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# THE ISOLATION OF 2-(2-HYDROXY-7-METHOXY-1,4-BENZOXAZIN-3-ONE)β-D-GLUCOPYRANOSIDE FROM *ZEA MAYS**

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**Abstract**—A previously unknown compound, identified as a 1,4-benzoxazinone derivative, was isolated from the roots of corn plants. By comparison with other known 1,4-benzoxazinones and the use of infrared, mass, nuclear magnetic resonance and ultraviolet spectrophotometry and synthetic procedures the newly discovered compound was identified as 2-(2-hydroxy-7-methoxy-1,4-benzoxazin-3-one)β-D-glucopyranoside.

## INTRODUCTION

IN 1955 Virtanen and Hietala isolated a compound possessing antifungal activity from rye seedlings, which they identified as 2-(3H)-benzoxazolinone (I).[1] In the same year Koyama and Yamato isolated and identified 6-methoxy-2(3H)-benzoxazolinone (II) from *Coix Lachryma-Jobi*.[2] (II) was also isolated from corn and wheat seedlings and shown to possess antifungal activity and to be involved in the resistance of corn plants to attack by the European corn borer.[3,4]

(I) and (II) are reported to be degradation products produced during the isolation procedure from 2,4-dihydroxy-1,4-benzoxazin-3-one (III) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (IV).[5] (III) and (IV) have been shown to exist in the plant as the 2-glucosides; the sugar is enzymatically removed during the isolation procedure.[6,7]

(III) and (IV) possess antifungal activity, but the related glucosides are rather inactive. Several investigators have implicated (III), (IV) and the related glucosides in the detoxification of simazine and atrazine in plants.[8–10]

We wish to report the isolation and identification of a previously unknown 1,4-benzoxazin-3-one derivative from corn roots, 2-(2-hydroxy-7-methoxy-1,4-benzoxazin-3-one)β-D-glucopyranoside. A preliminary communication on this work has been published.[11]

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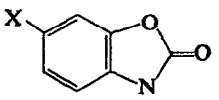
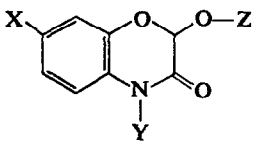
[1] A. I. VIRTANEN and P. K. HIETALA, *Acta Chem. Scand.* **9**, 1543 (1955).
[2] T. KOYAMA and M. YAMATO, *J. Pharm. Soc. Japan* **75**, 699 (1955).
[3] E. E. SMISSMAN, J. B. LAPIDUS and S. O. BECK, *J. Org. Chem.* **22**, 220 (1957).
[4] A. I. VIRTANEN, P. K. HIETALA and O. WAHLROOS, *Arch. Biochem. Biophys.* **69**, 486 (1957).
[5] A. I. VIRTANEN and P. K. HIETALA, *Acta Chem. Scand.* **14**, 499 (1960).
[6] O. WAHLROOS and A. I. VIRTANEN, *Acta Chem. Scand.* **13**, 1906 (1959).
[7] E. HONKANEN and A. I. VIRTANEN, *Acta Chem. Scand.* **14**, 504 (1960).
[8] R. H. HAMILTON and D. E. MORELAND, *Science* **135**, 373 (1962).
[9] R. H. HAMILTON, *Weeds* **12**, 27 (1964).
[10] W. ROTH and E. KNUESLI, *Experentia* **17**, 312 (1961).
[11] H. E. GAHAGAN and RALPH O. MUMMA, *Chem. Ind.* 1967 (1966).

## RESULTS

Mature corn roots were extracted in a Soxhlet with a chloroform methanol solvent. An aqueous extract of these crude concentrated corn lipids contained an aromatic glycoside which was partially purified by Florisil column chromatography. Final purification was achieved by preparative thin-layer chromatography and re-crystallization from water yielding a white crystalline compound melting at 228–235°, possessing optical activity and analysing for $C_{15}H_{19}NO_9$.



