Natural Inhibitors of Germination and Growth IV
Compounds from Fruit and Seeds of Mountain Ash *(Sorbus aucuparia)*

Ulrike Oster, Inge Blos, and Wolfhart Rüdiger

Botanisches Institut der Universität München, Menzinger Straße 67, D-8000 München 19, Bundesrepublik Deutschland

Z. Naturforsch. **42c,** 1179–1184 (1987); received August 3, 1987

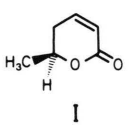
Dedicated to Professor Hermann Schildknecht on the occasion of his 65th birthday

Abscisic Acid, Parasorbic Acid, Isopropylmalic Acid, Dormancy, *Amaranthus caudatus*, *Lepidium sativum*

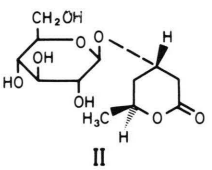
Extracts from ripe fruit and seeds of *Sorbus aucuparia* inhibit germination of other seeds, tested with *Amaranthus caudatus* and *Lepidium sativum*. The main compound of the "neutral fraction" of fruit extracts is the lactone compound parasorbic acid (4–7 mg/g fresh weight) which inhibits germination at concentrations $\geqq 5\times10^{-4}$ M. The "acid fraction" contains abscisic acid (1.3–2.5 µg/g fresh weight) and isopropylmalic acid (1.0–1.5 µg/g fresh weight) as germination inhibitors. Whereas abscisic acid inhibits germination of *L. sativum* at concentration $\geqq 5\times10^{-7}$ M, germination of *A. caudatus* is inhibited only at concentrations $\geqq 10^{-5}$ M. This is probably due to differences in uptake of the acid because abscisic acid methylester inhibits germination of both species at concentrations $\geqq 5\times10^{-7}$ M. Isopropylmalic acid belongs to a structural type of germination inhibitors which had already been detected in oat extracts.

Introduction

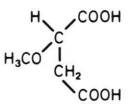
Inhibitory activity for seed germination in ripe fruit and seeds has been known since a long time; the active compounds have been called "blastokolins" [1] before elucidation of their structure. High "blastokolin" activity has been found in the berries of the mountain ash *(Sorbus aucuparia)* and attributed to parasorbic acid [2] the structure of which had been elucidated as **I** [3–5]. Parasorbic acid had already been obtained in 1859 from the juice of mountains ash berries by Hofmann [6], like other unsaturated lactones [7] it exhibits multiple biological activities including growth inhibition of some microorganisms and animal fibroblast investigated in tissue culture [2]. According to Tschesche *et al.* [8], **I** is formed during extraction by elimination of glucose from the naturally occurring glucoside parasorboside (**II**) which itself has little or no antibiotic activity. Since seeds of *Sorbus aucuparia* have a high degree of dormancy (see [9]) we asked whether other compounds with "blastokolin" activity may occur in *S. aucuparia*. We describe here the analysis of extracts from fruit and seeds of *S. aucuparia* with respect to compounds active as seed germination inhibitors.



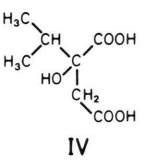
I



II



III



IV

Reprint requests to Prof. Dr. W. Rüdiger.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/1100–1179 $ 01.30/0

Materials and Methods

Isolation of germination inhibitors

Ripe berries of *Sorbus aucuparia* were collected, immediately frozen and stored at −18 °C until extraction. 500 g berries were briefly ground and the resulting brei was boiled with 2.5 l water under reflux for 24 h. The clear filtrate was adjusted to pH 8 with $NaHCO_3$; extraction with diethyl ether at this pH and subsequently at pH 1 was performed as previously described for *Rosa canina* [12]. The neutral fraction was further purified on silica gel Si 60 (column 24 × 2,5 cm) with a step gradient of 0–75% ethyl acetate in *n*-hexane. The bioactivity was eluted at 50% ethyl acetate.

Further fractionation of the acid fraction on Sephadex LH-20 was performed as previously described [12], for results see Fig. 4. Compounds of fraction S-1 were extracted into diethyl ether. The residue of the ether phase was further fractionated by distillation. Residual parasorbic acid (fraction S-1A) was removed at 70 °C/0.5 Torr. The residue of this distillation was treated with diazomethane and then again distilled at 70 °C/0.5 Torr. This yielded fraction S-1B which was further fractionated by chromatography on silica gel Si 60 (column 7 × 0.7 cm) with a gradient of 45–50% diethyl ether in hexane. Fractions with bioactivity were analyzed by GC/MS (see Fig. 5). Fraction S-2 was lyophilized and treated with diazomethane. Chromatography on silica gel Si 60 with a gradient of 50–85% ethyl acetate in *n*-hexane yielded one active fraction which eluted at 55–60% ethyl acetate. Only abscisic acid methyl ester was detected in this fraction by GC/MS.

