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SYNTHESES AND KINETIC EVALUATION OF HYDROXAMATE-BASED PEPTIDE INHIBITORS OF GLYOXALASE I

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Abstract: Hydroxamate-containing tripeptide analogs resembling a reactive intermediate in glyoxalase I catalysis were prepared by solution methods and were found to be competitive inhibitors of the enzyme from *Saccharomyces cerevisiae*. Electronic properties of the hydroxamate functionality as well as those of the expected intermediates in the enzyme-catalyzed reaction were compared.

The ubiquitous glyoxalase system converts toxic endogenously-produced 2-oxoaldehydes, such as methylglyoxal (MG), into 2-hydroxyacids, utilizing glutathione (GSH) as an essential cofactor (Figure 1). This system is composed of two enzymes.[1,2] The first enzyme, glyoxalase I (*S*-lactoylglutathione methylglyoxal lyase (isomerizing) EC 4.4.1.5, GlxI) is a zinc metalloprotein which catalyzes the isomerisation of the hemithioacetals formed non-enzymatically between GSH and the cytotoxic α-ketoaldehydes. Glyoxalase II (*S*-2-hydroxyacyl-glutathione hydrolase, EC 3.1.2.6, GlxII) hydrolyses the GSH-thioester product of the GlxI reaction, regenerating GSH. It is believed that a major physiological role for this enzyme system is as a detoxification pathway for cytotoxic MG.[3]

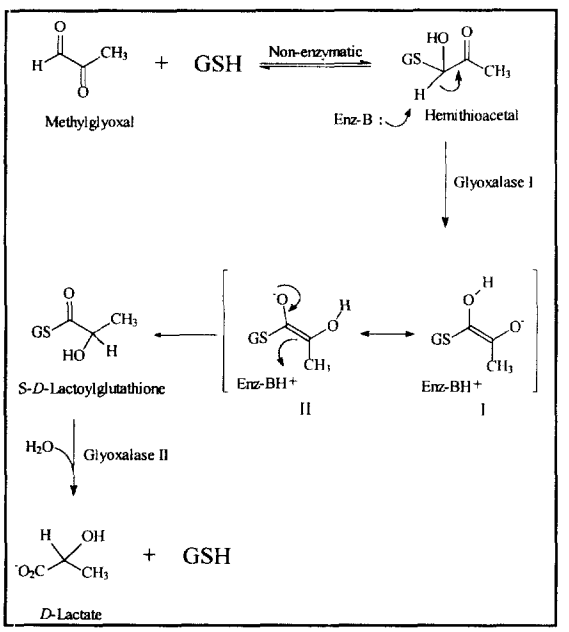

Figure 1. The glyoxalase system.

Inhibitors of GlxI have been found to exhibit anti-proliferative effects on cancerous cells as well as on bacterial and parasitic infections,[2] a result possibly due to the inability of the blocked glyoxalase system to detoxify cellular MG. Recent reports have indicated that human tumor cell lines contain functional glyoxalase I and varying levels of glyoxalase II.[4] Hence, potent glyoxalase I inhibitors could prove to be valuable therapeutic agents, in addition to providing information about the active site of glyoxalase I.

A variety of *S*-alkylated glutathione analogs as well as nonpeptide-based compounds resembling the possible enediol(ate) reaction intermediate of glyoxalase I have been investigated as GlxI inhibitors.[5,6] The synthesis of

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glutathione derivatives containing a variety of *S*-linked hydroxamate moieties has recently been reported and these analogs have proven to be effective competitive inhibitors of the yeast and human glyoxalase I enzymes.[7] These compounds were also found to be slowly hydrolyzed by GlxII.

