Isolation of an Anti-*Fusarium* Substance Present in Intact Rye Seedlings

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After it was shown in this laboratory that 6-methoxybenzoxazolinone is formed in wheat and maize plants and benzoxazolinone in rye plants from precursors through enzymatic reactions and ensuing chemical changes [1], a glucoside $C_{14}H_{17}O_9N$ (m. p. 186–187°C) could be isolated from rye seedlings. From this glucoside the aglucone $C_8H_7O_4N$ (m. p. 152°C) is formed when the seedlings are crushed [2]. The aglucone is converted into benzoxazolinone $C_7H_5O_2N$ under liberation of formic acid [2]. In young wheat and maize plants the corresponding methoxy derivative, the glucoside $C_{15}H_{19}O_{10}N$ (m. p. 168–170°C) was found as an original compound from which the aglucone $C_9H_9O_5N$ (m. p. 156–157°C) was enzymatically formed [3]. 6-Methoxybenzoxazolinone $C_8H_7O_3N$ is a reaction product of the latter. The glucosides have only a very weak anti-*Fusarium* activity, whereas the aglucones inhibit the growth of *Fusarium nivale* in an oatmeal-glycerol nutrient medium already at a concentration of 0.1 % and benzoxazolinone at a concentration of 0.06 % [3].

Because the extract of rye seedlings, after the enzyme activity had been destroyed by boiling intact seedlings with 70 % ethanol, inhibits the growth of *Fusarium nivale* at concentrations corresponding to 1 to 1.5 g of fresh weight per ml substrate, the seedlings have to contain antimicrobial substances which as such are present in the seedlings and not formed only after the seedlings are crushed. The rye seedlings used in this work were of the varieties Pekka, Ensi, and Kungsråg. They were collected in November and December on the field.

On dividing the 70 % alcohol extract of the seedlings into ether-soluble neutral material, ether-soluble weakly acidic material (soluble in 1 N NaOH), ether-soluble acids (soluble in sodium bicarbonate), and water-soluble material (not extractable with ether from acidified water solution), about half of the total activity of the alcohol extract is found in the ether-soluble weakly acidic material, and the other half in the water-soluble material.

In the water-soluble material, part of the activity was extractable with *n*-butanol. On attempts to fractionate the water-soluble material by paper chromatography, the activity could not be recovered. Also on standing in the refrigerator for two months the activity was lost.

The ether-soluble weakly acidic material was shown to contain an active component moving with an R_F-value of 0.86 in *n*-propanol:ammonia (7:3) on Whatman No. 4 paper (desc.). This component was purified at first by cellulose-column chromatography in the *n*-propanol-ammonia solvent, and then by adsorption chromatography on a silica-gel column. The silica-gel was previously equilibrated against water-vapour of the pressure 5.6 mm Hg at 20°C, and the active material was eluted with ethanol-free chloroform containing rising amounts (2–50 %) of ether. Because the material was found to be subject to autoxidation the following steps were carried out in a CO_2 atmosphere, and illumination of the samples was avoided. The chloroform-ether fractions were evaporated to dryness *in vacuo* and dissolved in methanol. After addition of 20 % of water the solution was cooled and the inactive material crystallizing was filtered off. The filtrate was again evaporated to dryness *in vacuo* and the residue was chromatographed on a cellulose column in a solvent consisting of water-saturated benzene containing 0.2 % of methanol. The R_F-value of the active component in this solvent on Whatman No. 4 paper was 0.87 (asc.). The active fractions were evaporated to dryness *in vacuo*. The resulting slightly yellow-green oil was found to solidify at −14 to −15°C, and it melted at −12.5 to −11°C. The oil was crystallized from methanol at −40 to −50°C using an apparatus described by Kajanne [4]. After separation of the crystals they were allowed to melt, and the resulting oil was filtered at −10 to −5°C through a micro filter. After drying over P_2O_5 at +20°C and 0.01 mm Hg pressure, the oil solidified at −14°C and melted at −12 to −11°C. The yield was 68 mg oil from 255 g fresh weight of seedlings. Considering the amount of oil in the mother liquor after crystallization the total amount of oil in

the plants was calculated to be about 1.5 mg per g fresh weight.

Some of the properties of the substance are listed below:

Boiling point: At a pressure of 0.01 mm Hg signs of boiling were observed at about 170°C, with simultaneous darkening of the sample.

Empirical formula: Found: C 76.16; H 10.61; O 12.96. Calc. for $C_8H_{14}O$: C 76.2; H 11.1; O 12.7. Calc. for $C_8H_{13}O$: C 76.8; H 10.4; O 12.8.

Molecular formula: For the molecular weight, determined by the camphor method, the value 270–290 was found. Accordingly, the molecular formula is $C_{16}H_{28}O_2$ or $C_{16}H_{26}O_2$ with the calculated molecular weight of 252 or 250.

UV-absorption: In *cyclo*hexane the substance exhibits one maximum, at 236 mμ, $\varepsilon = 3020$.

Acidic properties: The substance dissolves in aqueous sodium carbonate solution and in sodium hydroxide, being precipitated on acidifying.

Hydroxyl content: The substance is esterified by acetylchloride. The product of catalytic hydrogenation reacts with one mole of acetyl chloride per mole substance.

Catalytic hydrogenation at room temperature with PtO_2: Two moles of hydrogen per mole substance are rapidly consumed.

Reducing properties: The substance decolorizes permanganate rapidly in sodium carbonate solution. One of the products from permanganate oxidation was identified as azelaic acid, $HOOC-(CH_2)_7-COOH$, by isolation and mixed melting point determination. The substance is also oxidized by periodic acid at room temperature within a few minutes. It exhibits a reducing action upon 2,6-dichlorophenolindophenol and reduces Hg(I)-nitrate and bromine slowly. It is autoxidized in the air, the reaction is light-catalyzed. The products of autoxidation seem to contain peroxides (liberation of iodine from iodide).

Other reactions: With silver ions the substance seems to form a complex. No colour formation with ferric chloride was observed. With diazotized *p*-nitroaniline a yellow colour appeared on making the solution alkaline. With 2,4-dinitrophenylhydrazine the substance is reacting, as shown by chromatography and spectrometry of the reaction mixtures, though no crystalline product could be obtained. No carboxylic acid-, lactone-, or ester-reaction could be obtained with the substance.

On the basis of the oxidation products the isolated substance contains a straight saturated chain of nine C-atoms. The other properties listed above suggest that the substance contains: two aliphatic double bonds, one C–OH group, and one C=O group.

The substance isolated inhibits the growth of *Fusarium nivale* to 50 % at a concentration of 0.5 to 0.7 mg per ml of an oat-meal-glycerol medium. On increasing the concentration to 2.4 mg/ml the inhibition increases to 85 %. Above this concentration the inhibition *vs.* concentration curve becomes parallel to the concentration axis, depending on the poor solubility of the substance in aqueous medium.

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