For investigation of seeds, berries were frozen with liquid nitrogen and pestled in a mortar. Seeds were removed by this treatment from the frozen pulp, collected, washed with water to remove residual pulp, and dried on filter-paper. 14.8 g seeds were obtained from 1000 g berries. The seeds were boiled with 50 ml water under reflux for 15 h. Parasorbic acid was determined in this aqueous extract by HPLC.

Quantitative analyses

Quantitation of the germination inhibitors was performed either by high performance liquid chromatography (HPLC) for the free acids or by gas chromatography (GC) for the methyl esters.

HPLC analyses were performed with a Gynkothek instrument 300 C equipped with a spectral photometer model SP-6V as detector. Separation of inhibitor compounds was achieved on a reversed-phase column (Lichroprep RP-8, 150 × 5 mm) with either water, acidified with H_3PO_4 to pH 3, flow rate 2 ml/min (for parasorbic acid) or with methanol/1% aqueous H_3PO_4 flow rate 1.5 ml/min (40:60), for abscisic acid. Detection was always by UV absorption at 215–220 nm.

GC analyses were performed on a Hewlett-Packard gas chromatography (model 5890) equipped with a fused-silica capillary column (SE 52, length 25 m). Flow rates of the carrier gas helium was 2 ml/min. Injection was splitless. The oven temperatur was programmed from 50 to 250 °C at 5°/min. The flame ionization detector was set at 250 °C.

Analysis by GC/MS was performed with a quadrupol mass spectrometer (MAT 4500, Finnigan), under the following conditions: electron energy 70 eV, temperature of the ion chamber was 120 °C, scan 33–350 *m/z*. The mass spectrometer was coupled to a gas chromatograph (model 9611, Finnigan), equipped with a fused-silica capillary column (SE 54, length 30 m). The injector temperature was 240 °C, analysis were performed with a temperature program at 5°/min from 50 to 250 °C.

The biotest was performed either with 25 seeds of *Amaranthus caudatus* in 1.0 ml test solution as described earlier [10] or with 15 seeds of *Lepidium sativum* in 1.5 ml test solution under otherwise identical conditions.

Results and Discussion

For isolation of bioactive compounds, we used an established method the outlines of which are given in Fig. 1. It corresponds to the method which had successfully been applied already to investigation of oat husks [10, 11] and fruit and seeds of roses [12]. The main compound of the neutral fraction is parasorbic acid which can easily be purified by column chromatography on silica gel. We identified the compound by mass spectrometry. Mass spectra have been reported for the glucoside parasorbide and some derivatives thereof [8] but not for parasorbic acid. Therefore we present here the mass spectrum and the fragmentation pattern of parasorbic acid (Fig. 2). The mass spectrum is identical with that of an authentic sample which we synthesized from sorbic acid with hydrogen bromide and then water according to Kuhn and Jerchel [3]. For quantitative determination of parasorbic acid, the crude extract can be directly applied to HPLC. Typical chromatograms for extracts from fruit and seeds are given in Fig. 3. We determined values of 4 to 7 mg parasorbic acid per g fresh weight of fruit and 0.08 to 0.12 mg per g fresh weight of seeds for various batches of berries. Bioactivity of the neutral fraction corresponds with the amount of parasorbic acid determined in this fraction (Table I). We conclude therefore that germination inhibition of this fraction is only due to the presence of parasorbic acid.

The acid fraction which contains only 5–10% of bioactivity of crude extracts was applied to a column