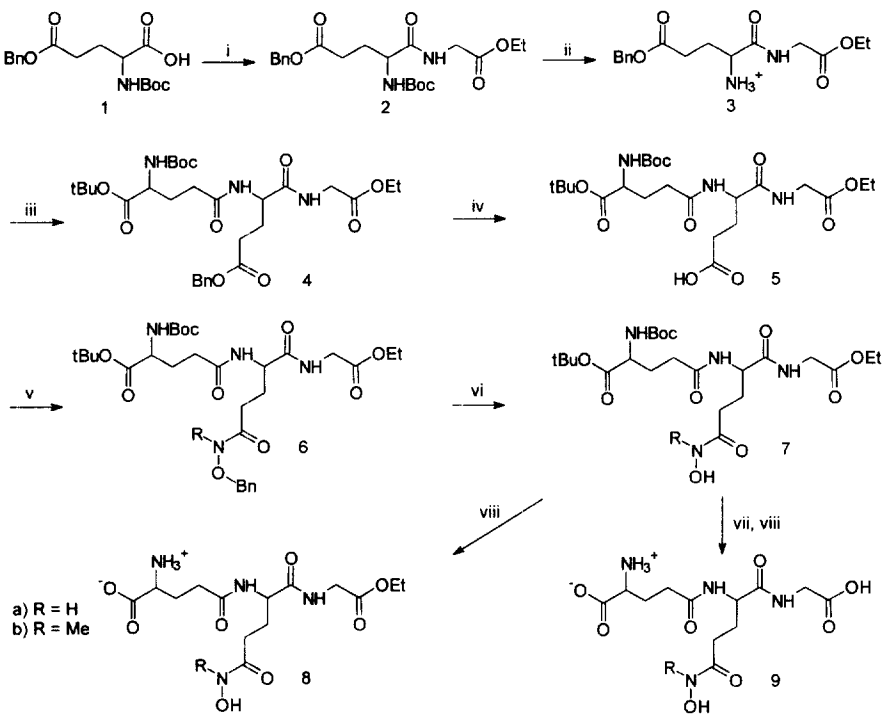
Because of our continuing interest in the mechanistic characterization of GlxI,[8] we felt that compounds which might resemble either of the two possible enzyme-catalyzed reaction intermediates (**I** and **II**; Figure 1) could be useful probes of the GlxI reaction. In addition these compounds could aid in the development of therapeutically useful GlxI inhibitors. In order to obtain a "matched set" of compounds that selectively resembled either intermediate **I** or **II**, a carbon-based hydroxamate linkage seemed appropriate. Triose phosphate isomerase, an enzyme generating an enediol(ate) intermediate, has been successfully probed by hydroxamate-containing substrate analogs.[9] As well, the carbon-based hydroxamate linkage would be expected to stabilize the analog to hydrolysis by the second enzyme of the system, GlxII, and therefore extend the metabolic lifetime of the inhibitor in an intact biological system such as a tumor cell. Therefore unsubstituted and *N*-methyl substituted tripeptide hydroxamates resembling the possible reactive intermediate **II** were prepared. Based on earlier work which indicated that glycyl ethyl esters are more readily transported across the cellular membrane,[7a,10] ethyl ester derivatives were also studied in various cell assays.

Chemistry

Preparation of the tripeptide hydroxamates and their ethyl esters is outlined in Scheme 1. Coupling of glycine ethyl ester and *N*-*t*-Boc-*L*-glutamic acid-γ-benzyl ester (**1**) in the presence of triethylamine and HBTU at room temperature[11] gave compound **2**. Removal of the Boc group by treatment with excess TFA in methylene chloride afforded compound **3**. Coupling of **3** with *N*-*t*-Boc-*L*-glutamic acid-α-*t*-butyl ester in the presence of triethylamine gave tripeptide **4**. Removal of the benzyl group was achieved by transfer hydrogenation with ammonium formate and palladium on activated carbon.[12] Purification by silica gel chromatography (0.2% acetic acid/ ethyl acetate) afforded tripeptide **5** in an overall reaction yield of 54%. Compound **5** was dissolved in acetonitrile at –20 °C in the presence of triethylamine and the appropriate protected hydroxylamine.[13] The reactions were initiated by addition of HBTU and gave **6a** and **6b** in yields of approximately 90%. Removal of the *O*-benzyl protecting group was achieved by transfer hydrogenation ($NH_4^+HCO_2^-$/ 10% Pd-C) to give **7a** and **7b**. Removal of the Boc and *t*-butyl ester groups by treatment of **7a** and **7b** with excess TFA in methylene chloride at room temperature for 2–3 h gave the hydroxamate analogs **8a** and **8b**. Hydroxamates **9a** and **9b** were generated by lithium hydroxide hydrolysis of the ethyl ester and TFA mediated cleavage of the Boc and *t*-butyl ester groups of **7a** and **7b**. Purification was effected using reversed phase HPLC to give **8a**, **8b**, **9a**, and **9b** in yields of 40–65%.[14]

