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Award Number: DAMD17-99-1-9275

TITLE: Inhibition of Tumor Cells that Over-Express nGST

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REPORT DATE: July 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

20011203 064

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	**2. REPORT DATE** July 2001	**3. REPORT TYPE AND DATES COVERED** Annual (1 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE
Inhibition of Tumor Cells that Over-Express nGST

5. FUNDING NUMBERS
DAMD17-99-1-9275

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

In the second year of this three year project, we have discovered an additional class of molecules that will serve as substrates for human glutathione transferase, producing as products cytotoxic alkylating agents of nucleic acids and/or proteins critical to cell function. This provides additional evidence that it is possible to make antitumor prodrugs that will selectively kill multidrug resistant breast cancer cells by using glutathione transferase as a *drug factory* inside tumor cells. Specifically we have demonstrated that (a) the glutathione conjugate of 2-crotonyloxymethyl-2-cyclohexenone (COMC) is unlikely to fully account for *in vitro* antitumor activity of COMC, (b) that COMC, in the presence of glutathione, undergoes glutathione transferase (GSTP1-1)-catalyzed conversion to a reactive exocyclic enone spe;cies, and (c) this species covalently modifies model nucleic acids, which is the likely basis for the tumor toxicity of COMC. We are now in the process of testing the *in vitro* tumoricidal activities of COMC and related compounds, in collaboration with Dr. Julie Eiseman, University of Pittsburgh Cancer Center. *Key words: glyoxalase I, glutathione transferase, N-hydroxycarbamoyl esters of glutathione, 2-crotonyloxymethyl-2-cyclohexenone, DNA alkylating agents.*

14. SUBJECT TERMS Breast cancer			**15. NUMBER OF PAGES** 61
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	**18. SECURITY CLASSIFICATION OF THIS PAGE** Unclassified	**19. SECURITY CLASSIFICATION OF ABSTRACT** Unclassified	**20. LIMITATION OF ABSTRACT** Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Donald J. Creighton 7/19/01

PI - Signature Date

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Introduction

The objective of the proposed research program is to develop a novel new class of anti-tumor agents that will, in effect, turn the enzymes that normally confer resistance to anticancer drugs into "drug factories" inside breast cancer cells. The proposed anti-tumor agents do not specifically target rapidly dividing cells and, therefore, might not exhibit the side effects so commonly associated with cancer chemotherapy. The proposed agents are a new class of cyclic N-hydroxycarbamates that will capitalize on differences in the activities of glutathione(GSH)-dependent enzymes between normal cells and cancer cells in order to generate high levels of selective tumoricidal activity. Specifically, the carbamates are designed to function as cell-permeable substrates for the π-isoform of glutathione transferase (πGST). The resulting N-hydroxycarbamoyl esters of GSH are predicted to serve both as powerful mechanism-based competitive inhibitors of the detoxifying enzyme glyoxalase I (GlxI), and as slow substrates for the thioester hydrolase glyoxalase II (GlxII). The hypothesis that the πGST substrates will selectively inhibit the growth of breast tumor cells versus normal cells is founded on three observations. First, GlxI plays a critical detoxification role in cells by converting the metabolite methylglyoxal to nontoxic *D*-lactate. Therefore, inhibitors of GlxI should inhibit cells by causing the build-up of intracellular methylglyoxal. Second, πGST is often expressed at *higher* levels in human breast tumors than in peritumoral tissues. This is particularly true of cells exhibiting the multi-drug resistance phenotype. Thus, the GlxI inhibitors should be generated more rapidly in tumor cells exposed to the πGST substrates. Third, GlxII is often expressed at *lower* levels in cancer cells than in fully differentiated normal cells. Therefore, the GlxI inhibitors should be less susceptible to catalyzed hydrolysis inside tumor cells. The latter two observations suggest that the steady-state concentrations of the GlxI inhibitors in breast tumor cells will exceed that in normal cells, resulting in tumor-selective toxicity. In order to test this hypothesis, we will pursue the following specific aims: (1.) To synthesize and test cyclic compounds of the following general type as substrates for πGST: YCON(OH)R, where Y equals different leaving groups and R equals different alkyl or aryl substituents. (2.) To determine the competitive inhibition constants of the resultant GSH-conjugates (GSCON(OH)R) with GlxI and the kinetic properties of the conjugates as substrates for GlxII. (3.) To determine the rates at which YCON(OH)R diffuse into MCF7 human breast cancer cells and Adr^R MCF7 cells (over-expressing πGST), and form GlxI inhibitors of the type GSCON(OH)R. (4.) To measure the efflux rates of GSCON(OH)R from MCF7 and Adr^R MCF7 cells. (5.) To evaluate the IC_{50}s of YCON(OH)R with MCF7 and Adr^R MCF7 cells for comparison with the intracellular concentrations of GSCON(OH)R with these cell lines.

Body

Overview: In the review of our first year progress report, the reviewer commented that the review process would be easier, if we clearly stated the tasks accomplished and placed less emphasis on the technical details of the project. We have responded to these comments by including an "*overview*" section briefly describing the work accomplished and a "*detailed account*" section for those interested in the technical aspects of our work.

We have now accomplished tasks 1 and 2 related to the synthesis and testing of compounds that serve as substrates for glutathione transferase, producing as products tight-binding inhibitors of the methylglyoxal detoxifying enzyme glyoxalase I:

Task 1. To identify substrates for glutathione transferase (GST) that give as products inhibitors of glyoxalase I.

Task 2. To determine the competitive inhibition constants of the resulting products of the GST reaction as inhibitors of glyoxalase I and as substrates for glyoxalase II.

Published papers describing this work can be found in Appendix I. An additional paper is in preparation. This work provides the experimental and conceptual foundation for a fresh approach to inhibiting breast tumors that exhibit the multi-drug resistance phenotype.

The work associated with tasks 3 and 4 are now in progress:

Task 3. To measure the influx and efflux rates of the GST substrates with breast cancer cells.

Task 4. To evaluate the IC_{50} values of the GST substrates with breast cancer cells.

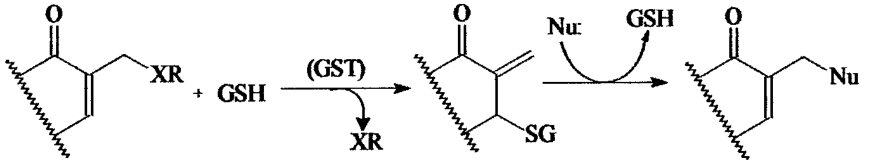
This work is being done in collaboration with Dr. Julie Eiseman at the University of Pittsburgh Cancer Center, as originally planned.

At the end of the first year's work we also asked for approval to pursue a fifth task that arose from a collaboration between this laboratory and Professor Bruce Ganem's laboratory at Cornell University. This task is related to accomplished tasks 1 and 2 and expands the number of compound we are testing for tumoricidal activity against breast cancer:

Task 5. To evaluate the antitumor agent 2-crotonyloxymethyl-2-cyclohexenone (COMC) as a substrate for GST, producing as a product a glutathione adduct that is an inhibitor of glyoxalase I.

A published paper and a submitted paper describing this work may be found in Appendix II. In the PI's judgment, this is some of the most important work to come out of the second year of this research program, because these studies *overturn* the accepted view that the antitumor activity of COMC is exclusively due to inhibition of glyoxalase I. Rather, the antitumor activity of COMC, and compounds like it, most likely arise primarily from the formation of a highly reactive exocyclic enone that is formed during the GST-catalyzed conversion of COMC to its corresponding glutathione adduct. We believe that cytotoxicity arises from the covalent modification of cellular nucleic acids and/or proteins by the exocyclic enone. So What? This could mean that breast tumor cells that over-express GST, as part of the multi drug resistance phenotype, will be particularly sensitive to COMC in comparison to peritumoral tissue that expresses low levels of GST. In addition to the compounds described in the original proposal, we will also test the selective tumoricidal activity of COMC *in vitro* in collaboration with Dr. Eiseman.

Detailed account. In the second year of this three year project we have tested the hypothesis that the known tumoricidal activities of COMC is due, in part, to its glutathione transferase (GST)-catalyzed conversion to a highly reactive exocyclic enone, which kill cells by reacting with intracellular proteins and/or nucleic acids, **Eqn. 1**.



(1.

where GSH = glutathione (γ-Glu-Cys-Gly); Nu: = nucleophiles (DNA, proteins)

If correct, compounds of this type might be highly selective antitumor agents, on the basis of differences in the expression levels of pi-GST in normal cells versus some types of breast cancer cells.

The above hypothesis emerged from our recent discovery that COMC (**1a**) is an excellent substrates for pi-GST and forms covalent adducts with model dinucleotides according to Eqn 1.

(1) Glutathione Adducts of COTC (2-crotonyloxymethyl-(3,4,5- trihydroxy)-2-cyclohexenone) and COMC (1a) Are Weak Inhibitors of Glyoxalase I.

Inhibition of glyoxalase I by glutathione adducts of COTC and COMC are unlikely to account for the cytotoxicity of COTC and COMC [Hamilton *et al.* (2000), Appendix II]. While we have demonstrated that transition state analog inhibitors of glyoxalase I retard the growth of both murine and human tumors in culture,^{1} the K_i values with human glyoxalase I are in the submicromolar concentration range. In addition, the IC_{50} values are approximately proportional to the K_i values, such that a relatively weak enzyme inhibitor with a $K_i = 0.16$ μM gives an IC_{50} value >100 μM. On this basis, COMC should exhibit very poor potency, because the corresponding GSH conjugate is a weak inhibitor of the enzyme ($K_i = 106$ μM). This is contrary to reported IC_{50} values in the range 0.5-19 μM for established murine and human tumor cell lines.^{2} We have confirmed in our laboratories that COMC is indeed a very potent inhibitor of B16 melanotic melanoma in culture ($IC_{50} = 0.04$ μM), Fig. 3.

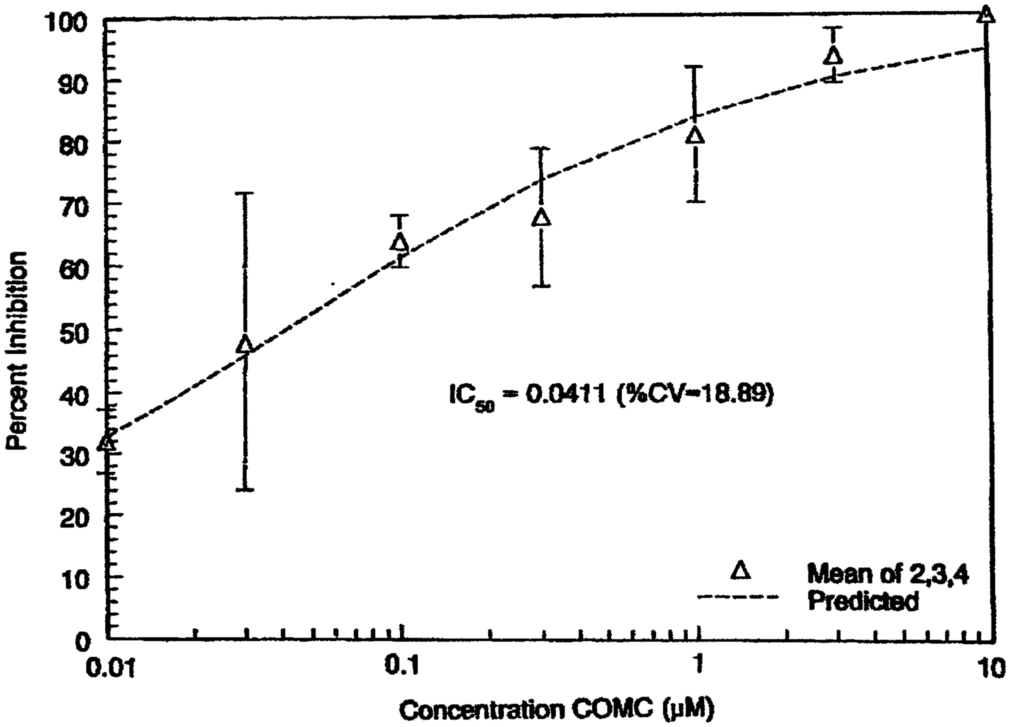
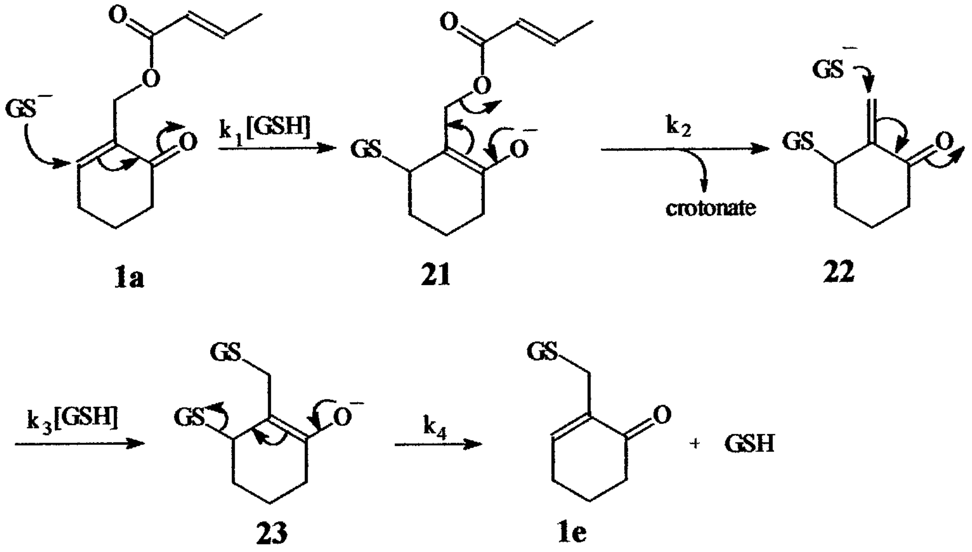


Fig. 3. Dose-response curve for B16 melanotic melanoma cells in culture with **1a** (COMC).

Moreover, when the GSH conjugate of COMC is indirectly delivered into B16 cells as the [glycyl, glutamyl] diethyl ester (a prodrug strategy developed by this laboratory [1]) there is little inhibition of cell growth (IC$_{50}$> 400 µM). *This suggests that cytotoxicity does not arise simply from the presence of the conjugate inside the cells, but must be due either to unconjugated COMC or to a reactive intermediate formed during conjugate addition of GSH to COMC.*

(2) The Reaction of COMC (**1a**) with GSH Involves the Formation of a Reactive Intermediate, on the Basis of Kinetic Measurements and Chemical Trapping Experiments.

A variety of mechanisms have been proposed to account for the reaction of COMC with GSH. On paper, the simplest mechanism is a direct, S_N2 displacement of the crotonate ester by the nucleophilic thiol of GSH. However, carboxylic esters are only moderate leaving groups, and the α,β-unsaturated enone system would be expected to undergo much more rapid conjugate addition reactions with thiols. Thus, a stepwise 1,4-addition/β-elimination mechanism can be envisioned in which GSH first adds to the endocyclic enone function of COMC **1a** via enolate **21** to form an intermediate exocyclic enone **22.** The exocyclic enone then subsequently reacts with another molecule of GSH to give the endocyclic enone **1e** (Scheme 3). Exocyclic enones like **22** are particularly reactive Michael acceptors, and could function as carcinostatic agents by reacting with nucleic acids and proteins critical to cell function.



Scheme 3

Kinetic studies. Experimentally, the reaction of excess GSH with **1a** to give **1e** follows a simple first-order decay with no evidence of any intermediate (Fig. 4, trace A):

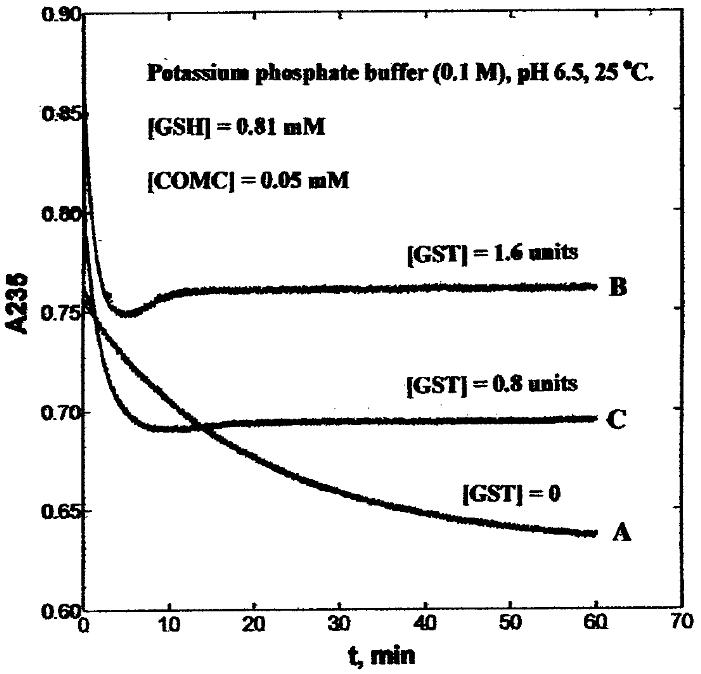


Fig. 4. *Time course for the loss of* ***1a*** *(0.05 mM) in the presence of GSH (0.81 mM) (A) in the absence of GST ($k = 0.0514 \pm 0.0002$ min^{-1}) and (**B**) in the presence of 1.6 units of GST ($k_1 = 0.641 \pm 0.019$ min^{-1}; $k_2 = 0.315 \pm 0.015$ min^{-1}) and (C) 0.8 units of GST ($k_1 = 0.451 \pm 0.0063$ min^{-1}; $k_2 = 0.135 \pm 0.010$ min^{-1}).* Different end-point absorbances are due to different background absorbances of enzyme protein. Conditions: phosphate buffer (0.05 M), pH 6.5, 25 °C

Nevertheless, this observation would still be consistent with the mechanism shown in Scheme 3, provided that the formation of the exocyclic enone is rate determining.

During the course of these studies, we made the chance observation that in the presence of human placental glutathione transferase (pi-GST) the rate of conversion of **1a** to **1e** is composed of a rapid, enzyme-dependent, initial phase and a slower enzyme-independent first-order phase (Fig. 4, traces B and C). This can be explained in terms of the mechanism shown in Scheme 3, wherein pi-GST catalyzes the rapid formation of exocyclic enone **22**, which subsequently reacts with free GSH in solution to give **1e.** The shape of the kinetic trace indicates that the molar absorptivity of **22** is less than that of **1e**.

