# 3-Methyleneoxindole: An Affinity Label of Glutathione *S*-Transferase pi Which Targets Tryptophan 38[†]

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ABSTRACT: The compound 3-methyleneoxindole (MOI), a photooxidation product of the plant auxin indole-3-acetic acid, functions as an affinity label of the dimeric pi class glutathione *S*-transferase (GST) isolated from pig lung. MOI inactivates the enzyme to a limit of 14% activity. The *k* for inactivation by MOI is decreased 20-fold by *S*-hexylglutathione but only 2-fold by *S*-methylglutathione, suggesting that MOI does not react entirely within the glutathione site. The striking protection against inactivation provided by *S*-(hydroxyethyl)ethacrynic acid indicates that MOI reacts in the active site region involving both the glutathione and the xenobiotic substrate sites. Incorporation of [$^3$H]MOI up to ∼1 mol/mol of enzyme dimer concomitant with maximum inactivation suggests that there are interactions between subunits. Fractionation of the proteolytic digest of [$^3$H]MOI-modified GST pi yielded Trp38 as the only labeled amino acid. The crystal structure of the human GST pi−ethacrynic acid complex (2GSS) shows that the indole of Trp38 is less than 4 Å from ethacrynic acid. Similarly, MOI may bind in this substrate site. In contrast to its effect on the pi class GST, MOI inactivates much less rapidly and extensively alpha and mu class GSTs isolated from the rat. These results show that MOI reacts preferentially with GST pi. Such a compound may be useful in novel combination chemotherapy to enhance the efficacy of alkylating cancer drugs while minimizing toxic side effects.

Glutathione *S*-transferases (EC 2.5.1.18) are a ubiquitous group of detoxification enzymes with broad substrate specificity, catalyzing the conjugation of glutathione to electrophilic substrates (*1*). They are also involved in the transportation of nonsubstrate hydrophobic compounds within the cell. The cytosolic glutathione *S*-transferase isoenzymes are currently divided into at least eight classes (named alpha, kappa, mu, omega, pi, sigma, theta, and zeta) on the basis of their physical and chemical properties (*2*−*8*). The alpha and mu classes from rat liver are extensively characterized, and each includes several isoenzymes; homo- or heterodimers are known to form within a given class (*1*, *9*).[1] The pi class has only one member: a homodimer of a distinctive subunit.

A number of studies have shown that glutathione *S*-transferases (GST)[2] are overexpressed in certain tumor cells compared to the corresponding normal tissues (e.g., refs *11*−*16*). These enzymes are thought to be important in the development of resistance to chemotherapeutic agents because they detoxify these compounds; therefore, the inhibition of certain GSTs could increase the efficacy of treatment and reduce the dose of medication required. Variation exists in the extent of the GST concentration increase in tumors among the several classes of GST. Increases of the mu and alpha class isoenzymes have been reported (*1*, *13*, *17*, *18*); however, most changes in activity are attributed to higher concentrations of class pi GST in the tumor cells (*12*, *19*−*24*). Since GSTs also constitute an important cellular defense against xenobiotic compounds, generalized inhibition of GSTs could lead to unacceptable toxic side effects. The development of inhibitors *specific* for particular isoenzymes of GST may lead to novel modes of combination chemotherapy.

The aim of this project is to use affinity labeling to develop an inhibitor specific for GST pi and to evaluate the distinctive features of the active site of this GST isoenzyme. The compound investigated here is 3-methyleneoxindole (MOI), a photooxidation product of the plant auxin indole-3-acetic acid (Figure 1). MOI inactivates mandelonitrile lyase from almonds (*25*) with the reaction suggested to involve Michael addition to the exocyclic double bond. Since the pi class GST enzyme is noted for its ability to catalyze Michael additions

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[1] The nomenclature of rat glutathione *S*-transferases presented by Hayes and Pulford (*1*) based on that of Jakoby et al. (*10*) designates class alpha subunits 1a, 1b, and 2 as rGSTA1, rGSTA2, and rGSTA3, respectively. Class mu subunits 3 and 4 are designated rGSTM1 and rGSTM2, respectively. The class pi subunit is designated as 7.

[2] Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DTT, dithiothreitol; GS-EA, glutathione−ethacrynic acid conjugate; GST pi, class pi glutathione *S*-transferase; GS-MOI, glutathione−3-methyleneoxindole conjugate; hGST P1-1, human class pi glutathione *S*-transferase; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MOI, 3-methyleneoxindole; NEM, *N*-ethylmaleimide; PTH, phenylthiohydantoin; TER-117, γ-glutamyl-(*S*-benzyl)cysteinyl-D-phenylglycine; TFA, trifluoroacetic acid.

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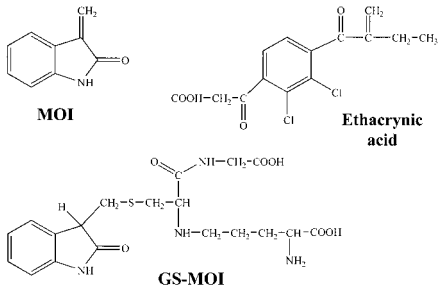


FIGURE 1: Structure of the affinity label 3-methyleneoxindole (MOI) and two compounds that afford protection for GST pi from inactivation by the label. The inactivation rate of the affinity label was strikingly decreased with the addition of the glutathione−3-methyleneoxindole conjugate (GS-MOI) and derivatives of ethacrynic acid.

(particularly with ethacrynic acid) (*1*), we postulated that this isoenzyme might react best with MOI.

We present here the results of the affinity labeling of the pi class GST by the plant auxin derivative MOI. MOI targets Trp38 and has a binding site similar to that occupied by the electrophilic substrate ethacrynic acid. MOI was also tested with other isoenzyme classes and found to be more potent and specific toward the pi class GST. A preliminary version of this work has been communicated (*26*).

## EXPERIMENTAL PROCEDURES

*Materials.* Frozen pig lungs (untrimmed) were purchased from Pel Freez Biologicals (Rogers, AR). Sephadex G-50, 2,4-dinitrophenol, ethacrynic acid, glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), *S*-hexylglutathione, dithiothreitol (DTT), indole-3-acetic acid, and *N*-bromosuccinimide were purchased from Sigma Chemical Co., St. Louis, MO. Trifluoroacetic acid (TFA) was purchased from Aldrich Chemical Co, Milwaukee, WI, Liquiscint from National Diagnostics, Atlanta, GA, and [5-$^3$H]indole-3-acetic acid from American Radiolabeled Chemicals Inc., St. Louis, MO. All other chemicals were of reagent grade.

*Synthesis of MOI.* 3-Methyleneoxindole was prepared by the conversion of indole-3-acetic acid to 3-bromooxindole-3-acetic acid with *N*-bromosuccinimide, followed by decarboxylation and elimination of bromide. MOI was synthesized as described by Petrounia et al. (25), based on the method of Hinman and Bauman (*27*, *28*), except that 3-bromooxindole-3-acetic acid was left to precipitate overnight from the reaction mixture at room temperature and then filtered prior to the next stage of the synthesis.

A portion of the final product was dissolved in methanol (2.5 mg/mL) and purified by HPLC. The sample (300 $\mu$L loaded per run) was purified using a Varian 5000 LC HPLC (Varian, Walnut Creek, CA) equipped with a 0.46 × 25 cm reverse-phase Vydac $C_{18}$ column (Vydac, Hesperia, CA) equilibrated in 15% acetonitrile and 0.1% TFA and eluted isocratically at a flow rate of 1 mL/min. MOI eluted at 15.2 min. The eluate was monitored at 254 nm with fractions containing MOI (as judged by the typical double absorbance maximum at 248 and 253 nm), combined, and lyophilized (*28*).

