Synthesis and Kinetic Evaluation of S- and N-Substituted Cysteinylglycines as Inhibitors of Glyoxalase I

Philip A. Lyon and Robert Vince*

Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455.
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Eight S- and N-substituted L-cysteinylglycines were prepared by condensation of *S*-benzyl-L-cysteinylglycine or *S*-(*p*-bromobenzyl)-L-cysteinylglycine with glutaric anhydride, succinic anhydride, or the appropriately blocked and activated amino acids. In contrast to the previously prepared S-substituted glutathiones, all of the title compounds exhibited noncompetitive inhibition of yeast glyoxalase I. A kinetic evaluation under Yonetani–Thorell conditions established the existence of two binding sites on the glyoxalase I enzyme.

The inhibition of glyoxalase I as a means of controlling the metabolism of α-ketoaldehydes such as methylglyoxal has been well documented in previous reports from this laboratory.[1] Since the substrate for glyoxalase I is a hemimercaptal adduct of methylglyoxal and reduced glutathione, S-substituted glutathione derivatives exhibited effective inhibition of the enzyme.[2-4] During in vivo tests, metabolism by glutathionase enzymes inactivated these inhibitors. Subsequently, it was reported that replacement of the γ-glutamyl moiety with a glutaryl group rendered the glutathione analogues resistant to metabolism by glutathionase.[2] This modification of the glutamyl residue still allows for effective binding to the enzyme. For example, some glutarylcysteinylglycines were later used successfully as ligands for affinity chromatography purification of glyoxalase I.[5]

Other structural modifications of the γ-glutamyl moiety of glutathione are possible and may result in greater enzyme inhibition. Systematic alteration of the glutamic acid side chain would permit assessment of the binding requirements essential for inhibition of glyoxalase. In this communication, variations in both the N- and S-substituents of cysteinylglycine and the effects of these modifications on glyoxalase inhibition are examined. Specifically, the kinetic parameters for the interaction of these analogues with the glyoxalase binding site are reported.

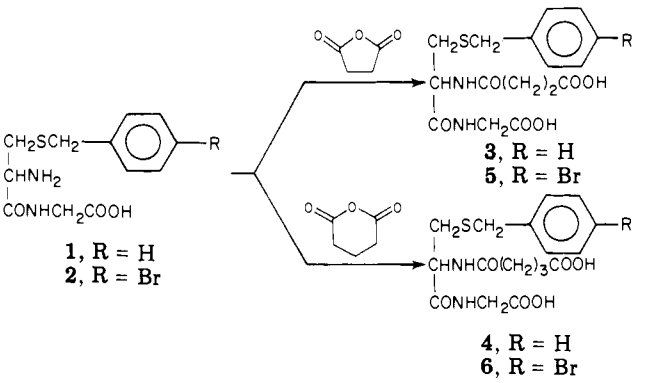
Chemistry. *S*-Benzyl-L-cysteinylglycine (**1**)[6] was allowed to react with a solution of succinic or glutaric anhydride in glacial acetic acid to produce succinyl-*S*-benzyl-L-cysteinylglycine (**3**) or glutaryl-*S*-benzyl-L-cysteinylglycine (**4**) (Scheme I). Similarly, *S*-(*p*-bromobenzyl)-L-cysteinylglycine (**2**) was converted to succinyl-*S*-(*p*-bromobenzyl)-L-cysteinylglycine (**5**)[1] and glutaryl-*S*-(*p*-bromobenzyl)-L-cysteinylglycine (**6**).

Scheme II depicts the synthesis of compounds in which the γ-glutamyl moiety of glutathione was replaced by a side chain lacking the terminal carboxyl group. Thus, *N*-carbobenzoxy-β-alanine (**7**) and *N*-carbobenzoxy-4-aminobutyric acid (**8**) were converted to the corresponding activated esters with equimolar amounts of *N*,*N*-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide in dioxane. The activated esters were coupled with the N terminus of the cysteinylglycine dipeptides, **1** and **2**, in aqueous solution. In this manner, four N-substituted cysteinylglycines, **9**–**12**, were prepared. The final compounds, **13**–**16**, were obtained by selectively deblocking the amines with glacial acetic acid saturated with hydrobromic acid.

