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DEGRADATION OF DIAZINON BY 2,4-DIHYDROXY-7-METHOXY-2H-1, 4-BENZOXAZIN-3(4H)-ONE IN MAIZE

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Abstract—A cyclic hydroxamate, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA), was isolated and identified from shoots of 6-day-old corn seedlings grown in the dark. From 100 g of plant tissue 100 mg of DIMBOA were isolated. This hydroxamate was very effective in catalysing the hydrolysis of the pyrimidinyl organophosphate insecticide, diazinon (*O*, *O*-diethyl-*O*-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] phosphorothioate) to 6-methyl-2-(1-methylethyl)-4-hydroxypyrimidine and diethyl phosphorothioic acid. The optimum pH for hydrolytic activity was 5 and at pH values equal to or higher than the pK_a of the hydroxamic group (6.95) most of the activity was lost.

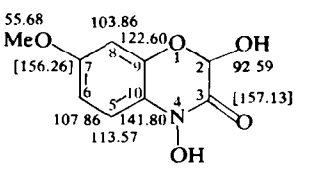
INTRODUCTION

Hydroxamic acids are known to reactivate organophosphate-inhibited acetylcholinesterase [1] and to catalyse the hydrolysis of certain organophosphates [2]. Cyclic hydroxamic acids have been isolated from the cereal grasses, maize, wheat and rye [3–14] and are found in these plants mainly as glucosides. Upon injury of the plant cells, the glucosides are hydrolyzed enzymatically by β-glucosidases to yield glucose and the corresponding aglucones [5–17]. The cyclic hydroxamate, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA), was shown to be an active agent in the resistance of maize to the European corn borer [18–28] and was also demonstrated to be a factor in resistance of corn to stalk rot [29–32] and to leaf blight [33, 34], resistance of wheat to stem rust [35, 36], and resistance of corn to attack by the corn leaf aphid [37]. Tolerance of corn to 2-chloro-*s*-triazine herbicides has likewise been attributed to the presence of DIMBOA [38–47]. Cyclic hydroxamic acids isolated from corn have recently been demonstrated to be mutagenic to *Salmonella typhimurium* [48]. Although cyclic hydroxamic acids derived from plants have been studied extensively over the past two decades, no evidence has been presented concerning the reactions of these compounds with organophosphorus insecticides. In the present paper we report the degradation of a pyrimidinyl organophosphate insecticide, diazinon, by DIMBOA isolated from corn, as well as additional information on the purification and properties of this hydroxamate.

RESULTS AND DISCUSSION

In a previous study [49] the *in vitro* degradation of diazinon by subcellular fractions of corn was found to be due partly to the presence of an active glutathione *S*-transferase system. The major degradative system which results in up to 72% diazinon degradation could not be identified as any of the enzymes known to occur in animals or plants which metabolize pesticides. Upon trypsin digestion, there was no loss of degradative activity suggesting the presence of a non-enzymatic catalytic system. Subsequent purification and characterization revealed that the factor responsible for diazinon hydrolysis was DIMBOA, a compound reported in corn, wheat and rye seedlings [5, 10, 13, 50]. The identity of the diazinon hydrolysis factor was established as DIMBOA by rigorous comparison of the isolated factor with authentic DIMBOA by a variety of techniques. Data from ^{13}C and ^{1}H NMR, IR and EI-MS [*m/e* 211.0516 (obs.); 211.0680 (calc.)] were identical to those obtained from an authentic sample.

The ^{13}C NMR spectrum was determined in dimethyl sulfoxide-d_6 in a 10-mm sample tube at 15 MHz. Chemical shifts are in ppm relative to TMS. Assignments were made by comparison with lit. values for similar carbon groups [50]; those in brackets are tentative and may be reversed.



The elemental analysis (C 50.99, H 4.67, O 37.59, N 6.50, obs.) was also the same as that of the authentic sample. Both DIMBOA and the isolated compound gave a deep blue color (λ_{max} 590 nm) with ferric chloride reagent [52]. Finally, TLC also substantiated the rest of our data, i.e. the substance isolated from

