Isozyme-Specific Glutathione-S-Transferase Inhibitors: Design and Synthesis

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Glutathione-S-transferase (GST) isozyme-selective inhibitors were designed by an empirically guided strategy. In the first phase, literature data were used to select C-terminal modifications which generated maximum variation in the catalytic efficiency (V_{max}/K_m) for glutathione (GSH) analogs used as substrates with different rat GSTs. Also, on the basis of literature data, the sulfhydryl group was functionalized with a selection of alkyl and aryl groups to maximize potential isozyme specificity. Affinity chromatography sorbents were prepared from these which showed isozyme selectivity for both rat tissue and recombinant human GST isozymes. Some of these compounds also showed selective inhibition of GST activity in catalysis of the reaction of 1-chloro-2,4-dinitrobenzene with GSH. In the second phase, electronic effects were explored through synthesis of an isostructural series of S-benzyl GSH ligands with different substituents on the aromatic ring. GST isozyme specificity for these ligands, measured by binding to derivatized sorbents, varied substantially, with hydrophobic substituents favoring the human GST M1a isozyme and electronegative moieties favoring GST P1. In the third phase, information obtained from testing both series of compounds was combined and used to prepare GSH analogs with chemical features responsible for isozyme specificity at both the C-terminus and the sulfur. This approach gave two new compounds which showed improved potency while still maintaining selectivity in the inhibition of GSTs. A detailed discussion of the logic used in the selection of functional groups for maximum potency and selectivity is included.

Introduction

The family of glutathione-S-transferases (GSTs) utilizes the abundant intracellular tripeptide glutathione (GSH, γ-glutamylcysteinylglycine) to neutralize electrophilic toxins of both exogenous and endogenous origin.[1] At least eight different types of human GSTs have been identified from isozyme families called α, μ, π, and θ, defined by biochemical properties[2] and sequence homology.[3] GSTs appear to be a major contributor to resistance to chemotherapeutic drugs in many cancer tissues, which often show elevated levels of these enzymes when compared to healthy tissue.[4] Many cancers also show different distributions of GST isozymes compared to those seen in normal tissue,[5] which suggests that the use of selective GST inhibitors could provide tumor-directed potentiation of conventional cancer chemotherapeutic agents. Potentiation by an isozyme-nonselective inhibitor, such as ethacrynic acid, has previously been observed in both tissue culture and animal models.[6]

To explore isozyme selectivity, we prepared a first set of analogs on the basis of previous work by Adang[7] and Askelof.[8] Adang *et al.* synthesized a series of glutathione analogs, containing a sulfhydryl group, in which the C-terminal glycine was replaced with different amino acids. These compounds were then used as cosubstrates in reactions catalyzed by four rat α and μ GSTs with 1-chloro-2,4-dinitrobenzene (CDNB) as the electrophilic substrate, and the kinetic parameters V_{max} and K_m for each isozyme and substrate were derived. We used this information to select candidate C-terminal amino acids which showed maximum isozyme selectivity in Adang's study.[9] Work by Askelof *et al.* had previously shown that potency and selectivity of S-functionalized GSH analogs as inhibitors of rat GSTs varied with the length of n-alkyl groups pendant on the sulfur. Other studies had also shown that various aromatic compounds functioned as GST substrates. We combined several alkyl and aryl S-functionalization groups with the C-terminal modifications to give a matrix of target molecules. Of these, 25 were synthesized by us by solution-phase peptide synthesis, as previously reported.[10] Purification of the compounds proved to be quite difficult, so that crude materials were used for functional testing. First, the GSH analogs were immobilized with standard techniques.[11] Affinity chromatography was then performed, and the results were analyzed by reverse-phase HPLC.[12,13] The second test of the new compounds was as inhibitors in the reaction of recombinant human GSTs with glutathione and CDNB.[14] Improved synthetic techniques, purification procedures, and characterization of the more interesting of these previously reported compounds, as well as two improved inhibitors, are given below.

Results and Discussion

In the design of our original set of analogs,[10] we used Adang's kinetic data for the C-terminal amino acid GSH analogs by constructing a table of V_{max}/K_m, which is a qualitative measure of the microscopic affinity of the substrates for the enzymes.[9] See Table 1. Seven candidate C-terminal modifications were selected which had the maximum variance of these values, compared with those obtained with glutathione, for each enzyme. Valine, aspartic acid, phenylglycine, histadine, alanine, β-alanine, and 4-aminobutyric acid were selected for our initial study. Because we wanted these compounds to behave as inhibitors and affinity ligands rather than as substrates, we functionalized the sulfur with one of five alkyl or aryl groups. Affinity chromatography studies with rat GSTs[15] confirmed our theory regarding the utility of the V_{max}/K_m ratios in the design of selective ligands. Different alkyl and aryl groups pendant on the sulfur of the compounds

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Table 1. Ratios of V_{max}/K_m for Purified Rat GST Isozymes Conjugating CDNB to Different C-Terminal-Modified Glutathione Analogs[a]

	rat GST			
GSH analog	1-1(α)	2-2(α)	3-3(μ)	4-4(μ)
Gly	90.0	46.7	96.7	50.0
Val	2.2	0.7	4.5	1.0
Asp	14.8	32.0	27.0	8.3
PheGly	41.5	2.8	20.3	1.8
Phe	16.7	7.5	24.0	10.5
Lys				
His			5.0	1.0
Ala	8.3	2.0	95.0	25.5
D-Ala	55.0	46.6	42.5	10.3
β-Ala	57.5	14.8	260.0	90.0
4-ABu	21.7	5.3	93.3	13.3

[a] Val, Asp, PheGly, His, Ala, β-Ala, and 4-ABu were selected for our initial study.

also provided some noticeable selectivity to the affinity supports, but the effect varied depending on the C-terminal modification, and no obvious trends were seen. Highly selective affinity separations of individual human recombinant GST isozymes were also achieved with some of these sorbents, particularly for human M1a and P1 GSTs[16] (μ and π classes, respectively). Interestingly, these types are among the GSTs predominantly expressed in many cancers.[17] The C-terminal modifications having the largest variance of V_{max}/K_m values with rat GSTs, phenylglycine and β-alanine, also proved to be the best for selective chromatography and inhibition of recombinant human GSTs.

