ene–hexane extractable radiolabeled methylthio adduct formed was measured by the procedure of Bartsch et al.[2,16] Control experiments were carried out with heat-denatured enzyme.

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S-Carbobenzoxyglutathione: A Competitive Inhibitor of Mammalian Glyoxalase II

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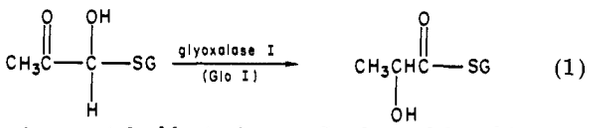
An effective competitive inhibitor of mammalian glyoxalase II has been synthesized and studied. The compound, *S*-carbobenzoxyglutathione, is almost totally inactive as an inhibitor of mammalian glyoxalase I. This is in marked contrast to other glyoxalase II competitive inhibitors, which in general are even more effective against glyoxalase I. *S*-Carbobenzoxyglutathione has found utility as an affinity ligand for the purification of rat liver glyoxalase II, and it may well have use in the study of the glyoxalase enzymes in vivo.

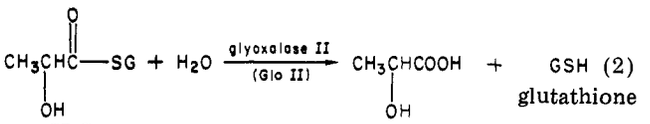
The widely distributed glyoxalase system[1-6] catalyzes the following reactions:



(1)

hemimercaptal adduct of methylglyoxal and glutathione

S-D-lactoylglutathione (SLG)



(2)

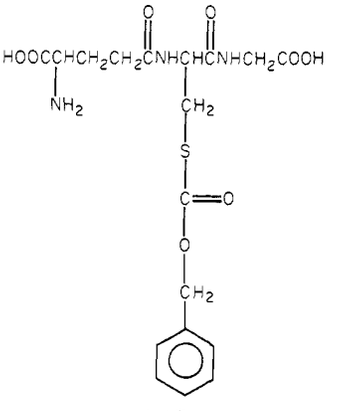
glutathione

SLG

D-lactic acid

Over the many years of study of the enzymes of the glyoxalase system, Glo I and Glo II (EC 4.4.1.5 and 3.1.2.6, respectively), a number of potential inhibitors of these enzymes have been synthesized and studied in vivo and in vitro.[7-12] Some of these inhibitors have antitumor activity.[8,9,12] Most of the compounds that serve as effective inhibitors have a structural relationship to the substrates of Glo I and Glo II; consequently, they generally act as competitive inhibitors of both of these enzymes. Thus, the most effective inhibitory compounds have been various alkyl, aryl, and phenacyl thioethers of GSH. An example, *S*-(*p*-chlorophenacyl)glutathione is an excellent inhibitor of both Glo I and Glo II;[7,13] it has been utilized in Glo II active-site mapping studies and as an affinity ligand in the purification of Glo II.[13,14]

While it might be anticipated that various alkyl and aryl thioesters of GSH might serve as inhibitors of the glyoxalase enzymes, the thioesters often serve as substrates for Glo II and, in general, are ineffective inhibitors of Glo I in crude systems or in vivo.[15] While searching for better ligands for the affinity chromatographic purification of rat liver Glo II, we synthesized a new inhibitor of Glo II. The compound, *S*-carbobenzoxyglutathione (CBG), is not hydrolyzed by Glo II, does not inhibit Glo I, and is a potent competitive inhibitor of Glo II. This is the first report, to our knowledge, of a competitive inhibitor of Glo II that is inactive toward Glo I from various sources. Further, the compound has been used effectively as an affinity ligand in a two-step total purification of rat liver Glo II.[16]



CBG

Results and Discussion

The synthesis of CBG was carried out under conditions that preclude condensation of carbobenzoxy chloride with the single free amino group of glutathione; thus, the pH of the aqueous phase of the reaction mixture was kept at pH 7.8 or lower. Oxidation of glutathione to oxidized glutathione could be largely prevented by carrying out the

