Synthesis and Study of Glutaryl-S-(ω-aminoalkyl)-L-cysteinylglycines as Inhibitors of Glyoxalase I

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Glutaryl-S-(8-aminooctyl)-L-cysteinylglycine and glutaryl-S-(10-aminodecyl)-L-cysteinylglycine have been prepared by a seven-step procedure as potential ligands for affinity chromatography purification of mouse liver glyoxalase I. Both compounds exhibited nonlinear, mixed-type inhibition of the enzyme. The decyl derivative was a more effective inhibitor than was the octyl analog.

The purification of glyoxalase I from mouse liver has recently been accomplished by workers in this laboratory.[1] A major step in the purification procedure was the use of affinity chromatography[2] employing the glyoxalase I inhibitor S-(ω-aminodecyl)glutathione (SADG)[3] as the ligand. It was found, however, that this technique could not be satisfactorily utilized during early stages in the purification due to a rapid degradation of the ligand material and could be used only after the enzyme had been partially purified by other procedures. The degradation of the SADG ligand was attributed to the presence in the more impure enzyme preparations of glutathionase (γ-glutamyl transpeptidase) and cysteinylglycinase, which can cause hydrolysis of glutathione and its S-substituted derivatives.[4]

It was therefore of interest to prepare compounds which would be inhibitors of glyoxalase I, would be capable of serving as affinity chromatography ligands, and would still be resistant to the action of certain degradative enzymes. Such compounds would potentially be useful in allowing the affinity chromatography technique to be employed at an earlier stage in the enzyme purification process and hopefully would shorten and improve the overall isolation procedure.

Vince and coworkers have found that S-benzylglutathione is degraded by glutathionase, whereas glutaryl-S-(p-bromobenzyl)-L-cysteinylglycine is not affected by glutathionase but does act as an inhibitor of glyoxalase I.[4] In view of these observations it was thought that compounds combining certain structural features of the glutaryl compounds reported by Vince and of the S-(ω-aminoalkyl)glutathiones might satisfy the requirements of the desired compounds. Thus the synthesis of glutaryl-S-(ω-aminoalkyl)-L-cysteinylglycines was undertaken. The octyl and decyl derivatives were the compounds of choice since these chain lengths provided the most potent inhibitors among the S-(ω-aminoalkyl)glutathione series.[3] These new compounds were to be investigated for their inhibitory effect on glyoxalase I, with subsequent evaluation as potential affinity chromatography ligands, through linkage to an appropriate support through the ω-amino group. This report describes the synthesis and preliminary enzymatic study of glutaryl-S-(ω-aminoalkyl)-L-cysteinylglycines.

Chemistry. The reaction sequence employed for the synthesis of these compounds is shown in Scheme I. The Gabriel reaction was utilized for introduction of the ω-amino group on the alkyl chain. The phthalimidoalkyl chain was attached to the sulfhydryl group of the cysteine moiety early in the sequence with subsequent attachment of the glycine and glutaric acid groups.

The formyl group was chosen to protect the amino group of S-(ω-phthalimidoalkyl)-L-cysteine. The formation and subsequent cleavage of the N-formyl derivative was accomplished by the method of Sheehan and Yang.[5,6] The use of the formyl protective group combined with the subsequent use of N,N'-dicyclohexylcarbodiimide (DCC)[7,8] as a coupling reagent for peptide bond formation has been shown to give little or no racemization when optically active amino acid residues are involved.[6] The introduction of the glycine residue by the DCC method provided good yields of the fully protected dipeptide. The glutaric acid moiety was attached in good yield by reaction of compounds **4a** and **4b** with glutaric anhydride.[4]

