Augmentation of Human and Rat Lenticular Glutathione in Vitro by Prodrugs of γ-L-Glutamyl-L-cysteine

Herbert T. Nagasawa,*[,†,‡] Jonathan F. Cohen,[‡] Ann M. Holleschau,[§] and William B. Rathbun[§]

Medical Research Laboratories, VA Medical Center, Minneapolis, Minnesota 55417, and the Departments of Medicinal Chemistry and Ophthalmology, University of Minnesota, Minneapolis, Minnesota 55455

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A marked age-related decrease in glutathione (GSH) levels as well as depression of γ-glutamylcysteine synthetase activity are factors that are believed to render the aged lens more susceptible to oxidative stress and, therefore, to cataractogenesis. Providing γ-L-glutamyl-L-cysteine, the dipeptide precursor of GSH, would effectively bypass the compromised first step in its biosynthesis and should protect the lens from GSH depletion. Accordingly, some bioreversible sulfhydryl-, amino-, and C-terminal carboxyl-protected prodrug forms of this dipeptide were prepared. Sulfhydryl protection was in the form of an acetyl thioester, while the carboxyl group was protected as the ethyl ester. These prodrugs were evaluated for their GSH-enhancing activity in cultured human and rat lenses in vitro using an assay that measured the incorporation of [^{14}C]glycine into lens GSH. Ethyl *S*-acetyl-γ-L-glutamyl-L-cysteinate (**2**) raised GSH levels in human lenses by 25% and in rat lenses by >150%. These data suggest that **2** may have potential as an anticataract agent since ethyl γ-L-glutamyl-L-cysteinate (**1a**), the des-*S*-acetyl analog of **2,** had been shown (by others) to protect against experimental rodent cataracts. GSH augmentation by **1a** was 2% in human lenses and 25% in rat lenses, considerably less than that shown by **2**.

The sulfhydryl-containing tripeptide, glutathione (GSH; γ-L-glutamyl-L-cysteinylglycine), is generally recognized as the major and essential cellular antioxidant that protects ocular lenses from oxidative stress elicited by ultraviolet radiation, free radicals, and/or reactive electrophilic metabolites of substances with xenobiotic origin. It is noteworthy that a healthy ocular lens contains the highest concentrations of GSH of any mammalian tissue.[1] The concentration of GSH is 6-fold higher in the single cell layer forming the epithelium than in the enclosed bulk of the lens.

The biosynthesis of GSH is known to be catalyzed by two enzymes acting in tandem, viz., γ-glutamylcysteine synthetase (γ-GCS; EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3).[2] The reaction catalyzed by γ-GCS was shown to be rate-limiting in the lens due in part to the paucity of L-cysteine.[3] In cultured rat and human lenses, the rate of GSH biosynthesis was directly proportional to uptake of L-cysteine, indicating that the uptake of this amino acid was the ultimate, rate-limiting factor in lenticular GSH biosynthesis.[3,4] Moreover, the activity of γ-GCS was found to be 35−100-fold lower in the lenses of humans, Old World monkeys, and apes than in the lenses of prosimians (lower primates) and nonprimates.[5,6]

Harding[7] and others[4] have observed an *age-related* decrease of GSH levels in the human lens, due in part, to a 4-fold decrease in L-cysteine uptake with aging. Additionally, an age-related decrease of γ-GCS activity was observed in human, Old World monkey, and orangutan lenses, but not in the lenses of prosimians or several nonprimates.[8−10] These factors render the aging human lens more susceptible to oxidative stress and, ostensibly, to cataractogenesis.

The low levels of GSH in aging lens might, therefore, theoretically and practically be restored by two therapeutic strategies implemented singly or in concert, viz., (a) by providing γ-L-glutamyl-L-cysteine (γ-GluCys, **1b**), the dipeptide precursor of GSH, thereby effectively bypassing the compromised first step in its biosynthesis, or (b) by supplying GSH itself. More correctly, GSH and/or its dipeptide precursor must be made available in pharmacologically and biochemically compatible *prodrug* forms, since their ionic charges prevent their uptake by cells for biological utilization.

It has been reported[11] that the dipeptide prodrug, γ-L-glutamyl-L-cysteine monomethyl ester (γ-GluCysOMe), prevented buthionine sulfoximine (BSO)-induced cataracts in ICR mice by bypassing the latter's inhibitory action on γ-GCS. Similar results were shown also for the corresponding monoethyl ester, γ-GluCysOEt (**1a**).[12] Moreover, instillation of liposome encapsulated **1a** to the eyes of streptozotocin-induced diabetic rats prevented the depletion of lens GSH and, hence, cataract formation.[13] The isopropyl and hexyl esters of **1b** have been proposed as more lipophilic prodrug forms of this dipeptide. However, the presence of a free SH group in compounds such as **1a** severely limits the formulation of aqueous pharmaceutical dosage forms because of the facile air oxidation of such SH groups in solution.[14]

Accordingly, we prepared and herein describe some sulfhydryl-, amino-, and C-terminal carboxyl-protected prodrug forms of the dipeptide **1b** (Chart 1) and show that the sulfhydryl-protected prodrugs are highly stable in physiological saline solution. One of these prodrugs, viz., **2** (vide infra), also significantly augmented GSH when incubated with human and rat lenses in vitro, suggesting that **2** may be useful as a topical anticataract agent. A new synthetic method for the preparation of **1a** and **1b** is also described.