Results and Discussion

The peptides were tested as inhibitors of glyoxalase I from *Saccharomyces cerevisiae* and as inhibitors and possible substrates of bovine liver glyoxalase II. A summary of the inhibition of the yeast glyoxalase I by hydroxamates **8**–**9**, is listed in Table 1.[15] Figure 2 contains a plot illustrating a sample of the analyzed data for



Scheme 1. (i) glycine ethyl ester, HBTU, Et_3N, CH_3CN, 90%; (ii) TFA, CH_2Cl_2; (iii) *N*-*t*-Boc-*L*-glutamic acid-α-*t*-butyl ester, HBTU, Et_3N, CH_3CN, 76%; (iv) $NH_4^+HCO_2^-$/10% Pd-C, MeOH, 79%; (v) BnO-NHR, (R = H, Me), HBTU, Et_3N, CH_3CN, –20 °C, 90%; (vi) $NH_4^+HCO_2^-$/10% Pd-C, MeOH, 70–80%; (vii) LiOH, THF/H_2O (3:1), 0 °C, 85%; (viii) TFA, CH_2Cl_2, 40–65%.

9b. No time dependence was noted for the inhibition of yeast GlxI by **9a** or **9b**. The *C*-linked hydroxamate, **9b**, has a K_i approximately 35 times lower than the reported *S*-linked hydroxamate (*S*-(*N*-hydroxy-*N*-methylcarbamoyl)glutathione).[7] Hydroxamate **9b** also binds ~250-fold tighter than the K_m of the substrate of GlxI, GSH-MG hemithioacetal,[16] and >1000-fold better than the product of the GlxI reaction, *S*-lactoylglutathione.[7a] In comparison phosphoglycolohydroxamate (PGH), which has been an useful probe of rabbit muscle TIM (K_i = 4 μM), binds ~150-fold tighter than dihydroxyacetone phosphate and ~78-fold tighter than *D*-glyceraldehyde-3-phosphate.[17]

To compare the effectiveness of these inhibitors to those previously studied, one of the most potent glyoxalase I inhibitors, *p*-bromobenzylglutathione (**10**) was also tested. The inhibition results obtained for **10** were similar to those previously reported.[18] The activity of **10** may be due to its ability to make use of a hydrophobic region in the active site of GlxI.[1] This appears to be substantiated by the recently reported structure of the human GlxI with *S*-benzyl-glutathione bound.[19] The comparable activities of **9b** and **10** are interesting in that **9b** lacks the extensive hydrophobic portion for contribution to binding to the enzyme. Compound **9b**, which contains a *N*-methyl group, binds more tightly to the enzyme than **9a**, perhaps indicative of additional hydrophobic contributions which could be made in this compound. This may indicate that analogs containing

more hydrophobic *N*-linked moieties could increase the affinity of the inhibitor for the enzyme. This has been observed for the *S*-linked hydroxamates studied previously.[7b]

Table 1. Inhibition of *S. cerevisiae* Glyoxalase I

9b	1.9 ± 0.2	3.9 ± 0.3
8b	ND[3]	140.5 ± 3.3
9a	92.9 ± 2.2	209.6 ± 15.6
8a	ND	1624.6 ± 52
10	4.3 ± 0.5	8.2 ± 0.1

[1]K_i values were determined by measuring the enzymatic activity with no inhibitor and 3 additional inhibitor concentrations, at each of 6 substrate concentrations between 0.1 mM and 2.0 mM. Each point was measured in triplicate and each set of K_i measurements performed in triplicate, except **10**, which was tested in duplicate.
[2]IC_{50} values were determined by measurement of the activity with 0.5 mM substrate. For **8a** and **8b**, seven different inhibitor concentrations were measured. For the other three inhibitors, the three values measured in the K_i determination, with 0.5 mM substrate, were used to determine the IC_{50}.
[3]ND = Not determined.