The method was scaled down by 100-fold for synthesis of radioactive MOI. [5-$^3$H]Indole-3-acetic acid (250 $\mu$Ci with a specific activity of 20 Ci/mmol) was dissolved in 3 mL of *tert*-butyl alcohol with 0.066 g (0.38 mmol) of unlabeled indole-3-acetic acid. Under argon, *N*-bromosuccinimide (0.13 g) was added over a 1 h period and the procedure followed as before. The 3-bromooxindole-3-acetic acid was collected by centrifugation rather than filtration due to the low amounts. The product was then dissolved and extracted as before to synthesize the [$^3$H]MOI. The specific radioactivity of the product was (9.23 ± 0.11) × $10^{11}$ cpm/mol, where $\epsilon_{248nm}$ = 14 820 $M^{-1}$ $cm^{-1}$ and $\epsilon_{253nm}$ = 14 250 $M^{-1}$ $cm^{-1}$ (*25*) were used for MOI in 100 mM citrate buffer, pH 5.5. All stock solutions of MOI were dissolved in methanol to yield a maximum concentration of 5 mM. At higher concentrations of MOI in methanol, the compound precipitates, probably as a dimer or higher polymer (*28*).

*Synthesis of GS-MOI.* GS-MOI was prepared by reaction of MOI with glutathione. At room temperature and under an atmosphere of argon, 0.28 mmol of glutathione was dissolved in 170 mL of 25% methanol, and the pH was adjusted to 5−5.5 with 1 M potassium hydroxide. To the stirred solution was added 0.34 mmol of solid MOI in portions over a 20 min period, and the reaction mixture was stirred for an additional 2 h or until the yellow color of MOI had dissipated. The reaction mixture was filtered to remove a small white precipitate due to MOI polymerization and concentrated under reduced pressure at 30 °C to about 25 mL. GS-MOI was obtained as a fluffy, white powder following lyophilization. The dry powder was washed with 2 × 30 mL portions of ether to remove residual MOI and dried in vacuo or under a stream of dry argon. The yield was 0.26 mmol (93%); anhydrous MW = 452. The dry solid is stable for months when stored desiccated at −20 °C.

*Characterization of GS-MOI.* Proton NMR of 8 mM GS-MOI was performed in 50 mM potassium phosphate buffer/$D_2O$, pD 7.2, HDO reference at 4.7 ppm: $\delta$ 6.9−7.3 ppm (4H), Ar−H; $\delta$ 4.1−4.4 ppm (1H), Cys-α-H (observed as two sets of signals due to GS-MOI diastereomers); additional signals from the glutathione backbone observed between 1.9 and 3.7 ppm. At pD 5.4 a characteristic signal for the C-3 proton of the indole ring is observed at 3.8 ppm, which slowly exchanges with solvent deuterium. This exchange is too rapid to be observed at pD >6.4. There was no evidence for the vinylic protons of MOI at 6.4 ppm. Reverse-phase chromatography (RP-HPLC) was carried out using a Rainin solvent delivery system, Model HPXL, Model 288 detector at 254 nm, Rainin C-18 Dynamax-60A column, and a Hitachi Chromatointegrator Model D-2500. A mobile phase of 35% acetonitrile/0.1% TFA was used, at a flow rate of 1 mL/min. RP-HPLC of a freshly prepared 0.2 mM sample of GS-MOI in 50 mM phosphate buffer, pH 7.0, gives a single peak for GS-MOI with a retention time of 2.6 min. On the basis of NMR and RP-HPLC analysis, GS-MOI was estimated at >90% purity. Buffered aqueous stock solutions of GS-MOI at pH 7 have a half-life of about 3 days at 25 °C and at least 1 week at −20 °C. The formation of MOI is detected by its retention time of 7.1 min.

*Synthesis of Ethacrynic Acid Conjugates.* The glutathione−ethacrynic acid conjugate (GS-EA) was synthesized by mixing ethacrynic acid (in ethanol with a final concentration of 10 mM) with excess glutathione (final concentration of 40 mM) in 80 mM phosphate buffer at pH 7.4. The reaction mixture was incubated at 25 °C for 2 h and then purified by HPLC based on the method of Dixit et al. (*29*). The reaction mixture was applied batchwise (1 mL/purification) to a 0.46

× 25 cm Vydac $C_4$ reverse-phase column equilibrated with 100% solvent A (solvent A, 0.1% TFA in water; solvent B, acetonitrile). At a flow rate of 1 mL/min, the compounds were separated by a 10 min linear gradient from 0 to 10% solvent B, followed by a 15 min gradient to 25% and then by a 30 min isocratic elution of 25% B, and finally a gradient to 100% solvent B in 15 min. The eluate was monitored at 270 nm with the conjugate eluting between 42 and 46 min. The GS-EA conjugate was lyophilized, neutralized, and dissolved in water. The concentration was determined using $\epsilon_{270nm} = 5.7\ mM^{-1}\ cm^{-1}$ (*30*).

*S*-(Hydroxyethyl)ethacrynic acid (conjugate of ethacrynic acid and $\beta$-mercaptoethanol) was synthesized by mixing ethacrynic acid (in ethanol with a final concentration of 4 mM) with excess $\beta$-mercaptoethanol (final concentration of 750 mM) in 100 mM phosphate buffer, pH 7.7. The reaction mixture was incubated at 25 °C for 30 min and then lyophilized twice (to remove the volatile $\beta$-mercaptoethanol). *S*-(Hydroxyethyl)ethacrynic acid was dissolved in water and the concentration determined as for GS-EA.

*Enzyme Purification*. All purification procedures were performed at 4 °C. The pig lung GST pi enzyme was purified using a modified version of the method of Dirr et al. (*31*), as described previously (*32*), where $\epsilon_{280nm}^{0.1\%} = 1.26\ M^{-1}\ cm^{-1}$. GST isoenzymes (1-1, 2-2, 3-3, and 4-4) were purified from rat livers, as described in Hu et al. (*33*). The procedures yield GSTs at greater than 90% purity as evaluated by SDS−PAGE and reverse-phase HPLC (Vydac $C_4$ reverse-phase column, using a 50 min gradient of 30−60% acetonitrile containing 0.075% TFA).

*Enzyme Assay*. As a standard assay, the GST pi activity was measured using the method of Habig et al. (*34*). The assay solution contained glutathione (final concentration, 2.5 mM), CDNB (1 mM), and GST pi in 0.1 M phosphate buffer, pH 6.5, in a final volume of 1 mL (2.5% ethanol). A Hewlett-Packard 8453 spectrophotometer was used to monitor formation of the glutathione−CDNB conjugate at 340 nm ($\Delta\epsilon_{340nm} = 9.6\ mM^{-1}\ cm^{-1}$) at 25 °C and the rate corrected for spontaneous nonenzymatic conjugation of glutathione and CDNB. The transfer of glutathione (0.63 mM) to ethacrynic acid (0.2 mM) in 0.1 M phosphate buffer, pH 6.5 (2.5% ethanol), was monitored at 270 nm at 25 °C (*35*), where $\Delta\epsilon_{270} = 5.0\ mM^{-1}\ cm^{-1}$ (*34*).

