Natural Inhibitors of Germination and Growth, V
Possible Allelopathic Effects of Compounds from *Thuja occidentalis*

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Dedicated to Professor Otto Kandler on the occasion of his 70th birthday

Allelopathy, *Amaranthus caudatus*, Germination Inhibition, *Lepidium sativum*, *Thuja occidentalis*, Volatile Compounds

Volatile compounds which are released from fresh leaves of *Thuja occidentalis* inhibited germination of seeds of *Amaranthus caudatus* and *Lepidium sativum*. The volatile compounds were obtained by a vacuum method applied to the leaves, by direct analysis of the content of secretory organs and by solvent extraction of leaves. The bioactive compounds proved to be monoterpenes. The highest bioactivity were found for alcoholic compounds followed by ketones, esters and finally hydrocarbons. Non-volatile germination inhibitors which were extracted with hot water were abscisic acid (3–4 µg/g fresh weight of leaves), and two oxidation products of thujone, 2-[2′-acetyl-1′-isopropyl]cyclopropylacetic acid ("Thujaketosäure") and 3-isopropyl-5-oxohex-2-enoic acid. These compounds could also be prepared from thujone. The relationship of these compounds with possible allelopathic effects of *Thuja occidentalis* is discussed.

Introduction

The term "allelopathy" has been widely used in the original sense of Molisch [1] as negative or positive biochemical interactions between all types of plants, but distinct from competition [2]. Natural compounds which are responsible for this type of interactions have been called allelochemicals (review [3]). Distribution of allelochemicals can occur (1) by excretion from the roots of one plant and diffusion to the roots of other plants, (2) by leaching from the leaves (*e.g.* by rain) and accumulation in the soil surrounding the plant or (3) *via* the gas phase if the compound is volatile enough [4]. Diffusion in the soil seems to be a prerequisite for mechanisms (1) and (2). It is doubtful whether volatile compounds according to (3) can be accumulated in the soil to concentrations which are high enough to exert allelopathic effects. A direct interaction of gaseous compounds with surrounding plants is more likely.

Volatile compounds are released from a great variety of plants especially in desert areas [5]. The effective compounds are – as far as they are identified – mainly monoterpenoids and phenolic compounds. Phytotoxic effects of such compounds may include interaction with membranes [6], with energy metabolism [7] or with plant hormones [8].

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Our interest in possible allelochemical effects started with the coincidental observation that seeds of garden cress seemed to be inhibited or at least retarded in their germination in the immediate neighbourhood of *Thuja occidentalis*. Several volatile monoterpenes have been described as compounds from *Thuja* sp. [9–15]. The present paper correlated volatile compounds released from *Thuja occidentalis* with the inhibitory effect upon seed germination of garden cress (*Lepidium sativum*) and foxtail (*Amaranthus caudatus*). The possible allelopathic effect of non-volatile compounds was also investigated.

Experimental

Equipment

Gas chromatograph HP 5890 equipped with a 25 m × 0.32 mm i.d. fused silica capillary column coated with 0.52 µm cross-linked SE 54. Carrier gas 2 cm^3/min He; temperature program, 50–250 °C with 5 °C/min, injection splitless, Detection FID. For the volatile compounds the injection and detector temperature was 150 °C. For the non-volatile compounds the injection and detector temperature was 250 °C.

GC/MS. Finnigan 4500 (quadrupol) linked on-line to an INCOS data processing system; directly coupled to a Finnigan GC. J + W 25 m × 0.25 mm i.d. fused silica capillary column coated with 0.35 µm bonded DB 5; carrier gas

2 cm^3/min He. Temp. program 50–250 °C, 5 °C/min; injector and transfer-line temp., 250 °C; injection splitless; detection TIC; ionization energy 70 eV.

Biotest with Lepidium sativum

a) Volatile compounds: The bottom of Petri dishes (diameter 12 cm) was covered with filter paper which was then moistured with dist. water (3.5 ml). Subsequently, 35 seeds of *L. sativum* were evenly distributed on the filter paper so that a free place for a small Petri dish (diameter 2 cm) was left. The small dish was filled with fresh pieces of leaves of *Thuja occidentalis* (20–200 mg). The large Petri dish was tightly closed so that volatile compounds released from the leaves could not escape. The closed system was then incubated in the dark at 28 °C for 48 h. The length of primary roots was then determined in comparison with water controls without *Thuja* leaves (average root length: 18 mm); the results were expressed as percent inhibition (see [32]).

b) Non-volatile compounds: A solution of the compound in either Et_2O or Me_2CO was evenly distributed on a filter paper which covered the bottom of a Petri dish (diameter 4.5 cm). After evaporation of the solvent, the filter paper was moistured with 1.5 ml dist. water and 15 seeds of *L. sativum* were evenly distributed on the filter paper. Incubation and determination of seed germination was as under a).

