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UPTAKE AND TRANSLOCATION OF EXOGENOUSLY APPLIED 7-OXYSTEROLS IN BARLEY†

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Key Word Index—*Hordeum vulgare*; Poaceae; barley; exogenous application; resistance inducers; sterols; 7-hydroxy- and 7-oxosterols.

Abstract—7β-Hydroxy- and 7-oxocholesterol as well as [3α,7α-$^2H_2$]-7β-hydroxysitosterol and [3α-^{2}H]-7-oxositosterol were sprayed on the primary leaves of 7-day-old barley plants. A higher uptake of the 7-oxosterols compared to the 7β-hydroxysterols and a higher uptake of the cholesterol compared to the sitosterol derivatives was observed. The results also indicated that the uptake of [3α,7α-$^2H_2$]-7β-hydroxysitosterol was dependent on the concentration whereas the uptake of [3α-^{2}H]-7-oxositosterol was rather independent of the concentration on the leaf surface. No significant differences of the concentrations of [3α,7α-$^2H_2$]-7β-hydroxysitosterol and [3α-^{2}H]-7-oxositosterol were detected in the leaves when the plants were harvested 3 days or 4 weeks after the treatment. The sprayed 7-oxysterols were never detected in the roots. [3α,7α-$^2H_2$]-7β-hydroxysitosterol and [3α-^{2}H]-7-oxositosterol were also applied to the roots of 11-day-old plants. The data indicated the higher uptake of [3α-^{2}H]-7-oxositosterol compared to [3α,7α-$^2H_2$]-7β-hydroxysitosterol and the transport of both into the leaves.

INTRODUCTION

7α-Hydroxy- (**10**), 7β-hydroxy- (**13**) and 7-oxositosterol (**16**) as well as the corresponding cholesterol derivatives induced resistance toward the fungal pathogen *Puccinia hordei* Otth in barley (*Hordeum vulgare* L. cv "Mammut") [1]. Compounds **10**, **13** and **16** also exhibit resistance inducing activity in the host–parasite system *Hordeum vulgare* L. cv "Abed Binder"/*Puccinia striiformis* West. (Seifert, K. *et al*., unpublished results). When spraying the primary leaves with solutions of these compounds two days before the challenge inoculation with the fungal pathogens a different reduction in the infection sites has been reported [1]. The 7-hydroxyderivatives of sitosterol and cholesterol exhibit a much higher resistance inducing activity than the corresponding 7-oxoderivatives. The sitosterol derivatives 7α-hydroxy- (**10**), and 7-oxositosterol (**16**) show approximately the same activity as the analogous compounds of cholesterol. 7β-Hydroxysitosterol (**13**) shows a higher activity than 7β-hydroxycholesterol (**19**).

At present it is not clear whether the applied 7-oxysterols remain on the leaf surface of the plants or can penetrate into the leaves. If the uptake can be observed the following questions arise: (1) Is the higher protection against the pathogens caused by the 7-hydroxyderivatives of sitosterol and cholesterol compared to the corresponding 7-oxoderivatives dependent on the higher uptake? (2) Is the different resistance inducing activity of 7β-hydroxysitosterol (**13**) and 7β-hydroxycholesterol (**19**) in accordance with their uptake? (3) Is the uptake of the sterol derivatives dependent on their concentration on the leaf surface? (4) Are the barley leaves able to metabolize the penetrated resistance inducers and if they can, might the resistance inducers be transported in the roots?

In this paper we report *in vivo* studies on the uptake and translocation of exogenously applied 7β-hydroxy- and 7-oxoderivatives of sitosterol and cholesterol in the barley cultivar "Mammut" with the aim of clarifying the above questions. The endogenous sterols and 7-oxysterols of untreated plants were also analysed. All data were determined by means of GC and GC-MS experiments.

