Natural Inhibitors of Germination and Growth, III
New α-Pyrones from Seeds of *Rosa canina*

Edith Lohaus, Claudia Zenger, and Wolfhart Rüdiger

Botanisches Institut der Universität München, Menzinger Str. 67, D-8000 München 19

and

Edmund Cmiel

Institut für Physikalische und Theoretische Chemie der Technischen Universität München, Lichtenbergstr. 4, D-8046 Garching

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Dedicated to Professor Dr. O. Kandler *on the Occasion of His 65th Birthday*

3-Methyl-l-oxa-bicyclo (4,1,0)hept-5-en-2-one-4,6-dicarboxylic Acid Dimethylester, 3-Methyl-2H-pyran-2-one-4,6-dicarboxylic Acid

The new α-pyrone derivative II was isolated in crystalline form from extracts of dormant seeds of *Rosa canina*. It inhibits germination of seeds of *Amaranthus caudatus* completely, but reversibly, at conc. $> 2.5 \times 10^{-5}$ M. Its chemical structure was elucidated by mass spectrometry, ^{1}H- and ^{13}C-NMR spectroscopy to be 3-methyl-l-oxa-bicyclo (4,1,0) hept-5-en-2-one-4,6-dicarboxylic acid dimethylester (**II**). It is derived from 3-methyl-2H-pyran-2-one-4,6-dicarboxylic acid by cyclopropanation with diazomethane. The parent compound which is a natural product of the seeds of *Rosa canina* was isolated as its dimethyl ester **I** the structure of which was elucidated by mass and NMR spectroscopy. **I** shows less inhibition of seed germination than **II**. The reference α-pyrones dimethyl-6-methyl-2H-pyran-2-one-3,5-dicarboxylate (**III**), 3-methoxycarbonyl-5-methoxycarbonylmethyl-6-methyl-2H-pyran-2-one (**IV**), and isodehydracetic acid methylester (**V**) did not show any inhibitory activity. The relationship of II with threo-dihydrohematinic acid (**VII**) is discussed.

Introduction

The phenomenon of dormancy is found in the seeds of many plants. There can by many reasons for dormancy [1]. Of special interest are germination inhibitors, *i.e.* compounds which inhibit germination at higher concentrations and partially inhibit root and shoot growth at lower concentrations. Inhibition of this type of compounds is reversible, *i.e.* germination, root and shoot growth is normal after removal of the compound.

A quantitative bioassay for germination inhibitors was developed by us [2] using seeds of *Amaranthus caudatus;* percentage of germination and length of radicle were used for quantitation. With this assay, several germination inhibitors were isolated, inter alia *n*-pentane-1,3,4-tricarboxylic acid (**VII,** dihydrohematimic acid) from *Avena sativa* [3].

In the course of investigation of the highly dormant seeds of *Rosa canina,* we isolated a new α-pyrone which acts very efficiently as germination inhibitor. Although the compound turned out to be an artificial product of the isolation procedure it deserves interest because its structure bears some resemblance to dihydrohematinic acid. The isolation, structural elucidation and some properties of the new compound and of the parent natural product are described here.

Reprint requests to Prof. Dr. W. Rüdiger.

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Materials and Methods

Isolation of the α-pyrones I and II

Mature fruits of *Rosa canina* were collected near Munich during August and September. The plant material was either extracted immediately or stored at −18 °C until use. Compounds with inhibitor activity were only detected in the seeds but not in the pulp. For large scale preparations, it was preferred to extract broken fruits and remove more contaminats by subsequent chromatographic methods rather than to mechanically separate seeds from the pulp at first.

500 g of frozen fruits (corresponding to 38 g seeds) were briefly ground (5s) in a Waring blendor to release the seeds. The whole mixture was then extracted with boiling water [2l] for 48 h in a Soxhlet extractor. The aqueous solution (about 1.8 l) was adjusted to pH 8 with $NaHCO_3$ and repeatedly extracted with diethylether. The ether phase which mainly contained neutral compounds without much bioactivity

was discarded. The aqueous phase was then acidified to pH 1 with H_2SO_4 and exhaustively extracted with diethylether in a perforator. The aqueous phase which did not show any more bioactivity was discarded. The ether phase which contained acidic compounds was dried with Na_2SO_4 and evaporated to dryness in vacuo.