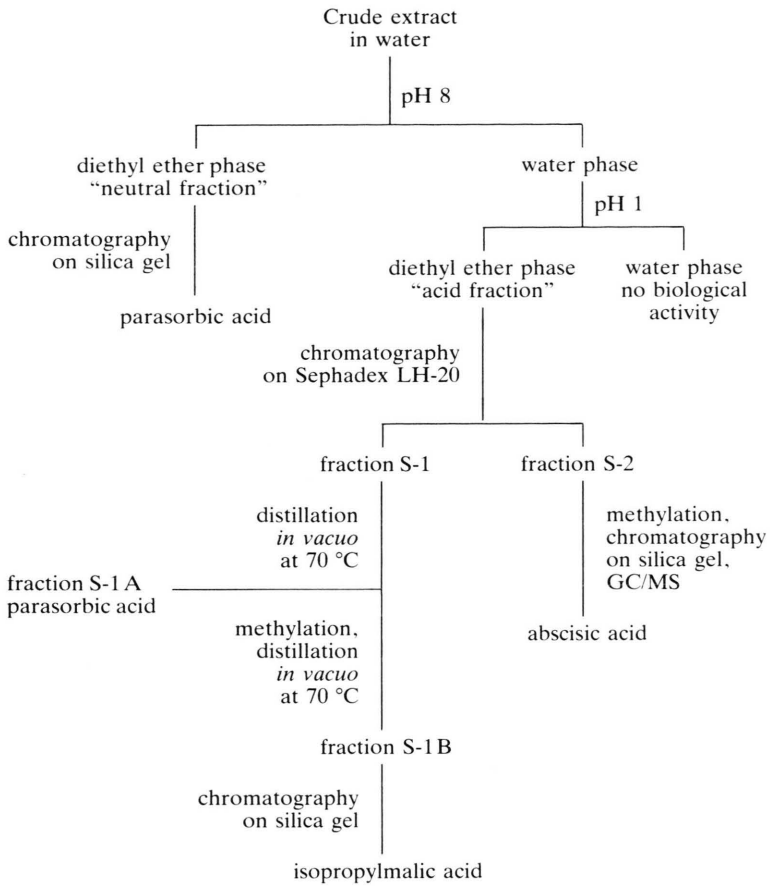
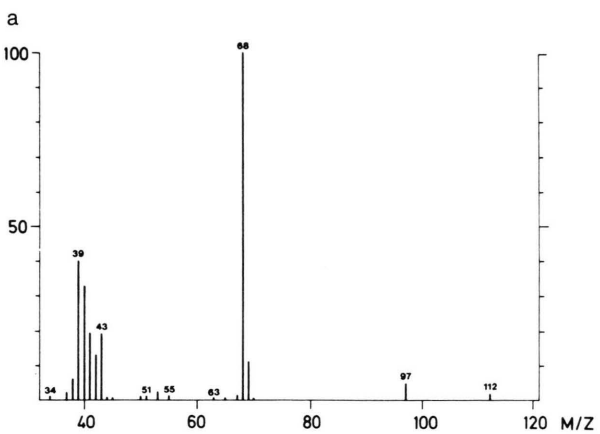


Fig. 1. Scheme of isolation of seed germination inhibitors from fruit of mountain ash *(Sorbus aucuparia)*.



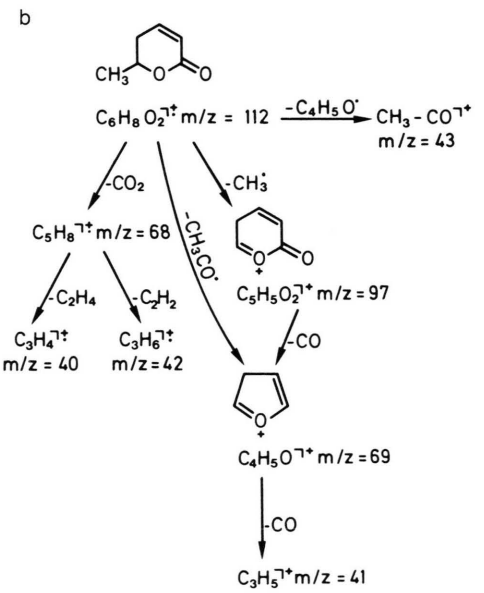


Fig. 2. Mass spectrum and fragmentation of parasorbic acid from *Sorbus aucuparia*. a: Mass spectrum obtained in the GC/MS system (see Materials and Methods) of the neutral fraction after purification by chromatography on silica gel (see Fig. 1). b: Fragmentation pattern deduced from data of Fig. 2a.

Table I. Bioactivity of the neutral fraction after chromatography on silica gel (see Fig. 1) and authentic parasorbic acid. Numbers are % inhibition of germination of *Amaranthus caudatus* seeds.

