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BIOTRANSFORMATION OF 2-BENZOXAZOLINONE AND 6-METHOXY-BENZOXAZOLINONE BY *FUSARIUM MONILIFORME*

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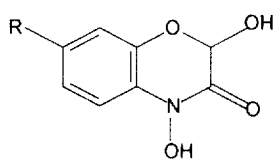
Key Word Index—*Fusarium moniliforme*; fungi; hydroxamic acid; MBOA; BOA; corn; malonamic acid; detoxification.

Abstract—*Fusarium moniliforme*, a fungus that commonly produces a symptomless endophytic association with most corn cultivars and inbred lines, was previously shown to catabolize 6-methoxy-benzoxazolinone (MBOA) and 2-benzoxazolinone (BOA), biologically active compounds produced by corn and known to be fungistatic. Studies were undertaken to isolate and identify the breakdown products of this decomposition. For each benzoxazolinone, a novel acid was isolated by column chromatography from 48-h old cultures of *F. moniliforme*. MS, NMR, and IR spectral data were used to identify the compounds as N-(2-hydroxyphenyl) and N-(2-hydroxy-4-methoxyphenyl) malonamic acids, products of BOA and MBOA, respectively.

INTRODUCTION

Cyclic hydroxamic acids with the 1,4-benzoxazin-3-one skeleton are secondary metabolites found in several grasses (Gramineae), including corn, wheat, and rye [1]. In the intact plant, the hydroxamic acid exists as a glucoside, but the aglycone is rapidly cleaved by a β-glucosidase upon disruption of cellular integrity. These compounds are abundant during seedling development [2] and are considered defense chemicals because of their wide range of biological activity against specific crop pests [3, 4, 5]. The most abundant hydroxamic acids in corn are 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) **1** and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) **2** [1]. These compounds are unstable in aqueous solutions and are rapidly converted to 2-benzoxazolinone (BOA) **3** and 6-methoxy-benzoxazolinone (MBOA) **4**, respectively [6]. The benzoxazolinones are also inhibitory to several fungal plant pathogens including *Helminthosporium turcicum* Passerini [7], *Septoria nodorum* (Berk.) Berk. [3], *Microdochium nivale* (Fries) Samuels and Hallett (*F. nivale* (Fr.) Ces.) [8], and *Fusarium moniliforme* J. Sheldon [9].



1. R=H DIBOA
2. R=OMe DIMBOA

F. moniliforme is one of the most common fungi associated with corn (*Zea mays* L.), usually as a symptomless endophyte [10, 11]. In addition to producing several potent mycotoxins on corn used for human and livestock food- and feedstuffs, it can also act as a pathogen during its early association with corn seedlings [10, 11]. One of the least understood aspects of the *F. moniliforme* complex on corn is the seedling blight disease that sporadically expresses itself on various corn cultivars and inbred lines [11]. The relationship of the benzoxazolinones to the endophytic colonization and occasional seedling disease development is unknown. We previously reported that the nature of the inhibition of *F. moniliforme* to the benzoxazolinones was fungistatic, and that *F. moniliforme* can rapidly degrade the benzoxazolinones to products that had reduced biological activity to *F. moniliforme* [9]. In the present paper, we describe the isolation and structural identification of the biodegradation products of BOA and MBOA by *F. moniliforme*.

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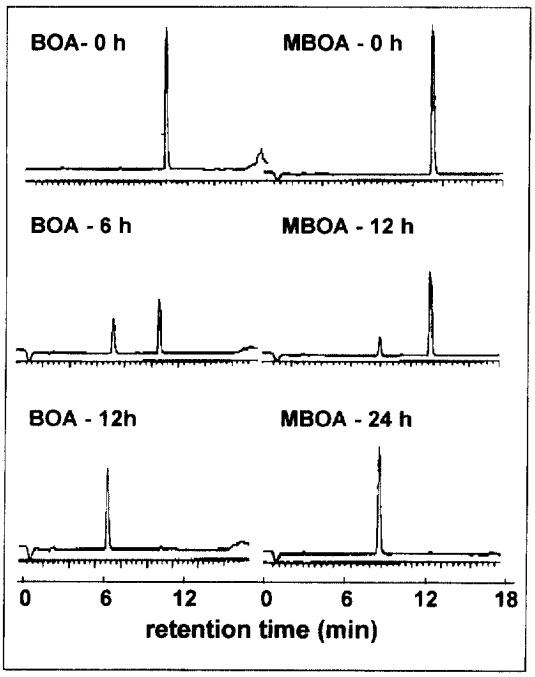


Fig. 1. HPLC chromatograms of extracts from *F. moniliforme* cultures in which MBOA and BOA was added at 1.0 mM concentrations, indicating the transformation of each benzoxazolinone, and the appearance of the products.

