Design, Synthesis, and Evaluation of Latent Alkylating Agents Activated by Glutathione *S*-Transferase

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In search of compounds with improved specificity for targeting the important cancer-associated P1-1 glutathione *S*-transferase (GST) isozyme, new analogs **4** and **5** of the previously reported glutathione *S*-transferase (GST)-activated latent alkylating agent *γ*-glutamyl-α-amino-*β*-[[[2-[[bis[bis(2-chloroethyl)amino]phosphoryl]oxy]ethyl]sulfonyl]propionyl]-(*R*)-(−)-phenylglycine (**3**) have been designed, synthesized, and evaluated. One of the diastereomers of **4** exhibited good selectivity for GST P1-1. The tetrabromo analog **5** of the tetrachloro compound **3** maintained its specificity and was found to be more readily activated by GSTs than **3**. The GST activation concept was further broadened through design, synthesis, and evaluation of a novel latent urethane mustard **8** and its diethyl ester **9**. Interestingly, **8** showed very good specificity for P1-1 GST. Cell culture studies were carried out on **4**, **5**, **8**, and **9** using cell lines engineered to have varying levels of GST P1-1 isozyme. New analogs **4** and **5** exhibited increased toxicity to cell lines with overexpressed GST P1-1 isozyme. The urethane mustard **8** and its diethyl ester **9** were found to be not as toxic. However, they too exhibited more toxicity to a cell line engineered to have elevated P1-1 levels, which was in agreement with the observed *in vitro* specificity of **8** for P1-1 GST isozyme. Mechanistic studies on alkaline as well as enzyme-catalyzed decomposition of latent mustard **3** provided experimental proof for the hypothesis that **3** breaks down into an active phosphoramidate mustard and a reactive vinyl sulfone. The alkylating nature of the decomposition products was further demonstrated by trapping those transient species as relatively stable diethyldithiocarbamic acid adducts. These results substantially extend previous efforts to develop drugs targeting GST and provide a paradigm for development of other latent drugs.

Introduction

A major cellular defense mechanism utilizes a tripeptide, glutathione (*γ*-glutamylcysteinylglycine, GSH), which acts as a scavenger molecule able to couple to and neutralize many toxic electrophiles, including chemotherapeutic agents. Such coupling is mediated by a family of enzymes named glutathione *S*-transferases (GSTs) (EC 2.5.1.18). At least 10 different types of human GSTs have been identified from four different isozyme families named A, M, P, and T. It has been observed that many types of cancer tissue often have elevated levels of GSTs compared to corresponding healthy tissue.[1] There are a few reports demonstrating the association of GSTs with resistance to alkylating agents, a major class of anticancer drugs.[2−4] A chemotherapeutic agent that takes advantage of this intrinsic property of many types of cancer cells may prove to be a valuable anticancer drug.

Interestingly, different cancers show different GST isozyme distribution, but it is most often the P1-1 isozyme that is elevated to the greatest extent in many types of cancer tissue.[5,6] As a result, an isozyme selective drug can be targeted to a specific cancer tissue on the basis of its GST isozyme distribution profile. In principle, targeting of GSTs can be accomplished in two ways. The first way is to develop isozyme specific/selective GST inhibitors. Several laboratories,[7−9] including ours,[10] have reported the synthesis and evaluation of a variety of GSH analogs as GST inhibitors, which exhibited varying affinities toward different GST isozymes. The second way, on which this article is based, involves the use of an appropriately functionalized GST analog that would serve both as a modified GSH substrate and a latent alkylating agent. In the past, on the basis of this latter concept, we designed and successfully demonstrated[11] that the glutathione-linked nitrogen mustards *γ*-glutamyl-α-amino-*β*-[[[2-[[bis[bis-(2-chloroethyl)amino]phosphoryl]oxy]ethyl]sulfonyl]-propionyl]glycine (**2**) and *γ*-glutamyl-α-amino-*β*-[[[2-[[bis[bis(2-chloroethyl)amino]phosphoryl]oxy]ethyl]-sulfonyl]propionyl]-(*R*)-(−)-phenylglycine (**3**) are cleaved by physiological concentrations of GSTs at physiological temperature and pH to release a toxic phosphorodiamidate mustard **7b** (Scheme 1). As an extension of this work, we designed, synthesized, and evaluated two new compounds **4** and **5** (Scheme 1) which provide more GST P1-1 isozyme selectivity. More importantly, because of its potential therapeutic importance, we succeeded in broadening the concept further by the design, synthesis, and evaluation of novel GST P1-1 selective urethane mustards **8** and **9** (Scheme 2), enabling the concept to be applied to a much wider range of toxic moieties than the phosphorodiamidate mustards previously reported.

Design

(a) Phosphorodiamidate Mustards. In all the structures generated to date for the different GST families,[12−15] a Tyr residue is present proximal to the S atom of the Cys in glutathione. It has been speculated that the Tyr is in the phenoxide form and that its function is to facilitate the deprotonation of the sulfhy-

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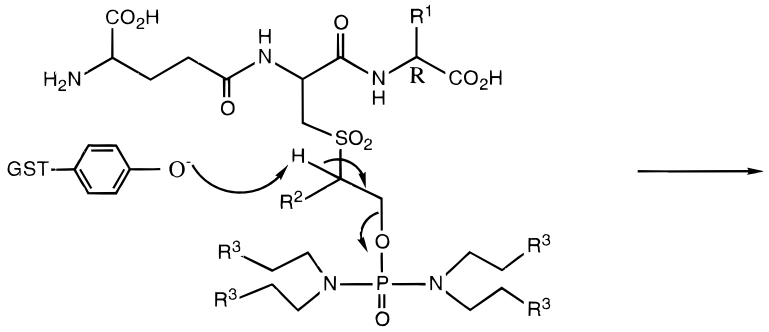
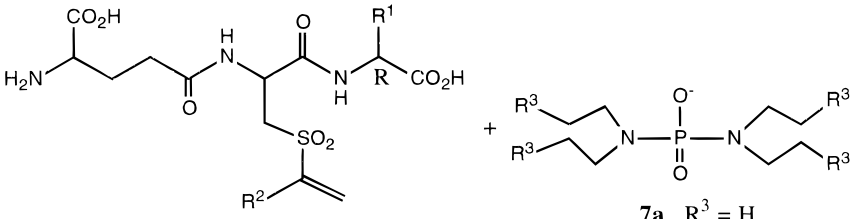
Scheme 1. Proposed Mechanism of GST-Mediated Cleavage of **1**−**5**



1, $R^1 = R^2 = R^3 = H$ (TER-230)
2, $R^1 = R^2 = H$, $R^3 = Cl$ (TER-231)
3, $R^1 = Ph$, $R^2 = H$, $R^3 = Cl$ (TER-286)
4, $R^1 = H$, $R^2 = Ph$, $R^3 = Cl$ (TER-296)
5, $R^1 = Ph$, $R^2 = H$, $R^3 = Br$ (TER-338)



6a, $R^1 = R^2 = H$
6b, $R^1 = Ph$, $R^2 = H$
6c, $R^1 = H$, $R^2 = Ph$

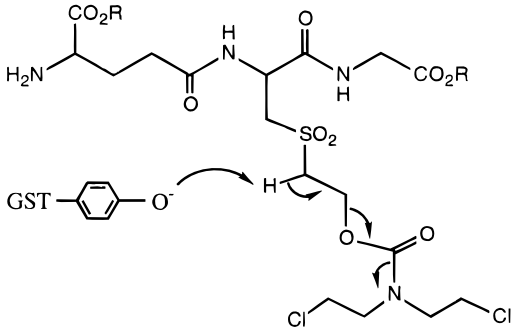
7a, $R^3 = H$
7b, $R^3 = Cl$
7c, $R^3 = Br$

dryl group in GSH, making it able to react with electrophilic species.[16] By taking advantage of this active-site geometry, we previously designed compounds **1**−**3** (Scheme 1) where an active methylene group was placed adjacent to the glutathione sulfur in such a way that the Tyr phenoxide (Tyr7 in the case of GST P class and Tyr6 in case of GST M class) would be able to abstract one of those acidic methylene protons. Such a deprotonation should trigger a β-elimination, resulting in the cleavage of the phosphorodiamidate moiety **7**.[11]

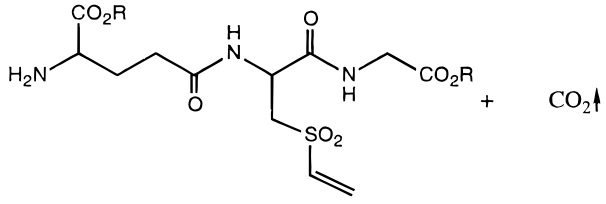
To lend additional support to this hypothesis, a computer model of **3** (TER-286) at the active site of GST P1-1 isozyme was generated (Figure 1). The model shows that it is possible to accommodate **3** in the active site of this enzyme in such a way that the Tyr7 is close to the active methylene protons adjacent to the sulfone group in **3**.

