Synthesis and Evaluation of *N*-Alkanoyl-*S*-benzyl-L-cysteinylglutamic Acid Esters as Glyoxalase I Inhibitors and Anticancer Agents

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Received October 14, 1982, from the *Samuel M. Best Research Laboratory, Massachusetts College of Pharmacy and Allied Health Sciences, Boston, MA 02115.* Accepted for publication February 3, 1983. ‡ Present address: G. D. Searle and Company, Chicago, IL 60680.

Abstract □ Esters of several glutathione analogues have been synthesized in which the glycine moiety was replaced by straight-chain fatty acids and the mercapto group was benzylated. Two of the derivatives (sufficiently water-soluble for the assay) were found to inhibit glyoxalase I to a greater extent than did *S*-methylglutathione. The two glyoxalase I inhibitors did not inhibit P388 lymphocytic leukemia in mice, however.

Keyphrases □ *N*-Alkanoyl-*S*-benzyl-L-cysteinylglutamic acid esters—synthesis, anticancer activity in the P388 leukemia screen, glyoxalase I inhibition □ Anticancer agents—potential, *N*-alkanoyl-*S*-benzyl-L-cysteinylglutamic acid esters, activity in the P388 leukemia screen, glyoxalase I inhibition

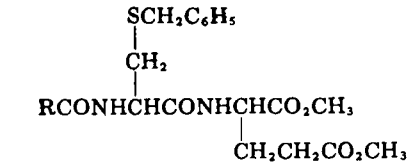
S-Alkyl and *S*-aryl glutathiones are potent competitive inhibitors of glyoxalase (1). The glyoxalase enzyme system converts methylglyoxal to lactic acid in the presence of the cofactor glutathione (2). Since methylglyoxal and other α-keto aldehydes are known to be carcinostatic (3), it was suggested that inhibitors of the glyoxalase system may create a build-up of methylglyoxal in the cells and thus inhibit cellular growth (4). The fact that glutathione concentration in the cells is rapidly increased (5) just prior to cell division suggests that the glyoxalase system is involved in the regulation of cell growth by maintaining a proper concentration of methylglyoxal (1). The high concentration of lactic acid (6) and deficiency of methylglyoxal (7) in cancer cells suggests further that such cells, having lost the ability to maintain the necessary balance of methylglyoxal, continue to grow at an uncontrolled rate. Inhibitors of the glyoxalase system that result in a build-up of cellular methylglyoxal could, therefore, function as anticancer agents. To some extent this has been realized. Some of the *S*-alkyl and *S*-aryl glutathione inhibitors of the glyoxalase system exhibited cytotoxic activity against L1210 leukemia and KB cells in tissue culture (8). However, α-hydroxy thiol esters of glutathione inhibited glyoxalase I but lacked antileukemic activity against L1210 lymphoid leukemia (9).

The rapid metabolism of *S*-substituted glutathiones by glutathionase rendered the *S*-alkyl and *S*-aryl glutathiones inactive when tested *in vivo* (10). Glutathione and *S*-substituted derivatives are rapidly hydrolyzed by two enzymes, glutathionase and cysteinylglycinase (11). In an attempt to find glutathione analogues that might be resistant to enzymatic hydrolysis, a series of analogues (I) having the glycine residue replaced by a fatty acid was synthesized. The diester function was not successfully converted to the carboxyl function, however. It was also considered possible that such a derivative would have greater lipophilicity and possibly provide a more strongly bound enzyme-inhibitor complex. A series of *S*-(ω-phthalimidoalkyl)glutathiones and *S*-(ω-aminoalkyl)-glutathiones, for instance, showed greater inhibition of glyoxalase I with greater lipophilicity (12).

