# Identification of Metabolites of the Herbicide Safener Benoxacor Isolated from Suspension-Cultured *Zea mays* Cells 3 and 24 h after Treatment†

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The metabolic fate of the herbicide safener benoxacor was studied in suspension cultures of *Zea mays* (cv. Black Mexican Sweet) 3 and 24 h after treatment (HAT) with [$^{14}$C]benoxacor. Following purification by reversed phase HPLC, metabolites were subjected to $^{1}$H-NMR or mass spectrometry for structural analysis. A catabolic α-hydroxyacetamide derivative was detected as well as several amino acid conjugates either containing GSH or presumably derived from GSH. A relatively abundant di(GSH) conjugate, which consisted of two GSH molecules linked to benoxacor, was biosynthesized following the apparent addition of a suitable activating group to the benzoxazine-ring C2 atom of the previously described mono(GSH) conjugate. The remaining metabolites identified were all presumably derived from either the mono(GSH) or di(GSH) conjugates of benoxacor. A disaccharide conjugate was identified as *S*-*O*-(diglycoside)GSH and was apparently formed by one or more glycosyl transferases which used the mono(GSH) conjugate as a substrate. Two additional metabolites identified were the mono- and di(γ-GluCys) conjugates of benoxacor, the formation of which was most likely the result of peptidase activity on the mono- and di(GSH) conjugates of benoxacor, respectively. An *S*-(*S*′-Cys)GSH conjugate was also identified and was likely formed by both peptidase and dipeptidase activities on the di(GSH) conjugate. Metabolite structures and postulated pathways of their biosynthesis *in vivo* are presented.

**Keywords:** *Benoxacor; herbicide safener; CGA-154281; glutathione S-transferase; BMS cells; xenobiotic metabolism; xenobiotic detoxification; maize; glutathione conjugation; glutathione conjugate metabolism; dichloroacetamide, substituted benzoxazine; herbicide safener metabolism*

## INTRODUCTION

Benoxacor is a dichloroacetamide herbicide safener used in maize to protect seedlings against toxic effects of the α-chloroacetamide herbicide metolachlor which may occur under specific environmental conditions. Benoxacor detoxifies metolachlor by increasing the activity of GST isozymes which metabolize metolachlor to a nonphytotoxic glutathione conjugate (Viger *et al.*, 1991; Dean *et al.*, 1991; Fuerst *et al.*, 1993; Irzyk and Fuerst, 1993). Benoxacor also increases the activity of GST isozymes in a model system of maize cell suspension cultures (Miller *et al.*, 1994).

We have previously described the metabolism of benoxacor in maize cell suspension cultures 1 HAT with [$^{14}$C]benoxacor (Miller *et al.*, 1996). Formylcarboxamide and carboxycarboxamide derivatives were oxygenated metabolites of benoxacor, while the most abundant metabolite was the mono(GSH) conjugate of benoxacor (Miller *et al.*, 1996). We postulated that the formylcarboxamide catabolite was biosynthesized from benoxacor by a GST-catalyzed dechlorination mechanism and that the carboxycarboxamide metabolite formed through oxygenation of the formylcarboxamide catabolite. The mono(GSH) conjugate was apparently biosynthesized by GST isozymes that used either an α-chloroacetamide derivative or the formylcarboxamide catabolite as a substrate. This evidence demonstrated that a xenobiotic capable of increasing GST activity in maize cell suspension cultures was itself metabolized by GSTs.

To determine if the metabolic fate of the mono(GSH) conjugate of benoxacor in maize is similar to the metabolic fate of other well-studied xenobiotic glutathione *S*-conjugates (*e.g.* herbicide−glutathione conjugates), we decided to determine the structure of benoxacor metabolites isolated from maize cells at two time points subsequent to the 1 HAT time point previously described (Miller *et al.*, 1996). Therefore, the objectives of the present study were to (1) isolate and purify by reversed phase HPLC the most abundant or most efficiently attainable [$^{14}$C]benoxacor metabolites present 3 and 24 HAT, (2) subject the purified metabolites to $^{1}$H-NMR or mass spectral analyses for structural elucidation, and (3) propose pathways by which the metabolites were biosynthesized *in vivo*.

## MATERIALS AND METHODS

**Chemicals**. Reference standard CGA-245097 [4-(hydroxyacetyl)-3,4-dihydro-3-methyl-2*H*-1,4-benzoxazine] was synthe-

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**Table 1. Reversed Phase HPLC Gradients Used for the Separation and Purification of Benoxacor Metabolites Isolated from BMS Cells 3 and 24 HAT**

| gradient | conditions[a] |
|---|---|
| 1 | 0−5 min, 0% B; 5−35 min, 0−100% B; 35−60 min, 100% B |
| 2 | 0−3 min, 0% B; 3−6 min, 0−30% B; 6−40 min, 30% B isocratic grad |
| 3 | 0−2 min, 0% B; 2−2.5 min, 0−5% B; 2.5−12 min, 5% B isocratic grad; 12−32 min, 5−30% B; 32−40 min, 30% B isocratic grad |
| 4 | 0−2 min, 0% B; 2−2.5 min, 0−5% B; 2.5−22 min, 5% B isocratic grad; 22−40 min, 5−30% B |
| 5 | 0−20 min, 0% B; 20−20.5 min, 0−5% B; 20.5−40 min,5% B isocratic grad |
| 6 | 0−3 min, 0% B; 3−8 min, 0−50% B; 8−40 min, 50% B isocratic grad |

[a] Solvent A = $H_2O$; solvent B = acetonitrile.

sized by Ciba Corp. All other chemicals used in this study were obtained from the same sources as previously described (Miller *et al.*, 1996).