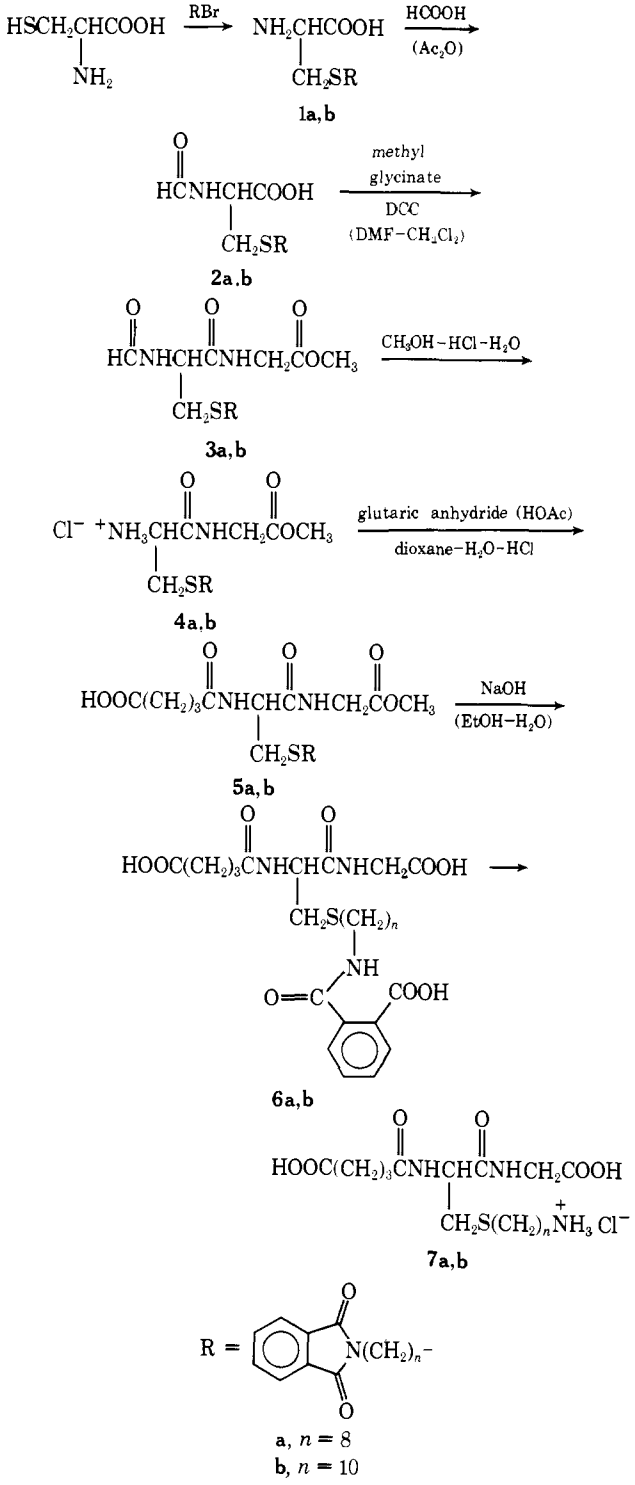
Considerable difficulty was encountered in the removal of the phthalimido and methyl ester protective groups from compounds **5a** and **5b**. Attempts at selective hydrazinolysis[9] of the phthalimido group with a variety of solvents (ethanol,[10,11] methanol,[12,13] and DMF–H_2O[3]), selective mild acidic hydrolysis[14–16] of the ester, and simultaneous removal[17] of both groups were all unsuccessful. Thin-layer chromatography (TLC) showed multiple spots in all cases. Removal of both groups was accomplished by two-stage hydrolysis.[9] Mild basic hydrolysis of the ester was performed with the simultaneous rupture of the phthalimido group to form an N-substituted phthalamic acid. The latter compound was then degraded to phthalic acid and the aminoalkyl peptide hydrochloride by mild acidic hydrolysis in dioxane–water. The final hydrolysis proceeded only very slowly at room temperature, as shown by TLC, but was complete after 30 min on a steam bath. Lower temperatures or even shorter reaction times may suffice for this reaction, since under the conditions used, a small amount of impurity was obtained as evidenced by a minor ninhydrin-positive spot on TLC. This small amount of impurity could generally be removed by recrystallization from ethanol–ether.

Other synthetic routes to the desired compounds were initially attempted using different protective groups for the amino and sulfhydryl groups of cysteine, such as the benzyloxycarbonyl[7,18] and thiazolidine[5] groups. Low yields of intermediates were generally obtained and the intermediate compounds, particularly with the benzyloxycarbonyl protective agent, were often isolated only as oils or were quite resistant to crystallization. The sequence eventually employed appeared to be superior to others attempted for these particular compounds, even though a procedure using an S-protective group would be more amenable to introduction of a variety of S-substituents by replacement of the protective group at a late stage in the sequence.

Compounds **1**, **4**, **5**, **6**, and **7** were isolated with varying amounts of water of hydration. Compounds **4** and **7** were hygroscopic, with the octyl derivative being noticeably more so than the decyl counterpart. Compounds **1**, **5**, and **6**, while not perceptibly hygroscopic, were all isolated from aqueous solutions. The reaction scheme used provided overall yields of 24% for the octyl compound and 33% for the decyl compound from cysteine, based on the individual yields of the intermediate products.