Model building of **1a** into the X-ray crystal structure of pi-GST tentatively suggests a mechanism for catalysis that is analogous to the one proposed for conjugate addition of GSH to ethacrynic acid, Fig. 5.

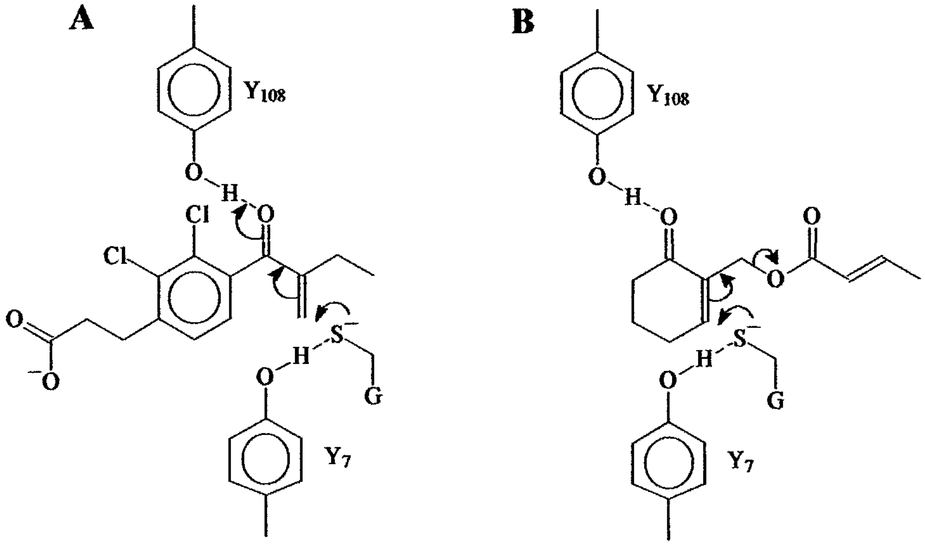
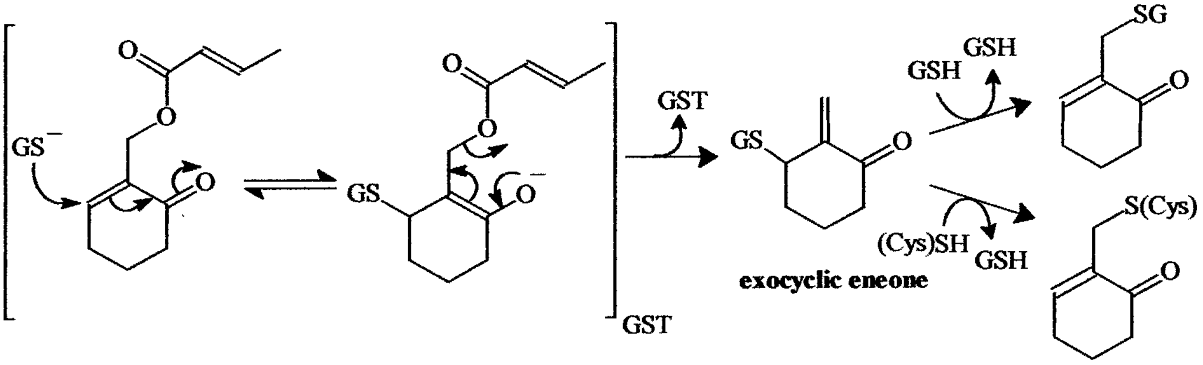


Fig. 5 . *Proposed reaction mechanisms for glutathione transferase-catalyzed formation of the adducts between (A) GSH and ethacrynic acid*[3] *and (B) GSH and COMC (**1a**).*

Intermediate trapping. In order to confirm that the exocyclic enone is the immediate product of catalysis, the transferase reaction was carried out in the presence of equimolar amounts of GSH and cysteine, and the ratio of the two thiol addition products **1e** and **24** determined by reverse-phase HPLC. Judging from the ratio of first-order rate constants for the enzymic and nonenzymic processes, the amount of enzyme-derived product formed in the enzyme-catalyzed pathway was estimated to be about 90 percent of the total product yield. Moreover, the interconversion of thiol adducts **1e** and **24** proved minimal: in the presence of cysteine (2.5 mM), glutathione adduct **1e** (0.5 mM) formed less than 1% of **24** over 27 h, as determined by HPLC. Thus, the product ratio observed in the enzymic reaction must reflect the true relative rates of reaction of **22** with GSH and cysteine. If intermediate **22** dissociates from the surface of the enzyme and reacts with the free thiols in solution, the product ratios for the enzymic and nonenzymic processes should be identical (Scheme 4).



However, if the transferase catalyzes all steps in the conversion of **1a** to **1e**, the ratio of **1e** to the cysteine adduct **24** should be much higher in the reaction mixture containing transferase. This follows from the fact that the enzyme is highly specific for GSH as a substrate.[4]

Experimentally, the product ratios for the two systems are identical, within experimental error, indicating that **22** is the product of catalysis (Fig. 6).

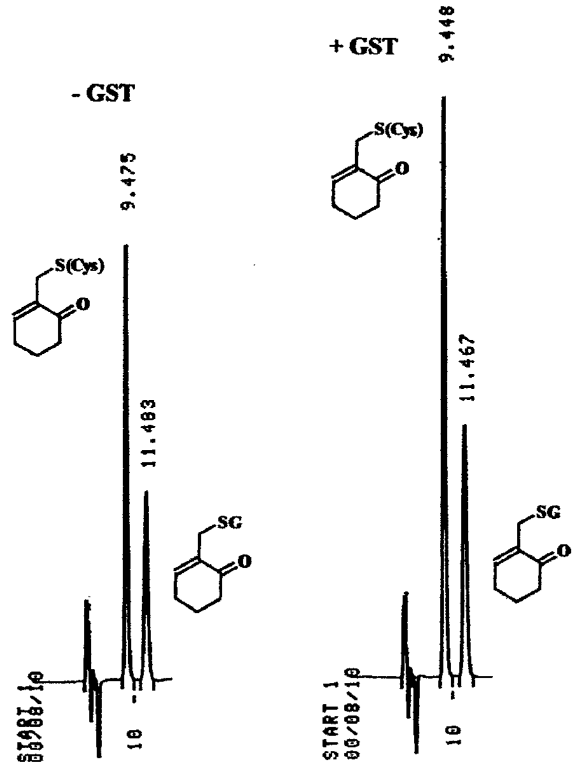


Fig. 6. *Relative amounts of* ***24*** *(~ 9.5 min) and* ***1e*** *(~ 11.5 min) observed by reverse phase HPLC after 30 min incubation of reaction mixtures initially composed of* ***1a*** *(0.1 mM), cysteine (0.5 mM), GSH (0.5 mM), 0.1 M potassium phosphate (pH 6.5) and EDTA (0.05 mM), 25 °C in the absence, or in the presence of pi-GST (1.6 units).* Running solvent: 25% methanol in water containing 0.25% acetic acid.

(3) Exocyclic Enone **22** Can Be Isolated and Identified by NMR

The chemical identity of the intermediate was confirmed by briefly incubating **1a** in the presence of GSH and GST, quenching the reaction mixture in dilute acetic acid, and resolving the components by reverse-phase HPLC, as shown in Fig. 7.

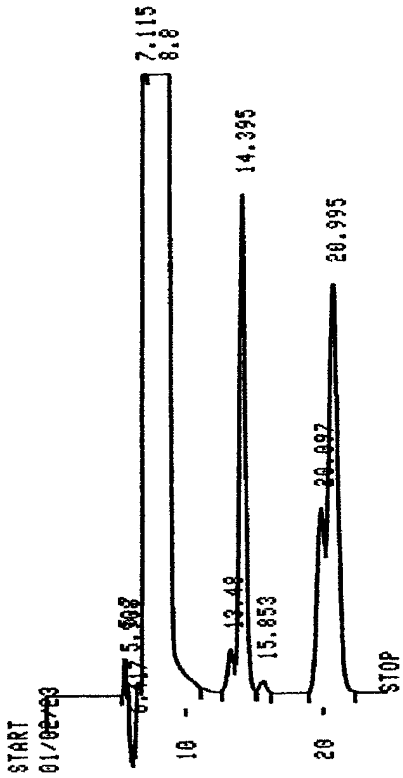


Fig. 7. *Elution profile from a reverse-phase HPLC column of a reaction mixture 40 seconds after combining GSH (0.5 mM), GST, and COMC* ***1a*** *(0.1 mM) in 0.1 M phosphate buffer pH 6.5.* The peak at 21 min corresponds to adduct **1e**, the peak at 20.1 min is tentatively identified as the exocyclic enone **22,** and the peak at 14.4 min. is crotonic acid. Running solvent: 5% methanol in water containing 0.5% acetic acid.

The compound corresponding to the 20.1 min peak is identified to be the exocyclic enone. When neutralized and combined with cysteine, this compound gives a new peak in the chromatogram that comigrates with authentic **24**. Moreover, the first order rate constant leading to **24** is similar in magnitude to that associated with the slow phase of the transferase-catalyzed conversion of **1a** to **1e**, Fig. 4. The 600 MHz ^{1}H NMR spectrum of the putative intermediate species is consistent with exocyclic enone **22**, Fig. 8.

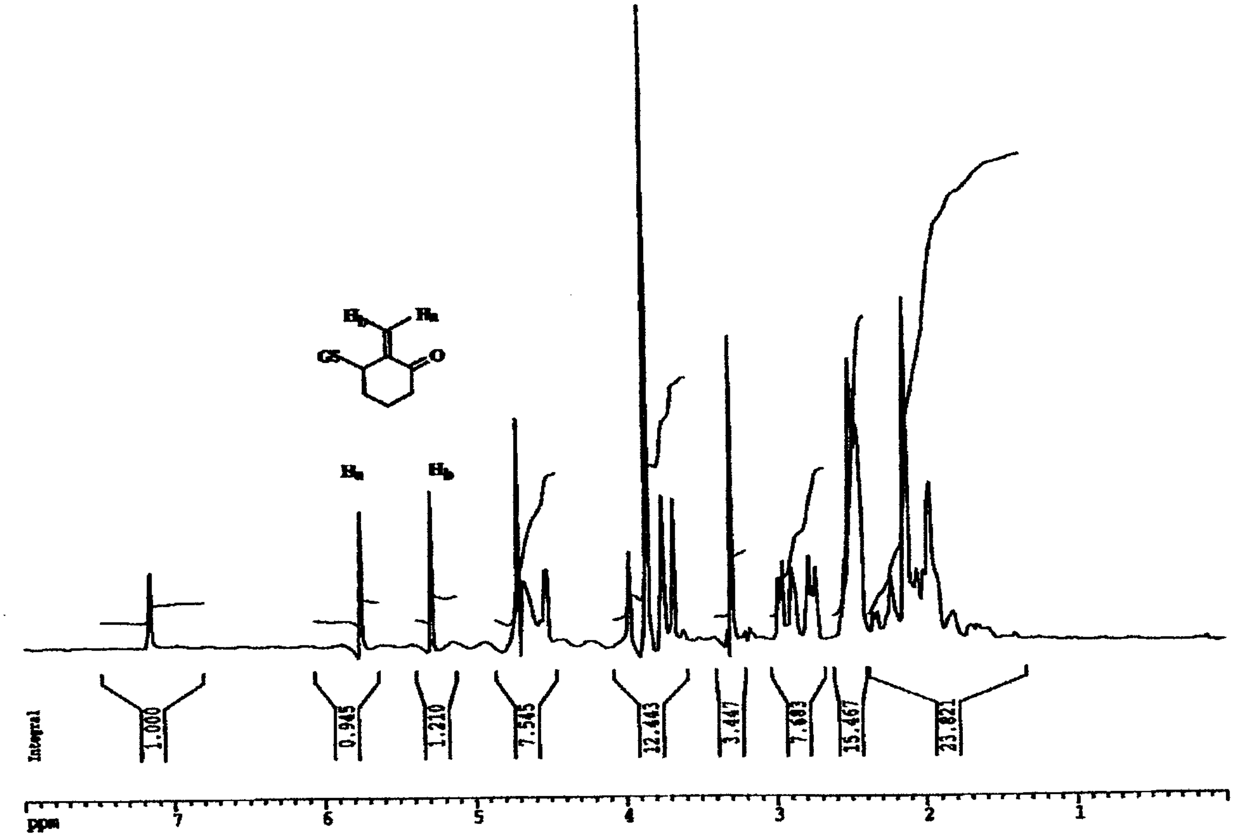


Fig. 8. *The 600 MHz ^{1}NMR spectrum of the putative exocyclic enone **22** corresponding to the 20.1 min peak in the HPLC elution profile of Figure 7.* The resonance at 7.16 ppm is due to the presence of adduct **1e**. The other resonances in the spectrum are those expected for S-substituted GSH derivatives[5].

The vinyl proton resonances at 5.76 and 5.29 ppm are characteristic of geminal vinylic hydrogens, and are consistent with published NMR spectra of several closely related exocyclic α-methylene cyclohexanones.[6]

(4) The Exocyclic Enone Intermediate **22** Reacts with Model Nucleotides.

In principle, the cytotoxicity of the substituted endocyclic enones could result from alkylation of DNA and/or proteins critical to cell function. Using COMC **1a** to test this hypothesis, the dinucleotides ApA, GpA and CpC were separately incubated with 0.5 mM GSH, 0.1 mM **1a** and 0.5 units pi-GST. After two hours, the incubation mixtures were analyzed by matrix assisted laser desorption mass spectometry (e.g., Fig. 9). In all cases, significant concentrations of nucleotide adducts as well as GSH adducts were detected.

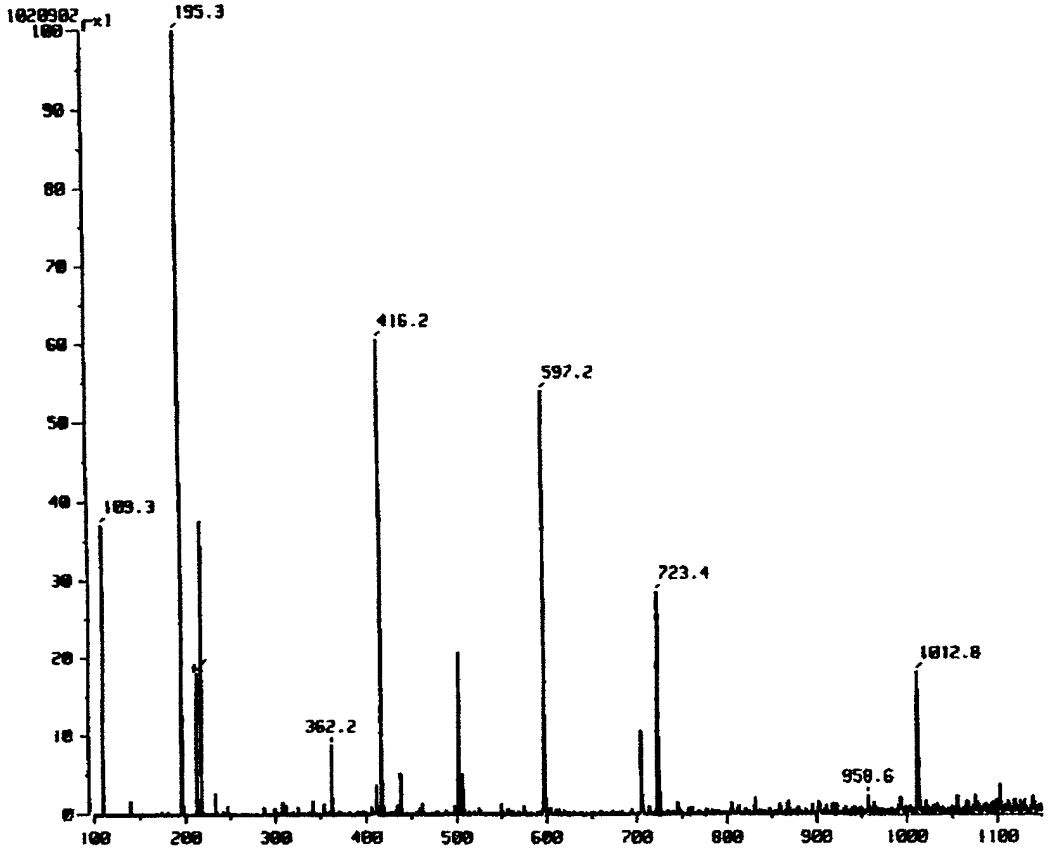
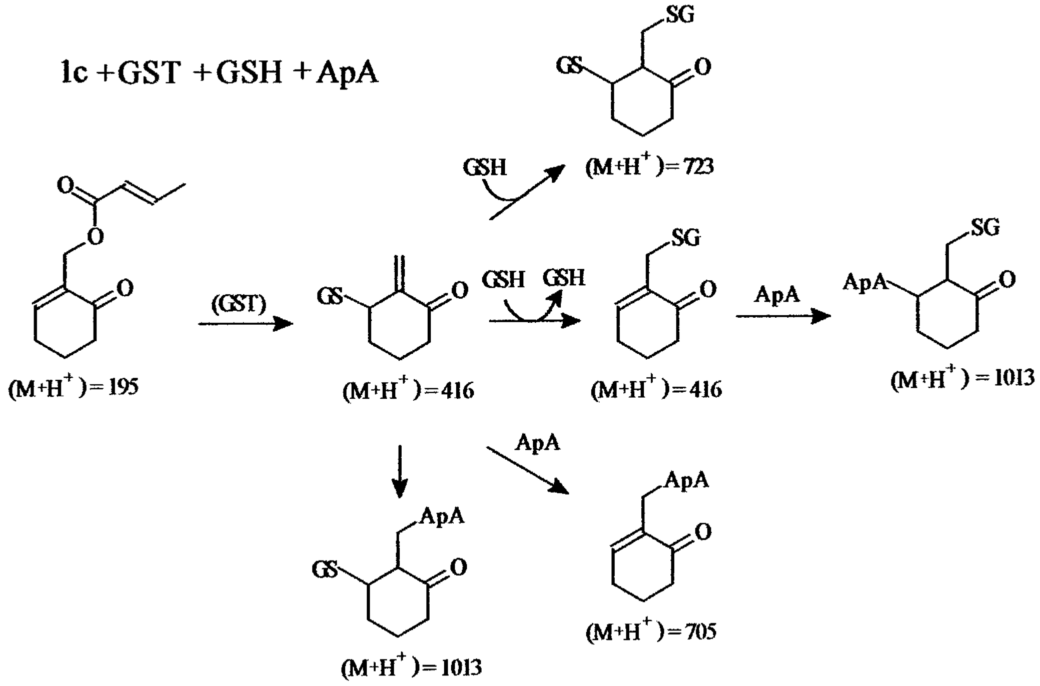


Fig 9. *MALDI spectrum of a two hour incubation mixture composed of ApA, GSH (0.5 mM), **1a** (0.1 mM) and GST (0.5 units), phospate buffer (50 mM, pH 7), 25°C.*

A reaction pathway consistent with the observed products is shown in Scheme 5.