*Reaction of GST pi with MOI.* GST pi (0.4 mg/mL) was incubated with MOI (75−250 $\mu$M) in 90 mM phosphate buffer at a final pH of 7.7 at 25 °C (10% methanol). In every case, aliquots of the reaction mixture were removed at specified times and assayed for enzymatic activity. To determine their effects on the rate constant for inactivation of GST pi by MOI, ligands were preincubated in the reaction mixture for 10 min prior to MOI addition (200 $\mu$M final concentration). All ligands were dissolved in buffer except dinitrophenol, *tert*-stilbene oxide, and bromosulfophthalein, which were dissolved in dimethyl sulfoxide (10% of final reaction mixture), and the $\beta$-mercaptoethanol-treated MOI and *S*-(hydroxyethyl)ethacrynic acid, which were dissolved in methanol. Controls containing the ligand and enzyme (without MOI) were run in parallel with the test sample to assess any effect of the ligands on the enzyme activity.

*Characterization of Modified GST pi−MOI.* GST pi (0.4 mg/mL) inactivated with MOI, as previously described, was characterized. Apparent kinetic constants were determined using the CDNB-based or ethacrynic acid-based assay method described previously. For determination of the $K_m$ of glutathione, the CDNB concentration was maintained at 2.5 mM with the concentration of glutathione varied from 0.02 to 1 mM. For the determination of the $K_m$ for CDNB, the glutathione concentration was maintained at 3 mM while the CDNB was varied from 0.013 to 5 mM. For determination of the $K_m$ of ethacrynic acid, the glutathione concentration was maintained at 0.625 mM with the concentration of ethacrynic acid varied from 0.003 to 0.25 mM. Kinetic constants were determined by linear least-squares analysis of double reciprocal plots of substrate concentration versus enzymatic rate of conjugation. Circular dichroism of modified enzyme (0.15 mg/mL) and control enzyme (0.15 mg/mL), in 0.02 M phosphate buffer, pH 6.5, were obtained using a Jasco model J710 spectropolarimeter from 200 to 250 nm with a 0.1 cm path length cylindrical quartz cell. An average of five measurements of each of triplicate samples (adjusted for protein concentration) were recorded as the spectrum for control enzyme and for modified enzyme.

*Incorporation of [$^3$H]MOI.* The reaction mixture contained GST pi (0.4 mg/mL), [$^3$H]MOI (200 $\mu$M), and 90 mM phosphate buffer at a final pH of 7.7. The mixture was incubated at 25 °C for various time periods after which excess MOI was removed by the gel centrifugation method of Penefsky (*36*), in which the reaction mixture was loaded sequentially onto two 5 mL G-50 Sephadex columns and centrifuged.

The protein concentration was estimated using the Bio-Rad protein reagent (Bio-Rad, Hercules, CA) based on the method of Bradford (*37*) with pure GST pi as the standard. The amount of radioactivity present was measured using a Tri-Carb liquid scintillation counter, Model 1500.

*Chymotrypsin Digest of the MOI-Modified GST pi and Peptide Purification*. GST pi (0.4 mg/mL) was inactivated with [$^3$H]MOI as described previously. *N*-Ethylmaleimide (NEM) was added to a final concentration of 10 mM, and the solution was incubated for 5 min at 25 °C. Solid urea (9 M final concentration) was added, and the solution was incubated for 30 min at 25 °C. The enzyme solution was then dialyzed against 10 mM phosphate buffer, pH 6.5. The enzyme solution was lyophilized, resolubilized in 250 $\mu$L of 8 M urea in water, and incubated at 37 °C for 2 h. Water (750 $\mu$L) was then added to the solution to dilute the urea to 2 M. Chymotrypsin (5% w/w) was added, and the solution was incubated for 2 h at 37 °C. A second aliquot of chymotrypsin was added, and the solution was incubated for another 2 h at 37 °C.

The chymotrypsin digest was applied to a Vydac $C_{18}$ reverse-phase column equilibrated with 15% solvent B (solvent A, 0.1% TFA in water; solvent B, 0.075% TFA in acetonitrile). At a flow rate of 1 mL/min, the peptides were separated by a 90 min linear gradient from 15% to 35% solvent B followed by a 50 min gradient to 45% and then by a 30 min gradient to 100% solvent B. The eluate was monitored at 220 nm with 1 mL fractions collected. Aliquots of each fraction were tested for radioactivity.

The radioactive peptide peak from the chymotrypsin digest was pooled, lyophilized, redissolved in 250 $\mu$L of 8 M urea in 10 mM phosphate buffer, pH 6.5, and then diluted to 1 mL with 10 mM phosphate buffer, pH 6.5. *Staphylococcus*

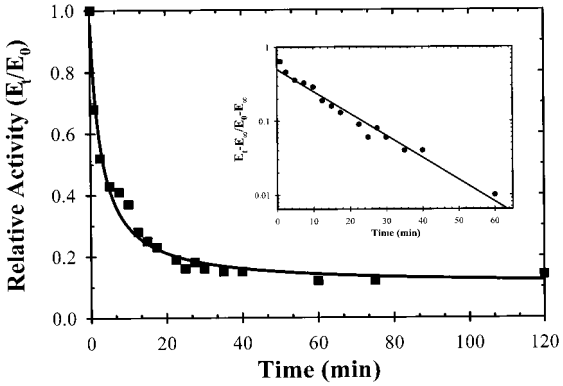


FIGURE 2: Inactivation of GST pi by 200 $\mu$M MOI. Relative activity ($E_t/E_0$) was determined at each time point (where $E_0 = 31$ $\mu$mol min$^{-1}$ mg$^{-1}$). Inset: $k_{obs}$ is calculated from a semilogarithmic graph of $(E_t - E_\infty)/(E_0 - E_\infty)$ versus time ($E_\infty = 4.34$ $\mu$mol min$^{-1}$ mg$^{-1}$). For 200 $\mu$M MOI $k_{obs} = 0.071$ min$^{-1}$.

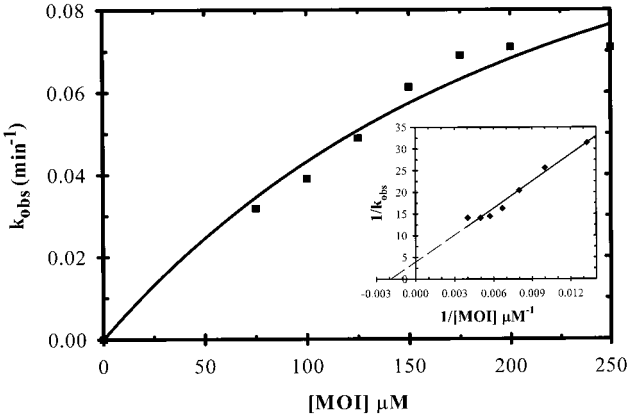


FIGURE 3: Inactivation of GST pi as a function of MOI concentration. GST pi was incubated with increasing concentrations of MOI and the $k_{obs}$ determined (75−250 $\mu$M) as described in Figure 2. Inset: A double reciprocal plot produces a linear relationship from which $K_I = 520 \pm 17$ $\mu$M and $k_{max} = 0.25 \pm 0.01$ min$^{-1}$.

*aureas* V8 protease was added twice at 8% w/w, and the solution was incubated at 30 °C for a total of 18 h. The peptide solution was then filtered through a Centricon YM-10 membrane to remove the protease. The resulting filtrate was loaded onto the HPLC (Vydac $C_{18}$ column) and eluted at a flow rate of 1 mL/min with a 90 min linear gradient from 15% to 35% solvent B followed by a 100 min gradient to 45% and then by a 10 min gradient to 100% solvent B. The eluate was monitored at 220 nm with 1 mL fractions collected. Aliquots of each fraction were tested for radioactivity.

*Sequence Determination of the Separated Peptides.* The amino acid sequences of the peptides were determined on an Applied Biosystems Model 470A gas-phase protein/peptide sequencer, equipped with a Model 120A phenylthiohydantoin analyzer. The amount of Trp in cycle 3 was compared to the commercial peptides Asp-Leu-Trp-Gln-Lys and Tyr-Pro-Trp-Gly from Sigma Chemical Co.

*Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) of the Isolated Modified Peptides.* Samples were purified by HPLC as previously described, lyophilized, and then redissolved in 50 $\mu$L of 50% acetonitrile/0.1% TFA. The samples were then mixed with saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile. The spotted sample (1 $\mu$L) was allowed to air-dry on the plate and then run on a Bruker (Billerica, MA) BIFLEX III MALDI-TOF-MS, operating in reflector mode using delayed extraction.

## RESULTS

*Inactivation of GST pi by MOI.* MOI (200 $\mu$M) inactivates GST pi at pH 7.7 in a time-dependent manner, reaching a constant level of 14% activity after 30 min (Figure 2). No further decrease was observed at longer times or with the addition of fresh MOI. Removal of excess MOI at 20 min (21% residual activity) prevents further inactivation of the enzyme and, without the addition of MOI to the reaction mixture, no decrease in enzyme activity is observed, showing that inactivation is caused by the addition of MOI. The rate of inactivation is influenced by pH, in that as the pH increases above pH 5.5, the rate of inactivation increases until pH 8.3 when the enzyme denatures.

The rate and degree of inactivation caused by 200 $\mu$M MOI were compared when the residual activity was measured using two different substrates (CDNB and ethacrynic acid) to determine whether there was any differential effect on the regions within the xenobiotic binding site. The enzyme activity is similarly affected when assayed using either substrate, and the percent residual activity is also consistent using different substrates. For example, with CDNB there is 42.4 ± 0.7% residual activity at 5 min inactivation, 29.4 ± 2.3% at 10 min, and 14.1 ± 1% at 30 min; with the substrate ethacrynic acid at 5 min there is 42% residual activity, at 10 min 29%, and at 30 min 13% residual activity.

The rate of inactivation as a function of MOI concentration (75−250 $\mu$M) was determined (Figure 3). Rates were calculated assuming a limiting value of 14% residual activity (i.e., $E_\infty/E_0 = 0.14$). A double reciprocal plot (Figure 3, inset) was used to calculate $K_I = 520 \pm 17$ $\mu$M and $k_{max} = 0.25 \pm 0.01$ min$^{-1}$. It was not possible to test rates of inactivation with higher MOI concentrations since MOI has been reported to polymerize in concentrated solutions (*28*).

*Characterization of MOI-Modified GST pi.* Apparent kinetic constants for the substrates glutathione, CDNB, and ethacrynic acid were determined for the native and MOI-modified GST pi (GST pi reacted with MOI, which has 14% residual activity). For CDNB, the $K_m$ values were 0.79 ± 0.06 and 0.65 ± 0.41 mM, respectively, for native and modified GST pi; for glutathione, the $K_m$ values were 0.11 ± 0.03 and 0.10 ± 0.03 mM, respectively, for native and modified GST pi. The native and modified GST pi samples had the same $K_m$ value for ethacrynic acid; 0.04 ± 0.01 mM. There were no significant differences between the two samples, showing that the residual active enzyme does not exhibit altered affinity for any of these substrates.

Circular dichroism spectra were determined for the modified and native enzyme (data not shown). No significant differences were found, suggesting that neither unfolding of the enzyme nor appreciable changes in the enzyme's secondary structure cause the loss of enzyme activity.

*Effects of Ligands on the Inactivation Rate of GST pi.* Glutathione *S*-transferase ligands were added to the reaction mixture at concentrations at least five times their reported $K_m$ or $K_I$ to determine their effect on the inactivation rate constant of 200 $\mu$M MOI (Table 1). The results are expressed as ratios of the inactivation rate constant measured in the presence of a particular ligand ($k_{+L}$) to the rate constant

Table 1: Effect of Ligands on the Inactivation of GST pi[a]

| no. | ligand added to reaction mixture | $k_{+L}/k_{-L}$ |
|---|---|---|
| 1 | none | 1.00 ± 0.10 |
| 2 | *S*-hexylglutathione (5 mM) | 0.05 ± 0.01 |
| 3 | *S*-hexylglutathione (1 mM) | 0.26 ± 0.03 |
| 4 | *S*-(*p*-nitrobenzoyl)glutathione (5 mM) | 0.29 ± 0.09 |
| 5 | *S*-methylglutathione (5 mM) | 0.52 ± 0.03 |
| 6 | *S*-methylglutathione (10 mM) | 0.55 ± 0.06 |
| 7 | *S*-methylglutathione (5 mM) + 2,4-dinitrophenol (5 mM) | 0.31 ± 0.02 |
| 8 | 2,4-dinitrophenol (5 mM) | 0.79 ± 0.15 |
| 9 | *t*-stilbene oxide (0.1 mM) | 0.94 ± 0.22 |
| 10 | GS-EA (0.2 mM) | 0.22 ± 0.06 |
| 11 | GS-EA (1 mM) | 0.06 ± 0.03 |
| 12 | *S*-(hydroxyethyl)ethacrynic acid (0.2 mM) | 0.45 ± 0.03 |
| 13 | *S*-(hydroxyethyl)ethacrynic acid (0.8 mM) | 0.10 ± 0.04 |
| 14 | GS-MOI (0.2 mM) | 0.36 ± 0.05 |
| 15 | GS-MOI (1 mM) | 0.00 ± 0.01 |
| 16 | *S*-hydroxyethyl-MOI (1 mM) | 0.18 ± 0.05 |
| 17 | estradiol disulfate (0.1 mM) | 1.00 ± 0.20 |
| 18 | bromosulfophthalein (0.1 mM) | 1.07 ± 0.04 |
| 19 | HEPES (100 mM) | 1.09 ± 0.13 |
| 20 | MES (100 mM) | 0.90 ± 0.13 |

[a] GST pi (0.4 mg/mL) was incubated in 90 mM phosphate buffer, pH 7.7, at 25 °C with 200 $\mu$M MOI in the presence and absence of ligands. Rate constants were calculated by the method illustrated in Figure 2, inset, where $E_\infty/E_0 = 0.14$. The results are expressed as a ratio of the inactivation rate constant measured in the presence of a ligand ($k_{+L}$) to the rate constant measured in the absence of any ligand ($k_{-L}$) ± standard error, where $k_{-L} = 0.071$ min$^{-1}$.

measured in the absence of any ligand ($k_{-L}$) ± the standard error.

*S*-Hexylglutathione is known to bind within the glutathione and electrophilic binding sites of GSTs (*38*, *39*) with a $K_I$ of 35 $\mu$M (*40*). *S*-Hexylglutathione markedly decreases the inactivation rate at 1 and 5 mM (Table 1, lines 2 and 3), showing that when the glutathione and electrophilic substrate binding sites are both occupied, MOI is less able to react with GST pi. The protection is concentration dependent with 5 mM protecting better than 1 mM against inactivation. These results clearly show that the target of MOI is within or near the active sites of GST pi. MOI-modified GST pi no longer binds to an *S*-hexylglutathione−agarose affinity column, consistent with labeling within the active site of the enzyme.

To evaluate whether MOI is binding in the glutathione active site, *S*-(*p*-nitrobenzoyl)glutathione and *S*-methylglutathione were tested. *S*-(*p*-Nitrobenzoyl)glutathione is an aryl derivative of glutathione, which binds within the glutathione active site and part of the electrophilic substrate binding site. With 5 mM *S*-(*p*-nitrobenzoyl)glutathione added to the reaction mixture together with MOI and GST pi, the rate of inactivation decreases significantly (line 4) but not as much as in the case of *S*-hexylglutathione at the same concentration.