Biotest with Amaranthus caudatus

The test was performed as under b) but with only 1 ml dist. water and 25 seeds of *A. caudatus*. Evaluation of germination was as under a). Average root length was 25 mm.

Isolation and identification of volatile compounds

a) 10 g ground leaves of *Thuja occidentalis* were placed in a 250 ml round-bottom glass flask which was then connected to 3 U-tubes in series and finally a vacuum apparatus. The U-tubes were cooled with liquid nitrogen. Vacuum of 9 mbar was applied for 8 h. The compounds which condensed in the U-tubes were then eluted with 15 ml Et_2O. After drying with Na_2SO_4, the Et_2O solution was directly analyzed by gas chromatography and mass spectrometry.

b) The secretory organs of *Thuja* leaves were punctured with a syringe under a microscope. The liquid which entered the syringe by capillary forces was collected from 80 secretory organs and immediately dissolved in 2 ml Et_2O. After drying with Na_2SO_4, the solution was directly analyzed by GC and MS.

c) For extraction with organic solvents, leaves (10 g) of *Thuja occidentalis* were frozen with liquid nitrogen and pulverized in a mortar with further addition of liquid nitrogen. Et_2O (10 ml) was added while still frozen. After thawing, the leaf powder was squeezed out under Et_2O. The Et_2O solution (8 ml) was poured upon a small silica gel column (0.5 cm, ∅ 6.5 cm) which had been equilibrated with Et_2O. The fractions eluted with Et_2O were analyzed by GC and MS. Some experiments were performed with chilling of Et_2O throughout the procedure, other experiments were performed at room temperature. The results were identical. Extraction with acetone was performed correspondingly with 5 g of *Thuja* leaves.

Isolation and identification of non-volatile compounds

500 g leaves of *Thuja occidentalis* were homogenized in 2.5 l H_2O and the homogenate was boiled under reflux for 24 h. The aqueous solution was cleared by filtration, adjusted with $NaHCO_3$ to pH 8.0 and extracted with Et_2O in a perforator. The organic phase contained the same monoterpenes which had been extracted directly with organic solvents from the leaves (see Table I). The aqueous solution was then acidified with HCl to pH 1.0 and extracted again with Et_2O. The residue of the ether phase was dissolved in MeOH and loaded upon a column of Sephadex LH-20 (8 × 40 cm) which had been equilibrated with H_2O. The column was eluted with H_2O. The bioactivity of each fraction (8 ml) was determined. The first region which contained bioactivity comprised fractions 40–60, the second region fractions 110–130.

The fractions of region 1 were combined, adjusted to pH 1 and extracted with Et_2O in a perforator. The residue of the Et_2O solution was methylated with CH_2N_2 and then further fractionated by distillation at 0.05 Torr. The active fraction (50–70 °C) was then purified by horizontal distillation in a stream of nitrogen. The fraction with the high-

est bioactivity still contained 25 compounds at this stage. This fraction was then further fractionated by column chromatography on silica gel (Lichroprep Si 60, Merck Darmstadt). Elution was achieved with hexane containing increasing amounts of ethyl acetate. Bioactivity eluted at 40–45% ethyl acetate. The active fraction contained 4 compounds which were characterized by GC/MS.

Compound 1 (**20a,** retention time 19.6) MS *m/z* (rel. int.): 198 (2), 180 (5), 167 (7), 166 (10), 125 (16), 124 (46), 123 (30), 109 (42), 96 (12), 95 (35), 81 (17), 79 (8), 74 (8), 71 (9), 69 (21), 68 (12), 67 (11), 59 (10), 55 (16), 53 (10), 43 (100), 41 (29), 39 (12).

Compound 2 (**20b,** retention time 19.9) MS *m/z* (rel. int.): 198 (2), 180 (2), 167 (5), 166 (9), 125 (13), 124 (58), 123 (25), 109 (54), 97 (6), 96 (13), 95 (39), 81 (18), 79 (8), 74 (7), 71 (9), 69 (20), 68 (12), 67 (10), 59 (10), 55 (15), 53 (10), 43 (100), 41 (27), 39 (11).

Compound 3 (**21a,** retention time 21.7) MS *m/z* (rel. int.): 198 (1), 180 (5), 167 (8), 166 (50), 139 (14), 138 (9), 125 (15), 124 (20), 123 (92), 121 (23), 109 (28), 108 (16), 107 (15), 96 (16), 95 (86), 81 (28), 79 (18), 77 (8), 74 (12), 69 (9), 67 (26), 59 (18), 55 (22), 53 (20), 43 (100), 41 (35), 39 (20).

