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BIOSYNTHESIS OF 1,4-BENZOXAZIN-3-ONES IN *ZEA MAYS**

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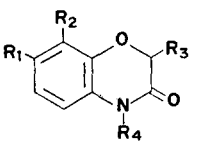
Key Word Index—*Zea mays*; Gramineae; biosynthesis; 1,4-benzoxazinones; benzoxazolinones; cyclic hydroxamic acids; DIMBOA; 6-MBOA; anthranilic acid.

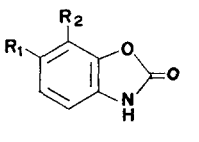
Abstract—^{14}C- and ^{15}N-anthranilic acid are incorporated into the 1,4-benzoxazin-3-ones in maize seedling leaves with low dilution of the isotope. *o*-Aminophenol and 3-hydroxyanthranilic acid are not incorporated and are probably not intermediates. The cyclic hydroxamic acid and lactam members of the 1,4-benzoxazin-3-one group of compounds are readily interconverted.

INTRODUCTION

A GROUP of 1,4-benzoxazin-3-ones was isolated from maize, wheat and rye several years ago by Virtanen *et al.*,[1-3] and additional members of this family of compounds have been isolated from or detected in maize since that time.[4-6] Compound Ih, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one(DIMBOA), has been identified as a feeding deterrent for the European corn borer[7] and has been shown to inhibit germination of spores of the phytopathogenic fungus *Helminthosporium turcicum*.[8] High concentrations of Ih are associated with resistance of cereal grasses to fungi.[9-11]



(I)



(II)

(Ia) $R_1 = R_2 = R_4 = H$; R_3 = glucosido
(Ib) $R_1 = R_2 = R_4 = H$; $R_3 = OH$
(Ic) $R_1 = R_2 = H$; R_3 = glucosido; $R_4 = OH$
(Id) $R_1 = R_2 = H$; $R_3 = R_4 = OH$
(Ie) R_1 = OMe; $R_2 = R_4 = H$; R_3 = glucosido
(If) R_1 = OMe; $R_2 = R_4 = H$; $R_3 = OH$
(Ig) R_1 = OMe; $R_2 = H$; R_3 = glucosido; $R_4 = OH$
(Ih) R_1 = OMe; $R_2 = H$; $R_3 = R_4 = OH$
(Ii) $R_1 = R_2$ = OMe; $R_3 = R_4 = OH$
(IIa) $R_1 = R_2 = H$
(IIb) R_1 = OMe; $R_2 = H$
(IIc) $R_1 = R_2$ = OMe

In the intact plant, the glucosides predominate, but following mechanical injury to the cells, a glucosidase catalyzes hydrolysis to the 2-hydroxy compounds. The 2,4-dihydroxy compounds (cyclic hydroxamic acids) are unstable, especially at pHs above the pK_a of the

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hydroxamic acid group, and undergo quantitative decomposition to the corresponding benzoxazolinones and formic acid.[1,12] The lactams are stable under mild conditions and do not undergo this degradation.

Reimann and Byerrum[13] have concluded from feeding experiments that the aromatic ring of DIMBOA is derived from the shikimic acid pathway and carbons 2 and 3 of the oxazine ring from carbons 2 and 1, respectively, of ribose. We have performed additional feeding experiments to identify intermediates between the shikimic acid pathway and the benzoxazinone end products.

RESULTS

Isotopically labeled compounds were administered to seedling leaves through the cut ends. In some experiments Ih was then isolated and crystallized, while in others, it was degraded to 6-methoxybenzoxazolinone (6-MBOA) (IIb).[1,12] For ring-labeled aromatic precursors incorporated by a direct route, the specific activity of IIb should be equivalent to that of Ih.

TABLE 1. SYNTHESIS OF BENZOXAZINONES FROM LABELLED PRECURSORS

Expt.	Compound fed	Sp. act. cpm/μmol	Sp. act. of isolated compounds (cpm/μmol) Ih	IIb	If
1	^{14}C-Anthranilic acid	38 100		779	378
2	^{15}N-Anthranilic acid	38*	2·7*		
3	^{14}C-Shikimic acid	$15{\cdot}4 \times 10^6$	6660		
4	1-^{14}C-Ascorbic acid	$5{\cdot}0 \times 10^6$	34		
5	1-^{14}C-Ribose	$9{\cdot}8 \times 10^6$	1330		
6	^{14}C-*o*-Aminophenol	25 000		none	
7	^{14}C-*o*-Aminophenol + phenylhydrazine + ascorbic acid	39 400		none	
8	^{14}C-Anthranilic acid + phenylhydrazine + ascorbic acid	58 500		1216	
9	^{3}H-3-Hydroxyanthranilic acid	$2{\cdot}0 \times 10^6$	<100		

* Atom % excess ^{15}N.