Scheme 5. *Possible routes to the various species detected by MALDI spectroscopy.*

Taken together, these observations support the view that the cytotoxicity of COMC **1a** and its derivatives could result from covalently modification of intracellular nucleic acids and/or proteins critical for cell survival.

Key Research Accomplishments (during the second year)

- Demonstrated that inhibition of intracellular glyoxalase I by the glutathione conjugate of COMC is unlikely to fully account for *in vitro* antitumor activity of COMC.
- Demonstrated that COMC in the presence of glutathione undergoes glutathione transferase (GSTP1-1)-catalyzed conversion to a reactive exocyclic enone species.
- Demonstrated that this species covalently modifies model nucleic acids, which is the likely basis for the tumor toxicity of COMC.

Reportable Outcomes (years 1 and 2)

Manuscripts in print or in press

1. Diana S. Hamilton and Donald J. Creighton, "Using GSTP-1 to Generate Mechanism-based Competitive Inhibitors of the Anticancer Target Enzyme Glyoxalase I," Chemico-Biological Interactions, 133, 355-359, 2001.

2. Avinash Kalsi, Malcolm J. Kavarana, Tianfenu Lu, Dale L. Whalen, Diana S. Hamilton, and Donald J. Creighton, "Role of Hydrophobic Interactions in Binding S-(N-Aryl/Alkyl-N-hydroxycarbamoyl)glutathiones to the Active Site of the Antitumor Target Enzyme Glyoxalase I," J. Med. Chem., Vol. 43, No. 21, 3981-3986, 2000.

3. C. Frederick M. Huntley, Diana S. Hamilton, Donald J. Creighton, and Bruce Ganem, "Reaction of COTC with Glutathione: Structure of the Putative Glyoxalase I Inhibitor," Org. Lett., Vol. 2, No. 20, 2000.

4. Diana S. Hamilton, Zhebo Ding, Bruce Ganem, and Donald J. Creighton, "Studies on the Antitumor Activity of 2-Crotonyloxymethyl-2-cyclohexenone: Mechanism of the Glutathionly Transferase-Catalyzed Addition of Glutathione," J. Med. Chem., in press.

Presentation at scientific meetings that describes work accomplished:

1. D.J. Creighton, "Tumor-selective Anticancer Strategies: Using GSTP1-1 to Generate Enediol Analogue Inhibitors of Glyoxalase I," GST 2000: International Conference on Glutathione Transferases, Uppsala University, Uppsala Sweden, 19-23 May 2000.

2. Diana S. Hamilton, Zhebo Ding, Bruce Ganem, and Donald J. Creighton, "Possible Mechanistic Basis of the Antitumor Activity of 2-Crotonyloxymethyl-2-cyclohexenone,"
34th ACS Middle Atlantic Regional Meeting (MARM), Towson University, Towson, Maryland, May 30 - June 1, 2001.

3. Diana S. Hamilton, Dan Fabre, Zhebo Ding, Bruce Ganem, and Donald J. Creighton, "GSTP1-1 Catalyzes the Addition of Glutathione to the Antitumor Agent 2-Crotonyloxymethyl-2-cyclohexenone Via a Highly Reactive Exocyclic Enone Intermediate," Gordon Research Conferences: Enzymes , Coenzymes and Metabolic Pathways, West Kingston, Rhode Island, July 22 - July 27, 2001.

4. D.J. Creighton, "Biochemical Basis of the Antitumor Activity of Endocyclic Eneones," University of Pittsburgh Cancer Center, April 19, 2001.

Conclusions

There are two observations of central importance in this work. First, the GSH adduct formed with COMC is a weak competitive inhibitor of human glyoxalase I and, therefore, this phenomenon is unlikely to fully account for the reported tumoricidal activities COMC. Human glutathione transferase (GSTP1-1) catalyzes the conversion of COMC to the corresponding GSH adduct by a multi-step process involving the formation of a highly reactive exocyclic enone intermediate, which is the immediate product of enzyme catalysis. Conceivably, tumoricidal activity might arise from the reaction of this electrophilic species with proteins and nucleic acids critical to cell survival. Indeed, covalent adducts between COMC and model polynucleic acids, in the presence of human GSTP1-1, have been detected by mass spectrometry (Fabris, Creighton and Ganem, unpublished). Enzyme-catalyzed formation of the exocyclic enone probably involves general acid/base catalysis by Tyr(108) and Tyr(7), as these residues have been implicated to play a role in catalyzing nucleophilic addition of GSH to the eneone ethacrynic acid. This hypothesis is consistent with the observation that COMC and ethacrynic acid have similar kinetic properties with the transferase. Finally, the fact that GSTP1-1 catalyzes the formation of the cytotoxic exocyclic enone is of considerable interest from a cancer control perspective, as there are often large differences in GSTP1-1 activity between breast tumors and peritumoral tissue that might contribute to the selective tumoricidal activities of compounds like COMC.

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APPENDIX I. Published papers related to Tasks 1 and 2

1. Diana S. Hamilton and Donald J. Creighton, "Using GSTP-1 to Generate Mechanism-based Competitive Inhibitors of the Anticancer Target Enzyme Glyoxalase I," Chemico-Biological Interactions, 133, 355-359, 2001.

2. Avinash Kalsi, Malcolm J. Kavarana, Tianfen Lu, Dale L. Whalen, Diana S. Hamilton, and Donald J. Creighton, "Role of Hydrophobic Interactions in Binding S-(N-Aryl/Alkyl-N-hydroxycarbamoyl)glutathiones to the Active Site of the Antitumor Target Enzyme Glyoxalase I," J. Med. Chem., Vol. 43, No. 21, 3981-3986, 2000.

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Chemico-Biological Interactions 133 (2001) 355–359

Chemico-Biological Interactions

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Using GSTP1-1 to generate mechanism-based competitive inhibitors of the anticancer target enzyme glyoxalase I

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Abstract

Substrates for GSTP1-1 that produce as products inhibitors of the methylglyoxal detoxifying enzyme glyoxalase I might exhibit selective tumoricidal activity against multidrug resistant (MDR) tumors, as the transferase is often overexpressed at high levels in these tumors. In support of the feasibility of this strategy, we have discovered that selected *N*-hydroxy carbamoyl esters ($ClC_6H_4OC(O)N(OH)R$, where $R = ZC_6H_4(Z = H, Cl, Br)$) are slow substrates for human placental GSTP1-1, producing as products powerful, mechanism-based competitive inhibitors of human glyoxalase I ($GSC(O)N(OH)R$, where $GS = $ glutathionyl). In contrast, the transferase did not exhibit detectable activity with carbamoyl esters in which $R = $ alkyl.

Keywords: Carbamoyl esters; Glyoxalase I inhibitors; GST substrates; MDR tumors

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The glyoxalase enzyme system has been the focus of much recent attention as a potential target for antitumor drug development [1,2]. This enzyme system, composed of the isomerase glyoxalase I (GlxI) and the thioester hydrolase glyoxalase II, promotes the glutathione(GSH)-dependent conversion of cytotoxic methylglyoxal to D-lactate [3,4]. We recently demonstrated that both S-(*N*-aryl/alkyl *N*-hydroxycarbamoyl)glutathione derivatives (1) are powerful competitive inhibitors of human GlxI with K_i values in the nanomolar concentration range [5–7].

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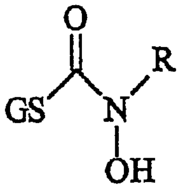
PII: S0009-2797(00)00216-7

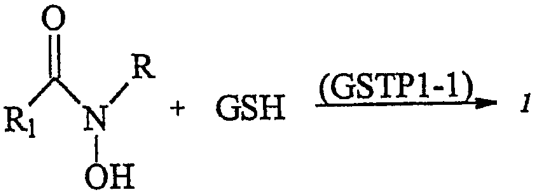


1 (R = C_6H_4, C_6H_4Cl, C_6H_4Br, $(CH_2)_nCH_3$ (where n = 0 - 6))

Tight binding appears to result from the fact that these compounds are stable intermediate analogues of the enediolate intermediate that forms along the reaction coordinate of the enzyme [8]. The diethyl ester prodrugs of the *N*-aryl derivatives inhibit the growth of both murine and human tumors in vitro [9] and in vivo [10]. Tumor toxicity appears to be due to inhibition of intracellular GlxI, which results in elevated concentrations of methylglyoxal [9]. Taken together, these results emphasize the potential importance of GlxI as a novel antitumor target.

Like the glyoxalase enzymes, the glutathione *S*-transferases (GSTs) play an important detoxification role in cells by catalyzing the addition of GSH to different electrophilic xenobiotics [11,12]. Among the many different members of the GST family of enzymes, the GSTP1-1 isozyme is of particular interest from a cancer control perspective. This isozyme is significantly elevated in solid human tumors of the colon, lung and particularly breast with only a few tumors showing abnormally low levels of this isozyme [13]. Tumors exhibiting the multidrug resistance (MDR) phenotype can have GSTP1-1 activities that are 15-fold higher than normal [14]. Several different laboratories are in the process of developing specific inhibitors of GSTP1-1 in order to overcome drug resistance [15].

In contrast, we aim to take advantage of the high levels of GSTP1-1 activity in MDR tumor cells by using the transferase to generate cytotoxic enediol analogues of the GlxI reaction in situ. In order to test this hypothesis, carbamoyl esters **2** were synthesized using methods previously described by this laboratory [5–7] and evaluated as substrates for human placental GSTP1-1, Eq. (1).



2 (R_1 = p-ClC_6H_4O, CH_3CH_2S)

The transferase was purchased from Sigma Chemical Co. and salts and free GSH were removed by ultrafiltration. The carbamoyl esters (**2**) were separately incubated in the presence and in the absence of 2 U of GSTP1-1 for 69 h under the condition given in the legend to Fig. 1. The solvent was removed in vacuo and the residue resolved on a reverse-phase C 18 column using a methanol/water mixture, containing 0.25% acetic acid, as a running solvent. The amount of GSH conjugate formed was determined from a comparison of the integrated intensity of the peak, corresponding to that of authentic GSH conjugate, with standard curves of peak area versus concentration. The activities of the different substrates with the transferase were computed from the amount of conjugate formed in the presence of enzyme minus that formed in the absence of enzyme.

The results of these preliminary studies suggest a tentative structure-activity correlation, namely, that carbamoyl esters in which both R and R_1, are aromatic functions are slow substrates for GSTP1-1, Fig. 1. This correlation is based on the observation that no significant activity was found with any of the *N*-alkyl carbamoyl esters having p-chlorophenol as a leaving group. Moreover, no activity was

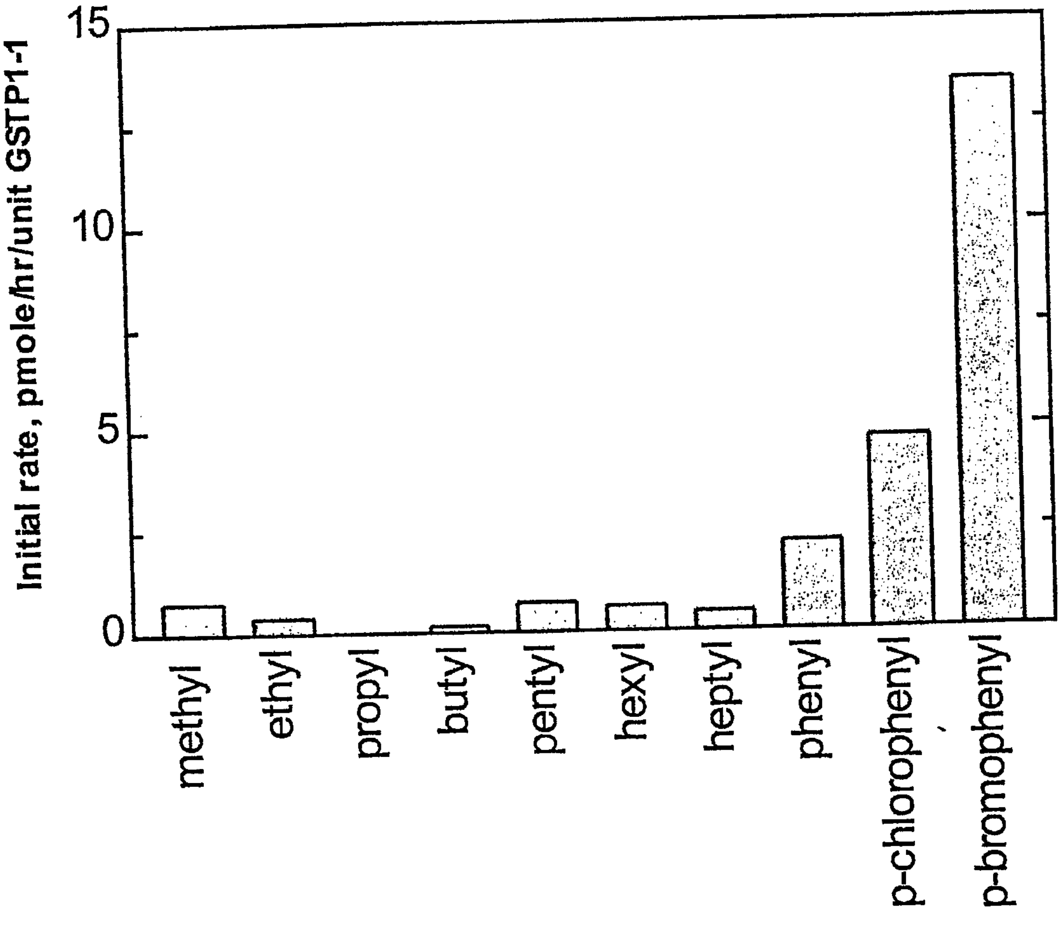


Fig. 1. Comparison of the activities of human GSTP1-1 with the carbamoyl esters shown in Eq. (1), where R_1 = p-chlorophenol. Conditions: Sodium Phosphate buffer, pH 7.5, containing 30% (v/v) ethanol and 0.05 mM ethylenediamine tetraacetate; 1 mM GSH, 0.4 mM carbamoyl ester, human placental GSTP1-1 (2.5 U using 2,4dinitrochlorobenzene as substrate), 22°C.

detected with the *N*-p-chlorophenyl derivative having ethanthiol as a leaving group. However, significant activity was found for the *N*-aryl derivatives in which p-chlorophenol is the leaving group. Apparently, the diaryl carbamoyl esters bind to the transferase in such a way as to allow significant transition state stabilization by the active site residues. We are currently attempting to improve the substrate properties of the carbamoyl esters by optimizing substrate structure using molecular docking methods with the known x-ray structure of human GSTP1-1.

This is the first demonstration that carbamoyl esters can be designed to serve as substrates for GSTP1-1, giving as products enediol analogue inhibitors of GlxI. This is the basis of a potentially novel tumor-selective anticancer strategy.

Acknowledgements

This work was supported by a grant from the US Army Medical Research and Material Command (Breast Cancer Research Program).

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Role of Hydrophobic Interactions in Binding *S*-(*N*-Aryl/Alkyl-*N*-hydroxycarbamoyl)glutathiones to the Active Site of the Antitumor Target Enzyme Glyoxalase I

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JOURNAL OF
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Volume 43, Number 21, Pages 3981–3986

J. Med. Chem. **2000,** *43,* 3981−3986 **3981**

Role of Hydrophobic Interactions in Binding *S*-(*N*-Aryl/Alkyl-*N*-hydroxycarbamoyl)glutathiones to the Active Site of the Antitumor Target Enzyme Glyoxalase I

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Received April 6, 2000

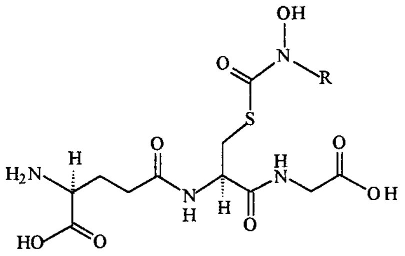
Hydrophobic interactions play an important role in binding *S*-(*N*-aryl/alkyl-*N*-hydroxycarbamoyl)glutathiones to the active sites of human, yeast, and *Pseudomonas putida* glyoxalase I, as the log K_i values for these mechanism-based competitive inhibitors decrease linearly with increasing values of the hydrophobicity constants (π) of the *N*-aryl/alkyl substituents. Hydrophobic interactions also help to optimize polar interactions between the enzyme and the glutathione derivatives, given that the K_i value for *S*-(*N*-hydroxycarbamoyl)glutathione ($\pi = 0$) with the human enzyme is 35-fold larger than the interpolated value for this compound obtained from the log K_i versus π plot. Computational studies, in combination with published X-ray crystallographic measurements, indicate that human glyoxalase I binds the syn-conformer of *S*-(*N*-aryl-*N*-hydroxycarbamoyl)glutathiones in which the *N*-aryl substituents are in their lowest-energy conformations. These studies provide both an experimental and a conceptual framework for developing better inhibitors of this antitumor target enzyme.

