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THE REDUCTION OF 2,4-DIHYDROXY-7-METHOXY-1,4-BENZOXAZIN-3-ONE BY THIOLS

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Key Word Index—*Zea mays*; Gramineae; 1,4-benzoxazin-3-one; thiols; reduction.

Abstract—2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a naturally occurring hydroxamic acid involved in pest resistance of cereals, was reduced by thiols to the corresponding lactam. Kinetic studies showed that the reactive species are undissociated DIMBOA and thiolate anion. Possible mechanisms for the reaction are discussed in the light of relative reactivities of DIMBOA and a compound lacking the 7-methoxy substituent, and results from molecular orbital calculations.

INTRODUCTION

Hydroxamic acids isolated from extracts of Gramineae such as wheat, maize and rye [1] inhibit bacterial [2] and fungal [3] growth, as well as insect development and reproduction [4, 5]. These compounds have been suggested as resistance factors of maize against the European corn borer [4], and of cereals against aphids [6]. The main hydroxamic acid in maize is 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA, **1**) [7]. It was recently shown that DIMBOA inhibits energy transfer reactions in chloroplasts and mitochondria [8, 9], a fact that may account for its widespread toxicity [8]. These enzymatic inhibitions were shown to be partly due to the reaction of DIMBOA with sulfhydryl groups of the enzymes [9]. To gain further insight into these biochemical reactions we undertook the study of the reaction of DIMBOA with thiols. A preliminary account of this study has been published [10].

RESULTS

Spectroscopic description of the reaction

The reaction of DIMBOA with thiols was followed between pH 5 and 12 through the spectral changes in the 240–310 nm region. In this pH range the main product was the corresponding lactam, **5**.

At pH about 8, the starting spectrum was that of dissociated DIMBOA with λ_{max} at 290 nm. As the reaction proceeded this absorption peak gradually decreased, a peak at 259 nm corresponding to the lactam gradually increased and an isosbestic point at 271 nm was produced. At pHs below 6, the starting spectrum was that of undissociated DIMBOA with λ_{max} at 263 and 286 nm, the absorption of the product at 259 nm thus being partially hidden by the reactant. At pHs above 9, thiolate anion absorbs strongly below 280 nm [11] and the absorption of the product remained hidden. Acidification of this solution to pH 8 revealed the absorption at 259 nm due to the product. The reaction was hence followed in all cases at 290 nm, where the lactam shows negligible absorption. Additionally, at pH around 8 the reaction could equivalently be monitored at 259 nm.

Kinetics

DIMBOA disappeared in the presence of an excess of thiol with pseudo-first order kinetics. The rate law for the reaction is given by equation (1). DH_2 represents

$$-d(DH_2)_t/dt = k_{obs}(DH_2)_t = k_1(DH_2)_t + \bar{k}_2(DH_2)_t(RSH)_t \quad (1)$$

DIMBOA as a diprotic acid and RSH represents a thiol. Subscripts 't' indicate total concentrations (formalities); k_1s are first order rate constants for the pH-dependent decomposition of DIMBOA [12]; $\bar{k}_2$s are apparent second order rate constants for the reaction of DIMBOA with thiols, and were determined from the slope of plots k_{obs} vs. thiol concentration. Figure 1 shows such plots at different pHs for the reaction of DIMBOA with mercaptoethanol. Figure 2 shows the dependence of $\bar{k}_2$ on pH at a given concentration of thiol. The curves are bell-shaped with maxima at pH 8.3 for the reaction with mercaptoethanol, and 6.7 for the reaction with cysteine methyl ester.

The distribution of species present in a solution of DIMBOA and mercaptoethanol or cysteine methyl ester, were calculated using pK_a values from the literature [12, 13]. The products $(DH_2)(RS^-)$ and $(DH^-)(RSH)$ varied with pH in the same way as the experimental curves of Fig. 2. The following identities can thus be established, where the subscripts 'e' indicate effective concentrations, k_2 true pH-independent second order rate constants for the reaction, and 'f' molar fractions.

$$\bar{k}_2(DH_2)_t(RSH)_t = k_2(DH_2)_e(RS^-)_e \text{ or } k_2(DH^-)_e(RSH)_e \quad (2)$$

$$k_2 = \bar{k}_2/f_{DH_2}f_{RS^-} \text{ or } \bar{k}_2/f_{DH^-}f_{RSH} \quad (3)$$

