A STUDY ON THE BIOSYNTHESIS OF 2,4-DIHYDROXY-7-METHOXY-2H-1,4-BENZOXAZIN-3-ONE

Thesis for the Degree of Ph. D.

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Jessica E. Reimann

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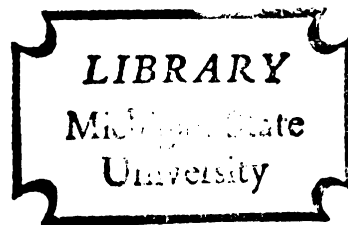

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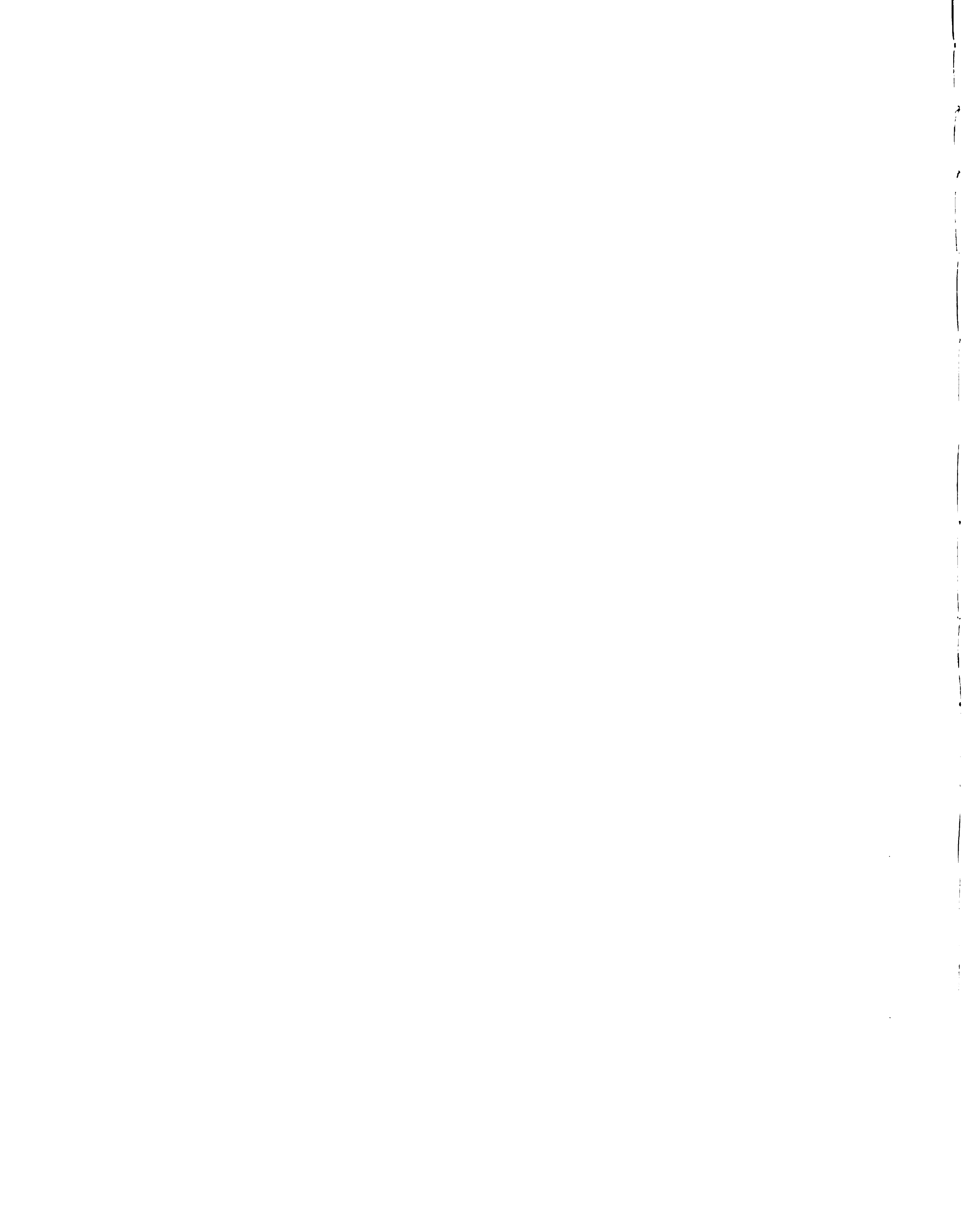

ABSTRACT

A STUDY ON THE BIOSYNTHESIS OF
2,4-DIHYDROXY-7-METHOXY-2H-1,4-BENZOXAZIN-3-ONE

by Jessica E. Reimann

The compound 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA), found in corn and other grasses, contains two unique structures of biosynthetic interest, an oxazine ring system and a cyclic hydroxamate structure. A study of DIMBOA was undertaken since there have been no previous investigations on the biosynthesis of these structures in higher plants. Information concerning the mode of formation of this compound would permit comparison with pathways operative in other systems and contribute to an understanding of the mechanisms of oxazine ring and cyclic hydroxamate biosynthesis in general.

The roots and seeds were removed from 6-day old etiolated corn seedlings and labeled compounds, which might serve as precursors to DIMBOA, were administered hydroponically to the plants through the excised stems. Absorption of the metabolite was essentially complete within five hours. The nutrient solution was replaced with water at the end of this time and the plants were allowed to metabolize the administered compound for an additional 21 hours.

DIMBOA was isolated from the plants and the extent of isotope incorporation from the labeled precursor was measured. The distribution of the incorporated C^{14} in the molecule was also determined by degrading DIMBOA in a manner permitting the isolation of carbons in positions 2 and 3, the methoxyl group and the aromatic ring carbons.

DIMBOA was converted to 6-methoxybenzoxazolinone with the liberation of formic acid in the first step of the degradation procedure. The formic acid originates from the 2 position of the molecule. Treatment of 6-methoxybenzoxazolinone with hydriodic acid produced CO_2 from the 3 position of DIMBOA, CH_3I from the methoxyl group and 2,4-dihydroxyaniline, representing the aromatic ring. The compounds resulting from the degradation procedures, or in some cases their stable derivatives, were subjected to total combustion and the CO_2 produced was isolated and counted as $BaCO_3$.

Of a number of metabolites tested, quinic acid-U-C^{14}, L-methionine-methyl-C^{14} and D-ribose-1-C^{14} were most readily incorporated.

The degradation of DIMBOA by the procedure described indicated that 99.5% of the C^{14} in the compound from plants fed uniformly labeled quinic acid was located in the benzenoid ring. The ready incorporation of quinic acid and the specific labeling pattern suggests that the synthesis of the aromatic ring of the compound proceeds by the shikimic acid pathway.

Essentially all of the radioactivity incorporated from methionine-methyl-C^{14} was found in the methoxyl group of DIMBOA. The O-methyl group most probably is formed by the conventional transmethylation process from methionine.

Ribose-1-C^{14} was significantly incorporated into DIMBOA and 62.5% of the radioactivity was associated with the carbon in the three position. Two-carbon metabolites which might be related to an intermediate formed from the 1 and 2 carbons of ribose such as acetate-1-C^{14},

glycine-2-C^{14} and glycolate-1-C^{14} were not incorporated into the 2 and 3 positions of DIMBOA. These results suggest that the two carbons of the heterocyclic ring are derived from the 1 and 2 carbons of ribose through the formation of a ribose intermediate.

The significance and implications of these results are discussed and a reaction sequence is postulated for the biosynthesis of DIMBOA.

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INTRODUCTION

The cyclic hydroxamate, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA), present in corn and wheat plants, was first isolated and characterized by Virtenen and coworkers (1,2). A closely related analogue, 2,4-dihydroxy-1,4-benzoxazin-3-one was isolated from rye seedlings by these same workers and the structure established by synthesis of the compound (3). The similarity in chemical properties suggested that the compound obtained from corn was the 7-methoxy derivative of 2,4-dihydroxy-1,4-benzoxazin-3-one. Additional characterization by Hamilton *et al*. (4) confirmed the postulated structure.

DIMBOA exists in plants as a monoglucoside and is converted to glucose and the aglucone on rupturing of the plant tissues, presumably through the release of a glycosidase (2).