Anthranilic acid, labeled with ^{14}C in the ring or with ^{15}N in the amino group, is incorporated into the benzoxazinones with low dilution of the isotope (Table 1), indicating a rather direct route of incorporation. Chromatographic examination of extracts of leaves to which ^{14}C-anthranilic acid had been administered failed to reveal labeled intermediates between anthranilic acid and the benzoxazinones. When ^{14}C-shikimic acid was administered, however, radioautograms of thin-layer plates prepared from ether extracts of the leaves showed that both anthranilic acid and 3-hydroxyanthranilic acid were labeled. These compounds were identified on the TLC plates by comparison of R_fs and fluorescence under UV light with authentic samples. The possibility that anthranilic acid is converted to the benzoxazinones via 3-hydroxyanthranilic acid and *o*-aminophenol was then considered.

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When ^{14}C-*o*-aminophenol and ^{3}H-3-hydroxyanthranilic acid were administered to seedling leaves, there was no incorporation of the label into the benzoxazinones (Table 1). Inclusion of phenylhydrazine to inhibit enzymatic oxidation of *o*-aminophenol[14] and ascorbic acid to prevent nonenzymatic destruction did not result in incorporation of ^{14}C into the benzoxazinones. In the control experiment, phenylhydrazine and ascorbic acid had no effect on the incorporation of ^{14}C-anthranilic acid.

In experiments with cell-free extracts of seedling leaves, ascorbic acid stimulated incorporation of anthranilic acid into Ih.[15] A plausible mechanism for deriving the carbons of the oxazine ring from ascorbic acid can be written[15] but 1-^{14}C-ascorbic acid was far less effective as a precursor of Ih in feeding experiments than was 1-^{14}C-ribose. A stepwise degradation similar to that carried out by Reimann and Byerrum[13] also showed that the label from 1-^{14}C-ascorbic acid was incorporated into Ih in a nearly random fashion, but that 40% of that from 1-^{14}C-ribose was found in C-3 of the oxazine ring. This is not substantially different from the 62·5% incorporation reported by Reimann and Byerrum[13] in a similar experiment.

TABLE 2. INTERCONVERSION OF LABELLED BENZOXAZINONES IN FEEDING EXPERIMENTS

Expt.	Compound fed	Sp. act. (cpm/μmol)	Sp. act. of isolated compounds (cpm/μmol) Ig	Ie	IIb	If	Ib	IIa
1	Glucose-U-^{14}C	27·55 × 10^6	5688	4829	1617	443		
2	Glucose-U-^{14}C	27·55 × 10^6	5200	2895	1825	672		
3	Ig	5688 (1617)*			832	702		
4	Ie	4829 (443)*			8	211		
5	Ie	4507 (996)*	82	947	45	546		
6	Ih	1825			310	297		
7	If	193			31	51		
8	Ib	28 000			950	1133	14 000	1701
9	Ib	36 000			1041	1424	22 500	5175
10	Ib	36 000	1314	3285	787	803		

* Specific activity of the aglucone moiety of the glucoside.

To study the interconversions of Ih, If [2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA)] and their glucosides, labeled materials were isolated from seedling leaves to which ^{14}C-glucose had been administered (Table 2). After determination of the specific activities of the isolated glucosides, portions were enzymatically hydrolyzed, and the specific activities of the aglucones were determined. As might be expected, the specific activities of the glucosides were considerably higher than those of the aglucones. When the ^{14}C-labelled glucoside of DIMBOA was re-administered, both If and IIb were labeled, with approximately the same specific activity. The specific activity of these products is far higher than can be accounted for by conversion of the glucose moiety of the labeled glucoside, indicating that the aglucone moiety of the glucoside has been reduced to the lactam. When Ie was administered, the specific activity of the isolated MBOA was relatively low, but some oxidation of the lactam to the cyclic hydroxamic acid had occurred. In another experiment with ^{14}C-Ie, -Ig and -Ie were isolated. After determination of the specific activities of the glucosides, they were enzymatically hydrolyzed, and the specific activities of the aglucones were determined.

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[15] C.-C. L. TU, Ph.D. Thesis, Iowa State University (1970).