Compound 4 (**21b,** retention time 22.8) MS *m/z* (rel. int.): 198 (1), 180 (2), 167 (5), 166 (27), 139 (7), 138 (5), 125 (6), 124 (8), 123 (39), 121 (12), 109 (13), 108 (12), 107 (6), 96 (8), 95 (39), 81 (15), 79 (10), 77 (4), 74 (7), 69 (4), 67 (13), 59 (8), 55 (11), 53 (10), 43 (100), 41 (19), 39 (10).

The fractions of region 2 were also combined, adjusted to pH 1 and extracted with Et_2O in a perforator. The residue of the Et_2O phase was methylated with diazomethane and investigated by TLC (solvent hexane/ethyl acetate 15:85). Abscisic acid Me ester (R_f = 0.69) was identified by comparison with the authentic compound. The Me ester of ABA was also identified by GC/MS and comparison with an authentic sample. Quantification of ABA Me ester was achieved by calibration of the GC peak.

*Preparation of "Thujaketosäure" (**20**)*

The hot solution of 30 g $KMnO_4$ in 400 ml H_2O was added to 10 g thujone. The mixture was shaken for 20 min and then stirred at room temperature for further 5 h. After filtration, the clear solution was adjusted to pH 7 with dil. H_2SO_4 and evaporated to dryness. The solid white residue was then extracted with absolute ethanol. After evaporation of the ethanol solution, the oily residue was dissolved in diethyl ether. The desired acids were then extracted from ether into 5% $NaHCO_3$ and after acidification to pH 1 back into diethyl ether. The ether phase was then washed with water to neutrality and dried over Na_2SO_4. Evaporation of the clear ether phase yielded colorless crystals (0.72 g).

*Preparation of 3-isopropyl-5-oxohex-2-enoic acid methyl ester (**21**)*

200 mg "Thujaketosäure" was refluxed with water for 66 h. After this time the solution was adjusted to pH 8 with 5% $NaHCO_3$ in water and extracted with diethyl ether. Then the solution was adjusted to pH 1 with dil. H_2SO_4 and again extracted with diethyl ether. This ether extract was washed three times with water and dried over Na_2SO_4. The diethyl ether was evaporated and the residue methylated with CH_2N_2. GC-MS analyzes showed only one product (**21a**).

a) ^1H NMR, ppm (number of protons, multiplicity): 5 (1H, s), 3.65 (3H, s), 2.75 (2H, t), 2.6 (2H, t), 2.38 (1H, s), 2.15 (3H, s), 1.05 (6H, d).

b) ^{13}C NMR, ppm (DEPT): 209 (q), 169 (q), 167 (q), 114 (=C–H), 52 ($-CH_3$), 43 ($-CH_2$), 37 ($-CH_3$), 31 (–C–H), 24 ($-CH_2$), 22 ($-CH_3$), 21 ($-CH_3$).

Results and Discussion

Volatile compounds

We tested at first the possibility that volatile compounds which are released from the leaves of *Thuja occidentalis* might be transported *via* the gas phase to cress seeds and inhibit germination. For this purpose, we placed pieces of *Thuja* leaves in a small Petri dish. The small Petri dish was placed in a larger Petri dish which contained moist filter paper and cress seeds. The large Petri dish was sealed with a cover so that a closed room was created in which volatile compounds of *Thuja* occidentalis could come into contact with the cress seeds only *via* the gas phase.

As shown in Fig. 1, increasing amounts of *Thuja*

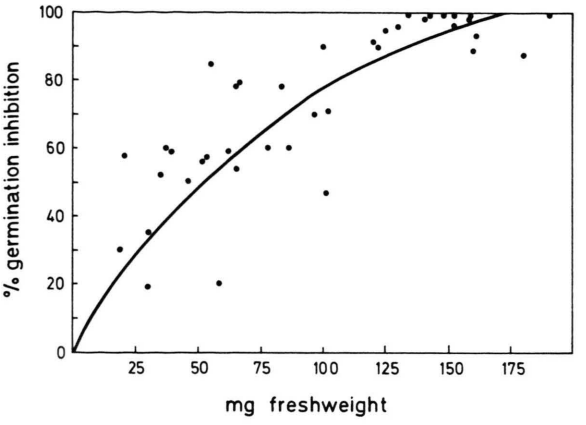

Fig. 1. Inhibition of germination of seeds of *Lepidium sativum* by volatile compounds released from leaves of *Thuja occidentalis*. Increasing amounts (given as mg fresh weight) of *Thuja* leaves were placed together with the seeds in a closed Petri dish. Percent inhibition of germination was determined after 48 h *via* length of primary roots.

leaves induced increased inhibition of germination. A strict correlation between fresh weight of leaves and inhibition of germination cannot be expected because the leaves contain variable numbers of secretory organs. Nevertheless, the experiment demonstrates that volatile compounds of *Thuja* leaves can penetrate the gas phase and induce inhibition of germination in cress seeds.

