was homogenized in 5 mL of ice-cold Tris-HCl (50 mM, pH 7.4) with a Polytron. The homogenate was centrifuged a first time at 460g for 3 min and the supernatant was recentrifuged at 22 400g for 20 min. The resulting supernatant was discarded, and each pellet was resuspended in 3 mL of buffer and centrifuged again at 22 400g for 20 min. The resulting pellet was again suspended in 3 mL of buffer, rehomogenized, divided into 0.5-mL fractions, and stored at -20 °C for at least 24 h before use. For the inhibition studies, the thawed membrane preparations were diluted with 20 volumes of ice-cold buffer, and 900-μL aliquots were incubated at 0 °C for 60 min with [^{3}H]flunitrazepam (76.9 Ci/mmol, NEN, final concentration of 0.4 nM) and varying concentrations of the test compound ranging from 10^{-5} to 5×10^{-10} M (final concentrations for a total volume of 1 mL). Nonspecific binding was measured in the presence of 10 μM nonradioactive flunitrazepam and represented 10–15% of the total binding. Incubations were terminated by adding 3 mL of cold buffer to each incubation tube, filtering through Whatman GF/B glass fiber filters, and washing each filter three times with 5 mL of cold buffer. Radioactivity retained on the filters was counted in 10 mL of Aquasol scintillation solution with an LKB Wallac 1215 Rackbeta 2 counter. Each value was determined in triplicate. IC_{50} values (the concentration of ligand inhibiting 50% of flunitrazepam binding) were determined by Hofstee analysis.

The acyl azide **7** was not tested since, under the conditions necessary to achieve and maintain solubilization (aqueous HCl, buffer), the molecule was not stable.

In Vivo Studies. Baboons. *Papio papio* baboons (5–10 kg) either naturally sensitive to intermittent light stimulation (ILS) (25 cps) of convulsions or nonsensitive to ILS were used.[26] EEG was registered by means of brain-implanted electrodes[26] connected to a Grass polygraph recorder. β-Carbolines (1–10 mg/kg) were suspended in 200–400 μL of saline and dissolved by the addition of the minimum amount of concentrated hydrochloric acid and the resulting solution was diluted to 1–2 mL with saline. All solutions were injected intravenously (iv). A compound was judged convulsant if it induced convulsions in nonphotosensitive baboons without the use of ILS. Proconvulsant compounds, without inducing convulsions in nonphotosensitive baboons, made these animals sensitive to ILS presented 1 min after drug administration and also induced convulsions in photosensitive animals without the use of ILS. A decrease in the latency time of the onset of a convulsion under ILS as well as enhanced proconvulsive symptoms (a higher score in the Killam scale of convulsant activity[26]) was also used as criterion for judging the proconvulsant character of a compound. Anticonvulsant compounds blocked the response to ILS in photosensitive animals, increased the latency time, and/or diminished the animal's score on the Killam scale.

Mice. Convulsion Studies with β-CMC (9). Male Swiss mice (25 g, 10 per group) were used to assess the pharmacological activity of β-CMC. Thus, β-CMC (solutions prepared as for baboons) was administered subcutaneously at doses up to 20 mg/kg alone, 10 min before pentylenetetrazol treatment (convulsive and subconvulsive doses) or 10 min before β-CCM treatment. For studies in conjunction with diazepam, β-CMC was injected 10 min after diazepam administration but 10 min before pentylenetetrazol.

Conflict Studies with β-CMC. The punishment–reward conflict experiments used in assessing the anxiolytic or anxiogenic properties of drugs have been previously described in detail.[8] Briefly, mice were deprived of food until they attained 80% of their free-feeding weight. They were then trained in a Skinner box to press a lever to receive a food pellet. After attainment of stable pressing rates of approximately eight presses/min, mice were submitted to 15-min daily sessions divided such that, during the first and last 5-min periods, each lever press resulted in a food pellet (nonconflict) while, during the central 5-min period, each lever press was similarly rewarded with a food pellet but also concomitantly punished with an electric foot shock (conflict). After 2 weeks of training on this 15-min paradigm, mice showed stable day-to-day performances. Anxiolytic drugs (diazepam) produce increased rates of lever pressing during the conflict period relative to control, while anxiogenic drugs (β-CCM) result in decreased rates of lever pressing during this period. β-CMC was injected alone (sc, up to 20 mg/kg) or 10 min after diazepam (1 mg/kg, sc). Groups of seven to ten mice were used for each experiment.