Biochemistry. Various mechanisms have been proposed for the action of glyoxalase I. These include a two-substrate mechanism,[19] in which glutathione and methylglyoxal (pyruvaldehyde) serve as substrates, a one-substrate mechanism,[20] where the hemimercaptal adduct of glutathione

Scheme I



and methylglyoxal is the substrate, and an alternative one- or two-substrate branch mechanism.[21] The latter mechanism was found to best describe the action of the enzyme; the one-substrate (hemimercaptal) branch appears to predominate except at very low hemimercaptal and free glutathione concentrations. In the present inhibition study, the enzymatic reaction rates were measured at several different fixed levels of the hemimercaptal substrate with varying concentrations of glutaryl-*S*-(ω-aminoalkyl)-L-cysteinylglycine (**7a,b**). Due to its known inhibitory effect,[20] free glutathione was held constant in all cases.

It has been reported[4] that replacement of the α-amino group of S-substituted glutathiones by a hydrogen results in compounds which are inhibitors of glyoxalase I but are

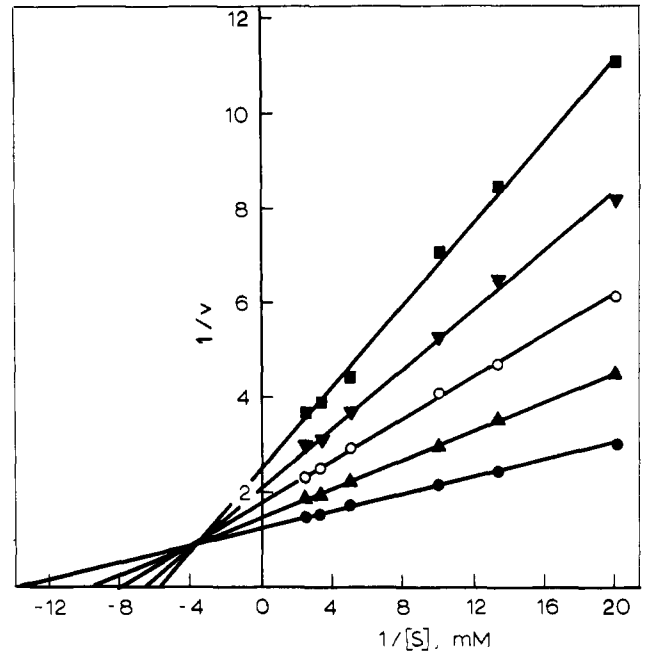


Figure 1. Double reciprocal plot showing inhibition of glyoxalase I by glutaryl-*S*-(10-aminodecyl)-L-cysteinylglycine (**7b**). Concentrations of **7b**: ●—●, zero; ▲—▲, 1.0 m*M*; ○—○, 2.0 m*M*; ▼—▼, 3.0 m*M*; ■—■, 4.0 m*M*. *v* = ΔOD per minute; S = $CH_3COCHOH$-SG; concentration of free glutathione, 0.3 m*M*.

less potent than the glutathione counterparts. Consistent with this report, compounds **7a** and **7b** were found to be inhibitors of glyoxalase I but are less effective than the corresponding *S*-(ω-aminoalkyl)glutathiones. The results of the studies with the two glutaryl compounds prepared were similar, but the decyl derivative **7b** was found to be a more potent inhibitor than was the octyl derivative **7a**, which is in keeping with the previous results for the glutathione series.[3]

A double reciprocal plot (Figure 1) obtained from the study of **7b** indicates a mixed type of inhibition.[22] Plots of $[I]\alpha/1 - \alpha$ vs. substrate concentration (where α = velocity of inhibited reaction/velocity of uninhibited reaction)[19,22] and of percent inhibition vs. substrate concentration at various levels of inhibitor (**7b**) indicate that the inhibition is more competitive at substrate levels less than 0.2 m*M* and more noncompetitive at substrate levels greater than 0.2 m*M*. This change in inhibitory mode is particularly pronounced at inhibitor levels greater than 2 m*M*, while at levels less than 2 m*M*, the inhibition appears more consistently competitive. However, under the same reaction conditions which were used in this study, SADG gave more nearly competitive inhibition.[1]

A Dixon plot[22] (Figure 2) for **7b** indicates nonlinear (parabolic) inhibition, as do replots of slopes and of intercepts (from the double reciprocal plot) vs. inhibitor concentration. Such parabolic inhibition curves indicate the possibility of involvement of two molecules of inhibitor to produce an I^2 term in the rate equation.[23] Nonlinear inhibition was not observed for SADG.[1,3] An apparent K_i of approximately 1.4 was determined for **7b** from the intersection of lines in the Dixon plot while a value of about 4.3 was obtained for **7a.** As the substrate concentration was increased from 0.05 to 0.4 m*M* in these studies, the concentration of **7b** required for 50% inhibition ranged from approximately 2 to 3 m*M* and the value of [I]/[S] for 50% inhibition decreased from 41.0 to 7.2.

