The Precursors of 6-Methoxybenzoxazolinone in Maize and Wheat Plants, their Isolation and Some of their Properties

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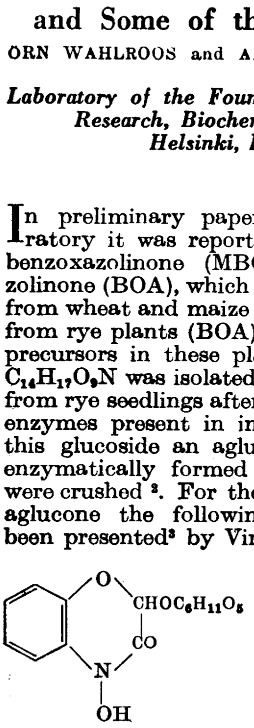
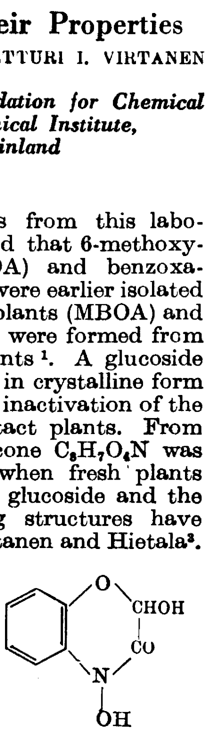
In preliminary papers from this laboratory it was reported that 6-methoxybenzoxazolinone (MBOA) and benzoxazolinone (BOA), which were earlier isolated from wheat and maize plants (MBOA) and from rye plants (BOA), were formed from precursors in these plants [1]. A glucoside $C_{14}H_{17}O_9N$ was isolated in crystalline form from rye seedlings after inactivation of the enzymes present in intact plants. From this glucoside an aglucone $C_8H_7O_4N$ was enzymatically formed when fresh plants were crushed [2]. For the glucoside and the aglucone the following structures have been presented[3] by Virtanen and Hietala[3].



Glucoside
$C_{14}H_{17}O_9N$



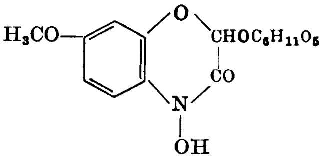
Aglucone
precursor of BOA
2,4-dihydroxy-1,4-benzoxazin-3-one

The glucoside

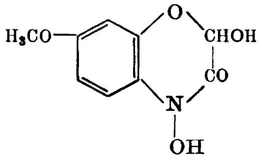
The isolation of the glucoside from maize seedlings. 2.8 kg fresh weight of maize seedlings (grown in a greenhouse, about 10 days old, plants about 10 cm high, green parts) were put into 7 l of boiling water, keeping the temperature of the water above 80°C. When all seedlings were added (about 15 min), the mixture was cooled with tap water. It was filtered, the seedlings were homogenized in water of room temperature and filtered. The combined filtrates were evaporated *in vacuo* to about 0.5 l. The concentrate was extracted six times with *n*-butanol. The butanol extract was evaporated *in vacuo*, water was added to 0.5 l, and the suspension was extracted five times with ether. 56 g of carbon were added to the water phase (the carbon had previously been washed with 2 N hydrochloric acid and water, deactivated with cyanide according to Schramm and Primosigh [4] and washed with water). It was mixed for 30 min and filtered (washed cellulose powder as filter aid). The carbon was washed on the filter with 0.5 l of distilled water, and was eluted with 1.5 l of a mixture containing benzene, methanol, and water 3:7:1 v/v. The eluate was evaporated to dryness *in vacuo*, the residue dissolved in a small amount of water, and chromatographed on a polyamide column (5.5 × 34 cm) using water as solvent. The polyamide powder was mixed into water and allowed to stand for 2 h, occasionally stirring, before it was poured into the column. It was washed in the column with 700 ml of methanol and 1.5 l of water before chromatographing. At the solvent front a small amount of ninhydrin-positive material appeared, and immediately after that the precursor. Other UV-absorbing material remained in the column. The red coloured fractions containing the precursor were pooled, evaporated to dryness *in vacuo* and rechromatographed on a cellulose powder column (7.5 × 27 cm) with *n*-butanol saturated with water. The cellulose powder was washed with 2 N hydrochloric acid, distilled water, hydroxy-quinoline-containing methanol, to remove heavy metal ions, and methanol, and was air-dried before packed into the column. First a red front emerged from the column. It contained part of the precursor. After it a colourless front emerged, containing the precursor. This crystallized from the colourless fraction when evaporated *in vacuo*. It was recrystallized from absolute ethanol. Yield: 1.23 g of red material, containing a large amount of the precursor, 1.22 g of crystalline crude preparation (pink), and 0.143 g of colourless narrow prisms, m. p. 168–170°C (decomp.).

