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

H. Schabdach, S. Johnec, U. Steiner and K. Seifert

Lehrstuhl für Organische Chemie 1/2, NW II, Universität Bayreuth, D-95440 Bayreuth, Bundesrepublik Deutschland
Institut für Pflanzenkrankheiten und Pflanzenschutz, Universität Hannover, Herrenhäuser Str. 2, D-30419 Hannover, Bundesrepublik Deutschland
Firma Ingenieurgesellschaft Wasser- und Tiefbau m.b.H. Bitterfeld, Zörbiger Str., D-06749 Bitterfeld, Bundesrepublik Deutschland

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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} \text{ mol/l in } 1\% \text{ aqu. ethanol}) followed, 2 days later, by challenge inoculation with the fungal pathogens. The results indicate that 7a- and 7ß-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...
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), stigmaster-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 μE 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 (1 cm²) were coated with 20 μl droplets of the 10⁻⁴ mol/l sterol solutions and measuring the 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 ¹H - ¹³C NMR spectra on a Bruker AC-300 spectrometer. TLC on silica gel sheets (cyclohexane-EtOAc (1:1), 0.25 mm, Polygram R SILG/UV254). 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-Hydroxysterigmat-5-ene-7-one (1)

Rₚ 0.38; m.p. 149–152 °C; IR νₘₐₓ 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-ene-7-one (2)

Rₚ 0.36; m.p. 169–170 °C; ref. (Chicoye et al., 1968) m.p.172 °C; IR νₘₐₓ 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)

Rₚ 0.29; m.p. 169–172 °C; IR νₘₐₓ 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)

Rₚ 0.28; m.p. 175 °C; ref. (Kumar et al., 1987) m.p. 176–178 °C; IR νₘₐₓ 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)

Rₚ 0.22; m.p. 198–200 °C; ref. (Fukuyama et al., 1988) m.p. 202–204 °C; IR νₘₐₓ 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)

Rₚ 0.22; m.p. 183–184 °C; ref. (Kumar et al., 1987) m.p. 184–186 °C; IR νₘₐₓ 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 = 6.5 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.
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-sitosteryl-benzoate and cholesterylbenzoate with ten molar equivalents of CrO₃/3,5-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-ketocholesterolbenzoate either with NaBH₄ in the presence of CeCl₃ in THF/MeOH (2:1) afforded the 7β-hydroxylated compounds (yields 97-98%) or with LiB[CH₂(C₂H₅)₃]₃H (L-Selectride) in THF the 7α-hydroxylated compounds (yields 60-62%). Deprotection of the 7β-hydroxy- and the 7α-hydroxysterolbenzoates 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 13C NMR data for 1-6 were based on 1H-, 13C-, 13C APT-, and 1H,13C COSY-experiments. According to 13C APT and 1H,13C 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>
<thead>
<tr>
<th>Treatment</th>
<th>Barley/Puccinia hordei</th>
<th>Wheat/Puccinia striiformis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of infection sites</td>
<td>% Reduction* in infection sites</td>
</tr>
<tr>
<td>1 % aqu. ethanol</td>
<td>64 (±14)b</td>
<td>19</td>
</tr>
<tr>
<td>3β-Hydroxystigmast-5-en-7-one (1)</td>
<td>52 (±13)</td>
<td>17</td>
</tr>
<tr>
<td>3β-Hydroxycholest-5-en-7-one (2)</td>
<td>53 (±14)</td>
<td>17</td>
</tr>
<tr>
<td>Stigmast-5-ene-3β,7β-diol (3)</td>
<td>34* (± 7)</td>
<td>47</td>
</tr>
<tr>
<td>Cholest-5-ene-3β,7β-diol (4)</td>
<td>44* (± 8)</td>
<td>32</td>
</tr>
<tr>
<td>Stigmast-5-ene-3β,7α-diol (5)</td>
<td>36* (± 7)</td>
<td>44</td>
</tr>
<tr>
<td>Cholest-5-ene-3β,7α-diol (6)</td>
<td>35* (± 6)</td>
<td>45</td>
</tr>
</tbody>
</table>

* Values significantly different from values for control plants (p ≤ 0.05) according to Student's t-test.

* 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. None 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 broadening 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 aminobutyric 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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