(I) X=H
(II) X=$OCH_3$



(III) X=H, Y=OH, Z=H
(IV) X=$OCH_3$, Y=OH, Z=H
(V) X=$OCH_3$, Y=OH, Z=$C_6H_{11}O_5$
(VI) X=$OCH_3$, Y=H, Z=$C_6H_{11}O_5$
(VII) X=$OCH_3$, Y=H, Z=H

Spectroscopic data on the newly isolated glycoside and the previously published information on 1,4-benzoxazin-3-one derivative suggested that the compound might be 2-(2-hydroxy-7-methoxy-1,4-benzoxazin-3-one)β-D-glucopyranoside (VI). To test this theory 2-(2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)β-D-glucoside (V) was isolated from corn seedlings and the hydroxamic acid function was chemically reduced with either sodium hydrosulfite or zinc and acetic acid to an amide function. The reduced product and the isolated glycoside (VI) were chromatographically identical and had the same melting points and mixed melting point (228–235°). The u.v., i.r., NMR, and mass spectra were obtained for the isolated glycoside and compared to a number of analogous 1.4-benzoxazin-3-one derivatives. In each case the spectra of the isolated glycoside and of the reduced product (VI) were identical.

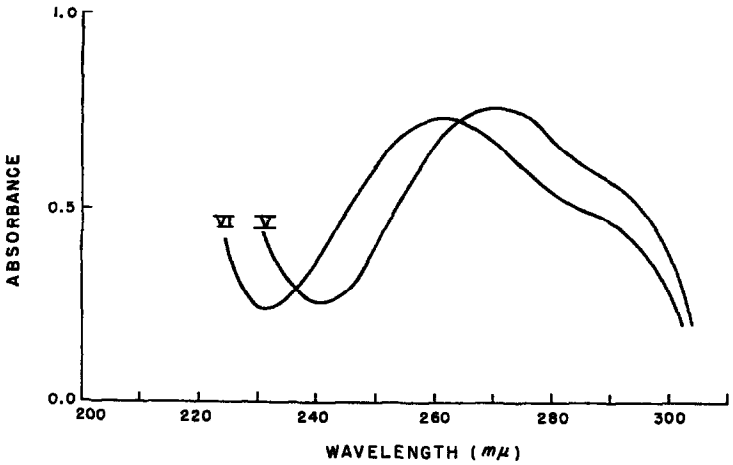



FIG. 1. ULTRA VIOLET SPECTRA OF (V) AND (VI).

The u.v. spectra of (V) and (VI) are shown in Fig. 1 and the absorption maxima are compared to those of some other 1,4-benzoxazin-3-ones in Table 1. The reduction of (V) ($\lambda_{max}$=270 nm) to produce (VI) ($\lambda_{max}$=262 nm) causes a slight hypsochromic shift. The presence of the sugar or a change from a hydroxamic acid function to an amide has little effect on the u.v. absorption.

TABLE 1. ULTRA VIOLET SPECTRA: SUMMATION OF ABSORPTION, MAXIMA

| Compound | $\lambda_{max}$ (nm) | Adsorptivity coefficient |
|---|---|---|
| Isolated glycoside (VI) | 262, sh 288 | 10,700 |
| Reduced product from (V) | 262, sh 288 | 10,400 |
| (VII) | 266, sh 288 | 10,000 |
| (V) | 270, sh 290 | — |
| (IV) | 264, sh 288 | 10,300 |
| 2-Dichloroacetamido-5-methoxyphenol | 266, 298 | — |

Infra-red spectra were obtained of the isolated and reduced glycoside (VI) and of (V). The most significant spectral shift in the change from a hydroxamic acid is the well-defined shift of the carbonyl absorption from 6·05–6·10 $\mu$ to 5·95 $\mu$.

Table 2 summarizes the NMR absorptions of (IV), (V), and (VI) and a schematic interpretation of the spectra of (V) and (VI) is presented in Fig. 2. Broad absorption attributable to the sugar protons was observed between 5·3 and 8·0 $\tau$. The C-1 proton of the hexose absorbed as a doublet at 5·37 $\tau$ ($J$=8 c/s). The coupling constant (8 c/s) requires the C-1 and C-2 protons of the hexose to be transdiaxial; suggesting a β-glucopyranoside. The chemical shift of the C-1 proton (5·37 $\tau$) is closer to that of β-D-glucopyranoside (5·50 $\tau$) than that of β-D-glucopyranoside (5·96 $\tau$) (values in deuterated dimethyl sulfoxide). This downfield shift from that expected for a β-glycoside indicates that the proton is deshielded probably by its close proximity to the heterocyclic ring.