Of the first series of inhibitors designed with C-terminal modifications, several have now been isolated in >90% purity. Inhibition studies with these purified compounds showed selective inhibition of μ and π classes with potencies in the range of 10^{-5}–10^{-7} M for the best compounds. See Figure 1.

S-benzyl GSH analogs were among the best of these compounds tested. Work by Meyer *et al.*[18] had previously shown that a series of aromatic alkylating agents functionalized with electron-withdrawing groups had differing affinities as substrates for various rat GST isozymes. Other work by Jakoby *et al.*[19] further showed that the rates of the enzyme-catalyzed reaction of glutathione with differing aromatic alkylating agents showed good correlation with Hammet[20] σ parameters. On the basis of this work, we reasoned that the attachment of different substituted benzyl moieties to the sulfur of glutathione would yield inhibitors or affinity ligands which would differentially inhibit or bind different GST isozymes. A second series of seven different para-substituted benzyl glutathiones was therefore prepared. The substituents were methyl, *tert*-butyl, fluoro, methoxy, chloro, and nitro as well as hydrogen. The crude peptides were used to make affinity sorbents,[10] and these sorbents were tested as previously described.[15,21] Ligands with hydrophobic para substituents such as *tert*-butyl or methyl showed an increased preference for the recombinant human M1a GST isozyme, while electron-withdrawing substituents such as nitro or chloro provided preferential retention of P1. These distinctive isozyme selectivities of the immobilized ligands are apparent in plots of the Hammet σ meta values[20] against the log of the fraction of that isozyme bound (of total enzymes bound) for the four out of five of the sorbents which bound GST isozymes. See Figure 2. With the exception of the *p*-methyl-functionalized sorbent, the overall trend was linear. There may be a more complex specificity for methyl groups in this region of the ligand. Previous work here showed a marked preference of rat A1 GST for α,α-dimethylbenzyl GSH, used as an affinity ligand.[15] These methyl groups do little to perturb the electronic configuration in the aromatic ring, being at the benzylic carbon, and a related phenomenon may be why the result with the *p*-methyl sorbent reported here does not correlate well with the other values. No clear correlation was seen when the σ para values were used (data not shown). Two benzyl substituents which had little steric bulk, F and H, did not bind any of the GSTs appreciably. Although the data are limited to a small number of tests on sorbents prepared with impure peptides, the observed trends suggest that inductive effects are influencing the interaction. This information proved to be quite useful in the design of improved inhibitors.

STRUCTURES AND INHIBITION VALUES FOR GSH ANALOGS

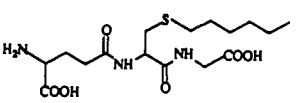
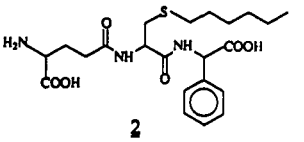
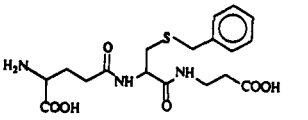
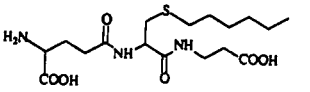
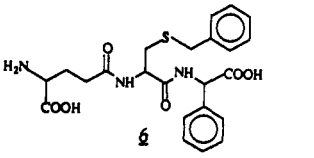
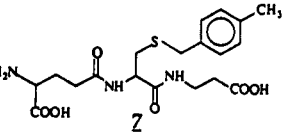
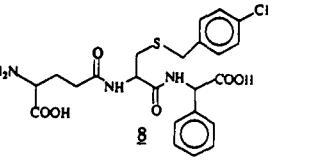
Structure	Inhibition, in μ Moles			
	A1	M1a	M2	P1
 1	0.84	2.0	36.0	10.0
 2	5.8	41.0	97.0	0.85
 4	360.0	22.0	26.0	710.0
 5	43.0	11.0	42.0	550.0
 6	24.3	57.8	184	0.42
 7	43.0	2.1	20.0	40.0
 8	14.7	15.1	48.3	0.12

Figure 1. Enzyme inhibition values for compounds 1–8 with the four different recombinant human GST isozymes tested. The K_i values were determined by the method of Flatgaard *et al.*[14] The error in each measurement is ±30%.