† VA Medical Center.
‡ Department of Medicinal Chemistry.
§ Department of Ophthalmology.
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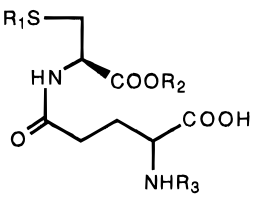
Chart 1



Compound No.	R_1	R_2	R_3
1a	H	Et	H
1b	H	H	H
2	CH_3CO	Et	H
3	CH_3CO	Et	CH_3CO
4	CH_3CO	H	H
5	CH_3CO	H	CH_3CO

Results

Chemistry. In the synthesis of **1a**, it was observed that physical manipulation of this product resulted in partial oxidation to its disulfide form, even when these operations were conducted under N_2. Because of this extreme susceptibility to air oxidation of the sulfhydryl group, it was rationalized that the SH group of **1a** (as well as the free SH groups of other biochemical precursors of GSH) must be derivatized to a bioreversible functionality. Accordingly, the *S*-acetyl derivative of **1a**, viz., **2**, was prepared, such acetyl thioesters being readily hydrolyzable in vivo by nonspecific esterases.[15]

Scheme 1 outlines the synthetic route to ethyl *S*-acetyl-γ-L-glutamyl-L-cysteinate (**2**), which starts by coupling of the sulfhydryl-masked cysteine derivative ethyl 2(*R*,*S*)-methylthiazolidine-4(*R*)-carboxylate (**6a**) as its trimethylsilyl derivative with the synthon, phthaloyl-L-glutamic anhydride.[16] The crude product (not shown in Scheme 1) was not isolated, and the phthaloyl protective group was removed directly by hydrazinolysis. The resulting ethyl 3-(γ-L-glutamyl)-2(*R*,*S*)-methylthiazolidine-4(*R*)-carboxylate (**7a)** was isolated and subjected to extensive purification by ion exchange chromatography. Opening the thiazolidine ring of **7a** provided the free sulfhydryl compound γ-GluCysOEt (**1a)**. This ring-opening reaction with $Hg(OAc)_2$[17] was found to be extremely sensitive to reaction conditions, with lower temperatures resulting in recovery of the thiazolidine starting material and excess reagent or higher temperatures leading to byproduct formation with attendant low yields.

Although **1a** could be isolated at this stage (indeed, this was accomplished in several runs), because of its sensitivity to air oxidation (vide supra) a more prudent approach was to selectively acetylate the free sulfhydryl group of crude **1a** with minimal acetylation of the α-amino group of the γ-glutamyl moiety to provide the sulfhydryl-protected ethyl *S*-acetyl-γ-L-glutamyl-L-cysteinate (**2**) directly. Regioselective acetylation was effected by subjecting the dipeptide ester **1a** to careful, low-temperature reaction with thioacetic acid in batchwise additions and monitoring the reaction course by NMR analysis of the products formed. Although formation of 3−5% of the bis-acetylated dipeptide ester (**3**) was indicated, the latter could be separated by physical means, and purification by reverse-phase column chromatography gave pure **2** in 15% overall yield from **7a**. An additional 37% of a slightly less pure product was also isolated. The synthesis of *S*-acetyl-γ-L-glutamyl-L-cysteine (**4**) followed similar paths (Scheme 1, b series), giving the regioselectively *S*-acetylated **4,** isolated ultimately as the crystalline hydrochloride. The corresponding *N*,*S*-bis-acetylated dipeptides **3** and **5** were readily prepared from crude **1a** and **1b**, respectively, using standard acetylation conditions with Ac_2O and aqueous $NaHCO_3$ as base.

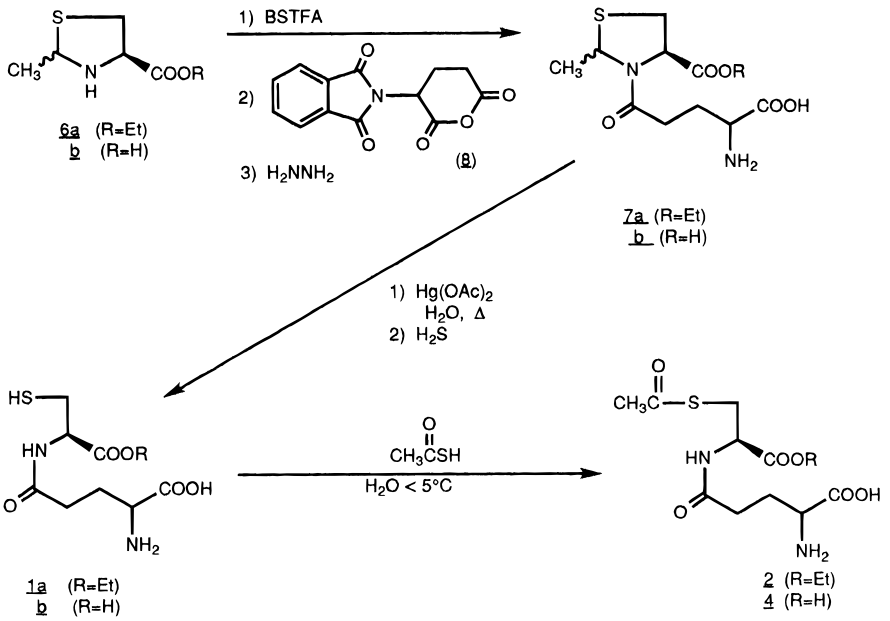
The γ-glutamylated compounds **2**, **4**, **7a**, and **7b**, on positive ion chemical ionization mass spectrometry (+tive CI/MS) all showed the presence of a prominent *m*/*z* 130 ion due to protonated pyroglutamic acid, which is diagnostic for γ-glutamylated (as opposed to α-glutamylated) amino acids[18] and/or γ-glutaminated amines.[19] All had base peaks at *m*/*z* 84.