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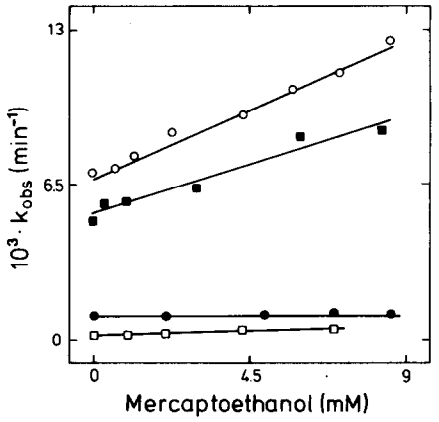


Fig. 1. Variation of the observed pseudo-first order rate constants for the reaction of DIMBOA with mercaptoethanol, with thiol concentration at pH 5.0 (□), 12.1 (●), 7.2 (■) and 8.1 (○).

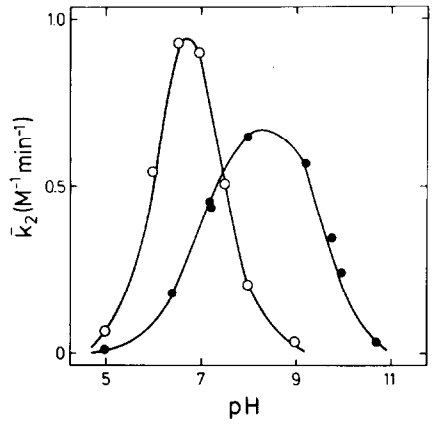


Fig. 2. pH dependence of the apparent second order rate constant $\bar{k}_2$ for the reaction of DIMBOA with mercaptoethanol (●) or with cysteine methyl ester (○). Values of $\bar{k}_2$ were determined from the slopes of graphs of k_{obs} against thiol concentration (see Fig. 1).

In order to distinguish between the two situations presented, k_2 values for a series of thiols were determined. For mercaptoethanol and cysteine methyl ester, k_2s were obtained from the least-squares fit shown in Fig. 2. For cysteine, dithiothreitol, thiolactic acid and mercaptoacetic acid, k_2s were determined from one point experiments using equation 3 for DIMBOA and thiolate anion. A linear correlation was obtained when log k_2 was plotted against the corresponding pK_a of the thiol [11, 13, 14] (Fig. 3), indicating that the reactive species were undissociated DIMBOA and thiolate anion.

Comparative reactivity studies

The reactivity towards thiols of DIMBOA was compared with that of DIBOA (a naturally occurring analogue of DIMBOA lacking the methoxy group). pK_a values of both hydroxamic acids were determined under the same experimental conditions. At 44°, the pK_a of DIMBOA was 6.74 ± 0.05 and that of DIBOA 6.79 ± 0.04.

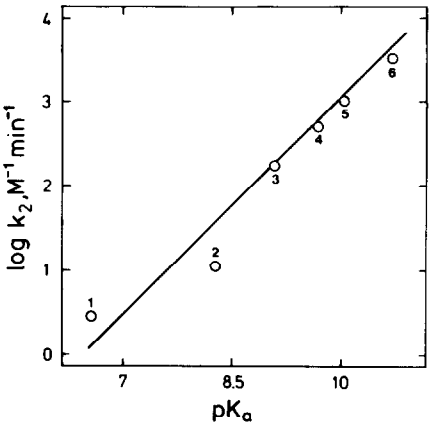


Fig. 3. Dependence of the logarithm of the true second order rate constant for the reaction of DIMBOA with thiols on the pK_a of the thiol. The true second order rate constant k_2 for each thiol was determined by least-squares fitting of $\bar{k}_2$ to equation

$$k_2 = \bar{k}_2 \left[\frac{(H^+)^2 + K_1(H^+) + K_1K_2}{(H^+)^2} \right] \cdot \left[\frac{(H^+) + K_3}{K_3} \right]$$

where $K_1 = 1.25 \times 10^{-7}$ and $K_2 = 1.26 \times 10^{-11}$ are the dissociation constants of DIMBOA [12], and K_3 is the dissociation constant of the thiol. Thiols used were: cysteine methyl ester (1), cysteine (2), dithiothreitol (3), mercaptoethanol (4), thiolactic acid (5) and mercaptoacetic acid (6).