Design of **3** was based on earlier work from several laboratories,[7−9] but primarily from our laboratory,[10] where the C-terminal (*R*)-(−)-phenylglycine GSH analogs were discovered to exhibit a high degree of potency and specificity in inhibition of P1-1 GST isozyme. Interestingly, these GSH variants exhibited different isozyme selectivity in the context of the latent alkylating agent: **1** and **2** were M1a-1a selective and **3** was equally selective to both P1-1 and A1-1 GST isozymes. As expected, only **2** and **3** were cytotoxic. The placebo compound γ-glutamyl-α-amino-β-[[[2-[[bis(diethylamino)phosphoryl]oxy]ethyl]sulfonyl]propionyl]glycine (**1**), which contains the diethylamine group instead of bis-(2-chloroethyl)amine, was found to be noncytotoxic although as readily cleaved by GST as its toxic analogs, thereby suggesting that the cytotoxicity is due to the liberation of chlorinated species **7b**. The human breast

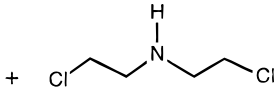
Scheme 2. Proposed Mechanism of GST-Mediated Cleavage of **8** and **9**



8, R = H (TER-322)
9, R = C_2H_5 (TER-324)



6a, R = H
6d, R = C_2H_5



10

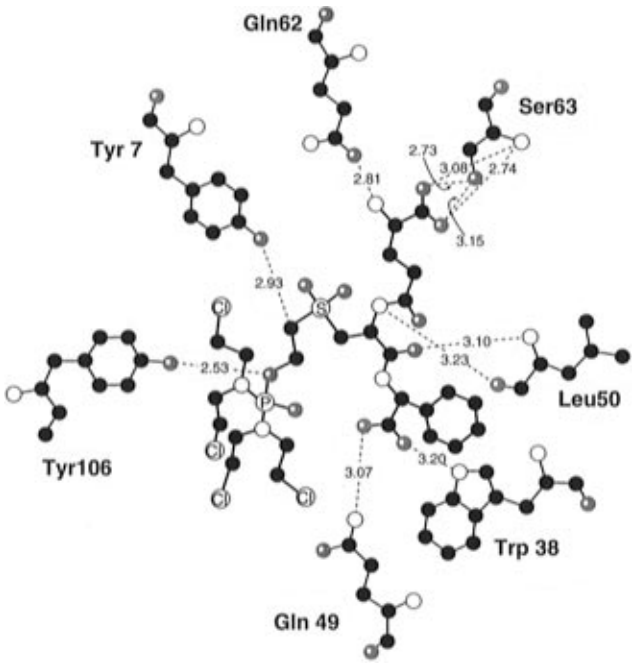


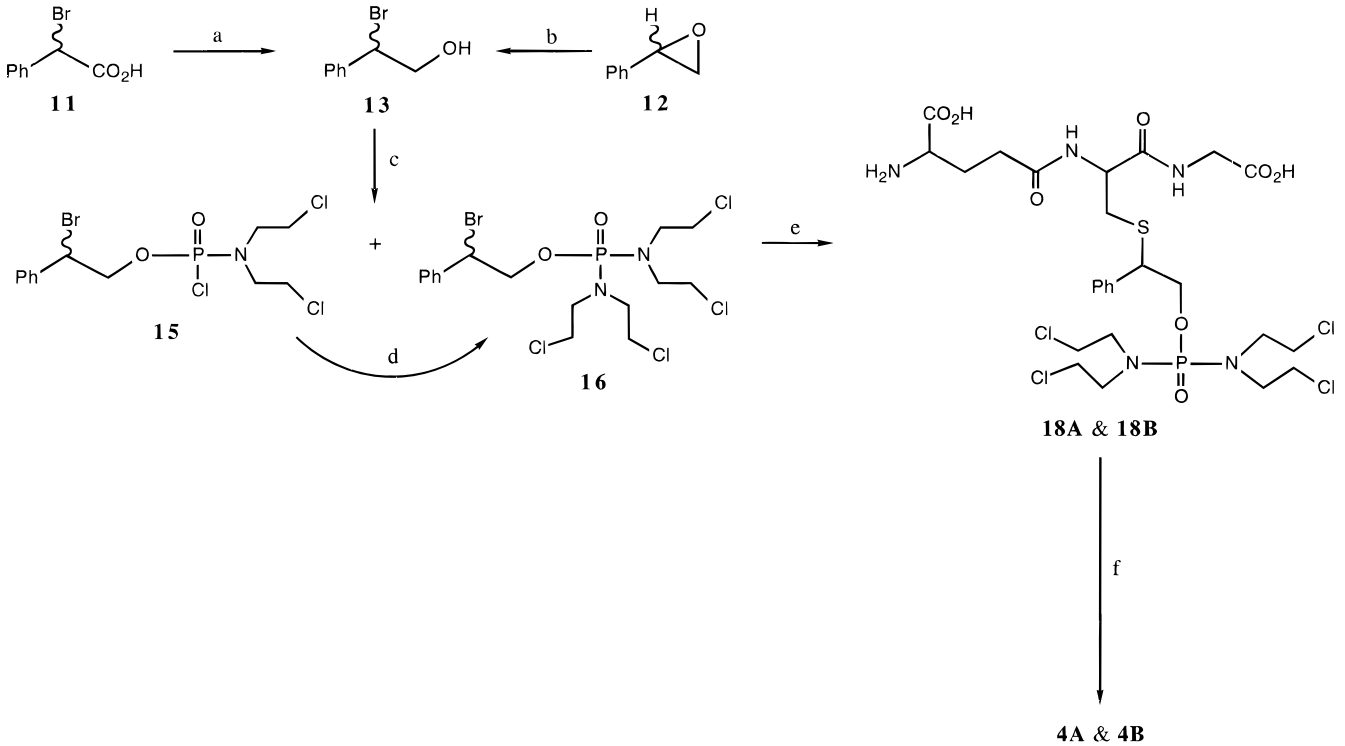
Figure 1. Schematic two-dimensional representation of **3** (TER-286) at the active site of the GST P1-1 isozyme. Indicated are the potential hydrogen bond sites as well as the distance between the the phenoxide oxygen of Tyr7 of the enzyme and the methylene group adjacent to the sulfone group in **3**.

tumor cell line MCF-7, transfected with GST P1-1 gene, showed 2-fold increased sensitivity to **3** as compared to **2**, which correlated well with the increased cleavage rates observed with P1-1 GST *in vitro*. **3** not only appears to be effective against cancer in a variety of animal models but also seems to be minimally toxic to bone marrow GM progenitor cells.[17,18] It is of interest to note that a GST P1-1 selective inhibitor TER-199 (γ-glutamyl-*S*-benzylcysteinyl-(*R*)-(−)-phenylglycine diethyl ester)[10] has been shown in preclinical (*in vivo*) studies not only to potentiate cytotoxicity of common therapautic drug[19] but also to accelerate the recovery of bone marrow cells, as well as circulating neutrophils and platelets, following their suppression by cancer chemotherapy.[18] Interestingly, compound **3** as well as one of its decomposition products (see Scheme 1) bear structural similarities (same peptide backbone) to TER-199 and may therefore have beneficial effects with regard to myelostimulation.

Since GST P1-1 is elevated in many types of cancer cells, we continued our efforts to design more P1-1 selective analogs and synthesized compounds **4** and **5** (Schemes 1, 3, and 4). Compound **4** differs from M1a-1a specific **2** by having a phenyl group on the methylene group adjacent to the sulfone. The design of **4** was based on our speculation that introduction of a bulky phenyl group at the sulfone α-carbon would change its selectivity from M1a-1a to either A1-1 or P1-1 GST isozymes. With a desire to enhance the toxicity of **3**, we also synthesized and evaluated its tetrabromo analog **5** (Scheme 1 and 4).

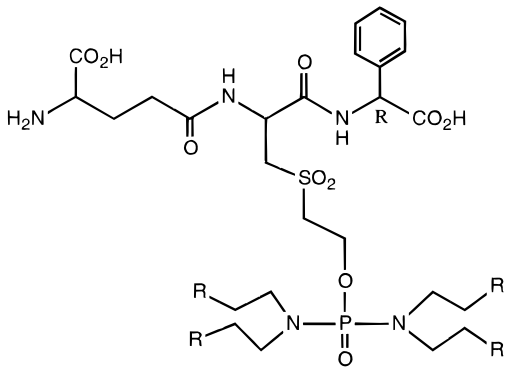
(b) Urethane Mustard. Introduction of a urethane protecting group on the toxic bis(2-chloroethyl)amine should deactivate its alkylating ability at physiological pH and temperatures. By combining this attractive feature with our GST activation concept, we designed a novel urethane mustard **8** and its diethyl ester **9** containing a latent alkylating group (Scheme 2). It was anticipated that **8** or **9**, upon GST-assisted proton abstraction, would trigger a β-elimination with concomitant decarboxylation to release a toxic nornitrogen mustard, bis(2-chloroethyl)amine (**10**)[20] (Scheme 2). A literature survey revealed that there are at least two examples that utilized a similar concept. The first

Scheme 3. Synthesis of Compound **4**, the α-Phenyl Analog of **3**[a]



[a] (a) BH_3·THF, THF; (b) bromotrimethylsilane, CH_3CN; (c) $POCl_3$, bis(2-chloroethyl)amine hydrochloride (**14**), Et_3N, CH_2Cl_2; (d) **14**, Et_3N, CH_2Cl_2 or EtOAc; (e) glutathione (**17**), NaOH, H_2O, EtOH, CH_3CN, pH 9−10; (f) AcOH, aqueous H_2O_2, CH_3CO_3H.

Scheme 4. Synthesis of Compound **5**, the Tetrabromo Analog of **3**[a]

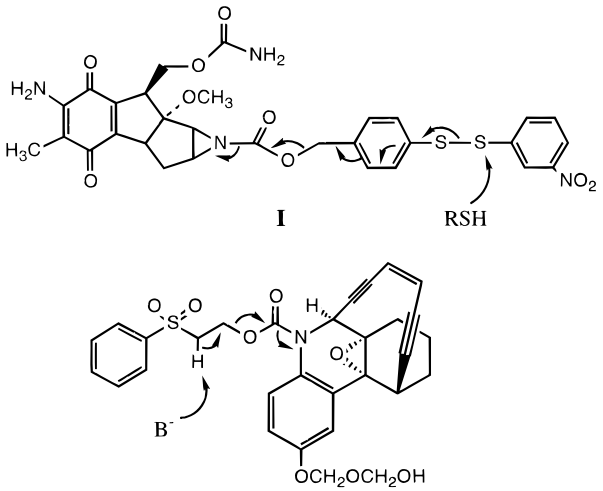


3 (TER-286), R = Cl

a

5 (TER-338), R = Br

[a] (a) LiBr, acetone/acetonitrile.