The premise that protective acylation of the sulfhydryl group of **1a** and **1b** would lead to products that are stable to air oxidation was borne out by analysis of **2** and **4** dissolved in isotonic saline. Thus, the proton NMR spectrum of **2 (**or **4)** dissolved in 0.9% NaCl in D_2O remained unchanged for 43 days at room temperature, after which time it was not further analyzed (see Supporting Information). However, in 0.1 M deuterated phosphate buffer (pD 7.4) containing 0.9% NaCl, some evidence for solvolysis of the *S*-acetyl group of **4** was seen by the appearance of an acetate peak at δ 1.94 with concomitant disappearance of the peak due to the *S*-acetyl group at δ 2.36 after 4 days at room temperature (see Supporting Information). The height of this acetate peak did not exceed 8% even after 28 days. However, the stability of **2** in the phosphate-buffered system was considerably less than for **4**. After 24 h, 11−12% of the thioester had solvolyzed (see Supporting Information), and after 29 days at room temperature, only 66**%** of the thioester remained intact. Disulfide formation was also evident.

Augmentation of Lenticular GSH in Vitro. In order to determine whether these derivatives of **1b** were in fact serving as prodrugs of GSH when incubated with human and rat ocular lenses in vitro, it was necessary to devise an assay system to measure their incorporation into lens GSH. The multiple steps required to introduce ^{14}C-labeled L-glutamic acid or ^{35}S-labeled L-cysteine into these dipeptides precluded their synthetic incorporation into **2** or **4** via Scheme 1 for use in tracer studies, and a more general assay specifically applicable to these dipeptide prodrugs was needed.

The enzyme glutathione synthetase catalyzes the second step in GSH biosynthesis, viz., the coupling of γ-GluCys (**1b**) to glycine to form the tripeptide.[2] If the dipeptide derivatives depicted in Chart 1 were in fact behaving as prodrugs of **1b** itself, i.e., if the acetyl group(s) and/or the ethyl carboxylate group of these compounds were being hydrolyzed by lenses to **1b**, then these prodrugs, in the presence of glycine, should stimulate the biosynthesis of GSH resulting in an increase in lens GSH levels relative to controls without the prodrugs. By utilizing ^{14}C-labeled glycine and measuring the extent of incorporation of its radioactive label into lens GSH, the assay specifically links the dipeptide prodrugs to the biosynthesized GSH.[20] Any increase found in the levels of *radioactive* GSH would, therefore, represent de novo biosynthesis, lending persuasive support that the dipeptide released from the prodrug was being incorporated into lens GSH.

Scheme 1



The generality of the method is also attractive. For example, the stimulation of de novo GSH biosynthesis by *cysteine* prodrugs can also be evaluated using this assay method since, as alluded to earlier, the rate-limiting step in GSH biosynthesis involves this sulfhydryl amino acid. This assay developed in our laboratories[21] was used to determine GSH augmentation in lenses by these prodrugs of γ-GluCys.

Incubation of isolated human and rat ocular lenses with [^{14}C]glycine and the dipeptide derivatives **2, 3,** and **4** raised the levels of radiolabeled GSH in human lenses when compared to control, paired, contralateral lenses incubated without the test compounds (Figure 1). In contrast, **1a** and **5** were not effective in augmenting GSH levels in the cultured human lens. However, the variable postmortem times for lens excision (5−15 h) and the donors' variable ages and unknown medical history may have been factors. Although the control lenses from rats were not always contralateral, the augmentation of rat lens GSH by **2** was particularly impressive. Compounds **4** and **5** were equally as effective as **1a**, while compound **3** had no effect on rat lens GSH.

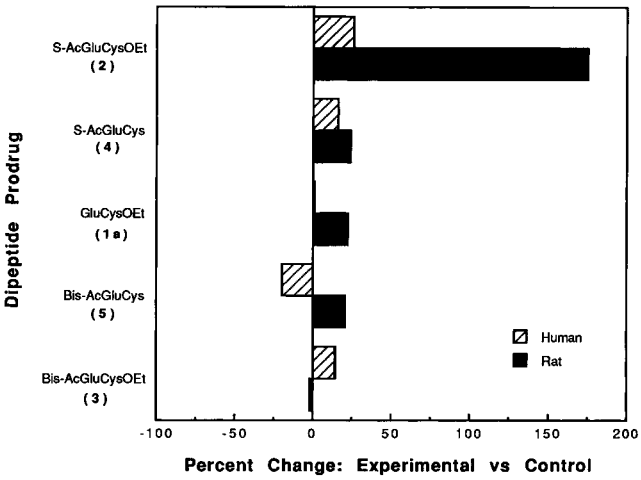


Figure 1. Incorporation of [^{14}C]glycine into GSH in cultured human and rat lenses in the presence of γ-L-glutamyl-L-cysteine prodrugs. For the human lens (n = 4−6), the contralateral lens served as "no-drug" controls. For the rat lens (n = 8−12) however, the lenses were pooled and were not always contralateral. The accuracy of the method using GSH as the standard was greater than 95%.

The comparative efficacy of γ-GluCysOEt (**1a**) and its *S*-acetylated derivative **2** in this in vitro system was of particular interest, since **1a** has already been shown to be an effective anticataract agent in experimental rodent cataracts.[11−13] The results shown in Figure 1 suggest that compound **2**, with over 150% stimulation of GSH biosynthesis in the rat lens and 25% stimulation in the human lens, may be an even more effective aniticataract agent than **1a**, particularly as a topical agent, with such topical formulations now being possible due to the intrinsic stability of **2** over **1a** in solution.

Discussion and Conclusions

Cataract, a disease of the ocular lens, is characterized by opacities that result from a change in the refractive index at affected loci due to influx of water to form "lakes". The interface between such relatively hydrated areas with low refractive index and the unaffected anhydrous areas with higher refractive index scatters and reflects incident light causing apparent opacities termed cataracts.[22] One function of lenticular GSH is to maintain the integrity of the plasma membrane responsible for the continual efflux of water from this anhydrous tissue, a function impaired by low levels of GSH.[23,24] In addition, the age-related decrease in lenticular GSH due to low levels of γ-GCS accompanied by increased susceptibility to oxidative stress suggests that augmentation of lens GSH by supplying its dipeptide precursor, viz., **1b**—*as prodrug forms*—constitutes a rational therapeutic approach for the prevention of cataracts.