Rotarod Studies with β-CMC. The rotarod-deficit was evaluated on a 2.5-cm diameter wooden rod rotating at 4 rpm. Mice were placed backwards on the rotating rod in such a way that they had to turn around to face the movement. Mice were pretrained for a 5-min period 30 min before the beginning of the experiment. The ability of each mouse to stay on the rod for 1 min was then pretested just before the first drug treatment. The occasional mouse not succeeding this pretest was eliminated. Mice were then injected with diazepam (2 mg/kg, sc) followed 10 min later by β-CMC (up to 20 mg/kg, sc) or saline and tested every 10 min during the next hour. Each experiment was repeated on 10 mice.

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Registry No. 1, 74214-62-3; 6, 73834-74-9; 7, 73834-75-0; 8, 73834-77-2; 9, 91985-74-9; 10, 73834-76-1; 11, 91985-75-0; 12, 95935-49-2; 13, 95935-50-5; 14, 95935-51-6; 15, 95935-52-7.

S-(Nitrocarbobenzoxy)glutathiones: Potent Competitive Inhibitors of Mammalian Glyoxalase II

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Three potent competitive inhibitors of mammalian liver glyoxalase II, the *S*-(*o*-, *m*-, and *p*-nitrocarbobenzoxy)-glutathiones, have been synthesized and studied. The K_i values of the ortho, meta, and para isomers, as inhibitors of rat liver glyoxalase II, were 15, 9, and 6.5 μM, respectively. While showing marked competitive inhibition of glyoxalase II, the glutathione derivatives were almost inactive as inhibitors of glyoxalase I. For example, with the para isomer, $[I]_{0.5}$ values for rat liver glyoxalase I and II were 925 and 12 μM, respectively. This is in marked contrast to other glyoxalase II competitive inhibitors, which in general are even more effective against glyoxalase I. The *S*-(*o*-, *m*-, and *p*-nitrocarbobenzoxy)glutathiones have found utility as affinity ligands for the purification of rat liver glyoxalase II and may well have use in the study of the glyoxalase enzymes in vivo.

The glyoxalase system[1–6] catalyzes the reactions given in eq 1 and 2. Throughout the study of the enzymes of the glyoxalase system, Glo-I and Glo-II (EC 4.4.1.5 and 3.1.2.6, respectively), several inhibitors of these enzymes

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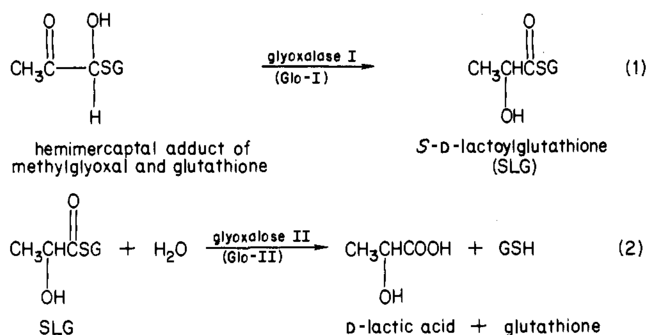
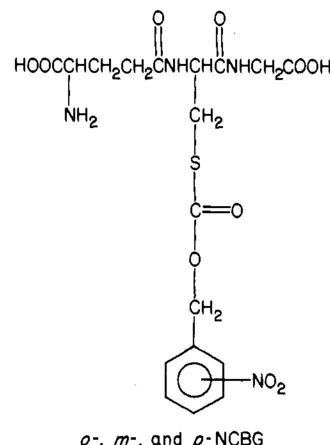


have been synthesized and studied in vivo and in vitro.[7-12] Most of the compounds that serve as effective inhibitors have a structural relationship to the substrates of Glo-I and Glo-II; consequently, they generally act as competitive inhibitors of both of these enzymes. In order to study certain aspects of the glyoxalase system, such as the role of SLG in metabolism,[13,14] it was desirable to synthesize a competitive inhibitor of Glo-II that was relatively inactive toward Glo-I.

In a previous communication from this laboratory, we reported on the synthesis and inhibitory activity of *S*-carbobenzoxyglutathione (CBG) on mammalian (rat liver) Glo-II.[15] To our knowledge this was the first report of a competitive inhibitor of Glo-II that was relatively inactive as an inhibitor of Glo-I. CBG, K_i = 65 μM, has been successfully used as an affinity ligand in the two-step total purification of rat liver Glo-II.[16]

As a part of our continuing study of the glyoxalase enzymes, other potentially useful competitive inhibitors of Glo-II have been sought. It was felt that by exploring structural and/or functional group variations of the inhibitor CGB, even more effective competitive inhibitors of Glo-II might be synthesized. Our initial focus has been on the effects of the addition of a single nitro group to the aromatic ring portion of the inhibitor CBG, yielding three different compounds: the *S*-(*o*-, *m*-, and *p*-nitrocarbobenzoxy)glutathiones (*o*-NCBG, *m*-NCBG, *p*-NCBG, respectively). We have synthesized and purified all three of these isomers; all three have proven to be potent competitive inhibitors of rat liver Glo-II, with K_i values significantly lower than that for CBG. Further, as with CBG, they have been successfully utilized as affinity ligands for purification of rat liver Glo-II by coupling to Sepharose 4B.[16] In the case of *p*-NCBG as the ligand for affinity chromatography, Glo-II can be eluted from the columns by a 50 μM solution of *p*-NCBG.