Results and Discussion

Each modified glutathione was assayed for its ability to inhibit yeast glyoxalase I enzyme. The dissociation constants of the enzyme inhibitor complex (K_i) obtained from double recipricol plots[7] (Figure 1) are tabulated in

Scheme I



Scheme II

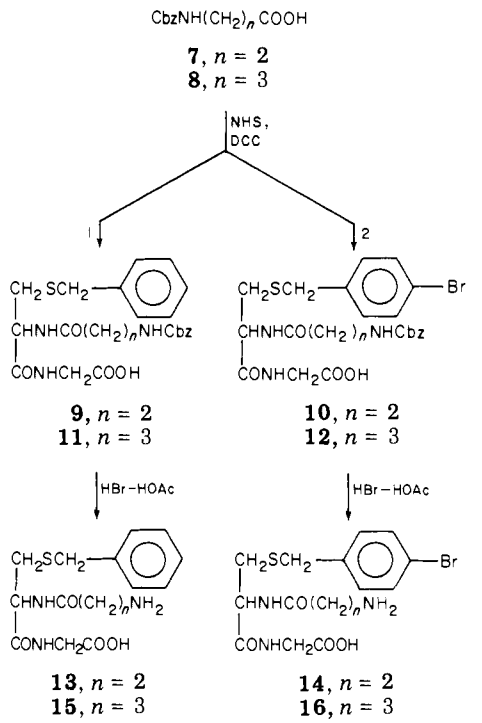


Table I. Replacing the *S*-benzyl group with *S*-*p*-bromobenzyl in every instance greatly enhanced the binding of the inhibitor to the enzyme. Previous observations[1] suggested the existence of a hydrophobic region on the glyoxalase which is accessible to the S-substituent of glutathione. The hydrophobic character of the bromine significantly increases the affinity of the benzyl S-substituent for this nonpolar site. This effect is further illustrated by the unusually high inhibition of glyoxalase observed with *S*-(*p*-bromobenzyl)-L-cysteinylglycine (**2**) when compared with the inactive *S*-

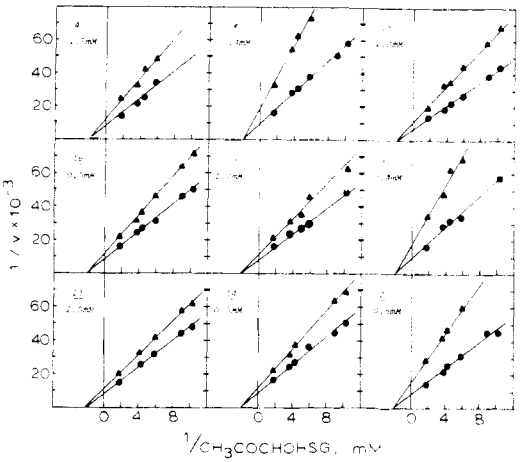
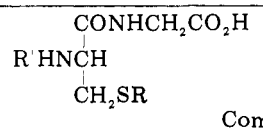


Figure 1. Double reciprocal plots showing inhibition of glyoxalase I by S- and N-substituted cysteinylglycines. For each curve the concentration of inhibitor is given in the left panel. The concentrations of hemimercaptal substrate, $CH_3COCHOHSG$, were calculated using the dissociation constant, 3.1×10^{-3} M, previously determined for the equilibrium reaction:[2] (●—●) no inhibitor present; (▲—▲) inhibitor present at concentration indicated. The enzymatic assay is described in the Experimental Section.