In order to identify the volatile compounds, fresh leaves of *Thuja* were placed in a flask which was connected to a vacuum pump *via* an U-tube. The U-tube was cooled with liquid nitrogen while the leaves were placed under vacuum. Volatile compounds which condensed in the U-tube were dissolved in diethyl ether and analyzed by gas chromatography (see Fig. 2a) and mass spectrometry. By comparison with data from the literature [16–20] the compounds turned out to be monoterpenes as listed in Table I.

Table I. Quantitation of volatile Compounds of *Thuja occidentalis*. Compounds were either directly taken from secretory organs with a syringe (A) or released from intact leaves *in vacuo* and recovered by condensation at liquid nitrogen temperature (B) or extracted from ground leaves with the indicated solvents (C). Values are means of 2–3 determinations. Numbering of compounds according to increasing retention time (see Fig. 2).

Compound	A	B	C			
			Water 100 °C	Water 20 °C	Diethyl ether	Acetone
Total terpenoids [μmol · g f.w.$^{-1}$]	n.d.	n.d.	23.5	5.5	19.7	19.1
Percentage of single terpenoids (total terpenoids = 100%)						
1. α-Thujene	<0.3	<0.1	–	–	–	–
2. α-Pinene	1.5	1.8	0.4	0.9	1.8	2.0
3. Camphene	1.4	1.3	0.4	0.9	1.5	1.3
4. Sabinene	5.4	8.7	–	1.1	6.5	7.4
5. Myrcene	1.9	1.8	0.4	0.9	0.9	1.9
6. *p*-Cymene	<0.2	<0.1	–	–	–	–
7. Limonene	1.3	1.3	0.6	0.9	1.1	1.3
8. γ-Terpinene	–	–	0.5	0.4	–	–
9. Fenchone	12.0	14.8	20.7	14.6	12.4	11.1
10. α-Thujone	63.0	60.8	55.6	63.3	62.0	62.6
11. β-Thujone	7.7	7.4	11.4	7.5	7.6	7.4
12. Campher	1.7	1.1	2.4	3.4	1.2	1.0
13. Borneol	<0.1	<0.1	0.4	1.5	–	–
14. Terpinen-4-ol	<0.1	<0.1	4.5	3.5	–	–
15. α-Terpineol	<0.2	<0.1	0.8	0.5	–	–
16. Fenchyl acetate	<0.5	<0.1	–	–	–	–
17. Bornyl acetate	2.8	1.0	1.5	0.3	3.9	2.6
18. Thymol	<0.2	<0.1	0.2	–	–	–
19. Terpinyl acetate	1.3	0.3	0.3	0.2	0.9	1.0

