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The maize benzoxazinone DIMBOA reacts with glutathione and other thiols to form spirocyclic adducts

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ABSTRACT

Maize, wheat and other grasses synthesise large quantities of benzoxazinones and their glucosides, which act as antifeedant and allelopathic agents. These activities are probably due to the electrophilic nature of the aglycones, however, the mechanism of their action is unclear. In biological systems, glutathione (GSH) is the major electrophile-reactive compound so the reaction of the major maize benzoxazinone DIMBOA with GSH was studied. GSH reacts with DIMBOA to form eight isomeric mono-conjugates and eight isomeric di-conjugates. Through NMR studies with the model thiol 2-mercaptoethanol, these were structurally elucidated as unusual spirocycles. Similar reactivity was observed with proteins, with cysteinyl thiols being modified by DIMBOA. The thioether bonds formed were stable and not easily reduced to the parent thiol. DIMBOA can therefore readily deplete GSH levels and irreversibly inactivate enzymes with active-site cysteine residues, with clear implications for potentially toxic effects when young grasses are ingested, whether by insect pests or humans.

1. Introduction

Certain grasses, including the important cereal crops wheat, maize and rye, can synthesise large amounts of benzoxazinoid secondary metabolites, with particularly high levels being present in seedlings (Macías et al., 2009). Benzoxazinoids have also been found in wholemeal wheat and rye bread, albeit at lower levels (Pedersen et al., 2011) and are therefore of potential human dietary significance. The major benzoxazinoid in maize and wheat is DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one; Fig. 1), and in rye, DIBOA (2,4-dihydroxy-2*H*-1,4-benzoxazin-3-one), with other related compounds also being present. These reactive compounds are stored as glucosides in the vacuole and on damage of the tonoplast (e.g. during herbivore attack or on wounding) mix with plastidic glucosidase to release the aglycone (Niemeyer, 2009). These aglycones can also be found in the apoplast (Ahmad et al., 2011) and the rhizosphere. There is good evidence that benzoxazinoids in aerial tissues act as antifeedants, while benzoxazinones secreted from the roots act as allelopathic agents. How these compounds exert such effects is less certain. It is clear that these compounds are electrophilic, with DIMBOA, the best studied benzoxazinone, reacting well with thiols (Atkinson et al., 1991; Niemeyer et al., 1982; Pérez and Niemeyer, 1985), and also reacting with amines (Pérez and Niemeyer, 1989a). Despite these previous investigations, the multiplicity of electrophilic centres in DIMBOA and the lack of complete physical characterisation of the resulting adducts means that the nature of these adducts and therefore the molecular mechanism of DIMBOA-induced effects is not clear. An early study focused on the reaction of DIMBOA with excess ethanethiol at pH 8.0 and elevated temperature (45 °C) (Niemeyer et al., 1982). From this three products were isolated in low yield with each having a UV spectrum similar to that of DIMBOA. The structures of the products were proposed to be ring-opened DIMBOA with addition of thiol to the ketoaldehyde group, and/or reduction of the hydroxamic acid moiety to a lactam (Fig. 1). Later work from the same group examined the kinetics of the reaction of DIMBOA with thiols, and here the formation of a product with λ_{max} = 259 nm and negligible absorbance at 290 nm was observed (Pérez and Niemeyer, 1985). This was

Abbreviations: CID, collision-induced dissociation; DIBOA, 2,4-dihydroxy-2*H*-1,4-benzoxazin-3-one; DIMBOA, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one; DIM_2BOA, 2,4-dihydroxy-7,8-dimethoxy-2*H*-1,4-benzoxazin-3-one; GSH, glutathione; 2-HMBOA, 2-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one; 4-HMBOA, 4-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one; ME, 2-mercaptoethanol; TCEP, tris(2-carboxyethyl)phosphine.

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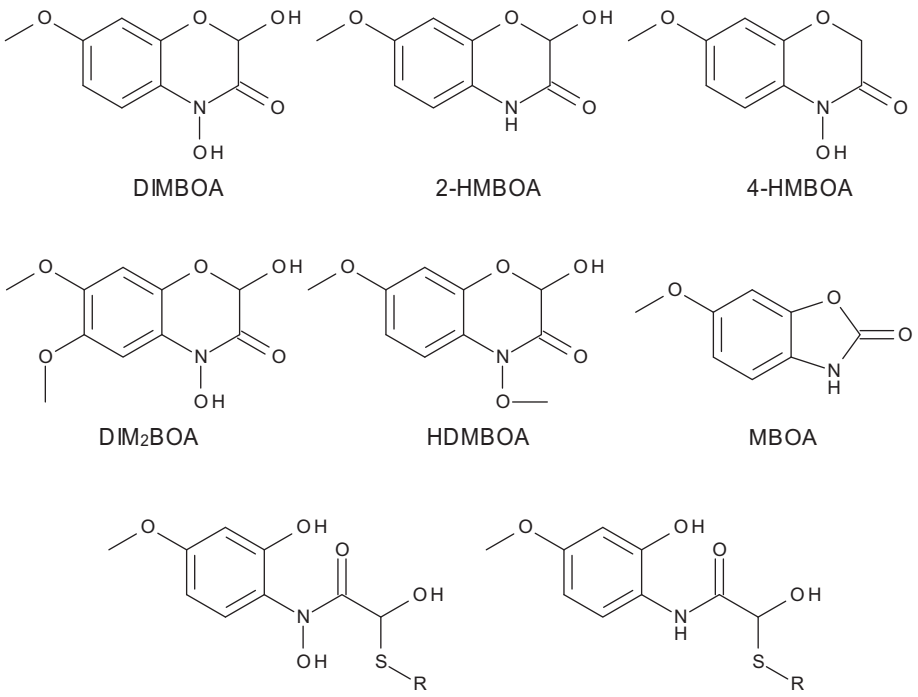

