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Cyclic Hydroxamic Acids and Related Compounds from Maize. Isolation and Characterization*

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ABSTRACT: Two cyclic hydroxamic acids have been isolated from seedlings of the inbred strain of maize (CI31A). The major component, 2,4-dihydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one, is accompanied by small amounts of the analog lacking a methoxyl group. The related lactam 2-hydroxy-7-methoxy-1,4-(2H)-benzoxazin-3-one has been isolated from older plants. All three compounds occur in the intact plants as glucosides. This is the first report of the natural occurrence of this lactam or its glucoside.

Two cyclic hydroxamic acids which occur in cereal grasses have been described (Wahlroos and Virtanen, 1959; Virtanen and Hietala, 1960; Hietala and Virtanen, 1960). These compounds occur mainly as glucosides (Figure 1) from which the aglucones are rapidly released by enzymatic hydrolysis after the crushing or homogenization of the plants, although small amounts of the free aglucone V have been isolated from maize (Wahlroos and Virtanen, 1964). The aglucones, in turn, decompose in water to benzoxazolinones (Honkanen and Virtanen, 1961; Bredenberg *et al.*, 1962). Isolation of 2(3)-benzoxazolinone (III) from rye seedlings by Virtanen and Hietala (1955) led to the later isolation of the cyclic hydroxamic acids. Rye seedlings were reported to yield I and II (Virtanen and Hietala, 1960; Hietala and Virtanen, 1960) but the methoxy compounds IV and V were isolated from wheat and maize seedlings (Wahlroos and Virtanen, 1959). These appear to be the only examples now known of hydroxamic acids occurring in higher plants, although there are numerous reports of hydroxamic acids isolated from microorganisms (*cf.* Emery, 1965).

The cyclic hydroxamic acids have attracted attention because of their relationship to several phenomena of agronomic importance. Elnaghy and Linko (1962) have suggested a correlation between cyclic hydroxamic acid content and stem rust resistance in wheat, while BeMiller and Pappelis (1965) suggest a similar correlation with resistance of maize strains to stalk rot. Tolerance of certain plants to 2-chloro-*s*-triazine herbicides has also been related to the cyclic hydroxamic acids (Roth and Knusli, 1961; Hamilton, 1964). A linear relationship between the logarithm of the cyclic hydroxamic acid content of various inbred strains of maize and the resistance of these strains to attack by European corn borer (*Ostrinia nubilalis*) larvae has been inferred (Klun and Brindley, 1966). Purified V has been shown to be a feeding deterrent for corn borer larvae when added to a defined nutrient medium (Klun *et al.*, 1967).

Despite widespread interest in the properties of the cyclic hydroxamic acids, to our knowledge only a single investigation of the biosynthesis of these compounds has been reported (Reimann and Byerrum, 1964). These authors determined the derivation of the carbon atoms of V; however, the mechanism of synthesis of the hydroxamic acid functional group ha not been investigated. In the investigation reported here, compounds related to V have been isolated and identified. The structural relationships of these compounds suggest possible biosynthetic and functional relationships.

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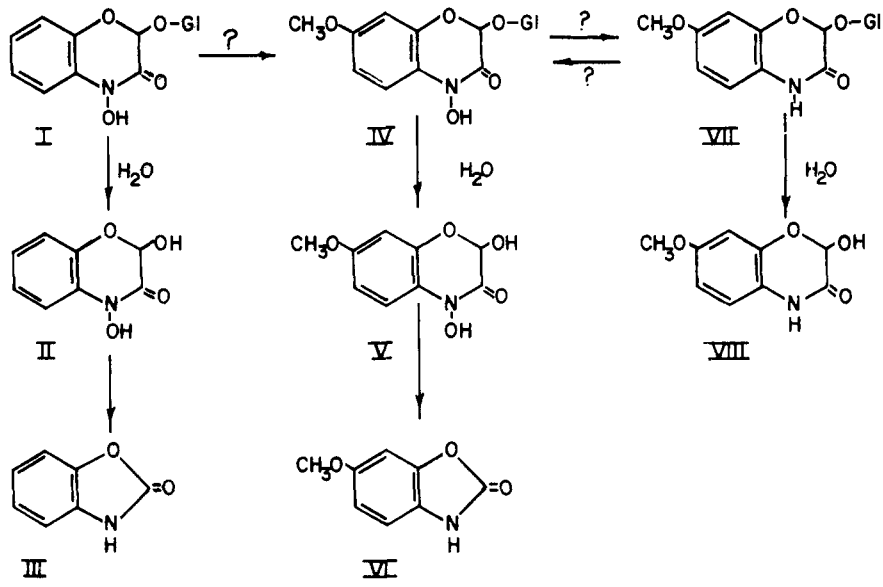