Introduction

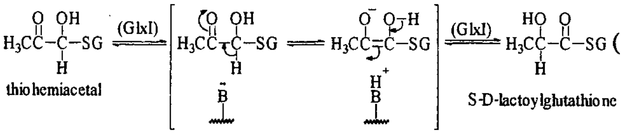
The glyoxalase enzyme system has been the focus of much recent attention as a potential target for antitumor drug development.[1,2] This enzyme system, composed of the isomerase glyoxalase I (GlxI) and the thioester hydrolase glyoxalase II (GlxII), promotes the glutathione (GSH)-dependent conversion of cytotoxic methylglyoxal to D-lactate.[3,4] The toxicity of methylglyoxal, a byproduct of normal cellular metabolism,[5] appears to arise from its ability to cross-link proteins and to form adducts with DNA.[6,7] We recently demonstrated that selected *S*-(*N*-aryl-*N*-hydroxycarbamoyl)-glutathiones **1a**−**c** (Chart 1, Table 1) are powerful competitive inhibitors of human GlxI[8,9] and that the [*glycyl,glutamyl*] diethyl ester prodrugs inhibit the growth of both murine and human tumors in vitro[10] and in vivo.[11] Tumor toxicity appears to be due to inhibition of intracellular GlxI, which results in elevated levels of methylglyoxal.[10] Thus, GlxI appears to be a potentially important antitumor target.

Understanding the nature of the binding interaction between the *N*-aryl-*N*-hydroxycarbamoyl esters of GSH and the active site of GlxI is clearly important, to provide a better basis for inhibitor design. Binding appears to result from two conceptually separate and distinct phenomena. First, the *N*-hydroxycarbamoyl ester function contributes to binding by modeling the stereoelectronic features of the tightly bound enediol intermediate and/or flanking transition states that form from the GSH−methylglyoxal−thiohemiacetal substrate, eq 1.[9]

Chart 1. Structures of *S*-(*N*-Aryl-*N*-hydroxycarbamoyl)glutathiones **1a**−**d** and *S*-(*N*-Alkyl-*N*-hydroxycarbamoyl)glutathiones **2a**−**h**



compd	R	compd	R	compd	R
1a	C_6H_5	*2a*	H	*2e*	$(CH_2)_3CH_3$
1b	$4\text{-}C_6H_4Cl$	*2b*	CH_3	*2f*	$(CH_2)_4CH_3$
1c	$4\text{-}C_6H_4Br$	*2c*	CH_2CH_3	*2g*	$(CH_2)_5CH_3$
1d	$4\text{-}C_6H_4I$	*2d*	$(CH_2)_2CH_3$	*2h*	$(CH_2)_6CH_3$

 (1)

Second, the *N*-aryl substituent also makes a major contribution to binding by interacting with a hydrophobic pocket in the active site, as indicated by the increase in binding affinity with increasing hydrophobicity of the *N*-aryl substituent. The recently determined high-resolution X-ray crystal structure of the homodimeric human enzyme in complex with enediol analogue **1d** confirms the presence of a hydrophobic binding pocket in the active site.[12] In the structure, a catalytically essential active site Zn^{2+} directly coordinates both oxygen atoms of the syn-conformation of the *N*-hydroxy-

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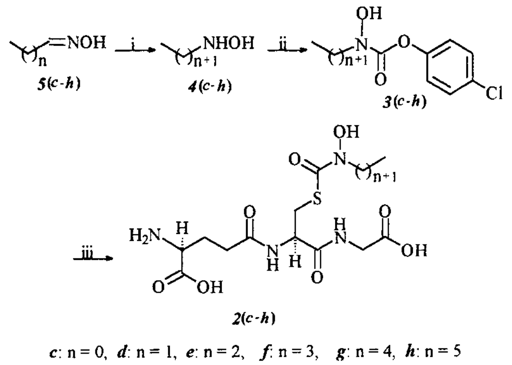
Scheme 1. Synthesis of *S*-(*N*-Alkyl-*N*-hydroxycarbamoyl)glutathiones **2c–h**[a]



[a] Reagents and conditions: (i) $(C_2H_5)_3SiH$; (ii) 4-chlorophenyl chloroformate; (iii) GSH/EtOH–H_2O, pH 9.

carbamoyl ester function, and the *N*-aryl substituent occupies a hydrophobic pocket composed of Phe 67A, Phe 62A, Leu 69A, Cys 60A, Phe 71A, Ile 88A, Leu 92A, Leu 174B, Leu 160B, Phe 162B, and Met 157B.

As part of a research program aimed at developing highly specific, tight-binding inhibitors of human GlxI, we have probed the dimensions and properties of the hydrophobic binding pocket using both experimental and computational methods. Essential to the conclusions of this study is a comparison of the inhibition constants of the *N*-aryl-*N*-hydroxycarbamoyl esters of GSH (**1a–c**) versus the homologous series of *N*-alkyl-*N*-hydroxycarbamoyl esters of GSH (**2a–h**) shown in Chart 1.

Chemistry and Enzymology

The *S*-(*N*-aryl-*N*-hydroxycarbamoyl)glutathione derivatives **1a–c** were prepared by reaction of GSH with the 4-chlorophenyl esters of the corresponding *N*-aryl-*N*-hydroxycarbamates, as previously described by this laboratory.[9] *S*-(*N*-Hydroxycarbamoyl)glutathione (**2a**) was prepared by an acyl-interchange reaction between *N*-hydroxycarbamate 4-chlorophenyl ester (**3a**) and GSH. The acylating reagent (**3a**) was prepared by reacting hydroxylamine with 4-chlorophenyl chloroformate. *S*-(*N*-Hydroxy-*N*-methylcarbamoyl)glutathione (**2b**) was prepared by a published method from this laboratory.[8] The *S*-(*N*-alkyl-*N*-hydroxycarbamoyl)glutathiones **2c–h** were synthesized as outlined in Scheme 1. The 4-chlorophenyl esters of the *N*-alkyl-*N*-hydroxycarbamates **3c–h** were prepared using a modification of the general method of Wu and Sun.[13] In a one-pot reaction mixture, triethylsilane was first used to reduce the alkyl oximes **5c–h** to the corresponding hydroxylamines **4c–h**, followed by reaction with 4-chlorophenyl chloroformate to give **3c–h**. Reaction of GSH with **3c–h** gave crude preparations of **2c–h**, which were purified to apparent homogeneity by differential precipitation or reverse-phase HPLC. NMR spectral assignments were based on comparisons with previously published NMR studies of GSH and its derivatives.[14]

Human erythrocyte GlxI was purified to homogeneity from outdated human blood by a published procedure.[15] *Pseudomonas putida* GlxI was purified from *Escherichia coli* BL21(DE3) transformed with pBTac1/GlxI,[16] using methods described elsewhere.[17]

Table 1. Inhibition Constants (K_i's) of *S*-(*N*-Aryl-*N*-hydroxycarbamoyl)glutathiones with Human Erythrocyte, Yeast, and *P. putida* GlxI[a]

		K_i (μM)		
compd	π[b]	human[c]	yeast[c]	*P. putida*
2b	0.54	1.7 ± 0.1	68 ± 5	86 ± 9
1a	2.13	0.16 ± 0.1	11 ± 1	28 ± 3
1b	3.04	0.046 ± 0.004	3.6 ± 0.3	16 ± 1
1c	3.25	0.014 ± 0.001	1.2 ± 0.2	10 ± 2

[a] Phosphate buffer (50 mM, pH 7), 25 °C. [b] Hansch hydrophobicity constants for R-substituents obtained from ref 20. [c] From ref 9.

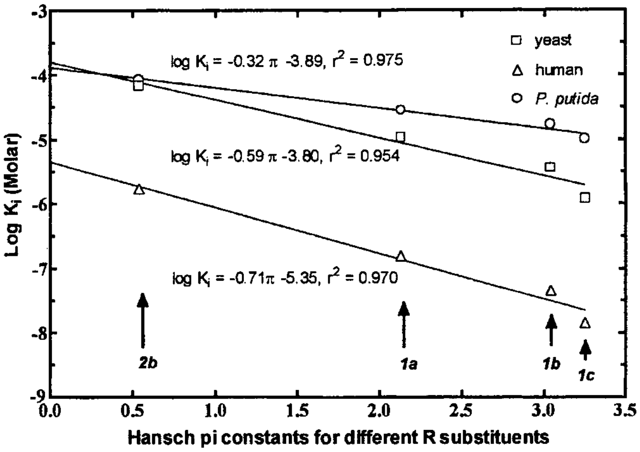


Figure 1. Log plot of competitive inhibition constants (K_i's) versus the Hansch hydrophobicity constants of the R-substituents for *S*-(*N*-methyl-*N*-hydroxycarbamoyl)glutathione (**2b**) and different *S*-(*N*-aryl-*N*-hydroxycarbamoyl)glutathiones **1a–c** with human, yeast, and *P. putida* GlxI. The data for human and yeast GlxI were taken from ref 9.

Results and Discussion

The presence of a hydrophobic binding pocket in the active site of yeast GlxI was first suggested by Vince and co-workers, on the basis of the progressive decrease in the inhibition constants of simple *S*-aryl and *S*-alkyl GSH derivatives with increasing hydrophobicity of the *S*-substituent.[18]

Role of Hydrophobicity in Inhibitor Binding. Indeed, the binding affinities of the enediol analogues **2b** and **1a–c** for human, yeast, and *P. putida* GlxI increase with increasing hydrophobicity of the *N*-substituent (Table 1), indicated by the inverse relationship between log K_i and the hydrophobicity constants (π) of the *N*-methyl and *N*-aryl functions, Figure 1.[19] The π constant is defined as the log of the *n*-octanol/water partition ratio for the *N*-aryl function, calculated according to the method of Hansch.[20] The slopes of the lines through the data measure the change in the free energy of transfer of the enediol analogues from aqueous buffer at pH 7 to the active sites of the enzymes *relative* to the change in the free energy of transfer of the analogues between buffer and *n*-octanol. Thus, the slopes for the yeast and human enzymes can be interpreted to indicate that the hydrophobicities of the respective binding pockets are about 60% and 70% that of *n*-octanol. The shallower slope for *P. putida* GlxI indicates a hydrophobicity about 30% that of *n*-octanol.

Polar/charged residues that are *not* present in the hydrophobic pockets of the human and yeast enzymes can explain the smaller apparent hydrophobicity of the

Table 2. Inhibition Constants (K_i's) of *S*-(*N*-Alkyl-*N*-hydroxycarbamoyl)glutathiones with Human Erythrocyte GlxI[a]

compd	π^b	K_i (μM)	compd	π^b	K_i (μM)
2a	0	183 ± 50	**2f**	2.70	0.17 ± 0.08
2c	1.08	1.18 ± 0.07	**2g**	3.24	0.016 ± 004
2d	1.62	0.80 ± 0.5	**2h**	3.78	0.018 ± 0.011
2e	2.16	0.18 ± 0.09			

[a] Phosphate buffer (100 mM, pH 7), 25 °C. [b] Hansch hydrophobicity constants for R-substituents calculated according to ref 20.

binding pocket in the bacterial enzyme. While there is no reported structural data for the yeast and bacterial enzymes, sequence comparisons with the human enzyme suggest the presence of analogous hydrophobic pockets in the active sites of the yeast and bacterial enzymes. In the human enzyme, Leu 92A and Ile 88A are 2 of 11 residues that compose the hydrophobic pocket identified in the X-ray crystal structure.[21] These two residues are replaced, respectively, by Lys and His residues in the sequence of amino acids that comprise the apparent hydrophobic pocket in the bacterial enzyme, presumably resulting in a lower overall hydrophobicity. In contrast, Leu 92A and Ile 88A are conservatively replaced by two Phe residues in the yeast enzyme, consistent with the similar observed hydrophobicities for the yeast and human enzymes.

The binding affinities to human GlxI of a homologous series of *S*-(*N*-alkyl-*N*-hydroxycarbamoyl)glutathiones **2a**−**h** (Chart 1) were determined for comparison with those of the *N*-aryl derivatives, Table 2. Initially, we anticipated that the slope of the log K_i versus π plot would be shallower than that observed with the *N*-aryl derivatives (Figure 1), because of the increasing conformational flexibility of the *N*-alkyl function proceeding from *N*-methyl to *N*-heptyl. Conformational flexibility (entropy) should negatively impact binding affinity, if the active site binds only a limited subset of *N*-alkyl conformers that exist in bulk solvent. By one estimate, for each degree of rotational freedom lost upon binding of a ligand to a protein, there is roughly a 9-fold decrease in binding affinity.[22] This effect will be less important proceeding from *N*-methyl to the *N*-aryl derivatives, as there is no systematic change in conformational flexibility of the *N*-substituent. However, contrary to expectation, the slopes for the two classes of compounds are identical within experimental error, Figure 2. This suggests either that there is little change in conformational flexibility of the *N*-alkyl derivatives upon binding to the enzyme *or* that there are compensating favorable enthalpic and/or entropic terms (due to solvent/protein reorganization), which are not reflected in the binding affinities of the *N*-aryl derivatives to the active site. *In either case, conformational flexibility appears not to adversely affect the binding affinity of the N-alkyl enediol analogues with the active site of GlxI.*

Role of Polar Interactions in Inhibitor Binding. The intercept values on the log K_i axis of Figure 1 reflect the contribution of polar interactions to binding affinity. These values are the hypothetical inhibition constants for an enediol analogue in which the *N*-aryl function is replaced by *N*-H ($\pi = 0$), a functionality that cannot interact with the enzyme hydrophobically. The difference in the intercept values indicates that polar interactions make a 25-fold greater contribution to binding affinity for the human enzyme than for the yeast and bacterial enzymes. Sequence comparisons indicate that one of the ligands (Gln 33) to the active site Zn^{2+} of the human enzyme is replaced by His in the yeast and bacterial enzymes.[21] Conceivably, this might indirectly influence the stability of the direct coordination interaction between the Zn^{2+} and the *N*-hydroxycarbamoyl ester function of the bound enediol analogues. The difference in binding affinities is less easily explained by differential interactions with the glutathionyl moiety, as the active site residues that interact with the glutathionyl backbone of the bound inhibitors are strictly conserved among the enzymes from the three different biological sources.[21]

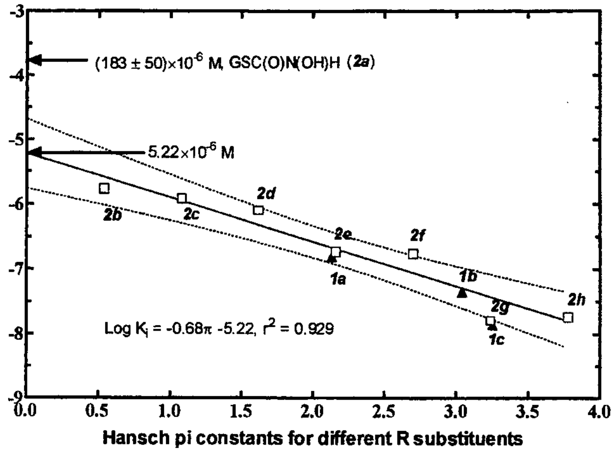
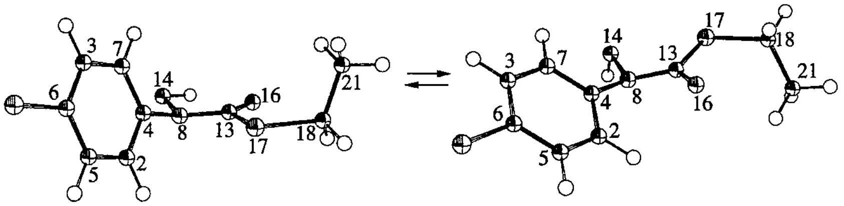


Figure 2. Log plot of competitive inhibition constants (K_i's) versus the Hansch hydrophobicity constants of the R-substituents for *S*-(*N*-alkyl-*N*-hydroxycarbamoyl)glutathiones **2b**−**h** (□) and *S*-(*N*-aryl-*N*-hydroxycarbamoyl)glutathiones **1a**−**c** (▲) with human GlxI. The solid line is the result of linear regression analysis of the data for *S*-(*N*-alkyl-*N*-hydroxycarbamoyl)glutathiones. The dashed lines demarcate the 95% confidence interval. Also shown is the experimentally determined inhibition constant for *S*-(*N*-hydroxycarbamoyl)glutathione (**2a**).

Of substantial interest is the observation that the experimentally measured value of the inhibition constant for *S*-(*N*-hydroxycarbamoyl)glutathione (**2a**, K_i = 183 μM) is 35-fold larger than the extrapolated value of 5.2 μM for this compound obtained from the intercept on the log K_i axis of the hydrophobicity plot of Figure 2. This strongly indicates that occupancy of the hydrophobic binding pocket somehow optimizes polar interactions between the enzyme and the bound ligand, perhaps by promoting alignments to the active site zinc ion. *This is the first evidence for a cooperative relationship between the polar and hydrophobic interactions associated with binding an enediol analogue to the active site of GlxI.*

Computational Studies. The binding of the *N*-aryl enediol analogues to the active site could be adversely affected by the presence of steric restrictions in the hydrophobic binding pocket. For example, the X-ray crystal structure of the binary GlxI·**1d** complex shows an orthogonal relationship between the plane of the phenyl ring and the plane of the *N*-hydroxycarbamoyl ester function.[12] The question of whether this might reflect steric bumping between the phenyl ring and active site residues was addressed using computational methods.



syn-conformer anti-conformer

Figure 3. Geometry-optimized structures of the syn- and anti-conformations of *S*-(*N*-4-chlorophenyl-*N*-hydroxycarbamoyl)-thioethane. Critical dihedral angles are given in Table 3.

Table 3. Critical Dihedral Angles (deg) for the Geometry-Optimized Syn- and Anti-Conformers of *S*-(*N*-4-Chlorophenyl-*N*-hydroxycarbamoyl)thioethane (Figure 3) Obtained at the HF/6-31G*Level of Theory

bonds	syn-conformer	anti-conformer
C(2)−C(4)−N(8)−C(13)	−90.75	−56.27
C(2)−C(4)−N(8)−O(14)	122.76	156.48
C(7)−C(4)−N(8)−C(13)	90.78	125.49
C(7)−C(4)−N(8)−O(14)	−55.71	−21.77
C(4)−N(8)−C(13)−O(16)	−160.16	21.18
C(4)−N(8)−C(13)−S(17)	23.01	−161.26
O(14)−N(8)−C(13)−O(16)	−14.29	168.32
O(14)−N(8)−C(13)−S(17)	168.89	−14.13
relative energy (kcal/mol)	0	−3.4

The energy-minimized syn- and anti-conformers of the model compound, *S*-(*N*-4-chlorophenyl-*N*-hydroxycarbamoyl)thioethane, were calculated by both semiempirical and ab initio methods, Figure 3. The conformation of the *S*-substituent of the less stable syn-conformer is nearly identical to that of the *S*-substituent of the syn-conformer of **1d** observed in the X-ray crystal structure. At the Hartree−Fock/6-31G* level of theory, the dihedral angle between the plane of phenyl ring and that of the *N*-hydroxycarbamoyl ester function is 91°, which is close to that observed in the X-ray structure, Table 3. Apparently, the geometry of the ground-state structure of the model compound in the gas phase is determined largely by steric interactions between the ortho-protons of the ring and the *N*-OH and *S*-ethyl functions. By inference, human GlxI likely binds a low-energy conformer of syn-**1d** in which the *N*-aryl function is orthogonal to the plane of the *N*-hydroxycarbamoyl ester function. *Thus, computational methods indicate that the conformation of the S-substituent of the enzyme-bound enediol analogue is a property of the ligand and not the result of steric interactions with the enzyme protein.*

Implications for Catalysis and Inhibitor Design. The inhibitor binding studies reported here indicate the presence of a large, diffuse hydrophobic binding pocket in the active site of human GlxI that plays a major role in binding the enediol analogues to the active site. Occupancy of this pocket is necessary in order to maximize polar interactions between the enzyme and bound enediol analogue. By inference, the methyl group of GSH−methylglyoxal thiohemiacetal substrate might participate in catalysis by helping to optimize interactions between active site residues and the enediol intermediate and flanking transition states. Each of these factors must be given careful consideration in the design of future mechanism-based competitive inhibitors of GlxI as potential antitumor agents.

