Isolation and Identification of 6,7-Dimethoxy-2-benzoxazolinone from Dried Tissues of *Zea mays* (L.) and Evidence of its Cyclic Hydroxamic Acid Precursor

Jerome A. Klun, Carl L. Tipton, John F. Robinson, Dennis L. Ostrem, and Morton Beroza[1]

A new benzoxazolinone, 6,7-dimethoxy-2-benzoxazolinone, was isolated from dried corn tissue and identified. Its structure was proved by synthesis. Evidence of its *in vivo* cyclic hydroxamic acid precursor, 2,4-dihydroxy-6,7-dimethoxy-2*H*-1,4-benzoxazin-3-one, was obtained by mass spectroscopy. Analysis by isotopic dilution of the dried tissue of four inbred varieties showed that the concentration of 6,7-dimethoxy-2-benzoxazolinone, depending on variety, ranged from 0 to 5.9 mg per g dry tissue when the corn was at the germination stage and from 0 to 2.1 mg per g dry tissue when the corn was at the seedling stage.

Two cyclic hydroxamic acids from the tissue of corn (*Zea mays* L.) were described by Tipton *et al.* (1967). These compounds exist in intact corn tissue as glucosides I and IV (Figure 1), but when plant tissues are crushed, the glucosides are hydrolyzed enzymatically to aglucones II and V. Then the labile aglucones decompose to benzoxazolinones III and VI (Brendenberg *et al.*, 1962; Honkanen and Virtanen, 1961). Interest in the cyclic hydroxamic acids has been generated because of their apparent significance in the resistance of cereal grasses to fungi (BeMiller and Pappelis, 1965; Elnaghy and Linko, 1962; Molot and Anglade, 1968), 2-chloro-*s*-triazine herbicides (Hamilton, 1964; Roth and Knusli, 1961), and insects (Klun and Robinson, 1969; Klun *et al.*, 1967).

ISOLATION AND IDENTIFICATION

6,7-Dimethoxy-2-benzoxazolinone was first observed as an unknown compound on thin-layer chromatograms (silica gel GF254; Brinkmann Instruments, Westbury, N.Y.) of extracts of dried corn tissue. The chromatographic properties of the compound were similar to those of 6-methoxy-2-benzoxazolinone (III) and 2-benzoxazolinone (VI). Therefore, to isolate a sufficient quantity of the unidentified compound for study, we germinated seeds of corn inbred B49 on moist filter paper in covered aluminum dishes at 29.4° C and 80% RH and then collected, froze, and air-dried (40° to 50° C) the coleoptile structures of these seeds. Twenty-five grams of dried coleoptile tissue was refluxed in 1 l. of water for 1 hr and filtered, and the filtrate was adjusted to pH 1 with concentrated HCl. The acidified filtrate was extracted with ethyl ether, and the extract was dried over Mg_2SO_4 and evaporated to dryness under reduced pressure. The resulting residue was dissolved in benzene-ethyl acetate (1 to 1 by volume). Then the unknown was isolated by preparative thin-layer chromatography (tlc) on plates of silica gel GF254 (0.25-mm layer) by developing the plant extract successively in chloroform-ethyl acetate-cyclohexane (4:4:2), cyclohexane-isobutyl alcohol (85 to 15), and chloroform-ethyl acetate (9 to 1). After each development, the plates were viewed under a short wavelength ultraviolet lamp, and the band corresponding to the R_f of the unknown, which appeared as a dark area, was scraped from the plate and eluted from the silica gel with 95% ethanol. In the first solvent system, the R_f (0.65) of the unknown was identical to that of 6-methoxy-2-benzoxazolinone (III). In the second solvent system, the unknown was resolved from III.