**Cell Culture Maintenance and Treatment with [$^{14}$C]-Benoxacor**. *Zea mays* cells (cv. Black Mexican Sweet) were maintained in MS liquid media as previously described (Miller *et al.*, 1994). Cells were treated with [$^{14}$C]benoxacor and harvested as previously described for large-scale metabolite isolations (Miller *et al.*, 1996).

**Metabolite Extraction and Preparation**. Harvested cells were extracted with 80% (v/v) acetonitrile, partitioned against chloroform, subjected to preparative solid-phase extraction, and further manipulated as previously described (Miller *et al.*, 1996).

**Preparative HPLC**. The HPLC instrumentation and methodology used in this study are the same as the instrumentation and methodology previously described (Miller *et al.*, 1996) except that TFA was omitted from the mobile phase. Aqueous-soluble metabolites extracted from cells treated for 3 and 24 h were initially subjected to gradient 1 (Table 1). Two peaks plus a small quantity of unmetabolized benoxacor, which copurified with the aqueous-soluble metabolites, were separated using this solvent system. The first peak was subjected to gradients 4 and 5 sequentially, while the second peak was subjected to gradients 2−4. Each separated radioactive peak was subjected to gradients 4 and 5 more than once until one detectable peak of radioactivity was present.

Chloroform-soluble metabolites from cells treated for 3 h were initially subjected to gradient 1. Separated metabolites were collected and individually subjected to gradient 6 followed by gradient 2. Metabolites were then subjected to gradient 2 more than once until one detectable peak of radioactivity was present. No attempt was made to purify chloroform-soluble metabolites present 24 HAT due to the relatively low level of chloroform-soluble radioactivity.

**Analytical HPLC**. The previously described analytical HPLC instrumentation and methodology used to purify metabolites from cells treated for 1 h with benoxacor (Miller *et al.*, 1996) were also used in this study. Samples partially purified by preparative reversed phase HPLC were subjected to analytical reversed phase HPLC using gradients 3−5 in sequence (Table 1). Samples were subjected to gradients 4 and 5 more than once until one detectable peak of UV absorbance was present.

**$^1$H-NMR and Mass Spectral Analyses of Metabolites**. Electron impact GC−MS, LC-ESI/MS, and $^1$H-NMR spectra of metabolites purified by analytical reversed phase HPLC were acquired using the same instrumentation and protocols as previously described (Miller *et al.*, 1996).

## RESULTS AND DISCUSSION

**Structural Analyses and Identification of Metabolites.** *α-Hydroxyacetamide Metabolite [4-(Hydroxyacetyl)-3,4-dihydro-3-methyl-2H-1,4-benzoxazine].* The electron impact GC−MS spectrum of this metabolite is presented in Figure 1. The molecular ion was present at *m/z* 207 with high mass fragment ions occurring at *m/z* 176, 149, 134, and 120. The *m/z* 176 fragment ion (M − 31) is due to the loss of a $CH_2OH$ group from the molecular ion. The remaining fragmentation pattern is the same as that which has been previously described for benoxacor (Miller *et al.*, 1996).

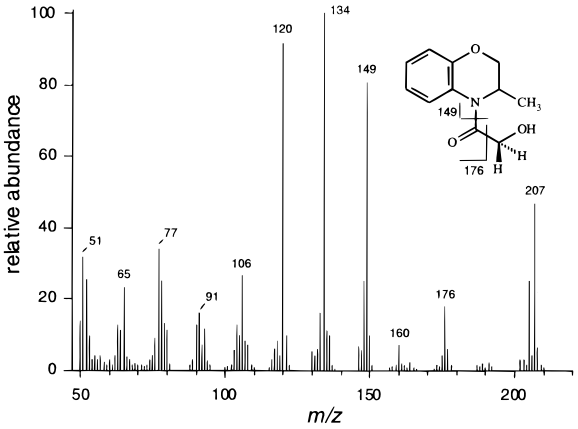


**Figure 1.** Electron impact GC−MS spectrum and deduced fragmentation pattern of the α-hydroxyacetamide metabolite. The GC−MS spectrum was identical to the reference standard (not shown).

The $^1$H-NMR spectrum (data not shown) of the extracted metabolite was identical to the spectrum of benoxacor except for the presence of a hydroxy proton at $\delta$ 3.43.