The observations that the α-amino group enhances binding[4] of glutathione analogs to the enzyme and that more

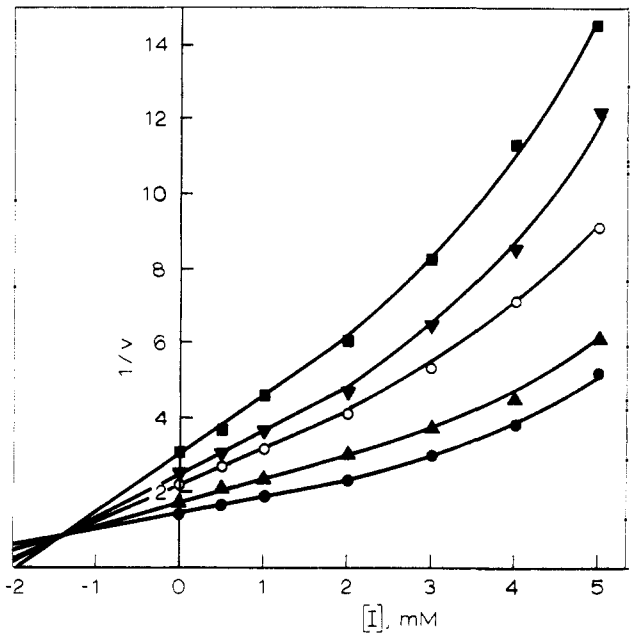


Figure 2. Dixon plot for glutaryl-*S*-(10-aminodecyl)-L-cysteinylglycine (I). Concentrations of substrate ($CH_3COCHOH$-SG): ●—●, 0.4 m*M*; ▲—▲, 0.2 m*M*; ○—○, 0.1 m*M*; ▼—▼, 0.075 m*M*; ■—■, 0.05 m*M*. v = ΔOD per minute; concentration of free glutathione, 0.3 m*M*.

nonpolar substituents attached to the sulfhydryl group of glutathione also increase the binding ability of such inhibitors[3,24] have been taken to indicate that at least two binding regions, one hydrophilic and one hydrophobic, are available near the active site of the enzyme. The data obtained in this study may suggest that the enzyme is able to simultaneously accommodate, in these two regions, two molecules of inhibitor or one molecule of inhibitor and one of substrate. This condition obviously would be more likely to arise at higher levels of inhibitor. Other interpretations of the data can also be made.

If such multiple binding with the inhibitor exists, it may be due to the lack of an α-amino substituent in **7b** which could result in weaker binding in the hydrophilic region than if an α-amino group were present. This could allow a second molecule of inhibitor or a molecule of substrate to occupy that site, while the first molecule of inhibitor remains bound at the hydrophobic region by virtue of the long alkyl chain. Thus, at low concentrations of inhibitor, the substrate may be competing with only one molecule of inhibitor, while at higher inhibitor levels it may be competing with two molecules of inhibitor in order to completely occupy the active site and be able to form product. The fact that SADG does not give rise to nonlinear kinetics may be due in part to the fact that the more inhibitory glutathione derivative was tested at concentrations approximately tenfold lower than was the glutaryl analog. The lower levels of inhibitor present in the system and the stronger binding afforded by the α-amino group in SADG could so reduce the probability of binding of a second molecule of inhibitor to the enzyme that the effects seen with **7b** would not be observed. More detailed investigations must be conducted, however, to determine whether multiple binding of the glutaryl analog, or of analog and substrate, with the enzyme can in fact occur and what effect free glutathione has if the two-substrate pathway is involved.

The use of affinity chromatography with *S*-(ω-aminoalkyl)glutathiones as ligands has led to the preparation of a homogeneous sample of glyoxalase I from mouse liver.[1] The compounds reported herein are sufficiently inhibitory to glyoxalase I (see ref 3) to warrant their use in similar purification studies. The resistance of the glutaryl group of the glutaryl-*S*-(ω-aminoalkyl)-L-cysteinylglycines to enzymic hydrolysis may negate preliminary purification steps required to remove hydrolyzing enzymes[4] and thereby simplify the purification process.[1] Analogs such as these may further prove to be of aid as probes in studying the mechanism of action of glyoxalase I.