After purification by tlc, the compound was recrystallized repeatedly from H_2O to yield 12 mg of light amber crystals; mp 180° C. The ultraviolet spectrum (95% ethanol) had maxima at 282, 233, and 215 mμ (Figure 2). The infrared spectrum (Figure 3), which had maxima at 3280 (N—H), 1765 (C=O), and 1630 and 1505 cm^{-1} (phenyl nucleus), was consistent with that of a benzoxazolinone. The mass spectrum of the compound showed the following diagnostic peaks, *m/e*: 195(P), 180 (P − CH_3). By high resolution peak matching (Consolidated Electrodynamic Corp., model 21-110B high-resolution mass spectrometer), the formula of the compound is $C_9H_9NO_4$ (calcd. 195.0532; found 195.0532). The nuclear magnetic resonance (nmr) spectrum (deuteroacetone) τ showed singlets at 6.21 and 7.03 (methoxyl protons) and two doublets centered at 6.73 (two vicinal aromatic protons, J, 8.1 cps and J, 8.2 cps). From these spectra we could identify the compound as one of the possible isomers: 6,7-, 4,7-, or 4,5-dimethoxy-benzoxazolinone (MW 195).

The 6,7 isomer (compound IX) was intuitively considered the candidate most likely to be consistent with the unknown. It was therefore prepared as shown in Figure 4. Nitration of 2,3-dimethoxyphenol (Aldrich Chemical Co.) was carried out in glacial acetic acid and nitric acid at 5° C, and the nitration product, 2,3-dimethoxy-6-nitrophenol, was reduced to the amine hydrochloride by the method of Henrich and Binkner (1913). Then 1.5 g of the 6-amino-2,3-dimethoxyphenol hydrochloride (highly susceptible to air oxidation; mp 150–160° C, decomposition) was reacted with urea in a fusion reaction (Smissman *et al.*, 1957) at 145° C for 2 hr under an N_2 atmosphere. The product was then dissolved in 1*N* HCl and extracted with six 100-ml portions of ethyl ether, which were combined and evaporated to yield a brown residue. The residue was chromatographed on a 25- X 375-mm column of 0.05 to 0.2-mm mesh silica gel with a solvent mixture of chloroform-ethyl acetate-cyclohexane (4:4:2). The fractions containing the desired product were then rechromatographed on thin-layer plates of silica gel GF254 by using the same solvent system. The product, which had an R_f identical to the plant-isolated compound used as reference material, was scraped from the thin-layer plates, eluted with 95% ethanol, and recrystallized from water with Norite to yield 20 mg of 6,7-dimethoxy-2-benzoxazolinone (IX); mp 180° to 181° C. The ultraviolet, infrared, and nmr spectra, and also the melting point and tlc properties of the synthetic product (IX),

[1] Entomology Research Division, Agricultural Research Service, USDA, Ankeny, Iowa, 50021, and Dept. of Zoology and Entomology and Biochemistry and Biophysics Dept., Iowa State University, Ames, Iowa 50010

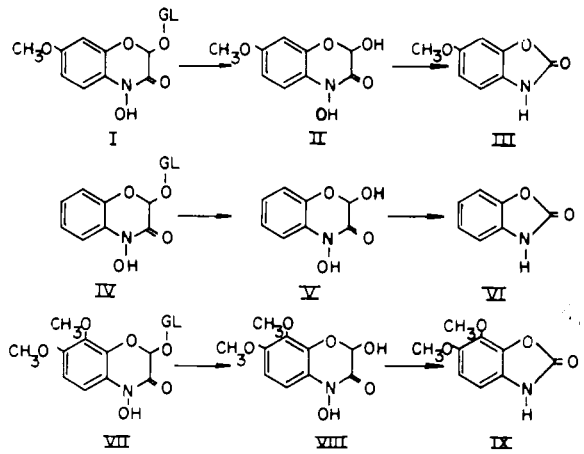

Figure 1. Formation of benzoxazolinones from precursors of cyclic hydroxamic acids in corn (*Zea mays* L.) [gl = glucose]