FIGURE 1: Structures of cyclic hydroxamic acids and related compounds found in young maize plants.

Experimental Section

Infrared spectra were obtained with a Beckman IR-8[1] spectrophotometer equipped with a beam condenser, with samples prepared in KBr pellets using a Beckman micropellet die. Only the most prominent absorptions, which were of significance in identifying the compounds, are reported. Molecular weights were determined from mass spectra, obtained with an Atlas CH 4 mass spectrometer.[2]

Chromatographic column eluates were monitored by recording absorption at 254 mμ with a Model UA ultraviolet analyzer, Instrumentation Specialities Co., Lincoln, Neb. Thin layer chromatography was carried out with plates prepared from silica gel G or GF_{254} (Brinkman Instruments, Inc., Westbury, N. Y.). Except where specified otherwise, the solvent was diethyl ether saturated with water–formic acid (99:1, v/v). Hydroxamic acids were detected by spraying with a reagent consisting of 50 g of $FeCl_3 \cdot 6\ H_2O$ in 500 ml of 95% ethanol made 0.1 N in HCl by addition of concentrated HCl. Powdered polyamide for column chromatography was a product of M. Woelm, Eschwege, Germany (Alupharm Chemicals, New Orleans, La.).

The isolations reported here started with seedlings or young plants of the inbred strain of maize (CI31A). Seedlings were grown to a height of approximately 15 cm in moist sand in wooden boxes. Although most of the isolations were carried out with etiolated seedlings grown in covered boxes, the same procedures have been used successfully with green seedlings. For some isolations, the whorl portions of plants grown in the field to a height of about 90 cm were used.

2,4-Dihydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one (*V*). In a typical isolation, 545 g (fresh weight; dry weight 6.9%) of etiolated seedlings that had been cut just above the surface of the sand was homogenized in a Waring Blendor with 800 ml of H_2O. The homogenate was filtered through six layers of cheesecloth, and the fibrous residue was squeezed dry. After standing for 1 hr at room temperature to allow enzymatic hydrolysis of the glucoside, the filtrate was divided into two portions in 1-l. erlenmeyer flasks, which were placed in a boiling water bath and swirled rapidly until the contents reached 65°. The filtrate was then cooled rapidly in an ice bath and the coagulated protein was removed by vacuum filtration using Whatman No. 42 filter paper. This filtrate was extracted with several portions of diethyl ether, and the combined extracts were dried over anhydrous $MgSO_4$ and evaporated to dryness on a rotary evaporator, yielding 0.785 g of brown residue.

The residue was washed with a small volume of chloroform–methanol (95:5, v/v), the solution was evaporated to dryness on a rotary evaporator, and the residue was set aside. The material that did not dissolve in chloroform–methanol was dissolved in a minimum quantity of warm acetone; hexane (Skelly B) was added until the solution became turbid. Upon standing, nearly pure product crystallized (0.173 g). Two recrystallizations from the same solvent yielded 0.108 g of material, which gave a single $FeCl_3$-positive spot on thin layer chromatography in ether–formic acid: mp 162°, $\lambda_{max\ (H_2O)}$ 262 mμ, shoulder at 285 mμ; after heating in water at 92°, 15 min: λ_{max} 283 mμ; mol wt 211; infrared spectra 3350, 3150, 1660–1670, and 1603

[1] The mention of firm names or trade products does not imply that they are recommended by the Department of Agriculture over other firms or similar products not mentioned.