Fig. 1. Structures of DIMBOA and related compounds discussed in this work.

assumed to be 2-HMBOA, the lactam derivative of DIMBOA (Fig. 1). However, it was subsequently shown that authentic 2-HMBOA retains absorbance at 290 nm (Atkinson et al., 1991). A later reaction of DIMBOA with mercaptoethanol was studied by NMR (Atkinson et al., 1991), but since the reaction products were not purified, the resulting complicated spectra were difficult to interpret. Furthermore, DIMBOA is relatively unstable, degrading to MBOA (6-methoxybenzoxazol-2-one; Fig. 1) via a poorly characterised pathway (Maresh et al., 2006), and as a result the nature of the most biologically active species is unclear. To understand the reason for the observed toxicity of benzoxazinones, and to be able to assess their dietary safety and also their utility for crop protection, the predominant reactions of these compounds under physiological conditions need to be determined. We have now elucidated the products of the reaction between DIMBOA and biological nucleophiles, which is likely to be a major route of metabolism *in vivo*. These findings suggest that DIMBOA exerts these 'toxic' effects through an irreversible alkylation of biologically relevant nucleophiles that can potentially occur at sites remote from the initial site of uptake.

2. Results and discussion

2.1. Reaction of DIMBOA with glutathione

As a component of a project exploring the metabolism of glutathione adducts of natural products (Dixon and Edwards, 2010), we synthesised glutathione adducts of DIMBOA. Analysis of the initial NMR spectra revealed a lack of aromatic signals suggesting that the resulting compounds were different from those expected based on the previous literature reports (see below). Given this discrepancy, we were curious as to the identity of the products and whether this might help elucidate the role of DIMBOA and related compounds *in vivo*. Consequently, we re-investigated these reactions in more detail.

Mixing maize-extracted DIMBOA (50 mM; $MH^+ = m/z$ 212.06; Supplementary data Fig. S1) with sub-stoichiometric amounts of GSH (20 mM) led to a rapid reaction affording a complex mixture of more hydrophilic, UV-absorbing products (Fig. 2A). In order to maintain physiological relevance mildly basic (pH 8) reactions conditions were used for all experiments. Significantly, DIMBOA reactivity reaches a peak near this pH (Pérez and Niemeyer, 1985). HPLC-mass spectrometric analysis identified these to be either mono-glutathionylated ($MH^+ = m/z$ 519.14) or di-glutathionylated ($[M+2H]^{2+} = m/z$ 404.61; M = 807 Da) conjugates of DIMBOA. The mass of the mono-conjugates showed the reaction was an addition while loss of water from the mass of the di-conjugates suggested that for these products, one thiol was introduced through addition, and the other through substitution of an OH group. Collision-induced dissociation (CID) fragmentation (Supplementary data Fig. S2) of the mono-conjugates gave fragments indicating a major neutral loss of 73 Da ($C_2H_3NO_2$) followed by further water loss. Other fragmentation showed neutral losses typical of GSH conjugates (75 and 129 Da). In contrast, all the di-conjugates showed abundant loss of intact GSH (neutral loss of 307 Da), and also showed neutral losses due to fragmentation of GSH (Supplementary data Fig. S3). Each conjugate had a similar UV absorption spectrum that was substantially different from those of DIMBOA and the other benzoxazinones used. In each case these showed an absorption peak at 258–260 nm (Fig. 3), matching the earlier observation of Pérez and Niemeyer (1985). However, the data obtained did not correlate with the previous assignment as 2-HMBOA. Purification of individual peaks by semi-preparative HPLC and subsequent HPLC analysis of the resolved peaks showed that for the mono-conjugate, four pairs of isomers were present, with the two components of each pair slowly interconverting (for each numbered peak in Fig. 2A, a and b suffixes identify the pairs of interconverting isomers). Such an observation would be consistent with the formation of three stereocentres, one of which is stereochemically labile. For the di-conjugate, individual peaks did not show any obvious interconversion. Although only five isomers