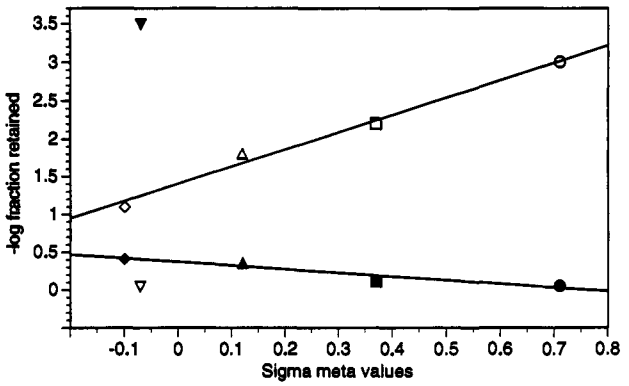


Figure 2. Hammet plot of σ meta values against fraction M1a (open symbols) and P1 (filled symbols) GSTs retained on affinity sorbents, calculated by dividing the area of the isozyme peak by the added area of all three peaks; para substituent on *S*-benzylglutathione: (●) NO_2, (■) Cl, (▲) OCH_3, (◆) *tert*-butyl, and (▼) CH_3.

In the third phase, two additional glutathione analogs were designed, incorporating chemical functionalities which showed similar isozyme specificity at the C-terminal and sulfur positions. Thus, γ-glutamyl-[*S*-(4-methylbenzyl)cysteinyl]-β-alanine (**7**) was expected to be a superior inhibitor for human recombinant M1a, while γ-glutamyl-[*S*-(4-chlorobenzyl)cysteinyl]-(*R*)-(−)-phenylglycine (**8**) was expected to be selective for human recombinant P1. When tested as before,[14] compound **7** proved to be 10 times more potent for M1a than **4**, while retaining good selectivity. Compound **8** also proved to be 3.4 times more potent for P1 than **6**, with similar maintenance of high selectivity. See Figure 1.

A crystal structure of rat liver μ GST shows that there is a pocket lined with hydrophobic amino acids close to where the sulfur of glutathione would bind,[22] and thus, the binding strength of an inhibitor is probably dependent on its ability to displace water molecules from this cavity. This mechanism would explain why **7** is a stronger inhibitor than **4**, although more subtle factors are probably responsible for the specificity.

The selectivity of many of our compounds is substantially better than that observed with commercially available (Sigma Co.) *S*-hexylglutathione (**1**) or ethacrynic acid,[6] which inhibits all of the isozymes roughly equally at potencies similar to our compounds.

Previous reports[23] showed that the glutathione ethyl esters are able to penetrate cellular membranes, becoming enzymatically saponified to glutathione once inside. Diethyl esters were therefore made from **2**, **6**, and **7**. Preliminary cell culture data show potentiation by **2** and **6** of the cytotoxicity of chlorambucil, a commonly prescribed cancer chemotherapeutic. These compounds do not inhibit GSTs *in vitro*, leading to the assumption that they are hydrolyzed in the cell to give potentiation. The diethyl esters of **1** and other nonselective glutathione-based GST inhibitors do not generally potentiate as well, which suggests that isozyme selectivity is important in the mechanism of potentiation. These results will be reported in detail elsewhere.[24]

Synthesis

All of the glutathione analogs described here were synthesized by solution-phase N^{α}-9-fluorenylmethoxycarbonyl (Fmoc)-mediated peptide synthesis, first described by Carpino[25] and reported preliminarily by us in the synthesis of **2** through **6**.[10] The novel aspect of our method is the utilization of protecting groups which are removable by mild base, without the harsh TFA treatment normally used. Recent reports show that carbonium ions generated with the TFA deprotection method alkylate electron-rich side groups present in peptide synthesis, even in the presence of scavengers.[26,27]

Since our initial report,[10] in which several GSH analogs were reported as crude preparations for affinity sorbent synthesis, improved synthetic and purification techniques have permitted the isolation of many pure GSH analogs. Column chromatography has been replaced by crystallization as the main method of purification of intermediates, and syntheses of as much as 21 g of pure GSH analog material have been performed.

Racemization of C-terminal phenylglycine containing GSH analogs was seen, and two compounds with similar mass spectra were isolated in each case. The compounds had different HPLC retention times. The (*R*)-(−)-phenylglycine-containing compounds all eluted later on reverse-phase HPLC systems than the corresponding *S*-(+) diastereomers. The product distribution for each pair of compounds was the same regardless of whether (*R*)-(−)- or (*S*)-(+)-phenylglycine was used in the starting materials, indicating a thermodynamic rather than kinetic product distribution. In the phenylglycine analogs containing *S*-hexyl or *S*-benzyl, the major product contains (*R*)-(−)-phenylglycine. When the sulfur was functionalized with 4-chlorobenzyl, the minor isomer contained (*R*)-(−)-phenylglycine. In two out of three cases, the (*R*)-(−)-phenylglycine analogs were the superior GST inhibitors, usually by several orders of magnitude. The (*S*)-(+)-phenylglycine-containing diastereomer of **6** could not be satisfactorily purified, so its inhibition of GSTs was not measured. The importance of the absolute configuration of the C- and N-terminal amino acids was shown in Adang's earlier work with the GSH analog substrates.[7] The absolute configuration of each amino acid in the samples was determined by hydrolysis of these GSH analogs followed by treatment with Marphy's reagent (1-fluro-2,4-dinitrophenyl-5-L-alaninamide) and HPLC.[28] The other two amino acids in these tripeptides, cysteine and glutamic acid, were not racemized according to the Marphy's reagent analysis. Building blocks containing the natural L-amino acids were used for these residues. Starting material **10** was also hydrolyzed and tested with Marphy's reagent, which showed that racemization of this benzylic amino acid α carbon does not occur during esterification. The lack of other stereoisomers observed during the isolation of the β-alanine-containing analogs also implies that racemization at the other chiral centers in the molecule is not occurring.