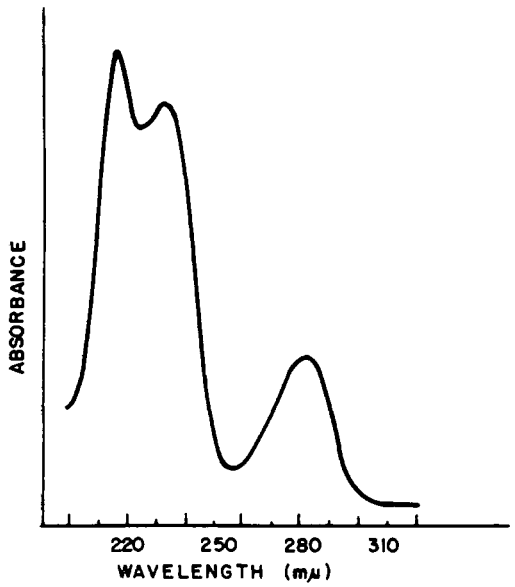

Figure 2. Ultraviolet absorption spectrum of 6,7-dimethoxy-2-benzoxazolinone in 95% ethanol

were identical to those of the compound isolated from corn; indicating that the plant isolated compound and the synthetic (IX) were identical in structure.

ISOTOPIC DILUTION ANALYSIS

C^{14}-labeled IX (57.31 μc/mM) was prepared by fusion of C^{14}-urea with 6-amino-2,3-dimethoxyphenol hydrochloride as described. Dried coleoptiles of germinated seeds and whole 6-in. field-grown seedlings (less roots) of four inbred varieties of corn were then analyzed by the technique of isotopic dilution (Klun and Robinson, 1969). The results of the analysis (Table I) showed that IX was not present in all varieties and that its concentration depended on the variety. Furthermore, its concentration is lower in the seedling stage of the corn than at germination. Compared with the concentrations of the benzoxazolinones III and VI (Table I) in the same varieties, the concentration of IX was less than the concentration of III, and the concentration of IX was greater or less than that of VI, depending on the variety and the stage of plant growth.

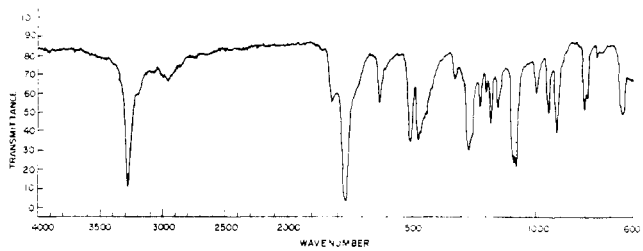

Figure 3. Infrared absorption spectrum of 6,7-dimethoxy-2-benzoxazolinone in micro KBr disc

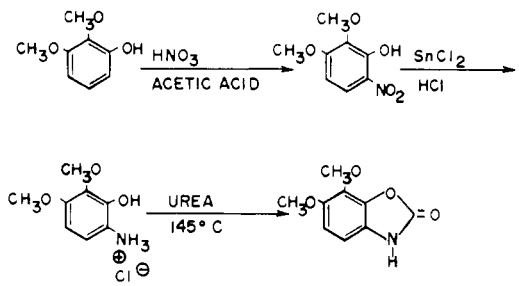

Figure 4. Synthesis of 6,7-dimethoxy-2-benzoxazolinone

Table I. Concentration of 6,7-dimethoxy-2-benzoxazolinone (IX) and Two Other Benzoxazolinones in Dried Corn Tissue at the Coleoptile and Seedling Stages

	Concentration of compound[a] (mg/g) in coleoptile			in seedling		
Variety	III	VI	IX	III	VI	IX
WF9	8.8	0.8	5.9	4.7	0.7	2.1
B52	10.2	1.4	1.7	7.7	1.3	1.1
Oh43	10.0	1.0	0	5.6	0.9	0
B49	7.4	0.4	2.9	9.6	0.6	1.0
Mean	9.8	0.9	2.6	6.9	0.9	1.1

[a] III = 6-methoxy-2-benzoxazolinone, VI = 2-benzoxazolinone, and IX = 6,7-dimethoxy-2-benzoxazolinone. Concentrations determined by the method of isotopic dilution of Klun and Robinson (1969). Expected deviation of the analytical method is ±0.03 mg/g.