The residue of the acid fraction was dissolved in 3 ml methanol and applied to a Sephadex LH-20 column (600 × 50 mm) which then was eluted with water at a flow rate of 90 ml/h. Fractions of 8 ml each were collected. The largest inhibitory zone was found in fractions 15–40. The combined fractions were lyophilized and the residue was methylated with diazomethane. For the preparation of deuterium-labelled compounds, deuterodiazomethane was used. As soon as the nitrogen formation had stopped, the excess of diazomethane was removed under reduced pressure. The main compound, citric acid trimethylester, crystallized from a small volume of diethylether and was removed by filtration. The supernatant was evaporated and the residue distilled at 0.5 torr. The compound investigated here was found in the fraction boiling between 100 °C and 150 °C.

Further purification was achieved by chromatography on silicia gel (Li-Chropep Si 60, Fa. Merck, Darmstadt) with hexane containing increasing amounts of ethyl acetate (5–100%). Fractions with the highest bioactivity which eluted at a concentration of 45–55% ethyl acetate were combined. These fractions contained according to GC-MS analysis (see below) only the α-pyrones I and II. By fractionated crystallization from hexane/ethylacetate (70:30, v:v), both compounds were obtained in pure form. The pyrone I crystallized at first from the mixture; the crystals were washed with ice-cold diethylether and recrystallized from hexane/ethylacetate. Purity of I was controlled by GC. The mother liquid was reduced in its volume. After crystallisation of mixtures of I and II, finally pure II was obtained. The crystals were also washed with a small volume of icecold diethylether and recrystallized from hexane-ethylacetate.

GC-MS

GC-MS analyses were performed on a Varian MAT 312 mass spectrometer with a combined EI/CI ion source connected to a Varian gaschromatograph model 3700. The mass spectrometer was operated under the following conditions: electron energy: 70V; ion source: 220 °C; scan: 34.5–400 *m/z*. High resolution data were obtained by peak matching in combination with a MAT SS 200 data system. The gas chromatograph was operated with a glas capillary column filled with OVl. The carrier gas was helium with a flow rate of 2 ml min^{-1}. The injector and detector temperature was 275 °C. The temperature of the column was programmed from 50 °C to 250 °C at 3 °C min^{-1}.

NMR-spectrometry

^{1}H-NMR spectra were obtained with a Bruker instrument model WP 80 FT and an Aspect 2000 data system. Spectra were run at 35 °C. ^{13}C-NMR spectra were recorded on a WM 400 Spectrometer, Fa. Bruker.

Bioassay

The test for inhibitory activity was performed as previously described [2] with seeds of *Amaranthus caudatus*. Seeds (from Bayerische Futtersaatbau GmbH, Ismaning, FRG) were germinated at 27 ± 1 °C. Percentage of germination and radicle length were determined after 48 h.

Synthesis of reference compounds

Dimethyl-6-methyl-2H-pyran-2-one-3.5-dicarboxylate (III) was prepared by condensation of methyl methoxymethylenecyanoacetate and methylacetoacetate according to Simonsen [4]. The methylmethoxymethylenecyanoacetate required was obtained by the method described by Bollemont [5].

m.p.: 96 °C.
MS: 226 (60, M^+), 211 (18, M^+-CH_3), 198 (38, M^+-CO), 195 (47, $M-OCH_3$), 194 (48, M^+-HOCH_3), 183 (22), 168 (18), 167 (38, $M^+-COOCH_3$); 166 (26, $M^+-HOOCCH_3$); 153 (24); 143 (18), 124 (12)
^{1}H-NMR: δ = 2.72 ppm (3 H); 3.92 (6 H), 7.25 (1 H)

3-Methoxycarbonyl-5-methoxycarbonylmethyl-6-methyl-2 H-pyran-2-one (IV) was a gift from Fa. BASF, Ludwigshafen.