At least two pathways can be postulated for the biosynthesis of this metabolite. Various aspects of both of these pathways have been previously discussed while describing the biosynthesis of the formylcarboxamide catabolite (Miller *et al.*, 1996). The first hypothetical pathway would involve cytochrome $P_{450}$ monooxygenase-catalyzed hydroxylation (Butt and Lamb, 1981; Jones and Caseley, 1989; Donaldson and Luster, 1991) of the dichloroacetyl α-carbon atom ensuing a reductive dehalogenation step. A second hypothetical pathway would involve reduction of the previously described formylcarboxamide catabolite (Miller *et al.*, 1996) by an aldehyde reductase. Miaullis *et al.* (1978) determined that a total-soluble protein extract from hepatic tissue of rat catalyzed the formation of *N,N*-diallylglycolamide from *N,N*-diallyldichloroacetamide if NADPH or NADH was added to an *in vitro* reaction mixture. It was hypothesized that biosynthesis of the *N,N*-diallylglycolamide metabolite proceeded through reduction of the *N,N*-diallylgloxylamide metabolite (analogous to the formylcarboxamide benoxacor catabolite). If NADPH or NADH was omitted from the reaction mixture, the *N,N*-diallylglycolamide metabolite was not detected.

The hydroxyacetamide metabolite of benoxacor was not detected in extracts from cells treated for 1 h with benoxacor, suggesting that formation of the formylcarboxamide metabolite is a precursory step. This also suggests that the formylcarboxamide metabolite is acting as a substrate for an aldehyde reductase, resulting in the formation of the α-hydroxyacetamide metabolite.

*Di(GSH) Conjugate [4-(Glutathione-S-acetyl)-3,4-dihydro-3-methyl-2H-2-(S-glutathionyl)-1,4-benzoxazine].* LC-ESI/MS of this metabolite yielded a protonated molecular ion at *m/z* 802 $(MH)^+$. The MS/MS daughter ion (+) spectrum of the *m/z* 802 parent ion is

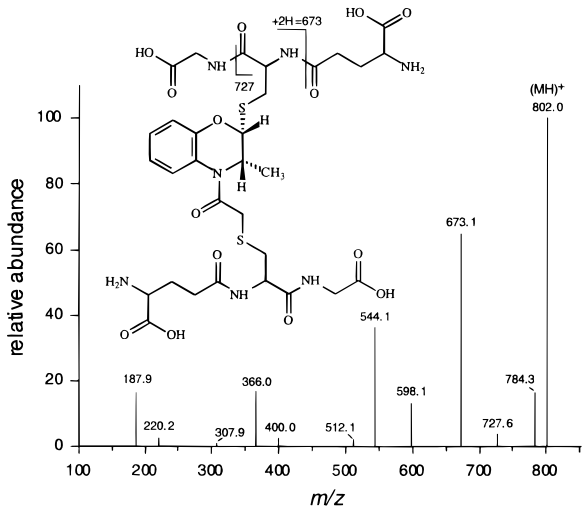


**Figure 2.** MS/MS daughter ion (+) spectrum and deduced fragmentation pattern of the *m/z* 802 parent ion obtained by LC-ESI /MS of the di(GSH) conjugate which was extracted from BMS cells 3 and 24 HAT.

depicted in Figure 2. In addition to the presence of $MH^+$ at *m/z* 802, high mass fragment ions were present at *m/z* 727, 673, 598, and 544. The fragment ions at *m/z* 727 $(MH - 75)^+$ and 673 $(MH - 129)^+$ suggest the loss of a glycine and $\gamma$-glutamyl moiety, respectively, from $MH^+$. The fragment ion at *m/z* 598 $(MH - 204)^+$ indicates the loss of both a glycine and a $\gamma$-glutamyl moiety from $MH^+$, while the fragment ion at *m/z* 544 $(MH - 258)^+$ corresponds to the loss of two $\gamma$-glutamyl moieties from $MH^+$. On the basis of the previously described fragmentation pattern, the *m/z* 802 molecular ion was tentatively assigned as benoxacor substituted with two GSH moieties.