Experimental and Computational Methods

Synthetic methods are outlined in Scheme 1. NMR spectra were taken on a GE QE-300 NMR spectrometer. Mass spectral data were obtained at the Midwest Center for Mass Spectrometry, University of Nebraska−Lincoln, and the Washington University Mass Spectrometry Resource, Washington University−Saint Louis. Elemental analyses were obtained at Atlantic Microlabs, Inc., Norcross, GA, and are within 0.4% of the calculated values unless otherwise indicated. Oximes **5c−h** were synthesized from the corresponding aldehydes by standard methods.[23] NMR and IR analyses of the oximes matched published standard spectra. All other reagents were purchased from Aldrich.

Competitive inhibition constants were calculated from the variation in the apparent K_m of GlxI with GSH−methylglyoxal−thiohemiacetal substrate in the presence of different concentrations of enediol analogue.[9] Apparent K_m values were obtained from computer fits of the initial rate data to the Michaelis−Menten equation.

The lowest-energy gas-phase conformation of *S*-(*N*-4-chlorophenyl-*N*-hydroxycarbamoyl)thioethane was calculated using the molecular modeling program Spartan (Wavefunction, Inc., Irvine, CA).

***N*-Hydroxy-*N*-heptylcarbamate 4-Chlorophenyl Ester (3h).** This compound was prepared by a modification of a published method.[23] Heptanal oxime **5h** (1.0 g, 0.008 mol) was dissolved in 10 mL chloroform in a dry, nitrogen-flushed vessel on ice. Triethylsilane (0.90 g, 0.008 mol) was added dropwise, followed by dropwise addition of 4-chlorophenyl chloroformate (1.47 g, 0.008 mol). The mixture was stirred overnight under a nitrogen atmosphere at room temperature. The crude mixture was purified by flash gel chromatography, eluting with chloroform−methanol (40:1 to 30:1). The solvent was removed in vacuo to give a colorless oil, which crystallized to white needles on standing. The product was recrystallized from ether−hexane. Yield: 40%. Mp: 54 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 0.86−0.90 (m, 3H), 1.27−1.32 (m, 8H), 1.67−1.72 (m, 2H), 1.78 (s, OH), 3.65 (t, $J = 7.2$ Hz, 2H), 7.06 (d, $J = 8.7$ Hz, aromatic-2H), 7.33 (d, $J = 8.7$ Hz, aromatic-2H). IR (KBr): 3250, 2920, 2840, 1670, 1480, 1420, 1220, 1160, 1080, 1010, 870, 810, 750 cm^{-1}. Anal. ($C_{14}H_{20}NClO_3$) C, H, N.

***N*-Hydroxy-*N*-hexylcarbamate 4-Chlorophenyl Ester (3g).** This compound was prepared by the same general method used to prepare **3h**. The product was recrystallized from ether−hexane as colorless plates. Yield: 28%. Mp: 61−62 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 0.86−0.91 (m, 3H), 1.2−1.4 (m, 8H), 1.6−1.8 (m, 2H), 3.66 (t, $J = 7.2$ Hz, 2H), 7.06 (d, $J = 8.7$ Hz, aromatic-2H), 7.33 (d, $J = 8.7$ Hz, aromatic-2H). IR (KBr): 3250, 2900, 2840, 1660, 1480, 1420, 1220, 1160, 1080,1010,870, 810, 750 cm^{-1}. Anal. ($C_{13}H_{18}NClO_3$) C, H; N: calcd, 5.15; found, 5.08.

***N*-Hydroxy-*N*-pentylcarbamate 4-Chlorophenyl Ester (3f).** This compound was prepared by the same general method used to prepare **3h**. The product was recrystallized from ether−hexane as white rhombahedral crystals. Yield: 46%. Mp: 47−48 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 0.91 (t, $J = 6.6$ Hz, 3H), 1.28−1.37 (m, 4H), 1.72 (m, 2H), 3.67 (t, $J = 6.9$ Hz, 2H), 6.66 (bs, OH), 7.07 (d, $J = 8.7$ Hz, aromatic-2H), 7.04 (d, $J = 8.7$ Hz, aromatic-2H). IR (KBr): 3250, 2920, 2845, 1660, 1480, 1420, 1210, 1160, 1080, 1005,865, 810, 745 cm^{-1}. Anal. ($C_{12}H_{16}NClO_3$) C, H; N: calcd, 5.43; found, 5.37.

***N*-Hydroxy-*N*-butylcarbamate 4-Chlorophenyl Ester (3e).** This compound was prepared by the same general method used to prepare **3h**, using chloroform–ethyl acetate (100:1) as a chromatographic solvent. The product was recrystallized from ether–hexane as white flat needles. Yield: 23%. Mp: 42–43 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 0.93 (t, J = 7.2 Hz, 3H), 1.37 (m, 2H), 1.68 (m, 2H), 3.66 (t, J = 6.9 Hz, 2H), 7.2 (bs, OH), 7.06 (d, J = 8.7 Hz, aromatic-2H), 7.333 (d, J = 8.7 Hz, aromatic-2H). IR (KBr): 3250, 2920, 2850, 1640, 1460, 1400, 1260, 1190, 1130, 1050, 1020, 980, 845, 790, 725 cm^{-1}. Anal. ($C_{11}H_{14}NClO_3$) C; H: calcd, 5.79; found, 5.73; N: calcd, 5.75; found, 5.83.

***N*-Hydroxy-*N*-propylcarbamate 4-Chlorophenyl Ester (3d).** This compound was prepared by the same general method used to prepare **3h**, using chloroform–ethyl acetate (100:1) as a chromatographic solvent. The product was crystallized from ether–hexane as colorless flat needles. Yield: 21%. Mp: 58–59 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 0.97 (t, J = 7.0 Hz, 3H), 1.76 (m, 2H), 3.64 (t, J = 7.0 Hz, 2H), 6.84 (bs, OH), 7.07 (d, J = 8.8 Hz, aromatic-2H), 7.33 (d, J = 8.8 Hz, aromatic-2H). IR (KBr): 3230, 2940, 2920, 2860, 1655, 1480, 1460, 1420, 1285, 1240, 1220, 1170, 1080, 1040,1005, 865, 810, 745 cm^{-1}. Anal. ($C_{10}H_{12}NClO_3$) C; H: calcd, 5.27; found, 5.32; N: calcd, 6.10; found, 6.05.

***N*-Hydroxy-*N*-ethylcarbamate 4-Chlorophenyl Ester (3c).** This compound was prepared by the same general method used to prepare **3h**, using chloroform–ethyl acetate (100:1) as a chromatographic solvent. The product was crystallized from ether–hexane as white needles. Yield: 16%. Mp: 67–68 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 1.31 (t, J = 7.0 Hz, 3H), 3.73 (t, J = 7.0 Hz, 2H), 6.40 (bs, OH), 7.08 (d, J = 8.8 Hz, aromatic-2H), 7.34 (d, J = 8.8 Hz, aromatic-2H). IR (KBr): 3250, 2970, 2910, 2880, 2860, 1665, 1480, 1425, 1270, 1220, 1170, 1080, 1030, 1005, 960, 865, 810, 745 cm^{-1}. Anal. ($C_9H_{10}NClO_3$) C; H: calcd, 4.67; found, 4.79; N: calcd, 6.50; found, 6.38.

***N*-Hydroxycarbamate 4-Chlorophenyl Ester (3a).** This compound was prepared by reacting hydroxylamine with 4-chlorophenyl chloroformate. The product was crystallized from ether–hexane as colorless plates. Yield: 34%. Mp: 137–139 °C. 300 MHz ^{1}H NMR ($CDCl_3$, TMS): δ 5.83 (bs, 1H), 7.11 (d, J = 8.7 Hz, 2H), 7.38 (d, J = 8.7 Hz, 2 H), 7.47 (bs, 1H). IR (KBr): 3300, 1725, 1465, 1260, 1210, 1080, 1005, 840 cm^{-1}. Anal. ($C_7H_6NClO_3$) C; H: calcd, 3.22; found, 3.34; N: calcd, 7.47; found, 7.39.

***S*-(*N*-Heptyl-*N*-hydroxycarbamoyl)glutathione (2h).** Into a stirring solution of 6.3 mL degassed, nitrogen-saturated ethanol:water (2:1) and **3h** (53 mg, 0.185 mmol) was placed a 6.3-fold excess of glutathione (563 mg, 1.16 mmol). The slurry was slowly brought to pH 9 by the dropwise addition of 6 N NaOH, during which the mixture became a homogeneous solution. The solution was placed under nitrogen and allowed to stand at room temperature for 24 h. The solution was brought to pH 3.5 with 6 N HCl and the solvent removed in vacuo. The white residue was suspended in 1 mL water, stirred for 9 h at room temperature, and the precipitate collected by centrifgation. This digestion procedure was repeated 4 more times. The dried white solid was then triturated 3 times with 0.8 mL portions of diethyl ether to remove unreacted **3h** and unreacted 4-chlorophenol. Yield: 35%. 300 MHz ^{1}H NMR (D_2O, pD 10.4, HOD reference): δ 0.88 (methyl-3H), 1.2–1.4 (m, alkyl-8H), 1.63 (m, alkyl-2H), 1.91 (m, Glu-$C_\beta H_2$), 2.40 (m, Glu-$C_\gamma H_2$), 3.07 (q, J = 9.0, 14.4 Hz, Cys-$C_\beta H_a$), 3.28 (q, J = 4.2, 14.4 Hz, Cys-$C_\beta H_b$), 3.32 (t, J = 6.0 Hz, Glu-C_αH), 3.59 (t, J = 6.6 Hz, N-CH_2), 3.75 (d, J = 17.1 Hz, Gly-$C_\alpha H_a$), 3.83 (d, J = 17.1 Hz, Gly-$C_\alpha H_b$), 4.53 (q, J = 4.2, 9.0 Hz, Cys-C_αH). FAB MS consistent with $C_{18}H_{32}N_4SO_8$. Anal. ($C_{18}H_{32}N_4SO_8$) C, H; N: calcd, 12.06; found, 11.92.

***S*-(*N*-Hexyl-*N*-hydroxycarbamoyl)glutathione (2g).** This compound was prepared by the same general method used to prepare **2h**, using a reaction time of 70 h. Yield: 66%. 300 MHz ^{1}H NMR (D_2O, pD 10.4, DSS): δ 0.86 (methyl-3H), 1.28 (m, alkyl-6H), 1.60 (m, alkyl-2H), 1.87 (m, Glu-$C_\beta H_2$), 2.36 (m, Glu-$C_\gamma H_2$), 3.04 (q, J = 9.0, 14.4 Hz, Cys-$C_\beta H_b$), 3.24 (q, J = 4.2, 14.4 Hz, Cys-$C_\beta H_a$), 3.27 (t, J = 6.6 Hz, Glu-C_α H), 3.57 (t, J = 6.9 Hz, N-CH_2), 3.72 (d, J = 17.1 Hz, Gly-$C_\alpha H_a$), 3.80 (d, J = 17.1 Hz, Gly-$C_\alpha H_b$), 4.50 (q, J = 4.2, 9.0 Hz, Cys-C_αH). FAB MS consistent with $C_{17}H_{30}N_4SO_8$. Anal. ($C_{17}H_{30}N_4SO_8$) C, H; N: calcd, 12.44; found, 12.28.

***S*-(*N*-Pentyl-*N*-hydroxycarbamoyl)glutathione (2f).** This compound was prepared by the same general method used to prepare **2h**. Yield: 53%. 300 MHz ^{1}H NMR (D_2O, pD 10.4, DSS): δ 0.87 (methyl-3H), 1.28 (m, alkyl-4H), 1.61 (m, alkyl-2H), 1.91 (m, Glu-$C_\beta H_2$), 2.39 (m, Glu-$C_\gamma H_2$), 3.04 (q, J = 9.0, 14.4 Hz, Cys-$C_\beta H_b$), 3.26 (q, J = 4.2, 14.4 Hz, Cys-$C_\beta H_a$), 3.35 (t, J = 6.3 Hz, Glu-C_αH), 3.57 (t, J = 6.9 Hz, N-CH_2), 3.73 (d, J = 17.1 Hz, Gly-$C_\alpha H_a$), 3.80 (d, J = 17.1 Hz, Gly-$C_\alpha H_b$), 4.52 (q, J = 4.2, 9.0 Hz, Cys-C_αH). FAB MS consistent with $C_{16}H_{28}N_4SO_8$. Anal. ($C_{16}H_{28}N_4SO_8$) C; H: calcd, 6.47; found, 6.38; N: calcd, 12.84; found, 12.71.

***S*-(*N*-Butyl-*N*-hydroxycarbamoyl)glutathione (2e).** This compound was prepared by the same general method used to prepare **2h**, using a reaction time of 115 h. The crude, acidified product was purified by flash chromatography on a silica gel column, using *n*-propanol:acetic acid:water (10:1:5) as an eluting solvent. The fractions containing the crude product were pooled, brought to dryness and further purified by reverse-phase column chromatography (Whatman μBondapak C_{18}, 0.78 × 30 cm), using 0.25% acetic acid and 35% methanol in water as an eluting solvent (retention volume: ~26 mL). The peak fractions were lyophilized to dryness to give the final product as a white powder. Yield: 35%. 300 MHz ^{1}H NMR (D_2O, pD 3.3, DSS): δ 0.89 (t, J = 7.3 Hz methyl-3H), 1.28 (m, alkyl-2H), 1.59 (m, alkyl-2H), 2.15 (m, Glu-$C_\beta H_2$), 2.51 (m, Glu-$C_\gamma H_2$), 3.14 (q, J = 8.4, 14.3 Hz, Cys-$C_\beta H_b$), 3.37 (q, J = 4.8, 14.3 Hz, Cys-$C_\beta H_a$), 3.65 (t, J = 7.0 Hz, N-CH_2), 3.79 (t, J = 6.6 Hz, Glu-C_αH), 3.94 (s, Gly-$C_\alpha H_2$), 4.62 (q, J = 4.8, 8.4 Hz, Cys-C_αH). FAB MS consistent with $C_{15}H_{26}N_4SO_8$. Anal. ($C_{15}H_{26}N_4SO_8 \cdot H_2O$) C: calcd, 37.86; found, 37.46; H: calcd, 5.87; found, 5.76; N: calcd, 13.58; found, 13.26.

***S*-(*N*-Propyl-*N*-hydroxycarbamoyl)glutathione (2d).** This compound was prepared by the same general method used to prepare **2e**. The flash chromatography column was eluted with *n*-propanol:acetic acid:water (70:25:30). The reverse-phase column was eluted with 0.25% acetic acid and 25% methanol in water (retention volume: ~23 mL). The peak fractions were lyophilized to dryness to give the final product as a white powder. Yield: 35%. 300 MHz ^{1}H NMR (D_2O, pD 3.3, DSS): δ 0.86 (t, J = 7.3 Hz, methyl-3H), 1.63 (m, J = 7.0, J = 7.3 alkyl-2H), 2.14 (m, Glu-$C_\beta H_2$), 2.51 (m, Glu-$C_\gamma H_2$), 3.12 (q, J = 8.4, 14.3 Hz, Cys-$C_\beta H_b$), 3.38 (q, J = 4.8, 14.3 Hz, Cys-$C_\beta H_a$), 3.61 (t, J = 7.0 Hz, N-CH_2), 3.79 (t, J = 6.2 Hz, Glu-C_αH), 3.94 (s, Gly-$C_\alpha H_2$), 4.62 (q, J = 4.8, 8.4 Hz, Cys-C_αH). FAB MS consistent with $C_{14}H_{24}N_4SO_8$. Anal. ($C_{14}H_{24}N_4SO_8 \cdot H_2O$) C; H: calcd, 6.15; found, 5.90; N: calcd, 6.50; found, 6.38.

***S*-(*N*-Ethyl-*N*-hydroxycarbamoyl)glutathione (2c).** This compound was prepared by the same general method used to prepare *2e*. The flash chromatography column was eluted with *n*-propanol:acetic acid:water (130:1:70). The reverse-phase column was eluted with 0.25% acetic acid and 5% methanol in water (retention volume: ~24 mL). The peak fractions were lyophilized to dryness to give the final product as a white powder. Yield: 48%. 300 MHz ^{1}H NMR (D_2O, pD 3.3, DSS): δ 1.16 (t, J = 7.0 Hz, methyl-3H), 2.15 (m, Glu-$C_\beta H_2$), 2.51 (m, Glu-$C_\gamma H_2$), 3.12 (q, J = 8.4, 14.3 Hz, Cys-$C_\beta H_b$), 3.38 (q, J = 4.8, 14.3 Hz, Cys-$C_\beta H_a$), 3.66 (t, J = 7.0 Hz, N-CH_2), 3.79 (t, J = 6.2 Hz, Glu-C_αH), 3.94 (s, Gly-$C_\alpha H_2$), 4.63 (q, J = 4.8, 8.4 Hz, Cys-C_αH). FAB MS consistent with $C_{13}H_{22}N_4SO_8$. Anal. ($C_{13}H_{22}N_4SO_8 \cdot H_2O$) C: calcd, 37.86; found, 37.46; H: calcd, 5.87; found, 5.76; N: calcd, 13.58; found, 13.26.