MS: *m/z* 240 (M^+, 23); 212 (M^+-CO, 2); 209 (M^+-OCH_3, 25); 208 (M^+-HOCH_3, 51); 198 (36); 181 ($M^+-COOCH_3$, 40); 166 (61); 153 (27); 149 (25); 139 (19); 121 (30); 79 (13); 59 (18); 43 (100).

^{1}H-NMR: δ = 2.6 ppm (3 H); 3.45 (2 H); 3.7 (3 H); 3.85 (3 H); 7.7 ppm (1 H).

Methyl-4.6-dimethyl-2H-pyran-2-one-5-carboxylate (isodehydracetic acid methylester) (**V**) was obtained from EGA.

MS: *m/z* 182 (M^+, 58); 167 (M^+-CH_3, 7); 154 (M^+-CO, 74); 151 (M^+-OCH_3, 37); 139 (42); 123 ($M^+-COOCH_3$, 37); 122 ($M^+-HOOCCH_3$, 54); 109 (21); 97 (10); 94 (12); 53 (19); 43 (100).

Results and Discussion

Identification of compound I

Both compounds present in the fractions with biological activity proved to be closely related α-pyrone derivatives. At first compound **I** was identified as dimethyl-3-methyl-2-H-pyran-2-one-4.6-dicarboxylate.

The molecular weight of **I** is 226, its molecular formular $C_{10}H_{10}O_6$ was determined by high resolution mass spectrometry.

Major fragment ions were observed at *m/z* 194 (M^+-CH_3OH), *m/z* 167 (M^+-CH_3COO; basepeak) and *m/z* 139 (*m/z* 167 − CO). Besides these fragmentations for carboxylic acid esters the primary loss of *m/z* 28 (CO) from the molecular ion indicated the presence of a lactone structure. The expulsion of carbon monoxide from a given molecular ion yielding a furan derivative is typical for α-pyrones [6]. The loss of CO was found with all reference compounds (see Material and Methods).

The ^{1}H-NMR and ^{13}C-NMR data of I are given in Table I.

The ^{1}H-NMR spectrum of **I** presented one singulet (6 H) at δ = 4.0 ppm for two methylester groups and one singulet at δ = 2.5 ppm characteristic of a methyl group in α-position to a carboxylic group. The signal at δ = 7.45 ppm belongs to one isolated proton attached to a double bond. The ^{13}NMR spectrum of **I** was more informative. The signal at δ = 161.5 ppm for the carbon in position 2 established the 2 H-pyran-2-one structure. In the coupled spectrum this signal gave a quartet with the long range C,H-coupling of 4 Hz due to the coupling with the methylenic group that therefore must be located at position 3. The C 5 showed a doublet centred at δ = 109.8 ppm with the ^{1}J C,H coupling constant of 178 Hz, due to the coupling with the isolated proton at position 5.

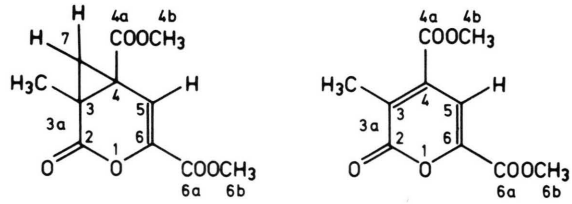
Table I. ^{1}H-NMR and ^{13}C-NMR data for compound **I** from RC 150[a].

Position	Chemical shift ^{1}H	Chemical shift ^{13}C
2		161.5 s
3		137.8 q (^2J = 4)
3a	2.5 s (3H)	15 q (^1J = 130)
4		134.7 s
4a		159.5 s
4b	4.0 s (3H)	53.2 q (^1J = 148)
5	7.45 s (1H)	109.8 d (^1J = 178)
6		145.5 s
6a		164.4 s
6b	4.0 s (3H)	53.4 q (^1J = 148)

[a] All spectra were determined in $CDCl_3$; ^{1}H-NMR at 80 MHz, ^{13}C-NMR spectra at 400 MHz. Chemical shifts are given as δ-values (TMS reference). Figures in parentheses refere to coupling constants in Hz.