TABLE 2. NMR SPECTRA: CORRELATION OF ABSORPTIONS AND PROTONS

| Proton | (V)* (N—OH) ($\tau$) | (VI)* (N—H) ($\tau$) | (IV)† ($\tau$) |
|---|---|---|---|
| $CH_3O$- | 6·27 | 6·27 | 6·23 |
| C-2 | 4·10 | 4·32 | 4·31 |
| C-5 | 2·61 doublet ($J$=9) | 3·07 doublet ($J$=9) | 2·73 doublet ($J$=9) |
| C-6 | 3·35 doublet ($J$=9) | 3·34 doublet ($J$=9) | 3·44 doublet ($J$=9) |
| C-8 | 3·26 | 3·25 | 3·38 |
| Anomeric proton | 5·39 doublet ($J$=7) | 5·37 doublet ($J$=7) | — |

* The solvent was deuterated dimethylsulfoxide.
† The solvent was deuterated methanol.

The absorption due to the methoxy protons (singlet, 6·27 $\tau$), C-6 (doublet, $J$=8 c/s, 3·35 $\tau$) and C-8 (singlet) of the ring system was approximately the same for (V) and (VI) (3·26 and 3·25 $\tau$). The C-5 proton absorbs at 2·61 $\tau$ (doublet, $J$=9 c/s) in (V), however, the

change from a hydroxamic acid to an amide causes an upfield shift of 0·46 ppm to 3·07 $\tau$ (doublet, $J=9$ c/s) in the isolated glycoside.

The absorption of the C-2 proton is affected by the change from hydroxamic acid to amide and is shifted from 4·10 $\tau$ (singlet) in (V) to 4·32 $\tau$ (singlet) in (VI).

The mass spectra of the isolated glycoside and the reduced product (VI) were identical and very similar to that of (V). Hamilton *et al.*[12] have reported that in the mass spectrum of (IV), under their conditions, the peak of highest mass number was P-16, corresponding to

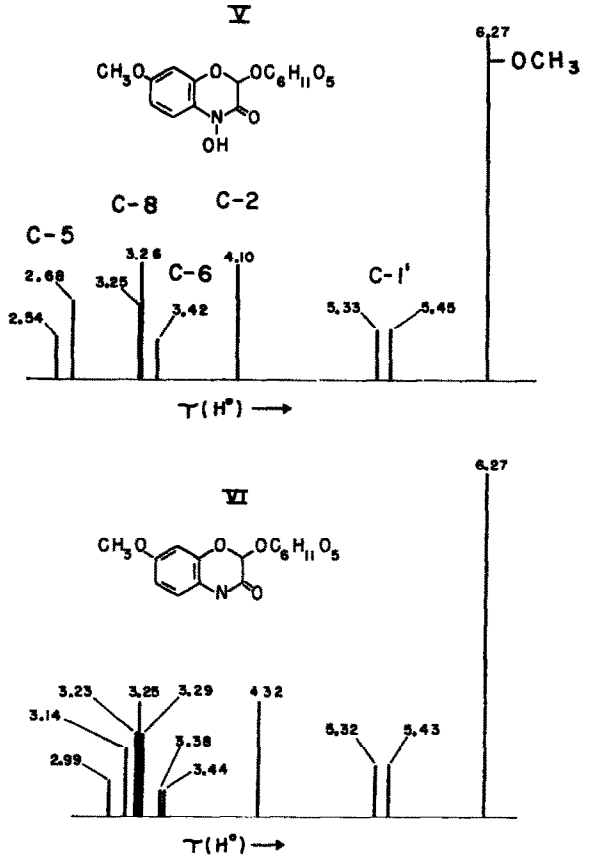



FIG. 2. SCHEMATIC REPRESENTATION OF THE NMR SPECTRA OF (V) AND (VI).

the loss of molecular oxygen. If this would occur in the case of (V), a fragment identical to (VI) would be produced and consequently, a spectrum very similar to that of (VI) would be expected.