Early workers reported the presence of 6-methoxybenzoxazolinone in corn (5,6), wheat (6) and grasses of genus *Coix* (7). However, the isolation of this degradation product of DIMBOA probably resulted as a consequence of the purification procedures employed.

A number of natural occurring cyclic hydroxamates have been isolated as products of mold metabolism (8,9,10,11,12). These compounds are of interest because they all possess antimetabolic properties. DIMBOA exhibits anti-bacterial, anti-fungal (13,2) and insecticidal (5) activity. Moreover, a number of synthesized hydroxamates are found to be antimetabolic in nature (14,15,16). The mechanism of this anti-metabolic behavior and its relationship to the hydroxamate structure has not, as yet, been elucidated.

A study of the biosynthesis of DIMBOA was of interest since it contains two biologically unique structures, an oxazine ring system and the cyclic hydroxamate moiety. There are few reports in the literature concerning the occurrence or the biosynthesis of these structures.

Compounds containing a phenoxazine ring system have been found in a group of pigments isolated from insects (17), in cinnabarin found in the fungus Coriolus sanguineas (18,19), and actinomycin isolated from Streptomyces antibioticus (20). It has been shown that tryptophan is incorporated into actinomycin (21) and the phenoxazinone found in insects (17). A product of tryptophan metabolism, 3-hydroxyanthranilic acid, is probably a closer precursor to these compounds since Sivak et al. (21) have obtained a cell free enzyme system which catalyzes the oxidative condensation of 4-methyl-3-hydroxyanthranilic acid to yield actinocinin.

Biosynthetic studies carried out on two of the cyclic hydroxamates isolated from molds, indicates that these compounds are formed by the condensation of two amino acids having a carbon skeleton appropriate for the final product. Aspergillic acid (22) is formed from leucine and isoleucine and myceianamiae (23) is synthesized from tyrosine and alanine.

There have been no previous investigations of either oxazine ring formation or cyclic hydroxamate biosynthesis in higher plants. Information concerning the biosynthesis of DIMBOA would permit comparison with pathways operative in other systems and contribute to an understanding of the mechanisms of oxazine ring and cyclic hydroxamate formation in general.

EXPERIMENTAL AND RESULTS

Growth of Plants and Administration of Labeled Compounds

Corn seeds, Michigan Hybrid 350, known to produce relatively large quantities of DIMBOA, were used in this study. In preparation for planting the seeds were washed three times in tap water then immersed in distilled water for 36 hours. Prior to planting, 150 mg of the water insoluble fungicide Ethyl Thiram* was added to the soak water and transferred with the seeds in the course of planting. The seeds were planted in Vermiculite[+] and grown in the dark at 30°C. Plants were kept moist with an inorganic nutrient solution (24) during the 6-day growth period.

The roots and seeds were removed from the 6-day old etiolated corn seedlings and isotopically labeled compounds, which might serve as precursors to DIMBOA, were administered hydroponically to the plants through the excised stems. The compounds were administered in a minimal volume of water and during the absorption period the cut stems were kept solvent by the addition of water as needed. Absorption of the metabolite was essentially complete within 5 hours.

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* A commercial heat-expanded mica.

\+ Bis (diethylthiocarbamoyl)disulfide obtained from Pennsalt Chemicals Corp., Philadelphia, Pa.

At the end of this time any nutrient solution remaining was decanted and the container and stems were rinsed three times with water. The amount of radioactivity that was not absorbed by the plants was determined by counting the combined solution and washes. Absorption of the labeled metabolites from the nutrient solution was at least 95% in all but two of the feeding experiments. L-methionine-methyl-C^{14} and calcium glycerate-3-C^{14} were 88% absorbed at the end of the feeding period.

The plants were allowed to metabolize the administered compound for an additional 21 hours at 30°C in the dark.

In the experiments where comparisons of isotope incorporation were to be made, the total pool of metabolite and the amount of radioactivity administered per plant was kept constant. The nutrient solution for a representative feeding of 500 corn stems contained 0.1 mmole of compound and 48 μc of radioactivity. The specific activity then of the labeled compound was 480 μc/mmole. Quinic acid-L-C^{14} and tryptophan-7a-C^{14} administered to plants had specific activities of 94 μc/mmole due to a limited availability of these compounds.

Isolation of DIMBOA

The isolation procedure for DIMBOA is a modification of that used by Hamilton et al. (4).

At the end of the 21 hour period of metabolism, the plant stems were homogenated in a Waring Blendor with water. The homogonate was allowed to stand for 45 minutes to insure a maximal liberation of DIMBOA

from its glucoside linkage. An equal volume of ethanol was added and the mixture homogenized again for an additional 10 seconds. After filtration through a Celite* pad, the alcoholic extract was reduced in volume on the rotatory evaporator. The concentrated extract (about 400 ml) was adjusted to a pH of 3.5 with 4 N phosphoric acid and extracted five times with an equal volume of peroxide-free diethyl ether. The ether fractions were reduced to a volume of 300 ml and partitioned with 100 ml of 5% sodium bicarbonate. The aqueous solution, containing DIMBOA, was acidified to a pH of 3.5 and ether extracted again as described above. The combined ether extracts were dried several hours over anhydrous sodium sulfate and then evaporated to dryness in vacuo. The residue was washed several times with 3 ml portions of anhydrous, peroxide-free diethyl ether, dissolved in a minimum amount of hot acetone and treated with Norite. Addition of Skelly C[+] to the filtered acetone with subsequent cooling resulted in the precipitation of grayish crystals. On recrystallization from acetone-cyclohexane, white needle crystals are obtained which melt at 163-164°C (decomp.)[++] (reported value 160-161° (4)). About 240 mg of pure DIMBOA can be obtained from 500 plant stems.

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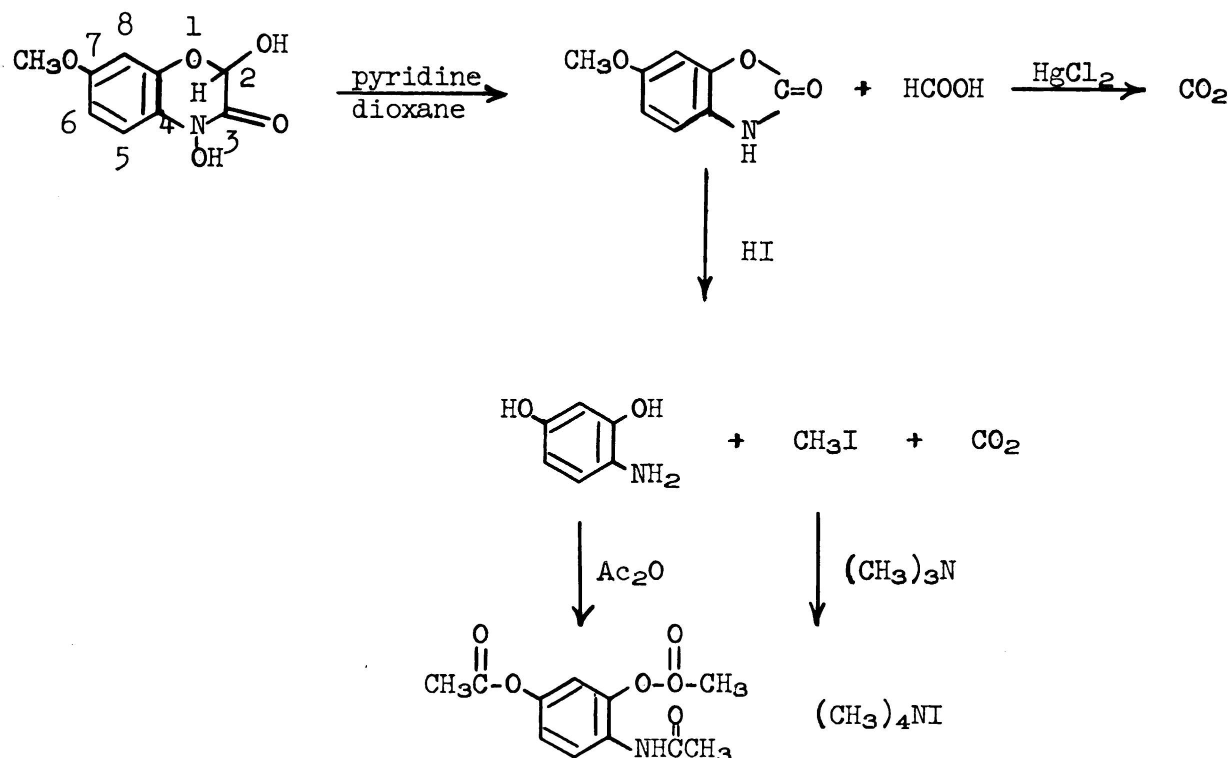
* Diatamecious earth obtained from Johns Manville.