[2] We are indebted to Dr. Thomas H. Kinstle, Department of Chemistry, Iowa State University, in whose laboratory the mass spectra were obtained, for helpful discussions.

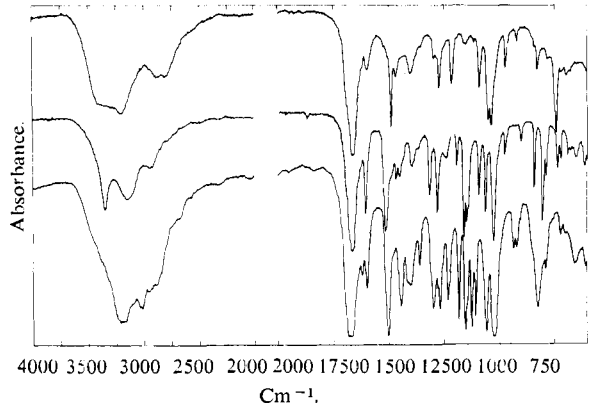

FIGURE 2: Infrared spectra of cyclic hydroxamates (II, top; V, middle) and a lactam (VIII, bottom) in KBr pellets.

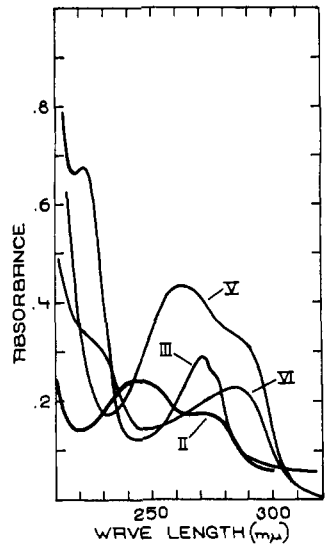

FIGURE 3: Ultraviolet absorption spectra of cyclic hydroxamic acids and the benzoxazolinones formed by heating in water. The absorbance of III is multiplied 2 × relative to the other spectra.

cm^{-1}. *Anal.* Calcd for $C_9H_9NO_5$: C, 51.19; H, 4.30; N, 6.63. Found: C, 51.13; H, 4.46; N, 6.72.

2,4-Dihydroxy-1,4(2H)-benzoxazin-3-one (II). Pooled chloroform–methanol-soluble fractions obtained during the purification of several batches of V were dissolved in acetone; hexane (Skelly B) was added until the solution was turbid. The crystals that formed upon standing were recrystallized from methanol.

DEAE-cellulose (20 g) (Applied Science Laboratories, State College, Pa.) was converted to the acetate form by treatment with a mixture of glacial acetic acid and 95% ethanol (1:1 v/v), washed thoroughly with 95% ethanol, and packed in a column (30-mm i.d.) to a height of 27 cm. The material that had crystallized from methanol was applied to this column in 95% ethanol and eluted with the same solvent. Two large, partly resolved peaks were observed, only the second of which formed a colored complex with ferric chloride. The tubes from this peak were pooled and evaporated to dryness. The product was crystallized, first by dissolving in acetone and adding several volumes of Skelly B, then by dissolving in diethyl ether and adding several volumes of cyclohexane. From the latter solvent colorless needles separated: mp 155°, $\lambda_{max\ (H_2O)}$ 253 mμ, shoulder at 285 mμ; after heating in water at 100° for 20 min: λ_{max} 271 mμ, shoulder at 277 mμ; mol wt 181; infrared spectra 3400, 3200, 2870, 2800, 1660, and 1595 cm^{-1}.