Mass spectra were obtained by placing the solid samples directly into the ionization chamber at high temperatures and low ionizing voltage. Consequently, the relative intensities of the peaks are somewhat variable. Under our conditions parent peaks were observed with (IV), the isolated glycoside, and the reduced product (VI); the peak of highest mass number observed with (V) was P-16. In spite of their low volatility parent peaks were

[12] R. H. HAMILTON, R. S. BANDURSKI and W. H. REUSCH, *Cereal Chem.* **39**, 107 (1962).

previously observed with glycosides.[13] A consideration of the spectra of the glycosides indicates that they probably fragment into a heterocyclic portion (m/e = 195) and a sugar portion (m/e = 179) (Fig. 3). The heterocyclic fragment appears to degrade via a benzoxazolinone fragment (base peak) and an aminophenol fragment (Table 3). The degradation of the aminophenol is consistent with previous work.[14]

Wahlroos and Virtanen determined that the sugar in (V) was glucose.[6] In view of the similarities between (V) and (VI), it was deemed probable that the sugar on (VI) was also glucose.

A small amount of (VI) was hydrolyzed with 1 N methanolic HCl. Trimethylsilyl ether derivatives were prepared of the hydrolyzate, which were then injected into the gas chromatograph. The sugar derivatives in the hydrolyzate chromatographed the same as the α- and

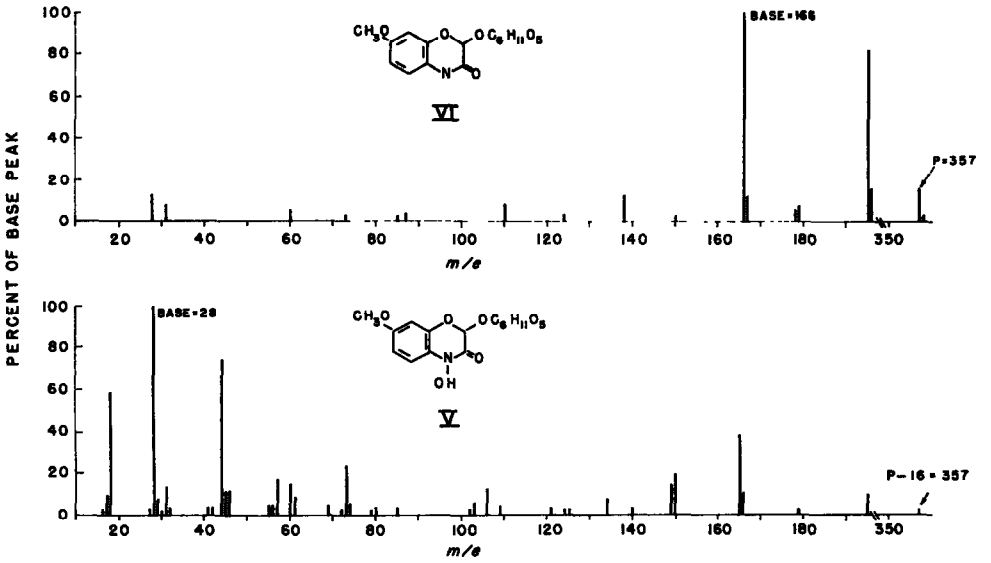



FIG. 3. MASS SPECTRA OF (V) AND (VI).

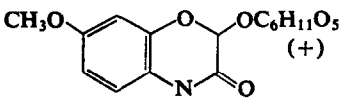
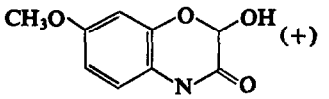
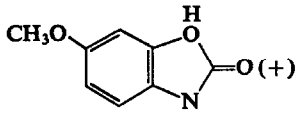
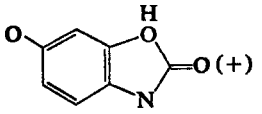
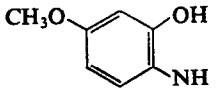
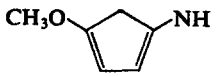
(All peaks less than 2·5 per cent were not included.)

β-methyl-D-glucopyranoside derivatives indicating that the sugar in (VI) is glucose. However, surprisingly, β-glucosidase did not appreciably hydrolyze (VI) and this supports the NMR evidence that the anomeric proton is partially shielded.

To lend further support to the proposed structure, we synthesized 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one. The synthesis was based on the procedure of Honkanen and Virtanen for the synthesis of 2-hydroxy-1,4-benzoxazin-3-one.[7] 5-Methoxy-2-nitrosophenol was reduced to the corresponding amine with sodium hydrosulfite. This was condensed with dichloroacetyl chloride to produce 2-dichloroacetamido-5-methoxyphenol, which was cyclized to 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one by heating in aqueous $NaHCO_3$ solution (see Fig. 4).