Experimental Section

General. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by Heterocyclic Chemical Corp. for all compounds listed in Table I for the elements indicated and were within ±0.4% of calculated values except where noted. The enzyme inhibition studies were conducted on a Beckman DBG recording spectrophotometer. Thin-layer chromatography was performed by the ascending technique using commercial silica gel G plates with fluorescent indicator (Eastman No. 6060) and cellulose plates (Eastman No. 6064). Solvent systems for TLC development included 1-butanol–glacial acetic acid–water (BAW, 4:1:1), 95% ethanol–ammonium hydroxide (EA, 7:3), and methanol. TLC plates were visualized by ultraviolet lamp, ninhydrin spray, and iodine vapor.

Organic Syntheses. The general methods of preparation of compounds listed in Table I are given below. The *N*-(8-bromooctyl)phthalimide and *N*-(10-bromodecyl)phthalimide were prepared by published procedures[3] and the melting points agreed closely with the literature values.[25]

Table I. Glutaryl-*S*-(ω-aminoalkyl)-L-cysteinylglycines and Intermediates

No.	n	Formula	Mp, °C	Yield, %	Analyses
1a	8	$C_{19}H_{26}N_2O_4S \cdot H_2O$	185–188 dec	85	C, H, N
1b	10	$C_{21}H_{30}N_2O_4S \cdot 0.5H_2O$	184–188 dec	95	C, H, N
2a	8	$C_{20}H_{26}N_2O_5S$	118–121	77	C, H
2b	10	$C_{22}H_{30}N_2O_5S$	118–121	73	C, H
3a	8	$C_{23}H_{31}N_3O_6S$	117–119	84	C, H, N
3b	10	$C_{25}H_{35}N_3O_6S$	123–126	68	C, H, N
4a	8	$C_{22}H_{31}N_3O_5S \cdot HCl \cdot 2.5H_2O$	81–83	92	C, H
4b	10	$C_{24}H_{35}N_3O_5S \cdot HCl \cdot H_2O$	84–86	87	C, H
5a	8	$C_{27}H_{37}N_3O_8S$	108–111	74	C, H, N
5b	10	$C_{29}H_{41}N_3O_8S \cdot H_2O$	110–113	91	C, H, N
6a	8	$C_{26}H_{37}N_3O_9S$	*a*	80	C, H, N
6b	10	$C_{28}H_{41}N_3O_9S \cdot H_2O$	*b*	92	H, N; C[c]
7a	8	$C_{18}H_{33}N_3O_6S \cdot HCl \cdot 2H_2O$	*d*	80	C, H, N
7b	10	$C_{20}H_{37}N_3O_6S \cdot HCl \cdot 0.5H_2O$	141–144 dec	94	C, H, N

[a] Slowly decomposes with bubbling from ca. 75 to 140°. [b] Slowly decomposes with bubbling from ca. 90 to 150°. [c] C: calcd, 54.79; found, 54.29. [d] Hygroscopic. Slowly decomposes with bubbling from ca. 75 to 120°.

S-(ω-Phthalimidoalkyl)-L-cysteines (1). These compounds were prepared in a manner similar to that reported for synthesis of S-(ω-phthalimidoalkyl)glutathiones.[3] L-Cysteine hydrochloride monohydrate (0.07 mol) and 280 ml of DMF were added to 140 ml of 1.0 *N* NaOH. *N*-(ω-Bromoalkyl)phthalimide (0.07 mol) dissolved in 140 ml of DMF was introduced to the reaction mixture dropwise over a period of 2 hr. A large amount of white precipitate formed during the addition and the mixture was stirred overnight at room temperature. The product was filtered, washed with EtOH, Me_2CO, and Et_2O, and dried in a heated vacuum desiccator. TLC (silica gel, BAW) indicated one component, which was ninhydrin-positive and uv-positive. Analytical samples were prepared by recrystallization from HOAc–H_2O.