Table I. Inhibition of Yeast Glyoxalase I by S- and N-Substituted Cysteinylglycines[a]



R	R'	Compd no.	K_i, mM
$-CH_2C_6H_5$	$-CO(CH_2)_3CO_2H$	4	3.0
$-CH_2C_6H_5$	$-CO(CH_2)_3NH_2$	15	4.1
$-CH_2C_6H_5$	$-CO(CH_2)_2CO_2H$	3	4.5
$-CH_2C_6H_5$	$-CO(CH_2)_2NH_2$	13	5.3
$-CH_2C_6H_5$	-H	1	Not active
$-CH_2C_6H_4Br$	$-CO(CH_2)_3CO_2H$	6	0.32
$-CH_2C_6H_4Br$	$-CO(CH_2)_3NH_2$	16	1.2
$-CH_2C_6H_4Br$	$-CO(CH_2)_2CO_2H$	5	0.4
$-CH_2C_6H_4Br$	$-CO(CH_2)_2NH_2$	14	1.5
$-CH_2C_6H_4Br$	-H	2	0.58

[a] The K_m for the glyoxalase substrate was 0.5 mM in each experiment.

benzyl-L-cysteinylglycine (1). Although 2 is a relatively potent inhibitor of glyoxalase, it would be rapidly metabolized in vivo by cysteinylglycinase enzymes.[1] It appears that the carboxyl group of the γ-glutamyl moiety contributes more to binding than the α-amine group when the K_i values of 15 and 16 are compared with 4 and 6. A slight decrease in inhibitory activity is noted when the glutamyl side chain is shortened by one methylene unit.

An unexpected observation from the glyoxalase inhibition studies revealed that all of the S- and N-substituted cysteinylglycines are noncompetitive inhibitors (Figure 1). In contrast, the S-substituted glutathiones displayed competitive inhibition. Figure 2 compares the double reciprocal plots of glutaryl-*S*-(*p*-bromobenzyl)-L-cysteinylglycine (6) and *S*-(*p*-bromobenzyl)glutathione. The difference in mechanism of inhibition suggested that the compounds with modified γ-glutamyl moieties were binding at a site other than the hemimercaptal substrate site. Further studies were conducted to clarify the existence of a second binding site on the glyoxalase enzyme.

It is known that glyoxalase I catalyzes the formation of *S*-D-lactoylglutathione from an equilibrium mixture of

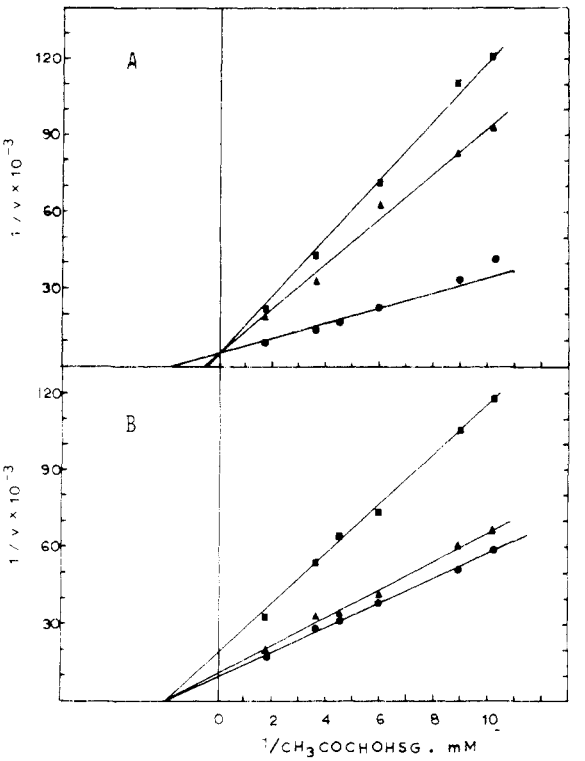


Figure 2. Double reciprocal plots showing competitive and noncompetitive inhibition of glyoxalase I. Concentrations of *S*-(*p*-bromobenzyl)glutathione (panel A): (●—●) 0 mM; (▲—▲) 0.01 mM; (■—■) 0.02 mM. Concentrations of glutaryl-*S*-(*p*-bromobenzyl)-L-cysteinylglycine (6) (panel B): (●—●) 0 mM; (▲—▲) 0.2 mM; (■—■) 0.4 mM.