The two carboxylic acid ester groups must be positioned at the remaining positions 4 and 6; the signal of the second group is downfield-shifted because of the neighbouring oxygen carbon atom.

Identification of the inhibitor II



I II

Mass spectrometric data and the ^{1}H and ^{13}C NMR data of the inhibitor II indicated close structural resemblence with the 2-H-pyran-2-one derivate I. The mass spectrum of the inhibitor as well as the spectrum of the deuteriumlabeled compound is presented in Fig. 1.

The high resolution data confirmed that the molecular ion *m/z* 240 corresponds to $C_{11}H_{12}O_6$; this suggests that **II** contains the elements of **I** plus an additional methylenic group. The mass spectrum is also characterized by the primary expulsion of carbon monoxide.

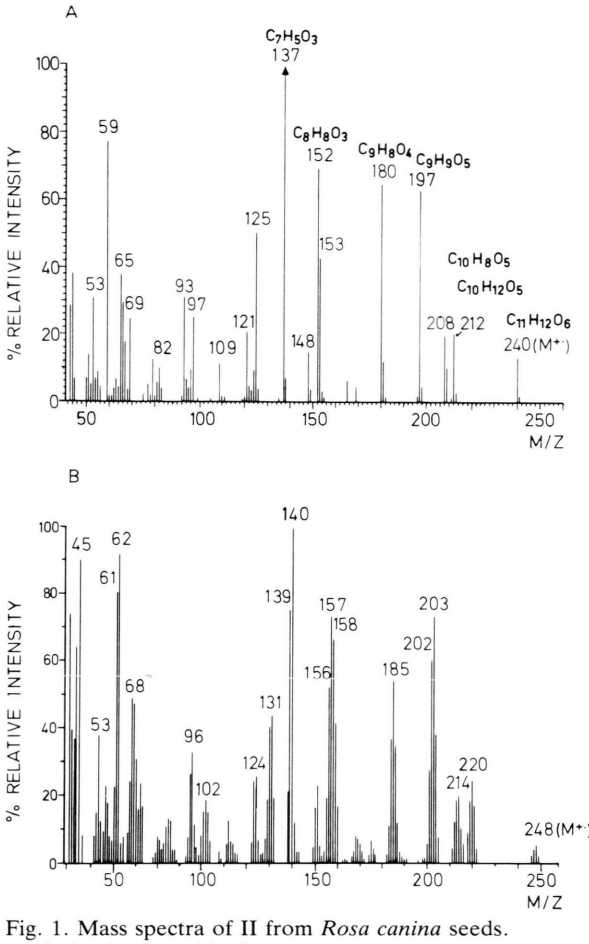
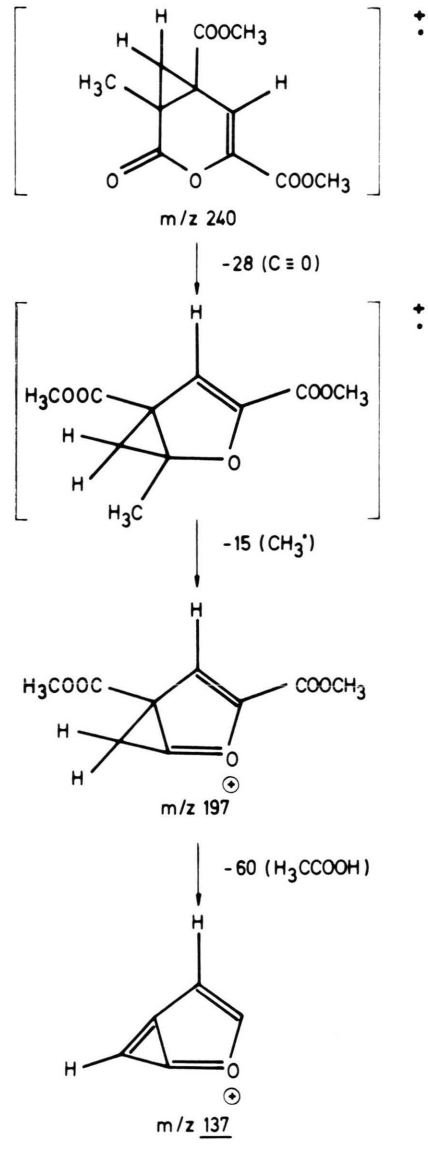