Again, some oxidation to the cyclic hydroxamic acid had occurred. The ratios of specific activities of the glucosides and aglucones isolated after the feeding are approximately 2, but the corresponding ratio for the glucoside administered is over 4·5, indicating that hydrolysis and reglucosylation of the administered glucoside has occurred. When DIMBOA and HMBOA were administered, interconversion also occurred.

^{14}C-Ib is metabolized by *N*-hydroxylation, methoxylation of C-7, or by a combination of both reactions, to form Id (isolated as IIa) If and Ih (isolated as IIb) (Table 2). Examination of the time-course of incorporation of the label from ^{14}C-Ib into the other benzoxazinones (Fig. 1) shows rapid incorporation into Id, followed by If, with Ih being labeled least rapidly.

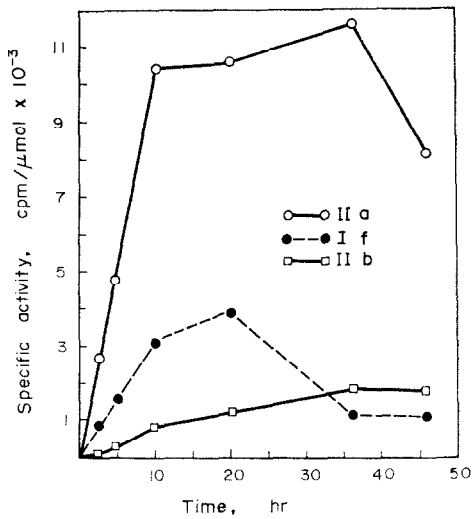

FIG. 1. INCORPORATION OF RADIOACTIVITY INTO THE BENZOXAZINONES Id (ISOLATED AS IIa), If, AND Ih (ISOLATED AS IIb) FOLLOWING ADMINISTRATION OF ^{14}C-Ib TO SEEDLINGS.

DISCUSSION

Reimann and Byerrum[13] suggested that the oxazine ring of Ih is formed by reaction of ribose or a ribose phosphate with an aromatic amine, followed by cyclization and modification of the heterocyclic ring. We have identified the aromatic amine involved as anthranilic acid. Neither 3-hydroxyanthranilic nor *o*-aminophenol is incorporated into the benzoxazinones. This makes unlikely the possibility that anthranilic acid is converted to another aromatic amine before reacting with a ribose phosphate and suggests that the next intermediate is *N*-(5'phosphoribosyl)anthranilate, also an intermediate in tryptophan biosynthesis.

Interconversion of the benzoxazinones by oxidation of the lactams Ib and If and reduction of the hydroxamic acid Ih has been demonstrated. The mechanism of neither reaction is known. The biosynthesis of a few hydroxamic acids of microbial origin has been investigated[16] and it has been shown that the hydroxylamine oxygen of hadicidin is derived from O_2. *N*-Hydroxylation reactions in mammalian liver are catalyzed by monooxygenases

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similar to those responsible for *C*-hydroxylation reactions.[17,18] The metabolic reduction of a hydroxamic acid has not been reported previously.

We have been unable to demonstrate interconversion of the lactam and hydroxamic acid forms of the benzoxazinones in cell-free extracts. This might be explained if the substrates for the interconversions are the glucosides, since the presence of an active glucosidase in the extracts prevents testing of the glucosides. Therefore, we have examined the results of feeding experiments in an attempt to determine if the glucosides must be hydrolyzed before oxidation or reduction *in vivo*. In expt. 5, Table 2, the ratio of specific activity of the glucoside fed to the aglucone portion of this glucoside is about 4·5, while the same ratios for the isolated glucosides are less than 2. Therefore, some hydrolysis of the administered glucoside has occurred. If complete hydrolysis and reglucosylation had occurred, however, one would expect essentially all the radioactivity of the isolated glucosides to be in the aglucones since the dilution of specific activity of the glucose moiety of the glucosides fed should be 10^3–10^4, as in expts. 1 and 2 (Table 2) in which glucose was fed. An alternative explanation, in which the glucose produced in the hydrolysis enters a very restricted compartment and is used for reglucosylation of the aglucones, is also possible. These results suggest, however, that the glucosides may be the substrates for the lactam-hydroxamic acid interconversion and that demonstration of these reactions in extracts may depend upon inhibition or removal of the glucosidase.

Ib is readily converted to Id, If and Ih. The changes in specific activity during a 48-hr feeding experiment are consistent with a scheme in which Ib is converted alternatively to Id or to If, with If being converted to Ih. Although the specific activities of the three products vary by only about a factor of 10, the amounts of the lactams in the leaves are much smaller than of the hydroxamic acids, so that most of the isolated radioactivity is found in the hydroxamic acids.