Conclusion

Empirically based methods have been used to develop several selective inhibitors of GSTs. The technique of developing enzyme inhibitors based on substrates is widely used in both nature and medicinal chemistry, owing to the principal of microscopic reversability in the Michaelis–Menten scheme. The technique of designing a systematically varied array of peptide analogs[29] with the catalytic efficiency ratios we have derived should be useful in other drug design projects where specific inhibition of one isozyme among a family of closely related enzymes is desired. Affinity chromatography showed isozyme-specific

binding preferences toward different sulfur pendant groups, and this information, in combination with earlier work, proved to be valuble in the design and synthesis of improved GST inhibitors. The isozyme selectivity of several of the compounds reported here is better than any previously reported in the inhibition of human GSTs.

Experimental Section

Materials. Methylene chloride (DCM), acetic acid, potassium hydroxide, sodium sulfate, and concentrated hydrochloric acid were reagent grade from J.T. Baker; DMF, ethyl acetate (EtOAc), petroleum ether, ethyl ether, ethanol, and acetonitrile were Omnisolve grade from VWR. THF, piperidine, DCC, sodium hydroxide, sodium carbonate, cysteine, β-alanine ethyl ester hydrochloride, (*S*)-(+)-phenylglycine, 4-chlorobenzyl chloride, diisopropylethylamine (DIPEA), triethylamine (TEA), TFA, TMS-Cl, and magnesium sulfate were from Aldrich. 9-Fluorenylmethoxycarbonyl succinimate ester (Fmoc-OSu) was purchased from Peboc (Gwynedd, U.K.). (*p*-Methylbenzyl)cysteine was purchased from Novabiochem (LaJolla, CA).

Affinity Chromatography. γ-Glutamyl-[*S*-benzylcysteinyl]-glycine analogs with *p*-chloro, -fluoro, -benzyl, -nitro, and -*tert*-butyl substituents on the cysteine *S*-benzyl group were synthesized with solution-phase methods as previously described.[10] The crude compounds were checked for the presence of the desired compound by FAB mass spectroscopy, and the analogs were then immobilized onto epoxy Sepharose 6B (Sigma) by their N-terminus[10] with the following procedure. Sepharose (0.66 g) was swollen with 10 mL of water for 15 min and then rinsed twice with 10 mL of water in a 15-mL sintered glass funnel. A solution of 100–200 mg of the tripeptide in 5 mL of ethanol and 10 mL of water was adjusted to pH 11–12 with 6 N NaOH in a 20-mL scintillation vial, and the rinsed resin was added. The mixture was gently agitated at 37 °C for 1 h, adjusted back to pH 11 if needed, and gently agitated overnight. The resin was then rinsed three times with 10 mL of water and treated with 0.1 mL of ethanolamine in 10 mL of water to cap the unreacted epoxy groups. A sample was removed for analysis, and the remainder was rinsed with 10 mL of 0.1 M NaOAc and 0.5 M NaCl buffer, pH 4.0, followed by 10 mL of 0.1 M Tris-HCl and 0.5 M NaCl buffer, pH 8.0. The resin was stored at 4 °C in this buffer. The resins were analyzed for glycine and glutamic acid content as previously described.[10] The loading ranged from 13–76 μm of peptide/g of dried support for these sorbents. Affinity chromatography was performed in 96 well microplates, as per Castro *et al.*,[15] as follows: microplates were pretreated by soaking in 0.1% polyoxyethylene sorbitan (Tween, Sigma), rinsed, and dried to prevent the enzymes from sticking to the plastic; 400 μL of a 50% aqueous slurry of each sorbent was pipetted into wells in a membrane-bottomed (1.2 μM Loprodyne) microplate (Pall Biosupport Silent Monitor), and the resulting microaffinity columns were equilibrated with two 150-μL portions of TE buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT (dithiothreitol, Sigma), and 100 μM PMSF ((phenylmethyl)sulfonyl fluoride, Sigma), pH 7.8) and two cycles of centrifugation at 650*g* (Beckman Model TJ-6) for 30 s at 20 °C. Next, 15 μg each of recombinant human A1, M1a, and P1 GSTs[30] dissolved in 75 μL of TE buffer was added to the semidry sorbents, and the plate was vibrated (Minimax, Fischer Co.) for 1 h at 4 °C. The sorbent microplate was placed above a standard Tween-treated microplate (Costar Co.), and the fluid was transfered by centrifugation at 650*g* for 30 s. To ensure full adsorption, the eluate was reapplied to the sorbents, and they were shaken for 10 min at 4 °C and centrifuged again. The flow-through eluate was saved for later analysis. The sorbents were washed by four cycles of centrifugation with 150 μL of TE buffer followed by six 150-μL washes with TEN buffer (TE buffer containing 200 mM NaCl). The retained GSTs were eluted with 150 μL of 10 mM *S*-hexylglutathione (1) in TEN buffer, and the eluate was analyzed by reverse-phase HPLC. The HPLC column was a 300-Å pore size, 5-μm particle size, 25-× 0.46-cm C-4 (Dynamax, Ranin); the flow rate was 0.6 mL/min, and detection was at 214 nm. Buffer A = 0.1% TFA and H_2O; B = 100% CH_3CN and 0.1% TFA. The time of the run was 70 min, with a gradient of: 0–32 min, 43–51% B; 32–42 min, 51–53% B; 42–43.5 min, 53–65% B; 43.5–45 min, 65% B; and 45–70 min, back to 43% B. The absorbance measured was 0–1.0, full scale. The sample injected was 25 μL, which contained roughly 3 μg of protein. The flow-through eluate for the *p*-fluoro and benzyl GSH sorbents contained all of the GSTs, and almost no protein was obtained during the attempted recovery of bound material with elution with 1, even though the loading of these peptides on the sorbent was comparable to the other sorbents. The HPLC traces of the applied mixture of GSTs, as well as the GSTs eluted from the *p*-chloro-, *p*-nitro-, *p*-methoxy-, *p*-*tert*-butyl-, and *p*-methylbenzyl GSH sorbents, are shown in the supplementary material.