\+ Skelly C was obtained from Skelly Oil Co., Kansas City, Mo.

++ Melting points are uncorrected.

Degradation Procedures

The extent of incorporation of a labeled compound into a product is a measure of its role as a precursor. However, incorporation alone is not sufficient evidence for the establishment or evaluation of a biosynthetic pathway. Since fed compounds may first be degraded in the plant to a general metabolite and then resynthesized into product, it is essential to show that the precursor is incorporated as an intact molecule. The maintenance of specific labeling patterns in the isolated product provides evidence that the administered compound was utilized directly in the biosynthetic pathway. For this reason, in those cases in which incorporation of labeled compound occurred, the position of the isotope in DIMBOA was determined by degradation to isolate specific carbon atoms or groups within the molecule. The degradation reactions devised permitted the isolation of carbons in position number 2,3, the methoxyl carbon and the aromatic ring carbons as indicated below.



DIMBOA, refluxed in a solution of dioxane and pyridine, forms 6-methoxybenzoxazolinone with the liberation of formic acid. The formic acid originating from the 2 position of the DIMBOA molecule was oxidized to $BaCO_3$. Treatment of 6-methoxybenzoxazolinone with hydriodic acid produced CO_2 from the 3 position of DIMBOA, methyl iodide from the methoxyl carbon and 2,4-dihydroxyaniline. The CO_2 was swept in a stream of nitrogen from the reaction mixture into a solution of $Ba(OH)_2$ to yield $BaCO_3$. Methyl iodide was converted to tetramethylammonium iodide. The 2,4-dihydroxyaniline was separated from the reaction mixture and treated with acetic anhydride. The product of this reaction was identified as 2,4-diacetoxyacetanilide which represented the aromatic ring carbons of DIMBOA.

<u>Conversion of DIMBOA to 6-methoxybenzoxazolinone and formic acid</u>

DIMBOA (80-120 mg), dissolved in 20 ml dioxane and 1.0 ml pyridine, was refluxed for 2 hours. The formic acid formed during the course of the reaction, and the solvents were distilled under reduced pressure into an iced receiving flask. After most of the solvent had been removed an additional 5 ml of dioxane was added and distilled from the reaction flask. To ensure a maximal recovery of formic acid, 5.0 ml of water containing 0.2 ml of glacial acetic acid was then added to the flask and distillation was continued until most of the solvent was removed and a slurry of the precipitated 6-methoxybenzoxazolinone remained.

The formic acid was converted to the sodium salt by addition of a concentrated sodium hydroxide solution to the distillate and the solvents were reduced in volume on the rotatory evaporator. The isolated formate was then oxidized to carbon dioxide according to the procedure of Sakami (25).

The aqueous solution, containing the formate, was adjusted to pH 3.0 with glacial acetic acid and transferred to a 50 ml flask with a side arm. The flask was equipped with a dropping funnel and a Vigreaux distillation head, which in turn was connected to enclosed tubes containing a saturated solution of $Ba(OH)_2$. The solution was heated and the system flushed with a stream of nitrogen for 30 minutes to remove any endogeneous CO_2. Thirteen ml of the oxidizing reagent, 8% mercuric chloride in acetate buffer, was added to the formic acid solution by means of the dropping funnel. Boiling was continued for 30 minutes, during which time the CO_2 liberated was swept in a N_2 stream into the $Ba(OH)_2$ trap and precipitated as $BaCO_3$. The $BaCO_3$ was collected in a special stainless steel funnel, washed with a small amount of carbonate-free water followed by methanol and allowed to dry. Discs of $BaCO_3$ prepared in this fashion are easily transferred intact to planchets for purposes of counting. Yields of 65-70% of $BaCO_3$ were obtained from the 2 position of DIMBOA.

Honkanen and Virtanen (26) reported the formation of formic acid on heating an aqueous solution of DIMBOA or its closely related analogue, 2,4-dihydroxy-1,4-benzoxazin-3-one. In order to establish which carbon atom gave rise to the formic acid, these investigations

synthesized the latter compound labeled with C^{14} at position 2. The formic acid, liberated on heating this synthesized compound, was found to be radioactive and thus originates from the carbon atom in the 2 position of the molecule. In view of the similarity in structure and chemical behavior of the two compounds, it is assumed that the formic acid liberated from DIMBOA also originates from the 2 position.

The amorphous 6-methoxybenzoxazolinone was recrystallized from hot water. The compound forms reddish-brown needles melting at 153-153.5°C (literature value 153-153.5°C (4)). The average yield obtained was 60%.

Conversion of 6-methoxybenzoxazolinone to CO_2, CH_3I and 2,4-dihydroxyaniline

A 50-100 mg sample of 6-methoxybenzoxazolinone was refluxed with 7 ml of hydriodic acid* for 3 hours. The flask was heated in a copper oxide fillings bath with a microburner. The liberated carbon dioxide and methyl iodide were swept by a slow stream of nitrogen through the Vigreaux distillation head into a series of traps. The first, a scrubber trap to remove iodine and hydrogen iodide, contained a 5% cadmium sulfate solution and a 2% solution of stannous chloride in 0.3 N hydrochloric acid. A saturated solution of barium hydroxide

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*HI for methoxy determination was purchased from Merck.

in the second receiving tube trapped carbon dioxide as barium carbonate was collected as previously described. The last trap, containing a 5% ethanolic solution of trimethylamine cooled in a dry ice-methylcellosolve bath, converted the methyl iodide to tetramethylammonium iodide. The quartenary ammonium salt precipitated on standing overnight. The precipitate was dried in vacuo and recrystallized from methanol and diethyl ether.

Yields of 80% were obtained for both the barium carbonate, originating from the 3 position of DIMBOA and for the methyl iodide, derived from the methoxyl group.

Preparation of 2,4-dihydroxyaniline derivatives

In order to isolate 2,4-dihydroxyaniline, the third product of the hydriodic acid reaction, it was necessary to prepare acetylated derivatives. The 2,4-dihydroxyaniline and its 4-methoxy analogue can not be isolated as such and their hydrochloride salts are of questionable purity. During the study to establish reaction conditions, highly chromaphoric compounds were isolated and assumed to be condensation products. The oxidative condensations of substituted O-hydroxyanilines to form phenoxazines are well known (27).

Preparation and identification of 2,4-dihydroxyacetanilide

Solid stannous chloride was added to the hydriodic acid reaction mixture until the solution was colorless. The copious yellow-red precipitate of stannous iodide was removed by filtration through a Buchner funnel and the residue washed with small portions of water. The filtrate and the combined washes were extracted with diethyl ether to remove excessive hydriodic acid.

Acetylation of the amine group was carried out in the following manner. Solid sodium carbonate was added to the aqueous solution to a pH of 4.5. The reaction mixture was constantly agitated by means of a magnetic bar and stirrer. Acetic anhydride (0.2 ml) was added followed by a concentrated solution of sodium hydroxide until the pH was adjusted to 7.5. After a period of 20 minutes the excessive stannous ion was removed from solution by precipitation as stannous hydroxide at a pH of 10. Acetic anhydride was then added dropwise until the pH reached 6.0 and the filtered solution was extracted with diethyl ether overnight in a continuous solvent-solvent extractor.

The ether phase, containing 2,4-dihydroxyanilide, was evaporated to dryness, and the product crystallized from ethyl acetate and ligroin*. If present in gram quantities, 2,4-dihydroxyanilide can be crystallized from water. Several recrystallizations were necessary to obtain white crystals having a melting point of 188-189°C.

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*All references to ligroin is the fraction boiling between 30-60°C.

The 2,4-dihydroxyacetanilide obtained from the reaction of 6-methoxybenzoxazolinone and hydriodic acid followed by acetylation was compared with an authentic sample of the synthesized compound. Comparison of the samples by paper chromatography in two solvent systems indicated that they were the same compound. The Rf values for both compounds on chromatograms developed in 20% ammonium sulfate and in the organic phase of a chloroform: methanol: 4% formic acid mixture (10:1:1) (28) were 0.60 and 0.08 respectively. Mixed samples developed as one spot. The compound was detected as blue spots by spraying the papers with a 2% solution of phosphomolybdlic acid followed by exposure to ammonia vapors (29).