*S*-Methylglutathione ($K_I$ 200 $\mu$M; *41*) is similar in structure to *S*-hexylglutathione, except that the alkyl side chain is shorter, thus not occupying the electrophilic substrate binding site appreciably. With 5 or 10 mM *S*-methylglutathione added to the reaction mixture, together with MOI and GST pi, the inactivation rate is decreased 2-fold (lines 5 and 6). With higher concentrations of *S*-methylglutathione, no additional protection is afforded. Since *S*-methylglutathione exerts a much less protective effect than does *S*-hexylglutathione, the reaction of MOI cannot take place entirely within the glutathione site.

The electrophilic substrate dinitrophenol (line 8) protects slightly against the reaction of MOI with GST pi. However, the addition of dinitrophenol and *S*-methylglutathione together (line 7) protect the enzyme against inactivation by MOI more than does either ligand alone.

*tert*-Stilbene oxide, another known GST electrophilic substrate, does not decrease the rate of GST pi inactivation by MOI (line 9) and thus is not binding in the same location as the MOI target site. These "protection" experiments show that MOI reacts within the interface of the hydrophobic and glutathione binding sites of the pig lung GST pi.

Ethacrynic acid inhibits GSTs ($K_I$ 11.5 $\mu$M; *30*) but is also a known electrophilic substrate, of which GST pi class is the most efficient at catalyzing the reaction. The striking protection against inactivation provided by the glutathione conjugate of ethacrynic acid (GS-EA, $K_I$ 1.5 $\mu$M; *30*) and *S*-(hydroxyethyl)ethacrynic acid (lines 10−13) indicates that MOI reacts in the active site region involving both the glutathione and the xenobiotic substrate sites. The protection afforded by *S*-(hydroxyethyl)ethacrynic acid is even more significant, since this compound is very similar in structure to ethacrynic acid; this result indicates that MOI reacts within the ethacrynic acid binding site.

The glutathione conjugate of MOI was initially tested for its potential to inactivate GSTs since it was postulated that it might bind within the glutathione binding site and release MOI locally. However, this compound by itself does not inactivate GST pi during 75 h over the pH range 6.5−8.3, at concentrations up to 1 mM. In the enzymatic assay using CDNB as substrate, GS-MOI acts as a competitive inhibitor with respect to glutathione; $K_I$ for GS-MOI is 0.27 ± 0.01 $\mu$M.

GS-MOI significantly decreases the rate constant for inactivation of GST pi by MOI (lines 14 and 15). *S*-Hexylglutathione (line 3) at 1 mM protects the enzyme less effectively than does GS-MOI (line 15) at the same concentration. The treatment of MOI with $\beta$-mercaptoethanol (line 16) resulted in a compound which, at 1 mM, significantly protected GST pi against MOI inactivation. However, this protective effect was less than that of GS-MOI, indicating that the entire glutathione site must be occupied in order for the product complex to bind effectively. The glutathione portion of GS-MOI is presumably bound within the glutathione binding site, consistent with the postulate that MOI reacts with an amino acid in the region of the glutathione and xenobiotic substrate site interface.

Several GST isoenzymes bind compounds at nonsubstrate binding sites. Whether inactivation was caused by MOI reacting at a steroid binding site (*1*, *35*, *40*) was assessed by including estradiol disulfate ($K_I$ 2.1 $\mu$M; *42)* in the reaction mixture. The compound did not influence the rate of inactivation (line 17), and therefore, such a nonsubstrate steroid site is not in the region of reaction of MOI.

Bromosulfophthalein is a large dye that binds in the ligandin site of GST pi with a $K_D$ of 1.1 $\mu$M (*43*). The ligandin site is within the electrophilic binding site and binds nonsubstrate compounds for transport (*43*, *44*). Bromosulfophthalein (line 18) does not reduce the rate of inactivation by MOI of GST pi and, thus, is not binding in the same area of the active site as MOI.

The buffer compounds *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) (resembling an elongated

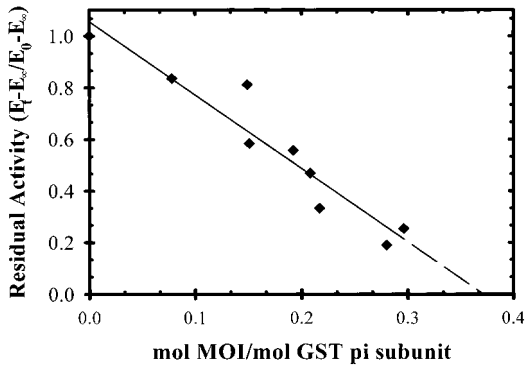


FIGURE 4: Incorporation of MOI into GST pi. The residual activity of MOI inactivated GST pi has been compared to the mol/mol ratio of [$^3$H]MOI to GST pi subunits.

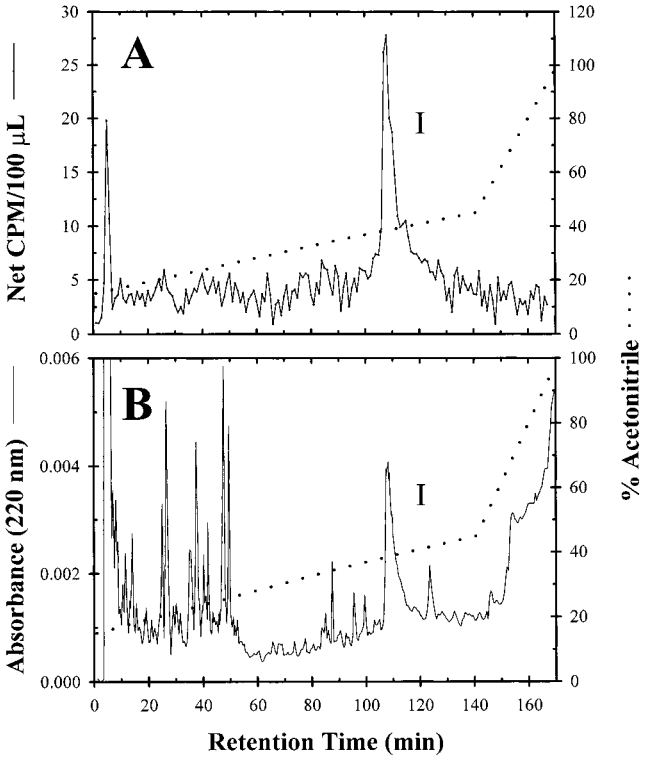


FIGURE 5: HPLC separation of GST pi modified with [$^3$H]MOI and digested with chymotrypsin. GST pi (0.4 mg/mL) was modified with 200 $\mu$M MOI and then digested with chymotrypsin, and the peptides were separated by HPLC using a $C_{18}$ column (as described in Experimental Procedures). (A) Net CPM in 100 $\mu$L of 1 mL fractions from the HPLC separation. (B) 220 nm absorbance profile. Peak I was pooled and further digested with V8 protease.

sulfate anion) (*45*) and 2-(*N*-morpholino)ethanesulfonic acid (MES) (*39*, *46*) bind at sites located in a surface cavity or between two subunits, at sites which are not the substrate binding site. The effect of HEPES and MES on inactivation of GST pi by MOI was determined (lines 19 and 20). Neither compound reduces the rate of inactivation by MOI, suggesting that these are not the sites at which MOI reacts.