General Methods. Mass spectra and elemental analyses were performed by the U.C. Berkeley Chemistry Department Analysis Services. HPLC purification and analysis used a Ranin Rabbit HP solvent delivery system with a LDC Milton Roy detector set at 225 nm. Two representative syntheses are given below for compounds 7, 8, and 9.

Synthesis of Starting Materials. Phenylglycine Ethyl Ester Hydrochloride, 10. (*S*)-(+)-Phenylglycine, 12 g (0.08 mol), was suspended in 200 mL of ethanol under argon, and 47 mL (0.64 mol) of thionyl chloride was added dropwise over 2 h. After 1 h, the solution went clear. After another hour, a precipitate began to form. The reaction was warmed to 40 °C and stirred overnight. The solution was stripped to a solid *in vacuo* and redissolved in 300 mL of ether. Crystals formed after 1 h, and these were collected and dried unver a high vacuum to yield 16 g (93%) of a white powder, mp 189 °C. ^1H NMR (300 mHz, $CDCl_3$): δ 1.8–1.9 (t, 3H), 4.8–5.0 (m, 2H), 6.05 (s, 1H), 8.2 (m, 2H), 8.5 (m, 3H), 10.6 (s, 3H). Anal. ($C_{10}H_{14}NO_2Cl$) C, H, N.

N^{α}-Fmoc-*S*-(4-chlorobenzyl)cysteine, 11. Cysteine, 26.3 g (0.217 mol), was suspended in 250 mL of ethanol under argon, and a solution of 12.2 g (0.217 mol) of KOH in 75 mL of ethanol was added. *p*-Chlorobenzyl chloride, 35 g (0.217 mol), was dissolved in 100 mL of acetone and added dropwise to the reaction mixture. Another 12.2 g of KOH in 75 mL of ethanol was added, and the reaction mixture was stirred for 3 h and then poured into 750 mL of water. The solution was neutralized with concentrated HCl, and the resulting white crystals were collected. Partial evaporation of the mother liquor gave more crystals. Both crops of crystals were combined and dried to yield 37.7 g (75%, 0.163 mol) of (4-chlorobenzyl)cysteine as white crystals, mp 205–209 °C dec. All of this material was dissolved in 250 mL of water and 75 mL of THF. Na_2CO_3, 35.4 g (0.163 mol), was dissolved in 300 mL of water, and half of this was added to the reaction mixture. Slight heating gave complete dissolution of the solids, and a solution of 55 g (0.163 mol) of Fmoc-OSu in 200 mL of THF and 80 mL of dioxan was added dropwise to the stirred mixture over 1 h. More of the Na_2CO_3 solution was added, as needed, to keep the pH above 8, and the cloudy solution was stirred for 3 h. The reaction mixture was poured into a separatory funnel and extracted with 200 mL of 1:1 ether–petroleum ether. The aqueous phase was acidified to pH 2 with concentrated HCl and extracted with two 200-mL portions of EtOAc. The combined organic phase was dried over $MgSO_4$, filtered, and evaporated. The resulting oil was subjected to high vacuum overnight and dissolved in a minimal amount of EtOAc. It was then brought to a cloud point with petroleum ether. Overnight chilling gave crystals which were collected and dried to yield 46.5 g (46% from cysteine) of material, mp 122–124 °C. Anal. ($C_{25}H_{22}NO_4SCl{\cdot}0.5H_2O$) C, H, N.

N^{α}-Fmoc-*S*-(4-methylbenzyl)cysteine, 12. (4-Methylbenzyl)cysteine, 20.6 g (0.09 mol), was suspended in 200 mL of water and 100 mL of THF. The pH of the solution was adjusted to above 9 by adding a portion of a solution of 21.5 g of Na_2CO_3 in 200 mL of water, and a solution of 30.3 g (0.09 mol) of Fmoc-OSu in 100 mL of THF was added dropwise over 1 h. The pH was maintained at above 9 by adding more of the Na_2CO_3 solution, as needed, and the mixture was stirred for 3 h. The solution was then extracted with two 100-mL portions of petroleum ether and 100 mL of ether. The aqueous layer was acidified to pH 2 with concentrated HCl and extracted with 200 mL of EtOAc. The organic phase was dried over Na_2SO_4 and reduced *in vacuo* to 50 mL in volume. Petroleum ether was added to a cloud point, and overnight chilling gave crystals which were collected and dried to yield 28.1 g (70%) of material, mp 152–154 °C. ^1H NMR

(300 mHz, $CDCl_3$): δ 2.3 (s, 3H), 2.9 (m, 2H), 3.7 (s, 2H), 4.2 (m, 1H), 4.4 (d, 2H), 5.6 (d, 1H), 7.2 (m, 9H), 7.6 (d, 2H), 7.8 (d, 2H). Anal. ($C_{26}H_{25}NO_4S$) C, H, N.