2(3)-Benzoxazolinone (III). The whorl portions of plants 90 cm tall were collected in the field, frozen in polyethylene bags, and stored in the frozen state. They were later thawed, dried, and ground in a Wiley mill. A 100-g portion of dried tissue was refluxed for 2 hr with 2 l. of water, filtered, and the filtrate was adjusted to pH 1 with concentrated HCl. The acidified filtrate was extracted with diethyl ether, and the extract was dried over $MgSO_4$ and evaporated to dryness. The resulting residue was dissolved in benzene–ethyl acetate (1:1, v/v). Compound III was isolated from this extract by preparative thin layer chromatography on plates of silica gel GF_{254}. As a reference compound, III synthesized by fusion of *o*-aminophenol and urea as described by Smissman *et al.* (1957) was used. Compound III and other ultraviolet-absorbing compounds were located as dark spots on the plates when viewed under a short-wavelength ultraviolet lamp. The extract was chromatographed successively in chloroform–ethyl acetate–cyclohexane (4:4:2, v/v), cyclohexane–isobutyl alcohol (85:15, v/v), and chloroform–ethyl acetate (9:1, v/v). After each development, the band corresponding in R_F to III was scraped from the plate and eluted with ethyl acetate–benzene (1:1, v/v). This solution was evaporated to dryness, redissolved, and applied to another plate for chromatography in the next solvent. After development in the third solvent, the material was eluted with 95% ethanol. The ultraviolet spectrum of this solution was identical with that of the synthetic material: λ_{max} 222–223 mμ, 274 mμ with a shoulder at 276 mμ; λ_{min} 240 mμ; infrared spectra 3240, 1775, 1738, and 1480 cm^{-1}, also identical with the synthetic material.

2-O-Glucosyl-7-methoxy-1,4(2H)-benzoxazin-2-one (VII). Whorl portions of corn grown in the field were used for this isolation, in which the procedure of Wahlroos and Virtanen (1959) for the isolation of IV was followed. A total of 26.4 kg of material was processed in several batches. The leaves were cut into small pieces and put in hot water, keeping the temperature above 80°. After heating for 15 min, the leaves and water were transferred to a large Waring Blendor and homogenized. The homogenate was squeezed through several layers of cheesecloth and the filtrate was concentrated *in vacuo* to a small volume. The sediment that appeared was removed by centrifugation, after which the filtrate was extracted six times with

1-butanol. The butanol was removed with a rotary evaporator, and the residue was dissolved in water and extracted several times with ether. The aqueous solution was treated with charcoal as described by Wahlroos and Virtanen (1959), then chromatographed on a polyamide column with water as the solvent. Ultraviolet-absorbing fractions that gave a positive ferric chloride test were pooled and concentrated to a small volume. A white precipitate which formed was collected by filtration; the filtrate was saved for the isolation of IV. The white precipitate was crystallized from absolute ethanol, yielding colorless crystals which gave only a weak positive test with $FeCl_3$: mp 250–251°; $\lambda_{max\ (H_2O)}$ 262 mμ (ε 7800); mol wt 357; infrared spectra 3439, 1665, 1515, 1070, and 1020 cm^{-1}, while IV[3] absorbed at 3400, 1680, 1600, 1503, 1060, and 1007 cm^{-1}. *Anal.* Calcd for $C_{15}H_{19}NO_9$: C, 50.42; H, 5.36; N, 3.92. Found: C, 50.12; H, 5.16; N, 3.93.

2-Hydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one (VIII). This compound was obtained by enzymatic hydrolysis of the glucoside with a crude protein preparation from corn seedlings. Four plants, about 25 cm tall, were ground with sand in a mortar containing 20 ml of sodium phosphate buffer (0.2 M, pH 6.0). The mixture was filtered with suction, and the residue was washed with an additional 30 ml of the buffer. A portion (2 ml) of this filtrate was placed on a column (2.4 × 27 cm) of Sephadex G-25 and eluted with the same buffer. The first ultraviolet-absorbing peak was assumed to contain the protein in the sample, including the desired glucosidase. A sample (101.3 mg) of the glucoside was dissolved in 10.2 ml of this protein solution and allowed to stand at room temperature. The progress of the hydrolysis was followed by measuring glucose released by use of glucose oxidase (Fleming and Pegler, 1963). The aglucone crystallized on the side of the container in nearly pure form. After being washed with water and air dried, the material was characterized: mp 198.5–200°; $\lambda_{max\ (H_2O)}$ 260 mμ (ε 10,500); mol wt 195; infrared spectra 3200, 1660, 1500, and 1020 cm^{-1}. *Anal.* Calcd for $C_9H_9NO_4$: C, 55.38; H, 4.65; N, 7.18. Found: C, 55.26; H, 5.16; N, 7.12.