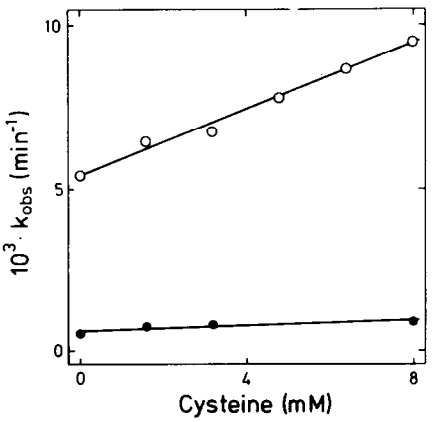


Fig. 4. Variation of the observed pseudo-first order rate constant for the reaction of DIMBOA (○) and DIBOA (●) with cysteine at pH 7.5 (phosphate 0.1 M) and 44 ± 0.5°.

Hence, it can be assumed that relative reactivity of both hydroxamic acids towards thiols (relative k_2) are equivalent to relative $\bar{k}_2$ at any given pH. A plot of k_{obs} vs. cysteine concentration (Fig. 4) shows the substantial influence of the 7-methoxy group upon reactivity towards thiols.

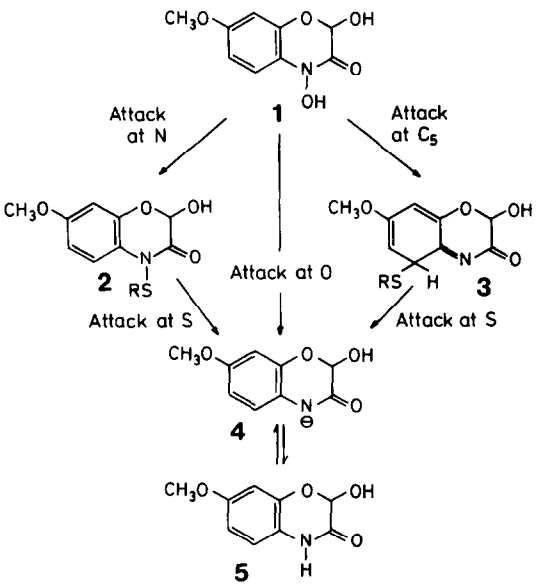
DISCUSSION

Recently it was reported that DIMBOA inhibits ATPase activity of chloroplast coupling factor (CF_1) and it was suggested that part of this inhibition was caused by

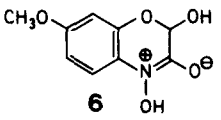
the reaction of DIMBOA with sulfhydryl groups on the enzyme. Sulfhydryl groups in proteins may vary widely in reactivity due to factors such as intrinsic nucleophilicity, local chemical microenvironment and steric hindrance [11, 15]. We undertook the study of the influence of these factors on the reaction of DIMBOA with thiols in aqueous solutions. In a previous communication [10] we reported that the main product of the reaction was the lactam of DIMBOA, **5**. The present work describes the dependence of the reactivity of DIMBOA towards thiols on pH, on the nucleophilicity of the thiol and on the effect of the 7-methoxy substituent. This work also shows that undissociated DIMBOA and thiolate anion are the most reactive couple.

The absence of ESR signals during the reaction suggests that the reaction is unlikely to proceed through a radical mechanism. Several ionic mechanisms can be envisaged that account for the results presented. DIMBOA has at least three electrophilic centres susceptible to attack by thiols to give its lactam (Scheme 1). It should be pointed out that all the species written may exist in aqueous solution in open hemiacetal form [10, 12, 16–18]. CNDO/2 molecular orbital calculations were performed to assess the relative reactivity of these centres. Nucleophilic superdelocalizability [19], a measure of the tendency of an atom to interact with a nucleophile, was higher for the hydroxamic nitrogen atom, indicating it as the most likely candidate for attack by thiolate anion. Attack at electrophilic nitrogen by thiols is not without precedent in the literature [20–22]. Intermediates similar to **2** have been isolated from the reaction of 7-methoxy-4-acetoxy-1,4-benzoxazin-3-one with ethanethiol [22].