Synthesis of GSH Analogs. N^{α}-Fmoc-α-benzyl-γ-glutamyl-*S*-(4-methylbenzyl)cysteinyl]-β-alanine Ethyl Ester, 13. β-Alanine ethyl ester hydrochloride, 4.1 g (0.027 mol), was dissolved in 50 mL of DCM under argon, and 11.9 g (0.027 mol) of 10 was added. TEA, 3.7 mL, was added, and a solution of 5.8 g (0.028 mol) of DCC in 50 mL of DCM was added dropwise over 1 h. The mixture was stirred overnight, and the DCC urea was removed by filtration. The solution was washed with two 100-mL portions of dilute aqueous HCl and dried over Na_2SO_4. The solution was reduced to a solid *in vacuo* and redissolved in 100 mL of EtOAc. Insoluble material was removed by filtration, and petroleum ether was added to a cloud point. The mixture was chilled to produce crystals which were dried under high vacuum to give 11.6 g (0.021 mol, 79% yield) of the protected dipeptide. This material was dissolved in 100 mL of 10% piperidine in DMF and stirred for 0.5 h. The solvent was removed *in vacuo* and subjected to high vacuum overnight. The resulting white solid was dissolved in 100 mL of DCM under argon, and 9.9 g (0.023 mol) of Fmoc-glutamic acid α-benzyl ester[9] was added. DCC, 5.1 g (0.024 mol), in 75 mL of DCM was added dropwise over 1.5 h, and the reaction was stirred overnight. The DCC urea was removed by filtration, and the solution was reduced to a solid *in vacuo*. The solid was dissolved in 150 mL of EtOAC, and petroleum ether was added to a cloud point. Overnight chilling gave crystals, which were dried and recrystallized from EtOAc and petroleum ether, as before. The crystals were dried under a high vacuum, yielding 11.6 g (72% from the dipeptide) of 13 as white crystals, mp 171–173 °C. ^1H NMR (300 mHz, $CDCl_3$): δ 7.7 (d, 2H), 7.6 (d, 2H), 7.4–7.2 (m, 9H), 6.7 (s, 1H), 7.5 (d, 1H), 5.8 (d, 1H), 5.2 (s, 2H), 4.4 (m, 4H), 4.2 (t, 1H), 4.1 (q, 2H), 3.6 (s, 2H), 3.4 (m, 2H), 2.8 (dd, 1H), 2.6 (dd, 1H), 2.5 (t, 2H), 2.3 (s, 3H), 2.2 (s, 2H), 1.9, 1.2 (t, 3H). *m/z* (rel intensity): 766.3 (MH^+, 100), 660.3 (10), 544.3 (6), 391.2 (6), 325.2 (20). Anal. ($C_{43}H_{47}N_3O_6S$) C, H, N.

γ-Glutamyl-[*S*-(4-methylbenzyl)cysteinyl]-β-alanine, 7. 13, 1.5 g (0.002 mol), was dissolved in 50 mL of THF and stirred at room temperature while 35 mL of 0.2 M aqueous NaOH was added. The reaction was stirred for 2 h, and the solution was extracted with petroleum ether. The solution was acidified to pH 4 with HOAc and extracted with EtOAc. The aqueous phase was filtered and lyophilized to give 400 mg (47% yield, 27% overall from β-alanine ethyl ester) of 7. A small amount of this was recrystallized from EtOH, H_2O, and EtOAc, mp 191–193 °C. ^1H NMR (300 mHz, DMSO-d_6): 8.4 (m, 3H), 7.2 (d, 2H), 7.1 (d, 2H), 4.4 (dd, 1H), 3.7 (s, 3H), 3.3 (dd, 1H), 3.2 (dd, 2H), 2.7 (dd, 2H), 2.5 (m, 3H), 2.3 (m, 6H), 1.8 (m, 2H). ^{13}C NMR (75 mHz, DMSO-d_6): δ 174.0, 172.5, 171.3, 171.0, 136.2, 135.8, 129.7, 53.5, 53.0, 35.2, 35.0, 34.5, 33.0, 31.8, 26.9, 20.7. *m/z* (rel intensity): 448.3, (MNa^+, 20), 426.3 (MH^+, 39), 237.1 (11), 215.1 (19), 207.2 (25), 185.2 (100). Anal. ($C_{19}H_{27}N_3O_6S \cdot 0.5H_2O$) C, H, N.