Results and Discussion

The procedure followed for isolation of V is a modification of that used by Wahlroos and Virtanen (1959). Thin layer chromatography of crude ether extracts of seedlings revealed the presence of small quantities of a second $FeCl_3$-positive material having a slightly higher R_F than V. Thin layer chromatography of a similar extract of rye seedlings, the source from which Virtanen and Hietala (1960) had isolated II, resulted in a single $FeCl_3$-positive spot at the same R_F as the minor component from maize. Both gave a purple color with $FeCl_3$, while V forms a deep blue complex. Purification of II from maize extracts was made difficult by the presence of the degradation product of V, 6-methoxy-2(3)-benzoxazolinone (VI) which has chromatographic and solubility properties very similar to those of II. Advantage was finally taken of the weakly acidic nature of the hydroxamic acid and separation from VI was achieved on an ion-exchange column. The purified II has no infrared absorption in the region 1700–1800 cm^{-1}, indicating it is free of contamination by benzoxazolinones, which absorb strongly in that region. The infrared spectra of II and V (shown in Figure 2) are very similar and consistent with the suggested structures.

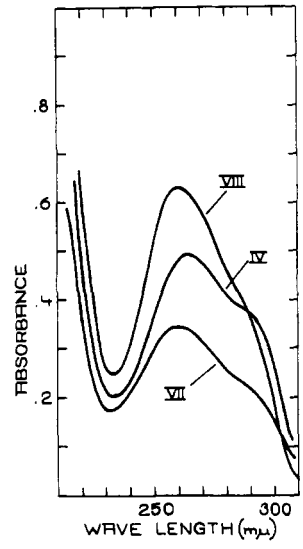

FIGURE 4: Ultraviolet absorption spectra, in 95% ethanol, of a cyclic hydroxamic acid (IV), the corresponding lactam (VIII), and the glucoside of the lactam (VII).

In Figure 3 are shown the ultraviolet spectra of II and V and of the products formed by heating them in aqueous solution. The formation of materials absorbing maximally at 272 and 284 mμ, respectively, is further proof of the identification of II and V (Wahlroos and Virtanen, 1959; Honkanen and Virtanen, 1961).

The occurrence of II was confirmed by isolation of 2(3)-benzoxazolinone (III) from plant material in which the cyclic hydroxamic acids had been allowed to decompose to the corresponding benzoxazolinones (Bredenberg *et al.*, 1962). Presumably II was present in the intact plants as the 2-*O*-glucosyl derivative (I), although we have not isolated the latter compound. The occurrence of small amounts of II in the seedlings suggests that IV may be synthesized in the plant by hydroxylation of I, followed by methylation of the resulting phenol. Attempts to detect such a phenolic compound or the expected degradation product, 6-hydroxybenzoxazolinone, have not yet been successful.

When maize plants grown in the field to about 90 cm in height (about 2 months from the time of planting) were extracted and worked up by the procedure of Wahlroos and Virtanen (1959), the lactam analogous to IV was obtained.

Enzymatic hydrolysis of this lactam glucoside

[3] Authentic sample obtained from Professor A. I. Virtanen.

yielded glucose and the water-insoluble aglucone. The ultraviolet spectra of VII and VIII (Figure 4) are very similar to those of IV and V. Similarly, Virtanen and Hietala (1960) report that reduction of II to the lactam causes only a small shift to a shorter wavelength in the ultraviolet absorption maximum. The molecular weights of the glucoside and aglucone were found to be 16 less than those of IV and V, respectively. The infrared spectrum of VIII (Figure 2) is consistent with a lactam structure and differs most obviously from the spectra of the hydroxamic acids in lacking the absorption band at 3350 or 3400 cm^{-1}. The biosynthesis of this group of related 1,4-benzoxazinones is being investigated, with particular attention to the metabolic relationships of the lactams and cyclic hydroxamates.

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