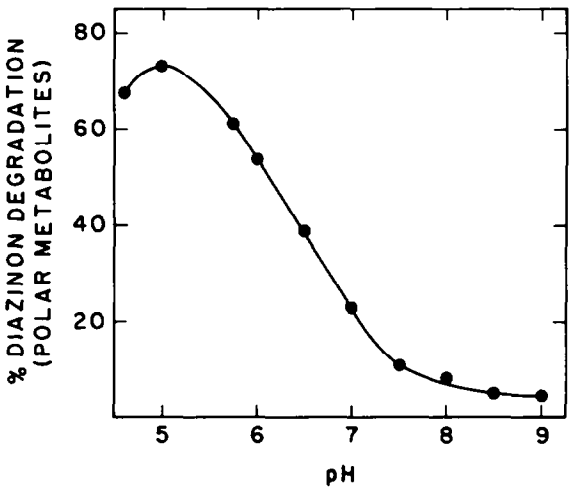


Fig. 1. Effect of pH on the degradation of diazinon by DIMBOA. Incubation conditions: 0.1 μmol diazinon; 5 μmol DIMBOA, at 37° for 2 hr, corrected for self-hydrolysis.

corn has the same R_f values as the authentic DIMBOA.

The catalytic activity of DIMBOA was measured in all instances as the % of the polar metabolites formed when DIMBOA was incubated with diazinon-[^{14}C]. This activity depended mainly on the species of the plant—all 3 corn cvs tested had high activity, while none of the other plants tested (beans, peas, lettuce, spinach, parsley, okra, squash, lima beans, coriander) had any activity—on the age of the plant (6-day-old corn seedlings had the highest activity and the activity decreased with age), portion of plant used (shoots had higher activity than roots), subcellular fraction used (activity was found only in the cytosol while there was no activity whatsoever in the particulate fractions), and the pH of the reaction mixture. Fig. 1 shows that the ability of purified DIMBOA to degrade diazinon reaches a maximum at *ca* pH 5 and declines steadily at higher pH values so that at pH 9.1 there is almost no measurable activity. This behavior of DIMBOA with pH is in agreement with earlier reports [47–52]. Tipton *et al.* [47] found that the pK_a of the hydroxamic group of DIMBOA is 6.95. Under alkaline conditions or when the pH of the solution is higher than the pK_a of the hydroxamic group, DIMBOA (**2**) is unstable and dissociates to form the phenolic compound (**3**). This reaction proceeds with the final quantitative decomposition of DIMBOA to yield methoxy benzoxazolinone (MBOA) (**4**) and formic acid (Scheme 1) [11, 15, 18, 19, 52, 53].

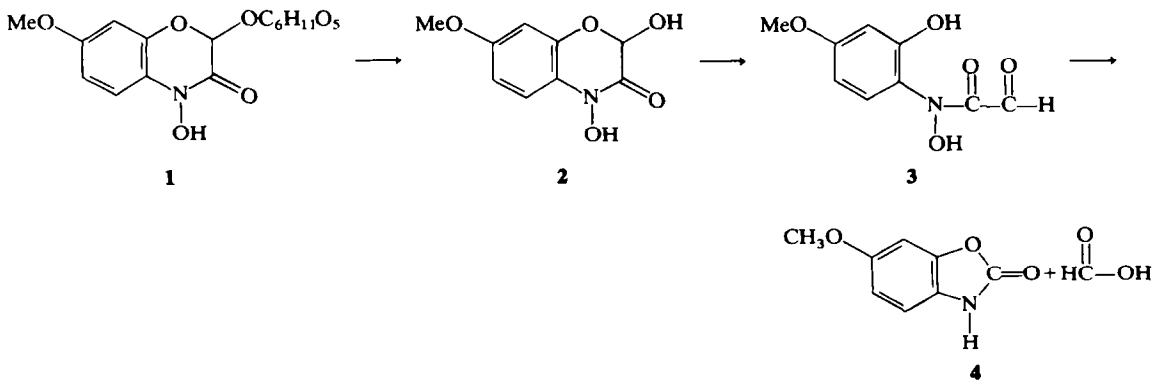
Among the insecticides DIMBOA appears to be relatively specific in catalysing the hydrolysis of diazinon. Another closely related pyrimidinyl organophosphate insecticide, etrimfos (*O, O*-dimethyl-*O*-[6-ethoxy-2-ethyl-4-pyrimidinyl] phosphorothioate) was not susceptible to hydrolysis by this catalyst. Also, DIMBOA would not degrade other organophosphate insecticides (malathion, parathion, monocrotophos, methidathion and EPN), carbamates (carbaryl, carbofuran and aldicarb) and pyrethroids (permethrin).

When purified DIMBOA was used in these assays, two metabolites were obtained in the aqueous layer: 6 - methyl - 2 - (1 - methylethyl) - 4 - hydroxypyrimidine (IMHP) and diethyl phosphorothioic acid (DEPTA) (Scheme 2). Both metabolites were identified by cochromatography with authentic standards. IMHP was removed from TLC plates and its structure confirmed by NMR and MS [49].

