Plant Disease Resistance Inducing Activity of 7-Oxo- and 7-Hydroxysterols

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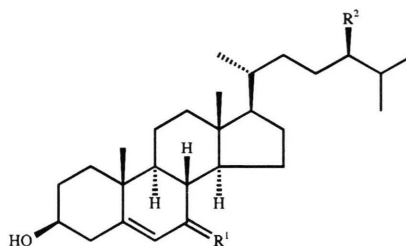*Dedicated to Professor Manfred Hesse on the occasion of his 60th birthday*

Hordeum vulgare, Triticum aestivum, Poaceae, 7-Oxo and 7-Hydroxysterols,
Induced Resistance

The 7-oxosterols **1-2** and the 7-hydroxysterols **3-6** induce resistance toward the fungal pathogens *Puccinia striiformis* West. and *Puccinia hordei* Otth in barley and wheat. Primary leaves of the plants were sprayed with solutions of the compounds (10^{-4} mol/l in 1% aqu. ethanol) followed, 2 days later, by challenge inoculation with the fungal pathogens. The results indicate that 7α- and 7β-hydroxylated epimers of β-sitosterol and cholesterol show the highest value of induced resistance (39–49% reduction of infection sites). No enhanced resistance toward the fungi *Erysiphe graminis* DC f. sp. tritici and hordei and *Cochliobolus sativus* Ito & Kuribayashi was observed.

Introduction

The enhancement of resistance of otherwise susceptible host plants by prior infections or by treating them with chemicals without alterations in the genome is termed induced resistance (Schönbeck *et al.*, 1993). Since Chester (1933) published the first review on this phenomenon numerous publications with different host-parasite systems have proven the efficiency of this type of protection against diseases caused by viruses, bacteria and fungi (Horsfall and Cowling, 1980; Kuc, 1987; Hammerschmidt, 1993). Induced resistance is distinguished from conventional chemical as well as biological procedures in plant protection by lack of toxicity of the inducing agents towards the pathogens, but depends on the stimulation of latent defence mechanisms of the plants. Although a number of resistance-inducing chemicals like salicylic acid (Mills and Wood, 1984), probeconazole (Sekizawa and Mase, 1981), isonicotinic acid (Kunz *et al.*, 1988), fatty acids (Cohen *et al.*, 1991) or metabolites of plants (Doubrava, 1988; Herger *et al.*, 1988) and microorganisms (Schönbeck *et al.*, 1981) are known, induced resistance is hardly used for the control of plant diseases until now (Sequeira, 1983; Steiner and Schönbeck, 1993)). Progress toward implementing this strategy has been limited by the availability of data concerning the efficiency and stability of induced resistance under natural infection pressure. Especially a broad spectrum of chemical compounds is missing which show activity in major crop plants like

1	$R^1 = O$	$R^2 = C_2H_5$
2	$R^1 = O$	$R^2 = H$
3	$R^1 = β\text{-OH}, α\text{-H}$	$R^2 = C_2H_5$
4	$R^1 = β\text{-OH}, α\text{-H}$	$R^2 = H$
5	$R^1 = α\text{-OH}, β\text{-H}$	$R^2 = C_2H_5$
6	$R^1 = α\text{-OH}, β\text{-H}$	$R^2 = H$

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monocots, induce resistance toward important pathogens as rust fungi, and can be applied practically. Hofferek (unpublished results) and Reiss (1986) reported on induced resistance to *Puccinia striiformis* in barley (*Hordeum vulgare* L.), after spraying the primary leaves with the extract of roots or leaves from barley. From the root extract Seifert *et al.* (unpublished results) isolated 3 sterol compounds closely related to β-sitosterol, which induce resistance to *Puccinia striiformis*. The preformed bioactive compounds were determined as 3β-hydroxystigmast-5-en-7-one (**1**), stigmast-5-ene-3β,7β-diol (**3**), and stigmast-5-ene-3β,7α-diol (**5**).

In this paper we report on the resistance inducing activity of β-sitosterol and cholesterol derivatives bearing a carbonyl or a hydroxy group in position 7. Induced resistance caused by sterol derivatives, to our knowledge, has not been described so far. The induced resistance toward rust fungi in barley and wheat was evident as impaired success of the fungal pathogens in infecting the plants.