***S*-(*N*-Hydroxycarbamoyl)glutathione (2a).** This compound was prepared by the same general method used to prepare **2h**, using a 3.5-fold excess of GSH and a reaction time of 4 h. The crude reaction mixture was treated with 4-pyridine disulfide in order derivatize any unreacted GSH to the mixed disulfide. This allowed a clean separation of the desired product by reverse-phase column chromatography using 0.25% acetic acid in water (retention volume: ~16 mL). The peak fractions were lyophilized to dryness to give the final product as a white powder. Yield: 30%. 300 MHz ^{1}H NMR (D_2O, pD 3.2, DSS): δ 2.15 (m, Glu-C$_\beta$H$_2$), 2.51 (m, Glu-C$_\gamma$H$_2$), 3.20 (q, J = 8.1, 14.3 Hz, Cys-C$_\beta$H$_b$), 3.43 (q, J = 4.8, 14.3 Hz, Cys-C$_\beta$H$_a$), 3.81 (t, J = 6.2 Hz, Glu-C$_\alpha$H), 3.95 (s, Gly-C$_\alpha$H$_2$), 4.66 (q, J = 4.8, 8.1 Hz, Cys-C$_\alpha$H). FAB MS consistent with $C_{11}H_{18}N_4SO_8$. Anal. ($C_{11}H_{18}N_4SO_8 \cdot H_2O$) C; H: calcd, 5.24; found, 5.17; N: calcd, 14.58; found, 14.36.

Acknowledgment. This work was supported by grants from the National Cancer Institute (CA 59612) and the U.S. Army Medical Research and Materiel Command.

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JM000160L

APPENDIX II: Papers published and in press related to Task 5

1. Diana S. Hamilton, Zhebo Ding, Bruce Ganem, and Donald J. Creighton, "Studies on the Antitumor Activity of 2-Crotonyloxymethyl-2-cyclohexenone: Mechanism of the Glutathionly Transferase-Catalyzed Addition of Glutathione," J. Med. Chem., in press.

2. C. Frederick M. Huntley, Diana S. Hamilton, Donald J. Creighton, and Bruce Ganem, "Reaction of COTC with Glutathione: Structure of the Putative Glyoxalase I Inhibitor," Org. Lett., Vol. 2, No. 20, 2000.

Studies on the Antitumor Activity of 2-Crotonyloxymethyl-2-cyclohexenone: Mechanism of the Glutathionyl Transferase-Catalyzed Addition of Glutathione

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NY 14853-1301

Abstract

The antitumor activity of 2-crotonyloxymethyl-2-cyclohexenone (COMC) has been attributed to the adduct of glutathione with COMC, which inhibits the methylglyoxal detoxifying enzyme glyoxalase I. In order to test this hypothesis, the adduct was independently synthesized and tested as an inhibitor of human erythrocyte glyoxalase I. While the adduct is indeed a competitive inhibitor of the enzyme, the inhibition constant ($K_i = 107\ \mu M$, pH 7) appears to be too high to account for reported IC_{50} values in the low micromolar concentration range for tumor cells in culture. An alternative hypothesis for tumor toxicity is presented, based on kinetic studies and trapping experiments, invoking a heretofore unrecognized exocyclic enone intermediate formed during either the nonenzymic or glutathione transferase-catalyzed conjugation of glutathione with COMC. The newly-detected intermediate is capable of covalently modifying proteins and/or nucleic acids critical to cell survival.

Key words: 2-crotonyloxymethyl-2-cyclohexenone, glutathione adduct, tumor toxicity, reaction mechanism.

Introduction

Endocyclic enones are of potential chemotherapeutic interest. In 1975, Umezawa and coworkers reported that the bacterial metabolite 2-crotonyloxymethyl-(4R, 5R, 6R)-4,5,6-trihydroxy-2-cyclohexenone (COTC, **1a**, Figure 1) exhibited potent antitumor activity *in vitro.*[1,2] Given its potential as a chemotherapeutic agent, five different synthetic routes to **1a** have so far been published.[3-7] Aghil *et al.* also observed that the simpler synthetic analog, 2-crotonyloxymethyl-2-cyclohexenone (COMC, **2a**) exhibited antitumor activity against a range of different murine and human tumors in culture, with **2a** being somewhat more potent ($IC_{50} = 0.5\text{-}19\ \mu M$) than **1a** ($IC_{50} = 3\text{-}44\ \mu M$).[8] Although the molecular mechanism of toxicity is unclear, the endocyclic enone function appears to be strictly required for activity.[8]

The observed antitumor activity of COTC and COMC has widely been attributed to the corresponding adducts with glutathione, **1b** and **2b**, respectively, which were thought to inhibit the methylglyoxal detoxifying enzyme glyoxalase I (Figure 1). This enzyme is known to play a critical detoxification role in cells by chemically removing cytotoxic methylglyoxal as S-D-lactoylglutathione.[9,10] Indeed, we recently demonstrated that conjugate **1b** is a competitive inhibitor of human erythrocyte glyoxalase I ($K_i = 183 \pm 6\ \mu M$).[11] Creighton and coworkers showed that enediol analogue inhibitors of glyoxalase I [13] having K_i values in the nanomolar concentration range inhibit the growth of both murine and human tumors in culture[12] and in tumor-bearing mice by inducing elevated concentrations of methylglyoxal in tumor cells. A similar mechanism might be operative in the cases of **1b** and **2b**.

However, the fact that **2b** is only a modest glyoxalase I inhibitor compared with the antitumor enediol analogues raises questions about the validity of this simple explanation. Further questions lingered about the chemical mechanism leading to the formation of **1b** and **2b** in the presence of glutathione. Since the most plausible mechanism involved two consecutive Michael reactions, it was of interest to ascertain whether the product of the first conjugate addition, an exocyclic enone, might be implicated in the antitumor activity of COTC and COMC. Here we report that the glutathione transferase (GST)-catalyzed conjugation of **2a** with GSH does indeed form a highly reactive exocyclic enone intermediate that can be isolated and characterized. That finding suggests that tumor toxicity might arise from covalent modification of critical cell components by the exocyclic enone.

Chemistry and Enzymology

The synthesis of COMC **2a** from 2-cyclohexenone was shortened from the seven-step procedure reported by Aghil *et al.*[8] to two steps (Scheme 1). The improved route utilized the known Baylis-Hillman reaction of commercially available 2-cyclohexenone to prepare 2-hydroxymethyl-2-cyclohexenone,[15] which was then crotonylated to **2a** following the literature procedure.[8] The GSH conjugate **2b** was prepared from **2a** by adapting the procedure for making **1b** from **1a**.[11] The NMR spectrum of **2b** featured the expected glutathionyl resonances and the downfield resonance (δ 7.12, triplet) characteristic of H3 in β,γ-unsubstituted 2-cyclohexenones. Human erythrocyte GlxI was purified to homogeneity from outdated human blood by a published procedure.[16] Human placental GSH transferase was purchased from Sigma Chemical Co. and salts and free GSH were removed by ultrafiltration. Units of transferase activity were determined using the substrate 1-chloro-2,4-dinitrobenzene.[17]

Results

The observations that lead to the above hypothesis are described below.

Inhibition Studies. Initially, our studies focused on testing the hypothesis that the greater antitumor activity of **2a** versus **1a** might reflect stronger inhibition of glyoxalase I by the GSH-conjugate **2b** than by conjugate **1b**. The binding affinities of S-substituted GSH derivatives for glyoxalase I are known to increase with increasing hydrophobicity of the S-substituent,[18,19] and a hydrophobic binding pocket has recently been identified near the active site of the human enzyme.[20] Therefore, **2b** might bind more tightly than **1b**, provided the ring systems occupy this hydrophobic pocket. Indeed, the inhibition constant of **2b** with human erythrocyte glyoxalase I ($K_i = 107 \pm 0.1$ μM, Fig. 2) is lower than that of **1b** ($K_i = 183 \pm 6$ μM),[11] potentially explaining the greater toxicity of **2a** versus **1a** toward tumor cells reported by Aghil *et al.*[8]

However, inhibition of glyoxalase I is unlikely to fully account for the tumor toxicities of **1a** and **2a**. While highly specific GSH-based, enediol analogue inhibitors of the enzyme do indeed inhibit the growth of L1210 and B16 melanotic melanoma in vitro (when delivered as the diethyl ester prodrugs), the K_i values for these compounds are in the submicromolar concentration range.[12] In addition, the IC_{50} values of the prodrugs are approximately proportional to the K_i values of the inhibitors, such that a relatively weak enzyme inhibitor with a $K_i = 0.16$ μM gives an IC_{50} value >100 μM for the corresponding prodrug. On this basis, **2a** would be predicted to have very poor potency, as **2b** has a $K_i = 107$ μM. This is contrary to observation ($IC_{50} = 0.5$-19 μM).[8]

Reaction Mechanisms. Another possibility is that conjugate addition of GSH to COMC involves a reactive intermediate that alkylates critical cell components. On paper, the simplest mechanism is a direct, S_N2 displacement of the crotonate ester by the nucleophilic thiol of GSH. However, carboxylic esters are only moderate leaving groups, and the α,β-unsaturated enone systems in **1a** and **2a** would be expected to undergo much more rapid conjugate addition reactions with thiols. Thus, a stepwise 1,4-addition/β-elimination mechanism can be envisioned in which GSH first adds to the endocyclic enone function of **2a** via enolate **5** to form an intermediate exocyclic enone **6.** The exocyclic enone then subsequently reacts with another molecule of GSH to give the endocyclic enone **2b** (Scheme 2). Exocyclic enones like **6** are particularly reactive Michael acceptors, and could function as carcinostatic agents by reacting with nucleic acids and proteins critical to cell function.

Initial Kinetic Studies. Experimentally, the reaction of excess GSH with **2a** to give **2b** follows a simple first-order decay with no evidence of any intermediate (trace C, Figure 3). This observation is consistent with the mechanism shown in Scheme 2, provided that the formation of the exocyclic enone is rate determining. During the course of these studies, we made the chance observation that the spectrophotometrically determined rate of conversion of **2a** ($\varepsilon_{235} = 11{,}000\ cm^{-1}M^{-1}$) to **2b** ($\varepsilon_{235} = 7500\ cm^{-1}M^{-1}$) is biphasic in the presence of human placental glutathione transferase (GSTP1-1), composed of a rapid, enzyme-dependent, initial phase followed by a slower enzyme independent first-order process (traces B and C, Figure 3). Apparently, the enzyme catalyzes the formation of exocyclic enone **6**, which subsequently dissociates from the surface of the enzyme prior to reacting with free GSH to give adduct **2b**, (Scheme 3). The shape of the progress curve obtained at 235 nm (Figure.3) would be comprehensible, if the absorbancy of **6** is less than that of **2b**.

Trapping of 6. As an additional test of the hypothesis, the transferase reaction was conducted in the presence of equimolar amounts of GSH and cysteine, under the conditions described in the legend to Figure 4. The product ratio **8**:**2b** was then determined by reverse-phase C_{18} HPLC. The product ratio in a control incubation without transferase was also determined. If intermediate **6** dissociates from the surface of the enzyme and reacts with the free mercaptans in solution, the product ratios for the two systems should be the same. However, if the transferase catalyzes all steps in the conversion of **2a** to **2b**, the ratio of **2b** to the cysteine adduct **8** should be much higher in the reaction mixture containing transferase, since the enzyme is highly specific for GSH as a substrate.[17] The amount of product formed by the enzyme is about 90%, on the basis of the ratio of first-order rate constants for the enzymic versus the nonenzymic control reactions (see legend, Figure 3). The product ratios for the two systems are identical, within experimental error (Figure 4). That adduct **2b** is stable in the presence of free cysteine under the buffer conditions used in the enzyme incubation is indicated by the fact that **2b** (0.5 mM) undergoes less than 1% conversion to adduct **8** in the presence of cysteine (2.5 mM) over 27 h, as determined by HPLC. Therefore, the product ratio observed in the enzyme incubation is under kinetic control and reflects the true relative rates of reaction of **6** with GSH versus cysteine. Taken together, these observations support the reaction mechanism depicted in Scheme 3, wherein **6** dissociates from the surface of the enzyme prior to reaction with free GSH.

Isolation and Characterization of ***6***. Brief incubation of a mixture of **2a**, GSH, and GST gives rise to a transient intermediate that can be isolated by reverse-phase HPLC, as shown in **Figure 5**. When combined with cysteine in buffered solution (pH 7), the intermediate gives rise to a new peak in the chromatogram that comigrates with authentic **8**. Moreover, the pseudo-first order rate constant for reaction of GSH (1.04 mM) with the intermediate species in potassium phosphate buffer (0.1 M, pH 6.5, 25 °C), obtained by following the *increase* in OD_{235}, is similar in magnitude (0.87 ± 0.08 min^{-1}) to that associated with the second phase of the transferase-catalyzed conversion of **2a** to **2b** (Figure 3). The estimated absorptivity of the intermediate (4270 $cm^{-1}M^{-1}$) significantly less than that of the final product **2b** ($\varepsilon_{235} = 7500$ cm^{-1} M^{-1}).

The 600 MHz ^{1}NMR spectrum of the intermediate species is consistent with the proposed structure of **6**, **Figure 6**. The vinyl proton resonances at 5.76 and 5.29 ppm are characteristic of geminal vinylic hydrogens, and are consistent with published NMR spectra of several closely related exocyclic α-methylene cyclohexanones.[22] The resonance at 7.16 ppm is due to the presence of adduct **2b**. The other resonances in the spectrum are those expected for S-substituted GSH derivatives.[23]

Comparisons with other substrates. The macroscopic kinetic constants of COMC **2a** with the transferase in buffered solution at pH 6.5 (25°C) are very close ($k_{cat} = 4.2\ s^{-1}$, $K_m = 46\ \mu M$) to those of ethacrynic acid ($k_{cat} = 3.3\ s^{-1}$, $K_m = 60\ \mu M$). The magnitude of k_{cat}/K_m for COMC **2a** exceeds that of COTC **1a** by 8.3-fold.

Summary and Discussion

Two observations in this work are of central importance. First, the GSH adducts **1b** and **2b** derived from COTC and COMC are weak competitive inhibitors of human glyoxalase I. For that reason, those adducts are unlikely to account fully for the reported tumoricidal activities of the corresponding endocyclic enones **1a** and **2a**.

Second, we have now shown conclusively that enone **6** is the primary product of human GSTP1-1 in the multi-step conversion of **2a** to the corresponding GSH adduct **2b**. Reaction of **6**, which is highly electrophilic, with nucleophilic groups on proteins and nucleic acids that are critical to cell survival may be responsible for the reported tumoricidal activity of **2a**. In support of that hypothesis, covalent adducts between **1a** and model polynucleic acids, in the presence of human GSTP1-1, have been detected by mass spectrometry (Fabris, Creighton and Ganem, unpublished).

Evidence is also presented that **2a** and ethacrynic acid exhibit similar kinetic properties with the transferase. Enzyme-catalyzed formation of **6** from **2a** probably involves general acid/base catalysis by Tyr(108) and Tyr(7), as these residues have been implicated in catalyzing nucleophilic additions of GSH to the enone group in ethacrynic acid.[24]

Finally, the possibility that **6** may be an antitumor agent that is released from **2a** by the action of GSTP1 is of considerable interest for several reasons. Compound **2a** (and by extension, the natural product COTC **1a**) represents a new class of a prodrug, whose function depends on a widespread family of glutathione-processing enzymes. Second, from a cancer control perspective, large differences in GSTP1-1 activity have been documented between normal cells and cancer cells, which might contribute to the selective tumoricidal activities of compounds like **2a**.[25-28] Finally, the possibility of designing tissue-specific and enzyme-specific forms of such prodrugs is apparent, which would be of significant potential use in targeted cancer chemotherapy.

Experimental Section

Proton and carbon-13 NMR spectra were recorded on a Bruker AF-300, a Varian VXR-400 or a Varian Unity-500 spectrometer. Chemical shifts were reported as δ scale in parts per million. Spectra obtained were referenced to residual deuterated solvent peaks. Electrospray mass spectra were acquired in the positive ion mode using a Micromass Quattro 1 Triple Quadrupole Tandem Mass Spectrometer at the Cornell University Mass Spectrometry Facility.

Synthesis of **2b** from **2a**. A solution of **2a** (12 mg, 0.061 mmol) was taken up in 2 mL of 0.1 M pH 7.5 sodium phosphate buffer. To the resulting solution was added reduced glutathione (Sigma, 15 mg, 0.05 mmol) and the solution placed in a 37°C water bath for 20 minutes. A 2cm x 2cm ion-exchange column was prepared using Dowex-1 resin (chloride form, 4% cross linked, 100-200 mesh). The reaction solution was added directly onto the column and washed with increasing concentrations of aqueous AcOH solutions (0-6% AcOH, 120 mL total eluent). The 5-6% AcOH fractions were pooled and the solvents removed to afford 24 mg of a hygroscopic white solid (95%, mp 95-102 °C, decomp.); R_f 0.50 (10:1:5 n-PrOH:AcOH:H_2O); ^1H NMR (300 MHz, D_2O) 7.12 (t, 1 H, J = 4.3 Hz), 4.50 (dd, 1 H, J=7.6, 5.3 Hz), 3.94 (s, 2 H), 3.88 (br. t, 1 H), 3.26 (s, 2 H), 2.93, 2.74 (ABX, 2 H, J = 5.0, 8.8, 14 Hz), 2.60-2.38 (2 m, 6 H), 2.21-2.12 (m, 2 H), 1.98-1.83 (m, 2 H); ^{13}C

NMR (300 MHz, D_2O) 203.3, 176.6, 174.4, 172.9, 172.2, 153.0, 134.2, 52.9, 52.7, 41.1, 37.9, 32.5, 30.9, 30.0, 25.7, 25.6, 22.2; EIMS *m/z* 416 (M+1, 100%).

Acknowledgment. This work was supported by grants from the U.S. Army Medical Research and Materiel Command to DJC and the National Institutes of Health (GM 24054) to BG. We thank Professor Dale L. Whalen for helpful discussions and Ms. Agnes Holmes for obtaining the 600 MHz ^{1}H NMR spectrum of **6**.