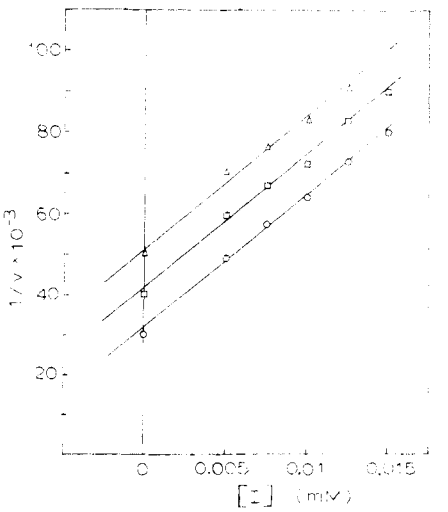


Figure 3. Reciprocal velocities of the glyoxalase reaction vs. concentration of *S*-(*p*-bromobenzyl)glutathione, [I], at constant substrate concentration (0.2 mM) and different fixed levels of free glutathione: (○—○) 0.1 mM; (□—□) 2.0 mM; (△—△) 5.0 mM.

glutathione, methylglyoxal, and their hemimercaptal adduct and that the hemimercaptal is the substrate for the enzyme.[8,9] Also, at high concentrations, free glutathione acts as a weak competitive inhibitor of glyoxalase.[2] A graphical procedure for determining whether kinetically indistinguishable binding sites are available for two inhibitors has been developed by Yonetani and Thorell.[10,11] A Yonetani–Thorell plot ($1/v$ vs. [I] at constant substrate and different fixed levels of glutathione) of *S*-(*p*-bromobenzyl)glutathione inhibition of glyoxalase I gave parallel

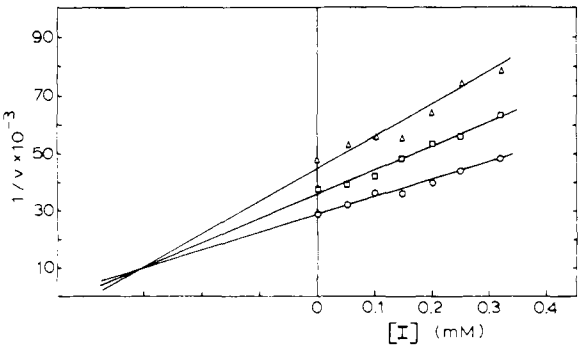


Figure 4. Reciprocal velocities of the glyoxalase reaction vs. concentration of glutaryl-*S*-(*p*-bromobenzyl)-L-cysteinylglycine, [I], at constant substrate concentration (0.2 mM) and different fixed levels of free glutathione: (○—○) 0.1 mM; (□—□) 2.0 mM; (△—△) 5.0 mM.

lines (Figure 3). A parallel pattern indicates that the inhibitor and glutathione are mutually exclusive as effectors of enzymatic activity.[12] This implies that glutathione, the hemimercaptal substrate, and *S*-(*p*-bromobenzyl)glutathione all bind to the same site, the active center of glyoxalase.

In a second experiment, a representative title compound, glutaryl-*S*-(*p*-bromobenzyl)-L-cysteinylglycine (**6**), was tested under Yonetani–Thorell conditions against free glutathione. As expected, nonparallel lines were obtained which converged near the horizontal axis (Figure 4). Intersecting lines indicate that the inhibitor and glutathione interact with different sites on the enzyme as suggested by the double reciprocal plots (Figure 1). An interaction coefficient (α), determined from the point of intersection in Figure 4, indicates that the binding is slightly synergistic.[10]

The results of these inhibitor studies were somewhat surprising in that such small modifications of the glutamyl moiety of glutathione would direct the molecule to an alternate binding site. In a recent report by Phillips and Norton,[13] glutaryl-*S*-(10-aminodecyl)-L-cysteinylglycine was found to exhibit mixed inhibition of mouse liver glyoxalase. The fact that we observe only noncompetitive inhibition with our substituted cysteinylglycines with yeast glyoxalase may reflect slight differences in the two enzymes. Further evidence for such a difference is suggested by the observed K_m values for the two enzymes. Thus, Phillips and Norton[13] and Kester and Norton[5] report a K_m of 0.057 mM for the hemimercaptal with mouse liver glyoxalase which is one order of magnitude lower than the K_m observed by us (0.5 mM) and by Vander Jagt and co-workers[16,17] (0.3 mM) with yeast glyoxalase.