*Incorporation of MOI by GST pi.* The amount of MOI reacting with the GST pi enzyme was estimated using radiolabeled MOI (Figure 4). The ratio of moles of MOI per mole of GST pi subunit extrapolates to about 0.4 when the enzyme is maximally inactivated (14% residual activity); thus, only one of the two subunits is modified when the enzyme is maximally inactivated. This suggests that the reaction of MOI in one active site influences the other subunit

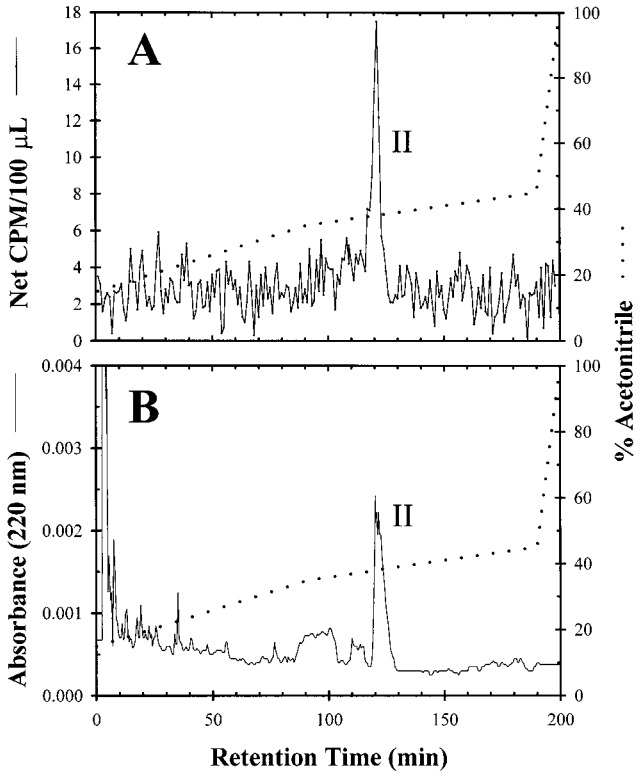


FIGURE 6: HPLC separation of chymotryptic [$^3$H]MOI-modified GST pi peptides digested with V8 protease. The MOI-modified chymotryptic peptides were further digested with V8 protease and separated by HPLC using a $C_{18}$ column (as described in Experimental Procedures). (A) Net CPM in 100 $\mu$L of 1 mL fractions from the HPLC separation of double digested modified GST pi. The peak labeled (II) was sequenced. (B) 220 nm profile of the double digested modified enzyme showing one major peak, eluting at 123 min, which correlates with the radioactivity.

of the dimer and reduces its ability to catalyze the transfer of glutathione.

*Isolation of Peptides from MOI-Modified GST pi.* After digestion of MOI-modified GST pi by chymotrypsin and fractionation of the digest by HPLC (Figure 5), two major radioactive peaks were identified. The first, smaller peak contained free radioactive MOI eluting in the void volume, while the second peak contained labeled peptide (elution volume of 109−111 mL). The radioactive peptide peak pool was a mixture containing a cross-linked peptide that was identified by amino acid sequence data and confirmed by MALDI-TOF mass spectrometry. Thus, the peptide peak from the chymotryptic digest of modified GST pi was subjected to a second digestion with V8 protease to decrease the size of the radioactive peptide and purify it further. The double digested sample was fractioned by HPLC (Figure 6) with a single radioactive peptide peak identified. Buffers at pH 6.5 were used during protease digestion and manipulation in order to maintain the stability of the MOI modification of the peptide.

*Characterization of Modified Peptide(s).* The results from several separately isolated modified peptides are summarized in Table 2. The peptide containing $E^{36}TW^{38}PPLKPSCL^{46}$ (corresponding to amino acids 36−46 of the known amino acid sequence) was identified as the site of labeling. Tryptophan 38 in cycle 3 was significantly lower in each sample sequenced. When the amount of tryptophan in cycle 3 was compared to the amount of PTH-amino acid derivative

Table 2: Representative Amino Acid Sequences of Modified Peptides Present from Three Different Experiments Showing a Decreased Amount of Trp in Cycle 3[a]

| | sample 1 | | sample 2 | | sample 3 | |
|---|---|---|---|---|---|---|
| cycle | amino acid | amount (pmol) | amino acid | amount (pmol) | amino acid | amount (pmol) |
| 1 | Glu | 35.5 | Glu | 293.2 | Glu | 32.7 |
| 2 | Thr | 9.9 | Thr | 159.4 | Thr | 13.7 |
| 3 | Trp[b] | 0.9 | Trp[b] | 1.8 | Trp[b] | 2.7 |
| 4 | Pro | 7.2 | Pro | 81.6 | Pro | 13.8 |
| 5 | Pro | 3.1 | Pro | 49.5 | Pro | 8.7 |
| 6 | Leu | 5.4 | Leu | 47 | Leu | 12.4 |
| 7 | Lys | 0.2 | Lys | 44.4 | Lys | 3.2 |
| 8 | Pro | 1.9 | Pro | 46.3 | Pro | 4.5 |
| 9 | Ser | 2.6 | Ser | 28.8 | Ser[e] | 3.9 |
| 10 | —[c] | — | —[c] | — | | |
| 11 | Leu | 2.7 | Leu[d] | 19.5 | | |
| 12 | Phe[d] | 1.5 | | | | |

[a] Pig lung GST pi (0.4 mg/mL) was incubated with 200 $\mu$M [$^3$H]MOI at pH 7.7, 25 °C. The modified enzyme was isolated, treated with NEM, and digested with chymotrypsin. Peptides were separated by HPLC, and then the modified peptide peak was redigested with V8 protease and separated by HPLC; the labeled peak was sequenced. The sequences are representative of the labeled peptide II of Figure 6 derived from three experiments. [b] The tryptophan level is significantly lower than expected. [c] Cysteine is expected from the known sequence. [d] End of peptide. [e] Low amounts, peptide not ended.

in the first cycle of sequencing (in this case Glu36), the samples in Table 2 have an average of 3.8 ± 2.3%. The average of all of the experimental sequencing data for the labeled peptide isolated from GST pi is 0.59 ± 0.32% of cycle 1, which is significantly lower than from commercial peptides (Asp-Leu-Trp-Gln-Lys and Tyr-Pro-Trp-Gly) with tryptophan in the third cycle (23.7 ± 0.78% of cycle 1). The amount of radioactivity applied to the sequencer correlates with the amount of PTH-Trp38 containing peptide detected from the sequencer. For example, sample 2 (Table 2) had 396 pmol applied to the sequencer (calculated from the specific activity of [$^3$H]MOI), similar to the amount of peptide identified. This sequence also exhibits the low level of tryptophan in cycle 3 and yielded some radioactivity in cycle 3 from the sequencer, correlating with the amino acid Trp38. Thus, Trp38 was identified as the amino acid labeled. Trp38 has previously been implicated in catalytic function (*47*).

The cysteine residues were not detected by sequencing; however, they are not likely to be the sites of reaction as the identification of cross-linked peptides was made by mass spectrometry. The controls for the experiment (no MOI added to the reaction mixture but manipulated the same way as in the MOI modification experiments) also contained the cross-linked peptides, indicating that MOI does not initiate the cross-link. Similar results were found with the compound *S*-(4-bromo-2,3-dioxobutyl)glutathione that labeled a Tyr of rGST 4-4 and had two cysteines being cross-linked (*48*). It is not possible from these results to determine whether the cross-link is intra- or intersubunit.

Chymotrypsin catalyzes the hydrolysis of peptide bonds following aromatic amino acids and, under the experimental conditions, should have catalyzed the hydrolysis of the peptide bond following Trp38. The absence of cleavage at this bond suggests that MOI is attached to Trp38 and is too large for the active site of chymotrypsin, thus preventing cleavage. The second digest with V8 protease would not remove the C-terminal Glu36 of the chymotryptic peptide (ETWPPLKPSCL) as V8 protease acts as an endopeptidase only.

