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BIOSYNTHETIC ORIGIN OF OXYGEN ATOMS IN DIMBOA FROM MAIZE: NMR STUDIES WITH $^{18}O_2$

SPECIAL PUBLICATION*

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Key Word Index—*Zea mays*; Gramineae; cyclic hydroxamic acids; DIMBOA biosynthesis; cytochrome P450 monooxygenases.

Abstract—Maize seeds were germinated in an atmosphere of $^{16}O_2/^{18}O_2$. 2,4-Dihydroxy-7-methoxy-1,4-benzoxazine-3-one (DIMBOA) was isolated and analysed by ^{13}C NMR spectroscopy. Heavy isotope shifts of ^{13}C signals indicated that all four oxygen atoms bonded to carbon atoms of DIMBOA are derived from molecular oxygen.

INTRODUCTION

1,4-Benzoxazine-3-ones are the predominant class of secondary metabolites in young cereal grasses [1]. In maize, 2,4-dihydroxy-7-methoxy-1,4-benzoxazine-3-one (DIMBOA) is important for resistance against insects [2], fungi [3] and bacteria [4]. DIMBOA is synthesized in large quantities [5] during the first days after germination and stored in the vacuole in a glucosylated form [6].

Radioactive labelling experiments have shown that DIMBOA is synthesized from a biosynthetic precursor of tryptophan [7]. More specifically, indole was recently identified as the committed precursor of DIMBOA (Fig. 1) [8]. Two reactions of the DIMBOA pathway, i.e. the C2-hydroxylation of benzoxazinone [9] and the *N*-hydroxylation of 2-hydroxy-benzoxazinone (HBOA) [10] have been studied *in vitro*. They both depend on the microsomal fractions of maize seedlings, molecular oxygen and NADPH. These monooxygenase reactions are reversibly inhibited by carbon monoxide which characterizes the responsible enzyme as cytochrome P450. The other enzymes involved in the biosynthesis of DIMBOA are unknown. A glucosylase required for vacuolar storage of DIMBOA has been described [11]. In this report we demonstrate that all four oxygen atoms bonded to carbon atoms of DIMBOA are introduced by monooxygenases.

RESULTS AND DISCUSSION

Maize seeds were germinated in the dark for 2 days at 28°. The seedlings were placed in a gas-tight container filled with a mixture of $^{16}O_2$ and $^{18}O_2$ at a ratio of 1:2 (v/v) to a pressure of 0.96 bar. The container was incubated in the dark for 48 hr.

DIMBOA was isolated according to a modification of the procedure of Bailey and Larson [11]. NMR spectra were recorded in deuterated methanol. The presence of ^{18}O induces an upfield isotope shift of the natural abundance ^{13}C NMR signal of adjacent carbon atoms [for a review, see 12]. The ^{13}C signals of DIMBOA from the experiment with the $^{16}O_2/^{18}O_2$ mixture are shown in the lower part of Fig. 2. The upper part shows a spectrum obtained after the addition of unlabelled DIMBOA for internal standardization. The signals for carbon atoms 3, 7, 8a and the methyl group show upfield satellites indicating the binding to ^{18}O. The ^{13}C signal for C-2 appears with two upfield satellites indicating the presence of isotopomers with one and two ^{18}O atoms bound at C-2. This indicates that all four oxygen atoms bound to carbon atoms of DIMBOA acquired isotope labelling from molecular ^{18}O. Isotope shifts and signal intensities are summarized in Table 1. With the exception of C-2, the relative intensities of the non-shifted sig-

* Papers appearing under the heading have received accelerated publication due to their particular significance in the field of biosynthesis.