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Figures

Figure 1. *Structures of COTC **1a** and COMC **2a** and their Corresponding Glutathione Conjugates **1b** and **2b**.*

Figure 2. *Reciprocal plot of the velocity of the glyoxalase I reaction (ΔOD_{240}) versus the concentration of GSH-methylglyoxal-thiohemiacetal ([H]) in the absence and in the presence of different concentrations of the GSH adduct **2b** (I).* In each kinetic run, the concentration of GSH was maintained at 0.2 mM by varying the total concentration of GSH and methylglyoxal on the basis of the dissociation constant for the thiohemiacetal ($K_{diss} = 2.2$ mM).[21] Conditions: sodium phosphate buffer (0.05 M), pH 7.0, 25 °C.

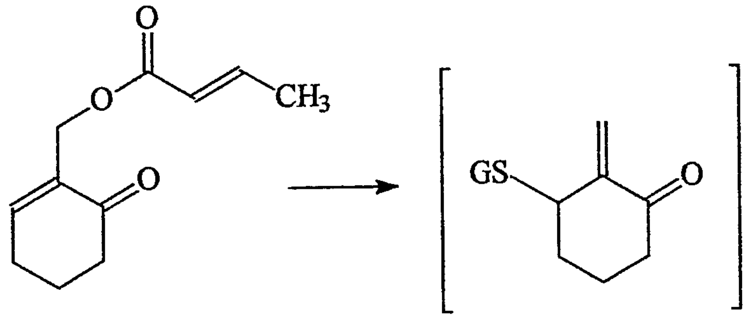
Figure 3. *Specrophotometrically determined rates of reaction of GSH (1.03 mM) with **2a** (0.05 mM) (A) in the absence GST ($k = 0.070 \pm 0.0002$ min^{-1}), **(B)** in the presence 1.8 units of GST ($k_1 = 0.882 \pm 0.055$ min^{-1}; $k_2 = 0.633 \pm 0.053$ min^{-1}),and (C) in the presence of 2.4 units of GST ($k_1 = 1.33 \pm 0.037$ min^{-1}; $k_2 = 0.685 \pm 0.024$ min^{-1}).* Rate constants were obtained by fitting the kinetic data to the equations for single (trace A) or double (traces B and C) exponential decays. Different end-point absorbancies are due to different background absorbancies of enzyme protein. Conditions: potassium phosphate buffer (0.1 M), EDTA (0.05 mM), pH 6.5, 25 °C.

Figure 4. *Elution profiles from a reverse phase HPLC column (Water's µBondapak C_{18}, 0.78 x30 cm) showing the relative amounts of **8** (~9.5 min) and **2b** (~11.5 min) observed (OD_{240}) after 30 min incubation of reaction mixtures initially composed of **2a** (0.1 mM), cysteine (0.5 mM), GSH (0.5 mM), potassium phosphate buffer (0.1 M), EDTA (0.05 mM), pH 6.5, 25 °C (A) in the absence, and **(B)** in the presence of GSTP1-1(1.5 units).* Running solvent: 25 % methanol in water containing 0.25% acetic acid.

Figure 5. *Elution profile (OD_{238}) from a reverse-phase HPLC column (Water's μBondapak C_{18}, 0.78 x30 cm) of a portion of a reaction mixture obtained 90 seconds after combining GSH (1.0 mM), GSTP1-1(5.3 units), and* ***2a*** *(5 mM) in 2.4 ml potassium phosphate buffer (0.1 M), EDTA (0.05mM), pH 6.5, 25 °C.* The reaction mixture was stopped at 90 seconds by the addition of trichloroacetic acid to 10% (v/v). The peak at 21 min corresponds to adduct **2b**, the peak at 20.1 min is tentatively identified as the exocyclic enone **6,** and the peak at 14.4 min. is crotonic acid. Running solvent: 1.5 % methanol in water containing 0.25% acetic acid.

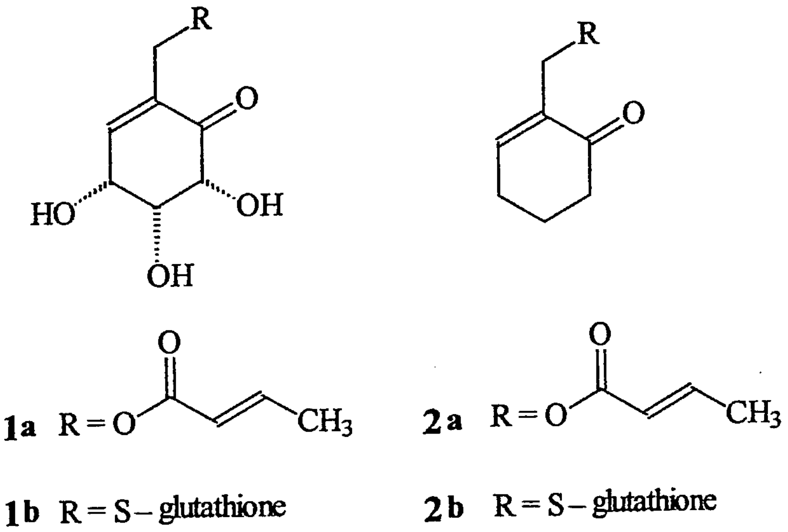
Figure 6. *The 600 MHz ^{1}NMR spectrum of the putative exocyclic enone* ***6*** *corresponding to the 20.1 min peak in the HPLC elution profile of Figure 5.*