Table 3: Comparison of GST Isoenzyme Activity and Rate of Inactivation with MOI[a]

| isoenzyme | % residual activity at limit | $k_{obs}$ (min$^{-1}$) × 10$^2$ |
|---|---|---|
| pi (7-7) | 14.0 | 7.1 ± 0.2 |
| alpha (1-1) | 32.4 | 2.2 ± 0.5 |
| alpha (2-2) | 20.0 | 1.0 ± 0.3 |
| mu (3-3) | 44.3 | 1.3 ± 0.3 |
| mu (4-4) | 29.6 | 2.7 ± 0.6 |

[a] GST isoenzymes (0.4 mg/mL) were incubated with 200 $\mu$M MOI, 25 °C and pH 7.7, until maximum inactivation. Rate constants were calculated by the method illustrated in Figure 2, inset, where $E_\infty$ for each isoenzyme is the residual activity at the limit.

Alternate cleavage sites of the two endopeptidases resulted in the peptide VVTMETWPPLKPSCL being identified, and it was one of these samples on which MALDI-TOF-MS was performed. MALDI-TOF-MS of the isolated modified peptide yields a major peak at *m*/*z* 2071 which correlates with the predominant peptide identified by sequencing (amino acids 32−46, 1701 Da) with the addition of the matrix compound α-cyano-4-hydroxycinnamic acid (190.17 Da), MOI (145 Da), oxidation of Met35 to the corresponding sulfoxide (16 Da), and one water (18 Da). The water is most likely due to hydrolysis of the imide ring of MOI under acidic conditions (with the addition of water). The modified peptide lacking α-cyano-4-hydroxycinnamic acid was also detected at *m*/*z* 1865.3. No peaks at high molecular weight were identified.

*Comparison of the Effect of MOI on the Activity of Five GST Isoenzymes.* Four other isoenzymes of GST were tested for inactivation by MOI: alpha class (1-1 and 2-2) and mu class (3-3 and 4-4) from rat liver as representative of mammalian GSTs.[3] MOI reacted with all of the isoenzymes; however, there were striking differences in the limiting residual activity and the rate of inactivation (Table 3). The residual activity was decreased to a greater extent in pi (14% residual activity) than in the other classes (20−44% residual activities). Furthermore, the inactivation rate of GST pi was 2−7 times higher than for the other isoenzymes. For example, when the isoenzymes were all incubated with 200 $\mu$M MOI, at 30 min, the GST 2-2 isoenzyme exhibited 94% residual activity, the 3-3 isoenzyme 91%, the 4-4 isoenzyme 60%, and the 1-1 isoenzyme 57%, while the pi enzyme had only 18% residual activity. These results indicate that MOI is much more effective in inactivating the pi enzyme than any of the other GST isoenzymes.

[3] Alignment of the rat and pig pi class GSTs shows an 84% identity plus 15% similarity in amino acid sequence, thereby justifying the comparison of these rat and pig GST isoenzymes. Comparison of all mammalian GST amino acid sequences within an isoenzyme category gives the following: for alpha class, type 1, 63% identity plus 18% similarity; for alpha class, type 2, 58% identity plus 20% similarity; for mu class, type 3, 76% identity plus 17% similarity; and for mu class, type 4, 76% identity plus 15% similarity. The close resemblance among all of the amino acid sequences of the glutathione *S*-transferases within a given isoenzyme category justifies the use of the rat GSTs as representative of the mammalian GSTs.

## DISCUSSION

Methyleneoxindole (MOI) is found in nature as the photooxidation product of the plant auxin indole-3-acetic acid. We have now demonstrated that this compound inactivates pig lung GST pi to 14% residual activity. Protection studies show that MOI is reacting at the interface of the glutathione and xenobiotic active sites, with Trp38 identified as the residue labeled. One mole of MOI is incorporated per mole of dimer concomitant with loss of activity.

The phenomenon of a compound binding only 1 mol per dimer with complete inactivation has previously been found for rGST 1-1 with *S*-[[(2,2,5,5-tetramethyl-1-oxy-3-pyrrolidinyl)carbamoyl]methyl]glutathione (*49*) and glutathionyl *S*-[4-(succinimidyl)benzophenone] (*50*) and for mGST A3-3 with 8,9-dihydro-8-(*S*-glutathionyl)-9-hydroxylaflotoxin B1 (*51*). These observations suggest there is cross-talk between the subunits so that when MOI is bound in one active site, it has a deleterious effect on the ability of the other site to bind substrate and catalyze conjugation. The MOI-modified enzyme's secondary structure (monitored by circular dichroism) does not show significant difference from the control, as was found for rGST 1-1 modified with glutathionyl *S*-[4-(succinimidyl)benzophenone] (*50*); therefore, the conformational change is very subtle. The two Trp38 residues are at least 35 Å away from each other, and therefore, MOI (with dimensions of approximately 6 × 5 Å) reacted with Trp38 on one subunit could not *sterically* block reaction of MOI with Trp38 on the second subunit. An induced conformational change in the second binding site, upon occupancy of the first site, could result in the dimer binding only 1 mol of ligand (*52*−*54*). The absence of any catalytic cooperativity for GST pi is well established; however, it is possible that, upon binding MOI at one site, the enzyme's conformation changes and thereby decreases accessibility for MOI binding and reaction. The regions surrounding the two tryptophan residues are flexible (*43*), and the enzyme may therefore bind MOI differently depending on conformation.

The inactivation of GST pi with MOI is time dependent and has a level of maximal inactivation similar to that observed using the substrates CDNB and ethacrynic acid. The $K_m$ values of the maximally inactivated enzyme for CDNB, ethacrynic acid, and glutathione showed no significant difference, indicating that binding to residual unreacted subunits was not affected. However, the 14% residual activity shows that the unreacted subunits have decreased $V_{max}$. Thus we propose that reaction of MOI at one subunit completely inactivates that subunit and markedly decreases $V_{max}$ of the second subunit for the catalytic reaction with normal substrates such as CDNB and ethacrynic acid.

The peptide labeled by MOI was identified by chymotrypsin and V8 protease digest and subsequent amino acid sequencing and MALDI-TOF-MS analysis. The specific amino acid labeled is Trp38. The PTH-Trp was missing from the Edman degradation sequence data, indicating that the amino acid at that position is not the normal tryptophan. The pig lung GST pi contains tryptophan residues at positions 28 and 38. Trp28 is located at the N-terminus of strand $\beta$2, while Trp38 is at the N-terminus of the helix $\alpha$2. The nonessential role of Trp28 in the binding of substrates has been confirmed by site-directed mutagenesis (*47*). In the human GST P1-1, Trp38 forms a hydrogen bond with the carboxylate of the glycine part of glutathione when complexed with glutathione sulfonate (*55*). Pig lung and human GST P1-1 amino acid sequences have 82% identity plus 9% similarity. An overlay of the backbone (*38*) shows that the folding topology of the subunits and the overall structure are very similar. In human and pig GST pi, the two conserved residues, Trp38 and Lys42, are involved in binding glutathione and have almost identical spatial positions in the crystal structure, despite the two-residue insertion in the pig GST pi sequence (*39*); therefore, it can be assumed that MOI would bind to the human enzyme in a manner similar to that of the pig GST pi.