Fig. 1. Mass spectra of II from *Rosa canina* seeds.
A. derivatisation with diazomethane.
B. derivatisation with deuterium-diazomethane.
The molecular formulae of the main ions obtained by high resolution mass spectrometry are indicated.



Further loss of *m/z* 15 (CH_3) followed by the elimination of $HOOCCH_3$ yields the base peak *m/z* 137. The stability of this fragment must be due to the complete conjugated system after the loss of the two side chains. The ^{1}H-NMR showed two additional doublets (2 H) at $\delta = 1.4$ and 2.3 ppm with a coupling constant of $J_{gem} = 4.8$ Hz; the corresponding signals in the ^{13}C-NMR spectrum appeared at $\delta = 27.5$ ppm. ^{1}H-NMR and ^{13}C-NMR data are given in Table II.

The spectroscopic data point to the presence of a cyclopropane ring in **II** which is absent in **I**. The large difference between the chemical shifts of the cyclopropane protons either could be due to the anisotropic efficiency on the *syn*-located proton exerted by the double bond or to the influence of the lactone ring [9]. Because of the high field shift of the methyl group to $\delta = 1.6$ ppm (^{1}H-NMR) and 13.5 ppm (^{13}C-NMR) respectively, the cyclopropane ring should be attached to the C 3 and C 4 atoms. This is also established by the further splitting of the methylene signals to a quartet with the long range coupling constant of 4 Hz in the decoupled ^{13}C-NMR spectrum. It also explains the high − field shift values of 33 and 34 ppm for the quarternary carbon atoms in 3 and 4 in comparison to those of compound **I.**

Table II. ^{1}H-NMR and ^{13}C-NMR data for the inhibitor **II**[a].

Position	Chemical shift ^{1}H	Chemical shift ^{13}C
2		160.5 s
3		33 s
3a	1.6 s (3H)	13.5 q ($^{1}J = 130$)
4		34 s
4a		166.5 s
4b	3.8 s (3H)	53 q ($^{1}J = 144$)
5	7.0 s (1H)	114.5 d ($^{1}J = 175$)
6		139 d ($^{2}J = 4$)
6a		168 s
6b	3.84 s (3H)	53.5 q ($^{1}J = 144$)
7	1.4 d 1H ($^{1}J = 4.8$) 2.3 d 1H ($^{1}J = 4.8$)	27.5 t ($^{1}J = 144$)

[a] All spectra were determined in $CDCl_3$. ^{1}H-NMR at 80 MHz, ^{13}C-NMR spectra at 400 MHz. Chemical shifts are given as δ-values (TMS reference). Figures in parentheses refere to coupling constants in Hz.

Table III. Effect of various α-pyrones on germination of seeds of *Amaranthus caudatus*. Quantitation of germination was achieved by determination of germination percentage and measurement of radicle length after 48 h germination [2], water controls beeing 100%. Concentrations were determined by gas chromatography with **III** and **IV** as reference compounds.

Pyrone Compound	Concentration [M]	Inhibition of germination [%]
I	1.0×10^{-4}	35
II	1.0×10^{-4}	100
	7.5×10^{-5}	75
	4.5×10^{-5}	50
	2.0×10^{-5}	55
III	1.0×10^{-4}	0–20*
IV	1.0×10^{-4}	0–20*
V	1.0×10^{-4}	0–20*

* The value 20% inhibition is not significantly different from the value of the water controls.