o-, *m*-, and *p*-NCBG

Results and Discussion

The syntheses of the nitro group isomers of CBG were carried out at a pH of 7.8 or lower. This prevented significant condensation of the nitrocarbobenzoxy chloride with the free amino group of glutathione. Because the reaction never proceeded to more than 40–50% completion, it was necessary to separate the isomers of NCBG from unreacted reactants and reaction byproducts. This purification was accomplished by a series of washes with diethyl ether and recrystallization from glacial acetic acid and water (1:1), or in the case of *o*-NCBG and *m*-NCBG, recrystallization from water proved most effective. The reaction need not be carried out under nitrogen, since little or no oxidized glutathione was formed during the course of the reaction.

Partially purified and homogeneous preparations of Glo-I[17] and Glo-II[16] from both mouse and rat livers were employed in the inhibition studies. Figure 1 shows the double-reciprocal (Lineweaver–Burk) plot of kinetic data, indicating strict competitive inhibition of rat liver Glo-II by *p*-NCBG. Similar plots were demonstrated for *o*-NCBG and *m*-NCBG. Dixon plots from kinetic data gave the respective K_i values, which along the corresponding $[I]_{0.5}$ values, are summarized in Table I.

Comparative inhibition studies of the new Glo-II inhibitors were conducted with Glo-I activity from both rat and mouse liver. In general, the inhibitors were much less inhibitory for Glo-I than for Glo-II. For example, with the para isomer, $[I]_{0.5}$ values for rat liver Glo-I and Glo-II were found to be 925 and 12 μM, respectively.

The nitro-substituted CBG compounds have proven to be potent competitive inhibitors of mammalian Glo-II (approximately 1 order of magnitude better than CBG); they are comparatively inactive as inhibitors of mammalian Glo-I; and they have been found useful in this laboratory as ligands for a facile affinity chromatographic purification of rat liver Glo-II. These inhibitors may find use in definitive in vivo studies of SLG metabolism and/or glyoxalase enzyme function.

Experimental Section

Glutathione was purchased from Sigma Chemical Co. *p*-Nitrocarbobenzoxy chloride and the *o*- and *m*-nitrobenzyl alcohols were purchased from Aldrich Chemical Co. Purification of both Glo-I[17] and Glo-II[16] have been previously reported. Crude rat and mouse liver cytosolic extracts were obtained by centrifugation (100000*g*) of the liver homogenates prepared in 10 mM phosphate buffer, pH 7.0, containing 20% glycerol. The final protein concentrations of the cytosolic extracts were ca. 20 mg/mL as determined by the procedure of Bradford.[18] *S*-D-Lactoylglutathione

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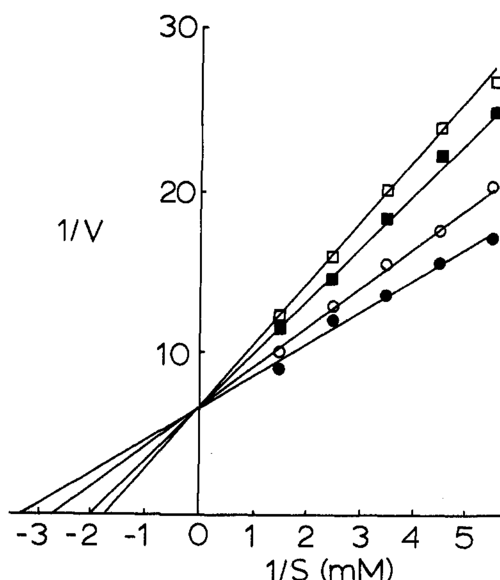

Figure 1. Lineweaver–Burk plot of the effects of *p*-NCBG on homogeneous rat liver glyoxalase II activity. The assay method is given in the Experimental Section. The substrate *S*-lactoylglutathione (SLG) was varied with the following constant levels of *p*-NCBG: (●) 0.0 μM, (○) 2.5 μM, (■) 5 μM, (□) 7.5 μM. Reaction velocity is in terms of micromoles of SLG hydrolyzed per minute.