Concentration of parasorbic acid [mM]	Neutral fraction [%]	Authentic parasorbic acid [%]
1.0	100	100
0.75	100	99
0.50	100	88
0.25	25	18

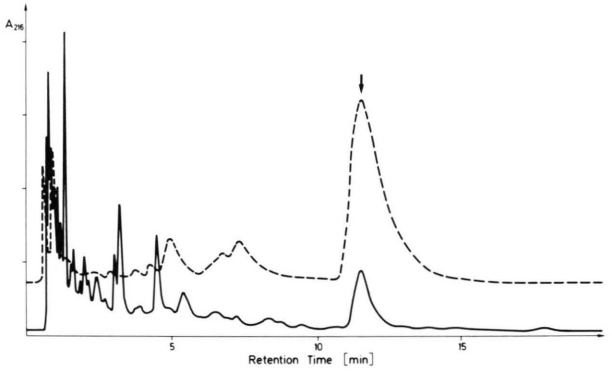


Fig. 3. HPLC chromatograms of crude extracts from fruit (– – – –) and seeds (———) of *S. aucuparia*. Detection by absorption measurement at 216 nm. Adsorbent: Lichroprep RP-8. Solvent: Water adjusted to pH 3 with phosphoric acid. The retention time of authentic parasorbic acid is indicated by the arrow.

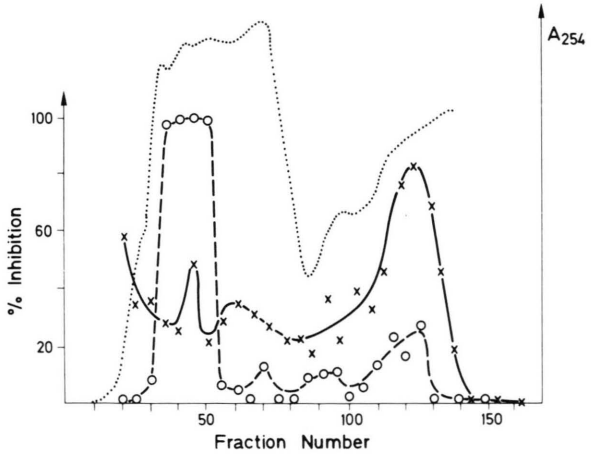


Fig. 4. Liquid chromatogram of the acid fraction (see Fig. 1) on Sephadex LH-20 with water as solvent. (·····) Absorbance at 254 nm; percent inhibition of *A. caudatus* (– – – –) and *L. sativum* (———).

of Sephadex LH-20. Two fractions with bioactivity, S-1 and S-2 could be eluted with water (Fig. 4). Further elution with water/methanol mixtures or pure methanol did not yield any further bioactivity. Interestingly, S-1 had the highest activity with seeds of *A. caudatus* whereas nearly no activity could be detected in S-1 with seeds of *L. sativum*. The activity of S-2, on the contrary, was high with *L. sativum* seeds and very low with *A. caudatus* seeds. This means that either one or the other bioactive fraction could easily be overlooked if test seeds of only one species are used.

Methylation of fraction S-2 with diazomethane caused disappearance of the difference in the biotests with *A. caudatus* and with *L. sativum*, *i.e.* the bioactivity after methylation is as high in *A. caudatus* as in *L. sativum*. The active compound of fraction S-2 was identified after methylation, chromatography and mass spectrometry as *abscisic acid*. The difference in sensitivity of the various seeds could also be demonstrated with authentic abscisic acid (Table II): *A. caudatus* seeds are relatively insensitive for free abscisic acid but sensitive for its methyl ester whereas *L. sativum* seeds have the same high sensitivity for free abscisic acid as for its methyl ester. Quantitative determination of abscisic acid can be achieved either by gas chromatography after methylation or by reversed phase HPLC of the free acid. The total bioactivity of fraction S-2 is identical within limits of error with the bioactivity of that amount of authentic abscisic acid which had been determined in S-2. This means that no other germination inhibitor is present in this fraction.

Fraction S-1 is still heterogeneous as revealed by gas chromatography after methylation. Further fractionation was achieved by distillation *in vacuo* which yielded still some parasorbic acid as the most volatile, bioactive compound. The residue was again

Table II. Bioactivity of fraction S-2 and authentic abscisic acid (ABA) as free acid and as methyl ester. Fraction S-2 contains ABA methyl ester. Numbers are % inhibition of seed germination.

Conc. of ABA [μM]	*Amaranthus caudatus* Fr. S-2	ABA-acid	ABA-ester	*Lepidium sativum* ABA-acid	ABA-ester
10	100	81	100	100	100
1	99	6	99	81	100
0.5	87	5	96	49	95
0.1	24	0	8	10	25

distilled after methylation and then further fractionated by chromatography on silica gel. The bioactive fraction S-1B from this column contained 3 compounds (see Fig. 5). They were identified by mass spectrometry as succinic acid, methoxymalic acid (**III**) and 2-isopropylmalic acid (**IV**). All compounds had been detected earlier in oat and other grains [11, 13], isopropylmalic acid also in human urine [14]. The latter is an intermediate in leucine biosynthesis.