The present results are consistent with the suggestion that two binding sites exist on the yeast glyoxalase I enzyme. The kinetic data demonstrate that glutathione, the hemimercaptal substrate, and the S-substituted glutathione all bind to the active center of glyoxalase, whereas the title compounds occupy a remote binding site. These sites apparently show a parallel response to changes in the nature of the S-substituent.

Experimental Section

General. Melting points were determined on a Mel-Temp apparatus and are uncorrected. Infared spectra were recorded on a Perkin-Elmer Model 237B spectrophotometer. The NMR spectra were obtained on a Varian A-60D or Varian T-60 spectrometer using tetramethylsilane as an internal standard. Thin-layer chromatograms were run on silica gel (Eastman chromatogram sheets with fluorescent indicator or EM Laboratories precoated glass plates with fluorescent indicator). TLC plates were developed with butanol–acetic acid–water (4:1:1) and spots were visualized by ultraviolet or ninhydrin spray.

***S*-(*p*-Bromobenzyl)-L-cysteinylglycine (2).** Dicarbobenzoxycysteinylglycine[6] (10 g, 15 mmol) was dissolved in liquid ammonia (80 ml) in a 200-ml round-bottom flask submerged in a dry ice–acetone bath. Metallic sodium was added to the rapidly stirred solution until a dark purple color persisted (about 3 g). Immediately, 8.25 g (33 mmol) of α,*p*-dibromotoluene was added and stirring was continued for 20–30 min, followed by the addition of 4.5 g (80 mmol) of ammonium chloride. The mixture was stirred for 10 min and the ammonia was allowed to evaporate. The white residue was dissolved in water (15 ml) and washed with ether (2 × 20 ml). The aqueous fraction was acidified and the white precipitate was collected by filtration and gave 10.3 g (99%) of product **2**. The crude product could be used in subsequent reactions without further purification. Samples for analysis and testing were recrystallized from water: mp 190–192 °C; TLC, single spot (R_f 0.39), ninhydrin positive. Anal. ($C_{12}H_{15}N_2O_3SBr$) C, H.

Succinyl-*S*-benzyl-L-cysteinylglycine (3). To a solution of 1.23 g (12.3 mmol) of succinic anhydride in 20 ml of glacial acetic acid was added 3.0 g (11.2 mmol) of *S*-benzyl-L-cysteinylglycine (**1**).[6] The mixture was stirred overnight at room temperature and then the solvent was removed in vacuo at 50 °C. The residue was dissolved in 25 ml of water and the product crystallized at room temperature. The solid was removed by filtration and gave 1.97 g. A second fraction of solid was obtained by concentrating the filtrate: 1.12 g. A total yield of 3.09 g (75%) of **3** was obtained: mp 150–151 °C; TLC, single spot by uv (R_f 0.57), ninhydrin negative; ir (KBr) 1715 (COOH). Anal. ($C_{16}H_{20}N_2O_6S$) C, H, N.

Succinyl-*S*-(*p*-bromobenzyl)-L-cysteinylglycine (5). To a solution of 0.55 g (5.5 mmol) of succinic anhydride in 15 ml of glacial acetic acid was added 1.73 g (5.0 mmol) of **2**. The solution was stirred overnight and the glacial acetic was removed in vacuo at 45 °C. The residue was crystallized from water and pure **5** was collected by filtration: yield 1.51 g (68%); mp 171–172 °C; TLC, single spot (R_f 0.56), ninhydrin negative. Anal. ($C_{16}H_{19}N_2O_6SBr$) C, H.

Glutaryl-*S*-benzyl-L-cysteinylglycine (4) and Glutaryl-*S*-(*p*-bromobenzyl)-L-cysteinylglycine (6). Compounds **4** and **6** were prepared as described previously. Spectral and physical properties were identical with reported values.