Table I—Physical Properties of the *N*-Alkanoyl-*S*-benzyl-L-cysteines [a]

R	Formula	Reaction Time, h	Yield, %	Melting Point, °C
C_2H_5	$C_{13}H_{17}NO_3S$	30	69	86.0–86.5
C_3H_7	$C_{14}H_{19}NO_3S$	40	63	115–116
C_5H_{11}	$C_{16}H_{23}NO_3S$	96	52	83–84
C_8H_{17}	$C_{18}H_{27}NO_3S$	96	51	88–89
$C_{10}H_{21}$	$C_{20}H_{31}NO_3S$	144	18 [b]	73–74
Dimethyl *N*-Alkanoyl-*S*-benzyl-L-cysteinylglutamates (I)				
C_2H_5	$C_{20}H_{28}N_2O_6S$	2	61	113–114
C_3H_7	$C_{21}H_{30}N_2O_6S$	2	59	93–94
C_8H_{17}	$C_{25}H_{38}N_2O_6S$	2	50	107–108

[a] Elemental analyses for C, H, N, and S were within ± 0.4% of the theoretical values for all compounds. [b] Recrystallized from *n*-hexane.



I: R = C_3H_7—$C_{10}H_{21}$

DISCUSSION

Chemistry—*S*-Benzyl-L-cysteine was readily converted to fatty acid amides (11) by reaction of the pyridinium salt of *S*-benzyl-L-cysteine with the fatty acid chloride in excess pyridine at 0°C. Yields of 18–69% were realized, with chain lengths ranging from 3 to 10 carbons with the longer chain acids giving progressively lower yields. Physical properties of the *N*-alkanoyl-*S*-benzyl-L-cysteines are recorded in Table I.

Condensation of the *N*-alkanoyl-*S*-benzyl-L-cysteines with dimethyl glutamate was carried out by conversion of the cysteine moiety to a mixed anhydride with isobutyl chlorocarbonate in the presence of *N*-methylmorpholine. Condensation with dimethyl glutamate hydrochloride in the presence of triethylamine took place readily at −10–0°C giving reasonable yields (50–61%) of I, in the case of the propanoyl, butanoyl, and octanoyl derivatives. Attempts to hydrolyze the methyl ester functions were unsuccessful. The reaction sequence is shown in Scheme I, and physical properties of I are listed in Table I.

Biological Testing—The method of Racker (13) for measuring inhibition of glyoxalase I was used. This was based on the formation of *S*-lactoylglutathione, catalyzed by glyoxalase I, which has high optical density at 240 nm. The rate of increase in absorption at 240 nm was measured, and the ratio of inhibitor concentration to substrate concentration at 50% inhibition was used to compare relative inhibitory properties of the compounds. To determine the concentration of inhibitor required for 50% inhibition, a plot of v_0/v_i *versus*

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Table II—Inhibition of Glyoxalase I

Compound	ID_{50}, mM	$[I/S]_{50}$[a]
S-Methylglutathione	6.10	9.84
Dimethyl *N*-propanoyl-*S*-benzyl-L-cysteinylglutamate	1.95	3.15
Dimethyl *N*-butanoyl-*S*-benzyl-L-cysteinylglutamate	1.90	3.06

[a] Ratio of inhibitor concentration to substrate concentration at 50% inhibition.

Table III—Antileukemic Activities in Mice[a]

Compound	Dose, mg/kg[b]	Weight Difference (T − C)	Median Survival Time (T/C), %[c]
Dimethyl *N*-propanoyl-*S*-benzyl-L-cysteinylglutamate	200	0.8	97
	100	0.3	100
	50	−0.2	110
Dimethyl *N*-butanoyl-*S*-benzyl-L-cysteinylglutamate	200	−0.2	96
	100	1.4	104
	50	1.1	104

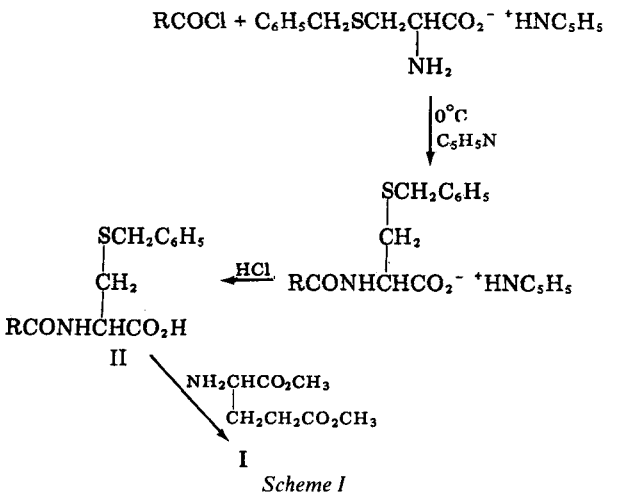
[a] CDF_1 mice were inoculated (5×10^6 cells) with P388 lymphocytic leukemia. [b] Drugs were administered intraperitoneally daily for 9 d; observations were continued for 30 d. [c] A T/C% value of >125 is considered a positive result.