RESULTS AND DISCUSSION

Endogenous sterols, 7-hydroxy- and 7-oxosterols

GC-MS analysis of 13-day-old plants of *Hordeum vulgare* L. cv "Mammut" revealed the presence of nine

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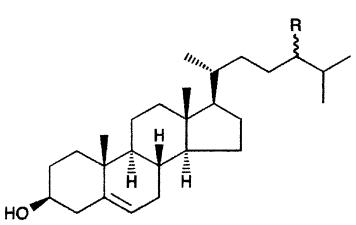
† Part 1 in the series "7-Oxysterol Induced Resistance toward Rusts in Barley".



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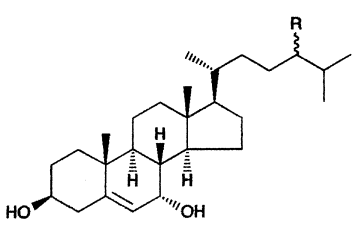

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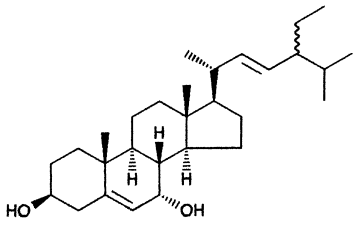

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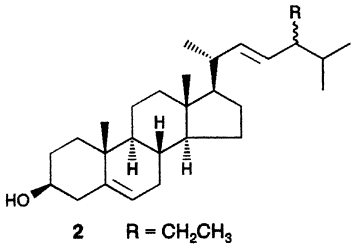

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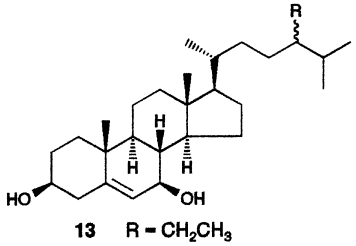

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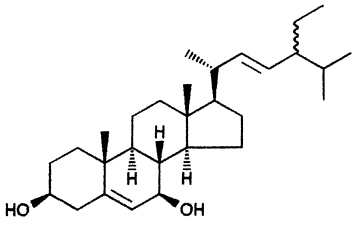

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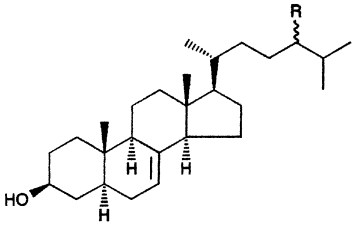

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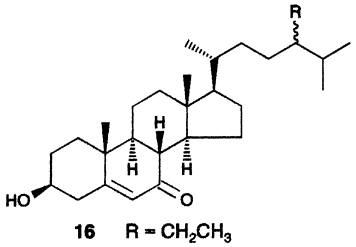

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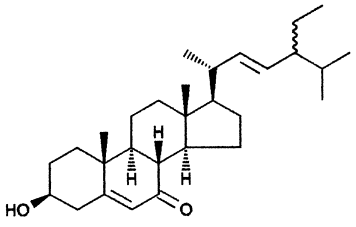

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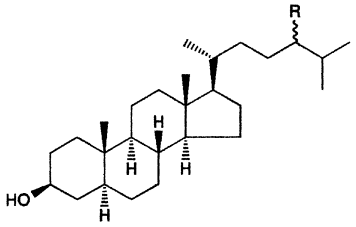

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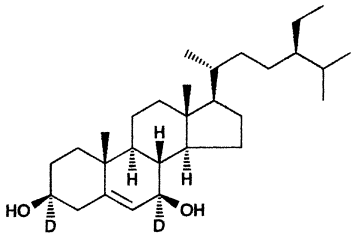

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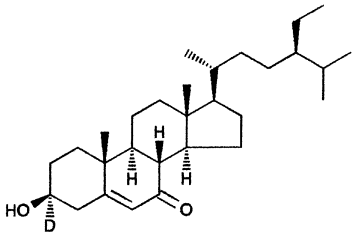

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sterols and nine 7-oxysterols. The sterols **1**–**9** were identified in both leaves and roots (Table 1). The 7-oxysterols **10**–**18** were only found in the roots (Table 2). No traces of the 7-oxygenated sterols were detected in the leaves.