Materials and Methods

Activity tests

Barley plants (*Hordeum vulgare* L.) cv. 'Mammut' and wheat plants (*Triticum aestivum* L.) cv. 'Astron' were grown in commercial compost in a greenhouse controlled at 24 °C/20 °C day /night temperature with 4 h supplementary lightning in the morning and evening to give a photoperiod of at least 16 h per day (light levels at plant height were approximately 200 to 250 $\mu E\ m^{-2}\ s^{-1}$) and 65–80% rel. humidity. Inoculation experiments were carried out with 7-day-old seedlings using field isolates of the fungi *Erysiphe graminis* DC f. sp. *tritici* and *hordei* Em. Marchal, *Puccinia striiformis* West., *P. hordei* Otth and *Cochliobolus sativus* Ito & Kuribayashi.

For fungitoxicity studies agar pieces (1cm²) were coated with 20 μl droplets of the 10^{-4} mol/l water solutions (1% ethanolic) of the sterol derivatives **1–6** before the spores of the biotrophic fungi were applied. Conidia of powdery mildew were incubated for 24 h at 20 °C in the darkness, the uredospores of the rusts at 100% rel. humidity at 15 °C in the darkness on the agar in petri dishes. Spore germination and germ tube elongation of 100 spores per treatment were measured microscopically. The effects on *C. sativus* were studied in agar diffusion tests applying 20 μl droplets of the 10^{-4} mol/l sterol solutions and measuring the mycelium growth compared to 1% ethanol water solution controls after 5 days.

The sterol derivatives **1–6** were tested for their ability to induce disease resistance on intact plants. The compounds were formulated in 1 % aqu. ethanol to give concentrations of 10^{-4} mol/l, 10^{-6} mol/l and 10^{-9} mol/l and sprayed on the lower and upper leaf surfaces of the primary leaves of 7-day-old plants with a hand held sprayer (3–4μl/ cm² leaf). The plants were challenged with the pathogens immediately or two days after the application of the sterol derivatives. With the conidia from 10-day-old colonies of *E. graminis* f. sp. *tritici* or f. sp. *hordei* the wheat and barley plants were inoculated in an inoculation chamber at 20–22 °C. To inoculate the wheat plants with *P. striiformis* and barley plants with *P. hordei* they were sprayed with uredospore suspensions (80.000 spores/ml) of the fungi followed by an incubation period of 24 h in an infection tent in the dark at 15 °C and high humidity. For the inoculation of *C. sativus* conidia suspensions (80.000 spores/ml) were used and the plants were maintained 48 h in the dark at high humidity at 20 °C. Plant disease severity was monitored by counting the infection sites 7 days after inoculation and the resistance inducing activity of the applied sterole derivatives was calculated in % disease reduction compared to control plants. The experiments were repeated three times with 30 plants per treatment. Results given below represent mean values of the experiments. Means were compared employing the Student's t-test. Plants treated with 1% aqu. ethanol served as controls.

Product analysis

General

Mps: uncorr.. IR spectra were recorded on a Bio-Rad FTS-40 spectrometer. The mass spectra were measured on a Varian MAT-312 spectrometer and the ^{1}H - ^{13}C NMR spectra on a Bruker AC-300 spectrometer. TLC on silica gel sheets (cyclohexane-EtOAc (1:1), .0.25 mm, Polygram R SILG/UV$_{254}$). Spots were visualized by UV (254 nm) and spraying with 'Rosenheim reagent'

(20 g SbCl₃ in 100 ml of CHCl₃-HOAc (3:1)) followed by heating at 110 °C. Column chromatography was performed on silica gel 60 (0.063–0.2 mm) with cyclohexane-EtOAc (1:1).

3β-Hydroxystigmast-5-en-7-one (1)

R_f 0.38; m.p. 149–152 °C; IR ν_{max}CHCl₃ cm⁻¹: 3613(OH), 1667(C=O); MS 70 eV m/z (rel. int.): 428 (M⁺, 100), 395 (12); ¹H-NMR (CDCl₃) δ (ppm): 0.65 (3H, s, H-18), 0.78 (3H, d, J = 6.7 Hz, H-26), 0.80 (3H, d, J = 6.7 Hz, H-27), 0.81 (3H, m, H-29), 0.90 (3H, d, J = 6.5 Hz, H-21), 1.16 (3H, s, H-19), 2.20 (1H, dd, J = 10.6 Hz, H-8), 3.65 (1H, m, H-3α), 5.65 (1H, J = 1.5 Hz, H-6); ¹³C NMR: see Table I.