[I] was made, where v_0 is the initial velocity of the uninhibited enzymatic reaction and v_I is the initial velocity of the inhibited reaction at various concentrations of inhibitor (Table II). The *N*-propanoyl- and *N*-butanoyl-*S*-benzyl-L-cysteinylglutamic acid esters both showed better inhibitory activities than did *S*-methylglutathione. The longer-chain amide proved to be too insoluble in water to be measured by this method.

Antileukemia testing[1] of the propanoyl and butanoyl amides was carried out in mice against P388 lymphocytic leukemia, according to the NCI protocol (14). Details regarding dose and survival times are listed in Table III. Neither of the compounds showed positive activity in this screen.

EXPERIMENTAL[2]

S-Benzyl-L-cysteine—L-(+)-Cysteine hydrochloride (36.0 g, 0.229 mol) was dissolved in a small amount of water, and 27.5 g (0.687 mol) of sodium hydroxide was added. Benzyl bromide (38.9 g, 0.229 mol) was added in a dropwise manner to the solution, and the mixture was shaken vigorously for 1 h. The unreacted benzyl bromide and benzyl alcohol were extracted with ether. The aqueous solution was acidified with 20% acetic acid, and the white crystals were collected and recrystallized from water, yielding 36.5 g (75%); mp 215–217°C [lit. (15) mp 213–214°C].



Scheme I

***N*-Alkanoyl-*S*-benzyl-L-cysteines**[3]—*S*-Benzyl-L-cysteine (31.65 g, 0.15 mol) in 180 mL of pyridine was cooled to 0°C. After 30 min, 13.02 mL (0.15 mol) of propanoyl chloride was added in a dropwise manner with vigorous shaking and ice cooling during 30 min. The mixture was shaken mechanically for 30 h at room temperature, and the excess pyridine was removed under reduced pressure. The syrupy residue was acidified with 10% HCl (Congo Red endpoint), and the oily product was extracted three times with ethyl acetate. The extract was dried (Na_2SO_4) and concentrated to a small volume using a rotary evaporator. The residual solution was treated with petroleum ether and stored at 0°C; recrystallization of the product from benzene gave 27.6 g (69%) of white needles, mp 86.0–86.5°C; IR(KBr): 3380 (N—H), 1735 (C=O), 1600 (C=O), and 1525 cm^{-1} (CNH).

***N*-Alkanoyl-*S*-benzyl-L-cysteinylglutamic Acid Dimethyl Esters**[3]—A solution of 5.35 g (0.02 mol) of *N*-propanoyl-*S*-benzyl-L-cysteine in 60 mL of dry tetrahydrofuran containing 2.20 mL (0.02 mol) of *N*-methylmorpholine was cooled to −15°C, and 2.80 mL (0.021 mol) of isobutyl chlorocarbonate was added with stirring. After 15 min, 4.66 g (0.022 mol) of L-glutamic acid dimethyl ester hydrochloride was added, followed by 3.0 mL (0.021 mol) of triethylamine in 60 mL of cold tetrahydrofuran. Stirring was continued at −10–0°C for 30 min and at 0°C for 1.5 h. The reaction mixture was diluted with 300 mL of cold water, and a yellow-green oil separated and was extracted three times with ethyl acetate. The extract was dried (Na_2SO_4) and concentrated to a small volume using a rotary evaporator. Crystallization resulted on storage at 0°C, and recrystallization from absolute ethanol gave 5.2 g (61% yield) of white crystals, mp 113–114°C; IR (KBr): 3300 (N—H), 1755 (C=O), 1735 (C=O), 1660 (C=O), 1640 (C=O), and 1545 cm^{-1} (CNH).

Inhibition of Glyoxalase I—The enzymatic reactions were carried out at room temperature in 0.05 M phosphate buffer, pH 6.6. A fresh solution of glutathione in distilled water was made before each assay. For each assay, the spectrophotometer cell contained a total volume of 3.0 mL, which was 5.0 mM in methylglyoxal and 0.869 mM in glutathione.