Chemical shift values obtained from one-dimensional $^1$H-NMR spectral data of this metabolite are as follows [the carbon atom numbering scheme of benoxacor can be found in Miller *et al.* (1996)]: $\delta$ 1.51 (C9, d, 3H), 2.43 (glu $\beta$C, c, 4H), 2.81 (glu $\gamma$C, c, 4H), 3.21 (cys $\beta$C, b, 1H), 3.44 (cys $\beta$C, m, 1H), 3.50 (cys $\beta$C, b, 1H), 3.62 (cys $\beta$C, m, 1H), 4.59 (C11, s, 2H), 4.90 (cys $\alpha$C, b, 1H), 4.95 (cys $\alpha$C, t, 1H), 5.61 (C2, s, 1H), 7.32 (C7 and C8, c, 2H), 7.57 (C6, c, 1H), and 7.90 (C5, c, 1H). Integration of the resonances in the spectrum (not shown) clearly indicated the presence of two glutathione moieties, which agrees with the previously described MS/MS spectral data. Linkage of the second glutathionyl moiety was assigned to the benzoxazine-ring C2 atom on the basis of the lack of methylene protons at $\delta$ 4.04 and 4.12 (Miller *et al.*, 1996) and the presence of a methine proton at $\delta$ 5.61. Vicinal coupling of the benzoxazine-ring C3 methine proton to the C2 methine proton was not clearly observed, suggesting that their dihedral angle approximates 90°. Although the data in Figure 2 were obtained from the di(GSH) conjugate isolated from cells treated for 24 h with benoxacor, this metabolite was also isolated from cells treated for 3 h with benoxacor. When the 3 HAT metabolite was subjected to mass spectral analysis, the resulting fragmentation pattern was identical to the fragmentation pattern in Figure 2. However, when the three HAT sample was subjected to $^1$H-NMR spectroscopy, the spectra and resulting chemical shift values obtained were identical to the chemical shift values listed above with one exception: in addition to the presence of a benzoxazine-ring C2 methine proton singlet at $\delta$ 5.61, there was a second more intense singlet at $\delta$ 5.65 which was also assigned to a benzoxazine-ring C2 methine proton. Based on resonance integration data, the $\delta$ 5.65 proton and the $\delta$ 5.61 proton were present in a molar ratio of about 7:1, respectively. The slight downfield shift of the benzoxazine-ring C2 methine proton from $\delta$ 5.61 to $\delta$ 5.65 ppm could be explained if the sample actually consisted of two component molecules which differed only in the configuration of the benzoxazine-ring C2 methine proton. These data suggest that two enantiomers of the di(GSH) conjugate are biosynthesized and that the presence of one of the enantiomers predominates 3 HAT while the other enantiomer predominates 24 HAT. Due to the lack of vicinal coupling of the C3 methine proton to the C2 methine proton, however, more direct NMR experiments (*e.g.* a NOESY experiment) are required before the presence and configuration of di(GSH) conjugate enantiomers can be confirmed.

The mono(GSH) conjugate is the most likely molecule to which a second GSH is added via a GST-catalyzed conjugation reaction. However, because the benzoxazine-ring C2 atom of the mono(GSH) conjugate is not electrophilic enough to be attacked by a glutathionyl thiolate anion, it must first be modified, or activated, in a way that increases its electrophilicity. One plausible reaction that could account for the alteration of electrophilicity at this site involves addition of a hydroxyl group via a cytochrome $P_{450}$ monooxygenase hydroxylation reaction (Figure 3-I). Cytochrome $P_{450}$ monooxygenases are commonly involved in the metabolism of xenobiotic molecules (Donaldson and Luster, 1991; Jones and Caseley, 1989) to hydroxylated derivatives. Other electronegative groups could function equally well to activate this carbon for nucleophilic attack by glutathione. The hydroxylated mono(GSH) conjugate in Figure 3-I allows one to speculate on one or more reaction sequences that could ultimately lead to the formation of the di(GSH) conjugate presented in Figure 2. Although three reaction sequences are postulated in Figure 3, there are no direct data to support that any of them result in the formation of the di(GSH) conjugate, so other reaction sequences might be possible. In the first sequence glutathione conjugation proceeds, via a GST-catalyzed $S_N1$ reaction, through a resonance-stabilized oxonium ion (Figure 3-II) formed after loss of a hydroxyl species from the mono(GSH) conjugate. Subsequent addition of GSH to the highly electrophilic oxonium ion C2 atom would result in the formation of the di(GSH) conjugate (Figure 3-III). In the second sequence, dehydration of the hydroxylated mono(GSH) conjugate would result in the formation of an unsaturated bond between C2 and C3 (Figure 3-IV). GST-catalyzed attack of glutathione on the C2 atom (Figure 3-IV), with concomitant proton addition to the C3 atom, would result in the formation of the di(GSH) conjugate (Figure 3-III). In this sequence, another enzyme may be involved to initially dehydrate the hydroxylated mono(GSH) conjugate prior to GST-catalyzed GSH conjugation. The last proposed sequence uses the hydroxylated mono(GSH) conjugate directly in a GST-catalyzed $S_N2$ reaction in which the C2 hydroxyl moiety is the leaving group and addition of GSH results in formation of the di(GSH) conjugate (Figure 3-III).