Summarizing our results toward these goals, we have devised a new synthesis for γ-GluCys (**1b**), as well as its monoethyl ester derivative **1a**, and have shown that regioselective *S*-acetylation of **1a** or **1b** provides potentially bioreversible derivatives that are highly stable in physiological saline solution over prolonged periods.

In addition, several of these prodrug forms of **1b** were found to be incorporated into GSH when incubated with

human and/or rat ocular lenses in the presence of ^{14}C-labeled glycine. The most effective compound of this series was **2**, the *S*-acetylated ethyl ester derivative of **1b**. Although no direct proof for the hydrolysis of the thioester or the carboxyester group in lenses is yet available, the method of assay[20,21] suggests that the incorporation of the resulting **1b** directly into GSH is a likely possibility, since complete degradation of these dipeptide derivatives to the component α-amino acids followed by de novo resynthesis of GSH from L-cysteine appears to be improbable.

Hazelton and Lang[25] and Chen et al.[26] have reported that GSH levels of liver, kidney, spleen, heart, lung, and blood were depressed in aged mice, although cysteine levels (exception, kidneys) were indistinguishable from that found in middle-aged and young mice. This suggested that the biosynthesis of γ-GluCys (**1b**) was sluggish in aged mice. Indeed, when these mice were treated with GSH monoethyl ester, a prodrug of GSH with anticataract properties,[27] tissue GSH levels returned to normal, undepressed levels.[28] The possibility that **2**, or any of the other γ-GluCys derivatives described in Chart 1, might also restore tissue GSH levels in aged mice appears to be worthy of further exploration.

Experimental Section

^{1}H-NMR spectra were recorded at ambient temperature on either GE-300 or Bruker AC-200 NMR spectrometers. Chemical shifts are reported as δ values (ppm). Mass spectra (CI, EI, or FAB) were obtained on a Kratos MS 25 mass spectrometer. For TLC analyses, Analtech silica gel GF and RPS-F plates were used. The solvent systems used for TLC were butanol:acetic acid:water (50:11:25) for normal phase, and acetonitrile:water (95:5 to 80:20) for reverse phase. The plates were visualized by spraying with ninhydrin solution and heating. Column chromatography was carried out using columns packed with octadecyl-functionalized silica gel (C-18 RPSG) (Aldrich) and Kieselgel 60 (230−400 mesh) silica gel (EM Science). Mercuric acetate was purchased from Fisher Scientific Co., thioacetic acid from Aldrich Chemical Co. or Sigma Chemical Co., and H_2S (gas) from Matheson Gas Products. Whenever the reactants or products contained a free sulfhydryl group, the reactions were conducted under a N_2 or Ar atmosphere.

Ethyl 2(*R*,*S*)-Methylthiazolidine-4(*R*)-carboxylate (6a). To a stirred solution of acetaldehyde (3.67 g, 83.2 mmol) in ice-cooled H_2O (30 mL) was added a solution of cysteine ethyl ester. [The latter was prepared by dissolving cysteine ethyl ester hydrochloride (14.1 g, 75.7 mmol) in ice-cooled H_2O (30 mL) and adding to it a 1 M aqueous NaOH solution (65 mL).] Since the mixture was cloudy and contained some precipitate, 50 mL of H_2O was added. The now homogeneous solution was kept in a refrigerator overnight and then extracted with 3 × 100 mL portions of EtOAc. The combined organic extracts were washed with 200 mL of saturated aqueous NaCl and dried over Na_2SO_4, and the solvent was evaporated to give 10.6 g of a light yellow oil. The oil was distilled through a small Vigreux column to give four fractions with identical NMR spectra (colorless oil, bp 56−61 °C/0.034 mmHg; reported[29] bp 80−81 °C/0.95 mmHg), $n^{23}_D = 1.4939$, in a total yield (63%) of 9.12 g: ^{1}H NMR ($CDCl_3$) δ 1.29 (t, 3H, C*H*$_3$CH$_2$), 1.49 and 1.60 (2d, J = 6.3 Hz and J = 6.1 Hz, 3H, C*H*$_3$CH), 2.2 (br s, 1H, N*H*), 2.9 and 3.3 (m, 2H, C*H*$_2$S), 3.81 (dd, J = 6.1, 9.2 Hz) and 4.2 (m, 1H, C*H*CO$_2$), 4.3 (m, 2H, C*H*$_2$CH$_3$), 4.56 and 4.79 (2q, J = 6.1 and 6.3 Hz, 1H, C*H*CH$_3$); mass spectrum (EI), m/z (rel intensity) 175 (M^+) (10), 160 (M − CH_3) (20), 102 (M − CO_2Et) (100). Anal. ($C_7H_{13}NO_2S$) C, H, N.

Ethyl 3-(γ-L-Glutamyl)-2(*R*,*S*)-methylthiazolidine-4(*R*)-carboxylate (7a). The trimethylsilyl derivative of **6a** (9.12 g, 52.1 mmol) in dry CH_3CN (90 mL) was prepared in situ by adding bis(trimethylsilyl)trifluoroacetamide (7.5 mL, 7.8 g, 30 mmol) and heating the mixture under reflux for 50 min.[30] After cooling to room temperature, *N*-phthaloyl-L-glutamic anhydride[16] (13.8 g, 53.1 mmol) was added, followed by dry CH_3CN (35 mL). After several days at room temperature, the solvent was evaporated in vacuo, keeping the temperature under 40 °C. To the resultant orange oil (37.1 g) was added with stirring 1.0 M Et_3N in MeOH (200 mL) and 2.0 M $NH_2NH_2 \cdot H_2O$ in 50% aqueous MeOH (70 mL). After solvent evaporation in vacuo, the resultant thick paste was suspended in H_2O (300 mL) and the solvent evaporated again to give 33.1 g of crude product (dark yellow paste) which was suspended in H_2O (350 mL) and acidified to pH 3.0 with 1.0 N HCl whereupon phthalhydrazide precipitated. This was removed by filtration, and the filtrate volume was reduced to ca. 200 mL by evaporation in vacuo when more phthalhydrazide precipitated. This was removed as above, and the filtrate was evaporated in vacuo to incipient dryness to give 24.6 g of a thick orange oil.