I

II

Figure 2. Structures of mitomycin C prodrug **I**, and dynemycin A analog prodrug **II**; note that prodrug activation mechanism by 1,6-elimination/β-elimination is illustrated.

example was the prodrug **I**[21] (Figure 2), where an anticancer drug mitomycin C was masked by an appropriately substituted benzyl carbamate disulfide. Upon treatment with a thiol, a thiophenoxide was generated that triggered 1,6-elimination, followed by decarboxylation to release the cytotoxic free mytomycin C. Surprisingly, *in vitro* studies showed that the prodrug **I** was 40−70-fold more toxic than mitomycin C. The authors speculated that the enhanced cytotoxicity of the prodrug **I** could be due to its lipophilic character and the ease with which it can penetrate into the cell and undergo subsequent fragmentation. The second example was the compound **II**[22] (Figure 2), where an analog of the enediyne anticancer drug dynemycin A was masked by a phenyl sulfone ethylene carbamate. Upon treatment with a base, a β-elimination with concomitant decarboxylation released the free dynemycin A analog. These examples clearly demonstrate the potential utility of a urethane mustard bridge and could be conveniently extended to the targeted and controlled delivery of other amine-containing anticancer drugs when it is beneficial to mask the reactive amino function of the drug during its transportation. Such latent drugs may be activated in a very specific, disease related, fashion in sharp contrast to prodrugs which are activated by mechanisms that are unrelated to the disease they are intended to treat.

Chemistry[23]

The compound **4** was synthesized as outlined in Scheme 3. 2-Bromophenethyl alcohol (**13**) was synthesized either by reduction of α-bromophenylacetic acid (**11**) or by treatment of styrene oxide (**12**) with bromotrimethylsilane. In the latter method, the major product was a polymer that was formed by the acid-catalyzed self-condensation of the initially formed **13**. As a result, **13** was subsequently prepared only by the first condition. Compound **16** was synthesized by treating **13** with phosphorus oxychloride and bis(2-chloroethyl)amine hydrochloride (**14**) in the presence of triethylamine. The major product obtained from this preparation was identified as **15**, but this could be converted to **16** by treatment with excess **14** and triethylamine. The sulfide **18** was synthesized by treating the bromide **16** with glutathione (**17**) at pH 9−10. Oxidation of sulfide **18** by treating with hydrogen peroxide and peracetic acid yielded the sulfone **4**. As expected, the sulfide **18** as well as the sulfone **4** were obtained as mixtures of diastereomers.[24] Our attempts to separate these diastereomers at the sulfide stage were unsuccessful. However, with some difficulty, we separated the diastereomeric sulfones **4A**[24] and **4B**[24] by HPLC.

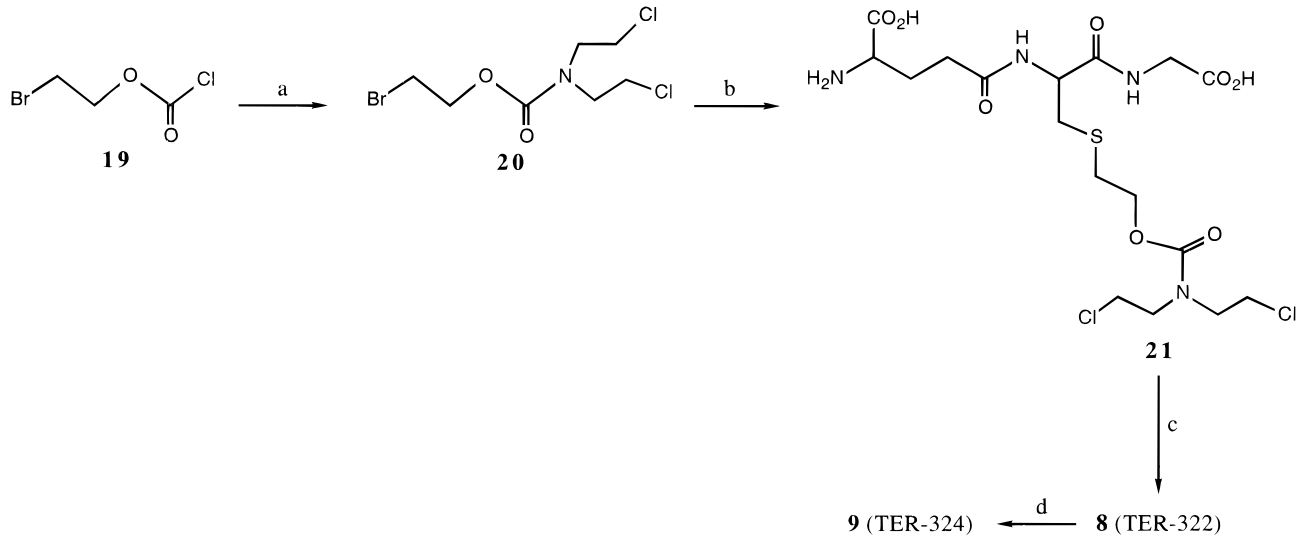
The tetrabromo derivative **5** was synthesized by treating the corresponding tetrachloro derivative **3** with lithium bromide as shown in Scheme 4, and the reaction progress was monitored by analytical HPLC. The reaction did not go to completion even after 46 h reaction time, and the mixture at that stage indicated the presence of varying amounts of monobromo, dibromo, and tribromo derivatives, along with a major amount (>50%) of tetrabromo derivative **5**. The reaction was stopped at 46 h reaction time because of the progressive appearance of other side products, and the compound **5** was isolated from the mixture by HPLC.

Synthesis of urethane mustards **8** and **9** was achieved as shown in Scheme 5. Treatment of 2-bromoethyl chloroformate (**19**) with **14** in the presence of triethylamine yielded the bromide **20**. Reaction of glutathione (**17**) with **20** at pH 9−10 gave glutathione conjugate **21**. Oxidation of the sulfide **21** with hydrogen peroxide and peracetic acid yielded the sulfone **8**. Esterification of the diacid **8** with thionyl chloride in the presence of ethanol yielded the corresponding diethyl ester hydrochloride **9**.

Results and Discussion

***In Vitro* Results.** Compounds **4**, **4A**, **4B**, **5**, and **8** were individually treated with physiological concentrations (usually 0.003−0.006 mM) of recombinant human A1-1, M1a-1a, and P1-1 GST isozymes at physiologically relevant pH (7.1−7.3) and temperature (37 °C). Control experiments without GSTs were run simultaneously. Aliquots of the reaction mixture were taken out at specific intervals and quenched with acetic acid to stop further decomposition, and the amount of cleavage was assayed by reversed-phase HPLC. The rates of decomposition were calculated by measuring the ratio of peak heights of test compound to a nonreactive internal

Scheme 5. Synthesis of Urethane Mustards **8** and **9**[a]



[a] (a) Bis(2-chloroethyl)amine hydrochloride (**14**), Et_3N, CH_2Cl_2; (b) glutathione (**17**), NaOH, H_2O, EtOH, CH_3CN, pH 9−10; (c) AcOH, aqueous H_2O_2, CH_3CO_3H; (d) $SOCl_2$, EtOH.

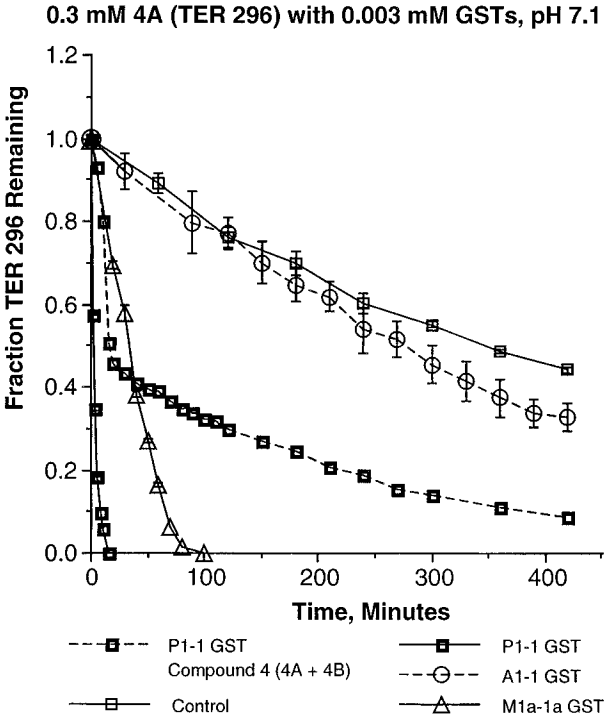


Figure 3. GST P1-1 catalyzed decomposition of **4** and differential cleavage of **4A** by a panel of GST A1-1, M1a-1a, and P1-1 isozymes; note that >50% of decomposition of **4** had occurred in first 20 min, but it took nearly 7 h to achieve an additional 40% decomposition. Also note that complete decomposition of **4A** had occurred in 20 min with GST P1-1 isozyme.