[13] K. BIEMANN, *Mass Spectrometry*, p. 349. McGraw-Hill, New York (1962).
[14] H. BUDZIKIEWICZ, C. DJERASSI and D. H. WILLIAM, *Interpretation of Mass Spectra of Organic Compounds*, p. 167. Holden Day, San Francisco (1964).

TABLE 3. PROPOSED MASS SPECTRAL DEGRADATION SCHEME FOR 2-(2-HYDROXY-7-METHOXY-1,4-BENZOXAZIN-3-ONE)β-D-GLUCOPYRANOSIDE

| Structure | Molecular formula | m/e |
|---|---|---|
|  | $C_{14}H_{19}O_9N$ (+) | 357 |
|  | $C_8H_9O_4N$ (+) | 195 |
|  | $OC_6H_{11}O_5$ (+) | 179 |
|  | $C_8H_8O_3N$ (+) | 166 (base peak) |
|  | $C_7H_5O_3N$ (+) | 151 |
|  | $C_7H_8O_2N$ (+) | 138 |
|  | $C_6H_8ON$ (+) | 110 |
|  | $CH_3O$ (+) | 31 |
|  | CO (+) | 28 |

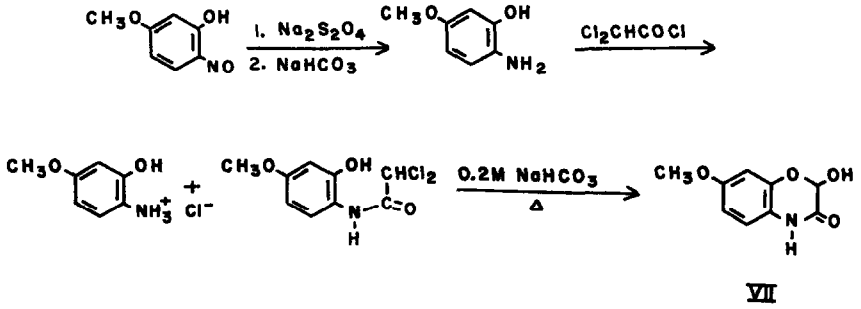



FIG. 4. SYNTHESIS OF 2-HYDROXY-1,4-BENZOXAZIN-3-ONE.

Four variations of the Koenigs–Knorr reaction was used in attempting to condense tetra-O-acetyl-α-D-glucopyranosyl bromide and the synthetic heterocycle. The reactions were all unsuccessful.

## DISCUSSION

The chemical and physical evidence clearly indicates that the newly isolated glycoside is 2-(2-hydroxy-7-methoxy-1,4-benzoxazin-3-one)β-D-glucopyranoside (VI). The discovery of (VI) and its similarity to (IV) and (V) permit speculation as to its function and origin. It may be an intermediate in the biosynthesis of (V), which accumulates after the plant reaches a certain stage of maturity. This is consistent with the proposal of Reimann and Byerrum that the C—N bond in (IV) is formed before the N-hydroxylation occurs.[15] The presence of (VI) suggests that the glucoside is formed before the N-hydroxylation occurs. The presence of (VI) could represent an actual change in the plant's metabolism whereby it is no longer able to perform the N-hydroxylation. Thus, it may be indicative of the maturity of the plant.

Alternatively, the decrease in concentration of (IV) and (V) with increasing age of the plant suggests that (VI) may be a possible catabolite of (V) which does not undergo any further degradation.

## METHODS

*Isolation of* 2-(2-*Hydroxy*-7-*Methoxy*-1,4-*Benzoxazin*-3-*One*)β-D-*Glucopyranoside* (*VI*)

Roots from mature Pennsylvania 812 corn plants were harvested, dried and comminuted. The roots (14·3 kg) were extracted for 24 hr in 600–800-g batches in a Soxhlet extractor using $CHCl_3$:$CH_3OH$ (1:1, v/v) as the solvent. The solvent was removed from the combined extracts under reduced pressure, leaving approximately 1 l. of a thick tar.

$CHCl_3$:$CH_3OH$:$HO_2$ (13:5:7, v/v/v) were equilibrated and the chloroform and aqueous phases were separated. Approximately 500 ml. of the tar were dissolved in 800 ml of the chloroform phase. This was extracted four times with 300 ml of the aqueous phase. The combined aqueous extracts were evaporated under reduced pressure, and the residue was taken up in 500 ml 95% ethanol. The ethanolic solution was filtered through a Buchner funnel and the filtrate evaporated under reduced pressure (Residue A).