The product thus obtained was purified by ion exchange chromatography using 140 g of Bio Rad cation exchange resin (AG 50WX4, H^+ form). The column was washed with H_2O (1 L) until the washings were colorless. It was eluted with 0.05 N aqueous NH_3 to give 12.8 g (81% yield) of **7a** as a light yellow solid: mp 166−168° C dec; ^{1}H NMR (D_2O) δ 1.36 (m, 3H, C*H*$_3$CH$_2$), 1.51 and 1.63 (2d, J = 6.2 Hz and J = 6.4 Hz, 3H, C*H*$_3$CH), 2.2 (m, 2H, C*H*$_2$CHNH$_2$), 2.7 (m, 2H, C*H*$_2$CO), 3.4 (m, 2H, C*H*$_2$S), 3.8 (m, 1H, C*H*NH$_2$), 4.2 (m, 2H, C*H*$_2$CH$_3$), 4.8 (m, 1H, C*H*CO$_2$H), 5.2 and 5.43 (m and q, J = 6.4 Hz, 1H, C*H*CH$_3$); mass spectrum (CI), m/z (rel intensity) 130 (15), 84 (100). Anal. ($C_{12}H_{20}N_2O_5S$) C, H, N.

Ethyl *S*-Acetyl-γ-L-glutamyl-L-cysteinate (2). A solution of **7a** (3.08 g, 10.1 mmol) in H_2O (50 mL) was stirred at 38 °C. Mercuric acetate (10.0 g, 31.4 mmol) in H_2O (50 mL) was added over 2 min. A milky-white, foamy precipitate formed immediately. The mixture was stirred for 2.5 h at 38−41 °C, then cooled to room temperature, and gaseous H_2S was bubbled through the stirred solution for 45 min. A black precipitate formed as soon as the H_2S was introduced. Nitrogen was bubbled through the stirred mixture for 30 min to remove H_2S by entrainment, after which time the solids were removed by filtration using Celite. Nitrogen was again bubbled through the filtrate, and after storage overnight in a refrigerator, the solvent was evaporated in vacuo, keeping the temperature under 40 °C. Addition of H_2O (ca. 75 mL) followed by solvent evaporation was repeated twice to give 2.16 g of pink solids. The NMR spectrum of the crude product showed no starting material and a doublet at δ 3.00 ppm assigned to the methylene group adjacent to the SH group of γ-L-glutamyl-L-cysteine ethyl ester (**2a**). This product was dissolved in H_2O (100 mL) and the solution cooled in an ice bath. Thioacetic acid (1.10 mL, 15.5 mmol) was added dropwise, and the mixture was shaken to dissolve all the reagent. The solution was then placed in a refrigerator, and after 3 days the solvent was evaporated in vacuo, keeping the temperature under 40 °C. Addition of H_2O (*ca.* 75 mL) and solvent evaporation was repeated twice to give 2.43 g of light yellow solids. NMR analysis showed that about 30% starting material still remained, based on the integration of the methylene multiplet at δ 3.0 ppm in the starting material vs the eight-line ABX pattern at about δ 3.5 ppm in the product. The crude product was therefore dissolved in ice-cooled water (80 mL) and the thioacetylation procedure was repeated twice when the NMR analysis showed less than 5% starting material remaining. During this acetylation procedure the pH was adjusted to between 3 and 4 with aqueous HCl or aqueous NaOH when necessary.

The product thus obtained was purified by column chromatography using 105 g of C-18 reverse-phase silica gel (RPSG). The column was washed with 500 mL each of H_2O:acetonitrile (1:3) and H_2O:acetonitrile (1:19). The product was placed on the column by dissolving it in H_2O, adding 1.1 g of RPSG, removing the solvent, and placing the coated RPSG on top of the column. The flask residues were scavenged by repeating this procedure with about 0.5 and then 0.2 g of RPSG. The column was eluted with H_2O:acetonitrile (1:19) and then with H_2O:acetonitrile (1:9). The representative ninhydrin-positive fractions collected were evaporated and analyzed by NMR

spectroscopy. The early fractions contained as much as 10% *N*,*S*-bis-acetylated **3,** while the later fractions showed decreased ester concentrations and some ester-hydrolyzed *S*-acetyl-γ-L-glutamyl-L-cysteine (**3**) plus some other unidentified material. The best fractions were combined and lyophilized to give 844 mg of light yellow solid, mp 147−149 °C dec (26% yield). The NMR spectrum showed a small doublet at about δ 1.6 ppm. This product was treated with decolorizing carbon, the mixture filtered, and the filtrate lyophilized again to give 491 mg (15% yield) of very pale pink solids whose NMR spectrum showed nearly complete absence of the doublet at δ 1.6 ppm. Another 1.2 g (37% yield) of less pure product was also isolated: ^{1}H NMR (D_2O) δ 1.25 (t, J = 7.1 Hz, 3H, CH_2CH_3), 2.10 (m, 2H, CH_2CH), 2.36 (s, 3H, CH_3CO), 2.45 (m, 2H, $COCH_2$), 3.26 (dd, J = 14.3 Hz, J = 7.1 Hz, 1H, SC*H*), 3.45 (dd, J = 14.3 Hz, J = 4.8 Hz, 1H, SC*H*), 3.81 (t, J = 6.2 Hz, 1H, $CHNH_2$), 4.20 (q, J = 7.1 Hz, 2H, CH_2CH_3), 4.65 (dd, J = 7.1 Hz, J = 4.9 Hz, 1H, C*H*NH); mass spectrum (CI), *m*/*z* (rel intensity) 130 (50), 84 (100). Anal. ($C_{12}H_{20}N_2O_6S$) C, H, N.