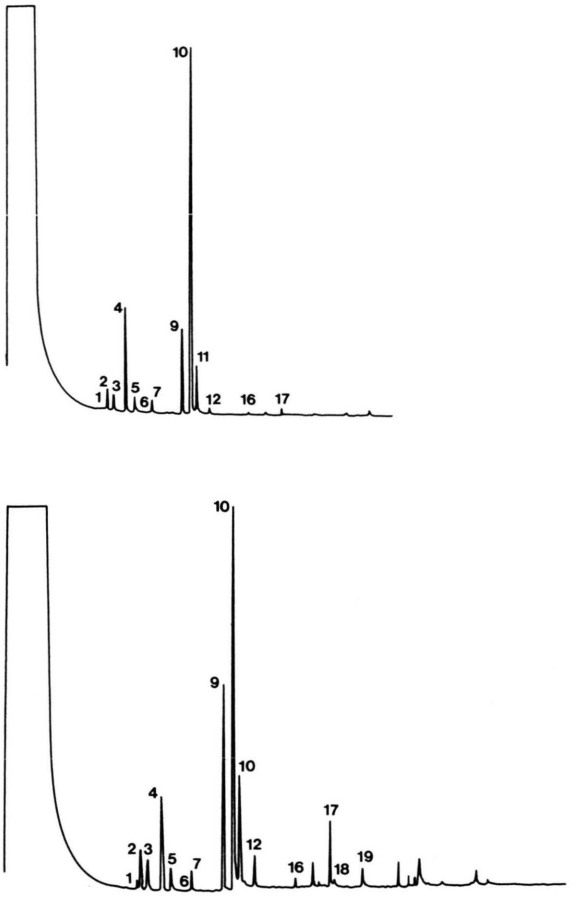

Fig. 2. Gas chromatogram of volatile compounds from leaves of *Thuja occidentalis*. Numbering according to increasing retention time; for identification see Table I. a) Compounds were released from intact leaves at low pressure *(in vacuo)* and condensed at liquid nitrogen temperature. The condensate was dissolved in diethyl ether and applied to gas chromatography. b) Fresh leaves of *Thuja occidentalis* were ground in a mortar under diethyl ether. The extract was applied to a silica gel column in order to remove pigments and lipids which interfere with gas chromatography. The eluate of the column was applied to gas chromatography.

The ratio of different monoterpenes obtained by the vacuum method was compared with the composition of the contents of the secretory organ. This content was obtained by introducing a syringe into the organ; the liquid which entered the syringe by capillary forces was immediately dissolved in diethyl ether and analyzed. For comparison, whole leaves were homogenized and extracted with different solvents (diethyl ether, acetone, water). With only few exemptions, the same compounds were obtained by extraction of whole leaves (see Fig. 2b) as by the previous methods. Quantitation (see Table I) shows the following results: Monoterpenoid alcohols are not present as such in the secretory organs but are found in the water extracts. The acetates of these alcohols are however present in the secretory organs; they are found in comparable amounts in extracts with organic solvents but less in water extracts and in the condensate of the vacuum method, probably due to their lower volatility. We identified the phenolic compound as thymol; it is not carvacrol as previously suspected [11, 12]. It is present in the secretory cells but is also not volatile enough to appear in the condensate of the vacuum method. The yield of the main compound, α-thujone, is relatively small after extraction with water at 100 °C. This is in part due to rearrangement to β-thujone (see Table I) and other reactions which will be discussed below. The hydrocarbons are – as expected according to their solubility – not or nearly not found in the water extracts but are present in comparable amounts in extracts with organic solvents and in the analysis of liquid content of secretory organs. An exemption is γ-terpinene: this hydrocarbon is not found in the secretory organs directly but is present (albeit in small amounts) in the water extracts. It is apparently formed by a rearrangement reaction in contact with water from α-thujene or sabinene [21, 22].

At next, we investigated the question whether only the identified compounds are responsible for inhibition of seed germination. For this purpose, *Thuja* leaves were placed in a desiccator together with an open Petri dish, filled with water so that volatile compounds could be spread in the gas phase and dissolved in the water. After 15 and 48 h, the water was analyzed for dissolved compounds (see Table II). This mixture of compounds was used in various dilutions for biotests with seeds of *Amaranthus caudatus*. We found the same inhibition for the mixture obtained from *Thuja* leaves and for the mixture of authentic compounds (not shown). We conclude therefore that the identified monoterpenes are the active compounds in our biotest system and that no other compounds which are active in our biotest are released to the gas phase from *Thuja* leaves.

Table II. Analysis of compounds which evaporated from leaves of *Thuja occidentalis* and were dissolved in water. Fresh leaves (40 g) were placed in a desiccator together with an open Petri dish containing 20 ml dist. water. After the indicated time of incubation (1. experiment: 15 h; 2. experiment: 48 h), the water was extracted with diethyl ether. The organic phase was then analyzed by gas chromatography.

	10^{-8} mol/g f.w.	%	10^{-8} mol/g f.w.	%
	15 h		48 h	
Fenchone	4.35	25.8	8.98	30.9
α-Thujone	11.10	65.8	17.10	58.8
β-Thujone	0.83	4.9	1.23	4.2
Campher	0.58	3.4	1.23	4.2
Borneol	–	–	0.20	0.7
Terpinen-4-ol	–	–	0.20	0.7
α-Terpineol	–	–	0.12	0.4
	16.86		29.06	

The identified monoterpenes and some related compounds were singly tested for their inhibitory activity with seeds of *Amaranthus caudatus* and *Lepidium sativum*. All compounds were applied in an amount of 10^{-6} mol per test vessel and in dilutions thereof. The highest concentration – presumed that the compounds are completely dissolved in water – was therefore 1 mM for *A. caudatus* and 0.66 mM for *L. sativum*. Only values >30% inhibition of germination for the highest concentration are listed in Table III. The hydrocarbons α-pinene, camphene, myrcene and limonene and the ketone β-thujone show a smaller inhibition and are therefore not listed. It is evident that the highest activity is found in the series of alcohols, followed by the ketones, acetates and finally hydrocarbons. There are further more differences related to the 3-dimensional structures as shown for the pairs borneol/isoborneol and α-thujone/β-thujone. Reynolds [24] found a different order of bioactivity in similar experiments with *Lactuca sativa:* With this species, germination was inhibited more by monoterpene ketones than by alcohols, followed finally by hydrocarbons.