N^{α}-Fmoc-γ-glutamyl-α-benzyl-[*S*-(4-chlorobenzyl)cysteinyl]-(*R*)-(−)-phenylglycine Ethyl Ester, 14. 10, 4.0 g (0.019 mol), was suspended in 100 mL of DCM, and 3.2 mL (0.019 mol) of DIPEA was added, causing the solid to dissolve. 11, 8.88 g (0.019 mol), was added, and 3.9 g (0.019 mol) of DCC was added dropwise as a solution in 75 mL of DCM over 1 h. The solution was stirred for 5 h, and the DCC urea was removed by filtration. The solution was reduced to a solid *in vacuo* and dissolved in 200 mL of EtOAc. The solution was washed with 100 mL of 0.1 M HCl, 100 mL of saturated $NaHCO_3$, 100 mL of water, and 100 mL of brine and dried over $MgSO_4$. The solution was filtered and reduced to about 50 mL, and petroleum ether was added to a cloud point. Overnight chilling gave crystals which were recrystalized again from EtOAc and petroleum ether to give 4.65 g (39%) of protected dipeptide as white crystals, mp 156–159 °C. A 25-g (0.04-mol) portion of this material was dissolved in 250 mL of DMF, and 30 mL of piperadine was added. The solution was stirred for 1 h, and the solvent was removed *in vacuo*. The yellow residue was subjected to a high vacuum overnight and dissolved in 250 mL of DCM under argon. Fmoc-glutamic acid α-benzyl ester,[10] 18.0 g (0.04 mol), was added and stirred until dissolved. DCC, 8.3 g (0.04 mol), was added dropwise as a solution in 100 mL of DCM over 1 h. After 1 h, the reaction mixture had solidified and an additional 300 mL of DCM and 100 mL of DMF were added. TLC (10% MeOH in DCM, aluminum-backed silica; EM Separations No. 5534.3) and visualization with UV showed that the deprotected dipeptide was still present and that all of the Fmoc-glutamic acid α-benzyl ester had been consumed. Another 14.4 g (0.035 mol) of Fmoc-glutamic acid α-benzyl ester and 6.6 g (0.032 mol) of DCC were added, and TLC showed completion of the reaction after another 2 h of stirring. The solids were removed by filtration, and the filtrate was reduced to a solid *in vacuo*. This material was dissolved in 400 mL of EtOAc, washed successively with 100 mL of 0.1 N HCl, saturated $NaHCO_3$, water, and brine, and dried over $MgSO_4$. The solution was reduced to 100 mL in volume, and petroleum ether was added to a cloud point. Overnight chilling gave crystals which were recrystallized to give 27.0 g (79%, 31% from 10) of 12 as white crystals, mp 164–167 °C. ^1H NMR (300 mHz, $CDCl_3$): δ 7.7 (d, 2H), 7.55 (d, 2H), 7.5 (m, 1H), 7.2–7.4 (m, 19H), 6.7 (s, 1H), 5.7 (dd, 1H), 5.4 (t, 1H), 5.1 (s, 2H), 4.6 (m, 1H), 4.4 (m, 3H), 4.0–4.2 (m, 3H), 3.6 (d, 2H), 2.6–2.9 (m, 2H), 2.2 (m, 2H), 1.8–2.0 (m, 2H), 1.5–1.7 (m, 1H), 1.1 (t, 3H). *m/z* (rel intensity): 848.3 (MH^+, 36), 666.4 (29), 541.4 (21), 527.3 (100), 449.4 (28), 429.1 (24), 361.1 (70). Anal. ($C_{47}H_{46}N_3O_6SCl \cdot 0.5H_2O$) C, H; N calcd, 4.90, found, 5.48.

γ-Glutamyl-[*S*-(4-chlorobenzyl)cysteinyl]-(*R*)-(−)-phenylglycine, 8, and γ-Glutamyl-[*S*-(4-chlorobenzyl)cysteinyl]-(*S*)-(+)-phenylglycine, 9. 14, 2.1 g (0.0024 mol), was dissolved in 75 mL of THF, and a solution of 0.8 g (0.02 mol) of NaOH in 75 mL of water was added. The solution slowly turned yellow and was stirred for 1 h, whereupon the reaction mixture was neutralized by the addition of 0.1 M HCl to pH 7. The solution was washed with 100 mL of petroleum ether, and the aqueous layer was reduced to a solid. Preparative HPLC (Ranin Dynamax C-4, 300 Å, 21 × 250 mm; 0.1% TFA, 0–50% CH_3CN over 30 min, 10 mL/min flow rate) gave 89 mg (7.2%, 2.3% from 10) of 8, mp 208–209 °C, and 75 mg (6.1%, 1.9% from 10) of 9, mp 190–193 °C, as fluffy white solids after lyophilization. 9 was characterized as follows. ^1H NMR (300 mHz, D_2O): δ 2.2 (m, 2H), 2.5 (t, 2H), 2.8 (dd, 1H), 2.9 (dd, 1H), 3.7 (s, 2H), 3.8 (t, 1H), 4.6 (dd, 1H), 5.7 (s, 1H), 7.3–7.5 (m, 9H). ^{13}C NMR (75 mHz, D_2O): δ 172.3, 172.1, 170.8, 170.2, 138.9, 138.1, 131.8, 131.5, 128.9, 127.9, 57.4, 53.0, 52.4, 34.2, 31.9, 27.2. *m/z* (rel intensity): 530.3 (MNa^+, 7), 508.3 (MH^+, 100), 200.1 (40), 185.2 (24), 173.2 (12), 152.2 (14), 125.1 (83). 8 also had *m/z* 530 (MNa^+). Analysis with Marphy's reagent, as previously described,[28] showed that 8 and 9 were identical except for the absolute configuration at phenylglycine. 8 Anal. ($C_{23}H_{26}N_3O_6SCl \cdot H_2O$) C, H, N. 9 Anal. ($C_{23}H_{26}N_3O_6SCl$) C, H, N.