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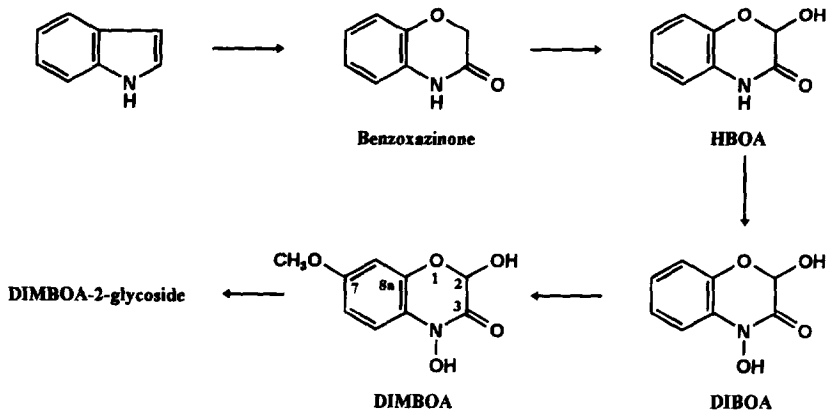

Fig. 1. Hypothetical scheme for biosynthesis of DIMBOA [8].

nals (i.e. with adjacent ^{16}O isotope) and the shifted signals (i.e. with adjacent ^{18}O, marked by an arrow in Fig. 2) are in the range of 1:2, thus reflecting faithfully the $^{16}O_2/^{18}O_2$ composition in the growth chamber.

If both oxygen atoms bonded to C2 are derived from molecular oxygen of 67% enrichment, the expected isotopomer composition would be 11% unlabelled, 44% single labelled and 44% double-labelled (i.e. [1,2a-$^{18}O_2$]DIMBOA). The NMR signature of C-8a indicates that O-1 has about 70% enrichment as expected. However, the deviation of the isotopomer signature at C-2 from the expected value as shown in Fig. 2 and Table 1 shows that the ^{18}O abundance of the hydroxy group at C-2 is significantly reduced. A loss of label from this position could occur by acid hydrolysis of DIMBOA glucoside [11].

The formation of the *N*-oxide motif in DIMBOA is catalysed by a cytochrome P450 monooxygenase using molecular oxygen. Our data show that all other oxygen atoms of DIMBOA are also derived from molecular oxygen, most probably by P450-type enzymes which have been shown to be involved in the biosynthesis of numerous secondary plant metabolites [13]. The sequence of the hydroxylation reactions is not yet known. A P450 gene family expressed specifically in maize seedlings has been described recently