The biosynthesis of dually substituted GSH conjugates of xenobiotics in both plant and animal tissue has been previously reported. A di(GSH) conjugate of pentachloronitrobenzene was isolated from peanut roots and identified by mass spectrometry (Lamoureux and

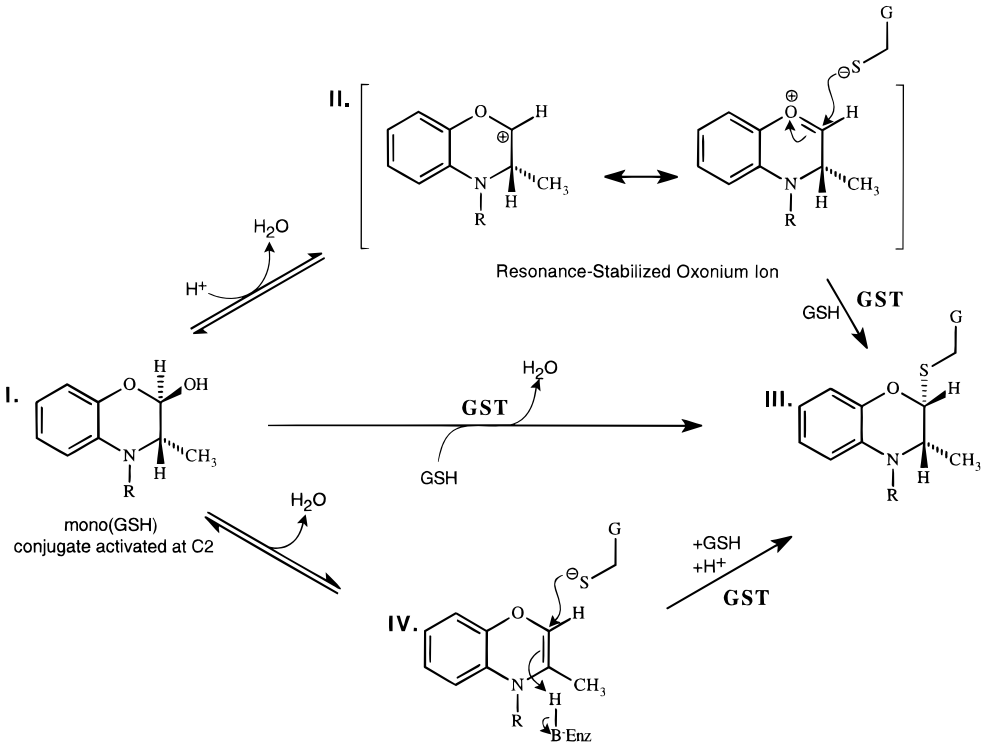


**Figure 3.** Hypothetical reaction sequences leading to the formation of the di(GSH) conjugate from the mono(GSH) conjugate. The initial metabolite is the mono(GSH) conjugate hydroxylated at C2 (I) to increase the electrophilicity of this atom sufficiently for nucleophilic attack by GSH. GST-catalyzed nucleophilic substitution of GSH for OH at C2 would result in the di(GSH) conjugate (III). Alternatively, loss of the hydroxyl group could form a resonance-stabilized oxonium ion (II) which could then undergo GST-catalyzed GSH addition to form the di(GSH) conjugate (III). Dehydration of I could form a mono(GSH) conjugate derivative with an unsaturated bond between C2 and C3 (IV). GST-catalyzed GSH addition to the C2 atom with concurrent proton addition to C3 would yield the di(GSH) conjugate (III).

Rusness, 1980, 1981). Dekant *et al.* (1988) determined that rat hepatic and renal GSTs catalyzed the formation of 1,4-bis(glutathion-*S*-yl)-1,2,3,4-tetrachlorobuta-1,3-diene from the mono(GSH) conjugate of hexachloro-1,3-butadiene. Similarly, Jones *et al.* (1985) determined that the di(GSH) conjugate of hexachloro-1,3-butadiene was formed in rat hepatocytes following administration *in vivo.* These data demonstrate that both plant and mammalian GSTs are able to utilize singly substituted GSH conjugates as substrates and catalyze the addition of a second molecule of GSH to form di(GSH) conjugates.

Our results clearly demonstrate that benoxacor and the mono(GSH) conjugate of benoxacor are suitable substrates for one or more maize GST isozymes. Whether or not this simply reflects the electrophilic nature of benoxacor or is a direct consequence of its mechanism of action is presently unknown.

To our knowledge, the present study is the first to report a dually substituted GSH conjugate of a plant xenobiotic confirmed by both $^1$H-NMR and mass spectrometry. This verifies not only that benoxacor increases GST activity in maize cell culture but also that it is metabolized by one or more GST isozymes to form both the mono- and the di(GSH) conjugates.

*S-(O-Glycosylglycoside) GSH Conjugate [4-(Glutathione-S-acetyl)-3,4-dihydro-3-methyl-2H-2-(O-glycosylglycoside)-1,4-benzoxazine].* LC-ESI /MS of this metabolite yielded a protonated molecular ion at $m/z$ 837. The MS/MS daughter ion (+) spectrum of the $m/z = 837$ parent ion is depicted in Figure 4. The fragment ion at $m/z$ 657 $(MH - 180)^+$ is due to the loss of one glycosyl moiety from $MH^+$, while the fragment ion at $m/z$ 495 $(MH - 342)^+$ is due to the loss of the diglycoside moiety from $MH^+$. The fragment ion at $m/z$ 420 is due to the loss of both the diglycoside and the glycine moiety, while the