The relatively high bioactivity of sabinene is probably due to a chemical reaction with water: When pure sabinene was incubated in water at pH 4.0 at 21 °C for 10 days, the following products were found: 62.3% terpinene-4-ol, 19.7% γ-terpinene, 6.6% α-terpinene, 3.8% terpinolene, 2.0% α-terpineol, 3.5% monoterpenes of unknown structure, and only 1.7% sabinene was left. The percentage of terpinene-4-ol is even higher if sabinene is incubated at higher temperatures. Our results agree with previous reports on the chemical reactivity of sabinene [21–23]. The corresponding incubation of limonene yields 10% α-terpineol and 90% limonene. All other tested monoterpenes – α-pinene, camphene, myrcene, fenchone, thujone, campher, borneol, terpinene-4-ol, α-terpineol, bornyl acetate, thymol, carvacrol, terpinyl acetate – were stable under the same conditions.

Non-volatile compounds

In order to detect those compounds which could possibly leach out from the leaves by rain and cause inhibition of germination beneath the *Thuja* plants, we investigated aqueous extracts of the leaves. Since extraction at 100 °C gives higher yields than extraction at 20 °C (4-fold higher for volatile compounds, see Table I), we concentrated our efforts upon hot water extracts. Separation into a neutral and an acidic fraction was achieved as previously [25–27] by extraction into diethyl ether at pH 8 and then at pH 1. The acid fraction which showed high bioactivity was further fractionated by chromatography on Sephadex LH-20. Bioactive compounds were eluted in 2 zones, zone I comprised fractions 40–60 and zone II fractions 110–130. The inhibitory activity of zone II was much higher with seeds of *Amaranthus caudatus*

Table III. Biotest with monoterpenes from *Thuja occidentalis* and related compounds. Dilution series of the respective compound were prepared in diethyl ether and pipetted onto filter paper in a biotest vessel. Immediately after evaporation of the diethyl ether, 1 ml water (for *A. caudatus*) or 1.5 ml water (for *L. sativum*) were added to filter paper and the seeds were placed upon the moistured paper. All values are concentrations for 50% inhibition of germination, determined as standardized (see Materials and Methods).

Compound	Concentration for 50% inhibition of germination [mmol/l] *Amaranthus caudatus*	*Lepidium sativum*
Fenchone	1.05	1.70
α-Thujone	0.52	n.d.*
β-Thujone	1.10	n.d.
Campher	0.92	1.02
Menthol	0.46	0.63
Thujyl alcohol	0.32	0.47
Borneol	0.50	0.70
Isoborneol	0.10	0.54
Terpinene-4-ol	0.38	1.48
α-Terpineol	0.45	1.01
Thymol	0.40	0.40
Carvacrol	0.28	0.69
Geraniol	0.11	0.66
Citronellol	0.18	0.49
"Thujaketosäure" (**20**)	0.55	1.10
"Thujaketosäure"-Me ester	0.38	0.32
3-Isopropyl-5-oxohex-2-enoic Me ester (**21**)	0.38	n.d.
Pinene	2.76	n.d.
Camphene	1.41	n.d.
Sabinene	1.36	n.d.
Myrcene	1.58	n.d.
Limonene	1.43	n.d.
Carvyl acetate	0.92	1.35
Bornyl acetate	0.96	1.30
Terpinyl acetate	2.75	n.d.

* n.d. = not determined.

than with those of *Lepidium sativum*. Such a property had been found previously [27] for abscisic acid (ABA). It turned out after methylation by TLC and by GC that zone II contained ABA. The bioactivity of zone II matched exactly the concentration ABA. We conclude that bioactivity of this fraction is exclusively due to its content of ABA. The calculated amount of ABA in *Thuja* leaves was 3–4 μg/g fresh weight. It is not yet known which function this plant hormone exhibits at such high concentrations in leaves of an evergreen plant during the summer period.