***N*-Formyl-*S*-(ω-phthalimidoalkyl)-L-cysteines** (2). *S*-(ω-Phthalimidoalkyl)-L-cysteine (0.06–0.07 mol) was dissolved in 140–150 ml of 97% formic acid and cooled in an ice bath. Acetic anhydride (50 ml) was added dropwise to the cold, stirring solution over 1 hr. After addition was complete, the solution was stirred overnight at room temperature. Ice water (300–350 ml) was added to the solution, causing precipitation of a light tan solid. After cooling in the refrigerator, the mixture was filtered and the solid was washed with cold water. The crude product was recrystallized from EtOH–H_2O and dried in a heated vacuum desiccator over P_2O_5. TLC (silica gel, MeOH and BAW) showed one spot, uv-positive and ninhydrin-negative. Analytical samples were prepared by recrystallization from EtOH–H_2O.

***N*-Formyl-*S*-(ω-phthalimidoalkyl)-L-cysteinylglycine Methyl Esters** (3). *N*-Formyl-*S*-(ω-phthalimidoalkyl)-L-cysteine (0.05 mol), dissolved in 120 ml of dichloromethane, was added to a solution of 0.06 mol of glycine methyl ester hydrochloride and 0.06 mol of triethylamine in a mixture of 170 ml of DMF and 170 ml of CH_2Cl_2. *N,N'*-Dicyclohexylcarbodiimide (0.05 mol) in 50 ml of CH_2Cl_2 was added to the reaction mixture at 0–5°. The reaction mixture was stirred at about 5° for 1 hr and then at room temperature for 2 days. The insoluble dicyclohexylurea which formed during this time was filtered and the filtrate was evaporated to low volume in vacuo. $CHCl_3$ (200 ml) was added to the residue; after cooling, the insoluble material was removed by filtration. About 100 ml of Et_2O was added to the $CHCl_3$ filtrate and the resulting solution was washed successively with 0.5 *N* HCl, H_2O, 5% $NaHCO_3$, and H_2O. The organic layer was dried over Na_2SO_4. After filtration of the drying agent, the solution was evaporated to low volume in vacuo and redissolved in the minimum amount of $CHCl_3$ by warming. The product was precipitated by addition of Et_2O. After cooling and filtering, the product was dried in a heated vacuum desiccator. Only one spot was shown by TLC (silica gel, MeOH), which was uv-positive and ninhydrin-negative. Analytical samples were prepared by recrystallization from $CHCl_3$–Et_2O.

***S*-(ω-Phthalimidoalkyl)-L-cysteinylglycine Methyl Ester Hydrochlorides** (4). *N*-Formyl-*S*-(ω-phthalimidoalkyl)-L-cysteinylglycine methyl ester (0.024–0.031 mol) was dissolved in about 100 ml of MeOH. A volume of 1.0 *N* methanolic HCl (prepared by diluting 4 ml of conentrated HCl to 48 ml with MeOH), sufficient to provide about 20–25% molar excess, was added to the dipeptide ester solution. The reaction mixture was stirred overnight at 45° and was then evaporated to low volume in vacuo. The residue was dissolved in MeOH and precipitated by addition of ether. The mixture was refrigerated and then filtered. The slightly hygroscopic product was washed with cold ether and dried in vacuo over P_2O_5. TLC (silica gel, MeOH and BAW) indicated only one spot, uv-positive and ninhydrin-positive. Recrystallization from MeOH–Et_2O provided the analytical samples.

Glutaryl-*S*-(ω-phthalimidoalkyl)-L-cysteinylglycine Monomethyl Esters (5). *S*-(ω-Phthalimidoalkyl)-L-cysteinylglycine methyl ester hydrochloride (0.023 mol) was added to a solution of sodium acetate (0.024 mol) in 150 ml of glacial HOAc, followed by addition of glutaric anhydride (0.027 mol). After about 12 hr of stirring at room temperature, an additional 0.003 mol of glutaric anhydride was added and stirring was continued for 24 hr at room temperature. After this time, the reaction mixture was evaporated to low volume in vacuo. Water was added to the residue and the mixture was agitated vigorously. After cooling, the light brown, solid material was filtered and washed with cold water. The crude product was reprecipitated from MeOH–H_2O and dried in vacuo over P_2O_5. TLC (silica gel, EA, MeOH, and BAW) indicated a single spot, uv-positive and ninhydrin-negative. Analytical samples were obtained by another reprecipitation from MeOH–H_2O.