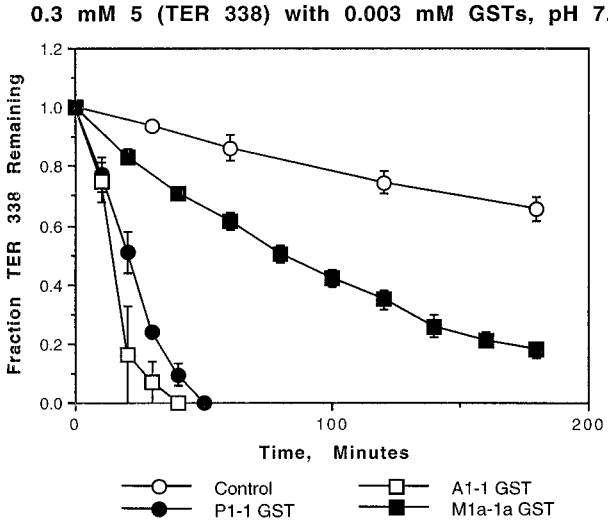


Figure 4. Differential cleavage of **5** by a panel of GST A1-1, M1a-1a, and P1-1 isozymes; note that complete cleavage of **5** had occurred in <50 min by both GST A1-1 and P1-1 isozymes.

standard (an oligopeptide DQQNAFYEILHOPN-NH_2) at specific time intervals, and the graphs were drawn accordingly. Interestingly, as shown in Figure 3, when a diastereomeric mixture of **4** was treated with GST P1-1 isozyme, it followed a decomposition pattern that is suggestive of a condition where only one of the diastereomers of **4** is preferentially and selectively cleaved faster by the isozyme. To confirm this interesting feature, we separated the diastereomer **4A** by a preparative HPLC and subjected it to the panel of GSTs. As anticipated, the rate of decomposition of **4A** by GST P1-1 isozyme was dramatic with 100% decomposition in just 20 min (see Figure 3). Interestingly, P1-1 accelerated decomposition of **4A** about 100 times faster than background, 80 times faster than A1-1, and about 11 times faster than M1a-1a (Figure 3). Contrarily, when the diastereomer **4B** was subjected to activation by GST P1-1, only about 20% decomposition was observed in 1 h (data not shown). Compound **5**, which is a tetrabromo analog of P1-1 and A1-1 specific **3**, was similarly subjected to the panel of three GST isozymes. **5** was found to be very reactive and was completely cleaved by P1-1 and A1-1 in about 45 and 30 min, respectively (Figure 4). In contrast, only 35−40% of **3** was cleaved by P1-1 and A1-1 in 45 min.[11] The most isozyme selective result was obtained from the *in vitro* GST assay of latent urethane mustard **8**. As shown in Figure 5, **8** exhibited very good activation by GST P1-1 isozyme and almost negligible activation by A1-1 and M1a-1a isozymes.

Cell Culture Results. The toxicity of compounds **3** (TER-286)[11] (included for comparison), **4A** (TER-296), **5** (TER-338), **8** (TER-322), and **9** (TER-324) was determined in MCF-7 cell lines transfected with either a P1-1-containing vector (*pi*) or a control (*neo*) vector. Trans-

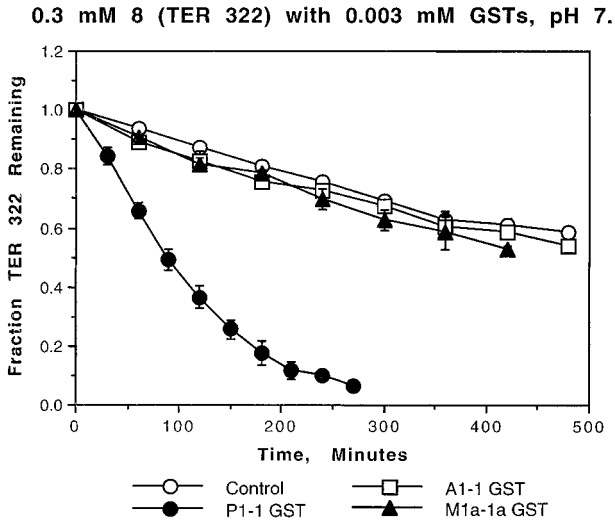


Figure 5. Differential cleavage of **8** by a panel of GST A1-1, M1a-1a, and P1-1 isozymes; note that only GST P1-1 isozyme could catalyze/accelerate the decomposition of **8**.

Table 1. Cytotoxicity Data for **3**, **4**, **5**, **8**, and **9**

cell line	treatment time, min	IC_{50}, μM[a]				
		3[b]	**4A**[c]	**5**[c]	**8**[c,d]	**9**[b,d]
MCF-7 *neo*	120	39.9 (4.4)	21.3 (7.8)	16.1 (2.7)	132.8 (29.5)	102.1 (10.8)
MCF-7 *pi*	120	14.4 (3.0)	13.5 (5.1)	11.4 (1.8)	85.8 (8.5)	68.8 (6.7)

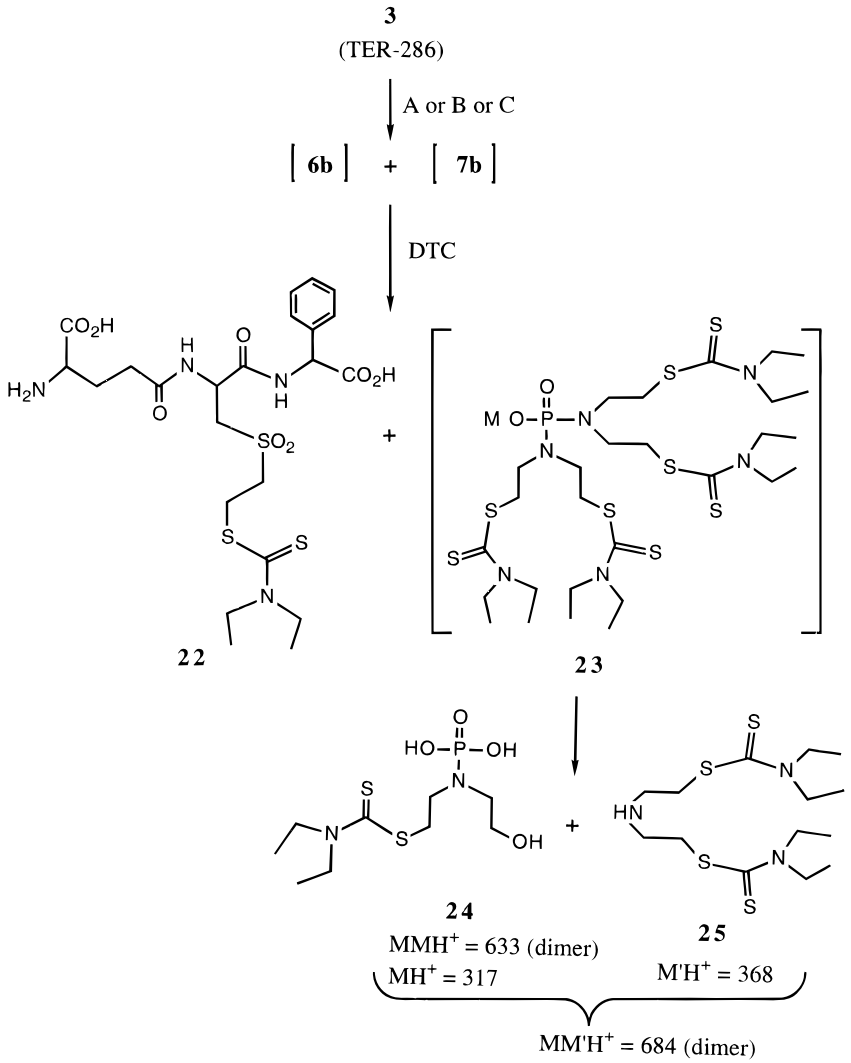
[a] Value is the mean of four or five experiments. Value in parentheses is the sem. [b] Difference between MCF-7 *neo* and MCF-7 *pi* is statistically significant, $p < 0.05$. [c] Difference between MCF-7 *neo* and MCF-7 *pi* is not statistically significant. [d] Difference between **8** and **9** is not statistically significant.

fection with P1-1 resulted in approximately 4-fold increase in P1-1 protein levels (2.5 μg/mg cytosolic protein) compared to the *neo* transfect (0.6 μg/mg/ cytosolic protein).[25] While it was too low for accurate determination of the GST activity in MCF-7 *neo* cells, previously published levels were approximately 7 nmol/ min per mg of protein; MCF-7 *pi* total GST π activity was reported to be approximately 90 nmol/min per mg protein.[26] Determination of GST *pi* activities in MCF-7 *pi* and MCF-7 *neo* cell lines was carried out again in our laboratory using the CDNB method.[27] Here again, the GST *pi* activity in MCF-7 *neo* cells was too low to be accurately measured and seemed to be less than 20 nmol/min per mg of protein. The GST *pi* activity in MCF-7 *pi* cells was about 176 nmol/min per mg of protein.[25] Glutathione (GSH) levels were not significantly different between MCF-7 *neo* and MCF-7 *pi*[28] and were similar to other MCF-7 cell lines transfected with μ or α GSTs (approximately 120 nmol/mg protein).[29] This rules out the possibility that the different sensitivities exhibited by *neo* and *pi* transfects to the test compounds is based on their GSH content. As shown in Table 1, all of the test compounds were found to be more toxic in the *pi*-containing MCF-7 cells than in the *neo*-containing MCF-7s, which was in good agreement with their *in vitro* GST P1-1 selectivities. The differential toxicity between MCF-7 *neo* and MCF-7 *pi* was statistically significant for **3** and **9**, but not for **4A**, **5**, and **8**. Lack of statistically significant differential toxicity for compounds **4A**, **5**, and **8** was due to inter-experimental variation since these compounds were more toxic to MCF-7 *pi* cell lines in all experiments. Less rigorous statistical analysis such as nonparametric determinations show such results to be highly significant. Among the test compounds, **3**, **4A**, and **5** were the most toxic while **8** and **9** were the least toxic to either cell line. One explanation for the disparity between *in vitro* enzymatic and cell culture results may be the relative ability of the different compounds entering the cell. Because of the presence of a hydrophobic phenyl ring in compounds **3**, **4**, and **5**, they may be entering cells more readily than **8** or **9**. It has been reported that glutathione diethyl esters can easily penetrate cellular membranes and be hydrolyzed enzymatically to glutathione once inside the cell.[19,30] Compound **8** was significantly less toxic in the cell culture model than could be anticipated from its *in vitro* specificity for GST P1-1 isozyme. This led us to examine its diethyl ester form (compound **9**) to see if it can exhibit improved toxicity. Compound **9** exhibited a 30% increase in toxicity in the cell culture model compared to compound **8** (Table 1), which was encouraging but not statistically significant. Since the differential toxicity between *pi* and *neo* transfects was maintained for **8** and **9**, the increased toxicity to *pi* transfects is unlikely to be a function of the cell's ability to internalize the compounds.