A commercial 40% (w/v) solution (10 mL) of methylglyoxal was diluted to 50 mL with distilled water, and the acidic components were removed by a Dowex 1-X8 (200–400 mesh, carbonate form) column. The solution was standardized by the method of Friedemann (16). Glyoxalase I was diluted to a concentration of 40 μg of protein/mL with 0.05 M phosphate buffer (pH 6.6) containing 0.1% bovine serum albumin.

Sufficient amounts of glyoxalase I were employed to give an easily measurable initial rate, which was followed by increase in absorption at 240 nm. Methylglyoxal, glutathione, and buffer were added to the cell and allowed to equilibrate for exactly 6 min before addition of the enzyme. The ratio of the inhibitor concentration to that of the substrate for 50% inhibition, $[I/S]_{50}$ was determined for each compound measured. To determine the concentration of inhibitor required for 50% inhibition (ID_{50}), a plot of v_0/v_I *versus* [I] was made, where v_0 is the initial velocity of the uninhibited reaction and v_I is the initial velocity of the inhibited reaction at various inhibitor (I) concentrations (17). At least two of the points on the plot were in the 30–70% inhibition range. Initial velocity measurements were determined by the change in absorbance, ΔO.D./time with readings at 1-min intervals for 3 min.

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[1] Testing was done at the National Cancer Institute; results were made available through the courtesy of Dr. Harry B. Wood, Jr.

[2] Melting points were taken in capillaries with a Mel-Temp block and are uncorrected. IR spectra were obtained with a Perkin-Elmer model 457 A grating spectrophotometer. The glyoxalase inhibition assay was determined with a Beckman model DB spectrophotometer. Elemental analyses were done by Dr. F. B. Strauss, Oxford, England. TLC was carried out using silica gel plates, and products were detected by exposure to iodine vapor. Organic reagents were supplied by J. T. Baker Laboratory Chemicals and Products, Eastman Organic Chemicals, or Fisher Scientific Co. Glyoxalase I, L-glutamic acid dimethyl ester hydrochloride, *S*-methyl-glutathione, and methylglyoxal were supplied by Sigma Chemical Co.

[3] Representative procedure.

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ACKNOWLEDGMENTS

Abstracted from a thesis submitted by C.-M. Jan to the Massachusetts College of Pharmacy and Allied Health Sciences in partial fulfillment of Master of Science degree requirements. Supported by funds from the John R. and Marie K. Sawyer Memorial Fund, Massachusetts College of Pharmacy and Allied Health Sciences.

Simple Radioligand Binding Assay for the Determination of Urinary Scopolamine

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Received November 2, 1982, from the **Human Pharmacology Institute, Ciba-Geigy, Tübingen, Federal Republic of Germany* and the ‡*Research Department of the Pharmaceutical Division, Ciba-Geigy, Basel, Switzerland.* Accepted for publication March 29, 1983.

Abstract □ A sensitive radioligand binding assay is described for the determination of scopolamine in human urine. As a measure for the drug concentration, the quantitative displacement of scopolamine of tritiated quinuclidinyl benzylate from rat brain receptors was used. The assay is sensitive to concentrations as low as 1.2 ng/mL, surpassed only by GC–MS techniques. It can be performed easily and quickly and does not include extraction procedures. Scopoline and scopine, possible metabolites of scopolamine, do not interfere with the assay. After transdermal administration of scopolamine, 34% of the drug is found in the urine. Of the total scopolamine excreted, 79% is conjugated to glucuronic and/or sulfuric acid and 21% is excreted in the unbound form.

Keyphrases □ Scopolamine—urinary determination, radioligand binding assay, □ Radioligand binding—simple assay, determination of urinary scopolamine □ Excretion, urinary—scopolamine, determination by radioligand binding assay

The lack of suitable methods for measuring low concentrations of scopolamine and other strongly acting parasympatholytic substances in biological fluids after therapeutic dosages in humans makes it difficult to obtain exact pharmacokinetic data on these drugs. Several methods exist for the determination of scopolamine, but these do not allow a rapid and sensitive measurement of low drug concentrations. GC (1) or acid–dye techniques (2) are not sensitive enough, while a GC–MS method (3) is not specific and involves difficult extraction procedures.