Ethyl *N*,*S*-Bisacetyl-γ-L-glutamyl-L-cysteinate (3). Crude γ-L-glutamyl-L-cysteine ethyl ester (**1a**, 1.31 g, 4.71 mmol) was acetylated in the same manner as for **5** below using 2.7 mL (2.9 g, 28 mmol) of acetic anhydride. The 1.53 g of crude product obtained, along with 0.34 g from another run, was purified by column chromatography using 105 g of C-18 reverse-phase silica gel (RPSG). The column was washed with methanol and then with water. The column was eluted with H_2O, followed by CH_3OH:H_2O (5:95), and finally with CH_3OH:H_2O (10:90) to give 0.59 g of the titled compound as a hygroscopic white solid: mp 66.5−68.5 °C; ^{1}H NMR (D_2O) δ 1.25 (t, J = 7.2 Hz, 3H, CH_3CH_2), 2.01 (s, 3H, CH_3CONH), 2.1 (m, 2H, CH_2CH_2CO), 2.34 (s, 3H, CH_3COS), 2.4 (m, 2H, CH_2CO), 3.21 (dd, J = 14.2 Hz, J = 7.4 Hz, 1H, SC*H*), 3.43 (dd, J = 14.2 Hz, J = 4.8 Hz, 1H, SC*H*), 4.18 (q, J = 7.1 Hz, 2H, CH_2CH_3), 4.30 (dd, J = 9.2 Hz, J = 4.8 Hz, 1H, $CHCH_2CH_2$), 4.62 (dd, J = 7.3 Hz, J = 4.8 Hz, 1H, $CHCH_2S$); mass spectrum (FAB), *m*/*z* 363 (MH^+). Anal. ($C_{14}H_{22}N_2O_7S\cdot0.5H_2O$) C, H, N.

3-(γ-L-Glutamyl)-2(*R*,*S*)-methylthiazolidine-4(*R*)-carboxylic Acid (7b). This product was prepared from 2(*R*,*S*)-methylthiazolidine-4(*R*)-carboxylic acid[31] (8.51 g, 57.9 mmol) essentially as described for the preparation of **7a**. The crude product thus obtained (24.6 g of a thick oil) was purified by ion exchange chromatography. The column packed with 400 g of Sigma anion exchange resin (SBR nuclear grade, OH^- form) was washed with H_2O (3 L), 1 M AcOH (2 L), and H_2O (4 L) to convert it to the acetate form before charging with the product. The column was eluted with 0.5 M AcOH and 1.0 M AcOH to give 8.10 g (51% yield) of the titled compound as an off-white solid: mp 190−192.5 °C dec; ^{1}H NMR (D_2O) δ 1.52 and 1.63 (2d, J = 6.3 Hz and J = 6.5 Hz, 3H, CH_3CH), 2.2 (m, 2H, CH_2CHNH_2), 2.7 (m, 2H, CH_2CO), 3.4 (m, 2H, CH_2S), 3.8 (m, 1H, $CHNH_2$), 4.8 (m, 1H, $CHCO_2H$), 5.4 (m, 1H, $CHCH_3$); mass spectrum (CI), *m*/*z* (rel intensity) 130 (32), 84 (100). Anal. ($C_{10}H_{16}N_2O_5S$) C, H, N.

***S*-Acetyl-γ-L-glutamyl-L-cysteine (4).** This product was prepared from **7b** (2.10 g, 7.60 mmol) in a manner similar to the preparation of **2**. The crude **4** (ca. 2 g) thus obtained was purified by column chromatography using 185 g of silica gel as follows. The column was washed with 2 L of 0.001 N aqueous HCl:1-propanol (1:10) and 500 mL of 0.001 N aqueous HCl:1-propanol (1:20). The product was placed on the column by dissolving it in H_2O, adding 2.0 g of silica gel, removing the solvent, and placing the coated silica gel on top of the column. The flask residues were scavenged by repeating this procedure with about 0.5 g of silica gel. The column was eluted with 0.001 N aqueous HCl:1-propanol (1:20) and then 0.001 N aqueous HCl:1-propanol (1:10). Representative ninhydrin-positive fractions were evaporated and the residues analyzed by NMR spectroscopy. The best fractions were combined and lyophilized. The early fractions contained some pyroglutamic acid and up to about 10% of the bis-acetylated **5**. The yield of pure **4** was 0.60 g, mp 192−194.5 °C dec (27%). Another 0.70 g (32% yield) of less pure product was also isolated: ^{1}H NMR (D_2O) δ 2.14 (m, 2H, CH_2CH), 2.37 (s, 3H, CH_3), 2.43 (m, 2H, $COCH_2$), 3.19 (dd, J = 14.2 Hz, J = 7.3 Hz, 1H, SC*H*), 3.48 (dd, J = 14.2 Hz, J = 4.5 Hz, 1H, SC*H*), 3.82 (t, J = 6.2 Hz, 1H, $CHNH_2$), 4.53 (dd, J = 7.3 Hz, J = 4.5 Hz, 1H, C*H*NH); mass spectrum (CI), *m*/*z* (rel intensity) 130 (30), 84 (100).