Glutaryl-*S*-[ω-(*o*-carboxybenzamido)alkyl]-L-cysteinylglycines (6). Glutaryl-*S*-(ω-phthalimidoalkyl)-L-cysteinylglycine monomethyl ester (1.7–2.7 mmol) was dissolved in 15–20 ml of absolute EtOH. To this solution was added a volume of 1.0 *N* NaOH sufficient to give 3 molar equiv of OH^- per mole of tripeptide ester plus a 10% excess. The resulting solution was stirred at room temperature for about 1.5 hr. The solution was cooled to 0–5° and then acidified to pH 7 with 1.0 *N* HCl. The solution was then evaporated to low volume in vacuo at room temperature. About 20 ml of H_2O was added to the residue and the solution was cooled in an ice bath. The solution was acidified to pH 3–3.5 with 1.0 *N* HCl in the cold, causing precipitation of a thick, white gummy material, which solidified upon refrigeration for 2–3 hr. The solid product was filtered and washed several times with cold H_2O and then dried at room temperature in vacuo over P_2O_5.

Glutaryl-*S*-(ω-aminoalkyl)-L-cysteinylglycines (7). Glutaryl-*S*-[ω-(*o*-carboxybenzamido)alkyl]-L-cysteinylglycine (0.8–2.0 mmol) was dissolved in 5–12 ml of *p*-dioxane. To this solution was added one-half volume of H_2O and sufficient 0.5 *N* HCl to provide a slight (5–7%) excess of the equimolar amount of acid. The solution was heated on a steam bath for 30 min and then cooled in an ice bath. TLC (silica gel, BAW and EA) indicated a uv-positive, ninhydrin-negative spot which corresponded to phthalic acid, a major uv-negative, ninhydrin-positive spot, and a much weaker uv-negative, ninhydrin-positive spot. The reaction mixture was evaporated to low volume in vacuo. *p*-Dioxane was added to the residue and the solution again was evaporated in vacuo. Upon addition of *p*-dioxane, an insoluble oily material formed, which, after standing in the refrigerator several hours, crystallized upon scratching and stirring with a glass rod. The resulting solid product was filtered and dried in vacuo over P_2O_5. TLC (silica gel, BAW and EA) of this material indicated that all phthalic acid had been removed and essentially one spot was seen, which was uv-negative and ninhydrin-positive, although in some cases, a small amount of impurity, uv-negative and ninhydrin-positive, was also detected. This impurity could usually be removed by recrystallization from EtOH–Et_2O. Analytical samples were afforded by recrystallization from EtOH–Et_2O.

Enzyme Inhibition Studies. The inhibitory effects of glutaryl-*S*-(ω-aminoalkyl)-L-cysteinylglycines on glyoxalase I were studied by established procedures.[1,3] The enzyme solutions used in this study were highly purified enzyme preparations obtained from the affinity column in the isolation of glyoxalase I from mouse livers.[1]

Substrate solutions of varying concentrations were prepared by adding reduced glutathione (GSH) and methylglyoxal to an imidazole–HCl buffer solution (100 m*M*, pH 6.8) containing 16 m*M* $MgSO_4$. Commercial 40% methylglyoxal solutions were employed after removal of acidic contaminants by passing through AG-1X8 resin (carbonate form). The methylglyoxal solutions were standardized by the method of Friedmann.[26] The concentration of the hemimercaptal ($CH_3COCHOH$-SG) at equilibrium was calculated using the dissociation constant $K = [CH_3COCHO][GSH]/[CH_3COCHOH\text{-}SG]$, assuming $K = 3.1$.[24] The level of free glutathione at equilibrium was maintained constant at 0.3 m*M* and the concentration of the hemimercaptal was varied from 0.05 to 0.40 m*M* for these studies.

The inhibitor solution was prepared by dissolving the glutaryl-*S*-(ω-aminoalkyl)-L-cysteinylglycine in H_2O, adding sufficient 1.5 *N* KOH (2 molar equiv) to raise the pH to about 7, and then adjusting the total volume with H_2O to obtain a final concentration of 120 m*M*.

Each reaction cell and the reference cell contained 2.9 ml of the substrate–buffer solution. In studying the inhibitor effects, varying amounts of inhibitor solution (6–125 μl) were added to the reaction cell; the reaction was initiated by the addition of a rate-limiting amount of the enzyme preparation. The production of *S*-lactoylglutathione was followed by measuring the increase in absorbance at 240 nm at 25° on a double-beam recording spectrophotometer. The initial rate of the reaction was determined by measuring the slope of the linear portion of the plot.

Acknowledgment. This investigation was supported by the Robert A. Welch Foundation of Texas, Grant No. B-133, and by Faculty Research of North Texas State University, Grant No. 35135.