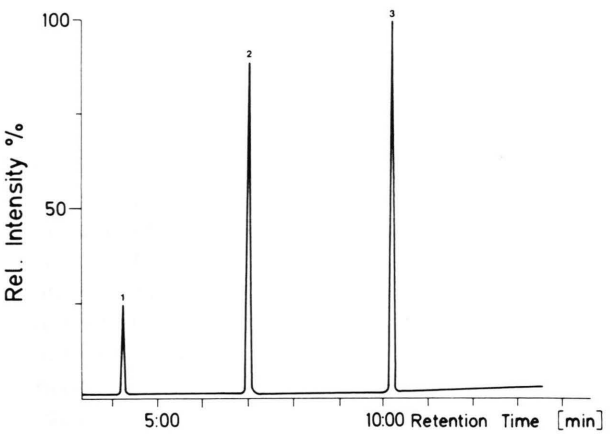


Fig. 5. Gas chromatogram of fraction S-1B (see Fig. 1). Detection in the GC/MS system (see Materials and Methods) *via* total ion current. 1 = Succinic acid methyl ester, 2 = methoxymalic acid methyl ester, 3 = isopropylmalic acid methyl ester.

Since authentic succinic acid had not shown any bioactivity in earlier experiments [11], we tested here isopropylmalic acid which was synthesized according to Yamashita [15] and methoxymalic acid which was prepared by methylation of malic acid (see [11]). Whereas methoxymalic acid did not show any significant bioactivity up to much higher concentrations than detected in the extracts (see Table III), we detected bioactivity for isopropylmalic acid. The bioactivity of the authentic compound applied in the concentration which was determined by quantitative gas chromatography in fraction S-1B corresponds within the limits of error with the activity of the total fraction S-1B (Table III). This means that this is the only germination inhibitor present in fraction S-1B.

Isopropylmalic acid has some structural features in common with di- and tricarboxylic acid derivatives detected as germination inhibitors in oat and rose extracts [11, 12]. It seems that several polar groups are required for bioactivity in a certain spatial arrangement linked to an unpolar backbone.

This aspect is being investigated further with synthetic model compounds.

Acknowledgements

This work was supported by the BMFT, Bonn (Az.: PTB 038519) and the Fonds der Chemischen Industrie, Frankfurt. We thank Mr. Chr. Harzer and Miss C. Zenger for careful technical assistance.

Table III. Bioactivity of fraction S-1B and authentic methyl esters of isopropylmalic and methoxymalic acid. Numbers are % inhibition of germination of *Amaranthus caudatus* seeds.

Concentration [mM]	Fraction S-1B	Isopropylmalic acid methyl ester	Methoxymalic acid methyl ester
0.75	100	100	n.d.
0.50	100	81	40
0.25	87	71	7

[1] A. Köckemann, Ber. dtsch. Bot. Ges. **52,** 523–526 (1934).
[2] R. Kuhn, D. Jerchel, F. Moewus, E. F. Möller, and H. Lettré, Naturwissenschaften **31,** 468 (1943).
[3] R. Kuhn and D. Jerchel, Ber. dtsch. chem. Ges. **76,** 413–419 (1943).
[4] R. Kuhn and K. Kum, Chem. Ber. **95,** 2009–2011 (1962).
[5] R. Lukeš, J. Jarý and J. Němec, Collect. czechoslov. chem. Commun. **1962,** 735.
[6] A. W. Hofmann, Liebigs Ann. Chem. **110,** 129–140 (1859).
[7] L. J. Haynes and E. R. H. Jones, J. Chem. Soc. **1946,** 954–957.
[8] R. Tschesche, H.-J. Hoppe, G. Snatzke, G. Wulff, and H. W. Fehlhaber, Chem. Ber. **104,** 1420–1428 (1971).
[9] K. Namwar and W. Spethmann, Allg. Forstl. Zentralblatt **36,** 937–943 (1985).
[10] R. Karl and W. Rüdiger, Z. Naturforsch. **37c,** 793–801 (1982).
[11] E. Lohaus, I. Blos, W. Schäfer, and W. Rüdiger, Z. Naturforsch. **37c,** 802–811 (1982).
[12] E. Lohaus, C. Zenger, W. Rüdiger, and E. Cmiel, Z. Naturforsch. **40c,** 490–495 (1985).
[13] E. Lohaus, I. Blos, and W. Rüdiger, Z. Naturforsch. **38c,** 524–530 (1983).
[14] M. Spiteller and G. Spiteller, J. Chromatogr. **164,** 319–329 (1979).
[15] M. Yamashita, J. Org. Chem. **23,** 835–837 (1958).