Nishihira et al. (*47*) used *N*-bromosuccinimide to chemically modify the two tryptophan residues of hGST P1-1. They found that Trp38 was important in the catalytic function and substrate binding of the enzyme. MOI is more specific than *N*-bromosuccinimide in that it only binds to Trp38 within the active site of GST pi. Failure of the modified enzyme to bind to an *S*-hexylglutathione affinity column is consistent with the lack of binding of Trp38 mutants (*47*). Furthermore, the inactivation of GST pi with 4-(fluorosulfonyl)benzoic acid (an affinity label of the glutathione active site) prevents binding to the affinity column when the enzyme is inactivated to 0.64 mol of reagent/mol of subunit (*32*). This was proposed to reflect an interaction between the two subunits, which is consistent with the results presented here, suggesting that two functional active sites are required for binding to the *S*-hexylglutathione affinity column.

The addition of several different ligands to the reaction mixture resulted in the conclusion that MOI is reacting within the active site and that it is near the glutathione and xenobiotic binding sites (not the steroid or buffer binding sites). The crystal structures of hGST P1-1 complexed with ethacrynic acid and TER-117 [$\gamma$-glutamyl-(*S*-benzyl)cysteinyl-D-phenylglycine] were compared. TER-117 is known to selectively and significantly inhibit hGST P1-1 compared to the other isoenzymes (*56*, *57*). The crystal structure of hGST P1-1 in complex with TER-117 (*39*; PDB identifier 10GS) shows that the indole ring of Trp38 is 3.04 Å from the benzoyl moiety (Figure 7A). Similarly, the crystal structure of hGST P1-1 in complex with ethacrynic acid (*58*; PDB identifier 2GSS) shows that the indole ring of Trp38 is less than 4 Å from ethacrynic acid (Figure 7B). Trp38 has close contacts with both TER-117 and ethacrynic acid; however, in complex with glutathione or glutathione conjugates, the side chain of glutathione binds closest (*58*; PDB identifier 3GSS). This explains why the glutathione derivatives protected from inactivation caused by MOI and why the ethacrynic acid derivatives also prevented reaction. Since Trp38 was identified as the target of MOI labeling, MOI is hypothesized to bind close to the binding site of TER-117 and of ethacrynic acid. Figure 7A shows the binding of TER-117 in the active site of hGST P1-1 (*39*), Figure 7B shows the binding of ethacrynic acid in the active site of hGST P1-1 (*58*), and Figure 7C shows MOI docked in the same site near Trp38, with the indole ring of Trp38 less than 4 Å from MOI.

Once MOI binds within the active site of GST pi, the closest residue is likely to be Trp38 on the basis of the comparisons of the crystal structures of the two inhibitors mentioned above. The reaction of MOI may occur via a

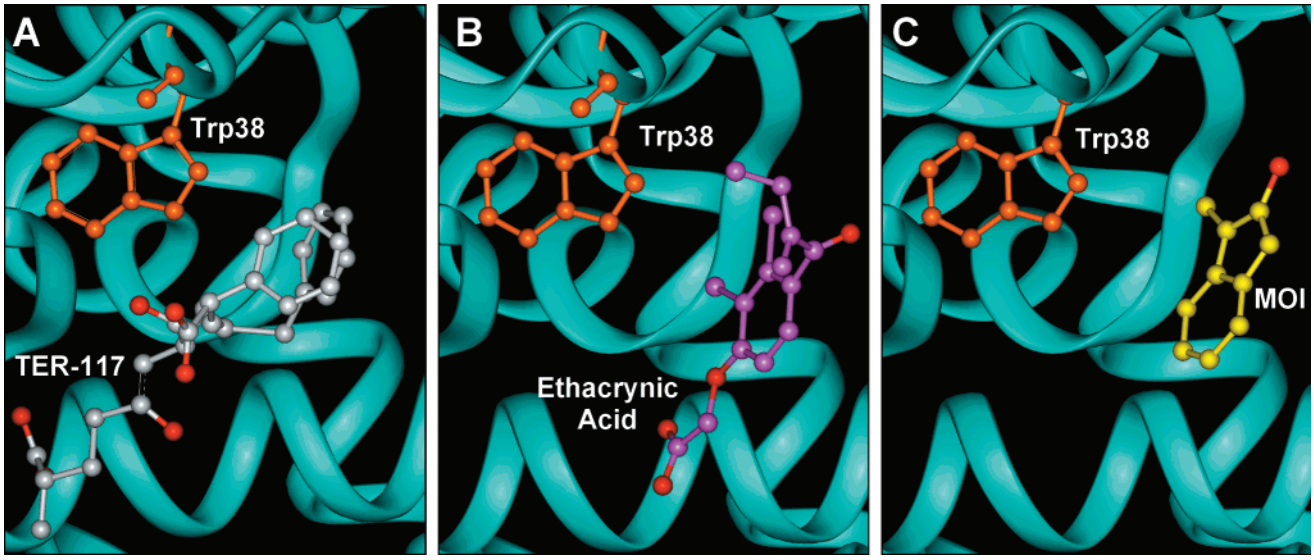


FIGURE 7: Part of the hGST P1−1 crystal structure showing Trp38. (A) Ribbon representation of the chain fold of the hGST P1-1 dimer complexed with TER-117 (*39*; PDB identifier 10GS). (B) Ribbon representation of hGST P1-1 complexed with ethacrynic acid (*57*; PDB identifier 2GSS). (C) Proposed binding of MOI in the hGST P1-1 dimer with the indole ring of Trp38 within binding distance of MOI. The benzene ring of MOI was overlaid on the benzene ring of ethacrynic acid. Oxygen is colored red to highlight the similarity of MOI to part of ethacrynic acid. The structures are colored as follows: cyan, hGST P1-1 dimer; orange, Trp38; white, TER-117; red, oxygen; pink, ethacrynic acid; yellow, MOI.

nucleophilic attack of the C2 of the indole ring of Trp38 on the exocyclic double bond of MOI in a manner analogous to the dimerization of tryptophan (*59*) or via a nucleophilic attack of the N1 of the indole ring of Trp38 on the $H_2C$═ moiety of MOI in analogy to the dimerization of MOI (*28*).

The inactivation rate and levels caused by MOI on each GST enzyme class tested were dissimilar, with the pi class inactivated more extensively and at the highest rate. The residue labeled, Trp38, is well conserved in GST class pi, and it forms part of the glutathione binding site along with Tyr7, Phe8, Arg13, Lys44, Gln51, Leu52, Gln64, Ser65, and Asp98 from the second subunit (*38*). A comparison of the sequences of the rat isoenzymes tested (alpha and mu classes) showed the absence of tryptophan in a position similar to that of Trp38 of the pig lung pi class enzyme. It was observed by Oakley and co-workers (*39*) that other GSTs do not have an equivalent Trp38 and Phe8 and proposed this as a reason for the lower inhibition of the alpha and mu classes by TER-117.

MOI is also a known inhibitor of glyoxylase I (*60*) (an enzyme which conjugates glutathione with methylglyoxal). The MOI inhibition of GST pi and glyoxylase may act in synergy in the cell and improve the effectiveness of chemotherapy, as was also proposed for the compound TER-117 (*61*). The significantly greater reactivity of MOI toward GST pi may aid in the development of combinational drugs to help in tumor treatment without the complete inhibition of the other GST classes, which are required for normal detoxification functions.

In summary, MOI acts as an affinity label for the pi class GST, causing an 86% reduction in the rate of catalysis, with 0.4 mol of MOI incorporated into the dimeric enzyme. MOI reacts with Trp38, a well conserved residue in the glutathione binding site of pi class GSTs. Although other GST isoenzymes are partially inactivated by MOI, neither the extent of inactivation nor the rate of inactivation is as striking as the effects produced by MOI on GST pi. This study indicates that GST pi is sufficiently different from other isoenzymes that it can be differentially inhibited.

## ACKNOWLEDGMENT

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