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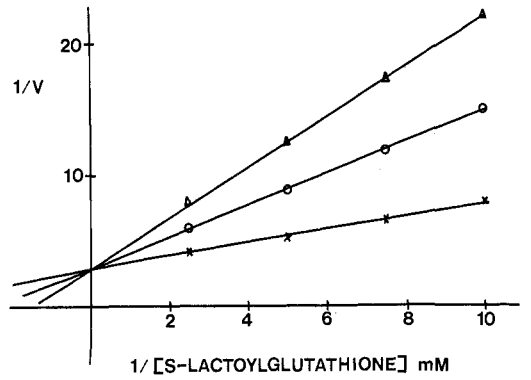

Figure 1. Lineweaver–Burk plot of the effects of *S*-carbobenzoxyglutathione (CBG) on homogeneous rat liver glyoxalase II activity. The assay method is give under Experimental Section. The *S*-lactoylglutathione (SLG) concentration was varied with the following constant levels of CBG: (×) 0.0 mM, (○) 0.1 mM, (△) 0.2 mM. Reaction velocity is in terms of micromoles of SLG hydrolyzed per minute.

condensation under nitrogen. The condensation of glutathione with carbobenzoxy chloride never exceeded 60–70% under a variety of conditions. It was therefore necessary to separate unreacted glutathione (and some oxidized glutathione) from product CBG by chromatography. Use of Sephadex G-15 in this regard was very effective; recovery of CBG from the reaction mixture was complete.

The inhibition studies were carried out by using various mammalian livers as sources of Glo I and Glo II. More detailed studies were performed on homogeneous rat liver Glo II[16] and homogeneous mouse liver Glo I.[17] As shown in Figure I, double reciprocal plots of kinetic data, employing pure rat liver Glo II, indicate that CBG is a strictly competitive inhibitor of Glo II. A Dixon plot from the data shown in the figure gave a plotted K_i value for CBG of 0.065 mM. Similar data were obtained for Glo II inhibitions by CBG when the enzyme source was crude liver extracts of rat, mouse, and sheep. When CBG was tested as a possible inhibitor of Glo I activity, with homogeneous liver Glo I or crude liver preparations from rat, mouse, or sheep as enzyme source, little or no inhibition was observed. For example, when a 12-fold higher CBG concentration than that giving >50% inhibition of Glo II was employed, <10% inhibition of Glo I activity was observed. This is in marked contrast to the inhibitory pattern found with inhibitors of Glo II previously described.[7,13,21]

While CBG has structural features similar to the thioesters of glutathione, the benzyloxycarbonyl group is sufficiently different that the compound is not hydrolyzed by Glo II. Various thioesters synthesized in this laboratory were subject to such thiohydrolase activity by Glo II and were not candidates for further study.

In addition to the successful utilization of CBG as an affinity ligand for the purification of rat liver Glo II,[16] it is possible that this compound will have other utilities. For example, *S*-lactoylglutathione (SLG), the product of Glo I activity, has been implicated as a mediator of a number of cellular processes.[22] *S*-Carbobenzoxyglutathione could possibly be used as an intracellular probe to promote the buildup of SLG by inhibiting its breakdown, thus permitting more definitive studies of the effects of SLG in metabolism.