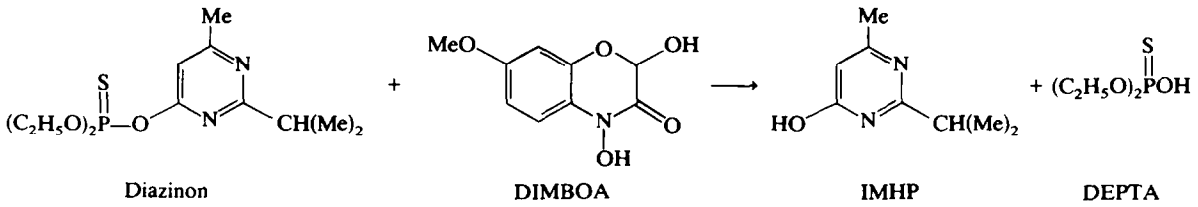
When the 22 000 g supernatant fraction was used for assays, another minor metabolite was detected in the aqueous layer, mainly when GSH was added to the incubation mixture. This metabolite has been tentatively characterized by cochromatography as *S*-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] glutathione which is formed by conjugation of GSH and the pyrimidinyl moiety of diazinon with the simultaneous cleavage of the phosphate ester bond [49].

The effect of DIMBOA concentration and time of incubation on diazinon degradation is shown in Table 1. Higher DIMBOA concentration results in larger amounts of diazinon being degraded. The same was true when the incubation period was less than 8–10 hr. At longer incubation periods, however, the amount of diazinon degradation increases very slightly, possibly indicating the breakdown of DIMBOA to the benzoxazolinone derivative **4** (Scheme 1).

Although it is desirable to compare the results obtained from our purification method to those of other investigators, it is very difficult to do so for a number of reasons. Different investigators have reported their findings on a different basis. Some have reported on a fresh and others on a dry wt basis. Some have used whole plants of different size and age and some have used portions of the plant. Some have grown the



Scheme 1. Chemical degradation of DIMBOA.



Scheme 2. Degradation of diazinon by DIMBOA.

plants under natural conditions and some have grown plants in darkness [15]. In our work, when corn was grown in the dark at room temperature for 6 days, 1 g of corn tissue resulted in *ca* 1 mg of purified DIMBOA. Unquestionably, the amount of DIMBOA per g of plant tissue is much higher than the amount purified since 0.5 g of plant tissue (1 ml of 22 000 g supernatant fraction) resulted in slightly higher diazinon metabolism than 1 mg of purified DIMBOA. This might be the result of suboptimal extraction procedures or the result of the breakdown of DIMBOA to MBOA during purification.

Many investigators [5, 7, 12, 15, 27, 54] have reported that DIMBOA is originally present in the uninjured plant cells as a glucoside (**1**). When the cells are injured during the purification process an enzyme (β-glucosidase) is released which splits the glucoside into glucose and the aglucone DIMBOA (**2**). At high pH and temperature DIMBOA is subsequently broken down to the corresponding methoxy benzoxazolinone (MBOA) (**4**) with the release of formic acid (Scheme 1). MBOA is chemically stable and has shown no biological activity against the European corn borer [18, 19] although its antifungal activity has been established in corn, rye and wheat [4, 50, 55].

It is obvious that the presence of DIMBOA in corn enhances the ability of the plant to survive adverse conditions brought about by insect attack (European corn borer, aphids) as well as fungal and bacterial diseases. Of equal importance is the ability of DIMBOA to dechlorinate 2-chloro-*s*-triazine herbicides (atrazine, simazine) to their 2-hydroxy derivatives which, in contrast to the parent compounds, are not phytotoxic to corn [38–40]. It is difficult, however, to assess the importance of diazinon degradation in corn by DIMBOA. It might be that this facile hydrolysis provides corn with a tolerance to high doses of diazinon, while the target insects are readily killed. It should be kept in mind, however, that all of our studies have been carried out *in vitro* and the above speculations might not hold true *in vivo*.

