ISOLATION AND CHARACTERIZATION OF A CYCLIC HYDROXAMATE FROM *Zea mays*[1]

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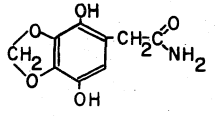
ABSTRACT

A sweet compound with phenolic properties has been isolated from corn seedlings. It yields 6-methoxybenzoxazolinone as a degradation product and appears to be the cyclic hydroxamate 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one.

Etiolated corn seedlings were noted to have a very sweet, saccharin-like taste. The compound responsible for the taste, here designated as corn sweet substance (CSS), has been isolated and characterized:



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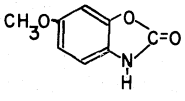

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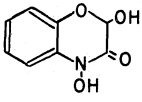

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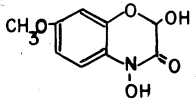

IV

CSS was identical with a substance previously isolated from corn seedlings by Suhadolnik (13), who suggested structure I, but this structure is inconsistent with the finding that the substance is converted readily to 6-methoxybenzoxazolinone II. The latter compound, II, has been directly isolated from corn seedlings (12,18), corn stalks (20), wheat seedlings (18), and grasses of genus *Coix* (9). Virtanen and Hietala (16) previously reported the isolation of benzoxazolinone itself from rye seedlings. Recent work by Virtanen and co-workers (7,8,17,19) indicates that the benzoxazolinone derivatives isolated from corn, wheat, and rye are artifacts formed by action of enzymes or heat during the isolation. The precursor from corn seedlings exists as a monoglucoside which is converted to the aglucone by treatment with press juice from the seedlings (19). This aglucone is identical with the sweet compound (CSS). Honkanen and Virtanen (8) confirmed by synthesis that structure III is the aglucone from rye seedlings, and Wahlroos and Virtanen (19) suggest that the precursor from corn is the glucoside of the analogous 6-methoxy derivative, IV. CSS was isolated in the present study by direct homogenization of the corn shoots with ethanol, which yielded about 0.5% of the

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seedling dry weight of CSS. CSS and its benzoxazolinone degradation products have been characterized, and the data confirm results of Virtanen and co-workers.

Experimental

Ultraviolet spectra were measured with a Beckman DU spectrophotometer, and infrared spectra with a Beckman IR-5 spectrophotometer. All melting points are uncorrected. Paper chromatography: with Whatman 3MM paper, ascending. Molecular weights were determined by the isothermal distillation method of Childs (2), or freezing-point depression of tert-butyl alcohol, as noted. Carbon and hydrogen were determined by the Pregl method, nitrogen by the Dumas method (11). Methoxyl groups were determined by the hydriodic acid method of Zeisel (15), and N-methyl by hydriodic acid pyrolysis (4). The C-methyl determination was by chromic-sulfuric acid oxidation to acetic acid (11), and active hydrogen by lithium aluminum hydride reduction (1)[3].

Isolation. Michigan 350 etiolated corn seedlings 5 days old (3.6 kg. fresh weight) were homogenized in a Waring Blendor with sufficient 95% ethanol to make the extract 80% ethanol. After filtration, the bright-yellow ethanol extract (20 l.) was concentrated *in vacuo* at 37°C. in a flash evaporator. The pH of the concentrated solution (about 800 ml.) was adjusted to 4.0 with 5*N* phosphoric acid, and it was extracted three times with 800-ml. portions of ethyl ether. The combined ether fractions were reduced in volume to 500 ml. and then partitioned twice with 100-ml. portions of 8% sodium bicarbonate in an atmosphere of hydrogen. The combined bicarbonate fractions were acidified (pH 4) and extracted three times with 200-ml. portions of ether. The combined acid ether extracts were dried several hours at 1°C. over anhydrous sodium sulfate, and then evaporated to dryness *in vacuo*. The resulting light-tan, semicrystalline residue was washed twice with ethyl ether (5 ml.), and dissolved in a minimum volume of hot acetone. Ligroin was added to the point of faint turbidity and, upon cooling, 2.50 g. of light-buff crystals were obtained. Upon recrystallization from the same solvent, 1.85 g. of almost-white anisotropic crystals, m.p. 160°–161°C. (decomp.) resulted. Analysis calculated for $C_9H_9O_5N$: C, 51.30; H, 4.27; N, 6.64; OCH_3, 14.65; 2 active H, 0.95%; m.w. or neut. equiv., 211. Found: C, 51.34; H, 4.43; N, 6.73; OCH_3, 14.05; active H (lithium aluminum hydride in