Columns (4 × 40 cm) were packed with 100 g of Florisil (60/100 mesh). Approximately 10 g of Residue A were dissolved in a small amount of $CHCl_3$:$CH_3OH$ (2:3 v/v) and placed on the column. Four fractions were eluted using the following $CHCl_3$:$CH_3OH$ mixtures: 8:1, v/v; 6:1, v/v; 4:1, v/v; 2:1, v/v. The residues remaining after removal of the eluting solvent were stored for several weeks, after which time an amorphous material consisting primarily of the compound being isolated separated from the 6:1 and 4:1 fractions.

Preparative thin-layer chromatography using Silica Gel G and $CHCl_3$:$CH_3OH$:$H_2O$ (65:25:4, v/v/v) as the developing solvent was used for the final purification. The compound was recrystallized several times from hot water and dried in an Abderhalden apparatus at 139° M.p. 228–235°, $[\alpha]_D^{24} = +46°$ (water) (Found: C, 50·56; H, 5·40; N, 4·40; $OCH_3$, 8·86. $C_{15}H_{19}NO_9$ required: C, 50·42; H, 5·36; N, 3·92; $OCH_3$, 8·65%).

*Isolation of* 2-(2,4-*Dihydroxy*-7-*Methoxy*-1,4-*Benzoxazin*-3-*One*)β-D-*Glucopyranoside* (*V*)

Pennsylvania 812 seed corn was germinated and grown for 7 days in a shallow pan lined with moistened filter paper. One pan yielded 1–1·5 kg, wet weight, of seedlings.

The extraction procedure was based on that of Wahlroos and Virtanen.[6] The seedlings were placed in a beaker containing boiling ethanol: water (1:1, v/v) for several minutes, homogenized in a Waring blendor in the ethanol : water solution and then strained through several layers of cheesecloth. The solution was filtered through a Buchner funnel with the help of Celite filter aid. The filtrate (3 l.) was extracted four times with 500 ml of butanol. The combined butanol extracts were evaporated under reduced pressure.

Final purification utilized preparative thin-layer chromatography followed by recrystallization from water.

*Isolation of* 2,4-*Dihydroxy*-7-*Methoxy*-2,4-*Benzoxazin*-3-*One* (*IV*)

The extraction procedure was based on that of Hamilton *et al.*[12] Seven-day-old Pennsylvania 812 corn seedlings were homogenized in a Waring blendor. Enough ethanol was added to make the homogenate approximately 60% ethanol; then the homogenate was allowed to stand for 30 min to allow enzymatic cleavage of the glucoside. It was strained through cheesecloth and filtered through a Buchner funnel with the help of Celite filter aid. The filtrate was made acid to litmus and extracted four times with one-fourth its volume of

[15] J. E. Reimann and R. U. Byerrum, *Biochemistry* 3, 847 (1964).

ether. The combined ether extracts were evaporated to dryness, the residue was dissolved in 50 ml of ether and was washed several times with water. After removing the ether, (IV) was recrystallized several times from hot acetone. All steps involving aqueous or alcoholic solutions were performed at 25° or less to retard decomposition of the aglucone.

*Methods of Analysis*

*Acid hydrolyses*: 1–3 mg of the compound plus 0·25–0·50 ml of 1·0 N methanolic HCl were placed in a small glass ampule which was then sealed and placed in the steam bath for 2–24 hr.

Detectional sprays (fuming nitric acid, 5% aqueous $FeCl_3$ and 20% $HClO_4$) were routinely used for the detection of compounds following thin-layer chromatography. Fuming nitric acid reacted instantaneously with (IV), (V), (VI) and (VII) producing a yellow colour. The 5% $FeCl_3$ spray reacted instantaneously with (IV) and (V) producing a violet color. Plates sprayed with 20% $HClO_4$ were heated at 110–120° for 10–15 min.

Trimethyl silyl ethers were prepared of sugar derivatives and hydrolyzates by the procedure of Sweely *et al*,[16] A Barber–Coleman Model 10 gas chromatograph with a 15% EGS column at 160° was used for the chromatography.