Experimental Section

Glutathione, Sephadex G-15, and carbobenzoxy chloride (benzyl chloroformate) were purchased from Sigma Chemical Co., Pharmacia Fine Chemicals Co., and Aldrich Chemical Co., respectively. Glo II from rat liver was purified to homogeneity by two passes of the liver cytosolic extract through a *S*-carbobenzoxyglutathione (CBG) affinity column. Details of this procedure are being reported elsewhere.[16] Pure mouse liver Glo I was obtained as previously described.[17] Crude sheep and mouse liver cytosolic extracts were obtained by centrifugation (100000*g*) of the liver homogenates prepared in 50 mM phosphate buffer, pH 7.0, containing 20% glycerol. The final protein concentrations of the cytosolic extracts were approximately 20 mg/mL as determined by the procedure of Bradford.[18] *S*-D-Lactoylglutathione was prepared and purified by the procedure of Uotila.[23]

***S*-Carbobenzoxyglutathione (CBG) Synthesis.** An ice-cooled solution of 1.84 g (6.0 mmol) of glutathione in 6 mL of an aqueous solution containing 10 mmol of sodium bicarbonate was covered with 18 mL of ether. Carbobenzoxy chloride (1.023 g, 6.0 mmol) was then added in a single portion. The mixture was kept at 4 °C with vigorous stirring. After 24 h, a transparent, gellike mass separated out; this material was then stirred with successive portions of absolute ethanol until a white, hygroscopic, crystalline solid was obtained. The solid was filtered, washed with absolute ethanol, and then dried in a vacuum desiccator. The material gave three ninhydrin-positive spots on cellulose thin-layer chromatography (1-butanol/acetic acid/water, 4:1:1; R_f = 0.038, 0.276, and 0.73). *S*-Carbobenzoxyglutathione, representing approximately 50% of the crude reaction mixture, corresponded to R_f 0.73.

Purification of CBG. Crude reaction mixture (0.3 g), dissolved in a minimal volume of water, was placed on a Sephadex G-15 column (1.5 × 50 cm), and the flow rate was adjusted to 1.5 mL/h using water as eluant. The early fractions gave two ninhydrin spots on cellulose thin-layer chromatography (1-butanol/acetic acid/water, 4:1:1), R_f 0.038 and 0.276, which corresponded to oxidized glutathione and glutathione, respectively. Later fractions gave one ninhydrin spot on thin-layer chromatography (R_f 0.73) and corresponded to CBG. These fractions (recovery was complete) were combined, dried in vacuo, and recrystallized from ethanol. The pure CBG had a melting point of >200 °C dec and gave a negative test for the presence of SH groups.[19] The overall yield of very hygroscopic product was approximately 50%. Cellulose thin-layer chromatography of the pure material in pyridine/water (4:1) and silica gel thin-layer chromatography in phenol/water (4:1) gave single ninhydrin spots, R_f 0.39 and 0.48, respectively. Elemental analyses were within ±0.4% of theory for C, H, and N as calculated for the monosodium salt, one-half hydrate of CBG ($C_{18}H_{22}N_3O_8NaS\cdot 1/2H_2O$): ^{13}C NMR (D_2O) δ 176.2 (s), 174.7 (s), 174.2 (s), 171.6 (s), 171.1 (s), 134.9 (s), 128.8 (d), 128.6 (d), 128.4 (d), 69.8 (t), 54.4 (d), 54.3 (d), 43.7 (t), 32.4 (t), 31.7 (t), 26.5 (t).

Glyoxalase I Inhibition Studies. Glyoxalase I activity was determined by the procedure of Racker,[20] as modified by Oray and Norton.[17] The reaction was initiated by the addition of an appropriate level of crude extract or pure Glo I. Inhibition by CBG was determined by comparing enzyme activities in reaction mixtures containing various levels of the inhibitor with the uninhibited activity.

Glyoxalase II Inhibition Studies. The thiohydrolase activity of Glo II (pure enzyme or crude extract) was determined at 25 °C by the rate of decrease of the absorption (240 nm) of 0.4 mM *S*-D-lactoylglutathione. Details of this assay have been reported.[21] Inhibition of CBG was determined as described for the Glo I inhibition studies.

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Registry No. Glo II, 9025-90-5; SLG, 41656-56-8; CBG, 85270-49-1; glutathione, 70-18-8; carbobenzoxy chloride, 501-53-1.

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