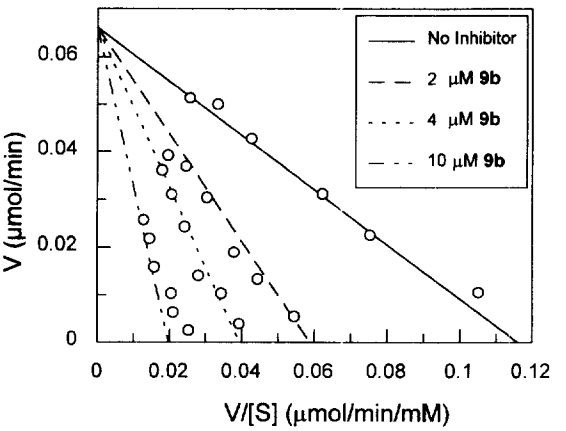


Figure 2. Eadie-Hofstee Plot for Inhibition of *S cerevisiae* Glyoxalase I by **9b**.

An electronic structure comparison of the hydroxamate functionality in **9b** compared to models of intermediates **I** and **II**, indicate that **9b** more closely resembles the charge distribution found in intermediate **II**. This may account for its binding affinity to GlxI (Figure 3).[20] To determine if **9b** is a substrate for GlxII, the hydroxamate was incubated with bovine liver GlxII and degradation was monitored by ^{1}H NMR and mass spectrometry.[21] No hydrolysis was observed over a 4 day period, and **9b** exhibited no detectable inhibition of GlxII at concentrations as high as 1.5 mM.[22]

Compounds **8a,b** and **9a,b** were tested for antitumor and antimalarial activity.[23] Testing against 60 cancer cell lines indicated that treatment with 100 μM **8a** or **9b** inhibited growth of CNS cancer (SNB-75) by 40%, whereas 100 μM **9a** inhibited growth of leukemia (HL-60 (TB)) by 46%, non-small cell lung cancer (NCI-H522) by 38%, renal cancer (A498) by 34%, and CNS cancer (SNB-75) by 31%. Compound **8a** (100 μM) inhibited growth of renal cancer (A498) by 31%. Antimalarial testing was performed with *Plasmodium falciparum* clones Sierra Leone (D6) and Indochina (W2), and compounds **9a,b** exhibited moderate activity (IC_{50} = 5–20 μM).

Compound **9b** will be useful in crystallographic investigations of the active site of GlxI. These studies may also elucidate whether inhibition by **9b** is due to its resemblance to intermediate **II**, or solely due to its direct interaction with the metal atom, as is often seen in hydroxamate inhibition of various metalloproteinases.[24] As well, it may be possible to utilize this hydroxamate analog and its retro-analog, the *N*-hydroxy-*N*-acetyl-*L*-2,4-diaminobutanoate-based tripeptide, to clarify the detailed changes in active site geometry occurring along the enzymatic reaction pathway. The retro-analog of **9b** is the subject of current synthetic efforts.