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Preparation and Properties of 5-Phenylphenoxymethylpenicillin

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Cycloaddition of azidoacetyl chloride to benzyl D-5,5-dimethyl-5-phenyl-2-thiazoline-4-carboxylate (**1a**) gave 5-phenyl-6α-azidopenicillanate (**2a**). By catalytic reduction of **2a** and reaction with phenoxyacetyl chloride, 5-phenyl-6-epiphenoxymethylpenicillin benzyl ester (**4a**) was obtained. Oxidation of **4a** gave the sulfoxide **6**, which was isomerized in the presence of DBN. The sulfoxide **7** with the normal configuration could be isolated but deoxygenation of the sulfoxide was not successful. Isomerization of **4a** with DBN, either with or without silylation of the side chain, gave a mixture from which 5-phenylphenoxymethylpenicillin benzyl ester (**5**) was isolated. Compound **5** was debenzylated to 5-phenylphenoxymethylpenicillin potassium salt (**8**). The antibacterial activity of **8** was low, whereas the 6-epimer **9** was inactive. Contrary to published information, the 5-phenylpenam derivative **4c** could be prepared by the same method.

Recently several penicillins with additional or modified substituents in the penam part of the molecule have been synthetized. Substituents like alkyl,[1,2] halogen,[3] methoxy,[4–8] methylthio,[6–8] hydroxybenzyl,[9] hydroxymethyl,[10] formyl,[11] carboxy,[11] or hydroxy[12] groups have been introduced on C-6 of the molecule. Penicillin analogs with two hydrogen atoms[13] or one or two acetoxymethyl groups[14] on C-2 instead of two methyl groups have been prepared.

Penam derivatives with a *tert*-butyloxycarbonyl[15] or a methylthio group[16] on C-5 have been described. These compounds did not have a carboxy group on C-3 and an acylamino side chain, which are necessary for antibiotic activity. The 5-phenylpenicillanic esters with a phthalimido or a succinimido group on C-6, prepared some time ago by Sheehan and coworkers,[17] were inactive. It should be noted, however, that the configuration at C-5 and C-6 was not determined and that penicillin methyl esters with an imido side chain, having a normal configuration, present a very low degree of activity.

In relation with our study of modified penicillins,[13] we wanted to examine the activity of 5-phenylphenoxymethylpenicillin having the same configuration as the natural product. For the preparation of this product we used the cycloaddition of azidoacetyl chloride to a thiazoline. This synthesis, which was discovered by Bose and coworkers,[18] yields a 5,6-*trans*-penicillin, which we have shown to have the 6-epi configuration.[19] The formation of methyl 5-phenyl-6-azidopenicillinate (**2b**), by reaction of azidoacetyl chloride with methyl 5,5-dimethyl-2-phenyl-2-thiazoline-4-carboxylate (**1b**) in the presence of triethylamine, has been described.[20] The configuration of **2b** has not been determined, but it was the same as that of the phthalimido- and succinimido-5-phenylpenicillanic acids prepared by Sheehan and coworkers.[17] As the preparation of **2b** and the transformation of **2b** into **4b** proceeded with satisfactory yields, we applied the same procedure to benzyl D-5,5-dimethyl-2-phenyl-2-thiazoline-4-carboxylate (**1a**). The benzyl ester of 5-phenyl-6-azidopenicillanic acid (**2a**) was obtained in fair yield (32%). Hydrogenation of **2a** in ethyl acetate in the presence of Adams catalyst gave benzyl 5-phenyl-6-aminopenicillanate (**3a**), which was transformed directly into benzyl 5-phenylphenoxymethylpenicillinate (**4a**) in 62% yield. We assume that **4a** had the 6-epi configuration in analogy with the course of the reaction with methyl 5,5-dimethyl-2-thiazoline-4-carboxylate.[18,19]

For the conversion of **4a** into a penicillin with normal configuration at C-6, we preferred the base-catalyzed isomerization of the sulfoxide derivative rather than the penicillin itself, because a more favorable ratio of normal to epi isomer was obtained with phenoxymethylpenicillin.[21] Only one product was obtained by oxidation of **4a** with *m*-chloroperbenzoic acid. The same product was formed upon oxidation of **4a** with ozone in acetone–water (1:1). Treatment of **6** with 1,5-diazabicyclo[4.3.0]non-3-ene (DBN) after silylating the amide side chain with *N,O*-bis(trimethylsilyl)acetamide (BSA) for 40 min at 0° gave a mixture of products with normal and epi configuration in the ratio 2:3. Treatment of **6** directly with DBN in the same conditions gave a