γ-Glutamyl-(*S*-hexylcysteinyl)-(*R*)-(−)-phenylglycine, 2, and γ-Glutamyl-(*S*-hexylcysteinyl)-(*S*)-(+)-phenylglycine, 3. 2 and 3 were prepared with methods analogous to those used for 8 and 9, using N^{α}-Fmoc-*S*-hexylcysteine[10] instead of 12. The synthetic procedures and yields of the protected tripeptide and the deprotected peptides, after purification, were similar to the synthesis of 7. Evaporation of the aqueous phase obtained after the alkaline deprotection of the analogous protected tripeptide, HCl neutralization, and petroleum ether wash gave a solid containing the desired products and NaCl. This was then triturated with hot EtOH, and the undissolved material was dissolved in water and desalted on a Ranin Dynamax C-4 300-Å 21- × 250-mm preparative HPLC column with 0.1% HOAc and 0–100% CH_3CN over 1 h (salt elutes first followed by peptide) to give 2 as a white solid, mp 198–199 °C. 3 was obtained by HPLC purification of the EtOH soluble material. For 2, *m/z* (rel intensity): 468.3 (MH^+, 100), 429.1 (10), 322.2 (12), 215.1 (5), 197.1 (26), 185.2 (63), 160.2 (49). The mass spectrum of 3 was nearly identical, and analysis with Marphy's reagent showed that 2 and 3 were identical except for the absolute configuration at phenylglycine. 2 Anal. ($C_{22}H_{33}N_3O_6S$) C, H, N. 3 Anal. ($C_{22}H_{33}N_3O_6S \cdot 1.5H_2O$) C, H, N.

γ-Glutamyl-(*S*-benzylcysteinyl)-β-alanine, 4. 4 was prepared with methods analogous to those used for 7, using N^{α}-Fmoc-*S*-benzylcysteine instead of 12, and purified by preparative open-column chromatography. A 23-g portion of protected tripeptide was deprotected by treating with 4.6 g of NaOH in 30 mL of water and 250 mL of THF. After 1 h, the solution was neutralized with concentrated HCl and poured into a separatory

funnel. Water, 250 mL, was added, and the solution was washed with 300 mL of petroleum ether. The aqueous layer was lyophylized and the residue washed with ethanol. The residue was dried and divided into three roughly equal portions, and each of these was purified by using a 3- × 7-cm bed of Bakerbond butyl C-4 40-μm 60-Å bulk packing in a low-pressure apparatus which we have previously described.[10] HOAc, 0.1%, with a gradient of 0–30% CH_3CN over 2.5 h was used, with a flow rate of 7 mL/min. The desired product eluted at 15%, and the product-bearing fractions from each run were pooled and lyophilized to give 3.7 g (29%) of **4** as a white powder, mp 189–193 °C. The compound was spectroscopically indentical to that previously isolated by us,[10] the melting point difference (lit.[10] mp 57–61 °C) is probably due to different salt forms of the compound.

γ-Glutamyl-(*S*-hexylcysteinyl)-β-alanine, 5. **5** was prepared with methods analogous to those used for **7**, using Fmoc-*S*-hexylcysteine[10] instead of **12**, and purified by preparative HPLC with the same methods as those used for **8**. A 2.5-g portion of fully protected tripeptide yielded 180 mg (13% yield from the protected tripeptide, with the overall yield about the same as for **7**) of **5** as a white solid, mp 172–176 °C, after HPLC purification. *m/z* (rel intensity): 429.0 (MNa^+, 15), 406.2 (MH^+, 100), 307.1 (14), 260.1 (12), 215.1 (69), 197.1 (33), 185.1 (82), 160.1 (25). Anal. ($C_{17}H_{31}N_3O_6S$·0.5HOAc) C, H, N.

γ-Glutamyl-(*S*-benzylcysteinyl)-(*R*)-(−)-phenylglycine, 6. **6** was prepared and purified with the same methods as those used to make **8** and **9**, using N^{α}-Fmoc-*S*-benzylcysteine instead of **11**. Two peptides were isolated upon HPLC purification; both products crystallized out of the HPLC effluent fractions as the acetonitrile was evaporated. **6** was the later eluting material and was isolated as white crystals, mp 202–205 °C. The yield was similar to that obtained for **7**. The minor product contained an inseperable impurity and could not be well characterized. For **6**, *m/z* (rel intensity): 496.0 (MNa^+, 7), 474.1 (MH^+, 100), 429.0 (10), 369.0 (10), 307.0 (40), 277.1 (51), 215.0 (58), 201.1 (38). The amino acid composition and absolute configuration of **6** were confirmed by analysis with Marphy's reagent. Anal. ($C_{23}H_{27}N_3O_6S$·HOAc) C, H, N.

γ-Glutamyl-(*S*-hexylcysteinyl)phenylglycine Diethyl Ester, 15. **2**, 212 mg (0.45 mm), was suspended in 30 mL of absolute EtOH, and 2 mL of TMS-Cl was added. The reaction was stirred for 1 h and neutralized with 5 mL of saturated $NaCO_3$. The reaction mixture was poured into 150 mL of water, and a gummy solid was collected by filtration. The solid was dried and dissolved in 4 mL of ethanol, and water was added until the solution became slightly turbid. This was loaded onto a C-18 preparative HPLC column (YMC Corp., 22 × 250 mm, 10 μm, 120 Å) and eluted, at 12 mL/min, with a gradient of 10–90% EtOH in pH 8.0 (diluted NH_4OH) water over 90 min. Fractions, 10 mL, were collected and assayed by TLC (silica plates, EM Science No. 5534, 85/10/5 DCM/MeOH/HOAc, visualized with ninhydrin spray) for purity. The pure fractions were pooled and partially evaporated, yielding a precipitate which was collected by filtration and dried to give 147 mg (62%) of white powder, mp 82–84 °C. *m/z* (rel intensity): 524.2 (MH^+, 100), 433.1 (7), 350.1 (19), 221.1 (6), 201.1 (9). Anal. ($C_{28}H_{41}N_3O_6S$) C, H, N.

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Supplementary Material Available: HPLC traces of the affinity chromatography results with the series of para-substituted benzylglutathione sorbents (7 pages). Ordering information is given on any current masthead page.

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