Precursors of Benzoxazolinone in Rye Plants

I. Precursor II, the Aglucone

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When benzoxazolinone (BOA) was isolated from crushed rye seedlings, it was at first regarded as a primary substance in these plants [1]. It was, however, difficult to understand why this ether-soluble compound could not be extracted from crushed plants with ether. Before long it was found that it is formed from a precursor (precursor II) which in turn is a product of an enzymatic splitting of a compound (precursor I) found in intact rye plants [2].

Glucosides proved to be primary precursors, the enzymatic hydrolysis of which led to the formation of the corresponding aglucones after the plants were crushed, or the plant sap was pressed[3]. BOA was formed from the aglucone in rye plants and MBOA (methoxybenzoxazolinone) from the aglucone in wheat and maize plants by chemical transformation. If the enzymes are destroyed by placing intact plants in boiling water or boiling ethanol solution, or by keeping them in 70 % ethanol at −20°C for a longer time, the aglucone is no longer formed when plants are crushed. Only the glucoside (precursor I) is to be found in plants treated in this way.

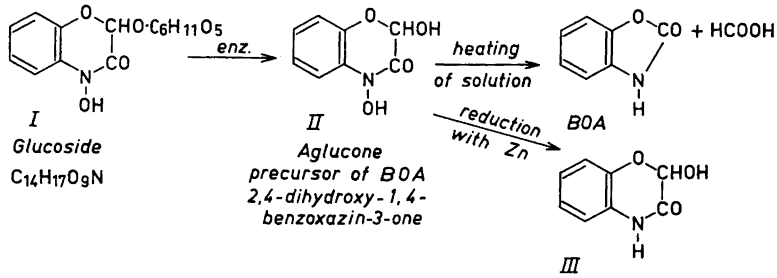
When experimental results accumulated, Virtanen and Hietala [3,4] found that only the splitting off of glucose takes place in the enzymatic decomposition of the glucoside. Soon thereafter they were able to propose the probable structure for the glucoside (I) in rye and the aglucone (II) formed from it. At the same time evidence was obtained that BOA is formed from the aglucone by the liberation of formic acid.

A proof for structure II for the aglucone was obtained by reducing the oxime group to the imido group (III) [4]. This compound was synthesized by Honkanen and Virtanen [5] from *ortho*(dichloroacetamido)-phenol by heating in a dilute sodium bicarbonate solution. Recently Honkanen and Virtanen [6] also succeeded in synthesizing the aglucone, and thus proved its structure.

Wahlroos and Virtanen [7] have described the isolation of the glucoside and aglucone from maize plants and demonstrated that these precursors of MBOA have anologous structures to those of the precursors of BOA in rye plants.

The spectroscopic identification of precursor II (the aglucone) of BOA in the press juice of rye plants. 5 ml of juice were pressed from 13 g of 8-day-old rye seedlings. The juice was heated in a water bath for 2 min (the temperature rose to about 70°C) to coagulate proteins. No noticeable amount of BOA was formed by this treatment. The juice was filtered through a hard filter paper. The clear solution was pipetted into two test tubes, 2 ml into each. The one was heated in a water bath for 1 h. Both water solutions were extracted four times with 2 ml of ether. Five different samples were then obtained: 1. The original juice. 2. The ether extract obtained from the juice heated for 1 h. 3. The water solution of the above. 4. The ether extract obtained from the unheated juice. 5. The water solution of the above.

All the solutions were diluted with water to 1:100 (the ether had been evaporated before this at room temperature). The curves in Fig. 1 show the UV-absorption curves of the solutions. Water was used as control solution in all measurements.



The examination of the spectra: The ether extract spectra of the heated and the unheated juice differ completely from each other. Spectrum 2 is that of BOA (max. 270 mμ and min. 245 mμ). If spectra 4 (ether extract) and 5 (water solution after ether extraction), obtained from the unheated juice, are summed, a spectrum nearly identical with spectrum 1 is obtained. This is not the case if spectra 2 and 3 are summed. Spectrum 4 is thus derived from the substance from which BOA is formed on heating the water solution.

In agreement with the spectroscopic investigation reported above, a paper chromatographic analysis (*iso*propanol:ammonia:

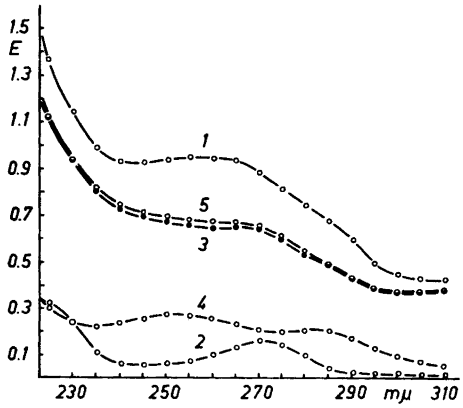


Fig. 1. UV spectra of solutions 1—5 mentioned in the text.

water 8:1:1, Whatman No. 4 paper) of solutions 1—5 showed that a substance (R_F 0.23) is found in ether extract 4. This substance had disappeared in ether extract 2, which on the other hand contained the BOA spot moving near the solvent front. When again it was found that the ether-soluble precursor II is not present in intact rye seedlings if these are placed in boiling water or ethanol, it was obvious that the substance was formed enzymatically from the primary precursor on crushing. This substance was found to be a glucoside and precursor II the aglucone formed from it.