EXPERIMENTAL

Materials. Maize seeds of the inbred variety CI31A were obtained from Dr. W. A. Russell, Department of Agronomy, Iowa State University.

Ring-labeled ^{14}C-anthranilic acid was prepared from 1-^{14}C-*o*-nitrotoluene by oxidation of the methyl group with neutral potassium permanganate[19] and reduction of the nitro group with H_2 over 5% Pd on charcoal.[20] The product, isolated as the crystalline hydrochloride, was characterized by PC in two solvents (*n*-BuOH–conc. HCl-H_2O, 100:20:39, R_f 0·8;[21] MeOH–C_6H_6–$CHCl_3$–H_2O, 2:1:1:1, R_f 0·9). Radioautography after PC in the latter solvent indicated no detectable radioactive impurity. The UV spectrum of the product was identical with that of an authentic sample. ^{15}N-Anthranilic acid was prepared by Hofmann degradation of ^{15}N-phthalimide. ^{14}C-*o*-Aminophenol was prepared by nitration of ^{14}C-phenol with $Cu(NO_3)_2$ in glac. HOAc[22] followed by catalytic hydrogenation and recrystallization from abs. EtOH. ^{14}C-Ib was synthesized from this product by reaction with dichloroacetylchloride as described by Honkanen and Virtanen[23] except that a large excess of triethylamine was added to neutralize the HCl formed by reaction of the acid chloride.

Isolation of benzoxazinones. Ih was isolated as described previously.[4] For the isolation of If, IIb, Ib and IIa, seedling leaves and stems (10 g) were ground with sand and 1 ml H_2O, allowed to stand 30 min at room temp., then extracted with Et_2O. The Et_2O solution was evaporated to dryness, 10 ml H_2O was added to the residue, and the mixture was refluxed for 50 min. The sample was again evaporated to dryness, and the residue fractionated by TLC on Silica Gel GF_{254} with Et_2O saturated with H_2O as the solvent. R_fs are 0·44

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for If, 0·55 for Ib, 0·65 for IIb and 0·75 for IIa. The 4 bands were scraped from the plates and extracted with Et_2O. For the isolation of the glucosides Ie and Ig, finely cut seedling leaves and stems (10 g) were homogenized in a mechanical blender (Waring Blendor) with cold acetone (− 18°). The cold acetone extract was collected by suction filtration and evaporated to dryness under vacuum at 35°. Then, 2 ml H_2O was added to the residue, the insoluble material was discarded, and the aqueous solution was applied to a Sephadex G10 column. The column was eluted with water at a flow rate of 100 ml/hr and monitored by measurement of absorbance at 254 nm. After 3 minor peaks, a major peak appeared which was collected and concentrated to a small vol. under vacuum at 35°. This material was chromatographed on Whatman No. 3 filter paper with the solvent *n*-BuOH–EtOH–conc. NH_4OH–H_2O, 90:10:1:9. Dark bands seen at R_f 0·05 corresponding to Ig and at 0·35 corresponding to Ie when the papers were viewed under long-wavelength UV light were cut out and eluted with H_2O.

Enzymatic hydrolysis of glucosides. Etiolated seedling leaves (16 g) were homogenized with 8 ml of 0·2 M phosphate buffer (pH 7·0) in an ice-jacketed blender for 2 min, and the homogenate was quickly filtered through 6 layers of cheese cloth. The filtrate (16 ml) was applied to a Sephadex G25 column and eluted with the same buffer solution at 4°. The first UV-absorbing peak was collected and used as a glucosidase preparation. Et_2O extraction and TLC of the ether-soluble material showed that the Sephadex column separated all the benzoxazinones from the protein solution. The glucosides in 1 ml 0·2 M phosphate buffer, pH 7·0, were mixed with 2 ml of the protein solution and allowed to stand at room temp. for 30 min, after which the aglucones were extracted into Et_2O.

Feeding experiments. Etiolated 7- or 8-day-old seedlings (15–18 cm high) were cut off at the stem 1 cm above the seed, in H_2O. The cut stems were placed in an aqueous solution of the radioactive compound, in a small beaker, and illuminated with one regular and one Gro-Lux fluorescent lamp, both 15 W, at a distance of about 30 cm, at room temp. The radioactive solution was taken up in 1–2 hr, then small quantities of H_2O were added, as needed, for the duration of the feeding.