In higher plants the 24-ethyl sterols consist predominantly of the 24α-epimer, whereas the 24-methyl sterols are often a mixture of the 24α- and the 24β-epimer [2–8]. In our laboratory the epimeric 24-alkyl sterols could not be separated by GC [9]. Therefore, the 24α-configuration was presumed for the 24-ethyl sterols **1**–**4** and their 7-oxygenated derivatives **10**, **11**, **13**, **14**, **16** and **17**, whereas the 24-methyl sterols **5**–**8** and the 7-oxygenated 24ξ-methylcholesterol derivatives **12**, **15** and **18** may be an epimeric mixture.

To our knowledge 22-dihydrospinasterol (**3**), sitostanol (**4**), 24ξ-methyl-22-dehydrocholesterol (**6**), 24ξ-methyllathosterol (**7**) and 24ξ-methylcholestanol (**8**) were detected for the first time in barley. Sitosterol (**1**), stigmasterol (**2**), 24ξ-methylcholesterol (**5**) and cholesterol (**9**) have been frequently described as constituents of barley [10–18]. Although the order of relative abundance of **1**, **2** and **5** in both tissues was in agreement with published data [10–13, 15, 16, 18–21], the percentages of **1**, **2** and **5** were found to vary from some previously published values in the leaves [10–12, 16, 19, 20] and roots [13, 18, 20]. However, this may be at least partially explained in terms of the use of different cultivars, tissues of different ages, and different growth conditions.

Table 1. Sterol composition (%) in *Hordeum vulgare* L. cv "Mammut". The sterols were detected as their TMSi ethers. Values are the average of three aliquots ±S.E.

Sterols	R_I	% Leaves	Roots
Sitosterol (**1**)	3307	45.6±0.68	44.0±1.02
Stigmasterol (**2**)	3251	23.8±1.63	22.4±1.49
22-Dihydrospinasterol (**3**)	3361	7.53±0.19	1.24±0.09
Sitostanol (**4**)	3319	2.05±0.14	5.11±0.11
24ξ-Methylcholesterol (**5**)	3215	17.3±1.14	25.9±0.35
24ξ-Methyl-22-dehydrocholesterol (**6**)	3156	0.89±0.04	0.28±0.01
24ξ-Methyllathosterol (**7**)	3267	1.18±0.03	traces
24ξ-Methylcholestanol (**8**)	3225	0.57±0.05	0.59±0.05
Cholesterol (**9**)	3109	1.08±0.05	0.48±0.02

Table 2. 7-Hydroxy- and 7-oxosterol composition (%) of the roots in *Hordeum vulgare* L. cv "Mammut". The 7α-hydroxy- (**10**–**12**), 7β-hydroxy- (**13**–**15**) and 7-oxosterols (**16**–**18**) amount to 100%, respectively. The 7-oxysterols were detected as their TMSi ethers. Values are the average of three aliquots ±S.E.

7-Oxysterols	R_I	%
7α-Hydroxysitosterol (**10**)	3289	55.3±0.52
7α-Hydroxystigmasterol (**11**)	3229	23.8±0.72
7α-Hydroxy-24ξ-methylcholesterol (**12**)	3205	20.9±0.19
7β-Hydroxysitosterol (**13**)	3420	54.5±0.67
7β-Hydroxystigmasterol (**14**)	3347	27.0±0.61
7β-Hydroxy-24ξ-methylcholesterol (**15**)	3334	18.5±0.19
7-Oxositosterol (**16**)	3579	49.5±1.82
7-Oxostigmasterol (**17**)	3522	22.0±0.97
7-Oxo-24ξ-methylcholesterol (**18**)	3478	28.5±1.21