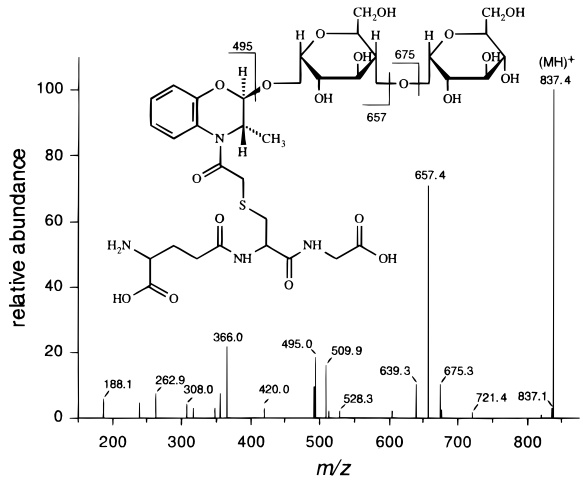


**Figure 4.** MS/MS daughter ion (+) spectrum and deduced fragmentation pattern of the $m/z$ 837 parent ion generated by LC-ESI/MS of the *S*-(*O*-glycosylglycoside)GSH conjugate which was extracted from BMS cells 3 HAT.

fragment ion at $m/z$ 366 is due to the loss of the diglycoside and the $\gamma$-glutamyl moiety from $MH^+$. *O*-Glycosylation at the benzoxazine-ring C2 atom is consistent with the hypothesized existence of the hydroxylated mono(GSH) conjugate presented in Figure 3. However, NMR spectroscopy of this metabolite was unsuccessful, so the carbon atom to which the disaccharide moiety is linked to the mono(GSH) conjugate, as well as the configuration of the anomeric carbon atoms of the diglycoside, is unknown.

A UDP-glycosyltransferase is presumably responsible for glycosylating the mono(GSH) conjugate. Therefore, hydroxylation of the mono(GSH) conjugate would ini-

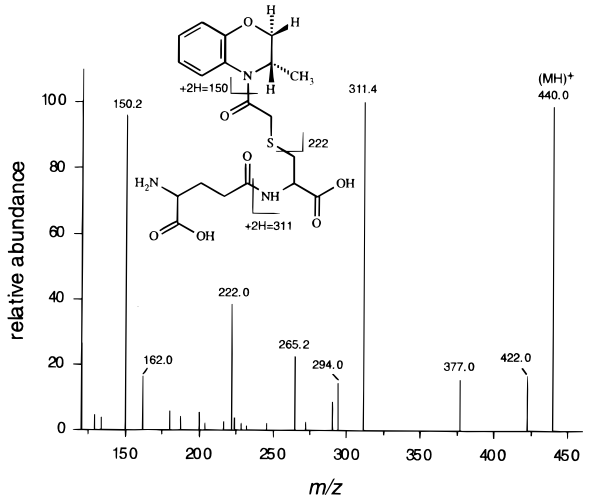


**Figure 5.** MS/MS daughter ion (+) spectrum of the *m/z* 440 parent ion derived from LC-ESI/MS of the mono(γ-GluCys) conjugate which was extracted from BMS cells 24 HAT.

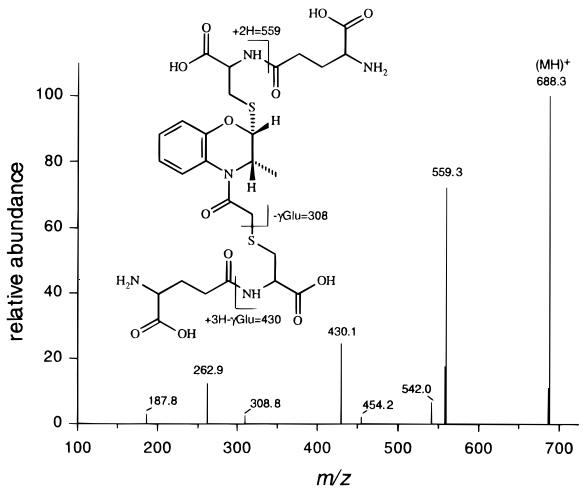


**Figure 6.** MS/MS daughter ion (+) spectrum and deduced fragmentation pattern of the *m/z* 688 parent ion derived from LC-ESI/MS of the di(γ-GluCys) conjugate extracted from BMS cells 24 HAT.

tially be required. In addition to glutathione conjugation, glycosylation of plant xenobiotics is a commonly observed detoxification reaction. Many xenobiotic (*e.g.* herbicides) molecules in plants are known to undergo glycosylation following cytochrome $P_{450}$ monooxygenase-catalyzed hydroxylation reactions.

Shore *et al.* (1995) previously demonstrated that rat hepatic GSTs enhanced the conjugation of GSH to 1-*O*-clofibryl glucuronide forming *S*-[*p*-(chlorophenoxy)-2-(methylpropanoyl)]glutathione. Not only did a GST use the 1-*O*-clofibryl glucuronide as a substrate but it also catalyzed the nucleophilic displacement of the glucuronic acid moiety. However, the relatively rapid appearance [15 min for the mono(GSH) conjugate vs 3 h for the glycosylated mono(GSH) conjugate] and comparatively high abundance of the mono(GSH) conjugate in maize cells following benoxacor treatment (Miller *et al.*, 1996) suggest that the mono(GSH) conjugate is the substrate for a glycosyltransferase rather than benoxacor.