The development of radioligand binding techniques for studies of drug interactions with cholinergic receptors, using tritiated quinuclidinyl benzylate (I) as the radioligand (4), allows (analogous to RIA) the measurement of drugs having strong affinity to muscarinic receptors. Using tritiated I we developed a radioligand binding assay for the determination of the very low urinary concentrations of scopolamine that are found after transdermal administration of the drug.

EXPERIMENTAL

Preparation of Rat brain Homogenate—Male rats (Tif RAI f SPF) of 150–250-g body weight were used. After being sacrificed by decapitation, the brains were removed and the cerebral cortices dissected on ice and homogenized[1] for 30 s, at position 5, in 20 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.3). The homogenate was centrifuged at 26,000×*g* for 10 min, and the pellet was resuspended in the same volume of fresh buffer; this step was repeated three times. All procedures were performed at 4°C. The final pellet was suspended in 20 volumes of Tris-HCl and divided into 1-mL aliquots, which were rapidly frozen in liquid propane (~−160°C) and stored at −30°C. (After storage at −30°C for up to 3 months no loss of binding capacity was observed.) For the radioreceptor assay, the 1-mL aliquots were thawed for 5 min in a water bath (37°C) and diluted 17-fold by addition of ice-cold Tris-HCl. Membrane suspensions were not used in the assay for longer than 12 h after thawing.

Radioligand Binding Assay—The radioreceptor assay was performed at room temperature in 5-mL polystyrene tubes in a total volume of 2 mL of Tris-HCl containing brain membranes [~100 μg of protein as determined according to Lowry *et al.* (5)], 0.2 nM [^{3}H]-I[2] (33 Ci/mmol), and a 50-μL urine sample.

After 30 min, the incubation was terminated by filtration through a glass-fiber filter (2.4 cm diameter[3]) under reduced pressure. The filters were rinsed twice with 5-mL ice-cold Tris-HCl. After incubation of the filters for 30 min with 1-mL of tissue solubilizer[4] (diluted 1:1 with propanol), 10 mL of acidified scintillation fluid[5] was added and the radioactivity was determined in a liquid scintillation counter[6]. Nonspecific binding was defined by 10^{-7} M unlabeled I[7]. Scopolamine hydrobromide trihydrate[8] was used as a standard.

For the calibration curve, different concentrations of scopolamine were added to blank urine and tested as described above. The displacement (in percent) of ^{3}H-QNB from the muscarinic receptors in the brain homogenate was plotted on logit–log scale against the scopolamine concentration. Unknown urinary scopolamine concentrations were calculated from the calibration curve. To determine the total scopolamine (conjugated and free drug), urine samples were incubated at 37°C for 3 h with an equal volume of 1 M acetate buffer (pH 4.75) and 7.5 μL of β-glucuronidase–sulfatase[9]/mL of the mixture. The pH was adjusted to 7.3 with NaOH before the binding assay was performed.

Scopolamine Medication and Collection of Urine—Scopolamine was administered transdermally to nine healthy volunteers as a transdermal therapeutic system with scopolamine[10]. The drug system was applied on the skin surface behind the ear. Each system contains 1.5 mg of scopolamine base and can deliver ~0.5 mg of drug over 72 h. The system is programmed to release 0.15 mg as a loading dose during the first few hours; afterward, ~5 μg of scopolamine/h pass through the skin into the blood circulation. The drug system was applied for 64 h, delivering ~0.45 mg of scopolamine. Urine was collected in plastic bottles before the medication, in 12-h periods during the application, and for 8 × 6 h after removal of the drug. Aliquots were stored at −20°C in plastic tubes until assayed. For each subject, an individual urine blank for the calibration of the assay was collected before the medication.

[1] Polytron; Kinematika, Luzern, Switzerland.

[2] New England Nuclear, Boston, Mass.
[3] GF/B; Whatman.
[4] Soluene; Packard.
[5] Aquasol; Packard.
[6] Tri-Carb 460C; Packard.
[7] Synthesized by Dr. A. Storni, Ciba-Geigy, Basel, Switzerland.
[8] EGA Chemie, Steinheim, West Germany.
[9] Calbiochem.
[10] Scopoderm TTS; Ciba-Geigy.

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