Mechanistic Studies. Some experimental proof of the GST activation concept was presented in our preliminary communication[11] where the compounds **1** and **2** were individually subjected to both base-(chemical) and enzyme-catalyzed decomposition and the decomposition products identified by mass spectrometry. **1** and **2** gave mass peaks attributable to vinyl sulfone **6a** (*m/z* 366 amu) and their respective phosphorodiamidate **7a** (*m/z* 231 amu, MNa^+), and the monoaziridinium (*m/z* 275 amu) and diaziridinium (*m/z* 333 amu, MNa^+) derivatives of **7b** (Scheme 1). Additionally, in a separate experiment, **1** was subjected to a base-catalyzed (chemical) decomposition and the decomposition product **7a** was isolated and characterized (^{1}H-NMR and mass spectrometry). The vinyl sulfone **6a** could not be isolated in pure form probably because of its inherent reactivity/instability. Control experiments in which other proteins, such as ovalbumin and human serum albumin (HSA), showed no activity combined with the GST isozyme selectivity for decomposition of test compounds not only supported the proposed mechanism but also ruled out the possibility of a nonspecific protein-dependent degradation.

We present here additional experimental proof of the concept. Our main aim during the present study was to probe whether the anticipated decomposition products can be trapped as relatively stable diethyl dithiocarbamate adducts.[31] Experiments were designed to provide proof of not only the decomposition pattern but also the alkylating ability of the decomposition products. We have selected compound **3** (TER-286) for this important study because it has been the most extensively studied member of the family.[17,18] In a separate experiment, as shown in Scheme 6, **3** was first subjected to a base-catalyzed chemical decomposition in the presence of diethyldithiocarbamic acid (DTC) at pH 11−12 while monitoring the reaction by HPLC. **3** decomposed completely within 1 h. Purification of the mixture by HPLC led to the isolation of the vinyl sulfone−DTC adduct **22** (t_R = 17.23 min, *m/z* 591 amu, MH^+) and an

Scheme 6. Base- (Chemical) and GST P1-1 Isozyme-Catalyzed Decomposition of **3** (TER 286) in the Presence of Diethyldithiocarbamic Acid (DTC), Trapping Unstable Decomposition Products **6b** and **7b** as Stable DTC Adducts **22**, **24**, and **25**



[a] (A) Acetonitrile/water (3:1), diethyldithiocarbamic acid sodium salt (DTC), 1 N Na_2CO_3 to pH 11−12; (B) acetonitrile/water (3:1), DTC, 1 N HCl to pH 7.2; (C) DTC, 0.0096 mM GST P1-1, phosphate buffer, pH 7.2.

impure fraction containing some amount each of phosphoramidate **24** and diethylamine−DTC adduct **25** (based on mass spectrometry; see the Experimental Section). Obviously, **24** and **25** must have formed from the probably unstable phosphorodiamidate−DTC adduct **23**.[32] Further structural proof of **22** was established from its ^{1}H-NMR, HRMS, and elemental analysis (see the Experimental Section). As anticipated, ^{31}P-NMR of **22** did not show any phosphorus-31 peak, thereby confirming the fact that the phosphate portion of the molecule had been cleaved off. Convincingly, the presence of the phosphate fragment in the product mixture of **24** and **25** was evidenced by two phosphorus-31 peaks in its ^{31}P-NMR spectrum (data not shown).

In a separate experiment at pH 7.2, chemical decomposition of **3** in the presence of DTC resulted in only about 10−15% decompostion in 1 h and about 30% decomposition in 24 h periods (Scheme 6, condition B, data not shown).

To probe further, as shown in Scheme 6 and Figure 6, **3** was simultaneously subjected to decomposition in phosphate buffer at pH 7.2 under the following four conditions: a, with DTC and without GST P1-1; b, without DTC and without GST P1-1; c, with both DTC and GST P1-1 (Scheme 6); and d, without DTC and with GST P1-1.[11] As anticipated, only experiments c and d containing GST P1-1 isozyme exhibited decomposition of **3** roughly 3−4 times faster than control (experiments a and b) (see Figure 6). Similarly, as shown in Figure 6, generation of vinyl sulfone−DTC adduct **22** in experiment c was roughly 3 times faster than that in control (experiment a) and that the formation of DTC−adduct **22** was at a rate proportional to the decomposition of test compound **3**, thereby indicating that the major pathway of decomposition of the test compound is likely to be as proposed.

The above mechanistic studies clearly provided experimental proof to our hypothesis that GSTs catalyze the decomposition of the latent mustard **3** and its reported analogs. Isolation and characterization of DTC adducts **22**, **24**, and **25** demonstrated that these compounds are cleaved in the prescribed manner and the decomposition products **6b** and **7b** are indeed alkylating agents. With limited data in hand, it is premature to conclude firmly that the vinyl sulfone **6b**, which is a very good Michael acceptor, is nontoxic. However, previous

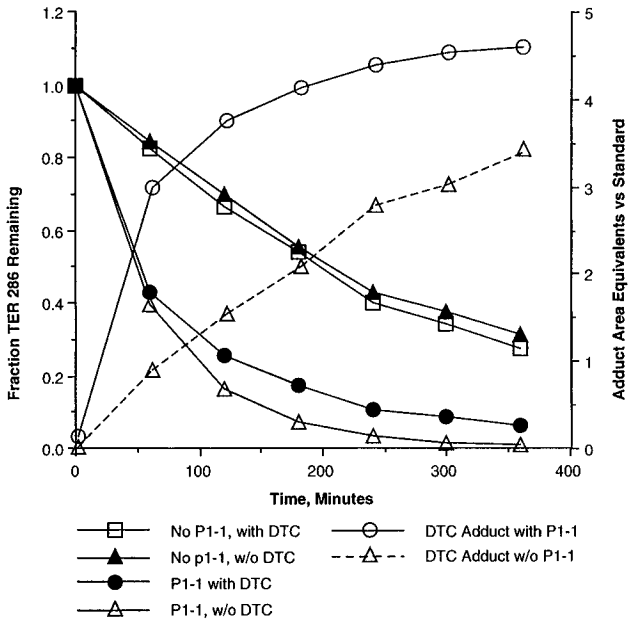


Figure 6. Differential cleavage of **3** (TER-286) with and without GST P1-1 isozyme in presence or absence of diethyldithiocarbamic acid (DTC); note that **3** had decomposed faster only in the experiments containing GST P1-1 isozyme. Also shown is the differential generation of vinyl sulfone−DTC adduct **22** from the decomposition reaction of **3** (TER 286) with or without GST P1-1 isozyme in presence of DTC; note that formation of DTC-adduct **22** was about 3 times faster in the experiment containing GST P1-1 isozyme and the formation of **22** was at a rate proportional to the decomposition of test compound **3**.

in vitro and cell culture studies on placebo compound **1**,[11] which was found to be noncytotoxic although readily cleaved by GST M1a-1a isozyme, suggest that the reactive vinyl sulfone **6b** may be quickly neutralized by a mild nucleophilic species (including water), or by any of more than 20 intracellular detoxification enzymes, to a nontoxic byproduct as soon as it is formed. The observed cytotoxicity of **3**, therefore, is apparently contributed solely by the phosphorodiamidate mustard fragment **7b**.

Conclusion

Two new analogs, **4** and **5**, of the previously reported GST-activated latent alkylating agent **3** have been designed, synthesized, and evaluated yielding a more selective compound for the key target GST P1-1 isozyme. The GST activation concept was further broadened through design, synthesis and evaluation of novel latent urethane mustards **8** and **9**. From *in vitro* enzymatic studies, **4A** and **8** were identified as selective for GST P1-1 isozyme which is known to be elevated in several types of cancer. Cell culture studies with MCF-7 cell lines transfected with high and low levels of GST P1-1 isozyme revealed that all the test compounds were more toxic to MCF-7 *pi* than to MCF-7 *neo* cell lines. However, a statistically significant differential toxicity between *pi* and *neo* transfects was achieved only by **3** and **9**. The tetrabromo derivative **5** was found not only to be more reactive and cytotoxic than the corresponding tetrachloro derivative **3** but also to have lost its statistically significant differential toxicity between *pi* and *neo* transfects. Mechanistic studies on base- (chemical) and enzyme-catalyzed decomposition of **3** provided experimental proof of the GST activation concept and the resulting fragmentation pattern. Trapping of transient decomposition products as relatively stable diethyldithiocarbamic acid adducts established the alkylating ability of those reactive species. Novel GST-activated urethane mustard compounds demonstrated the potential for targeted and controlled delivery of a wide variety of other amine-functionalized pharmaceutical agents into specific cancer and other tissues with elevated levels of a particular GST isozyme. This highly flexible latent drug activation paradigm may, in principle, be extended to other disease-associated enzymes, providing a substantial advantage over prodrugs that are activated in a manner unrelated to the disease tissue's properties. As an alternative to enzyme inhibition, therefore, this kind of latent drug shows considerable promise.