*Mono (γ-GluCys) Conjugate [4-(γ-L-Glutamyl-L-cysteine-S-acetyl)-3,4-dihydro-3-methyl-2H-1,4-benzoxazine].* LC-ESI/MS of this metabolite yielded a protonated molecular ion at *m/z* 440 $(MH)^+$. The MS/MS daughter ion (+) spectrum of the *m/z* 440 parent ion is presented in Figure 5. The fragment ion at *m/z* 311 $(MH - 129)^+$ is due to the loss of a γ-glutamyl residue from $MH^+$. The fragment ion at *m/z* 222 $(MH - 218)^+$ is due to the loss of both the γ-glutamyl and a cysteine (minus the sufur atom) residue from $MH^+$.

This compound, which was isolated from BMS cells 24 HAT, is a dipeptide conjugate resulting from the catabolism of the mono(GSH) conjugate of benoxacor. A peptidase enzyme, such as γ-glutamyltranspeptidase (Meister, 1983), probably removed the glycine residue from the mono(GSH) conjugate. Many plant species including maize, sorghum, barley, wheat, and soybean have been previously demonstrated to metabolize glutathione *S*-conjugates to γ-GluCys dipeptide conjugates (Lamoureux and Rusness, 1981, 1990). γ-GluCys conjugates of xenobiotics are generally considered to be the predominant dipeptide intermediates of glutathione *S*-conjugate catabolism in plants (Rennenberg, 1982), but the presence of CysGly conjugates, while uncommon, is sometimes detected (Riechers *et al.*, 1995).

*Di(γ-GluCys) Conjugate [4-(γ-L-Glutamyl-L-cysteine-S-acetyl)-3,4-dihydro-3-methyl-2H-2-(S-γ-L-glutamyl-L-cysteinyl)-1,4-benzoxazine].* A protonated molecular ion at *m/z* 688 was present in the LC-ESI /MS spectrum of this metabolite. The MS/MS daughter ion (+) spectrum of the *m/z* 688 parent ion is illustrated in Figure 6. The fragment ion at *m/z* 559 $(MH - 129)^+$ is due to the loss of one γ-glutamyl moiety from $MH^+$, while the fragment ion at *m/z* 430 $(MH - 258)^+$ corresponds to the loss of two γ-glutamyl moieties from $MH^+$. The fragment ion at *m/z* 309 $(MH - 380)^+$ corresponds to the loss of two γ-glutamyl moieties and one cysteine moiety from $MH^+$.

The formation of this metabolite, which was isolated 24 HAT, most likely resulted from catabolic reactions of the di(GSH) conjugate via a peptidase enzyme which removed the glycine residues from both of the glutathione moieties. Inasmuch as this compound is a disubstituted analog of the mono(γ-GluCys) conjugate, the peptidase enzymes responsible for its formation are probably the same in both instances. Although a cysteine conjugate was not observed in the present study, both the mono- and di(γ-GluCys) conjugates would be the most likely substrates for dipeptidase enzymes to remove the γ-Glu residues and form Cys conjugates.

*S-(S′-Cys)GSH Conjugate [4-(Glutathione-S-acetyl)-3,4-dihydro-3-methyl-2H-2-(S-cysteinyl)-1,4-benzoxazine].* LC-ESI/MS of this metabolite yielded a protonated molecular ion at *m/z* 616 $(MH)^+$. The MS/MS daughter ion (+) spectrum of the *m/z* 616 parent ion is depicted in Figure 7. The fragment ion at *m/z* 541 $(MH - 75)^+$ is due to the loss of a glycine moiety while the fragment ion at *m/z* 487 $(MH - 129)^+$ is due to the loss of a γ-glutamyl moiety from $MH^+$. The fragment ion at *m/z* 366 $(MH - 250)^+$ suggests the loss of both a γ-glutamyl moiety and the benzoxazine-ring C2 cysteine moiety from $MH^+$, while the fragment ion at *m/z* 309 $(M - 306)^+$ is due to the loss of the entire glutathione moiety from the molecular ion.