During the isolation of the glucoside its presence in different fractions was followed by treating the samples with fresh, crushed wheat seedlings, after which MBOA was determined. The homogenate was prepared by grinding 20 g of 7 to 10-day-old wheat seedlings in a homogenizer with 50 ml of water, and was sieved to obtain an even suspension. Each sample to be tested for the glucoside was mixed with 10 ml of this suspension. 10 ml of the suspension was used as a blank. The samples were allowed to stand for 30 min to 1 h at room temperature. They were kept in boiling water for 15 min and filtered. The filtrates were extracted with equal amounts of ether. Equal parts of the ether extracts were chromatographed one-dimensionally on paper with the solvent *n*-propanol-ammonia (25 %), 7:3. Pieces of the same size were cut out between R_F-values 0.6 and 0.8. They were eluted with equivalent volumes of absolute ethanol. The UV-spectra between 230 and 310 mμ were measured against the ethanol eluate of the chromatographic spot of the blank. The spectrum thus obtained shows the MBOA formed from the precursor in the preparation added.

A comparison of the glucoside in maize and wheat plants. Wheat seedlings (grown in a greenhouse, about 10 days) were put into boiling water. They were cooled, ground in water of room temperature, and filtered. The filtrate was extracted with ether and the waterphase remaining six times with n-butanol. A butanol extract was prepared in the same way from maize seedlings. Two-dimensional chromatograms were made from the butanol extracts obtained from maize and wheat, as well as from their mixture, with the solvents water-saturated n-butanol and 2 % acetic acid. In this solvent system the glucoside of maize and wheat moved as one spot. By chromatography it was also established that this precursor is not present in wheat seeds.



Glucoside
$C_{15}H_{19}O_{10}N$



Aglucone
precursor of MBOA
2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one

Properties of the glucoside. When dissolved in 25 % ammonia, the glucoside turns dark brown in a day. With ferric chloride it gives a blue colour. The UV-spectrum in water: Max. 270 mμ (K = 29.6). A knee at 282—283 mμ (K = 27.6). Min. 237 mμ.

Identification of the sugar part: The glucoside was kept for 2 h in a boiling water bath in 2 N hydrochloric acid. It was cooled, and extracted with ether. In the water phase the chloride-ions were exchanged to acetate-ions by pouring through a Dowex 1 column in acetate form. The effluent was evaporated to dryness *in vacuo*, dissolved in a small amount of water, and chromatographed parallel with glucose on Whatman No. 4 paper (aniline hydrogen phthalate spray). The sugar formed on hydrolysis was characterized as glucose by using different solvent systems: a) water-saturated n-butanol (ascending), b) n-butanol-glacial acetic acid-water, 63 : 10 : 27 v/v (descending), and c) water-saturated phenol (descending).

The nitrogen content of the glucoside (Kjeldahl determination, using permanganate). (Found: N 4.13. Calc. for $C_{15}H_{19}O_{10}N$: 3.85.) The methoxy content of the glucoside: 8.1 %, calc. for $C_{15}H_{19}O_{10}N$: 8.3. The molecular weight calculated from the obtained methoxy value was 382, calc. for $C_{15}H_{19}O_{10}N$: 373.