The isolation of the aglucone. 1 190 g of rye seedlings (Pekka), grown for 7 days in the light, were ground. The mass was allowed to stand for 1 h at room temperature to ensure the enzymatic decomposition of the glucoside (in fact the hydrolysis took place very rapidly). The mass was vigorously pressed, and 850 ml of juice was obtained. The juice was kept in two bottles in a water bath for 4 min during which the temperature rose to about 70°C. The filtered, clear solution was shaken twice with ether, twice with *n*-butanol, and finally twice with ether. The combined ether and butanol solutions were evaporated to dryness *in vacuo* (the temperature did not rise above 20°C). The residue was dissolved in 100 ml of water, the solution was shaken 3 times with 100 ml of ether. The ether solutions were evaporated, and 75 ml of ether was added to the residue. The unsoluble, reddish substance was filtered off, 250 mg of active charcoal was added, the mass was shaken, and the charcoal was filtered off after standing. 40 ml of *cyclo*hexane was added to the solution, and it was evaporated *in vacuo* until crystallization began. The crystals were separated, washed with *cyclo*hexane, and dried at low temperature. They were recrystallized in the same way from a mixture of ether and *cyclo*hexane, washed with *cyclo*hexane, and finally dried at 100°C. Yield 428 mg; m. p. 152°.

Properties of the aglucone

1. *Analysis.* Found: C 53.40; H 3.98; N 7.40; O 33.15. Calc. for $C_8H_7O_4N$: C 53.04; H 3.90; N 7.73; O 35.33.

2. It gives a strong blue-violet colour with $FeCl_3$.

3. Its R_F value with *iso*propanol-ammonia-water 8:1:1 is 0.23.

4. Formic acid and BOA are rapidly formed when a dilute water solution of the aglucone is heated in a sealed tube in a water bath. A strong formic acid reaction was obtained with chromotropic acid. Formic acid was determined quantitatively by a fractionated steam distillation.

5. It does not react with 2,4-dinitrophenylhydrazine at room temperature. Upon heating a dark precipitate is formed. This has not been further investigated.

6. A potentiometric titration with 0.0200 N NaOH gave 190 as the equivalent weight and a $pK = 7.02$ (calculated from the curve). On titration with acid no clear equivalent point could be noticed and hence the aglucone does not consume acid in the pH range of the titration. This indicates that it has not a betaine structure.

7. By paper chromatography *ortho*-aminophenol was found to be formed as the main

product on strong hydrolysis with hydrochloric acid. This compound was extracted with ether from the hydrolysate neutralized with $NaHCO_3$ and identified by the UV-spectrum. *Ortho*-aminophenol was formed also on alkali fusion.

8. Hydroxylamine is a product of strong acid hydrolysis.

9. On reduction with hydrogen iodide and red phosphorus (62 mg of substance, 30 mg of red phosphorus, and 0.5 ml of hydrogen iodide, heating for 4 h at 170°C) *ortho*-aminophenol and a smaller amount of glycine (about 3 to 4 mg) were formed. Amino compounds were separated in an Amberlite IR-120 column. *Ortho*-aminophenol was separated by ether extraction from the ammonia eluate, from which ammonia was evaporated *in vacuo*, and identified by the UV-spectrum. Glycine was identified in the remaining water solution on a two-dimensional paper chromatogram. Other amino acid spots were not observed.

10. Methylation with dimethylsulphate (73 mg of substance and a five-fold excess of dimethylsulphate) led to a reaction product which after ether extraction was recrystallized from water, m. p. 84–85°. Its UV-spectrum greatly resembled that of BOA. (Found: N 9.82. Calc. for $C_8H_7O_2N$: N 9.40.) The substance obtained is probably N-methylbenzoxazolinone, the m. p. of which has been reported to be 86°.

11. Reduction with zinc and hydrochloric acid (123 mg of aglucone, 2 g of zinc dust, and 0.1 ml of 1 % ferric chloride solution; dropwise addition of concentrated hydrochloric acid until the ferric chloride reaction disappeared) led to a reduction product with m. p. 198° without recrystallization, and 201–203° (corr.)[5] after recrystallization. Mol. wt. (Rast) 167; calc. for $C_8H_7O_3N$: 165. Since the substance no longer gives any colour reaction with ferric chloride, the NOH group has apparently been reduced to NH. The UV-spectrum of the reduction product is shown in Fig. 2.