This metabolite was extracted from maize cells 3 h after benoxacor treatment. Formation of this compound was probably catalyzed by a peptidase enzyme, which removed the glycine residue, and a dipeptidase, which removed the γ-glutamyl residue from one of the glutathione moieties of the di(GSH) conjugate. Because this metabolite was not subjected to $^1$H-NMR spectroscopy, it is not known which of the glutathione moieties of the di(GSH) conjugate was altered. Although not

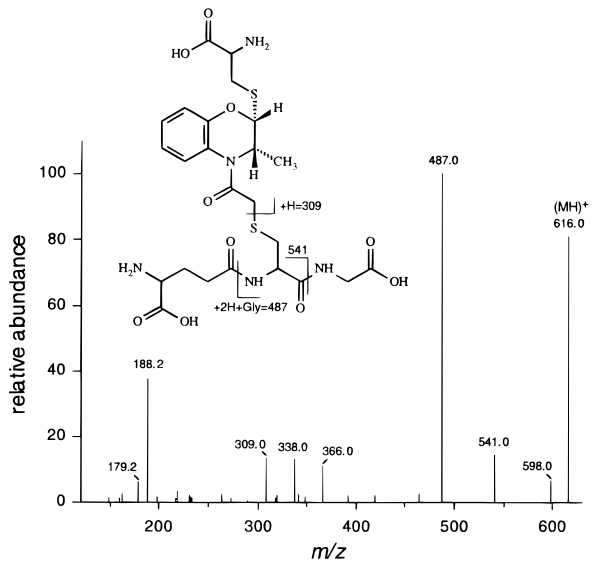


**Figure 7.** MS/MS daughter ion (+) spectrum and deduced fragmentation pattern of the *m*/*z* 616 parent ion generated by LC-ESI/MS of the *S*-(*S*′-Cys)GSH conjugate which was extracted from BMS cells 24 HAT.

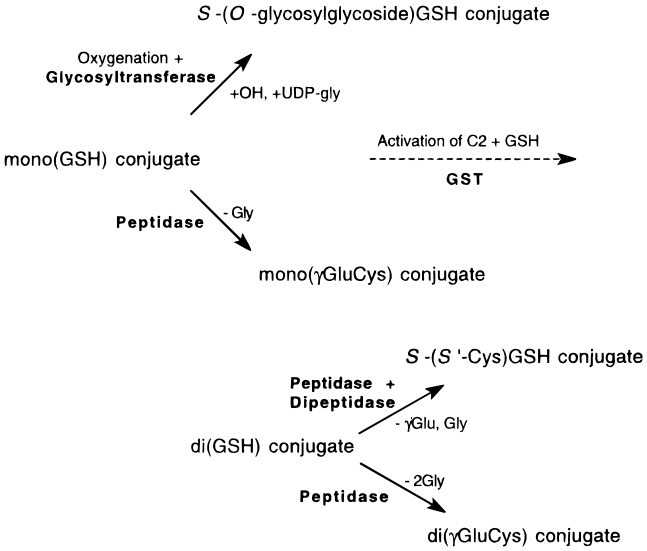


**Figure 8.** Postulated pathway of benoxacor metabolism in maize cells 3 and 24 HAT. The mono(GSH) conjugate, which was detected 3 and 24 HAT, is the initial metabolite in this pathway. Oxygenation or oxidation of the mono(GSH) conjugate could be followed by glycosyl or glutathione addition to form the *S*-(*O*-glycosylglycoside)GSH conjugate or the di(GSH) conjugate, respectively. Alternatively, peptidase enzymes could catabolize the mono(GSH) conjugate to the mono(γ-GluCys) conjugate. Analogously, peptidase enzymes could catabolize the di(GSH) conjugate to the di(γ-GluCys) conjugate or to the *S*-(*S*′-Cys)GSH conjugate.

detected in this study, it is logical that a *S*(*S*′-γ-GluCys)-GSH conjugate would be the precursory catabolic intermediate and serve as a substrate for a dipeptidase enzyme to form the *S*-(*S*′-Cys)GSH conjugate of benoxacor.

The postulated pathway of benoxacor metabolism in maize cells from three to 24 HAT is depicted in Figure 8. The mono(GSH) conjugate, which was present in maize cells 1, 3, and 24 h after treatment (Miller *et al.*, 1996), is the initial metabolite in this pathway. The mono(γ-GluCys) is probably formed by peptidase-mediated metabolism of the mono(GSH) conjugate. Alternatively, a UDP-glycosyltransferase metabolized the mono(GSH) conjugate to the *S*-(*O*-glycosylglycoside)-GSH conjugate following an enzyme-catalyzed hydroxylation reaction. Glutathione *S*-transferases catalyzed the addition of a second GSH to the the benzoxazine-ring C2 atom of the hydroxylated mono(GSH) conjugate to form the di(GSH) conjugate. The di(GSH) conjugate was then catabolized to either the di(γ-GluCys) conjugate via a peptidase enzyme or the *S*-(*S*′-Cys)GSH conjugate via both peptidase and dipeptidase enzymes.

## ABBREVIATIONS USED

BMS, Black Mexican Sweet; GC−MS, gas chromatography−mass spectrometry; GSH, reduced glutathione; GST, glutathione *S*-transferase; h, hour or hours; HAT, hours after treatment; LC-ESI/MS, liquid chromatography-electrospray ionization/mass spectrometry; MS/MS, mass spectrometry/mass spectrometry; $^1$H-NMR, proton nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; RP-HPLC, reversed phase high-performance liquid chromatography; TFA, trifluroacetic acid.

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