MASS SPECTRAL EVIDENCE

The benzoxazolinones III and VI found in dried corn tissue have been demonstrated to be degradation products of cyclic hydroxamic acid precursors II and V (Tipton *et al.*, 1967). It was assumed that the 6,7-dimethoxy-2-benzoxazolinone (IX) isolated from the dried corn tissue might be similarly derived from the cyclic hydroxamic acid precursor VIII. However, efforts to isolate VIII from fresh corn tissue were not successful. This lack of success was apparently caused by decomposition of VIII to IX during purification. The following approach was therefore used to establish the existence of VIII.

Corn (inbred B49) was planted in sand and grown for 10 days in the dark. The etiolated whole plants (608 g) were collected, homogenized in a Waring Blendor with 1 l. of water, allowed to stand 30 min at room temperature, and then squeezed through cheesecloth. The filtrate was extracted with ether which was dried over Na_2SO_4 and evaporated to dryness under vacuum. The residue (0.5401 g) was extracted at room temperature with 10 ml of chloroform-methanol (95 to 5) to obtain 0.1003 g of material containing a mixture of cyclic hydroxamic acids.

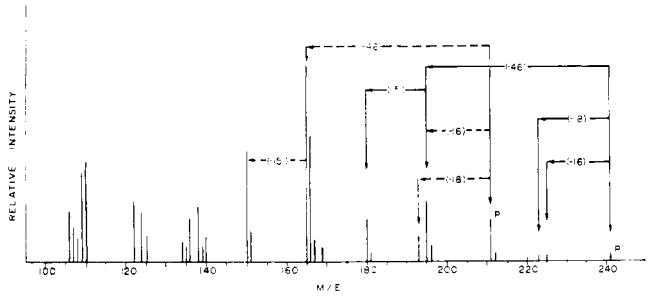

Figure 5. Mass spectrum of a mixture of cyclic hydroxamic acids isolated from corn. P and P^1 = assumed parent ions

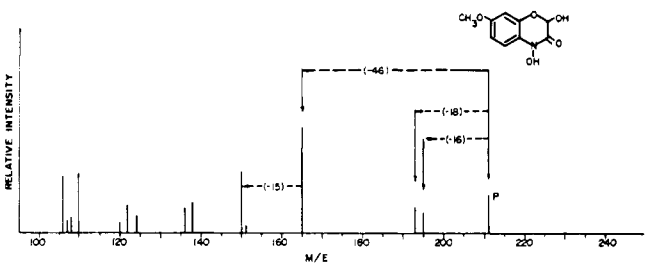

Figure 6. Mass spectrum of 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one (II). P = assumed parent ion