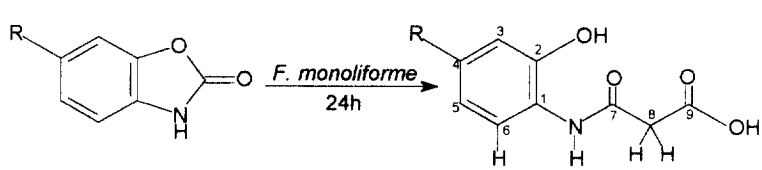
RESULTS AND DISCUSSION

The chromatographic peaks corresponding to BOA and MBOA disappeared completely within 24 h and a single new peak appeared in each culture (Fig. 1). After complete degradation of the parent compound, the medium was filtered and acidified to pH ~2.75, and extracted with ethyl acetate. The major products of BOA and MBOA were purified by silica gel column chromatography and the resulting compounds **5** and **6** were identified as the novel acids, N-(2-hydroxyphenyl) malonamic acid and N-(2-hydroxy-4-methoxyphenyl) malonamic acid, respectively.

The EI and DCI mass spectral data of **5** showed a $[M+H]^+$ peak at *m/z* 196, which corresponds to a molecular formula of $C_9H_9NO_4$. The base peak at *m/z* 109 was assigned to an ortho-hydroxylaniline moiety. The IR data established the presence of a carboxylic group (3200–2500 cm^{-1} broad band and 1702 cm^{-1}). The band at 1650 cm^{-1} was caused by the stretching vibration of the amide carbonyl. In the aromatic region of the ^1H NMR and ^{13}C NMR spectra of **5**, the chemical shifts and splitting patterns of protons were characteristic of a 1,2-disubstituted benzene derivative (Table 1) which were similar to those of substrate **3** [12]. The electron-donating substitution of the C-2 hydroxyl group caused the higher shielding effect observed at H-3 and H-5. Therefore, a double triplet at δ 6.83 ppm with a coupling constant $J = 8.0$ Hz was assigned to the signal of H-5, while a doublet at δ 6.91 ppm was assignable to H-3. The proton at C-6 was observed at δ 7.79, the lowest chemical shift of the proton on the aromatic ring, because of the deshielding effect of the electron-withdrawing substitution of the C-1 amide. The assignment of protons was confirmed by ^1H-^1H COSY spectral analysis.

The ^{13}C NMR and APT spectra showed two signals at δ 166.63 ppm and δ 169.98 ppm which were assigned to the carbonyl groups. The methylenic functionality at δ 42.70 ppm in the ^{13}C NMR spectra and the two proton singlet at δ 3.61ppm in the ^1H NMR spectrum suggested that the methylene group was juxta-positioned between the carbonyl groups. The broad, D_2O exchangeable singlet at δ 9.52 ppm was assigned to the amide proton, whereas neither phenolic or carboxylic acid hydroxyl protons were observed in the acetone-d_6 solution. NOESY experiments showed the interaction between NH and CH_2 and 6-H. The ^1H-^{13}C COSY experiment provided the evidence to confirm the assignments of secondary and tertiary carbons. The signals at δ 148.69 ppm and δ 127.53 ppm were assigned to C-2 and C-1, respectively, on the basis of the substituent-shift rule. Thus, the structure of metabolite **5** was established as N-(2-hydroxyphenyl) malonamic acid. MBOA was converted to N-(2-hydroxy-4-methoxyphenyl) malonamic acid **6** by *F. moniliforme* in a similar way to that of BOA to **5**. The structure of **6** was based on its analogy to **5**.