12. The aglucone did not consume hydrogen on catalytic hydrogenation with platinum in ethanol solution.

Conclusions: The results mentioned in paragraphs 7 and 9 can be understood only if nitrogen and carbon atoms outside the benzene ring are in the order —N—C—C—, and the nitrogen atom is attached to a carbon atom in the benzene ring. The formation of glycine in paragraph 9 is possible only if two oxygen atoms are bound to the terminal carbon atom.

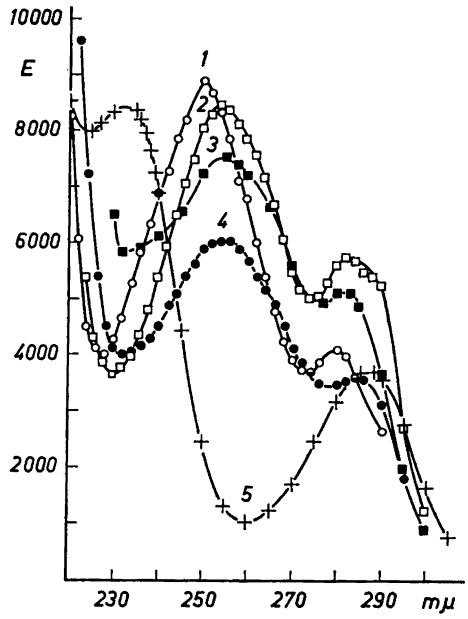
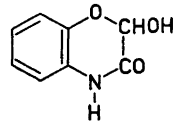
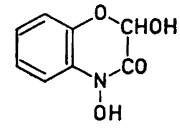


Fig. 2. UV spectra in ethanol solutions of

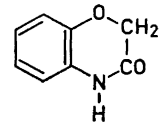


1. reduced aglucone,

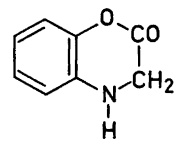


2. aglucone,

3. glucoside $C_{14}H_{17}O_9N$,



4. 2H-1,4-benzoxazin-3-(4H) one (synth.),



5. 3H-1,4-benzoxazin-2-(4H) one (synth.).

The formation of hydroxylamine mentioned in paragraph 8 and the positive Feigl-Amaral test [8] for an N—O bond suggests that the hydroxyl group is combined with the nitrogen atom and that the aglucone is a hydroxylamine derivative.

Taking into account these conclusions and the elementary composition of the aglucone, the possibility of an aliphatic side chain in the aglucone is ruled out, and a ring formed of nitrogen, carbon, and oxygen atoms is indicated. Structure II was suggested for the aglucone. This structure was supported by the similarity of the UV-spectra of the aglucone and its reduction product with 2H-1,4-benzoxazin-3-(4H) one synthesized by us (Fig. 2).

The structure of the aglucone could later be confirmed by synthesis by Honkanen and Virtanen [5,6], as already mentioned in this paper.

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II. Precursor I, the Glucoside

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The identification and isolation of precursor I (the glucoside). As reported in previous communications [1], the glucoside $C_{14}H_{17}O_9N$ was isolated from rye seedlings. It is a primary compound present in intact rye plants. The aglucone $C_8H_7O_4N$ which is the immediate precursor of benzoxazolinone (BOA) is formed from the glucoside enzymatically.

225 g of 10-day-old seedlings of Pekka rye were put into 1.5 l of boiling water, the solution was decanted, filtered, and evaporated to 250 ml *in vacuo*. Ether-soluble substances were removed by extraction, and a chromatogram was made from the water solution with water-saturated butanol. 150 μl were pipetted on the starting spot. The dark spot, R_F 0.57, visible in UV light, represented the glucoside sought, from which precursor II is formed enzymatically. The spectrum of the substance is very similar to that of the aglucone.

The isolation of larger amounts of the substance was performed by counter-current extraction, using the butanol-water solvent system. In another connection, one of us (H) will give a detailed account of the isolation. The m. p. of the glucoside was 186.5—187°C (uncorr.). R_F 0.57 in water-saturated *n*-butanol. (Found: C 49.04; H 4.98; N 3.81; O 41.04. Calc. for $C_{14}H_{17}O_9N$: C 48.98; H 4.99; N 4.08; O 41.95.) UV spectrum (in ethanol). Glucoside: max. 255 mμ, ε = 7 570, max. 281 mμ, ε = 5 160. Aglucone: max. 254 mμ, ε = 8 500, max. 282 mμ, ε = 5 800. The curves of the UV spectra are presented in the paper of Virtanen and Hietala [1].

The UV spectra of the glucoside and the aglucone are very similar. Already from this it was probable that the structure of the free aglucone was the same as that bound to the sugar glucosidically. This concept was supported by all other observations. Some of these which elucidate the