Preparation and identification of 2,4-diacetoxyacetanilide

In small quantities the monoacetylated derivative of 2,4-dihydroxyaniline tended to form an oil and proved difficult to isolate. For this reason the triacetylated derivative, 2,4-diacetoxyacetanilide, was prepared from the degradation product obtained from the radioactive feedings.

Triacetylation of 2,4-dihydroxyaniline could not be achieved by increasing the amount of acetic anhydride added to the stannous chloride treated reaction mixture. The large amounts of salts that were present could account for this since the ether extracted 2,4-dihydroxacetanilide acetylated readily. The degree of acetylation is pH dependent and complete conversion to 2,4-diacetoxyacetanilide was obtained by addition of acetic anhydride in excess to an aqueous

solution (pH 10.5) of the 2,4-dihydroxyacetanilide. There was no evidence on paper chromatograms for the formation of the tetracetylated derivate under these conditions.

The reaction solution was allowed to stand until the pH had dropped to 5. It was then partitioned 5 times with an equal volume of diethyl ether. The ether was removed by evaporation in vacuo; the oily residue was dissolved in hot benzene and treated with Norite. Ligroin was added to the filtered benzene to a definite cloudiness and on cooling overnight, 2,4-diacetoxyacetanilide crystallized from solution. On recrystallization from ether and ligroin, white feathery crystals are obtained (m.p. 113-114°C). The yields obtained averaged 18% of theory. An elemental analysis* gave the following results:

$C_{12}H_{13}NO_5$	Found :	C, 57.30%	H, 5.10%	N, 5.53%
(251.23)	Calculated:	C, 57.40%	H, 5.23%	N, 5.60%

The triacetylated product obtained from the degradation procedure and a synthesized sample of 2,4-diacetoxyacetanilide had the same Rf values on paper chromatograms in two different solvent systems. This Rf value was 0.81 for chromatograms developed in the organic phase from a mixture of isobutanol and 1 N formic acid (9:1) and 0.94 for chromatograms developed in chloroform, methanol and 4% formic acid (10:1:1) (28). Mixed samples developed as one spot. The compounds were located on the chromatograms by first spraying the papers with a saturated solution of hydroxylamine hydrochloride followed by 2 N alcoholic potassium hydroxide. The papers were dried in a 100°C oven for 5

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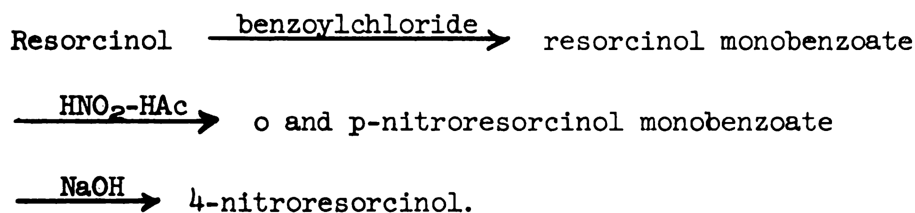
*Elemental analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Michigan.

minutes. Pink spots, corresponding to the position of the compound, developed after spraying the papers with a 1% solution of ferric chloride in 0.1 N hydrochloric acid (30).

Synthesis of 2,4-dihydroxyaniline Derivatives

The synthesis of the 2,4-dihydroxyaniline derivatives was undertaken so that a comparison could be made of the degradation product with an authentic sample of the compound. The synthesis was accomplished by preparing the 4-nitroresorcinol, followed by reduction of the nitro group to yield 2,4-dihydroxyaniline which was then acetylated to the desired derivative.

The 4-nitroresorcinol synthesis was carried out by the following reaction sequence according to the procedure of Kaufman and Kugel (31).



Reduction of the nitro group and the subsequent conversion of the 2,4-dihydroxyaniline to an acetylated derivative was performed in the same reaction flask.

The 4-nitroresorcinol (100 ml) was dissolved in 30 ml of boiling water and the nitro group reduced by the gradual addition of solid sodium hydrosulfite until the solution was decolorized. An excess of acetic anhydride was added and the solution shaken for 5 minutes. The product was extracted into diethyl ether, the ether evaporated to dryness and the residue dried over phosphorous pentoxide in a vacuum dessicator. The residue was dissolved in a small amount of ethyl acetate and a slow crystallization of 2,4-dihydroxyacetanilide effected by the addition of ligroin to produce a slightly turbid solution. A second crystallization yielded white needle crystals melting at 188-189°C. Vorozhtzov et al. (32) reported isolating this compound as brown crystals melting at 164°C. An elemental analysis gave the following results:

$C_8H_9NO_3$	Found :	C, 57.45%	H, 5.43%	N, 8.31%
(167.14)	Calculated:	C, 57.48%	H, 5.42%	N, 8.41%

The triacetylated derivative was obtained by adjusting the pH of the solution containing 2,4-dihydroxyaniline to a pH of 10.5 with 2 N sodium hydroxide prior to the addition of acetic anhydride. The residue, obtained from the ether extraction as described above, was dissolved in hot benzene and 2,4-diacetoxyacetanilide crystallizes on standing. On recrystallization from benzene or diethyl ether and ligroin white feathery crystals were obtained (m.p. 113-114°C, reported 113°C (32)).

Determination of Radioactivity

DIMBOA and the products of the degradation reactions were converted to $BaCO_3$ for C^{14} analysis in order to secure greater uniformity in counting conditions. The organic compounds were subjected to total combustion using the Van Slyke method (33) and the resulting CO_2 was isolated as $BaCO_3$ and prepared for counting as previously described.

$BaCO_3$ recovery studies were carried out on the compounds subjected to the combustion procedure to determine if the oxidation was essentially complete. Combustion of samples of DIMBOA, tetramethylammonium iodide and 2,4-diacetoxyacetanilide yielded $BaCO_3$ recoveries of 94.0%, 90.0% and 90.5% respectively. Due to small losses incurred on the sides of the CO_2 outlet tube these figures are considered adequate to give representative samplings of the compounds converted to $BaCO_3$.

DIMBOA, tetramethylammonium iodide and 2,4-diacetoxyacetanilide, isolated and purified as previously described, did not change in activity after additional purification steps.

The extent of incorporation into DIMBOA from a number of labeled compounds administered to the plants is shown in Table I. The compounds are listed in order of decreasing incorporation as judged by dilution. The dilution is defined as the ratio of the specific activity of the precursor to the specific activity of the isolated product.

Table II shows the distribution of C^{14} with regard to position 2, 3, the methoxyl carbon and the aromatic ring in DIMBOA isolated from plants fed the six precursors that were most readily incorporated. Within experimental limits, the carbon-14 analysis of the degradation products of DIMBOA accounted for all the radioactivity in the original molecule except in the case of the serine feeding. Only 84.0% of the total C^{14} content was recovered. Nevertheless, the data is included and interpreted since it is certain the loss was incurred in the analysis of the methoxyl group.

Duplicate feeding experiments with ribose-1-C^{14} were in good agreement with regard to the distribution of the C^{14} incorporated into the molecule. The values obtained from the two experiments for the fraction of total activity located in the 3 position of DIMBOA were 63.6% and 62.5%.

Measurements were made with a Tracerlab proportional flow counter and a Nuclear-Chicago Model 192x Ultrascaler equipped with an automatic sample changer and timer. The counting system was 38% efficient as determined with a standard sample of benzoic acid-C^{14}*. The counts were corrected for self-absorption.

Radioactivity measurements on the nutrient solution before and after administration to the plants were carried out with an end-window Geiger counter and a Nuclear Chicago Scaler Model 163.

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* Obtained from New England Nuclear Corp., Boston, Mass.