It is worthwhile noting that N-aryl malonamic acids can be formed from benzoxazolinone derivatives by biotransformation, as these acids have been shown to possess both plant growth regulatory [13] and fungicidal [14] activity. Procedures for the direct synthetic preparation of N-aryl malonamic acids involve refluxing the appropriate aniline with malonic acid, treating the aniline with diethyl malonates or with ethyl malonyl chloride followed by alkaline hydrolysis of the ester group, or by treating the Meldrum's acid with a silylated aniline followed by hydrolysis of malonic silyl ester [15]. Though a one-pot synthesis of N-aryl-malonamic acids was reported recently based on the reaction of *in situ* generated malonyl chloride



3. R=H BOA
4. R=OMe MBOA

5. R=H
6. R=OMe

Table 1. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data (acetone d_6 as int. standard) of compounds **5** and **6** (J in Hz in parenthesis)

	^{1}H (δ)		^{13}C (δ)	
Position	5	6	5[a]	6
1	—	—	127.53	124.12
2	—	—	148.69	154.73
3	6.91 *dd* (8.2, 1.6)	6.48 *d* (3.0)	117.53	103.83
4	7.00 *td* (7.8, 1.6)	—	126.31	159.25
5	6.83 *td* (8.0, 1.6)	6.43 *dd* (8.7, 3.0)	120.81	105.12
6	7.79 *dd* (8.0, 1.6)	7.50 *dd* (8.7, 0.6)	122.66	120.55
7, 9	—	—	166.63	166.84
			169.98	169.81
8	3.61 *s*	3.57 *s*	42.70	42.35
O—Me	—	3.73 *s*	—	55.79
NH[b]	9.52 *brd*	9.46 *brd*	—	—

* Assignments assisted by HETCOR spectra.
** Signal disappeared after adding D_2O

with aniline derivatives [16], the preparation of N-(*ortho*-hydroxyaryl) malonamic acids is difficult because the hydroxyl group is also sensitive to acetyl reagents. Therefore, it is significant that N-(*ortho*-hydroxyphenyl) malonamic acids can be easily biosynthesized from benzoxazolinone derivatives by *F. moniliforme*.

The biodegradation of BOA and MBOA with *F. moniliforme* may be an enzyme-catalyzed reaction, based on the observations that degradation of BOA and MBOA occurred more rapidly in cultures that had been previously exposed to low levels (0.5 mM) of the compound (Fig. 2). Several important classes of phytoalexins are known to be detoxified by enzyme-mediated schemes, e.g., the phytoalexin of *Nectria haematococca* [17]. The specific mechanisms of biotransformation of the benzoxazolinones will be the focus of future studies.

EXPERIMENTAL

Fungal isolates and culture conditions

The *F. moniliforme* isolate RRC38 [9] was used exclusively in this study. Two 500 ml cultures containing Difco Czapek-Dox broth (Difco Co., Detroit, Michigan, pH 7.2–7.4), and inoculated with a conidial suspension (1×10^6 conidia ml^{-1}) of RRC38, were grown for 24 h on an orbital shaker prior to addition of BOA or MBOA. One hundred mg of commercially available BOA (Aldrich Chemical Co. (Milwaukee, WI) or MBOA (Lancaster Synthesis (Windham, NH) was dissolved in 20 ml of ethanol, filter-sterilized (0.25 μm), and added to each culture. Earlier studies [9] demonstrated that this amount of absolute ethanol