***N*-(Carbobenzoxy-3-aminopropionyl)-*S*-benzyl-L-cysteinylglycine (9).** *N*,*N*-Dicyclohexylcarbodiimide (2.06 g, 10.0 mmol) was added to a chilled solution of 2.23 g (10.0 mmol) of carbobenzoxy-β-alanine (**7**) (prepared by the general procedure of Zahn and Hildebrand,[14] mp 114–115 °C) and 1.15 g (100 mmol) of *N*-hydroxysuccinimide in 25 ml of dry dioxane. The mixture was refrigerated overnight. The solid was removed by filtration and the filtrate was evaporated to an oil. Trituration in dry ether caused the oil to solidify. The white product was collected by filtration and gave 2.8 g (88%) of the activated ester which was used without further purification: mp 75–77 °C.

A solution of 1.92 g (6.0 mmol) of the *N*-hydroxysuccinimide ester of **7** in 15 ml of dioxane was added to a solution of 1.6 g (6.0 mmol) of **1** and 1.01 g (12 mmol) of sodium bicarbonate in 30 ml of water. The mixture was allowed to stand at room temperature for 72 h, concentrated to half volume at reduced pressure, acidified, and refrigerated overnight. The crude product was collected by filtration and gave 2.24 g. Recrystallization from ethanol–water gave pure **9**: 1.32 g (47%); mp 170–173 °C; TLC gave a single spot by uv (R_f 0.64), ninhydrin negative. Anal. ($C_{23}H_{27}N_3O_6S$) C, H, N.

***N*-(Carbobenzoxy-3-aminopropionyl)-*S*-(*p*-bromobenzyl)-L-cysteinylglycine (10).** Following the above procedure, 1.6 g (5 mmol) of the *N*-hydroxysuccinimide ester of **7** in 17 ml of ethanol was added to 1.74 g (5.0 mmol) of **2** and 0.84 g (10 mmol) of sodium bicarbonate in 40 ml of water. The same reaction conditions and work-up as above gave 1.4 g (50%) of pure **10**: mp 181–185 °C dec; TLC gave a single spot by uv (R_f 9.65), ninhydrin negative. Anal. ($C_{23}H_{26}N_3O_6SBr$) C, H, N.

***N*-(Carbobenzoxy-4-aminobutyryl)-*S*-benzyl-L-cysteinylglycine (11).** The known carbobenzoxy-4-aminobutyric acid (**8**)[15] was prepared by a general procedure[14] and converted to the *N*-hydroxysuccinimide ester (mp 141–144 °C) as described in the preparation of **9**. A solution of 1.67 g (5.0 mmol) of the above

activated ester was added to a solution of 1.34 g (5.0 mmol) of 1 and 0.84 g (10 mmol) of sodium bicarbonate in 40 ml of water. The mixture was allowed to stand at room temperature for 72 h and then concentrated to half volume at reduced pressure. The solution was acidified to pH 4 and then refrigerated overnight. The crude product was collected by filtration (2.14 g). Recrystallization from ethanol–water gave pure 11: 1.01 g (42%); mp 137–139 °C; TLC, single spot by uv (R_f 0.66), ninhydrin negative. Anal. ($C_{24}H_{29}N_3O_6S$) C, H, N.

N-(**Carbobenzoxy-4-aminobutyryl**)-*S*-(*p*-**bromobenzyl**)-L-**cysteinylglycine** (12). A solution of 1.67 g (5.0 mmol) of the *N*-hydroxysuccinimide ester of 8 in 17 ml of dioxane was added to a solution of 1.74 g (5.0 mmol) of 2 and 0.84 mmol of sodium bicarbonate in 40 ml of water. The same reaction conditions and work-up as described above gave 2.41 g of crude 12. Recrystallization from ethanol–water gave 1.1 g (40%) of pure 12: mp 123–124 °C; TLC, single spot (R_f 0.70), ninhydrin negative. Anal. ($C_{24}H_{28}N_3O_6SBr$) C, H, N.