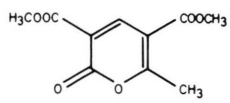
The mass spectrum of the deuterium labeled-compound **II** (see Fig. 1) showed an increase of the molecular weight of eight mass units and not, as expected for two carboxylic acid groups, of 6 mass units. Since diazomethane was used for the methylation of the carboxylic acids the possibility arose that cyclopropanation occurred by the reaction of the methylating reagent with the 2-H-pyran-2-one derivative **I**. It could be shown, that treatment of **I** with diazomethane followed by destillation at 150 °C under reduced pressure in the presence of catalytic amounts of copper yielded nearly quantitatively compound **II**. Cyclopropanation occurred with regioselectivity, the 5,6 double bond was not attacked. This result finally proved the structure of **II.**

The success of the cyclopropanation is in disagreement with earlier investigations on the reaction of diazomethane with pyranone derivatives. Treatment of 2-H-pyran-2-one-5-carboxylic acid chloride, even more reactive than the corresponding ester, with diazomethane only resulted in methylation in the 6-position [7]. According to [7], the presence of an electronegative substituent in α-position should be a necessary condition for the mentioned reaction. But the reference α-pyrones **III–IV** containing two carboxylic acid ester groups as well (see Materials and Methods) didn't undergo cyclopropanation even under stronger conditions, *e.g.* adding diazomethane at room temperature and heating the compound to 200 °C.

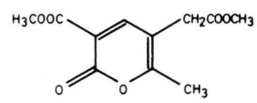
Biological activity

The germination inhibitory activity of **I** in the *Amaranthus* seed germination test is presented in Table III.

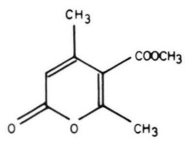
II affects the germination rate of *Amaranthus caudatus* seeds as well as the root length in a concentration range from 10^{-5} to 10^{-4} M (2.4 mg/l to 24 mg/l). Concentrations above 1.0×10^{-4} M inhibit seed germination completely. The germination inhibiting effect of **II** is reversible, that means not toxic to the



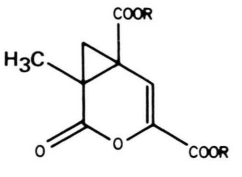
III



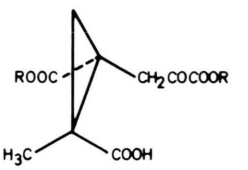
IV



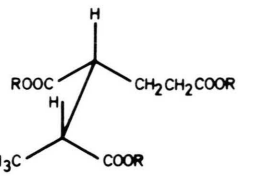
V



II



VI



VII

seeds. After transfer from the test solution to bidestilled water, germination started within 12–20 h.

The reference compounds **III, IV** and **V** did not show any activity as germination inhibitors. Compound **I** only showed a weak activity (concentration 1×10^{-4} M), but there are indications of a synergistic effect of **II** in the presence of the inhibitor **I**. From α-methylene-γ lactones with plant growth activity it is known, that the activity is enhanced a great deal if the methylenic double bond is replaced by a cyclopropane ring [8]. In case of the compounds discussed here, the increased inhibitory activity of **II** must be due to the cyclopropanation of the 2-H-pyran-2-one derivate **I**.

While considering possible structural requirements for inhibitory activity, we noticed an interesting relationship of **II** and threo dihydrohematinic acid (**VII**) which had been detected as germination inhibitor in oat [3]. Opening of the lactone ring of **II** leads to the tricaboxyl acid derivative **VI** which has a similar spatial structure as a certain conformation of **VII**. This conformation is certainly unfavored because of partial steric hindrance; it could however exist at a binding site of an enzyme or a receptor. The question whether the closed form of **II** or rather the open form **VI** acts as the proper germination inhibitor has still to be investigated.

Acknowledgements

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We thank the BASF AG, Ludwigshafen, for a gift of reference compounds.

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