The substantial rate increase caused by the 7-methoxy group in DIMBOA may be rationalized in terms of resonance structure **6**. This is borne out by molecular orbital calculations which show a higher polarization of the carbonyl bond and a lower electron density at the hydroxamic nitrogen atom in DIMBOA as compared with DIBOA. Similar situations have been reported where introduction of a 7-methoxy group in 1,4-benzoxazin-3-



Scheme 1.



ones enhances the reactivity of the hydroxamic nitrogen atom towards nucleophiles [22].

EXPERIMENTAL

Isolation of compounds. DIMBOA was isolated as described [2] from Et_2O extracts of 6-day-old seedlings of *Zea mays* L. cv T129s grown in a greenhouse at $25 \pm 3°$. Mercaptoethanol, dithiothreitol, thiolactic acid and mercaptoacetic acid (Sigma) were used without further purification. Cysteine methyl ester was obtained as described [13].

The reduction product of DIMBOA was isolated from Et_2O-extracts of the reaction of DIMBOA with dithiothreitol in KH_2PO_4 (0.1 M, pH 8) and was characterized as the corresponding lactam **5** by comparison of its UV, IR (KBr) and mass spectra with those reported previously [10].

Synthesis of 2-bromo-4-hydroxy-1,4-benzoxazin-3-one. A mixture of 1 g (0.006 moles) of 3,4-dihydro-4-hydroxy-1,4-benzoxazin-3-one (previously synthesized as described [23]) and 1.2 ml (0.012 moles) trichlorobromomethane in 10 ml CCl_4 was refluxed and stirred overnight. The reaction mixture was cooled to room temp., filtered and the filtrate concd to dryness. The residue was recrystallized from petrol to give (25% yield) the desired compound. The 60 MHz NMR spectrum (CCl_4, TMS) of the bromo compound was similar to that of the starting material except for the signal of the proton at C-2: in the initial compound it appeared at δ4.6 (*s*, 2H) whereas in the product it was shifted to δ4.8 (*s*, 1H).

Synthesis of 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA). A mixture of 100 mg (0.55 mmoles) 2-bromo-4-hydroxy-1,4-benzoxazin-3-one and 42 mg (0.5 mmole) $AgCO_3$ in 20 ml wet Et_2O was stirred for 3 hr at room temp., acidified to pH 5, filtered and the filtrate concd to dryness. The residue was recrystallized from petrol to yield DIBOA, which was characterized by comparison of its mp, R_f in TLC and UV spectrum with the lit. [24].

Kinetic measurements. To 10 ml of 0.1 M buffer soln of the desired pH, thiol was added to obtain an 8 mM reference soln. Five ml of this soln were pipetted into a tube containing enough solid DIMBOA to obtain a 0.08 mM soln (sample soln). The reactions were monitored in the 240–310 nm region using quartz cells thermostatted at $31 \pm 0.2°$. pH was measured before and after each reaction.

pK_a measurements. The pK_a values of DIMBOA and DIBOA were determined spectrophotometrically at $44 \pm 0.5°$ using buffers with $I = 0.1$. Spectra were recorded in the region 220–350 nm for each pH value. Isosbestic points were obtained up to pH 9 (at 272.5 nm for DIMBOA and 267.5 nm for DIBOA) indicating the presence in equilibrium of two absorbing species [25]. The analytical wavelengths employed were 260 nm for DIMBOA and 250 nm for DIBOA.

Electron spin resonance studies. ESR spectra were recorded with a Varian V4502 spectrometer. Measurements were performed at room temp. with $H_0 = 3340$ Gauss and $\nu = 9.5 \times 10^9$ Hertz. Under these conditions no signal was detected from a mixture of 0.02 M DIMBOA and 1 M mercaptoethanol in borate buffer pH 8 even after 10 min of reaction time.

Molecular orbital calculations. The CNDO/2 method [26, 27] was used with the geometry of one of the two independent molecules in the asymmetric unit of crystalline DIBOA [28]. The

methoxy group of DIMBOA was described with average crystallographic parameters [29], the rest of the molecule was identical to DIBOA.

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