7α-Hydroxy- (**11**), 7β-hydroxy- (**14**) and 7-oxostigmasterol (**17**) were not detected in barley up to now. The 7-oxygenated derivatives of sitosterol and 24ξ-methylcholesterol have been previously isolated from the roots of the barley cultivars "Bigo" and "Xenia" [1, 22]. The 7-oxysterols **10**–**18** were also identified in the roots of wheat (*Triticum aestivum* L. cv "Planet"), rye (*Secale cereale* L. cv "Danae") and the barley cultivar "Gunda" (König, M., unpublished results). They have been also found in the seeds of the cultural crop *Ornithopus sativus* [23] and in the roots of the Chinese drug *Euphobia fischeriana* [24]. The origins of 7-oxygenated sterols are still not clear. Either a non-selective autoxidation [25, 26] or a non-selective biological oxidation have been considered [24]. A selective biological oxidation has been also discussed [27].

In the case of barley roots the enzymatic oxidation of sterols to the 7-oxygenated sterols **10**–**18** by peroxidase is probable. The first formed 7-hydroperoxysterols were transformed into 7α-hydroxy-, 7β-hydroxy- and 7-oxosterols. Horseradish peroxidase is able to oxidize cholesterol to 7α-hydroxy-, 7β-hydroxy- (**19**) and 7-oxocholesterol (**20**) [28]. Peroxidase was detected in the barley roots [29]. A non-selective autoxidation is improbable due the fact that 7-oxygenated sterols were also detected after anaerobic work up of the barley roots. If autoxidation works during the isolation procedure, the detection of 7-oxysterols in the barley leaves would be expected. This was not the case.

Leaf application of **19**–**22**

To clarify whether the uptake of the resistance inducers is dependent on their functional group at position C-7, the leaves were sprayed with a solution of 7β-hydroxy- (**19**) and 7-oxocholesterol (**20**) or [3α,7α-$^2H_2$]-7β-hydroxysitosterol (**21**) and [3α-^{2}H]-7-oxositosterol (**22**). To determine the uptake of the cholesterol compared to the corresponding sitosterol derivatives, the leaves were sprayed with a solution of the 7β-hydroxysterols **19** and **21** or the 7-oxosterols **20** and **22**. The applied compounds **19**–**22** were clearly identified in the unwashed and washed leaves by GC-MS. The uptake of compounds **19**–**22** was determined by the ratio of the concentrations in the washed leaves to the concentrations in the unwashed leaves (Table 3). The values indicated that 46–72% of the applied

Table 3. Concentration (μg/g fr. wt) and uptake (%) of compounds **19**–**22** in the leaves of *Hordeum vulgare* L. cv "Mammut" after exogenous application (1 container). The 7-oxosterols were detected as their TMSi ethers. Values are the average of three independent experiments $\pm$s.e.

		μg/g fr. wt		
7-Oxysterols	R_I	Washed	Unwashed	%
7β-Hydroxycholesterol (**19**)	3243	4.22 $\pm$ 0.46	11.2 $\pm$ 0.76	38
7-Oxocholesterol (**20**)	3380	6.20 $\pm$ 0.71	11.5 $\pm$ 0.77	54
[3α,7α-$^2H_2$]-7β-Hydroxysitosterol (**21**)	3420	3.24 $\pm$ 0.36	11.4 $\pm$ 0.72	28
[3α-^{2}H]-7-Oxositosterol (**22**)	3679	4.79 $\pm$ 0.57	11.6 $\pm$ 0.78	41

7-oxysterols could not penetrate into the leaves and were washed from the leaf surface. If the plants were watered during the application period compounds **19**–**22** could not be detected in the leaves. Thus, they need an appointed time interval to penetrate into the leaves.