Purification of the active compound of zone I proved to be more difficult. As described in the experimental part, the following steps of purification were applied: methylation with diazomethane, distillation *in vacuo*, horizontal distillation and then column chromatography on silica gel of the respective bioactive fraction. The final active fraction contained still 4 compounds. This mixture consists of 2 pairs of compounds, **20a/b** and **21a/b.** Mass spectra within a pair are nearly identical. As shown in Fig. 3, the mass spectra of the two pairs are also closely related. We have to conclude that the compounds are 4 isomers. The molecular weight, deduced from EI and CI measurements, is 198; high resolution MS revealed the composition $C_{11}H_{18}O_3$. As expected from the isolation procedure the compounds are methyl esters of carboxylic acids as shown by fragments M-31/32 and *m/z* 59 and the fragment at *m/z* 117 after trimethylsilylation of the unesterified compound

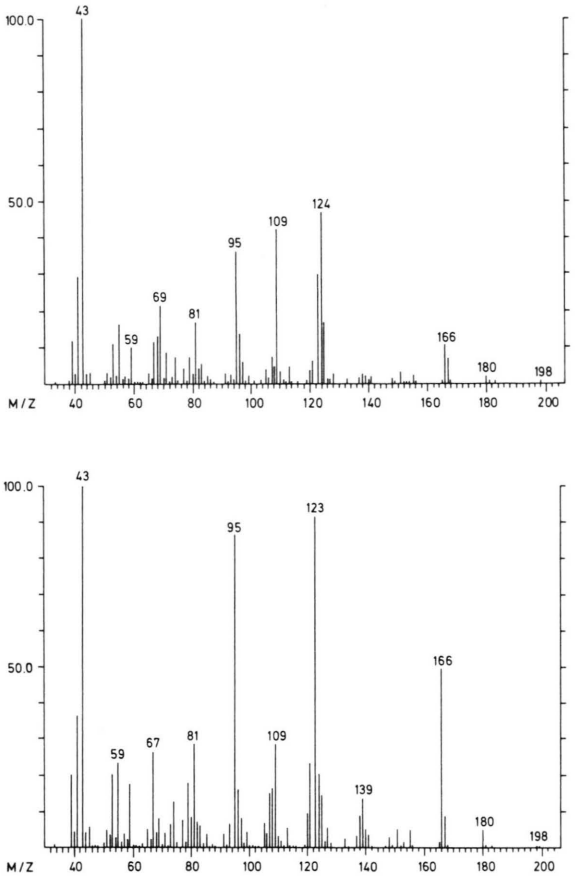

Fig. 3. Mass spectra of oxidation products of thujone. **20a:** "Thujaketosäure"-methyl ester, prepared by $KMnO_4$ oxidation from thujone. Retention time 17.9 min. Compound **20b** at 18.1 min has the identical mass spectrum. **21a:** Compound prepared from **20** with boiling water. Retention time 20.0 min. Compound **21b** at 21.1 min has the identical mass spectrum. For structures see Fig. 4.

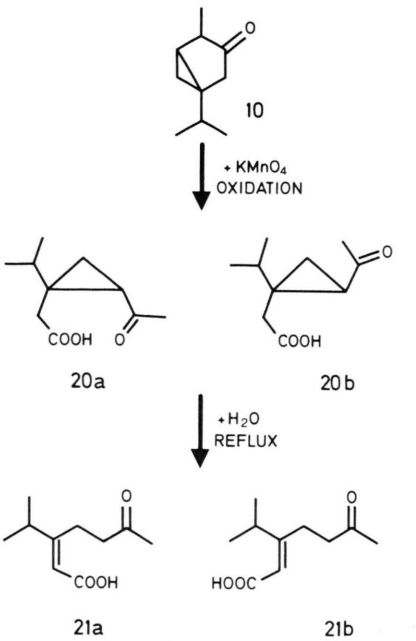

Fig. 4. Structures of thujone and its oxidation products. The reaction pathway is also indicated.

[28–30]. The fragment at m/z 180 (= M-18) points to the other oxygen function as either a hydroxy or carbonyl group. Further fragmentation is similar to that of several cyclic monoterpenes except for the intensity in the higher m/z range which is much smaller here. The compounds looked to us like oxidation products of a monoterpene.

Oxidation of thujone has been known since a long time [31] (see Fig. 4). We prepared the oxidation product 2-(1-isopropyl-2-acetylcyclopropane)acetic acid (**20,** Wallach's "Thujaketosäure"). Two of the 4 isomers isolated from *Thuja* leaves proved to be identical with the *Z* and *E* isomers **20a** and **20b.**

The other 2 isomers, contrary to "Thujaketosäure", gave a strong reaction with permanganate; they must contain therefore an oxidizable group, probably a C,C-double bond. The main product turned out to be a product of an opening reaction of the cyclopropane ring of "Thujaketosäure": it could be prepared from "Thujaketosäure" by treatment with boiling water. Structure **21** (see Fig. 4) was established by NMR spectra.