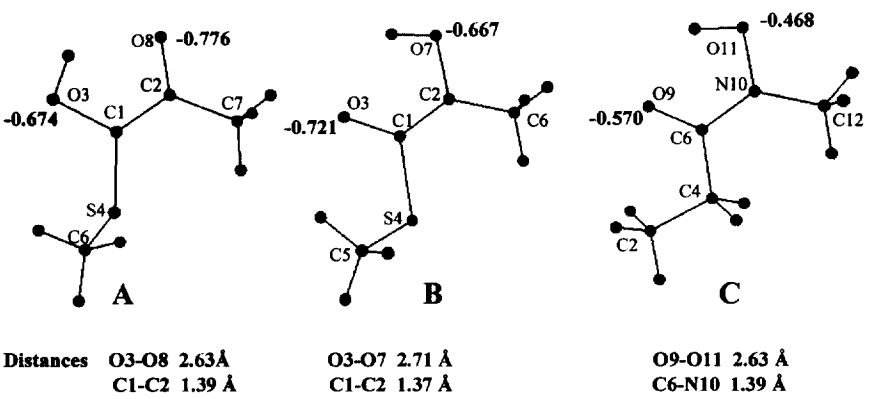


Figure 3. MP2/6-31+G*//B3LYP/6-31+G* level calculations on model compounds representing (**A**) intermediate **I**, (**B**) intermediate **II**, and (**C**) hydroxamate **9b**. Thiomethyl groups replaced GSH in **A** and **B**, and ethyl replaced the peptide in **C**. The electrostatic potential-derived charges on oxygens were calculated at the MP2/6-31+G* level using the CHelpG algorithm in Gaussian 94.

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References and Notes

1. Vander Jagt, D. L. In *Coenzymes and Cofactors VIII: Glutathione*; Dolphin, D.; Poulsin, R.; Avramović, O. Eds.; Wiley: N.Y., 1989; Part A, pp 597–641.
2. Thornalley, P. J. *Crit. Rev. Oncol./Hematol.* **1995**, *20*, 99.
3. (a) Vander Jagt, D. L. *J. Med. Chem.* **1975**, *18*, 1155. (b) Richard, J. P. *Biochemistry* **1991**, *30*, 4581.
4. (a) Hooper, N. I.; Tisdale, M. J.; Thornalley, P. J. *Leuk. Res.* **1987**, *11*, 1141. (b) Ayoub, F.; Zaman, M.; Thornalley, P.; Masters, J. *Anticancer Res.* **1993**, *13*, 151. (c) Tew, K. D.; Monks, A.; Barone, L.; Rosser, D.; Akerman, G.; Montali, J. A.; Wheatley, J. B.; Schmidt, D. E. Jr. *Mol. Pharmacol.* **1996**, *50*, 149.
5. (a)Vince, R.; Daluge, S.; Wadd, W. B. *J. Med. Chem.* **1971**, *14*, 402. (b) Jordan, F.; Cohen, J. F.; Wang, C.-T.; Wilmott, J. M.; Hall, S. S. *Drug Metab. Rev.* **1983**, *14*, 723. (c) Thornalley, P. J.; Ladan, M. J.; Ridgway, S. J. S.; Kang, Y. *J. Med. Chem.* **1996**, *39*, 3409.
6. (a) Douglas, K. T.; Gohel, D. I.; Nadvi, I. N.; Quilter, A. J.; Seddon, A. P. *Biochim. Biophys. Acta* **1985**, *829*, 109. (b) Barnard, J. F.; Honek, J. F. *Biochem. Biophys. Res. Commun.* **1989**, *165*, 118.
7. (a) Hamilton, D. S.; Creighton, D. J. *J. Biol. Chem.* **1992**, *267*, 24933. (b) Murthy, N. S. R. K.; Bakeris, T.; Kavarana, M. J.; Hamilton, D. S.; Lan, Y.; Creighton, D. J. *J. Med. Chem.* **1994**, *37*, 2161.
8. (a) Barnard, J. F.; Vander Jagt, D. L.; Honek, J. F. *Biochim. Biophys. Acta* **1994**, *1208*, 127. (b) Clugston, S. L.; Daub, E.; Kinach, R.; Miedema, D.; Barnard, J. F. J.; Honek, J. F. *Gene* **1997**, *186*, 103.
9. (a) Davenport, R. C.; Bash, P. A.; Seaton, B. A.; Karplus, M.; Petsko, G. A.; Ringe, D. *Biochemistry* **1991**, *30*, 5821. (b) Verlinde, C. L. M. J.; Witmans, C. J.; Pijning, T.; Kalk, K. H.; Hol, W. G. J.; Callens, M.; Opperdoes, F. R. *Protein Sci.* **1992**, *1*, 1578.
10. Anderson, M. E.; Powrie, F.; Puri, R. N.; Meister, A. *Arch. Biochem. Biophys.* **1985**, *239*, 538.
11. Dourtoglou, V.; Gross B. *Synthesis* **1984**, 572.
12. Anwer, M. K.; Spatola, A. F. *Synthesis* **1980**, 929.
13. Sulsky, R.; Demers, J. P. *Tetrahedron Lett.* **1989**, *30*, 31.

