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Incorporation of Anthranilate-d_4 into DIMBOA in Maize

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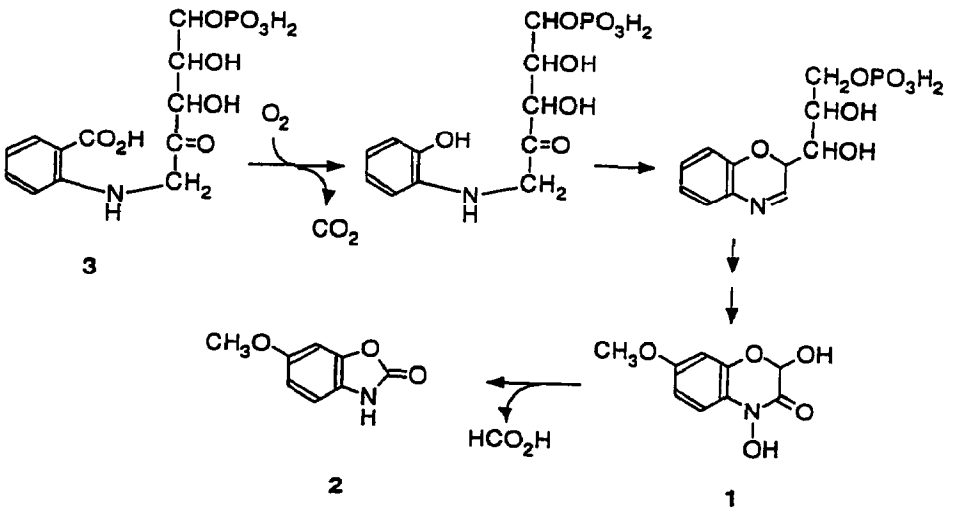
***Abstract*:** 2,4-Dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) is synthesized in maize from anthranilic acid-d_4 with high retention of deuterium. Retention of deuterium is only consistent with a mechanism of oxidative decarboxylation of o-carboxyanilinoribulose-5-phosphate or other anthranilate derivative via an intermediate in which oxygenation occurs at the carboxyl-bearing ortho carbon before decarboxylation.

The labile metabolite 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (**1**) (DIMBOA), a major defense of maize and wheat seedlings against microorganisms and insects, is stored as its glucoside and released at wound sites by a glucosidase.[1] DIMBOA subsequently decomposes to give the less toxic 6-methoxy-2(3H)-benzoxazolone (**2**) (MBOA) and formic acid.



Scheme 1

Previous studies on the biosynthesis of DIMBOA with ^{14}C-, ^{15}N-, and ^{3}H-labeled substrates have shown that the benzene ring and nitrogen atom of anthranilic acid are incorporated into DIMBOA and that the two carbons of the heterocyclic ring derive from C-1 and C-2 of ribose.[2,3] Anthranilic acid and ribose are also precursors of tryptophan in which the two heterocyclic ring carbon atoms are also derived from ribose C-1 and C-2, however [^{14}C]tryptophan is not significantly incorporated into DIMBOA.[2] It has been suggested that the DIMBOA pathway may proceed through 5-phosphoribosyl anthranilate and its Amadori rearrangement product, o-carboxyanilinoribulose-5-phosphate (3) (Scheme 1). These compounds are known to be intermediates in the tryptophan pathway in microorganisms[4] and in *Arabidopsis thaliana* .[5]

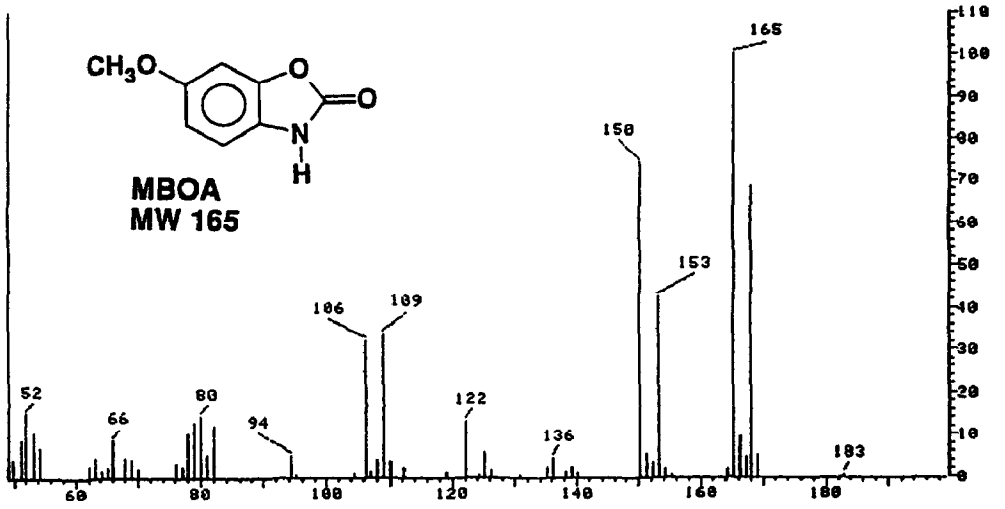


Figure 1. EI-MS of MBOA recovered from maize grown on anthranilic acid-d_4.