Table I. Incorporation of C^{14} into DIMBOA Isolated from Plants Administered Labeled Compounds

Compound Fed	Specific Activity of Compound Fed	Specific Activity of DIMBOA	Dilution
	mμc/mmole	mμc/mmole	
Quinic Acid-U-C^{14}	94,000	225.5	419
L-Methionine-methyl-C^{14}	480,000	367.0	1,306
D-Ribose-1-C^{14}	480,000	193.6	2,484
L-Serine-U-C^{14}	480,000	119.0	4,030
Glycine-2-C^{14}	480,000	99.6	4,820
Calcium Glycerate-3-C^{14}	480,000	35.5	13,520
DL-Tryptophan-7a-C^{14}	94,000	2.2	43,700
Sodium Acetate-1-C^{14}	480,000	0.9	533,000
Sodium Glycollate-1-C^{14}	480,000	0	---

Table II. Distribution of C^{14} in the DIMBOA Molecule from Labeled Precursors

Compound Isolated in Degradation	Quinic Acid-U-C^{14}		Methionine-methyl-C^{14}	
	Sp. Act. mμc/mmole	%	Sp. Act. mμc/mmole	%
DIMBOA	224.3	100.0	367.5	100.0
HCOOH (position 2)	0	0	0	0
CO_2 (position 3)	0.4	0.1	0	0
Tetramethylammonium iodide (methoxyl carbon)	7.1	3.1	377.5	102.7
2,4-Diacetoxyacetanilide (benzenoid ring carbons)	223.5	99.5	2.0	0.5

Ribose-1-C^{14}		L-Serine-U-C^{14}		Glycine-2-C^{14}		Calcium Glycerate-3-C^{14}	
Sp. Act. mμc/mmole	%	Sp. Act. mμc/mmole	%	Sp. Act. mμc/mmole	%	Sp. Act. mμc/mmole	%
193.2	100.0	119.0	100.0	99.6	100.0	35.5	100.0
7.5	3.9	0.1	0	0	0	0	0
120.8	62.5	0.2	0.1	0.1	0.1	1.9	5.3
11.0	5.7	97.6	82.0	97.8	98.2	13.2	37.2
60.9	31.5	2.3	1.9	0	0	22.2	62.5

DISCUSSION

The elucidation of biosynthetic pathways by the use of isotopically labeled compounds is based on two lines of evidence. First the tracer technique provide evidence that a labeled compound can be converted to the product. Other conditions being equal, the precursor incorporated with the smaller dilution is assumed to be closer to the product in the biosynthetic pathway. Precursors further removed from the end product may suffer large dilutions by endogeneous metabolites in multistep reactions and by conversion to other plant components. Dilution comparisons, however, can only be indicative since conditions are not equal and endogeneous pool sizes of possible precursors may vary considerably.

Demonstrations that the metabolite is incorporated intact provides the second line of evidence for a precursor role. As mentioned previously labeled compounds may be degraded to a general metabolite and then resynthesized into the product. The C^{14} tracer of the administered compound is redistributed and the labeling pattern becomes altered. The maintenance of specific labeling patterns is shown by the lack of C^{14} randomization into other carbon atoms and by recovery of the tracer from expected positions or groups. The results of this study are evaluated in the framework of this evidence.

The results obtained from the administration of a number of labeled compounds to corn plants indicates that the aromatic ring of DIMBOA is synthesized by the shikimic acid pathway, one of the known routes for the formation of aromatic rings in biological systems. Table I shows that, of the compounds administered, uniformly labeled quinic acid was incorporated into DIMBOA with the least dilution. Moreover, 99.5% of the C^{14} incorporated was found in the aromatic ring. The ready incorporation of quinic acid and the specific labeling pattern provide good evidence that the aromatic ring synthesis of DIMBOA proceeds by the shikimic acid pathway.

This pathway, leading to the formation of amino acids and benzoic acid derivatives was first established by using *E. coli* mutants by Davis and coworkers (34). Figure 1 shows the reaction sequence and the known intermediates in the shikimic acid pathway. More recently it has been demonstrated that the same precursors occur in the aromatic ring biosynthesis in higher plants and it would appear the overall pathway is essentially the same.

The formation of aromatic compounds from quinic and shikimic acids in higher plants is well documented. Neish and coworkers have shown that shikimic acid is a good precursor for lignins (35) and flavonoids (36) in wheat and maple plants. The conversion of quinic acid (37) and shikimate to the aromatic amino acids phenylalanine, tyrosine (38) and tryptophan (39) in higher plants has also been shown to occur.

Glucose

2-Keto-3-deoxy-7-phospho-D-gluco-heptonic acid

5-Dehydroquinic Acid

Quinic Acid

5-Dehydroshikimic Acid

Shikimic Acid

Shikimic Acid-5-P

3-Enolpyruvyl-shikimic Acid-5-P

Branch Point Compound

Prephenic Acid

Phenylalanine

Tyrosine

p-Hydroxybenzoic Acid

p-Aminobenzoic Acid

Anthranilic Acid

Tryptophan

Figure 1. Shikimic acid pathway.

An alternate pathway for the biosynthesis of aromatic compounds occurring in plants is the head to tail condensation of acetate units as described by Birch (40). The ring biosynthesis of DIMBOA, however, does not involve this pathway since acetate-1-C^{14} administered to corn plants was not incorporated.

As shown in Fig. 1, 5-dehydroquinic acid in the *E. coli* system results from the cyclization of a seven carbon sugar which in turn is formed by the condensation of phosphoenolpyruvate and erythrose-4-P. Nandy and Ganguli (41) have also reported that phosphoenolpyruvate and erythrose-4-P are optimal substrates for 5-dehydroshikimate biosynthesis in extracts of mung-bean seedlings. In an attempt to evaluate if 5-dehydroquinic acid arose in a similar manner in corn, glycerate-3-C^{14} was tested as a precursor. Calcium glycerate, absorbed by the plants, would presumably be oxidized by the enzyme D-glycerate dehydrogenase, known to be present in corn (42) and would then enter the shikimic acid pathway as phosphoenolpyruvate. On condensation of the labeled metabolite with erythrose-4-P and subsequent cyclization, the tracer carbon would become the two-carbon of the ring.

The results of this feeding experiment indicated that calcium glycerate was incorporated into DIMBOA and that 62.5% of the C^{14} incorporated was associated with the aromatic ring. This finding suggests that 5-dehydroquinic acid is biosynthesized by the conventional pathway in corn. Tracer studies, employing compounds that are readily shunted into cyclic pathways, must be interpreted cautiously since they immediately give rise to a number of labeled metabolites.

Ribose also contributed to the ring carbons of DIMBOA. This incorporation was probably due to the generation of shikimic acid precursors from ribose in the pentose pathway. Furthermore, it is suggested that ribose gave rise to both the 3 carbon and 4 carbon compounds utilized in the formation of 5-dehydroquinic acid.

The aromatic ring of DIMBOA obtained from plants fed ribose-1-C^{14} contained 60.9 mμc of radioactivity. This compares with 44.4 mμc* incorporated from glycerate-3-C^{14}. If ribose only gave rise to the three carbon precursor for shikimate biosynthesis the expected amount of C^{14} incorporation would be less than that incorporated from glycerate. The dilution of a phosphoenolpyruvate derived from ribose would be at least as great and in all probability greater than the dilution resulting from glyceric acid since ribose is further removed in the pathway. However, a greater incorporation might be expected if both the 3 carbon and 4 carbon precursors were derived from ribose. The data obtained by Sprinson and coworkers (43,44) on the labeling pattern in shikimic acid synthesized by Escherichia coli from labeled glucose and xylose support this hypothesis.

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* Assuming that only one isomer of the DL-glycerate is metabolized, the experimental figure was normalized for purposes of comparison.

An *E. coli* mutant grown on media containing D-xylose-1-C^{14} as the sole source of carbon accumulated labeled shikimic acid. Carbon-14 analysis of the individual carbon atoms indicated that the xylose-1-C^{14} was incorporated into shikimate with the same labeling pattern obtained when glucose-1-C^{14} and glucose-3-C^{14} served as the carbon source. Sprinson concluded that pentose-1-C^{14} was first converted to hexose-1,3-C^{14} by the formation of sedoheptulose through a transketolase reaction (TK), followed by a transaldolase reaction (TA). Plant metabolism of 5 carbon sugars in the pentose pathway gives similar results. Gibbs and Horecker (45) have shown that conversion of ribose-1-C^{14} by an enzyme preparation from peas yields hexosemonophosphate labeled almost entirely in the one and three carbons, in a ratio of 3:1. This was in accordance with the ratios Sprinson found in shikimate derived from xylose-1-C^{14}. The carboxyl carbon of shikimate contained 14% of the incorporated C^{14} and would correspond to the 3 carbon of the hexose; the 2 carbon of shikimate contained 60% and would be derived from the one carbon of hexose. This evidence suggests that phosphoenolpyruvate, which forms the carboxyl, 1 and 2 carbons of shikimate, is derived from hexose by glycolysis.