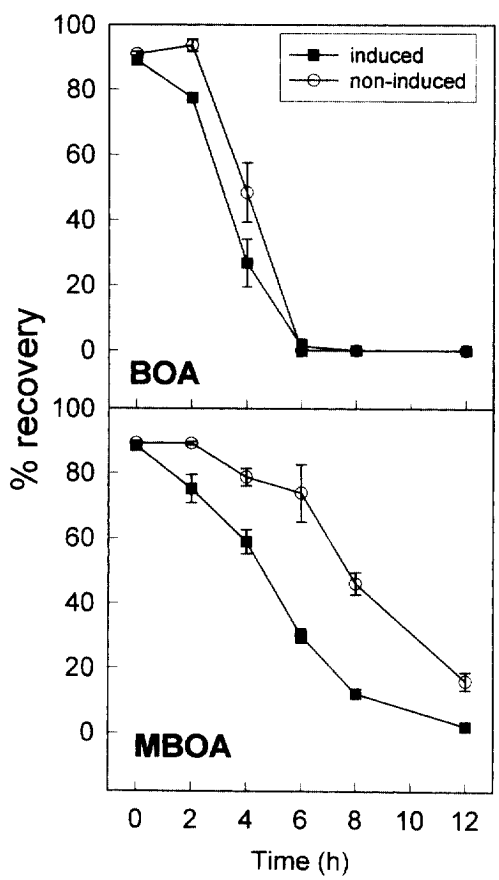


Fig. 2. Biotransformation of a 2.5 mM solution of BOA and MBOA by *F. moniliforme*. Figure compares cultures that had been previously exposed (induced, ■) to BOA or MBOA at 0.5 mM concentrations against cultures that were not prior exposed to the compounds (non-induced, ○).

had no adverse effect on the growth of *F. moniliforme*. [The conversion of BOA and MBOA to their malonamic acids has also been shown to occur when substrates were dissolved in equal concentrations of DMSO and acetone (Richardson, unpublished data).] Following the addition of MBOA and BOA to cultures of *F. moniliforme* (isolate RRC38), cultures were incubated on a rotary incubator for 24 h at room temperature. The amount of BOA and MBOA and their metabolites in the medium was monitored at 280 nm with a variable wavelength detector, usually for 24 h following an HPLC procedure previously described [9]. The media from these cultures were then used for isolation of products.

Extraction and isolation of metabolites

Fungal cultures were filtered through a Whatman no. 2 paper, the pH of the filtrate adjusted to ~2.75 with 1M HCl, and the samples were partitioned 2 times with 500 ml of ethyl acetate. The organic phase was reduced to dryness *in vacuo*. The residue (ca 140 mg) thus obtained was chromatographed on a column (1.8 × 20 cm) containing silica gel (30 g, 230–400 mesh) and eluted with $CHCl_3$:MeOH:HCOOH (100:3:0.1). After a highly pigmented (golden-yellow) band was eluted from the column, fractions with similar compositions were combined and evaporated. Purified compounds were obtained by recrystallization of the residues in CH_3OH:$CHCl_3$ (1:1). IR spectra were recorded on a Cygnus 100 FT-IR spectrometer and MS were determined with EI and DCI in a Finnigan MAT 8230 spectrometer. NMR spectra (400 MHz ^1H and 100 MHz ^{13}C) were performed in acetone-d_6 on a Varian 400 FT-NMR spectrometer. Compound **5**: ^1H,^{13}C NMR data are shown in Table 1. EI-MS (probe) *m/z* (rel. int): 196 $[M+H]^+$(3), 195 $[M]^+$(24), 177 $[M\text{-}H_2O]^+$(6), 151 $[M\text{-}CO_2]^+$(12), 133 (12), 135 (6), 110 (10), 109 (100), 80 (25), etc.; DCI-MS (probe) *m/z* (rel. int): 196 $[M+H]^+$(67), 152 $[M+H\text{-}CO_2]^+$(100), 134 $[M+H\text{-}CO_2\text{-}H_2O]^+$(54), etc.; IR ($\nu_{max}$ cm^{-1}): 2500–3200, 1702, 1651, 1615, 1590,1455 and 754. Compound **6**: ^1H,^{13}C-NMR data are shown on Table 1. EI-MS (probe) 70ev, *m/z* (rel. int): 226 $[M+H]^+$(5), 225 $[M]^+$(39), 207 $[M\text{-}H_2O]^+$(25), 181$[M\text{-}CO_2]^+$(21), 165 (24), 163 (19), 139 (100), 124 (80%), 110 (24), etc.; DCI-MS (probe) 70ev, *m/z* (rel. int): 226 $[M+H]^+$(34), 182 $[M+H\text{-}CO2]^+$(100), 164 $[M+H\text{-}CO_2\text{-}H_2O]^+$(35), 152 $[M+H\text{-}CO_2\text{-}O CH_3]^+$(60), etc.; IR ($\nu_{max}$ cm^{-1}): 2500–3200, 1697, 1643, 1605, 15280, 1450, 849 and 796.

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