3β-Hydroxycholest-5-en-7-one (2)

R_f 0.36; m.p. 169–170 °C; ref. (Chicoye *et al.*, 1968) m.p.172 °C; IR ν_{max}CHCl₃ cm⁻¹: 3609(OH), 1667(C=O); MS 70 eV m/z (rel. int.): 400 (M⁺, 100), 368 (14); ¹H NMR (CDCl₃) δ (ppm): 0.65 (3H, s, H-18), 0.83 (3H, d, J = 6.6 Hz, H-26), 0.84 (3H, d, J = 6.6 Hz, H-27), 0.89 (3H, d, J = 6.5 Hz, H-21), 1.17 (3H, s, H-19), 2.20 (1H, dd, J = 10.1 Hz, H-8), 3.64 (1H, m, H-3α), 5.66 (1H, d, J = 1.5 Hz, H-6); ¹³C NMR: see Table I.

Stigmast-5-ene-3β,7β-diol (3)

R_f 0.29; m.p. 169–172 °C; IR ν_{max}CHCl₃ cm⁻¹: 3607(OH); MS 70 eV m/z (rel. int): 412 (M⁺-H₂O, 100); ¹H NMR (CDCl₃) δ (ppm): 0.67 (3H, s, H-18), 0.79 (3H, d, J = 6.6 Hz, H-26), 0.81 (3H, d, J = 6.6 Hz, H-27), 0.82 (3H, m, H-29), 0.90 (3H, d, J = 6.4 Hz, H-21), 1.02 (3H, s, H-19), 1.35 (1H, m, H-8), 3.52 (1H, m, H-3α), 3.83 (1H, m, H-7α), 5.26 (1H, d, J = 1.6 Hz, H-6); ¹³C NMR: see Table I.

Cholest-5-ene-3β,7β-diol (4)

R_f 0.28; m.p. 175 °C; ref. (Kumar *et al.*, 1987) m.p. 176–178 °C; IR ν_{max}CHCl₃ cm⁻¹: 3605(OH); MS 70 eV m/z (rel. int.): 384 (M⁺-H₂O, 100); ¹H NMR (CDCl₃) δ (ppm): 0.67 (3H, s, H-18), 0.85 (6H, d, J = 6.6 Hz, H-26,27), 0.99 (3H, d, J = 6.9 Hz, H-21), 1.05 (3H, s, H-19), 1.38 (1H, m, H-8), 3.52 (1H, m, H-3α), 3.82 (1H, m, H-7α), 5.27 (1H, s, H-6); ¹³C NMR: see Table I.

Stigmast-5-ene-3β,7α-diol (5)

R_f 0.22; m.p. 198–200 °C; ref. (Fukuyama *et al.*, 1988) m.p. 202–204 °C; IR ν_{max}CHCl₃ cm⁻¹: 3605(OH); MS 70 eV m/z (rel. int.): 412 (M⁺-H₂O, 100); ¹H NMR (CDCl₃) δ (ppm): 0.67 (3H, s, H-18), 0.79 (3H, d, J = 6.4 Hz, H-26), 0.81 (3H, d, J = 6.5 Hz, H-27), 0.83 (3H, m, H-29), 0.91 (3H, d, J = 6.4 Hz, H-21), 0.97 (3H, s, H-19), 1.46 (1H, m, H-8), 3.56 (1H, m, H-3α), 3.83 (1H, br s, H-7β), 5.58 (1H, d, J = 4.1 Hz, H-6); ¹³C NMR: see Table I.

Cholest-5-ene-3β,7α-diol (6)

Rf 0.22; m.p. 183–184 °C; ref. (Kumar *et al.*, 1987) m.p. 184–186 °C; IR ν_{max}CHCl₃ cm⁻¹: 3607(OH); MS 70 eV m/z (rel. int.): 384 (M⁺-H₂O); ¹H NMR (CDCl₃) δ (ppm): 0.65 (3H, s, H-18), 0.83 (3H, d, J = 6.6 Hz, H-26), 0.84 (3H, d, J = 6.6 Hz, H-27), 0.89 (3H, d, J = 6.5 Hz, H-21), 0.96 (3H, s, H-19), 1.44 (1H, m, H-8), 3.55 (1H, m, H-3α), 3.82 (1H, br s, H-7β), 5.57 (1H, d, J = 5.3 Hz, H-6); ¹³C NMR: see Table I.

Table I. ¹³C chemical shifts of sterols **1–6** in CDCl₃ δ [ppm].