In C^{14} content the 3 carbon of shikimate corresponded to the 3 carbon of hexose-1,3-C^{14}. Erythrose labeled in the one carbon to the same extent as the 3 carbon of the hexose would arise by a 2 carbon group transfer reaction. The erythrose-1-C^{14} forms the 3,4,5 and 6 carbons of shikimic acid.

In summary, ribose-1-C^{14} is converted to fructose-1,3-C^{14} by way of sedoheptulose-1,3-C^{14}; fructose-1,3-C^{14} gives rise to phosphoenolpyruvate by glycolysis and to erythrose in the pentose pathway. Both precursors derived from ribose could be incorporated into shikimic acid. These interconversions are depicted in Figure 2.

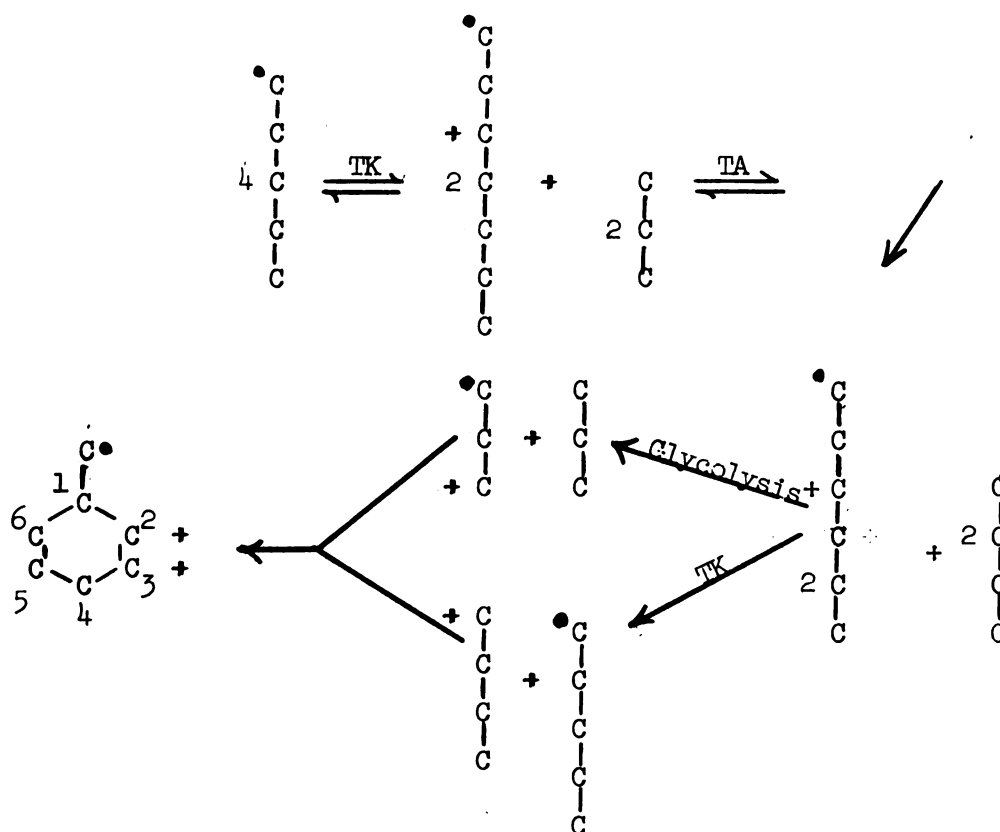

Figure 2. Proposed pathway for conversion of pentose to 3 and 4 carbon precursors of shikimic acid.

In order to confirm the double entry of ribose-1-C^{14} into the aromatic ring of DIMBOA it would be necessary to develop a degradation procedure permitting isolation and C^{14} analysis of the individual ring carbons.

The data obtained from the ribose-1-C^{14} feeding experiments further suggests that the pentose pathway is functioning according to the non-oxidative group transfer reactions rather than the oxidative pathway. If the oxidative pathway were operative the decarboxylation product of 6-phosphogluconate-1,3-C^{14} derived from ribose-1-C^{14}, as previously described, would be a pentose labeled in the 2 carbon. However, the carbon atom isolated from the 2 position of DIMBOA fed ribose-1-C^{14} presumably derived from the 2 carbon of ribose contained only 3.9% of the C^{14} incorporated into the whole molecule.

Tryptophan and its precursor anthranilic acid are the two nitrogen containing compounds occurring in the shikimic acid pathway for aromatic ring synthesis. Since DIMBOA is derived from quinic acid and possesses a nitrogen on the ring, it is possible that these three compounds share a common pathway. A common precursor might give rise to both DIMBOA and anthranilic acid or DIMBOA might be more directly derived from tryptophan. These possibilities are considered.

A number of nitrogen containing compounds occurring in plants are derived directly from tryptophan and contain the indole nucleus unchanged. However, Kowanko and Leete (46) have provided evidence for a tryptophan reaction leading to the quinoline moiety in the biosynthesis of quinine. A bond cleavage and bond formation occur in such a manner as to incorporate the two carbons of the side chain into the quinoline nucleus. DIMBOA could conceivably be derived from tryptophan in a similar manner and this possibility was tested experimentally by administering ring labeled tryptophan and uniformally labeled serine.

DL-tryptophan-7a-C^{14} administered to corn plants was incorporated into DIMBOA with a relatively large dilution of 43,700. Dilution of a metabolite to this extent does not suggest the compound was used as an immediate precursor.

There is evidence that the 3 carbon side chain of tryptophan is formed by a condensation reaction between serine and indole in peas and maize (47,48,49) as is the case in Neurospora. Assuming this mode of biosynthesis was operative, uniformally labeled serine was administered to corn plants. Degradation of the isolated DIMBOA indicated that there was no significant incorporation of C^{14} into the carbons of the heterocyclic ring. The participation of either the aromatic moiety or the side chain of tryptophan in DIMBOA biosynthesis appears highly unlikely.

Consideration of a common precursor for DIMBOA and anthranilic acid is necessarily speculative since the intermediates that must occur between 3-enolpyruvylshikimic-5-P and anthranilic acid have not, as yet, been identified.

The chemical structure of DIMBOA suggests that a likely precursor would have an amine group substituted in an ortho position to the hydroxyl on the ring. There are a few reports in the literature which suggest the existence of such an intermediate.

Pittard et al. (50) have isolated a number of phenolic and di-phenolic compounds which accumulate in cultures of an Aerobater aerogenes mutant requiring tryptophan, indole or anthranilic acid for growth. The chemical behavior of one compound isolated suggested the presence of o-dihydroxy, amino and carboxylic acid groups on the aromatic ring. The UV spectrum of the isolated compound, however, did not compare with a synthesized sample of 2-amino-3,4-dihydroxy-benzoic acid. The latter compound was very unstable. Free amino-phenols are notably unstable and if such a compound is a precursor to anthranilic acid or DIMBOA, it probably remains bound to the enzyme.

Trans 2,3-dihydro-3-hydroxyanthranilic acid has been isolated and identified from a Streptomyceses mutant fermentation mash (51). Due to the close chemical relationship to anthranilic acid, the authors suggested this compound might be an intermediate in the shikimic acid pathway.

Participation of a precursor to anthranilic acid has also been suggested for the biosynthesis of 2,3-dihydroxy-4-phenyl-quinoline (viridicatin), isolated from mycelium of Pencillium viridicatum. There is evidence that the compound is biosynthesized from phenyl-alanine and anthranilic acid (52). Tracer studies showed that phenyl-alanine was incorporated to the extent of 80% whereas only 3% of ring labeled anthranilic acid was utilized. The author attributed the low incorporation data to a large dilution of anthranilic acid which

resulted from the degradation of endogenous tryptophan. Mothes and Schutte (53) in a review article, provided an alternate explanation for the low anthranilic incorporation by suggesting that the compound incorporated into viridicatin was actually a precursor to anthranilic acid.