The hydrochloride was prepared by treatment of a crude sample with 1 equiv of 0.1 N aqueous HCl. This solution was then passed through a column of RPSG to remove lipophilic material by eluting with H_2O:CH_3CN (5:95). The product was dissolved in isopropyl alcohol, and any insoluble material was removed by filtration. Ether was added to the filtrate, and the solids that formed after cooling to −20 °C were removed. The filtrate was then evaporated, the resulting solids were taken up in water, and the solution was lyophilized to give the hydrochloride of **4** as a hygroscopic white solid: mp 68−70 °C; ^{1}H NMR (D_2O) δ 2.25 (m, 2H, CH_2CH), 2.38 (s, 3H, CH_3), 2.54 (m, 2H, $COCH_2$), 3.27 (dd, J = 14.3 Hz, J = 7.5 Hz, 1H, SC*H*), 3.50 (dd, J = 14.3 Hz, J = 4.6 Hz, 1H, SC*H*), 4.09 (t, J = 6.7 Hz, 1H, $CHNH_3$), 4.6 (m, 1H, C*H*NH). Anal. ($C_{10}H_{16}N_2O_6S\cdot HCl$) C, H, N.

***N*,*S*-Bisacetyl-γ-L-glutamyl-L-cysteine (5).** Crude γ-L-glutamyl-L-cysteine (**1b**, 1.59 g, 6.36 mmol) was dissolved in H_2O (20 mL), and the solution was stirred with ice bath cooling. A solution of $NaHCO_3$ (6.9 g, 82 mmol) in 80 mL of H_2O was added, followed by dropwise addition of acetic anhydride (3.6 mL, 3.9 g, 38 mmol) over 1 min. After 2 min a test for free thiol was negative. The solution was acidified to pH 2.5−3 with 6 N and then 1 N HCl. Removal of the solvent afforded 8.3 g of white solids whose NMR spectrum showed the presence of both *S*-acetyl and *N*-acetyl groups. The solids were extracted with 4 × 50 mL portions of hot absolute ethanol to give 1.71 g of product which, along with 0.36 g from another run, was purified by flash chromatography using 100 g of silica gel. The column was eluted with 1-propanol:0.02 N aqueous HCl (95:5), followed by 1-propanol:0.01 N aqueous HCl (90:10) to give 1.02 g of the titled compound as a hygroscopic white solid: mp 87−89 °C; ^{1}H NMR (D_2O) δ 2.0 (m, 2H, CH_2CH_2CO), 2.03 (s, 3H, CH_3CONH), 2.3 (m, 2H, CH_2CO), 2.37 (s, 3H, CH_3COS), 3.14 (dd, J = 14.1 Hz, J = 7.5 Hz, 1H, SC*H*), 3.48 (dd, J = 14.1 Hz, J = 4.4 Hz, 1H, SC*H*), 4.19 (dd, J = 9.1 Hz, J = 4.5 Hz, 1H, $CHCH_2CH_2$), 4.46 (dd, J = 7.5 Hz, J = 4.3 Hz, 1H, $CHCH_2S$). Anal. ($C_{12}H_{18}N_2O_7S$) C, H, N.

Stability Studies. These studies were directed toward evaluating the stability of the *S*-acetylated compounds **2** and **4** in saline solution as well as in phosphate buffer at physiological pH. Accordingly, **2** (and/or **4**) was dissolved in 0.9% NaCl solution in D_2O, and the proton NMR spectrum was monitored over time. There was no discernible change in the spectrum of **2** (or **4**) over the course of $1^1/_2$ months (see Supporting Information).

Compounds **2** and **4** were then dissolved in deuterated 0.1 M PO_4 buffer, the solution was adjusted to pD 7.4, and NaCl was added to a concentration of 0.9%. For **4** there was no apparent change after 1 or 2 days, but after 4 days a small peak appeared at δ 1.90 (NaOAc) which was about 2% of the height of the *S*-acetyl peak at δ 2.36. After a month this OAc peak was only 8% of the height of the *S*-acetyl peak. For **2**, the OAc peak was 19% of the height of the central peak of the stable triplet of the ethyl ester on day 1. This OAc peak grew fairly rapidly as the *S*-acetyl peak shrank. After 1 month, the OAc peak was 84% of the height of the reference peak. (see Supporting Information).

[^{14}C]Glycine Incorporation into Lens GSH in Vitro. The stimulation of [^{14}C]glycine incorporation into GSH in cultured human and rat lenses by the prodrug forms of **1a** was evaluated using the procedure developed by Holleschau et al.[21]

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Supporting Information Available: NMR spectra in 0.9% NaCl/D_2O of compounds **2** and **4** on days 1 and 43 after dissolution and spectra of the same compounds over time showing the solvolysis of the thiol ester in phosphate buffer at pD 7.4 (4 pages). Ordering information is given on any current masthead page.