Experimental Section

General information, including procedures for *in vitro* enzymatic and cell culture studies, are as previously reported.[11] The preparative HPLC column used was a YMC 18 ODS-AQ column (YMC Corp., Wilmington, NC; Catalog No. AQ-323-5). Unless otherwise noted, the buffers used for preparative HPLC were as follows: buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in 9:1 acetonitrile/water. After equilibration of the column with buffer A, elution of compounds was done by running a gradient of 0−100% buffer B at 12 mL/min over 120 min and the UV detector was set at 220 nm. The analytical HPLC column used was a Bakerbond C-18 column (VWR, Catalog No. JT7098-0); the buffers used for analytical HPLC were as follows: buffer A, 0.05 M $NH_4H_2PO_4$ in 95:5 water/acetonitrile; buffer B, 0.015 M $NH_4H_2PO_4$ in 3:7 water/acetonitrile. After equilibration of the column with buffer A, a gradient of 0−100% buffer B was run over 30 min and the UV detector was set at 214 nm. Retention times (t_R) reported were usually for >90% pure products by analytical HPLC. ^1H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker instrument at 400, 100, and 162 MHz, respectively (UC Berkeley, California); some ^1H NMR spectra were recorded on Nicolett instrument at 360 MHz (Acorn NMR Inc., Fremont, California); HRMS and elemental analysis were obtained from UC Berkeley. Mass spectral analyses of compounds were performed on a Finnigan matrix-assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometer using a 50 mM solution of α-cyano-4-hydroxycinnamic acid (Aldrich) and 0.01% TFA in 7:3 acetonitrile/water as the matrix. Bis(2-chloroethyl)amine hydrochloride, Borane−tetrahydrofuran complex, 2-bromoethanol, 2-bromethyl chloroformate, α-bromophenylacetic acid, bromotrimethylsilane, diethyldithiocarbamic acid sodium salt, diisopropylethylamine, hydrogen peroxide, lithium bromide, peracetic acid, styrene oxide, and thionyl chloride were obtained from Aldrich. Glutathione (reduced) was obtained from Sigma.

2-Bromophenethyl Alcohol (13): Condition a. A 100 mL (100 mmol) sample of a 1.0 M solution of borane−tetrahydrofuran complex was added via cannula to a solution of α-bromophenylacetic acid (**11**) (10.75 g, 50 mmol) in 50 mL of dry tetrahydrofuran at 0−5 °C under argon over 15 min. After the mixture was stirred at 5−10 °C for 3 h, it was rechilled and quenched with 100 mL of 10% acetic acid in methanol over 20 min. After 15 min of additional stirring, the mixture was concentrated *in vacuo*. The oily residue was redissolved in 300 mL of ethyl acetate, washed with water (2 × 250 mL) and brine (1 × 250 mL), and dried over anhydrous Na_2SO_4. Evaporation and purification by flash column chromatography (35- × 3.7-cm silica gel bed and eluted isocratically with methylene chloride) gave **13** (7.25 g, 72%) as a colorless viscous oil: ^1H NMR (300 MHz, $CDCl_3$) δ 2.53 (br s, 1H, O*H*, exchanged with D_2O), 3.90−4.11 (m, 2H, CHCH_2), 5.06 (m, 1H, C*H*CH$_2$), 7.25−7.45 (m, 5H, C_6H_5). This compound was found to be unstable and was used immediately after preparation.

Condition b. To a stirred solution of styrene oxide (**12**) (6 g, 50 mmol) in 50 mL of acetonitrile placed in a 2-neck reaction flask fitted with a reflux condenser and a dropping funnel under argon at room temperature was added bromotrimethylsilane (7.26 mL, 55 mmol) over 20 min. After an exothermic reaction which subsided within a few minutes, the mixture was stirred at room temperature under argon for 22 h. The mixture was concentrated *in vacuo*, and the residue was redissolved in 200 mL of ethyl acetate, washed successively with water (2 × 100 mL), 5% sodium bicarbonate (1 × 100 mL), water (1 × 100 mL), and brine (1 × 250 mL), and dried over anhydrous Na_2SO_4. Evaporation and purification by flash column chromatography (30- × 3.7-cm silica gel bed and eluted isocratically with 1:1 petroleum ether/methylene chloride) gave a colorless viscous oil (2.2 g, 22%) which was identical, by TLC and ^{1}H NMR, to that obtained from above. The major product obtained from this reaction was found to be, by ^{1}H NMR, a polymer probably formed by the acid-catalyzed self-condensation of the initially formed unstable **13**.

2-Bromophenethyl *N,N,N′,N′*-Tetrakis(2-chloroethyl)-phosphorodiamidate (16). To a stirred solution of freshly distilled phosphorus oxychloride (6.15 mL, 66 mmol) in 150 mL of dry methylene chloride at 0−5 °C in a reaction flask fitted with a pressure-equalizing dropping funnel under argon was added triethylamine (4.9 mL, 35 mmol) over 5 min. Then a solution of **13** (6.65 g, 33 mmol) in 33 mL of dry methylene chloride was added over a period of 4.5 h. After the mixture was stirred at room temperature for 17 h, it was rechilled to 0 °C and bis(2-chloroethyl)amine hydrochloride (**14**) (17.8 g, 100 mmol) was added as a solid in one lot. To this stirred suspension was added dropwise a solution of triethylamine (28 mL, 200 mmol) in 50 mL of methylene chloride over 2 h. The mixture was allowed to warm to room temperature and stirred for 3 days. Then, the mixture was suction filtered through a thin layer of Celite, and the filtrate was concentrated *in vacuo*. The brown oily residue was redissolved in 200 mL of ethyl acetate and suction filtered again to remove the insoluble triethylamine hydrochloride. The filtrate was concentrated *in vacuo*, and the oily residue, which still contains some triethylamine hydrochloride, was purified by flash column chromatography (48- × 4.3-cm silica gel bed and eluted isocratically with methylene chloride) to give 8 g (57%) of 2-bromophenethyl *N,N*-bis(2-chloroethyl)chlorophosphoramidite (**15**) as colorless viscous oil [^{1}H NMR (300 MHz, $CDCl_3$) δ 3.30−3.75 (m, 8H, $N(CH_2CH_2Cl)_2$), 4.50−4.75 (m, 2H, $CHCH_2O$), 5.10−5.20 (m, 1H, $CHCH_2O$), 7.30−7.50 (m, 5H, C_6H_5); MS *m/z* 446.66 (MNa^+)] and 5.5 g (32%) of **16** as colorless viscous oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.31−3.42 (m, 8H, $(N(CH_2CH_2Cl)_2)_2$), 3.52−3.63 (m, 8H, $(N(CH_2CH_2Cl)_2)_2$), 4.36 (t, 2H, J = 6.8 Hz, $CHCH_2O$), 5.09 (t, 1H, J = 6.8 Hz, $CHCH_2O$), 7.36−7.42 (m, 5H, C_6H_5); ^{31}P NMR (162 MHz, $CDCl_3$) δ 13.55 (s); MS *m/z* 552.58 (MNa^+).

Conversion of 15 to 16. A mixture of **15** (5.17 g, 12.2 mmol), **14** (4.46 g, 25 mmol), and triethylamine (7.0 mL, 50 mmol) in 120 mL of ethyl acetate was stirred at reflux temperature for 3 days, whereupon TLC indicated >95% conversion. The mixture was cooled to room temperature and suction filtered through a thin layer of celite. The solids were washed with 50 mL of ethyl acetate. The filtrate was washed successively with 100 mL each of 1 N HCl, 5% $NaHCO_3$, water, and brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo*. Flash column chromatography of the crude product as described above yielded 3.8 g (56.4%) of pure **16**, which was identical, by TLC, mass spectrometry, and ^{1}H NMR, to that obtained above.

