Tetrahedron Letters No.4, pp. 211-215, 1964. Pergamon Press Ltd.
Printed in Great Britain.

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

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(Received 18 November 1963)

The compound 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA), which exhibits antimetabolic activity, has been isolated from corn seedlings and the seedlings of some other grasses. DIMBOA has two unique structural features of biosynthetic interest. It contains a benzoxazinone ring system and it is a cyclic hydroxamate.

There are few reports in the literature of the oxazine moiety occurring in natural products. Compounds containing a phenoxazinone ring system have been found in a group of pigments isolated from insects (1), in cinnabarin found in the fungus Coriolus sanguineas (2,3) and actinomycin isolated from Streptomyces antibioticus (4). Studies on the biosynthesis of actinomycin indicate the phenoxazinone is formed by an oxidative condensation of two molecules of 3-hydroxy-4-methylanthranilic acid (5).

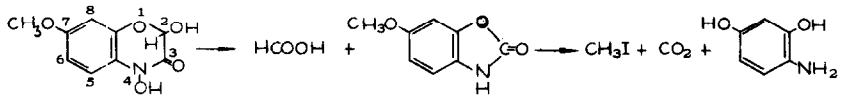
DIMBOA and a closely related analogue are the only cyclic hydroxamates isolated thus far from higher plants (6) although a number of such compounds have been found as products of mold metabolism. The biosynthesis of aspergillic acid (7) and mycelianamide (8) from amino acids suggests the hydroxamate moiety is formed by oxidation of a peptide bond. Cramer et al. (9) have presented evidence for the in vivo N-hydroxylation of 2-acetylaminofluorene in the rat. On the other hand, Emery (10) has suggested free N-hydroxyamino acids could serve as intermediates in the biosynthesis of cyclic hydroxamates.

A study of the biosynthesis of DIMBOA was therefore undertaken since no previous investigation of benzoxazinone or cyclic hydroxamate biosynthesis in higher plants has been made. In addition, information concerning the biosynthesis of DIMBOA might contribute to an understanding of the mechanism of benzoxazine and cyclic hydroxamate formation in general.

The results of the present study show that quinic acid-U-C^{14}, L-methionine-methyl-C^{14} and D-ribose-1-C^{14} are specifically and extensively incorporated into DIMBOA.

The roots and seeds were removed from 6-day old etiolated corn seedlings (Michigan Hybrid 350) 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 corn plants according to the procedure of Hamilton et al. (11).

In those cases in which incorporation of labeled precursors into DIMBOA was ascertained, the position of isotopic incorporation was determined by degradation to isolate specific carbon atoms or groups within the molecule. The degradation procedure devised permitted the isolation of carbons in positions number 2, 3, the methoxyl carbon and the benzenoid carbons.



DIMBOA, refluxed in a solution of dioxane and pyridine, formed 6-methoxybenzoxazolinone with the liberation of formic acid. The formic acid, originating from the 2 position of the DIMBOA molecule was oxidized to $BaCO_3$ according to the procedure of Sakami (12). 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. The CO_2 was flushed from the reaction mixture in a stream of N_2 which was swept through a $Ba(OH)_2$ solution 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 of DIMBOA.

All compounds to be analyzed for C^{14} were subjected to total combustion using the Van Slyke method (13). The resulting CO_2 was isolated and counted as $BaCO_3$.

From 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 into DIMBOA. The dilution, a measure of incorporation expressed as the ratio of the specific activity of the compound fed to the specific activity of DIMBOA, was 419 for quinic acid-U-C^{14}, 1,306 for methionine-methyl-C^{14} and 2,484 for ribose-1-C^{14}.

Degradation of DIMBOA by the procedure described, indicated that essentially all of the C^{14} in the compound from plants fed quinic acid-U-C^{14} was located in the benzenoid ring as shown in Table I. The ready incorporation of quinic acid and the specific labeling pattern suggests that the aromatic ring synthesis of the compound proceeds by the shikimic acid pathway. An intermediate in the biosynthesis of anthranilic acid from quinate and shikimate could be a more immediate precursor to DIMBOA and the source of the nitrogen in the hydroxamate group.

Essentially all of the radioactivity incorporated from methionine-methyl-C^{14} was found in the methoxyl group. This is consistent with the findings that the methyl carbon of methionine serves as a direct precursor for O-methyl groups synthesized in higher plants (14).

Isotope incorporation from ribose-1-C^{14} was located in the heterocyclic and the aromatic rings. Carbon in position 3 contained 62.5% of the total C^{14}.

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.

Ribose-1-C^{14} was also utilized in the shikimic acid pathway, perhaps with sedoheptulose as an intermediate, since 31.5% of the radioactivity of the molecule was associated with the aromatic ring.

Table I. DISTRIBUTION OF C^{14} IN DIMBOA FROM PLANTS FED QUINIC ACID-U-C^{14}, METHIONINE-METHYL-C^{14} AND RIBOSE-1-C^{14}

Compound Isolated in Degradation	Quinic Acid-U-C^{14}		Methionine-methyl-C^{14}		Ribose-1-C^{14}	
	Sp. Act. mμc/mmole	%	Sp. Act. mμc/mmole	%	Sp. Act. mμc/mmole	%
DIMBOA	224.3	100.0	367.5	100.0	193.2	100.0
HCOOH (position 2)	0	0	0	0	7.5	3.9
CO_2 (position 3)	0.4	0.1	0	0	120.8	62.5
Tetramethylammonium iodide (methoxyl carbon)	7.1	3.1	377.5	102.7	11.0	5.7
2,4-Diacetoxyacetanilide (benzenoid ring carbons)	223.5	99.5	2.0	0.5	60.9	31.5

From the evidence presented we conclude the aromatic ring of DIMBOA is biosynthesized from an intermediate derived from shikimic acid, the O-methyl group is formed by a transmethylation reaction from methionine and the heterocyclic ring carbons are derived from an intermediate formed from the 1 and 2 carbons of ribose.

The incorporation of these ribose carbons suggests a reaction sequence initiated by a Schiff base condensation of ribose and an amine

substituted shikimic acid intermediate, an Amadori rearrangement of the ribose followed by ring closure. Such a sequence of reactions is analogous to the biosynthetic pathway that has been postulated for the formation of the pyrrole ring of tryptophan (15) and the azine ring of pteridines (16,17). The biosynthesis of DIMBOA by a mechanism involving a Schiff base reaction further suggests that the carbon-nitrogen bond is formed prior to the N-hydroxylation reaction to yield the cyclic hydroxamate in the molecule.

Acknowledgements

We wish to thank Dr. S. A. Brown, National Research Council, Saskatoon, Canada for kindly supplying the quinic acid-U-C^{14}.

This investigation was supported in part by a grant (RG7862) from the National Institutes of Health, U. S. Public Health Service.

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