C-Atom	1	2	3	4	5	6
1	36.3	36.3	36.9	36.9	37.0	37.5
2	31.1	31.2	31.5	31.5	31.4	31.3
3	70.4	70.5	71.4	71.4	71.3	71.3
4	41.8	41.8	41.7	41.7	42.0	42.0
5	165.4	165.2	143.5	143.4	146.2	146.2
6	126.0	126.1	125.5	125.4	123.9	123.8
7	202.4	202.4	73.3	73.3	65.4	65.3
8	45.4	45.4	40.8	40.9	37.5	37.5
9	49.9	50.0	48.3	48.2	42.3	42.2
10	38.3	38.3	36.4	36.4	37.5	37.4
11	21.2	21.2	21.1	21.1	20.7	20.7
12	38.7	38.7	39.6	39.5	39.2	39.2
13	43.1	43.1	42.9	42.9	42.1	42.1
14	49.9	49.9	55.4	55.4	49.4	49.4
15	26.3	26.3	26.4	26.4	24.3	24.3
16	28.5	28.5	28.5	28.5	28.3	28.2
17	54.8	54.8	56.0	55.9	55.7	55.8
18	11.9	12.0	11.8	11.8	11.6	11.6
19	17.3	17.2	19.1	19.1	18.2	18.2
20	36.0	35.7	36.1	35.7	36.1	35.7
21	18.9	18.9	18.8	18.7	18.8	18.7
22	33.9	36.2	34.0	36.2	33.9	36.1
23	26.1	23.8	26.1	23.8	26.0	23.7
24	45.8	39.5	45.8	39.5	45.9	39.5
25	29.1	28.0	29.2	28.0	29.2	28.0
26	19.0	22.5	19.0	22.5	19.0	22.5
27	19.8	22.8	19.9	22.8	19.8	22.8
28	23.0		23.1		23.1	
29	12.0		12.0		12.0	

Results and Discussion

The sterols **1**, **3**, and **5** occur in the roots of *Hordeum vulgare* L. cv. 'Xenia' and *H. vulgare* L. cv. 'Bigo' in a concentration of 1–34 μg/g fresh weight. After spraying the primary leaves of barley (*H. vulgare* L. cv. 'Abed Binder' with one of the sterols **1**, **3**, and **5** induced resistance toward *Puccinia striiformis* could be detected (Seifert *et al.*, unpublished results). On the basis of these findings the sterols **1**, **3**, **5** and the analog compounds of cholesterol **2**, **4**, **6** were synthesized (Schabdach, 1992) and tested for resistance inducing activities. The synthesis (Kumar *et al.*, 1987) was realized as follows: Treatment of β-sitosterylbenzoate and cholesterylbenzoate with ten molar equivalents of CrO_3/3,5-dimethylpyrazole, prepared in situ at -20 °C, resulted in the corresponding ketones (yields 69–71%), which were deprotected with 0.1 M solution of NaOMe to give 3β-hydroxystigmast-5-en-7-one (**1**) and 3β-hydroxycholest-5-en-7-one (**2**) (yields 80–81%). The reduction of 7-keto-β-sitosterylbenzoate and

7-ketocholesterylbenzoate either with $NaBH_4$ in the presence of $CeCl_3$ in THF/MeOH (2:1) afforded the 7β-hxdroxylated compounds (yields 97–98%) or with $LiB[CHCH_3(C_2H_5)]_3H$ (L-Selectride) in THF the 7α-hydroxylated compounds (yields 60–62%). Deprotection of the 7β-hydroxy- and the 7α-hydroxysterylbenzoates with 0.1 M solution of NaOMe gave stigmast-5-ene-3β,7β-diol (**3**), cholest-5-ene-3β,7β-diol (**4**), stigmast-5-ene-3β,7α-diol (**5**), and cholest-5-ene-3β,7α-diol (**6**) (yields 89–91%). The assignments of the ^1H- and ^{13}C NMR data for **1-6** were based on ^1H-, $^1H,^1H$ COSY-, ^{13}C-, ^{13}C APT-, and $^1H,^{13}C$ COSY-experiments. According to ^{13}C APT and $^1H,^{13}C$ COSY experiments of **3** and **5** the assignments of the signals C-8, C-12, C-13, C-14, C-17, C-23, C-25, **3** and C-21, C-26 , **5** (Chaurasia and Wichtl, 1987) have to be interchanged.

Potential antifungal activities of the sterol derivatives **1–6** were examined in in-vitro tests proving the effects of the compounds on mycelium growth of the pertotrophic fungus *Cochliobolus sativus* and on spore germination and germ tube

Table II. Effect of 7-oxo- and 7-hydroxysterols **1-6** (10^{-4} mol/l in 1% aqu. ethanol) applied 2 days before challenge inoculation on the infection density of rust fungi on primary leaves of barley cv. 'Mammut' and wheat cv. 'Astron' (means based on 30 measurements per treatment in triplate experiments).

