced much higher micronucleus frequencies in mouse lymphoma L5178Y cells, while ω-hydroxyemodin induced lower micronucleus frequencies [310].

Kodama et al. [313] have reported that 2-hydroxyemodin showed a large electron-spin resonance signal in the presence of DNA, especially at alkaline pH. Coupled with generation of free radicals, hydrogen peroxide but not superoxide was formed. The active oxygen produced from 2-hydroxyemodin induced strand breaks [313]. In contrast,



Rhein **(51)** Aloe emodin **(52)** Emodin **(53)**

Physcion **(54)** Chrysophanol **(55)**

Bosch et al. [314] have demonstrated that the mutagenicity of emodin is unlikely due to the covalent interaction of a metabolite with DNA, because the specific activity of *Salmonella* DNA after incubation with [10-^{14}C]emodin did not remain constant after repeated precipitations. Very little radioactivity was detected in liver DNA from rats 72 h after treatment with labelled emodin. The covalent binding index (CBI) of emodin was calculated to be 10^4 times below the CBI of aflatoxin B_1. The authors concluded that the mutagenicity of emodin is due to a chemically stable, oxidized metabolite forming physico-chemical associations with DNA, possibly of the intercalative type [314].

Bruggeman and Van der Hoeven have reported that emodin was not active in the HGPRT forward mutation assay and in the SCE assay using V79 cells either with or without metabolic activation [297]. In contrast, Westendorf et al. reported that emodin was active in inducing HGPRT mutation in V79 cells, DNA-repair in primary rat hepatocytes, and transformation of C3H/M2 mouse fibroblasts [301]. Müller et al. have reported that emodin induced micronuclei and mutations at the TK-locus, and was also active in the comet assay in mouse lymphoma L5178Y cells [291, 315]. It was further reported that emodin bound noncovalently to DNA and inhibited topoisomerase II activity in L5178Y cells. Emodin also showed a high serum-protein binding affinity [316]. The inhibition of the catalytic activity of topoisomerase II was suggested to contribute to the genotoxicity and mutagenicity of emodin [317]. Emodin was cytotoxic at concentrations of 1–10 μg/ml and induced HGPRT mutation in the mouse mammary carcinoma cell line FM3A [318].

Physcion, the 3-methoxy analog of emodin, was also reported to induce mutations in *S. typhimurium* TA1537, but only after metabolic activation. Unlike emodin, physcion exhibited a weak mutagenic activity in *S. typhimurium* TA102, probably due to the formation of a different metabolite [299]. Physcion also was not genotoxic in mouse lymphoma L5178Y cells [316]. Physcion was metabolized by mouse and rat into emodin, chrysophanol, aloe-emodin, rhein, 6-hydroxyaloe-emodin, 6-hydroxyrhein, and, possibily, 6-methoxyaloe-emodin and 6-methoxyrhein [312].

Chrysophanol, the 3-dehydroxy analog of emodin, was reported to be mutagenic in *S. typhimurium* TA1537 [319] and TA2637 [298] with metabolic activation and was only weakly genotoxic in L5178Y cells [316]. Chrysophanol was suggested to be transformed to aloe-emodin as major metabolite by ω-hydroxylation catalyzed by cytochrome P450-dependent monooxygenases [310].

Aloe-emodin, the 3-hydroxymethyl analog of chrysophanol, was found to be mutagenic in *S. typhimurium* TA1537, TA1538 and TA98; it induced HGPRT mutations in V79 cells and DNA-repair in primary rat hepatocytes; transformed C3H/M2 mouse fibroblasts [301]; induced chromosome aberration in CHO cells [320, 321] and induced TK-mutations and micronuclei in mouse lymphoma L5178Y cells [315]. In *in vivo* studies using the mouse spot test and micronucleus in bone marrow cells of rats, no indication for a mutagenic activity of aloe-emodin was found. In an *ex vivo* test performed with hepatocytes of rats, aloe-emodin did not induce UDS as expression of DNA damage. After oral administration to rats, aloe-emodin could be detected in serum [320, 321]. Aloe-emodin was further found to be a topoisomerase II inhibitor [315, 317].

Rhein is a chrysophanol analog bearing a carboxylic group at position 3 instead of a methyl group in chrysophanol. As an anthraquinone-carboxylic acid, rhein did not show any mutagenic effect in *Salmonella* reverse mutation test and *E. coli* forward mutation test; in HGPRT mutation test in mammalian cells; chromosome aberrations in CHO cells; in micronucleus and chromosome aberration tests in bone marrow cells and in mouse spot test using melanoblast cells [320]. No mutagenicity of a decoction of *R. palmatum* rhizome was observed in *S. typhimurium* TA97, TA98, TA100, and TA102 at different concentrations [322].