Ultra violet spectra were obtained with a Cary Model 14 using ethanol as the solvent. A Perkin-Elmer Model 21 Recording Infrared Spectrophotometer was used to obtain the i.r. spectra. Samples were prepared either in KBr disks or as a thin film on a NaCl block.

A Varian Associates Model A-60 instrument was used to obtain the NMR spectra. Deuterated dimethylsulfoxide was used as the solvent; DDS (2,2-dimethyl-2-silapentane-5-sulfonate) was used as an internal standard. Spectra were taken at room temperature.

Mass spectra were obtained with a Nuclide Associates Model 12-90-G mass spectrophotometer. All the compounds were run as solids after being inserted directly into the ionization chamber. In order to obtain a molecular ion peak high temperatures (300–350° for (V) and (VI) and 250° for (IV)) and low ionizing voltages (20 eV) were used.

*Preparation of* 2-(2-*Hydroxy*-7-*Methoxy*-1,4-*Benzoxazin*-3-*One*)β-D-*Glucopyranoside* (*VI*)

(*a*) *Reduction with Zn and acetic acid.* Glucoside (V) (176 mg), was dissolved in dry ($CaSO_4$) acetic acid (10 ml). Powdered zinc (404 mg) was added and the mixture was refluxed for 25 mins. The filtrate was evaporated under reduced pressure, the residue dissolved in $H_2O$ and the $Zn^{++}$ removed with $H_2S$. The product was purified using preparative thin-layer chromatography yielding compound (VI), 21 mg (13 per cent yield).

(*b*) *Reduction with sodium hydrosulfite.* Glucoside (4) (150 mg), was dissolved in 15 ml $H_2O$. Sodium hydrosulfite (203 mg) was added slowly with constant stirring. The temperature was kept at 60–65° for 6 hr, then overnight at room temperature. The solution was evaporated to a small volume and preparative thin-layer chromatography of the residue was used to purify the product, yielding 32 mg (22 per cent yield) crystalline (VI).

*Preparation of* 2-*dichloroacetamido*-5-*methoxyphenol.* To a suspension of 5-methoxy-2-nitrosophenol (1 g) in 50 ml water, sodium hydrosulfite (3 g) was added slowly with constant stirring. The solution was heated at 60–65° for 15 min, cooled, neutralized (solid $NaHCO_3$), and extracted with 4 × 10 ml ethyl ether. The combined ether extracts were evaporated to dryness under $N_2$ and the residue taken up in 30 ml anhydrous ether. Dichloroacetyl chloride in anhydrous ether (3·5 ml of a 10% solution, v/v) was added dropwise with constant stirring. The blue precipitate of 2-amino-5-methoxyphenol hydrochloride was filtered, and the filtrate evaporated to a viscous residue under $N_2$. (VII) crystallized from the residue after standing overnight and was recrystallized from benzene and then acetone (0·20 g, 26 per cent yield).

*Preparation of* 2-*hydroxy*-7-*methoxy*-1,4-*benzoxazin*-3-*one* (*VII*). 2-Dichloroacetamide-5-methoxyphenol (200 mg) was added to 30 ml 0·2 M $NaHCO_3$, heated at 100° for 25 min, cooled, made acid to litmus with HCl, and extracted with 3 × 10 ml ethyl ether. The combined ether extracts were evaporated to 5 ml under $N_2$. Benzene (5 ml) was added and the mixture was allowed to stand until crystals appeared. The crude 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (51 mg, 32 per cent yield) was recrystallized from acetone.

*Attempts to synthesize* 2-(2-*hydroxy*-7-*methoxy*-1,4-*benzoxazin*-3-*one*)β-D-*glucopyranoside via the Koenigs–Knorr reaction.* Tetra-O-acetyl-α-D-glucopyranosyl bromide was synthesized[17] and reacted with (VII) in a Koenigs–Knorr type of reaction using a variety of conditions and catalysts.[18–21] All variations of the reaction were unsuccessful.

[16] C. C. SWEELY, R. BENTLY, M. MAKITA and W. W. WALLO, *J. Am. Chem. Soc.* **85**, 2497 (1963).
[17] R. U. LEMIEUX, *Methods in Carbohydrate Chemistry*, Vol. 2, p. 221. Academic Press, New York (1963).
[18] J. CONCHIE and G. A. LEVVY, *Methods in Carbohydrate Chemistry*, Vol. 2, p. 336. Academic Press, New York (1963).
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