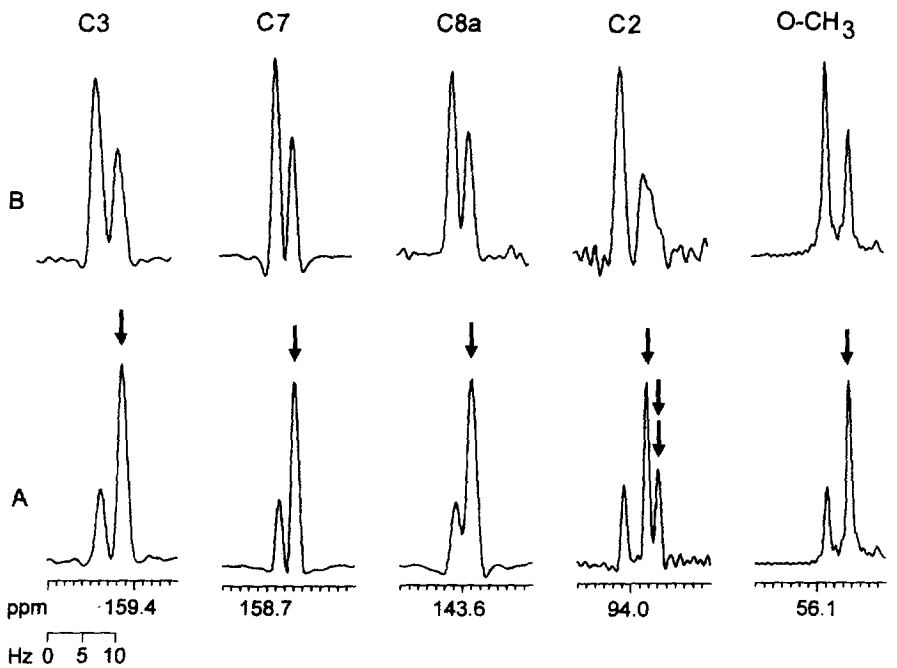

Fig. 2. ^{13}C NMR signals of DIMBOA. (A) DIMBOA isolated from seedlings germinated in an atmosphere containing 67% $^{18}O_2$; (B) after addition of an equal amount of unlabelled DIMBOA for internal standardization. The addition of the unlabelled material caused some decrease of resolution, probably by traces of paramagnetic ions. Single arrows indicate isotopomers with one ^{18}O atom bound to the respective carbon atom. The double arrow indicates the signal of [1,2a-$^{18}O_2$]DIMBOA.

Table 1. ^{13}C NMR analysis of DIMBOA from maize grown under an atmosphere of a $^{16}O_2/^{18}O_2$ (1:2; v/v)

Position*	Chemical shift† ppm	Isotope shift‡ $D^{13}C(^{18}O)$ ppb	Isotope shift‡ $D^{13}C(^{18}O_2)$ ppb	Isotopomer composition§ $[^{18}O_1]$ %	Isotopomer composition§ $[^{18}O_2]$ %
3	159.44	25		71	
7	158.68	19		70	
8a	143.61	19		69	
4a	123.25				
5	115.22				
6	108.85				
8	104.72				
2	94.01	26	39	55	22
O-CH_3	56.09	26		69	

*Signal assignments are based on two-dimensional homo- and heteronuclear NMR analysis (DQF-COSY, HMQC, HMBC).

† Chemical shifts are referenced to a solvent signal of methanol-d_4 at 49.0 ppm.

‡ An upfield shift in the ^{13}C NMR chemical shift upon ^{18}O isotopic substitution is given as a positive value according the convention used by Hansen [15].

§ Referenced to the sum of the ^{13}C NMR signal integrals of each carbon atom.

[14]. Whether these genes are involved in hydroxylations conducive to DIMBOA remains to be investigated.

EXPERIMENTAL

Plant materials. 50 g maize seeds (Blizzard, Ciba Geigy) were placed in wet germination paper and incubated in the dark for 2 days at 28°. The germinated seeds were placed in a 1 l beaker containing 200 ml of 50 mM MES, pH 5.8, and transferred to a 5 l gas-tight container partly filled with H_2O. The container was evacuated and was then filled with a mixture of $^{16}O_2$ and $^{18}O_2$ at a ratio of 1:2 (v/v) to a pressure of 0.96 bar. The container was incubated at 28° in the dark for 48 hr.

Isolation of DIMBOA. DIMBOA was isolated according to a modification of the procedure of Bailey and Larson [11]. Seedlings were ground in liquid N_2. H_2O (3 × wet wt of seedlings) was added, and the mixt. was incubated at room temp. for 1 hr to allow for hydrolysis of DIMBOA glucoside. The suspension was centrifuged for 20 min at 26000 *g*. The supernatant was extracted twice with one volume of EtOAc. The organic phase was evaporated to dryness under red. pres., and the resulting red-brown residue was dissolved in MeOH and chromatographed on HPLC.

HPLC. Performed on a Beckman system using a Merck LiChroCART RP-18 column (10 × 250 mm). The eluent contained 9% HOAc and 10% MeOH in H_2O (v/v). DIMBOA containing fractions were pooled and lyophylized.

NMR spectroscopy. NMR spectra were recorded in deuterated methanol using a Bruker DRX500 spectrometer equipped with a dual $^1H/^{13}C$ probe head. ^{13}C NMR spectra were measured as follows: 45° pulse (3 μsec); repetition time, 3.2 sec; spectral width, 29 kHz; data set, 64 kilo-words; temp., 10°; zero-filling to 128 kilo-words, and gaussian apodization prior to Fourier transformation; ^1H decoupling by WALTZ16 during acquisition and relaxation.

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