***γ*-Glutamyl-α-amino-*β*-[[[2-[[bis[bis(2-chloroethyl)amino]phosphoryl]oxy]-1-phenylethyl]thio]propionyl]glycine (18).** Glutathione (**17**) (3.07 g, 10.00 mmol) was dissolved in 40 mL of deionized water, and the pH was adjusted to between 9 and 10 by adding 1 N NaOH. To this stirred solution at room temperature was added a solution of **16** (3.46 g, 6.54 mmol) in 40 mL of ethanol. The resulting turbid mixture was made clear by adding 20 mL of acetonitrile, and the mixture was stirred at room temperature for 3 days. The mixture was neutralized to pH 5−6 with 10% acetic acid, and most of the organic solvent was removed *in vacuo*. The remaining aqueous mixture was lyophilized and purified by HPLC: after equilibration of the column with buffer A (0.1% TFA in water), an aqueous solution of the crude product was loaded onto the column. The column was eluted first with buffer A followed by a gradient of 0−50% buffer B (0.1% TFA in 9:1 acetonitrile/water) over 120 min at 12 mL/min. Fractions which appeared pure by TLC were combined and lyophilized to give 3.2 g (65%) of **18** as a white fluffy powder which was found by HPLC to be a 1:1 mixture of diastereomers **18A** and **18B**: mp 63−67 °C, t_R = 19.33 min (A), and 19.63 min (B); ^{1}H NMR (400 MHz, D_2O) δ 1.60−1.80 (m, 2H, $CHCH_2CH_2CON$), 2.08 (t, 1H, J = 7.8 Hz, $CHCH_2CH_2CON$), 2.13 (q, 1H, J = 6.6, 8.8 Hz, $CHCH_2CH_2CON$), 2.24−2.55 (m, 1H, $CHCH_2S$), 2.70 (dd, 0.5H, J = 5, 14 Hz, $CHCH_2S$), 2.83 (dd, 0.5H, J = 5, 14 Hz, $CHCH_2S$), 2.90−3.10 (m, 8H, $N(CH_2CH_2Cl)_2$), 3.20−3.35 (m, 9H, $N(CH_2CH_2Cl)_2$ + α-H), 3.40 (dq, 2H, J = 11, 17 Hz, $HNCH_2CO$), 3.95−4.05 (m, 1H, α-H), 4.05−4.10 (m, 2.5H, $SCHCH_2$), 4.17 (q, 0.5H, J = 5, 9 Hz, $SCHCH_2$), 7.05−7.15 (m, 5H, C_6H_5); ^{31}P NMR (162 MHz, D_2O) δ 28.73 and 28.77 (two s, 1:1.05); MS *m/z* 777.85 (MNa^+). Anal. ($C_{26}H_{40}Cl_4N_5O_8PS\cdot TFA\cdot H_2O$) C, H, N.

***γ*-Glutamyl-α-amino-*β*-[[[2-[[bis[bis(2-chloroethyl)amino]phosphoryl]oxy]-1-phenylethyl]sulfonyl]propionyl]-glycine (4).** To a stirred solution of **18** (755.5 mg, 1 mmol) in 10 mL of glacial acetic acid at room temperature was added 0.39 mL (2 mmol) of 30% H_2O_2. The reaction flask was covered with aluminum foil to exclude light, and the mixture was stirred at room temperature for 2 h when the mass spectrum of the crude indicated complete conversion to the sulfoxide. Next, 0.26 mL (1.25 mmol) of 32% peracetic acid in acetic acid was added to the mixture, and it was stirred at room temperature overnight, whereupon the mass spectrum of the crude indicated formation of the sulfone **4**. The mixture was lyophilized and purified by HPLC using the same column and procedure as for **18**, except that the column was eluted isocratically with 0.1% TFA in 3:7 acetonitrile/water to give 220 mg (28%) of **4** as a white fluffy powder which was found by HPLC to be a 1:1 mixture of diastereomers **4A** and **4B**: t_R = 22.17 min (A) and 22.48 min (B); ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.90−2.00 (m, 2H, $CHCH_2CH_2CON$), 2.15−2.35 (m, 2H, $CHCH_2CH_2CON$), 3.05−3.21 (m, 8H, $(N(CH_2CH_2Cl)_2)_2$), 3.25−3.60 (m, 11H, $(N(CH_2CH_2Cl)_2)_2$ + $HNCH_2CO$ + α-H), 3.67 (d, 1H, CH CH_2SO_2, J = 6.4 Hz), 3.82 (t, 1H, J = 6.8 Hz, CH CH_2SO_2), 4.40−4.45 (m, 1H, SO_2CHCH_2O), 4.50−4.61 (m, 1H, SO_2CHCH_2O), 4.70−4.85 (m, 1H, SO_2CHCH_2O), 5.02 (q, 1H, J = 5.2, 8.4 Hz, α-H), 7.30−7.50 (m, 5H, C_6H_5), 8.20 (br s, 2H, NH_2), 8.31 (t, 1H, J = 6 Hz, NH)), 8.45 (d, 1H, J = 8 Hz, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 25.72 (*β*-C, Glu), 30.80 (*γ*-C, Glu), 41.07 (*β*-C, Cys), 42.18 (Cl−C), 46.71 (α-C, Glu), 48.20 (q, P−N−C, J = 3.8, 9.1 Hz), 51.71 (α-C, Gly), 52.01 (α-C, Cys), 61.76 (Ph−C−SO_2), 66.77 (d, P−O−C, J = 8.3 Hz), 128.82 (*m*-C, phenyl), 129.19 (*p*-C, phenyl), 129.93 (*o*-C, phenyl), 130.60 (1-C, phenyl); 169.19 (C═O), 170.76 (C═O), 170.83 (C═O), 170.94 (C═O); MS *m/z* 788.32 (MH^+), 811.30 (MNa^+). Anal. ($C_{26}H_{40}Cl_4N_5O_{10}PS\cdot TFA\cdot H_2O$) C, H, N.

The diastereomers were carefully separated by HPLC (eluted isocratically with 0.1% acetic acid in 3:7 acetonitrile/water). The diastereomer **4A** was obtained (27 mg) as white pluffy powder: mp 114−118 °C; t_R = 22.17 min; ^{1}H NMR (360 MHz, CD_3CN/D_2O) (assignment of protons was done with the help of 2D (COSY) spectrum) δ 2.02 (q, 2H, J = 7.3, 14.0 Hz, $CHCH_2CH_2CON$), 2.37 (dt, 2H, J = 2.0, 7.3 Hz, $CHCH_2CH_2CON$), 3.16−3.33 (m, 9H, $(N(CH_2CH_2Cl)_2)_2$ + $CHCH_2SO_2$), 3.44−3.64 (m, 8H, $(N(CH_2CH_2Cl)_2)_2$), 3.66−3.72 (m, 2H, $CHCH_2CH_2CON$ + $CHCH_2SO_2$), 3.83 (d, 2H, J = 3.7 Hz, NCH_2CO_2H), 4.56−4.61 (m, 1H, SO_2CHCH_2O), 4.70−4.79 (m, 1H, SO_2CHCH_2O), 4.85 (dd, 1H, J = 5.6, 8.6 Hz, SO_2CHCH_2O), 4.90 (dd, 1H, J = 3.9, 9.4 Hz, $CHCH_2SO_2$), 7.46 (s, 5H, C_6H_5); ^{31}P NMR (162 MHz, CD_3CN/D_2O) δ 15.73 ppm; MS *m/z* 788.13 (MH^+), 833.2 ($M2Na^+$); HRMS calcd for $C_{26}H_{40}Cl_4N_5O_{10}PS$ + H^+ 786.1066, found 786.1072.

The diastereomer **4B** was obtained (25 mg) as a white powder: mp 116−120 °C; t_R = 22.48 min; ^{1}H NMR (360 MHz, CD_3CN/D_2O) (assignment of protons was done with the help of 2D (COSY) spectrum) δ 2.02 (q, 2H, J = 7.0, 14.4 Hz, $CHCH_2CH_2CON$), 2.38 (t, 2H, J = 8.2 Hz, $CHCH_2CH_2CON$),

3.17−3.29 (m, 8H, (N($CH_2CH_2Cl)_2$)$_2$), 3.45−3.53 (m, 9H, (N(CH_2-$CH_2Cl)_2$)$_2$ + $CHCH_2SO_2$), 3.63−3.73 (m, 2H, $CHCH_2CH_2CON$ + $CHCH_2SO_2$), 3.80 (d, 2H, J = 3.7 Hz, NCH_2CO_2H), 4.56−4.63 (m, 1H, SO_2CHCH_2O), 4.69−4.75 (m, 1H, SO_2CHCH_2O), 4.81−4.88 (m, 2H, SO_2CHCH_2O + $CHCH_2SO_2$), 7.45 (s, 5H, C_6H_5); ^{31}P NMR (162 MHz, CD_3CN/D_2O) δ 16.02 ppm; MS m/z 790 (M_3H^+), 833 (M_2Na^+); HRMS calcd for $C_{26}H_{40}Cl_4N_5O_{10}PS$ + H^+ 786.1066, found 786.1072.

γ-Glutamyl-α-amino-β-[[2-ethyl-*N,N,N′,N′*-tetrakis(2-bromoethyl)phophorodiamidate]sulfonyl]propionyl-(*R*)-(−)-phenylglycine (5). A solution of **3** (2.17 g, 2.76 mmol) and lithium bromide (13 g, 150 mmol) in 180 mL of 5:1 acetone/acetonitrile was stirred at gentle reflux temperature while the reaction progress was monitored by HPLC. After 46 h reaction time, there was >50% conversion to tetrabromo derivative **5** (51%, t_R = 15.95 min), along with varying amounts of unreacted starting material **3** (<1%, t_R = 13.46 min), monobromo (4%, t_R = 14.05 min), dibromo (13%, t_R = 14.63 min), and tribromo (24%, t_R = 15.20 min) derivatives. The mixture was concentrated *in vacuo*, and the residue was purified twice by HPLC: buffer A, 0.1% TFA in 1:4 acetonitrile/water; buffer B, 0.1% TFA in 9:1 acetonitrile/water; for the first purification, a gradient of 0−100% buffer B was run at 20 mL/min over 120 min. Fractions containing **5**, which were also contaminated with varying amounts of dibromo and tribromo derivatives, were pooled and repurified by running a gradient of 0−50% buffer B at 20 mL/min over 120 min. Here again a majority of fractions were obtained as mixtures of **5**, dibromo, and tribromo derivatives. These were mixed and kept for further purification. Fractions containing pure **5** were pooled and lyophilized to give 135 mg (5%) of a fluffy white powder: mp 75−93 °C; t_R = 25.24 min (94% pure); ^{1}H NMR (400 MHz, CD_3CN/D_2O) δ 2.19−2.36 (m, 2H, $CHCH_2CH_2CON$), 2.57−2.66 (m, 2H, $CHCH_2CH_2CON$), 3.44−3.65 (two m, 19H, $CH_2SO_2CH_2$ + ($NCH_2CH_2Cl)_2$)$_2$), 3.81 (dd, 1H, J = 5, 14.7 Hz, SO_2CH_2), 4.08 (t, 1H, J = 6.5 Hz, α-H), 4,45−4.50 (m, 2H, $SO_2CH_2CH_2O$), 5.12 (dd, 1H, J = 5, 8 Hz, α-H), 5.52 (s, 1H, C_6H_5CH), 7.47−7.55 (m, 5H, C_6H_5); ^{31}P NMR (162 MHz, D_2O/CD_3CN) δ 14.93 (s) and 15.10 (s). Anal. ($C_{26}H_{40}Br_4N_5O_{10}PS$·TFA·$H_2O$) C, H, N.