Table I. Inhibition[a] of Rat Liver Glo-II by S-Substituted Glutathiones: K_i and $[I]_{0.5}$ Values

inhibitor	K_i, μM	$[I]_{0.5}$, μM	inhibitor	K_i, μM	$[I]_{0.5}$, μM
CBG[b]	65	165	*m*-NCBG	9	15
o-NCBG	15	20	*p*-NCBG	6.5	12

[a] The substrate, SLG, was 0.4 mM. Details of the assay are presented in the Experimental Section. [b] Inhibition data are from a previous study in this laboratory.[15]

was prepared and purified by the procedure of Uotila.[19]

***o*- and *m*-Nitrobenzyl Chloroformate Synthesis.** Dry phosgene gas was bubbled into a flask (hood!) containing 75 mL of cold dioxane until 75 g of the phosgene was absorbed. To this solution was added 25 g of the appropriate nitrobenzyl alcohol dissolved in 40 mL of dioxane. The flask was stoppered and allowed to stand for 24 h at room temperature. The solution was then concentrated in vacuo at temperatures less than 40 °C. The oily residue was washed with dioxane and reconcentrated a total of three times. The solution was filtered before concentrating the third time. The oil was then dissolved in either hot hexane or heptane. Upon cooling the chloroformate reappeared as a semipure oil. No further purification was required for subsequent synthetic steps.

***S*-(*o*-, *m*-, and *p*-Nitrocarbobenzoxy)glutathiones (*o*-, *m*-, and *p*-NCBG) Synthesis.** To an ice-cooled solution of 1.80 g (5.87 mmol) of glutathione in 8 mL of water containing 0.678 g (8.08 mmol) of sodium bicarbonate was added 0.879 g (4.075 mmol) of the appropriate nitrocarbobenzoxy chloride dissolved in 4 mL of diethyl ether. The flask was stoppered and the mixture stirred vigorously at 4 °C. After 24 h, the ether layer was decanted and the remaining gel-like mass was washed two times with ether. The crude products were purified as described by one of the procedures below.

Purification of *p*-NCBG. The crude mixture was recrystallized three times in glacial acetic acid/water (1:1), allowing 24 h for the *p*-NCBG to crystallize. On the third recrystallization, the hot *p*-NCBG/acetic acid/water solution was washed three times each with 20 mL of ether. After crystallization was complete, the crystals were collected, washed with ether, and dried in vacuo (yield 50%). The pure *p*-NCBG had a melting point of >200 °C dec and gave a negative test for the presence of SH groups.[18] In contrast to CBG, the product was not hygroscopic. Cellulose thin-layer chromatography in 1-butanol/glacial acetic acid/water (4:1:1) gave a single ninhydrin positive/UV positive spot, R_f 0.68. Elemental analyses were within ±0.4% of theory for C, H, and N as calculated for the nonhydrated free acid ($C_{18}H_{22}N_4O_{10}S$).

Purification of *o*-NCBG and *m*-NCBG. Upon heating of the gel-like mass in a hot water bath, the gel melted. The desired product was then precipitated by lowering the pH to approximately 2 with hydrochloric acid. The product was then filtered and recrystallized three times in water. As with the *p*-NCBG during the third recrystallization, the hot solution was washed three times with 20 mL of ether. After crystallization was complete, the crystals were then filtered and allowed to cool as collected, washed with cold water, and dried in vacuo. Both the pure *m*-NCBG and *o*-NCBG had a melting point of >200 °C dec and gave a negative test for the presence of SH groups. The overall yield of the nonhygroscopic product was approximately 40% for both isomers. Cellulose thin-layer chromatography in 1-butanol/glacial acetic acid/water (4:1:1) gave a single ninhydrin positive/UV positive spot, R_f 0.71 and 0.72 for the ortho and meta isomers, respectively. Elemental analyses were within ±0.4% of theory for C, H, and N as calculated for the nonhydrated free acid ($C_{18}H_{22}N_4O_{10}S$) for both isomers.

Glyoxalase I Inhibition Studies. Glyoxalase I activity was determined by the procedure of Racker,[21] as modified by Oray and Norton.[17] The reaction was initiated by the addition of an appropriate level of Glo-I. Inhibition by the *o*-, *m*-, and *p*-NCBGs 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 26 °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.[22] Inhibition of the *o*-, *m*-, and *p*-NCBGs was determined as described for the Glo-I inhibition studies.

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Registry No. *p*-NCBG, 95998-72-4; *o*-NCBG, 95998-73-5; *m*-NCBG, 95998-74-6; Glo-I, 9033-12-9; Glo-II, 9025-90-5; glutathione, 70-18-8; *p*-nitrocarbobenzoxy chloride, 4457-32-3; *o*-nitrobenzyl alcohol, 612-25-9; *m*-nitrobenzyl alcohol, 619-25-0; *p*-nitrobenzyl alcohol, 619-73-8; phosgene, 75-44-5; *o*-nitrobenzyl chloroformate, 42854-99-9; *m*-nitrobenzyl chloroformate, 95998-71-3.

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