The mass spectra of the mixture of cyclic hydroxamic acids (Figure 5) and pure compound II (Tipton *et al.*, 1967) (Figure 6) were obtained with the solid-sample probe of the Perkin-Elmer Model 270 mass spectrometer at 70 eV. The spectrum of II (Figure 6) has a parent molecular ion (P) at *m/e* 211 and other peaks at 195 (P − oxygen), 193 (P − H_2O), 165 (P − HCOOH), and 150 (165 − CH_3). The mass spectrum of the mixture of hydroxamic acids (Figure 5) showed mainly peaks arising from II, its principal component (Table I, inbred B49), and other peaks consistent with the dimethoxy structure VIII. Thus, there was a weak peak at *m/e* 241 (P) corresponding to the molecular ion of VIII, and a series of peaks analogous to those seen in the mass spectrum of II, but they were 30 mass units higher because of the additional methoxyl group in VIII. The peaks at 225 and 223 represent the loss of oxygen and H_2O from the parent molecular ion, respectively. The loss of 46 *m/e* (HCOOH) from the parent ion *m/e* 241 gives a fragment at *m/e* 195, which also occurs in the mass spectrum of pure II. However, the ratio of intensities of the 195 and 193 peaks is much higher in the spectrum of the mixture of hydroxamic acids (Figure 5) than in the spectrum of the pure II (Figure 6), which indicates a contribution to the 195 peak from the fragmentation of the *m/e* 241 compound. The spectrum of the mixture of cyclic hydroxamic acids also has a peak at *m/e* 180 due to the loss of CH_3 from the *m/e* 195 species, and this peak is the analog of the *m/e* 150 peak in the mass spectrum of II. Thus, the mass spectral data indicate that the occurrence of IX in dried corn tissue results from the degradation of the cyclic hydroxamic acid precursor VIII. Presumably this precursor exists in the intact plant as a glucoside (VII), which has not yet been isolated.

CONCLUSION

A new compound, 6,7-dimethoxy-2-benzoxazolinone (IX), was isolated from the dried tissue of *Zea mays* (L.). It appears to be a degradation product of the cyclic hydroxamic acid, 2,4-dihydroxy-6,7-dimethoxy-2*H*-1,4-benzoxazin-3-one (VIII), which occurs in extracts of crushed fresh tissue. This hydroxamic acid is the dimethoxy analog of the cyclic hydroxamic acids, 2,4-dihydroxy-2*H*-1,4-benzoxazin-3-one (V) and 2,4-dihydroxy-7-methoxy-2*H*, 1,4-benzoxazin-3-one (II), previously isolated from corn. Because of the close structural relationship of these compounds, we suggest that a biosynthetic relationship may exist between the three cyclic hydroxamic acids of corn (II, V, and VIII) and that the resistance of corn to insect attack and to the 2-chloro-*s*-triazine herbicides, which has been attributed primarily to II, may be derived in part from V and VIII which also occur in resistant corn.

ACKNOWLEDGMENT

We thank Barbara Bierl for the nmr spectra and John Ruth for part of the mass spectra data used in this study. They are with the ARS, Entomology Research Division, Pesticide Chemicals Research Branch, Beltsville, Md.

LITERATURE CITED

BeMiller, J. N., Pappelis, A. J., *Phytopathology* **55,** 1237 (1965).
Brendenberg, J. B., Honkanen, E., Virtanen, A. I., *Acta Chem. Scand.* **16,** 135 (1962).
Elnaghy, M. A., Linko, P., *Physiol. Plant.* **15,** 764 (1962).
Hamilton, R. H., J. AGR. FOOD CHEM. **12,** 14 (1964).
Henrich, F., Binkner, H., *Berichte* **46,** 3380 (1913).
Honkanen, E., Virtanen, A. I., *Acta Chem. Scand.* **15,** 221 (1961).
Klun, J. A., Robinson, J. F., *J. Econ. Entomol.* **62,** 214 (1969).
Klun, J. A., Tipton, C. L., Brindley, T. A., *J. Econ. Entomol.* **60,** 1529 (1967).
Molot, P. M., Anglade, P., *Ann. Epiphyt.* **19,** 75 (1968).
Roth, W., Knusli, E., *Experientia* **17,** 312 (1961).
Smissman, E. E., Lapidus, J. B., Beck, S. D., *J. Amer. Chem. Soc.* **79,** 4697 (1957).
Tipton, C. L., Klun, J. A., Husted, R. R., Pierson, M., *Biochemistry* **6,** 2866 (1967).

Received for review January 2, 1970. Accepted May 15, 1970. Journal Paper No. J-6371 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Project No. 1648. Mention of a proprietary product in this paper does not constitute an endorsement of this product by the USDA.