Comparing the sitosterol with the cholesterol derivatives the ca 1.3-fold higher uptake of 7β-hydroxycholesterol (**19**) compared to [3α,7α-$^2H_2$]-7β-hydroxysitosterol (**21**) and the ca 1.4-fold higher uptake of 7-oxocholesterol (**20**) compared to [3α-^{2}H]-7-oxositosterol (**22**) was observed. The values also indicated the ca 1.4-fold higher uptake of the 7-oxosterols **20** and **22** compared to the 7β-hydroxysterols **19** and **21**, respectively. The data on the uptake of compounds **19**–**22** do not agree with the previously described resistance inducing activity of the corresponding compounds [1]. From this lack of agreement, a direct relationship between the uptake and the resistance inducing activity of the 7β-hydroxy- and 7-oxosterols can be excluded.

To analyse whether the uptake of the resistance inducers is dependent on their concentration on the leaf surface the barley plants from six containers, instead of one container, were sprayed with the same concentrated solution of [3α,7α-$^2H_2$]-7β-hydroxysitosterol (**21**) and [3α-^{2}H]-7-oxositosterol (**22**) (Table 4). Since the leaf area was 6-fold greater compared to the application to one container, significant lower concentrations of **21** and **22** were detected for the unwashed leaves. The data obtained by spraying plants in one container compared to six containers (Tables 3 and 4) permitted the determination of whether the uptake depends on the concentration on the leaf surface. The one container: six containers ratio of the uptake was 1.5 for **21** but 1.1 for **22**. Thus, only the uptake of [3α,7α-$^2H_2$]-7β-hydroxysitosterol (**21**) was significantly higher in the application to one container and, therefore, was dependent on the concentration on the leaf surface. The uptake of [3α,-^{2}H]-7-oxositosterol (**22**) was rather independent of the concentration on the leaf surface.

To determine the fate of compounds **21** and **22** which penetrated into the leaves, the plants from two of the six containers were not harvested at three days after the application period (normal test); instead the plants were grown for an additional four weeks in the greenhouse and watered normally until harvesting (long-term test). The values indicated that the uptake in the long-term test was almost identical with the uptake in the normal test (Table 4). In addition to this, when [3α,7α-$^2H_2$]-7β-hydroxysitosterol (**21**) was applied alone its oxidized derivative [3α-^{2}H]-7-oxositosterol (**22**) could be detected neither in the normal test nor in the long-term test in the leaves. It must be also pointed out that in all tests described above the compounds **19**–**22** could never be detected in the roots

Table 4. Concentration (μg/g fr. wt) and uptake (%) of compounds **21**–**22** in the leaves of *Hordeum vulgare* L. cv "Mammut" after exogenous application (6 containers). The 7-oxosterols were detected as their TMSi ethers. Values are the average of three independent experiments $\pm$s.e.

	Normal test			Long-term test	
	μg/g fr. wt			μg/g fr. wt	
7-Oxysterols	Washed	Unwashed	%	Washed	%
[3α,7α-$^2H_2$]-7β-Hydroxysitosterol (**21**)	0.39 $\pm$ 0.03	2.05 $\pm$ 0.18	19	0.36 $\pm$ 0.04	18
[3α-^{2}H]-7-Oxositosterol (**22**)	0.76 $\pm$ 0.06	2.08 $\pm$ 0.20	36	0.71 $\pm$ 0.06	34

of the treated plants. Together, these results may indicate the absence of any metabolization of the penetrated 7-oxysterols in the leaves.

Root application of compounds **21** *and* **22**

In the root application [3α,7α-$^2H_2$]-7β-hydroxysitosterol (**21**) and [3α-^{2}H]-7-oxositosterol (**22**) were clearly identified in the washed roots of the treated plants (Table 5). The ratio of the total content in the washed roots (data not shown) to the content in the application solution indicated the uptake of 3.5% for **21** and 5.3% for **22**. Since the ratio of **22** to **21** was 1 in the application solution but 1.5 in the washed roots, the uptake of [3α-^{2}H]-7-oxositostrol (**22**) was 1.5-fold higher compared with [3α,7α-$^2H_2$]-7β-hydroxysitosterol (**21**). Thus, compound **22** could penetrate into the root better than compound **21**.