Recently two groups of investigators (54,55) have obtained evidence through the use of bacterial mutants for the existence of such an intermediate occurring in the shikimic acid pathway. This new compound, indicated in Figure 1 as branch point compound, appears to extend the common pathway beyond shikimic acid-5-P and is a closer precursor to anthranilic acid. The identity of an anthranilic acid precursor and an evaluation of its role in the biosynthesis of DIMBOA must await further elucidation of intermediates in the shikimic acid pathway.

The 7-methoxyl carbon of DIMBOA appears to be formed by a transmethylation reaction from methionine. Essentially all of the radioactivity incorporated into DIMBOA from methionine-methyl-C^{14} was found in the methoxyl group. Serine and glycine, known methyl donors, were also utilized as precursors but to a lesser extent. The methyl carbon from the methionine feeding contained 377.5 mμc compared to 97 mμc from the serine and glycine experiments. It is assumed that the β-carbon of serine and the α-carbon of glycine are converted to the labile methyl group of methionine in the folic acid system, followed by a transmethylation reaction to form the methoxyl group of DIMBOA.

The evidence for biosynthesis of o-methyl groups in higher plants from this precursor are in accord with the findings of others. It has been shown that the methoxyl groups of ricinine (56) and lignins (57) are derived from methionine. By employing methionine doubly labeled in the methyl group with C^{14} and deuterium, Byerrum and coworkers (57) were able to demonstrate that the methyl group was transferred as a unit to form the methoxyl groups of lignin.

Calcium glycerate-3-C^{14} also provided methyl groups for the biosynthesis of DIMBOA as 37.2% of the incorporated radioactivity was associated with the methoxyl carbon. Presumably the glycerate was first converted to serine which in turn provided the carbon for methionine. This finding is in agreement with evidence presented by Hanford and Davies (58) that crude extracts of pea epicotyls are capable of converting 3-phosphoglycerate to 3-phosphoserine presumably by way of the intermediate 3-phosphohydroxypyruvic acid.

The experimental data considered thus far indicates that the aromatic ring of DIMBOA originates in the shikimic acid pathway and that the methoxyl group is derived from methionine. The remainder of this discussion is concerned with the formation of the heterocyclic ring based on the data collected during this study and on known metabolic pathways.

The results obtained on the degradation of DIMBOA from plants administered ribose-1-C^{14} suggest that the two carbon atoms completing the oxazole ring are derived from carbons 1 and 2 of ribose. Carbon 3 of DIMBOA contained 62.5% of C^{14} incorporated and presumably corresponds to the C-1 of ribose.

If the two carbons of the heterocyclic ring are derived from the 1 and 2 carbons of ribose, the question immediately arises as to the mechanism of entry. Does ribose give rise to a two carbon compound prior to a condensation reaction with the aromatic ring precursor or is ribose condensed intact with the precursor followed by a cleavage of a 3 carbon moiety?

Ribose is known to give rise to a number of two carbon compounds. Experimentally however, such two carbon compounds as acetate-1-C^{14}, glycine-2-C^{14} and glycolate-1-C^{14} were not incorporated into the 2 and 3 positions of DIMBOA. It is further assumed that glyoxylic acid is not a precursor since the administered glycolate-1-C^{14} would have been converted to glyoxylate-1-C^{14} by glycolic oxidase present in plants.

Ribose could give rise to an "active glycoaldehyde" intermediate bound to an enzyme or cofactor as in the case of transketolase reactions. In this reaction the carbon atom, corresponding to C-2 of ribose, bound to thiamine pyrophosphate becomes an anion and condenses with an aldehyde acceptor. In the formation of the heterocyclic ring of DIMBOA, if an analogous transfer occurred to a nitrogen (possibly an N oxide) of the anthranilic precursor, the carbon corresponding to the two position of ribose would be bonded to the nitrogen atom. This mechanism is not consistent with the findings, however, since the labeling pattern indicated this carbon was derived from C-1 of ribose.

An alternate possibility for a bound intermediate arising from ribose, allowing for an incorporation of ribose into DIMBOA consistent with the experimental findings, is a mechanism analogous to the formation of acetylphosphate. This mechanism would necessitate a phenolic ion attack of the acetyl group bound to thiamine pyrophosphate to form an amino substituted phenyl acetate intermediate, followed by oxidation of the methyl group and cyclization with the amine. This sequence of reactions however is not considered very likely. Although a two carbon precursor derived from ribose cannot be eliminated, a pathway consistent with the known mechanisms of ribose metabolism is not readily apparent.

The participation of the ribose molecule in the biosynthesis of heterocyclic rings in which only two of the carbons are retained in the final product has been demonstrated. The biosynthesis of the pyrrole ring of tryptophan, the imidazole of histidine and the azine moiety of pteridines are cases in which ribose reacts with other compounds to form intermediates but only carbons 1 and 2 are incorporated into the final ring structure.

The biosynthesis of tryptophan investigated in _E. coli_ extracts involves the formation of indole (59,60). This intermediate is formed by an enzyme catalyzed condensation of anthranilic acid and 5-phosphoribosyl-1-pyrophosphate, cyclization to form the pyrrole ring followed by cleavage of the 3 remaining ribose carbons. Isotope studies have shown that the carbons in position 2 and 3 of the pyrrole

ring of tryptophan are derived from C-1 and C-2 carbons of ribose respectively. Yanofsky postulated the mechanism of indole formation involved the formation of the ribotide of anthranilic acid conversion to a deoxyribulose intermediate by an Amadori type rearrangement followed by loss of the carboxyl carbon of anthranilic acid and ring closure. This mechanism is supported by evidence that has been obtained for the existence of the two postulated intermediates, N-(5-phosphoribosyl) anthranilic acid (61) and the 1-(o-carboxyphenyl-amino)-1-deoxyribulose-5-phosphate (62).

The biosynthesis of tryptophan and DIMBOA appear to have similar characteristics. Both compounds are formed by a condensation reaction of ribose with a nitrogen compound, derived from the shikimic acid pathway. Cyclization of this intermediate leads to the incorporation of the C-1 and C-2 carbons of ribose into a heterocyclic ring structure.

Ribose is also a precursor for two carbons of the imidazole ring of histidine. Moyed _et al_. (63) in tracer studies employing ribose-5-phosphate-1-C^{14} and adenine-2-C^{14} established that the N-1 and C-2 portions of the imidazole ring were derived from carbon 2 and an attached nitrogen atom of the purine base. Carbons 4 and 5 of the ring were formed from the C-1 and C-2 carbons of ribose. N-1-(5'-phosphoribosyl) adenosine triphosphate, presumably the condensation product of the first reaction of histidine biosynthesis, has been isolated and characterized (64).

Pteridine biosynthesis in biological systems represents a third example in which a heterocyclic ring is derived in part from the C-1

and C-2 carbons of ribose. Weygand and coworkers (65) presented ample evidence that all the carbon atoms of leucopterin, a pigment from butterfly wings, were derived from guanine and a pentose. Isotopic studies showed carbon atoms 2,4,9 and 10 of leucopterin were derived from guanine and carbons 6 and 7 from a pentose. Moreover, the C-8 of guanine was not incorporated into the pterin. These authors postulated a reaction sequence consistent with an earlier suggestion by Forrest (66) for the direct conversion of guanylic acid to the pterins, xanthopterin and leucopterin. These reactions are initiated by a cleavage of the imidazole ring of guanylic acid with elimination of carbon 8, to give 2,5,6-triamino-4-hydroxyl-N-6-(5'-phosphoribosyl)-pyrimidine (II); an Amadori rearrangement of the glycosylamine to give the 2,5,6-triamino-4-hydroxy-N-6-1'-(1'-deoxyribulose-5'-phosphate) pyrimidine (III) followed by ring closure to yield (2-amino-4-hydroxy-6-tetrahydropteridinyl) glycerol phosphate (IV). On cleavage of glycerol phosphate IV would give rise to 2-amino-4-hydroxy-6-tetra-hydropteridine (V) followed by xanthopterin (VI) and leucopterin (VII) in successive oxidation steps. The postulated sequence is shown in Figure 3.

There is considerable evidence for the conversion of guanylic acid to pteridine compounds in other systems including the biosynthesis of folic acid in micro organisms (67,68). Goto and Forrest (69) isolated and characterized a compound from E. coli whose properties were consistent with a possible intermediate (2-amino-4-hydroxy-6-pteridinyl) glycerol phosphate (VIII).