[3] Microanalyses for C, H, and N were conducted by Micro-Tech Laboratories, 8000 Lincoln Ave., Skokie, Ill. Methoxyl, C-methyl, N-methyl, and active hydrogen analyses were by Schwarzkopf Laboratories, 5619 37th Ave., Woodside 77, N.Y.

N,N-dimethylmorpholine), 1.09%; m.w. 212 (2) or 199 (freezing-point depression of tert-butyl alcohol); neut. equiv., 195. Paper chromatography: in water, *n*-butyl alcohol-27% acetic acid (1:1), *m*-cresol-water-acetic acid (50:2:48), or 73% phenol. The R_f values were respectively 0.90, 0.95, 0.75, and 0.88–0.92 as observed with a 253-mμ ultraviolet light, or brown spots developed after exposure to ammonia. Column chromatography (20 ml. 0.1*M* potassium phosphate buffer, pH 6.5, adsorbed on 25 g. Celite in a 1.8-cm.-diameter column using buffer-saturated ether as the mobile phase) yielded one peak eluted in the 18- to 27-ml. fractions as detected by 265-mμ absorbance and ferric chloride color. The ultraviolet spectrum was determined at several pH values (Fig. 1), and an infrared spectrum was obtained (Fig. 2).

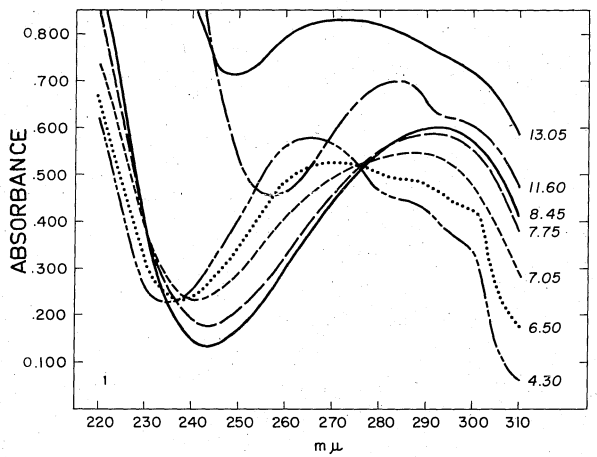

Fig. 1. Ultraviolet spectra of the corn sweet substance (CSS) as a function of pH. Buffers used were 0.1*M* potassium phosphate (4.30, 6.50, and 7.05), tris-(hydroxymethyl) amminomethane (7.75 and 8.45). A solution of 0.005*N* NaOH was used for a pH value of 11.60 and a solution of 0.1*N* KOH for pH value of 13.05 (10 μg. per ml. in a 1-cm. cell).

Formation of 6-Methoxybenzoxazolinone, Formic Acid, and Ammonia upon Alkaline Hydrolysis. CSS (50 mg.) was heated with 3 ml. of 1*N* sodium hydroxide and 0.001*N* ethylenediaminetetraacetic acid under hydrogen at 75°C. for 1 hour. Upon acidification (1*N* hydrochloric acid) and cooling, 15 mg. of red needles were obtained, m.p. 153°–154°. The product was recrystallized from hot water, yielding 9.2 mg., m.p. 153°–153.5°. It was not sweet; was ferric chloride-negative, slightly soluble in dilute alkali, and insoluble in dilute acid. During the hydrolysis, about 20% of theory of ammonia was evolved. In