The growth-inhibiting effect of the glucoside on *Fusarium nivale* was negligible. 6 mg per 1 ml of oat-glycerol nutrient solution, pH 6.5—6.8, caused about 50 % inhibition.

The aglucone

The isolation of the aglucone. 400 g fresh weight of maize seedlings (grown as mentioned above) were treated in a juice press. After 30 min the press juice was centrifuged for 40 min at 10 000 g and a temperature between 0 and 10°C. The clear supernatant was decanted and extracted twice with ether. The ether solution was dried for about 2 h with sodium sulphate, filtered, and evaporated at room temperature to a small volume. The precipitate thus obtained was recrystallized several times from methanol and then from an ether-*cyclo*hexane mixture. 75 mg of pink needle-like crystals, m. p. 156—157°C (decomp., gas evolution), were obtained.

Properties of the aglucone. Conversion into MBOA: A solution of the aglucone was made up in water to a concentration of 10 μg per ml. The UV-spectrum was measured: Maxima at 267 mμ (K = 34.7) and 292 mμ (K = 32.5), minima at 237 mμ (K = 5) and 280 mμ (K = 29.4). This solution was heated under reflux for 30 min in a boiling water bath and its UV-spectrum was measured once more.

Table 1. The growth-inhibiting effect of the aglucone on *Fusarium nivale*. Oat-glycerol nutrient solution, pH 6.5—6.8. Numbers refer to the diameter of the colony in mm.

Concentration mg/ml	Growth +						
	1	2	3	4	5	6	days
0 (contr.)	12	14	14	16	22	29	
0.5	10	10	14	15	16	22	
1.0	?	+	+	+	+	+	
1.5	+?	+?	+?	0	0	0	
2.0	0	0	0	0	0	0	
2.5	0	0	0	0	0	0	
3.0	0	0	0	0	0	0	

Table 2. R_F-values of the glucoside and the aglucone. Whatman No. 4 paper.

Solvent	R_F	
	The glucoside	The aglucone
H_2O-sat.*n*-BuOH	0.4—0.6 (tailing)	0.72—0.78
n-BuOH:AcOH:H_2O, 8:2:2	0.33	0.81
n-BuOH:MeOH:Bz:H_2O, 3:1:1:1	0.53	0.84
2 % AcOH in water	0.90	0.74
n-PrOH:NH_3(25 %), 7:3	0.30	decomposes

The spectrum then obtained was identical with the spectrum of MBOA, $E_{286} = 0.266$. Because the molar extinction of MBOA is 5 690 this absorbtion corresponds to 7.7 μg of MBOA per ml. From this concentration and that of the aglucone the following molecular weight for the aglucone is obtained: $\frac{10}{7.7} \cdot 165 =$ 214, calc. for $C_9H_9O_5N$ = 211.

When dissolved in 25 % ammonia, the aglucone turns yellow-brown within a few minutes. After 30 min the solution is green, and after about 2 h it is dark brown. This solution was chromatographed, and one of the products was chromatographically and spectrophotometrically identified with MBOA. With ferric chloride the aglucone gave a blue-violet colour.

The nitrogen content of the aglucone was 6.91 %, calc. for $C_9H_9O_5N$: 6.63. The methoxy content of the aglucone was 15.01 %; the molecular weight calculated from the methoxy content was 207, calc. for $C_9H_9O_5N$: 211.

The growth-inhibiting effect of the aglucone is nearly as high as that of MBOA and BOA (Table 1). The effect is mainly fungistatic. R_F-values can be seen in Table 2.

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1. Virtanen, A. I. and Wahlroos, Ö. *Suomen Kemistilehti B* **31** (1958) 402.
2. Virtanen, A. I. and Hietala, P. K. *Ibid.* **32** (1959) 38, 138.
3. Virtanen, A. I. and Hietala, P. K. *Ibid.* **32** (1959) 252.
4. Schramm, G. and Primosigh, J. *Ber.* **76** (1943) 373.

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