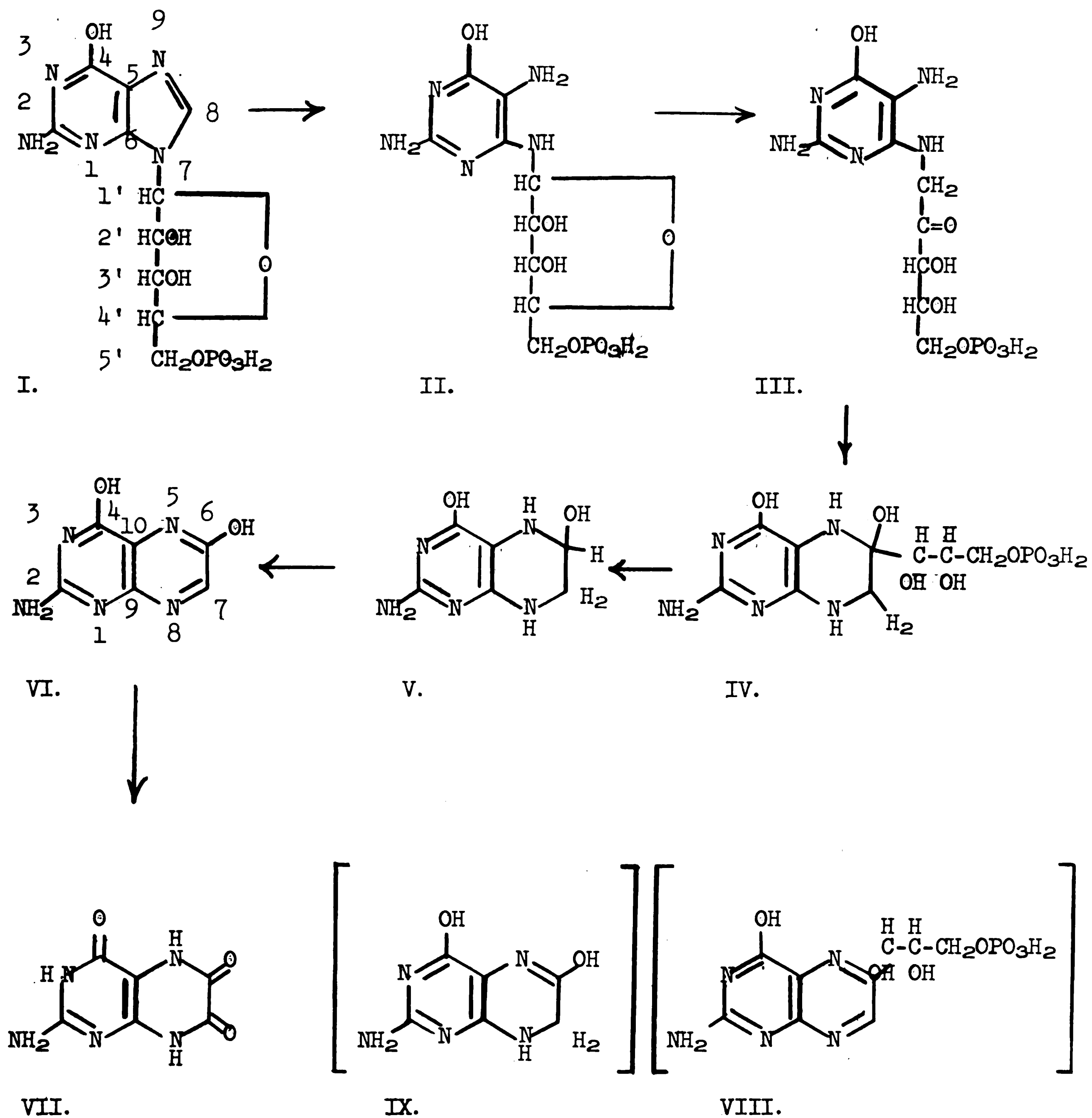

Figure 3. Postulated pathway for pteridine biosynthesis.

Experimental support for Weygand's postulated pathway was provided by Stuart and Wood (70). These workers synthesized the substituted 1-pyrimidinylamino-1-deoxypentulose III. Attempted isolation of this compound resulted in a cyclization to (2-amino-4-hydroxy-6-tetrahydro-pteridinyl) glycerol (IV). This cyclized product was readily oxidized in air with elimination of the triose to give 2-amino-4,6dihydroxy-7,8-dihydroxanthopteridine (IX). Prolonged air oxidation or treatment with $KMnO_4$ yielded xanthopterin (VI).

In regard to precursors and apparent mechanism of formation, the overall biosynthetic patterns of pteridines and tryptophan have distinct similarities. Both compounds are formed from a ribose substituted to an amine attached to a ring. The reaction sequence for this intermediate is similar in that the ribose is converted to deoxy-ribulose, followed by a cyclization reaction to form a heterocyclic ring with the inclusion of carbons 1 and 2 of ribose and finally the elimination of triose.

The formation of DIMBOA shares certain similarities with the biosynthetic pathways of tryptophan and pteridines. The evidence concerning precursors, obtained from this study, in conjunction with known pathways of heterocyclic ring formation, suggests the following reaction sequence for the biosynthesis of DIMBOA: formation of a ribosylamino derivative of an anthranilic acid precursor by a Schiff base condensation reaction; an Amadori rearrangement followed by cyclization of this intermediate to include carbons 1 and 2 of ribose in a heterocyclic ring; elimination of the triose moiety and oxidation of carbons 2 and 3 to the levels found in DIMBOA.

Unfortunately, no direct information was obtained in this study concerning the biosynthesis of the cyclic hydroxamate. However, the biosynthesis of DIMBOA by a mechanism involving a Schiff base reaction suggests that the carbon-nitrogen bond is formed prior to an N-hydroxylation reaction to yield the cyclic hydroxamate in the molecule.

There is no definitive evidence in the literature for the immediate precursor or for a biosynthetic mechanism in the formation of this structure. The biosynthesis of aspergillic acid (22) and mycelianamide (23) from amino acids suggests that the hydroxamate moiety is formed by oxidation of a peptide bond. Cramer (71) presented evidence for the *in vivo* N-hydroxylation of 2-acetylaminofluorene in the rat. He indicated, however, hydroxylation of the nitrogen may occur before acetylation, since the acetyl group is easily removed and replaced in the rat.

If cyclic hydroxamates are formed by oxidation of a peptide bond, then ribonic acid might be the immediate precursor in the synthesis of DIMBOA. On the other hand, the peptide bond could result from the oxidation of a Schiff base. Although the reaction has not been described in biological systems, the chemical conversion is known (72). A comparison of incorporation rates of ribonic acid-1-C^{14} and ribose-1-C^{14} into DIMBOA would shed some light on the formation of cyclic hydroxamates. If ribonic acid-1-C^{14} were not incorporated, a Schiff base oxidation mechanism would be indicated. However, if ribonic acid-

1-C^{14} proved to be a better precursor than ribose-1-C^{14}, two possibilities arise. The carboxyl could react with an amine to form the amide bond or it could react with an N-hydroxyamino group to give the cyclic hydroxamate directly (73).

The above consideration of possible pathways has not been exhaustive. Rather it has been an evaluation limited to those pathways which seemed most probable with regard to known metabolic mechanisms and the precursors utilized in DIMBOA biosynthesis.

SUMMARY

1. A degradation procedure was devised permitting the isolation of carbons in positions 2 and 3, the methoxyl group and the aromatic ring of DIMBOA.
2. A number of isotopically labeled compounds were administered to corn plants. The extent and position of C^{14} incorporation into DIMBOA was determined.
3. Quinic acid-U-C^{14} was incorporated into DIMBOA with a low dilution and 99.5% of the activity was associated with the aromatic ring. These results indicate that the aromatic ring biosynthesis of DIMBOA proceeds by the shikimate acid pathway.
4. Essentially all of the methyl C^{14} arising from methionine-methyl-C^{14} was found in the methoxyl group of DIMBOA. The O-methyl group most probably is formed by the conventional transmethylation reaction from methionine.
5. Ribose-1-C^{14} was significantly incorporated into DIMBOA and 62.5% of the radioactivity was associated with the carbon in the three position.
6. The significance and implications of these results are discussed and a reaction sequence is postulated for the biosynthesis of DIMBOA.

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