TOC Graphic



putative mediator
of antitumor activity

Fig. 1



1a R =

1b R = S – glutathione

2a R =

2b R = S – glutathione

Fig. 2

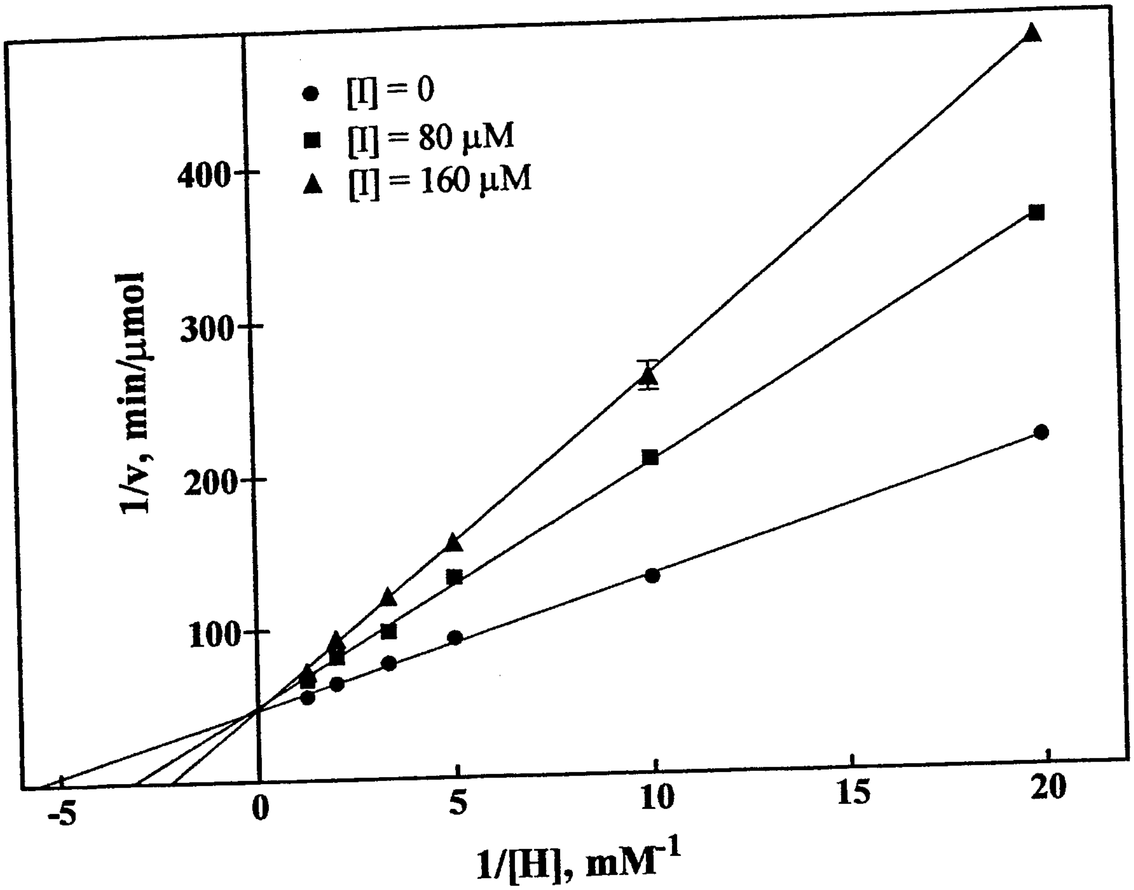


Fig. 3

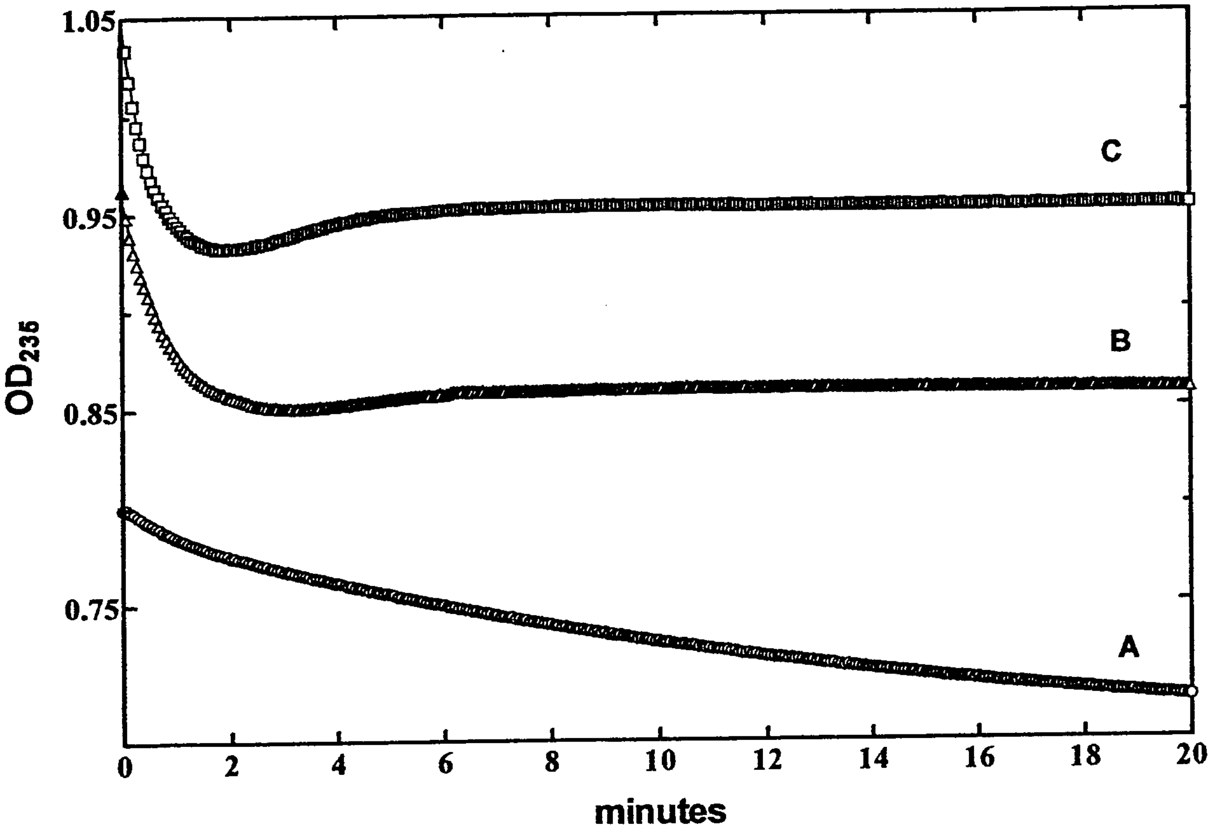


Fig. 4

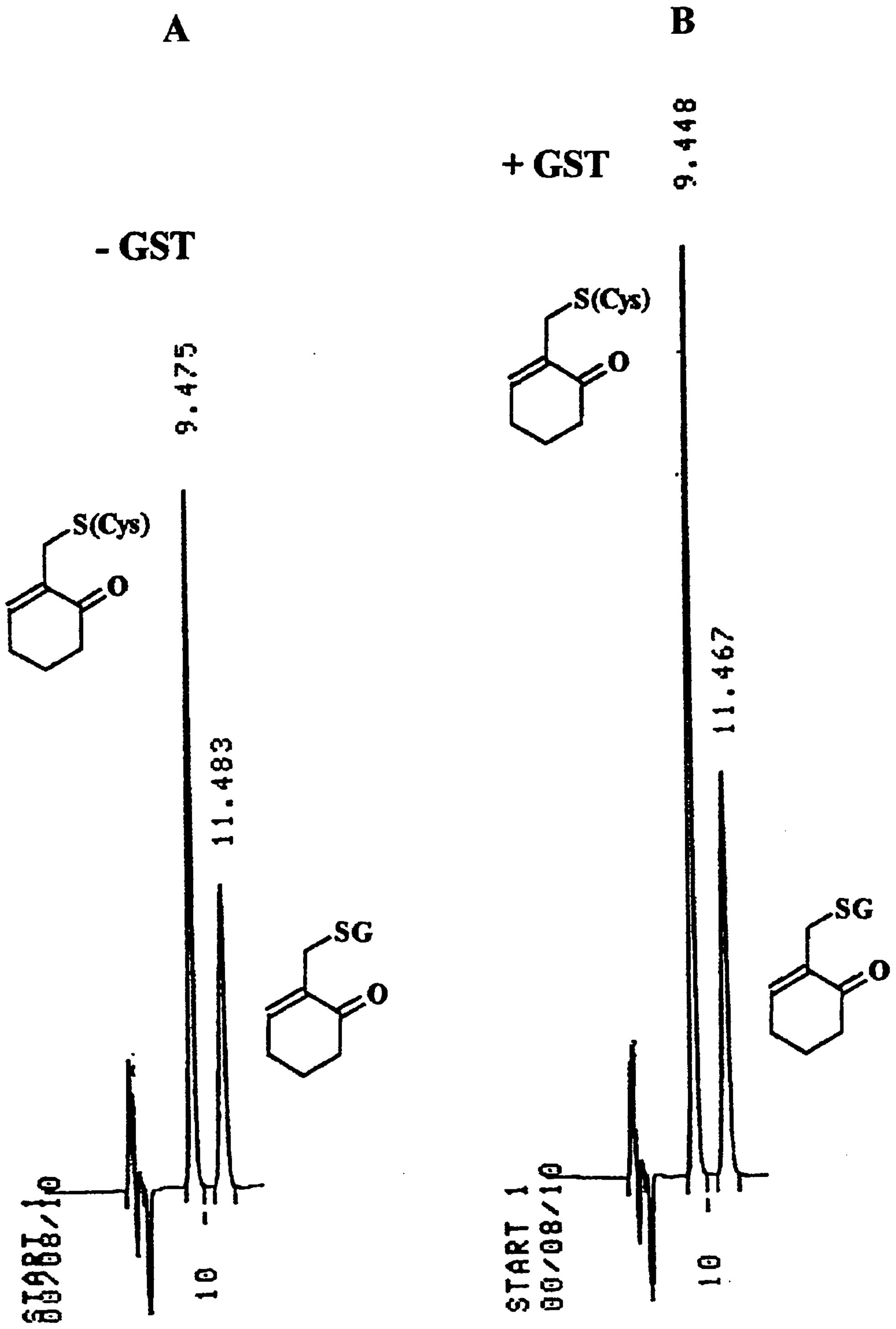


Fig. 5

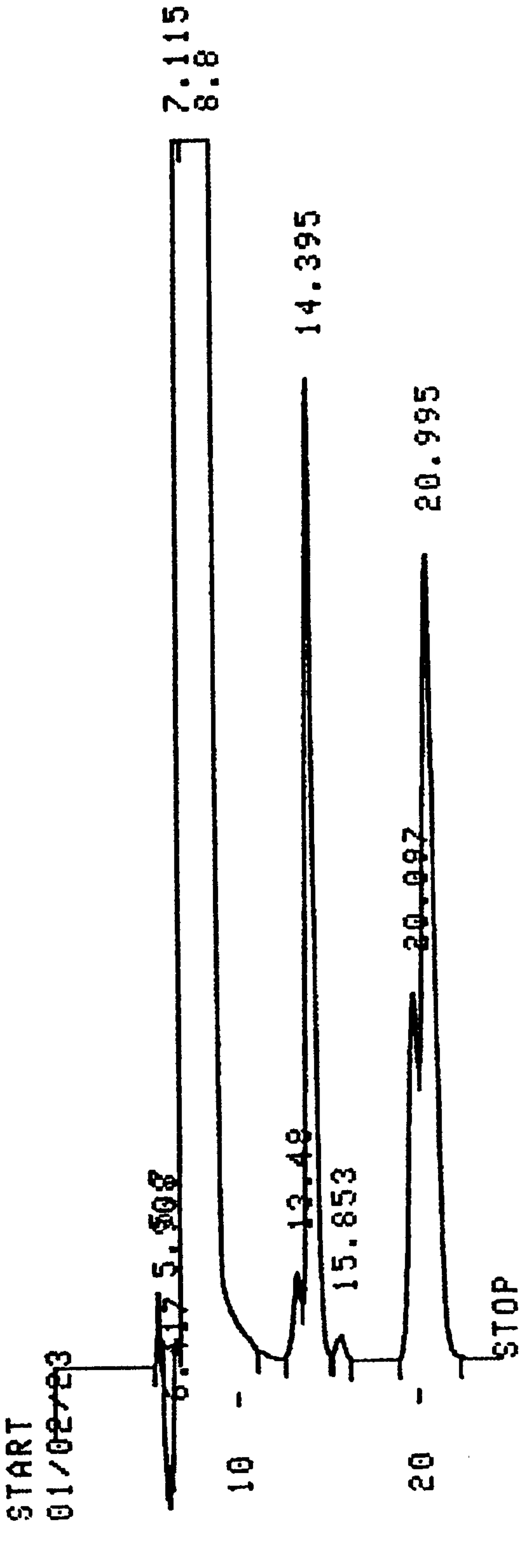

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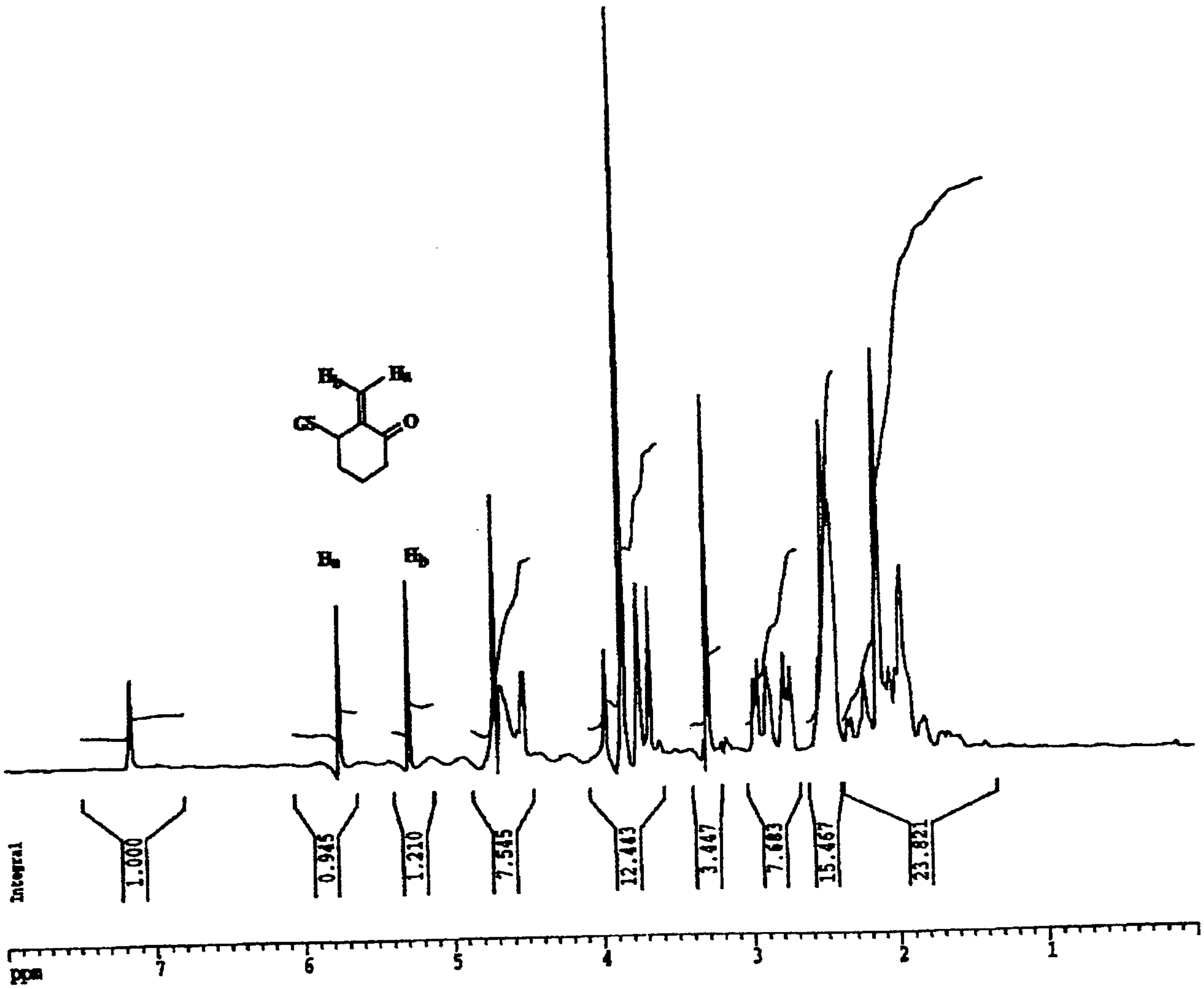
Fig. 6



Hb
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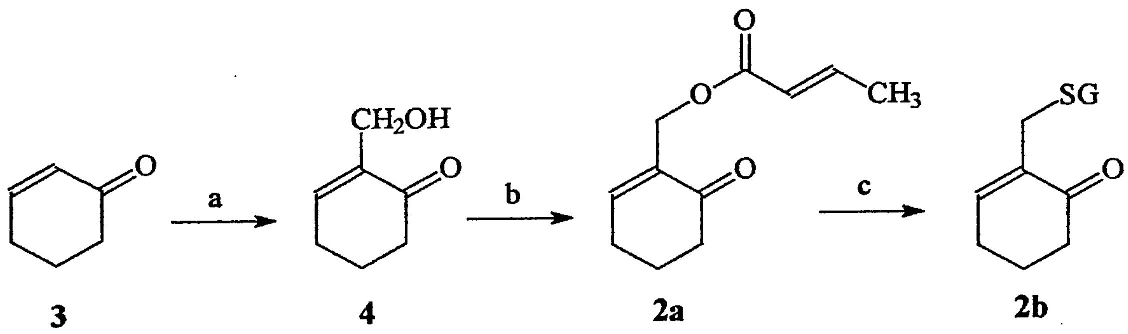


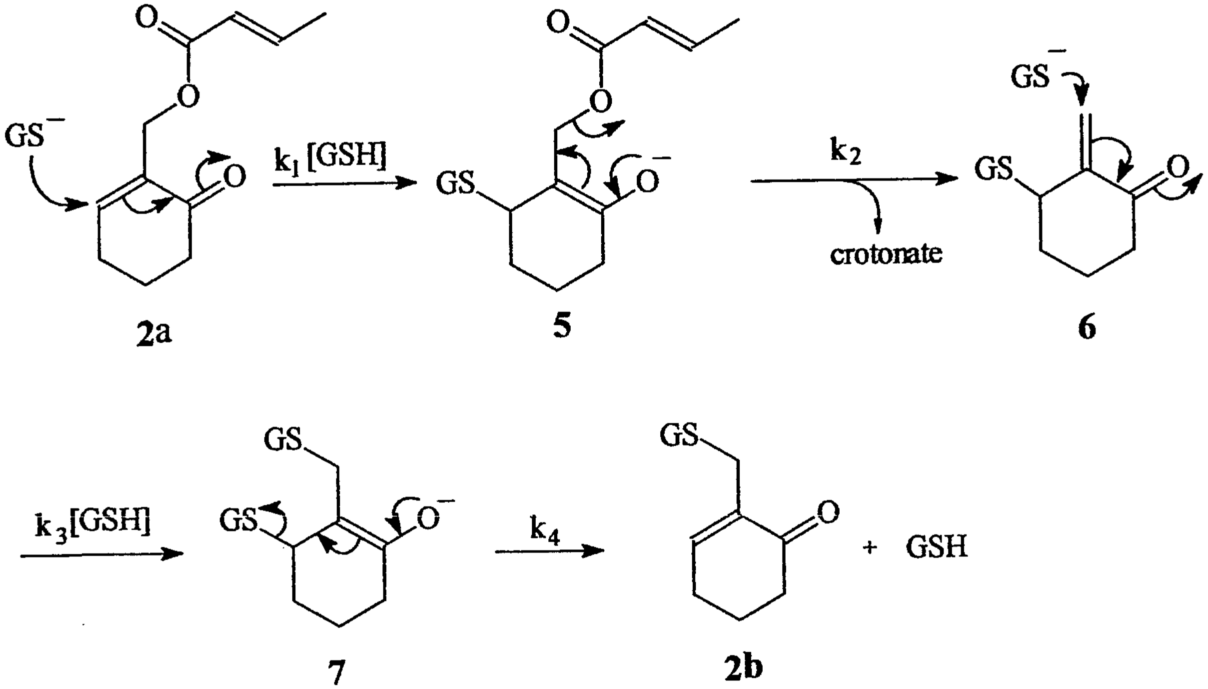
(a) DMAP, CH_2O, THF, rt 65%;

(b) crotonic anhydride, pyridine, DMAP, rt, 92%;

(c) glutathione, phosphate buffer (pH 7.5), 37 °C, 10 min, 85%.

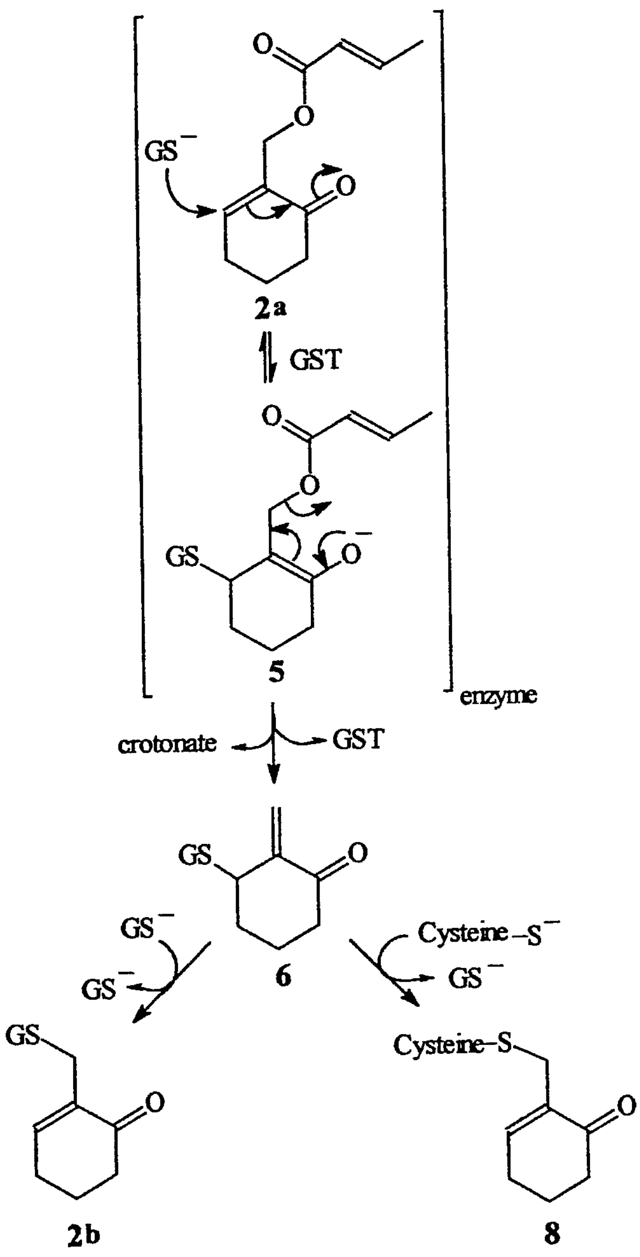
Synthetic Route to COMC **2a** *and its Glutathione Adduct* **2b**.

Scheme 1



Putative Reaction Mechanism for the Addition of Glutathione to **2a**.

Scheme 2



Mechanism for the Glutathione Transferase-Catalyzed Conjugation of Glutathione and Cysteine to COMC **2a** *via Intermediate* **6**

Scheme 3

Reaction of COTC with Glutathione: Structure of the Putative Glyoxalase I Inhibitor

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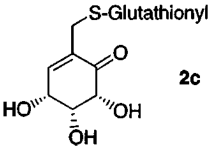
Received July 17, 2000



2c

The structure of the active glyoxalase I inhibitor derived from the *Streptomyces griseosporeus* metabolite COTC 1 has been conclusively identified by means of total synthesis as 2c. Human glyoxalase I is competitively inhibited by 2c (K_i = 183 ± 6 μM) but is not inhibited by 1 itself.

Cytotoxic methylglyoxal is removed from cells as its glutathione adduct by the enzymes glyoxalase I and II. In 1975, a new inhibitor of glyoxalase I having structure **1** was isolated from the culture broth of *Streptomyces griseosporeus* by Umezawa and co-workers.[1] Known as COTC, compound **1** was also reported to exhibit cytotoxic and cancerostatic activity with low toxicity.[2] The potential of COTC as an anticancer drug has attracted the attention of several synthetic research groups, and five successful total syntheses have now been reported.[3–7] Despite its prospective therapeutic significance, the mechanism of action of COTC remains incompletely understood.

Umezawa et al. noted that by itself COTC had no effect on glyoxalase I, even in the presence of the substrate, methylglyoxal.[1] However, in the presence of reduced glutathione (GSH), time-dependent inhibition of the enzyme was observed. In studies with rat Yoshida glyoxalase I, **1** exhibited an apparent IC$_{50}$ of 8.8×10^{-4} M in phosphate buffer containing methylglyoxal (0.13 M) and GSH (0.04 M). Consequently, the biological activity of COTC was thought to involve nucleophilic addition of GSH, an hypothesis later supported by the change in UV absorbance and loss of titratable SH groups noted when **1** was exposed to GSH in the absence of methylglyoxal.[8] While the reaction product of COTC with GSH has never been isolated and characterized, **1** does react with 2-mercaptoethanol and *p*-bromothiophenol to form thioethers **2a** and **2b**, respectively. Moreover, the structure of **2b** has been confirmed by X-ray crystallography.[2] On the basis of those findings, it has

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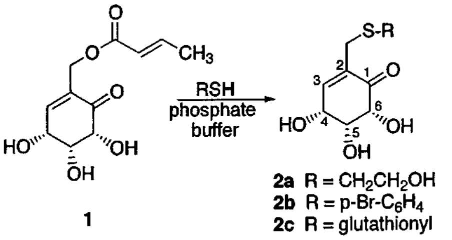
10.1021/ol006341z CCC: $19.00

been proposed that GSH combines with **1** to form **2c**. A variety of mechanisms have been suggested for this transformation, including direct displacement of crotonate,[8] two stepwise 1,4-addition/β-elimination reactions,[9] or 1,4-addition and sigmatropic rearrangement.[8] Such mechanistic speculation seemed premature in the absence of conclusive confirmation of the structure of the GSH reaction product. Here we report the isolation and purification of the GSH adduct of (−)-COTC and subsequent characterization of that structure as **2c**. In addition, we present quantitative kinetic information on the interaction of pure **2c** with glyoxalase I.



Freshly prepared synthetic (−)-**1** (13.5 mg)[7] was stirred with GSH (15.4 mg) in sodium phosphate buffer (pH 7.5) for 10 min at 37 °C and then poured onto an ion-exchange column (2 cm × 2 cm Dowex-1 resin)[10] and eluted with aqueous acetic acid to afford **2c** in 93% yield.[11] Besides the loss of crotonate and gain of glutathionyl resonances, the NMR spectrum of **2c** featured a singlet at δ 6.74 for H3 that is characteristic of β-unsubstituted cyclohexenones such as **1** and its congeners.

Adduct **2c** was a moderately potent competitive inhibitor of human erythrocyte glyoxalase I[12] (K_i = 183 ± 6 μM, Figure 1). Moreover, the inclusion of 0.2 mM COTC **1** in an assay cuvette did not reduce the initial rate of product formation beyond what would be expected from a small increase in the production of additional **2c**,[13] confirming that COTC does not inhibit the enzyme.

The fact that adduct **2c** is a moderately potent competitive inhibitor of glyoxalase I argues against an earlier suggestion that COTC functions simply by depleting levels of GSH needed to form the methylglyoxal hemithioacetal substrate.[8] Studies with a variety of S-substituted glutathionyl derivatives have established that K_i values decrease with increasing hydrophobicity of the sulfur substituent. These findings suggest the presence of a hydrophobic binding pocket in the active site of human glyoxalase I, which has recently been confirmed using high-resolution X-ray crystallographic analysis.[14] The consequent possibility that less polar analogues of **2c** may bind more tightly to the enzyme will be explored further in ongoing structure−activity studies in our laboratories.

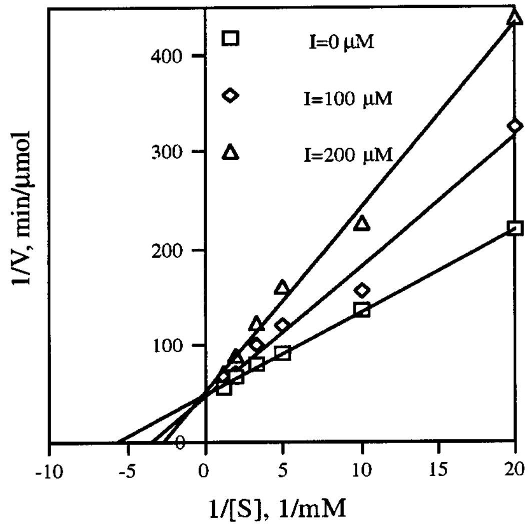


Figure 1. Reciprocal plot of the velocity of the glyoxalase I reaction (ΔA_{240}) versus the concentration of GSH-methylglyoxal thiohemiacetal ([S]) in the presence and absence of **2c** ([I]). In each kinetic run, the concentration of free GSH was maintained at 0.2 mM by varying the total concentration of GSH and methylglyoxal on the basis of the dissociation constant of the hemithioacetal (K_{diss} = 2.2 mM).[15] Conditions: 50 mM phosphate buffer, pH 7.0, 25 °C.

Acknowledgment. This work was supported in part by grants from the NIH (GM 24054, to B.G.; CA 59612, to D.J.C.) and the U.S. Army Medical Research and Materiel Command (to D.J.C.). Support of the Cornell NMR Facility has been provided by NSF and NIH.

OL006341Z

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(11) For **2c**: mp 210−220 °C dec; R_f 0.40 (10:1:5 *n*-PrOH:AcOH:H_2O); ^{1}H NMR (300 MHz, D_2O) δ 6.74 (s, 1 H), 4.51 (dd, 1 H, J = 4.8, 7.0 Hz), 4.43 (m, 2 H), 3.84 (s, 2 H), 3.75 (dd, 1 H, J = 6.4, 5.9 Hz), 3.41, 3.28 (AB q, 2 H, J = 14.0 Hz), 2.95, 2.76 (ABX, 2 H, J_{AB} = 14.0, J_{AX} = 9.1, J_{BX} = 4.8 Hz), 2.48 (m, 2 H, J = 3.8, 3.9, 2.7 Hz), 2.11 (dd, 2 H, J = 7.5, 6.5 Hz); ^{13}C NMR (300 MHz, D_2O) δ 196.3, 172.2, 169.6, 144.3, 130.6, 73.4, 72.5, 64.9, 51.4, 50.2, 39.6, 29.8, 28.5, 26.3, 23.3; MS ES *m/z* 464 (M + 1, 57%), 60 (100%).

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(13) The rise in [**2c**] in the assay cuvette was estimated from the second-order rate constant for formation of **2c** from GSH and **1** (k = 0.12 mM^{-1} min^{-1}, pH 7, 25 °C).

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