The key feature in the ^1H NMR spectrum are two triplets, representing 2 protons each, at 2.75 and 2.60 ppm which indicate the structural element $-CH_2-CH_2-$. This element does not occur in any of the isomeric structures which could theoretically also be derived from ring opening of "Thujaketosäure". The presence of two methylene groups is confirmed by the DEPT ^{13}C NMR spectrum. All other signals in the ^1H and in the ^{13}C NMR spectra are as well in agreement with the proposed structure. Due to the similarity in the mass spectra, we assume that the isomer mixture obtained from *Thuja* leaves contains the *Z* and *E* isomers **21a** and **21b.**

As shown in Table III, the biological activity of the oxidation products **20** and **21** is only slightly higher than that of the parent compound thujone. Nevertheless, the oxidation products could possibly contribute to the allelopathic effect of products from *Thuja* due to their lower volatility. A precondition is the rapid formation of the oxidation products from thujone under "natural conditions". In order to test this possibility, we investigated the transfer of liquid thujone into water *via* the gas phase and the formation of "Thujaketosäure" in water at room temperature. As shown in Fig. 5, saturation of water with thujone takes several days, only about 1% of thujone is oxidized to "Thujaketosäure" in parallel under these conditions. No unsaturated compound is formed at room temperature.

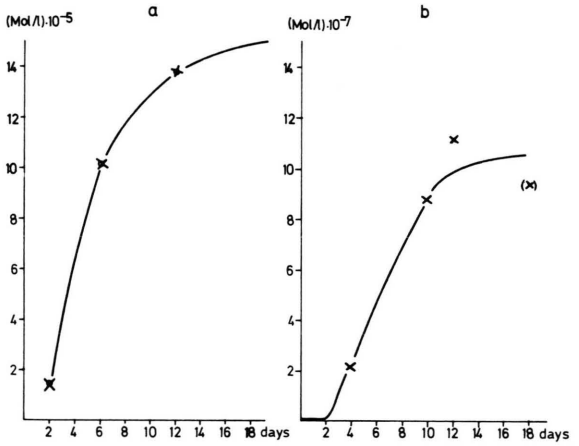

Fig. 5. Kinetics of increase in concentration of monoterpenes in water after application of thujone *via* the gas phase. a) Thujone, b) "Thujaketosäure".

We conclude that oxidation of thujone, dissolved in water, does occur in the presence of air. The yield of the oxidation product **20** is however so poor that it does not measurably contribute to the observed inhibitory action of thujone.

Even the saturated solution of thujone in water does not lead to complete inhibition of germination although thujone alone does lead to complete inhibition. In order to investigate this phenomenon in more detail, we performed the following experiment: Thujone was applied (A) *via* the filter paper in a Petri dish (as usual) and (B) *via* the gas phase without contact with the filter paper. For comparison, abscisic acid (ABA) was applied *via* the filter paper. The amount of both compounds was chosen so that germination was completely inhibited. After moisturing the filter paper, 25 seeds of *A. caudatus* were placed upon the filter paper. After 2 days, the content of thujone in the water proved again to be not sufficient for complete inhibition of germination. However, complete inhibition was observed in the experiment. Analysis of the seeds showed that thujone accumulated in the seeds even more than ABA (Table IV). No oxidation products **20** or **21** (see Fig. 4) were detectable in the seeds. This experiment demonstrates that lipid-soluble compounds like thujone can be accumulated in the seeds, probably in membranes or other lipid-containing compartments. Such accumulation can occur not only *via* a water phase but probably also directly *via* the gas phase. Even when conclusions drawn from "closed" laboratory

Table IV. Uptake of the germination inhibitors abscisic acid and thujone by seeds of *Amaranthus caudatus*. Application A: The inhibitor was applied to filter paper which was subsequently moistured with 1 ml water. Application B: The inhibitor was applied *via* the gas phase without direct contact to the moistured filter paper. Each vessel contained 25 seeds of *A. caudatus*. In all cases 100% inhibition of germination was achieved. Seeds of 40 vessels were collected after 2 days, extracted and analyzed.

Compound	Application	Applied amount per vessel [μmol]	Amount per seed after 2 days [pmol]	Percent of applied compound detected in 25 seeds after 2 days
Abscisic acid	A	0.1	0.5	0.01%
Thujone	A	2.5	330	0.33%
Thujone	B	2.5	335	0.34%

systems to the "open" system in the natural environment are dangerous, such a retainment mechanism could play a role in the allelopathic action of *Thuja occidentalis*. According to our data, possible chemical or biochemical transformation of volatile compounds from *Thuja occidentalis* into non-volatile, bioactive compounds seems to play only a minor – if any – role.

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