References

(1) Reddy, V. N.; Giblin, F. J.; Matsuda, H. Defense system of the lens against oxidative damage. *Red Blood Cell and Lens Metabolism*; Srivastava, S. K., Ed.; Elsevier/North Holland: New York 1980; pp 139−154.
(2) Deneke, S. M.; Fanburg, B. L. Regulation of cellular glutathione. *Am. J. Physiol.* **1989**, *257*, L163−L173.
(3) Murray, D. L.; Rathbun, W. B. Conditions for maximizing and inhibiting synthesis of glutathione in cultured rat lenses; an application of HPLC with radioisotope detection. *Curr. Eye Res.* **1990**, *9*, 55−63.
(4) Rathbun, W. B.; Murray, D. L. Age-related cysteine uptake as rate-limiting in glutathione synthesis and glutathione half-life in the cultured human lens. *Exp. Eye Res.* **1991**, *53*, 205−212.
(5) Rathbun, W. B. Glutathione synthesis in evolution: an Achilles' heel of human and other Old World simian lenses. *Ophthal. Res.* **1986**, *18*, 236−242.
(6) Rathbun, W. B.; Holleschau, A. M.; Alterman, L. Glutathione metabolism in primate lenses: a phylogenetic study of glutathione synthesis and glutathione redox cycle enzyme activities. *Am. J. Primatol.* **1994**, *33*, 101−120.
(7) Harding, J. J. Free and protein-bound glutathione in normal and cataractous human lenses. *Biochem. J.* **1970**, *117*, 957−960.
(8) Rathbun, W. B. Activity of glutathione synthesis enzymes in the rhesus monkey lens related to age: a model for the human lens. *Curr. Eye Res.* **1986**, *5*, 161−166.
(9) Sethna, S. S.; Holleschau, A. M.; Rathbun, W. B. Activity of glutathione synthesis enzymes in human lens related to age. *Curr. Eye Res.* **1983**, *2*, 735−742.
(10) Rathbun, W. B.; Holleschau, A. M. The effects of aging on glutathione synthesis enzymes in lenses of Old World simians and prosimians. *Curr. Eye Res.* **1992**, *11*, 601−607.
(11) Ohtsu, A.; Kitahara, S.; Fujii, K. Agent for raising glutathione levels. Jpn. Pat. 64-26516, Jan. 27, 1989.
(12) Ohtsu, A.; Kitahara, S.; Fujii, K. Anticataract properties of γ-glutamylcysteine ethyl ester in an animal model of cataract. *Ophthal. Res.* **1986**, *18*, 236−242.
(13) Ohtsu, A.; Kitahara, S.; Fujii, K. Anticataractogenic property of γ-glutamylcysteine ethyl ester in an animal model of cataract. *Ophthal. Res.* **1991**, *23*, 51−58.
(14) Garst, M. E., Allergan, Inc., Irvine, CA. Personal communication.
(15) Kahns, A. H.; Bundgaard, H. Prodrugs as drug delivery systems. 107. Synthesis and chemical and enzymatic hydrolysis kinetics of various mono- and diester prodrugs of *N*-acetylcysteine. *Int. J. Pharm.* **1990**, *62*, 193−205.
(16) Elberling, J. A.; Zera, R. T.; Magnan, S. D. J.; Nagasawa, H. T. *N*-Phthaloyl-L-glutamic anhydride (2-phthalimidoglutaric anhydride). *Org. Prep. Proc. Int.* **1978**, *11*, 67−70.
(17) Threadgill, M. D.; Gledhill, A. P. Synthesis of peptides containing *S*-(*N*-alkylcarbamoyl)cysteine residues, metabolites of *N*-alkylformamides in rodents and in humans. *J. Org. Chem.* **1989**, *54*, 2940−2949.
(18) Nagasawa, H. T.; Magnan, S. D. J.; Foltz, R. L. Determination of α- from γ-glutamyl dipeptides by chemical ionization mass spectrometry. *Biomed. Mass Spectrom.* **1982**, *9*, 252−256.
(19) Magnan, S. D. J.; Shirota, F. N.; Nagasawa, H. T. Drug latentiation by γ-glutamyltranspeptidase. *J. Med. Chem.* **1982**, *25*, 1018−1021.
(20) Since this procedure involves carboxymethylation of the sulfhydryl group and dinitrophenylation of the free amino group, respectively, of GSH, followed by HPLC separation and analysis of the radioactivity incorporated into this derivatized GSH, the hydrolysis of the protective groups on the dipeptides prior to incorporation into GSH can be assumed.
(21) Holleschau, A. M.; Rathbun, W. B.; Nagasawa, H. T. An HPLC radiotracer method for assessing the ability of L-cysteine prodrugs to maintain glutathione levels in the cultured rat lens. *Curr. Eye Res.*, in press.
(22) Rathbun, W. B. Glutathione in ocular tissues. *Glutathione: chemical, biochemical, and medical aspects*, Vol. III, Part B of the series Coenzymes and cofactors; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley: New York 1989; pp 467−510.
(23) Epstein, D. L.; Kinoshita, J. H. The effect of diamide on lens glutathione and lens membrane function. *Invest. Ophthal.* **1970**, *9*, 629−638.
(24) Epstein, D. L.; Kinoshita, J. H. Effect of methyl phenyldiazenecarboxylate (azoester) on lens membrane function. *Exp. Eye Res.* **1970**, *10*, 228−236.
(25) Hazelton, G. A.; Lang, C. A. Glutathione contents of tissues in the aging mouse. *Biochem. J.* **1980**, *188*, 25−30.
(26) Chen, T. S.; Richie, J. P.; Lang, C. A. Life span changes in acetaminophen detoxification in mice. *Drug Metab. Disp.* **1990**, *18*, 882−887.
(27) (a) Martensson, J.; Steinherz, R.; Jain, A.; Meister, A. Glutathione ester prevents buthionine sulfoximine-induced cataract and lens epithelial cell damage. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 8727−8731. (b) Maydani, M.; Martin, A.; Saatre, J.; Blumberg, J. B. The effect of glutathione (GSH) and GSH monoethyl ester (GSH-ME) on cataractogenesis in Emory mice and Sprague-Dawley rats. *FASEB J.* **1992**, *6*, 1906.
(28) Chen, T. S.; Richie, J. P.; Lang, C. A. Glutathione ester corrects the kidney glutathione and cysteine deficiencies in the aging mouse. *Toxicologist* **1990**, *10*, 320.
(29) Crabb, T. A.; Hall, M. J.; Williams, R. O. Proton magnetic resonance studies of compounds with bridgehead nitrogen atoms-XXVI. Configurational studies with derivatives of perhydrooxazolo[3,4-c]oxazole and the conformational analysis of perhydroimidazo[1,5-c]thiazole. *Tetrahedron* **1973**, *29*, 3389−3398.
(30) Nagasawa, H. T.; Elberling, J. A.; Shirota, F. N. Latentiated forms of the transport-inhibitory α-amino acid adamantanine. *J. Pharm. Sci.* **1980**, *69*, 1022−1025.
(31) Nagasawa, H. T.; Goon, D. J. W.; Muldoon, W. P.; Zera, R. T. 2-Substituted thiazolidine-4(*R*)-carboxylic acids as prodrugs of L-cysteine. Protection of mice against acetaminophen hepatotoxicity. *J. Med. Chem.* **1984**, *27*, 591−596.

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