N-(**3-Aminopropionyl**)-*S*-**benzyl**-L-**cysteinylglycine** (13). In a dry flask protected from moisture, 1.0 g of glacial acetic acid saturated with hydrobromic acid was added to 473 mg (1 mmol) of 9. The mixture was allowed to react 1 h or until bubbles were no longer produced. Dry ether (10 ml) was added and the mixture was refrigerated to crystallize as the salt. The solid was collected by filtration, quickly washed with fresh ether, and dissolved in 2 ml of 40% ethanol. The solution was neutralized with 1 N ammonium hydroxide and the solvent was removed at reduced pressure. The crude product was recrystallized from ethanol–water and gave pure 13: yield 256 mg (75%); mp 190 °C dec; TLC, single spot by uv (R_f 0.31), ninhydrin positive. Anal. ($C_{15}H_{21}N_3O_4S$) C, H, N.

N-(**3-Aminopropionyl**)-*S*-(*p*-**bromobenzyl**)-L-**cysteinylglycine** (14). Following the same general procedure described for the preparation of 13, 552 mg (1.0 mmol) of 10 gave 313 mg (75%) of pure 14 as a white solid: mp 218 °C darkens, 224–225 °C dec; TLC, single spot by uv, ninhydrin positive. Anal. ($C_{15}H_{20}N_3O_4SBr$) C, H, N.

N-(**4-Aminobutyryl**)-*S*-**benzyl**-L-**cysteinylglycine** (15). Following the general procedure described above, 487 mg of 11 gave 163 mg (47%) of pure 15 as white solid from ethanol–water: mp 205–208 °C dec; TLC, single spot by uv (R_f 0.30). Anal. ($C_{16}H_{23}N_3O_4S$) C, H, N.

N-(**4-Aminobutyryl**)-*S*-(*p*-**bromobenzyl**)-L-**cysteinylglycine** (16). Following the above procedure, 283 mg (0.50 mmol) of 12 was deblocked and gave 130 mg (65%) of pure 16 after one recrystallization from ethanol–water: mp 194–197 °C dec; TLC, single spot by uv (R_f 0.32), ninhydrin positive. Anal. ($C_{16}H_{22}N_3O_4SBr$) C, H, N.

Enzyme Inhibition Studies. A 40% methylglyoxal solution was purified and standardized as previously described.[2] Yeast glyoxalase I was obtained from Sigma Chemical Co. and was diluted to 20 μg/ml with 30% glycerin containing 0.1% bovine serum albumin. All enzymatic reactions were performed at 30 °C in 0.05 M phosphate buffer at pH 6.6. For each assay the cell contained 3.0-ml total volume and the rate of formation of product was followed by an increase in absorption at 240 nm using a Beckman Model 25 spectrophotometer. Methylglyoxal, reduced glutathione, inhibitor, and buffer were added to the cell and allowed to incubate for 3 min at 30 °C (to allow equilibration of hemimercaptal formation) before addition of enzyme. The concentration of hemimercaptal substrate, $CH_3COCHOH$-SG [SG = glutathione), at equilibrium was calculated from a quadratic equation using the dissociation constant, 3.1×10^{-3} M, previously determined for the equilibrium reaction.[2] A computer program was used to determine the line of best fit by the method of least squares.

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Syntheses and Antiinflammatory and Hypnotic Activity of 5-Alkoxy-3-(N-substituted carbamoyl)-1-phenylpyrazoles. 4[1]

Shoji Sugiura,* Sachio Ohno, Osamu Ohtani, Kihachiro Izumi, Tadashi Kitamikado, Hajime Asai, Kazuo Kato,

Research Laboratories, Maruko Seiyaku Company Ltd., Teramae-1212, Gejo-cho, Kasugai 486, Japan

Mikio Hori,

Gifu College of Pharmacy, 5-Mitahora, Gifu 502, Japan

and Hajime Fujimura

Gifu University School of Medicine, Tsukasa-cho-40, Gifu 500, Japan. *Received March 9, 1976*

5-Alkoxy-3-(N-substituted carbamoyl)-1-phenylpyrazoles were prepared and tested for antiinflammatory and hypnotic activity. Four compounds showed antiinflammatory activity and three possessed hypnotic properties.

In a previous paper,[1] the authors reported the synthesis of 3-substituted 5-methoxy-1-phenylpyrazoles and the biological activity of these compounds. The result showed that 5-methoxypyrazoles containing the N-substituted