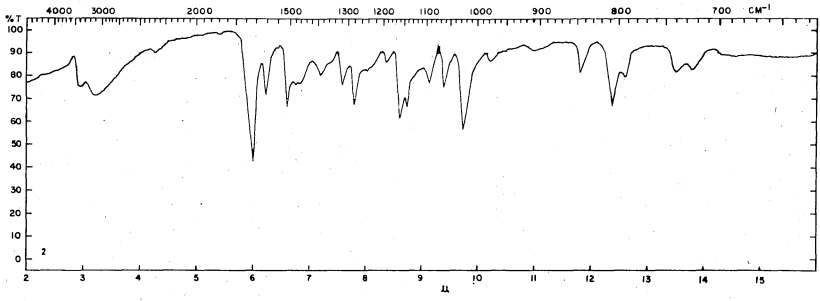

Fig. 2. Infrared spectrum of the isolated sweet substance from corn. A composite spectrum determined on a mull in Nujol "mineral oil" and hexachlorobutadiene.

other experiments, with prolonged hydrolysis, a maximum of about 46% of theory of ammonia was obtained in 5 hours. When the filtrate was steam-distilled, about 1.3 equivalent of volatile acid was obtained which was identified as formic acid by chromatography as the ammonium salt (ethanol-ammonia-water, 80:4:16) and by reduction to formaldehyde (5). The latter was detected with chromotropic acid (5). The red needles had λmax. 233 (ϵ, 10,100), λmax. 290 (ϵ, 5,675), and λmin. 255 mμ. Analysis calculated for $C_8H_7O_3N$: C, 58.18; H, 4.24; N, 8.48%; m.w., 165. Found: C, 57.85; H, 4.31; N, 8.73%; m.w., 214. The isolated sample was compared by infrared spectra, ultraviolet spectra, and mixed melting point with synthetic 6-methoxybenzoxazolinone (6,9) and found to be identical. Infrared spectrum (chloroform, main peaks only): 3,490 (w), 3,200 (w), 1,764 (s), 1,498 (s), 1,310 (m), 1,140 (m), 1,098 (m), 950 (m) cm.$^{-1}$.

N-Acetyl-6-Methoxybenzoxazolinone. CSS (77.5 mg.) was dissolved in dry pyridine (3 ml.), and acetic anhydride (7.5 ml.) was added (1). The large test tube was plugged with cotton and heated in a boiling-water bath for 10 minutes. After chilling, cold distilled water (about 30 ml.) was added and gray-white needles were obtained. These were collected on a filter, washed with cold water, and recrystallized from 70% ethanol. After recrystallization, 15.4 mg. of white crystals, m.p. 147°–148°C., were obtained. They were insoluble in 1*N* sodium bicarbonate and 1*N* hydrochloric acid, but were very soluble in methanol or ethanol. Analysis calculated for $C_{10}H_9O_4N$: C, 57.97; H, 4.35; N, 6.76; OCH_3, 14.97%; m.w. 207. Found: C, 57.85; H, 4.29; N, 6.97; OCH_3, 14.97%; m.w. 215. Infrared spectrum (CCl_4): 1,795 (s), 1,720 (m), 1,490 (m), 1,370 (w), 1,302 (s), 1,192 (w), 1,168 (w), 1,140 (w), 1,038 (s), 1,022 (m), 1,012 (m) cm.$^{-1}$.

N-Methyl-6-Methoxybenzoxazolinone. Dimethyl sulfate was shaken

with solid potassium carbonate and redistilled *in vacuo*. Methylation was carried out by two procedures, both yielding the same product. In the first method, 325 mg. of CSS were dissolved in methanol (20 ml.), and 0.75 ml. of saturated methanolic potassium hydroxide and 0.6 g. of dimethyl sulfate were added under nitrogen with stirring. The solution was brought to reflux, and cooled; sample then tested negative with ferric chloride. The solution had become acidic, so 0.2 ml. of saturated methanolic potassium hydroxide was added, and after standing 90 minutes, 80 ml. of water were added. The solution was cooled to 1°C. After 3 hours, the light-brown crystals were collected on a filter, washed with cold water, and dried at 60°C. *in vacuo*. The yield was about 161 mg., mp. 105°–107°C. The product was recrystallized from 50% methanol, yielding 100 mg. of light-tan platelets, m.p. 105°–107°C. The product was insoluble in water, dilute hydrochloric acid, and dilute alkali; but was soluble in methanol, ethyl ether, or chloroform.