When compounds **21** and **22** were applied to the roots, they were also detected together with their corresponding unlabelled compounds 7β-hydroxysitosterol (**13**) and 7-oxositosterol (**16**) in the leaves of the treated plants by GC-MS (Table 5). From the common occurrence of the labelled and unlabelled compounds in the leaves a deuterium-hydrogen exchange at the activated hydroxylated carbon atoms during the transport from the roots might be assumed. The leaf to root ratio of the concentration indicated that in the root application 1.2% of [3α,7α-$^2H_2$]-7β-hydroxysitosterol (**21**) and 1.6% of [3α-^{2}H]-7-oxositosterol (**22**) were found in the leaves.

The different fate of the exogenously applied compounds in the roots and leaves may be explained by the fact that the endogenous 7-oxygenated sterols have been found in the roots but never in the leaves of barley. Since in the leaves the biological routes to produce endogenous 7-oxysterols are absent, the barley leaves may be unable to metabolize the absorbed 7β-hydroxy- and 7-oxosterols. If the 7-oxygenated sterols are only biosynthesized in the roots, the biological pathways may be present to metabolize the absorbed 7-oxysterols [3α,7α-$^2H_2$]-7β-hydroxysitosterol (**21**) and [3α-^{2}H]-7-oxositosterol (**22**). At present it is not clear why in the root application 7β-hydroxysitosterol (**13**) and 7-oxositosterol (**16**) were also detected in the leaves. It will be important to clarify whether compounds **13** and **16** are endogenous root oxysterols or artifacts of the deuterium-hydrogen exchange.

Table 5. Concentration (μg/g fr. wt) of compounds **21**–**22** in *Hordeum vulgare* L. cv "Mammut" after exogenous root application. The 7-oxosterols were detected as their TMSi ethers. Values are the average of three independent experiments ±s.e.

	μg/g fr. wt	
7-Oxysterols	Roots	Leaves
[3α,7α-$^2H_2$]-7β-Hydroxysitosterol (**21**)	27.4±4.43	0.33±0.04*
[3α-^{2}H]-7-Oxositosterol (**22**)	42.0±6.77	0.67±0.08*

* Detected together with its unlabelled compound.

EXPERIMENTAL

Plant cultivation, treatment and harvest

Barley plants (*Hordeum vulgare* L. cv "Mammut") were grown in a greenhouse with an 8 h photoperiod of 10,000 lx at 21° (18° during the night) and 50–70% relative humidity. Plants assigned for leaf application or analysis of the sterols, 7-hydroxy- and 7-oxosterols were cultivated by sowing seeds in moist sand in plastic containers (39 × 29 × 6 cm) with holes in the bottom. For each container 40 g grains were evenly distributed over the surface and then covered with a thin layer of sand. The containers were placed in plastic bins and the plants were watered if necessary. Plants assigned for root application were cultivated by germination of barley seeds on moist paper tissue for 4 days. The paper tissue was removed and the seedlings were placed for 7 days on a nutrient soln according to Ref. [30].

For leaf application the containers with 7-day-old plants were sprayed 3 × a day at 8.00 a.m., 12.00 a.m. and 4.00 p.m. for 3 days with 1% aq. ethanolic soln of **19**–**22** (40 ppm per compound, 10 ml at a time). The sprayer was held at a distance of 10 cm from the outer plants and at the height of the tops of the leaves. During the application period, and for an additional 3 days, the plants were not watered. Instead, H_2O was placed in the plastic bins. For normal tests the plants were harvested afterwards. Plants assigned for long-term tests were watered normally for an additional 4 weeks and then harvested. For root application 11-day-old plants were removed from the nutrient soln and selected for their uniformity. The roots of about 80 plants were rinsed with H_2O and immersed for 48 h in 50 ml of 1% aq. ethanolic soln of **21** and **22** (100 ppm per compound). The plants were harvested immediately after the application period. Three independent experiments were carried out for each application.