14. **8a** HPLC (C18) Retention time: 28 min at flow rate 2 mL/ min, (5% CH_3OH/ 0.25% AcOH); H NMR (250 MHz, D_2O) δ: 4.19 (1H, dd, J_{AX} = 8.9 Hz, J_{AB} = 5.4 Hz), 4.05 (2H, q, J = 7.1 Hz), 3.89 (1H, d, J = 16.8 Hz), 3.82 (1H, d, J = 16.8 Hz), 3.62 (1H, t, J = 5.0 Hz), 2.25-2.45 (2H, m), 2.10-2.25 (2H, m), 1.70-2.25 (4H, m), 1.12 (3H, t, J = 7.1 Hz); ^{13}C NMR (250 MHz, D_2O) δ: 175.3, 174.5, 174.3, 171.9, 171.8 (C=O), 54.5, 53.5 (CH), 63.0, 41.8, 31.6, 29.0, 27.2, 26.4 (CH_2), 13.7 (CH_3); *m/z* (FABMS) 377.3600 (M+1, 100%). **8b** HPLC Retention time: 26 min at flow rate 3 mL/ min, (5% CH_3OH/ 0.25% AcOH); ^1H NMR (250 MHz, D_2O) δ: 4.18 (1H, dd, J_{AX} = 9.3 Hz, J_{AB} = 5.7 Hz), 4.03 (2H, q, J = 7.1 Hz), 3.88 (1H, d, J = 16.8 Hz), 3.80 (1H, d, J = 16.8 Hz), 3.59 (1H, t, J = 5.4 Hz), 3.05 (3H, s), 2.25-2.60 (4H, m), 1.70-2.10 (4H, m), 1.08 (3H, t, J = 7.1 Hz); ^{13}C NMR (250 MHz, D_2O) δ: 175.2, 174.6, 174.3, 171.8 (C=O), 54.5, 53.7 (CH), 41.9, 31.6, 28.3, 26.7, 26.5 (CH_2), 36.4, 13.7 (CH_3); *m/z* (FABMS) 391.1816 (M+1, 100%). **9a** HPLC Retention time: 20 min at flow rate 2 mL/ min, (0.2% AcOH); ^1H NMR (250 MHz, D_2O) δ: 4.18 (1H, dd, J_{AX} = 9.7 Hz, J_{AB} = 5.6 Hz), 3.87 (1H, d, J = 16.2 Hz), 3.79 (1H, d, J = 16.2 Hz), 3.66 (1H, s), 2.25-2.50 (2H, m), 1.70-2.20 (6H, m); ^{13}C NMR (250 MHz, D_2O) δ: 174.9, 174.2, 173.5, 172.5, 172.0 (C=O), 53.5, 53.1 (CH), 41.5, 31.4, 29.0, 27.2, 25.9 (CH_2); *m/z* (FABMS) 349.1357 (M+1, 100%). **9b** HPLC Retention time: 12 min at flow rate 4 mL/ min, (0.25% AcOH); ^1H NMR (250 MHz, D_2O) δ: 4.19 (1H, dd, J_{AX} = 9.7 Hz, J_{AB} = 5.6 Hz), 3.87 (1H, d, J = 16.1 Hz), 3.78 (1H, d, J = 16.1 Hz), 3.73 (1H, t, J = 5.2 Hz), 3.06 (3H, s), 2.25-2.50 (4H, m), 1.70-2.10 (4H, m); ^{13}C NMR (250 MHz, D_2O) δ: 175.0, 174.6, 174.5, 173.6, 173.2 (C=O), 53.7 (CH), 31.5, 28.3, 26.7, 26.2 (CH_2), 36.4 (CH_3); *m/z* (FABMS) 363.1515 (M+1, 100%).
15. Yeast GlxI (Grade IV, Sigma) was diluted in 50 mM sodium phosphate buffer, 30% glycerol, pH 6.6. GlxI assays were performed at 240 nm (ε = 2860 $M^{-1}cm^{-1}$)[25] in 50 mM degassed, sodium phosphate buffer, pH 7.0, 25 °C. To initiate each reaction, 0.1 units/mL of enzyme was added. GSH concentration was maintained at 0.1 mM, assuming a K_{diss} = 2.2 mM for the MG and GSH.[7a] Inhibitors **8a,b** and **9a,b** were dissolved in water and **10** in DMSO (final volume of DMSO of 1% (v/v) in the GlxI assays, which did not affect the GlxI enzymatic activity). Non-linear regression analysis and F-tests were utilized.