Taking into consideration the measured concentrations of anthraquinones in the human diet and their estimated daily intakes from vegetables, herbs and liquors, the genotoxic potency of the food matrix, emodin, chrysophanol and physcion do not represent a high priority genotoxic risk for human [291]. For aloe-emodin was assumed that a genotoxic risk for man is unlikely [321].

Bianthrones (dianthrones) [323, 324] and bianthrone glycosides [325, 326] from rhubarb are mainly derived from the five major anthraquinone derivatives physcion, rhein, emodin, aloe-emodin, and chrysophanol together with isobianthrones and heterobianthrones.

Senna leaves are known to be a mild laxative agent due to their content of bianthrone glycosides [327, 328]. After oral administration the glycosides were carried, unabsorbed, to the large intestine, where the active principle was released by bacterial enzymes [329, 330]. Sennoside A and sennoside B were found to be hydrolyzed by microbial β-glycosidase. The resulting sennidins were further reduced to give rheinanthrone as the laxative principle. The latter reduction is possibly performed by a reductase bound to cell membranes of intestinal bacteria [331–334].

Sennosides did not increase mutation frequencies in the *S. typhimurium* reverse mutation test and *E. coli* forward mutation test; in hypoxanthine HGPRT test in mammalian cells; in chromosome aberration test with CHO cells; in micronucleus test in mouse bone marrow cells [335]. After oral administration of senna extract at a dose of 2 g/kg to mice equivalent to about 120 mg rhein, there were no elevated levels of micronuclei in bone marrow cells [336]. However, positive results were observed with senna glycosides in *S. typhimurium*. Senna extracts demonstrated weak mutagenic activity in *S. typhimurium* TA100 in the presence of liver microsomes, and in TA97a and TA102 in the absence and presence of liver microsomes. A strong increase in mutant frequency was observed in *S. typhimurium* TA98 in the presence of liver microsomes [337].

An *in vivo* study has also shown that oral exposure of anthraquinone glycosides from *C. angustifolia* to mice did not induce significant numbers of chromosomal aberrations or aberrant cells [338]. Dietary exposure to high doses of senna glycosides to rats for 56 successive days did not induce aberrant crypt foci in colon mucosa or increase the incidence of aberrant crypt foci induced by 1,2-dimethylhydrazine [339–341].

Results from a two-year carcinogenicity study with sennosides in the rat did not indicate any relationship between long-term administration of senna extract and gastrointestinal, liver, kidney or adrenal tumors in the rat. Purified senna extract was given via the drinking water to rats of both sexes for 2 years at daily doses of 5, 15 and 25 mg/kg [342]. After intragastric administration there was no evidence of any embryolethal, teratogenic or fetotoxic action [343]. In the light of other data from animal and human metabolism or kinetic studies, human clinical trials and rodent carcinogenicity studies do not support concerns that senna laxatives pose a genotoxic risk to humans when

consumed under prescribed use conditions [344]. In contrast, a recent retrospective case-control study (1990–1995) suggested that senna showed high risk for urothelial cancer, especially for renal pelvis cancer [345].

6. Conclusions

Malignant tumors may be induced by environmental carcinogens, which can cause gene mutations and therefore activate the proto-oncogenes to oncogenes and inactivate tumor suppressor genes. The most promising preventive measures are to eliminate such compounds from the environment or to avoid contact. The total elimination of carcinogens from the environment, though, is not feasible because of their ubiquitous occurrence. Therefore, the most promising strategy to prevent cancer and to reduce carcinogenic risks is to reduce exposure against carcinogenic substances.

Mutagenicity, and carcinogenicity of several chemical constituents in medicinal herbs such as aristolochic acids, pyrrolizidine alkaloids, alkenylbenzene derivatives, and anthraquinones have been experimentally proven. The mechanisms of genotoxicity have been elucidated in detail. For example, the metabolic activation of aristolochic acids, pyrrolizidine alkaloids and alkenylbenzenes have been postulated and verified by isolation and structural identification of the postulated DNA adducts. On the basis of these results it may be concluded, that the carcinogens from medicinal herbs might also play a role as human cancer risk factors, but since the use of drugs known to containing the most potent carcinogens, is forbidden, this risk has been minimized or is restricted to an incidental use as was the case for the "Chinese herbs nephropathy" [5, 6].

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