The plants grown in the containers were harvested by cutting off the leaves just above the surface of the sand. The sand was removed from the roots by rinsing them with H_2O and the remaining parts of the leaves were separated. The leaves of the treated plants were divided into 2 groups. One group of the leaves remained unwashed, the other group and the roots were washed. From root treated plants the application soln was removed, the roots were rinsed with H_2O and then separated from the leaves. Roots and leaves of the treated plants were washed with H_2O containing commercial washing-up liquid and then rinsed with H_2O (3 ×). The wet roots and leaves were placed between 2 dry paper tissues until no more moisture

could be observed on the tissues. The leaves were cut in small pieces and homogenized in MeOH. The roots of the sand grown plants were immersed in liquid N_2 and powdered. The roots of the plants grown in the nutrient soln were worked-up as described for the leaves.

Extraction, chromatography and analysis of the sterols, 7-hydroxy and 7-oxosterols

Extraction was performed in MeOH (leaves 1 h, roots 24 h). The MeOH extracts were evaporated to dryness *in vacuo* and the residues were partitioned between H_2O and $CHCl_3$ (3×). The $CHCl_3$ phases were dried with Na_2SO_4. After filtration to remove the Na_2SO_4 the $CHCl_3$ phases were evaporated to dryness *in vacuo*. At least 3 aliquots (each 20–40 mg) were taken from the $CHCl_3$ phases for chromatography and analysis.

The sterols were separated from the 7-hydroxy- and 7-oxosterols by prep. TLC (silica gel, 1 mm) with cyclohexane–EtOAc (1:3). Authentic standards were run for the location of the bands. The spots were visualized by spraying with "Rosenheim reagent" (2.0 mg $SbCl_3$ in 100 ml of $CHCl_3$–HOAc, 3:1) or with a 1% soln of vanillin in 50% H_3PO_4 followed by heating. Bands containing the sterols or 7-hydroxy- and 7-oxosterols were scraped off and eluted with 20 ml of $CHCl_3$ (5×). The combined eluates were evaporated to dryness *in vacuo*. The samples were trimethylsilylated with *N*-methyl-*N*-trimethylsilyltrifluoroacetimide (10–50 μl, 12 h, room temp.) and subjected to FID-GC and GC-MS analysis (injection volume 0.4–1.2 μl).

The sterols, 7-hydroxy- and 7-oxosterols were analysed as their TMSi ethers on a DB-l fused silica capillary column (30 m × 0.3 mm, 0.1 μm film thickness, WCOT), temp. programmed from 80° (3 min) to 280° (15 min) at 3° min^{-1}. The operating conditions were: carrier gas He (FID-GC) and H_2 (GC-MS) at 2 ml min^{-1}, split ratio 1:20 (FID-GC), injector temp. 270°, detector temp. 290°.

The composition of the endogenous sterols, 7-hydroxy- and 7-oxosterols as well as the content of the exogenously applied 7β-hydroxy- and 7-oxosterols was determined according to their peak area by FID-GC (3 runs for each sample). For quantification of the exogenously applied 7β-hydroxy- (**19**, **21**) and 7-oxosterols (**20**, **22**) samples with known quantities of 7β-hydroxysitosterol (**13**) and 7-oxositosterol (**16**) were measured by FID-GC. The resulting peak areas were statistically analysed by the method of linear regression to give a linear calibration curve. Comparison of the peak areas determined by the calibration curve with the peak areas of the samples from the plant extracts yielded the absolute quantities of the applied compounds. By this method the different response of the FID to the two 7-oxygenated sterols was excluded. Compound identifications by GC-MS were based on R_I and MS (70 eV, EI-mode), in both cases by comparison with authentic samples and literature data. Compounds **19**–**22** were synthesized in our laboratory [1, 31].

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