16. Hamilton, D. S.; Creighton, D. J. *Biochim. Biophys. Acta* **1992**, *1159*, 203.
17. Collins, K. D. *J. Biol. Chem.* **1974**, *249*, 136.
18. Marmstål, E.; Mannervik, B. *FEBS Lett.* **1979**, *102*, 162.
19. Cameron, A. D.; Olin, B.; Ridderström, M.; Mannervik, B.; Jones, T. A. *EMBO J.* **1997**, *16*, 3386.
20. Calculations performed utilizing PC SPARTAN (Wavefunction Inc., Irvine CA) or Gaussian 94, Rev. C.3 (Gaussian, Inc., Pittsburgh PA). CHelpG is based on Breneman, C. M.; Wiberg, K. B. *J. Comp. Chem.* **1990**, *11*, 361.
21. NMR results were gathered under two separate conditions, with 2 or 10 units of bovine liver GlxII (Sigma). In both cases, 5 mM inhibitor (**9b**), in 100 mM potassium phosphate buffer in D_2O, pD 7.0 was utilized. The solutions were monitored at 23 °C for 4 days (*N*-methyl protons), and the enzyme activity monitored at intervals. Electrospray mass spectrometry data were collected on a Micromass Quattro II triple-stage quadrupole mass spectrometer. Two units of GlxII and 5 mM **9b** were dissolved to a total volume of 50 μL in 100 mM Tris-HCl, pH 7.4. The reaction was monitored at intervals over a 24 h period. NMR and mass spectral analysis indicated that **9b** is not hydrolyzed by bovine liver GlxII.
22. Inhibition of bovine liver GlxII by **9b** was studied by measuring the activity of the enzyme at inhibitor concentrations of 100 μM–1.5 mM. Studies were performed with 0.5 mM *S*-lactoylglutathione, 0.1 units of enzyme/mL, in 100 mM potassium phosphate buffer, pH 7.4, 25 °C. The decrease in the absorbance of the substrate was monitored at 240 nm for 90 seconds and the initial velocity determined from the slope.
23. (a) Anticancer testing at NCI was based on the method of Boyd, M. R.; Paull, K. D. *Drug Dev. Res.* **1995**, *34*, 91. (b) Antimalarial testing was performed by the method of Makler, M. T.; Ries, J. M.; Williams, J. A.; Bancroft, J. E.; Piper, R. C.; Gibbins, B. L.; Hinrichs, D. J. *Am. J. Trop. Med Hyg.* **1993**, *48*, 739. Chloroquine IC_{50} values against the D6 and W2 strains of *P. falciparum* are 30 nM and 142 nM, respectively.
24. Powers, J. C.; Harper, J. W. In *Proteinase Inhibitors*; Barrett, A. J.; Salvensen, G., Eds; Elsevier: New York, 1986; pp 219-298.
25. (a) Bergmeyer, H. U.; Graβl, M.; Walter, H.-E. In *Methods of Enzymatic Analysis;* Bergmeyer, H. U. Ed.; Verlag Chemie: Deerfield Beach, Florida, 1983; Vol. 2, pp 217–218. (b) Vander Jagt, D. L.; Han, L.-P. B.; Lehman, C. H. *Biochemistry* **1972**, *11*, 3735.