In the second method, 105.5 mg. of CSS were dissolved in acetone (40 ml.). Solid potassium carbonate (4 g.) and an excess of dimethyl sulfate (0.45 g.) were added to the solution under reflux; this resulted in a greenish color, changing to a pale, yellow-brown. After 1 hour of refluxing, another portion (0.45 g.) of dimethyl sulfate was added. After an additional hour, the solution was poured into 90 ml. of 1*N* sodium hydroxide and allowed to stand overnight at 1°C. The acetone was removed *in vacuo* and the solution was extracted twice with 90-ml. portions of ethyl ether. The combined ether fraction was dried (anhydrous sodium sulfate) and concentrated to dryness *in vacuo*. A light-brown, aromatic, ferric chloride-negative residue was obtained. The residue was recrystallized from aqueous ethanol, yielding 44 mg. of light-brown platelets, m.p. 96°–99°C. The infrared spectrum of this preparation was the same as that of the first preparation except for weak bands at 3,200 $cm.^{-1}$ and 3,450 $cm.^{-1}$. By sublimation at 110°/25 mm. Hg, white crystals were obtained from both preparations, m.p. 107°–108°. Analysis calculated for $C_9H_9O_3N$: C, 60.40; H, 5.03; N, 7.83; OCH_3, 17.31; NCH_3, 8.38%; m.w. 179. Found: C, 60.31; H, 5.18; N, 7.42; OCH_3, 17.07; NCH_3, 2.78%; m.w. 217. The ultraviolet spectra indicated λ max. at 235 and 290 mμ and λ min. at 255 mμ in 0.05*N* HCl or 0.05*N* NaOH. Infrared spectrum (CCl_4): 1,783 (s), 1,613 (w), 1,500 (s), 1,457 (m), 1,370 (m), 1,310 (w), 1,270 (m), 1,195 (m), 1,158 (s), 1,060 (m), 1,027 (m,w), 948 (m), 938 (m) $cm.^{-1}$

Discussion

The sweet substance (CSS), after isolation and crystallization, had

an m.p. of 160°–161°C. (decomp.). Paper and column chromatoggraphy revealed only one component, but paper chromatography (*n*-butyl alcohol-acetic acid-water) of crude corn extracts indicated the presence of another compound (R_f 0.50) giving the same ferric chloride color as the sweet compound (R_f 0.55). Urban (14) also detected these two compounds on chromatograms of corn-seedling extracts, and it appears the substance of lower R_f is the monoglucoside of CSS (15).

The ultraviolet-absorption spectrum (Fig. 1) showed a long-wave spectral shift of the 265-mμ acid peak as the pH was varied. In confirmation with the titration data, as determined from this shift, the pKa was 7.0. There was, in addition, a short-wave shift in the ultraviolet spectra and a considerable increase in absorbance at pH values above 8.5. This latter shift was immediate and CSS could be reisolated by acidification and extraction with ether.

A comparison of the alkaline hydrolysis product with synthetic 6-methoxybenzoxazolinone revealed that the ultraviolet spectra, infrared spectra, and melting points were identical (9,12). The red color of the isolated sample was probably due to traces of the oxidation products of 2-amino-5-methoxyphenol.

An examination of the ether derivative by nuclear magnetic resonance clearly showed the presence of an O-methyl and an N-methyl group. The N-methyl determination was low, but this is not unusual for heterocyclic compounds (10). Confirmation that the ether derivative is N-methyl-6-methoxybenzoxazolinone is that the melting point (107°), ultraviolet maxima (234 mμ, 290 mμ), and the carbonyl band in the infrared spectra correspond closely to the reported values of Koyama *et al.* (9). The melting point (6) and the absence of an N—H stretch, as well as the lack of a strong band in the 1200 $cm.^{-1}$ region, and the two carbonyl bands at 1795 $cm.^{-1}$ and 1720 $cm.^{-1}$, indicated that the acetate derivative of the sweet compound was N-acetyl-6-methoxybenzoxazolinone.

Mass thermal degradation of CSS *in vacuo* resulted in products of mass 195, 193, 165, 150, and 140. The product with mass 195 appeared to have lost oxygen, a rather unique degradation product. The loss of oxygen upon heating the cyclic hydroxamate aspergillic acid under N_2 with cupric chromite has been reported (3). A comparison of the infrared spectra of CSS (Fig. 2) with that found for the aglucone from rye (8) confirmed that they are analogs.

Acknowledgments

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