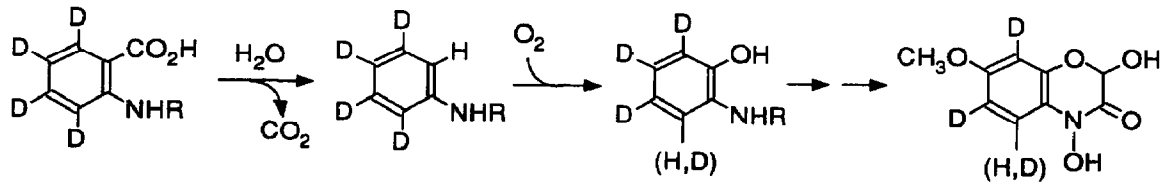
Anthranilic acid-d_4, prepared from the minor nitration isomer of ethyl benzoate-d_5 (Sigma, 99% isotopic purity), was used to study an early step in DIMBOA biosynthesis. Mass spectral analysis showed that the isotopic purity of the anthranilic acid-d_4 was 98.5% (94.1% d_4 species, 5.9% d_3). DIMBOA, isolated from maize shoots grown in Heller medium containing 2 mM anthranilic acid-d_4, was converted into MBOA by heating at 100° C for 1 hr in pH 7 phosphate buffer. MBOA was purified by TLC and analyzed by EI-MS (Fig. 1). Integrated intensities between m/z 165 and 169 (Table 1) show that 39% of the MBOA contains three deuteriums and is thus derived from exogenous anthranilate-d_4.

Table 1. Predicted and Observed Intensities of MBOA Molecular Ions

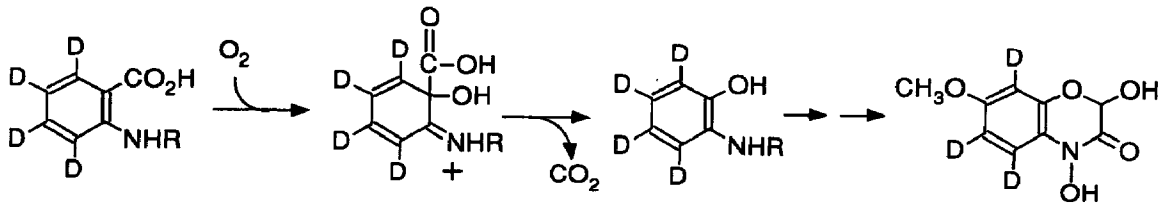
m/z	MBOA, predicted rel. intensity*	deuterated MBOA, predicted rel. int.*	Predicted sum	Observed rel. intensity
165	151.33	--	151.33	151.33
166	14.01	--	14.01	13.65
167	1.48	6.28 §	7.76	8.77
168	--	100.00	100.00	100.00
169	--	9.26	9.26	10.18

* Includes natural abundance ^{13}C, ^{15}N and ^{18}O.
§ From anthranilic acid-d_4, 94.1%, -d_3, 5.9%, with no deuterium loss (Scheme 2b).

Reimann and Byerrum[2] demonstrated that 3-hydroxyanthranilic acid is not incorporated into DIMBOA, ruling out introduction of the heterocyclic O-1 via oxygenation of the unsubstituted ortho site of anthranilic acid followed by decarboxylation. Two other sequences of decarboxylation and oxygenation are possible. One involves enzymatic decarboxyation to give phosphoribulosylaniline, followed by oxygenation to phosphoribulosyl-o-hydroxyaniline (Scheme 2a, R=5-phosphoribulosyl), and the other begins with oxygenation at the carboxyl-bearing carbon, followed by decarboxylation (Scheme 2b).



Scheme 2a



Scheme 2b

On enzymatic decarboxylation in ordinary water, phosphoribulosylaniline-d_4 would acquire one ortho ^1H. In subsequent reactions the ^1H and ^2H ortho hydrogens are indistinguishable leading to 50% retention of ^1H unless the rate determining step involves breaking the ortho C-H bond. The maximum kinetic isotope effect (K_H/K_D=6.4, 25°) would decrease ^1H retention to 13.5% only if the transition state involves the full effect of unsolvated C-H bond breaking. If hydrogen loss precedes the rate determining step, then exchange with the medium occurs before reaction leading to greater than 50% ^1H at the ortho position. Thus if phosphoribulosylaniline (Scheme 2a) were an intermediate in the biosynthesis of DIMBOA from anthranilate-d_4, at least 13.5% of the product would retain the ^1H introduced on decarboxylation and have m/z 167 (MBOA-d_2). In fact all but 1% of the intensity at m/z 167 can be accounted for by the natural abundance ^{18}O-containing ion of undeuterated MBOA plus the 5.9% trideuterated anthranilic acid in the deuterated anthranilic acid administered to maize shoots. Virtually no aryl ^1H is acquired from water in the conversion of ring-deuterated anthranilic acid into DIMBOA and subsequently MBOA, therefore oxygenation of anthranilic acid must occur at the carboxyl-bearing carbon before decarboxylation as shown in Scheme 2b.

ACKNOWLEDGMENT

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