Table 1. Effect of DIMBOA concentration and time of incubation on diazinon degradation

Incubation time (hr)	Diazinon degradation (%) DIMBOA concentration (μmol) 0.05	0.1	0.2	0.4	0.8	2.4
1	3.5	2.6	7.5	13.4	19.1	43.6
2	3.8	4.0	10.4	16.7	26.1	50.9
4	5.5	7.4	16.1	24.0	41.4	67.1
6	6.1	7.3	20.4	26.1	43.4	71.4
23	7.7	8.4	20.4	31.3	49.6	74.0
26	9.7	10.4	23.0	34.5	51.1	76.1
30	10.0	9.9	23.7	35.7	52.7	77.2
47	10.6	11.8	25.6	38.4	54.5	75.9
72	14.2	14.2	26.4	43.8	57.0	72.8

EXPERIMENTAL

Chemicals. Diazinon-[^{14}C], labeled in the ring at C-2 (sp. act. 36.3 μCi/mg), diazoxon, and 6-methyl-2-(1-methylethyl)-4-hydroxypyrimidine (IMHP) were generously supplied by CIBA-GEIGY. The *S*-[6-methyl-2(1-methylethyl-4-pyrimidinyl] glutathione was prepared by coupling reduced glutathione (GSH) with [6-methyl-2-(1-methylethyl)-4-pyrimidinyl] trimethyl ammonium chloride [56]. The K,*O*,*O*-diEt phosphorothioate was prepared by the method of ref. [56]. The cofactors NADPH and GSH were purchased from ICN Pharmaceuticals, BSA and trypsin (from bovine pancreas) were purchased from Sigma.

Plant materials. Seeds from 3 cvs of corn, *Zea mays* (L.) (Truckers Favorite hybrid, Golden Queen hybrid, and Silver Queen hybrid) were placed in trays containing a thick layer of wet paper towels and the trays kept in complete darkness. The paper towels were kept moist until the corn seedlings were removed (6 days). The shoots (whitish) and roots were separated and the roots washed with H_2O after separating and discarding the cotyledons.

Tissue preparations. Shoots and roots were homogenized separately in a Waring Blendor until a homogeneous thick liquid was obtained, using either H_2O or 0.1 M Pi buffer of various pHs. For all prepns, the vol. of buffer used for homogenizing was 1 ml/g of plant tissue. The crude homogenate was filtered through 6 layers of cheesecloth and then centrifuged at 22 000 g for 40 min.

Purification of DIMBOA. The 22 000 g supernatant fraction was extracted with 3–4 vol. of EtOAc. The EtOAc layer was then evapd by a gentle stream of air and the light brown ppt. obtained was taken up by a small vol. of either Me_2CO or EtOAc. Most of the ppt. did not dissolve and settled to the bottom of the flask as a white solid. The ppt. was then removed and put aside while the solvent layer was again evapd and the ppt. taken up with the same solvents. This procedure was repeated 3–4 times until only a heavy brown oily substance remained when the solvent was evapd. The solid material obtained was then purified using the method described in ref. [10]. The purified product was an off-white needle-like crystalline material, mp 161–162° (decomp.).

Confirmation of structure of DIMBOA. A variety of procedures was used for establishing the identity of the isolated hydroxamic acid. These procedures included: (a) ^{13}C and ^{1}H NMR, IR and MS of the unknown compound, (b) elemental analysis, (c) TLC using a wide range of solvent systems, (d) solubility properties. The same procedures were

used with an authentic standard sample of DIMBOA (obtained from Dr. D. E. Moreland, Crop Science, North Carolina State University) and the results compared.

Degradation of diazinon. (a) Incubation system. The reaction mixture consisted of 1 ml tissue prepn (22 000) g supernatant fraction), and 0.1 μmol of diazinon-[^{14}C] in a total vol. of 2 ml. Whenever purified DIMBOA was used the desired amount (0.1–2.4 μmol) was dissolved in a small vol. of Me_2CO (50 μl) and the vol. for the reaction mixture adjusted to 2 ml by addition of the appropriate buffer or H_2O. The reaction mixture was incubated for 2 hr (unless otherwise specified) at 37° and then extracted with 2 ml of C_6H_6. The two layers were separated and the radioactivity in 0.1 ml from each phase measured by liquid scintillation counting using Triton X-100 cocktail [58]. The efficiencies were corrected using *n*-hexadecane-[^{14}C] as the int. standard. The radioactivity in the aq. phase corresponding to the polar metabolites formed was used as a measure of degradation. All reactions were corrected for degradation in the absence of DIMBOA, and all incubations were duplicated. (b) Characterization of the metabolites. Metabolites of diazinon were separated by TLC as previously described [49].

Effect of pH on degradative activity of DIMBOA. Degradative activity of purified DIMBOA was tested by adjusting the pH of the incubation mixture in the range 4.7–9.1. The reaction mixture in this case consisted of 5 μmol of DIMBOA and 0.1 μmol of diazinon-[^{14}C] in a total vol. of 2 ml. The mixture was incubated for 2 hr at 37°.

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