Treatment	Barley/*Puccinia hordei*	
	Number of infection sites	% Reduction[a] in infection sites
1 % aqu. ethanol	64 (±14)[b]	
3β-Hydroxystigmast-5-en-7-one (**1**)	52 (±13)	19
3β-Hydroxycholest-5-en-7-one (**2**)	53 (±14)	17
Stigmast-5-ene-3β,7β-diol (**3**)	34* (± 7)	47
Cholest-5-ene-3β,7β-diol (**4**)	44* (± 8)	32
Stigmast-5-ene-3β,7α-diol (**5**)	36* (± 7)	44
Cholest-5-ene-3β,7α-diol (**6**)	35* (± 6)	45

Treatment	Wheat/*Puccinia striiformis*	
	Number of infection sites	% Reduction[a] in infection sites
1% aqu. ethanol	82 (±19)[b]	
3β-Hydroxystigmast-5-en-7-one (**1**)	69 (±14)	16
3β-Hydroxycholest-5-en-7-one (**2**)	71 (±12)	13
Stigmast-5-ene-3β,7β-diol (**3**)	48* (±10)	42
Cholest-5-ene-3β,7β-diol (**4**)	50* (± 6)	39
Stigmast-5-ene-3β,7α-diol (**5**)	42* (± 7)	49
Cholest-5-ene-3β,7α-diol (**6**)	42* (± 8)	49

* Values significantly different from values for control plants ($p \leq 0.05$) according to Student's t-test.
[a] Compared to plants treated with 1% aqu. ethanol.
[b] ± = Standard deviation of the mean.

elongation of the biotrophic fungi *Erysiphe graminis* f. sp. *hordei*, *E. graminis* f. sp. *tritici*, *Puccinia hordei* and *P. striiformis* by the method described in Materials and Methods. Non of the sterols inhibited colony growth of *Cochliobolus sativus* in the agar diffusion tests. Neither germination nor germ tube elongation of the biotrophics were affected by the applied concentrations of the sterols compared to the controls, and no morphological alterations of the germ tubes were observed.

Despite the absence of observable toxic effects on fungal development in vitro, the colony densities of *P. hordei* on barley plants and of *Puccinia striiformis* on wheat plants were significantly reduced up to 49% on plants challenge inoculated 2 days after application of the sterol derivatives **3–6**, but only slightly reduced after treatments with **1** and **2** (Table II).

Plants challenged immediately after sterol derivatives application were not protected. The necessity of a time interval indicate that changes in the metabolism of the host plants caused the reduced infection density of the plants. Therefore the involvement of a mode of action as described for tricyclazole which has no apparent effect on spore germination but preventing penetration of the fungus *Pyricularia oryzae* by inhibiting melanization within the appressoria formed only on the plant surface seems unlikely (Peterson, 1990). The degree of protection depended on the dosage of the applied sterol derivatives and on the frequency of application. Plants treated one time with 10^{-6} and 10^{-9} mol/l sterol derivative solutions were not protected. Both, barley and wheat plants showed no enhanced resistance toward *Erysiphe graminis* as well as barley toward *Cochliobolus sativus* after application of the sterols. These differences in the efficacy of protection could due to differences in the developmental pattern of the fungi. In contrast to *Cochliobolus sativus* and *Erysiphe graminis* which infect their host plant directly through the cuticle, the rust fungi develop a series of complex infection structures to infect the leaf parenchym. These development stages include the formation of appressoria on stomata, and in the intercellular space the formation of the substomal vesicle, infection hypha and haustorial mother cells before haustoria are established within the host cells (Mendgen and Deising, 1993). Possibly, the sterol derivatives activate selectively defense mechanisms of the plants which impaire the development or differentiation of these specific structures. Changes in the morphology or chemistry of the cuticula preventing the formation of appressoria on stomata may lead the fungus astray. In non-host plants rust fungi growth stopped during formation of the substomatal vesicle or of the haustorial mother cell (Fink *et al.*, 1990). More histological studies are required to elucidate the mode of action of this new group of chemicals broading the spectrum of substances capable to induce resistance against plant diseases.

Small quantities of the sterol compounds were required to induce resistance. The results indicate that 7α- and 7β-epimers induce more efficiently plant disease resistance compared to the corresponding 7-keto derivatives. This could result from differences in the uptake or recognition due to the binding to components of the cell wall matrix, plasmalemma or cytoplasmic receptors. Cohen *et al.* (1994) studied the effects of α, β and γ-isomers of aminobutrytic acid on late blight development (*Phytophthora infestans*) in tomato plants. They also reported an isomer specific induction of resistance. Precise experiments on the uptake and translocation of the bioactive sterol compounds in barley will be the aim of further investigations.

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