[(2-Bromoethoxy)carbonyl][bis(2-chloroethyl)]-amine (20). 2-Bromoethyl chloroformate (**19**) (5.6 mL, 50 mmol) was added to a stirred suspension of bis(2-chloroethyl)-amine hydrochloride (**14**) (9.8 g, 55 mmol) in 250 mL of dry dichloromethane at 0−5 °C under argon over 2 min followed by 28 mL (200 mmol) of triethylamine over 20 min. The mixture was stirred at 5−10 °C for 3 h and at room temperature for 18 h. The mixture was suction filtered, and the filtrate was concentrated *in vacuo*. The residue was dissolved in 200 mL of ethyl acetate and suction filtered to remove the triethylamine hydrochloride. The filtrate was successively washed with 100 mL each of 2 N HCl, 5% $NaHCO_3$, water, and brine. It was dried over anhydrous Na_2SO_4, filtered, and concentrated *in vacuo* to give 13 g of crude product as a colorless oil, which was further purified by flash column chromatography (31- × 3.7-cm of silica gel bed and eluted isocratically with dichloromethane) to give 12.5 g (85%) of **20** as a colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.55 (t, 2H, J = 5.7 Hz, $BrCH_2CH_2O$), 3.69 (s, 8H, ($NCH_2CH_2Cl)_2$), 4.43 (t, 2H, J = 5.7 Hz, $BrCH_2CH_2O$); MS m/z 316.31 (MNa^+). Anal. ($C_7H_{12}BrCl_2NO_2$) C, H, N.

γ-Glutamyl-α-amino-β-[[[2-[carbonyl[bis(2-chloroethyl)amino]oxy]ethyl]thio]propionyl]glycine (21). Glutathione (**17**) (6.14 g, 20 mmol) was dissolved in 100 mL of water, and the pH was adjusted to between 9 and 10 by adding 1 N NaOH. To this stirred solution at room temperature was added a solution of **20** (2.93 g 10 mmol) in 100 mL of 1:1 ethanol/acetonitrile, and the resulting clear colorless solution was stirred at room temperature under argon for 3 days, whereupon TLC of the mixture indicated completion of the reaction. The mixture was acidified to pH 5−6 with 10% acetic acid, and most of the organic solvent portion was removed *in vacuo*. The aqueous portion was lyophilized and purified by HPLC (buffer A, 0.1% TFA in 1:9 acetonitrile/water; buffer B, 0.1% TFA in 9:1 acetonitrile/water; eluted by running a gradient from 0−100% buffer B at an elution rate of 12 mL/min) to give 1.7 g (33%) of **21** as a white fluffy hygroscopic powder: mp 85−113 °C; t_R = 15 min; ^{1}H NMR (400 MHz, D_2O) δ 1.80 (q, 2H, J = 7.6 Hz, $CHCH_2CH_2CON$), 2.19 (dt, 2H, J = 7.3, 7.9 Hz, $CHCH_2CH_2CON$), 2.50−2.60 (m, 3H, $CHCH_2$-SCH_2), 2.77 (dd, 1H, J = 5, 14 Hz, SCH_2), 3.34−3.50 (m, 11H, $N(CH_2CH_2Cl)_2$ + $NHCH_2CO_2H$ + α-H), 3.95 (t, 2H, J = 6 Hz, SCH_2CH_2O), 4.24 (q, 1H, J = 5 Hz, α-H); MS m/z 542.69 (MNa^+). Anal. ($C_{17}H_{28}Cl_2N_4O_8S$·TFA·2.5H_2O) C, H, N.

γ-Glutamyl-α-amino-β-[[[2-[[[bis(2-chloroethyl)amino]-carbonyl]oxy]ethyl]sulfonyl]propionyl]glycine (8). To a stirred solution of **21** (0.519 g, 1 mmol) in 10 mL of glacial acetic acid at room temperature was added 30% H_2O_2 (0.39 mL, 2 mmol). The reaction flask was covered with aluminum foil to exclude light, and the mixture was stirred at room temperature for 4 h, when mass spectrum of the mixture indicated complete conversion to sulfoxide. Then, 0.26 mL (1.25 mmol) of 32% peracetic acid in acetic acid was added to the mixture, and it was stirred at room temperature for an additional 4 h, whereupon the mass spectral analysis of the mixture indicated formation of the sulfone **8**. The mixture was lyophilized and purified by HPLC as for **21** (except that the elution rate was 20 mL/min) to give 0.44 g (80%) of **8** as a hygroscopic fluffy white powder: mp 82−93 °C; t_R = 15.2 min; ^{1}H NMR (400 MHz, CD_3CN/D_2O) δ 1.90−2.00 (m, 2H, $CHCH_2$-CH_2CON), 2.31 (dt, 2H, J = 2.0, 7.5 Hz, $CHCH_2CH_2CON$), 3.38−3.66 (m, 14H, $N(CH_2CH_2Cl)_2$ + $NHCH_2CO_2H$ + CH_2-SO_2CH_2), 3.71 (dd, 1H, J = 3.5, 14.7 Hz, α-H), 4.37 (t, 2H, J = 5 Hz, $SO_2CH_2CH_2O$), 4.88 (dd, 1H, J = 3.4, 9.7 Hz, α-H); MS m/z 552.46 (MH^+). Anal. ($C_{17}H_{28}Cl_2N_4O_{10}S$·TFA·2$H_2O$) C, H, N.

γ-Glutamyl-α-amino-β-[[[2-[[[bis(2-chloroethyl)amino]-carbonyl]oxy]ethyl]sulfonyl]propionyl]glycine Diethyl Diester (9). To a stirred suspension of **8** (0.39 g, 0.7 mmol) in 28 mL of dry ethanol in a 100 mL reaction flask fitted with a reflux condenser under argon at room temperature was added thionyl chloride (1.1 mL, 15 mmol) from the top of the condenser. The resulting clear colorless solution was stirred at gentle reflux temperature for 2.5 h, whereupon mass spectral analysis of the mixture indicated formation of the diethyl diester. The mixture was concentrated *in vacuo*, and the gummy residue was purified by HPLC as for **21** to give 0.17 g (40%) of **9** as a hygroscopic fluffy white powder: mp 54−60 °C; t_R = 20.38 min: ^{1}H NMR (Varian 300 MHz, CD_3-CN/D_2O) δ 1.25 (t, 3H, J = 7.1 Hz, $CO_2CH_2CH_3$), 1.30 (t, 3H, J = 6.2 Hz, $CO_2CH_2CH_3$), 2.05−2.30 (m, 2H, $CHCH_2CH_2$-$CONH$), 2.53 (t, 2H, J = 7.1 Hz, $CHCH_2CH_2CONH$), 3.52−3.83 (m, 12H, $N(CH_2CH_2Cl)_2$ + $CH_2SO_2CH_2$), 3.92 (d, 2H, J = 2 Hz, $NHCH_2CO_2H$), 4.00 (dd, 1H, J = 4.5, 7.5 Hz, α-H), 4.17 (q, 2H, J = 7.2 Hz, $CO_2CH_2CH_3$), 4.26 (q, 2H, J = 7 Hz, $CO_2CH_2CH_3$), 4.50 (t, 2H, J = 5.3 Hz, $SO_2CH_2CH_2O$), 4.99 (dd, 1H, J = 3, 9 Hz, α-H); MS m/z 608.31 (MH^+), 631.26 (MNa^+). Anal. ($C_{21}H_{36}Cl_2N_4O_{10}S$·HCl) C, H, N.

Chemical Decomposition of 3: Condition A (Scheme 6). A mixture of **3** (1.24 g, 1.5 mmol) and diethyldithiocarbamic acid sodium salt (3.38 g, 15 mmol) was dissolved in 40 mL of 3:1 acetonitrile/water, and the solution pH was adjusted to 11 by addition of 1 N sodium carbonate (10 mL). The mixture was stirred at room temperature for 1 h whereupon HPLC analysis of the mixture indicated disappearance of **3** (t_R = ∼20 min) and appearance of a prominent product (t_R = ∼17 min) along with two or three other decomposition products whose retention times changed variously with time. The mixture was purified three times by HPLC (buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in 9:1 acetonitrile/water; eluted by running a gradient from 0−100% buffer B at an elution rate of 12 mL/min at 214 nm) to give a fraction (∼150 mg) containing some amount each of the decomposition products **24** (HRMS calcd for $C_9H_{21}N_2O_4P_1S_2$ + H^+ 317.0759, found 317.0753) (**24** also existed as a dimer with m/z 633 amu which was not a true molecular ion) and diethylamine−DTC adduct **25** (HRMS calcd for $C_{14}H_{30}N_3S_4$ + H^+ 368.1323, found 368.1323) (**24** and **25** also existed as a dimer with m/z 684 amu which was not a true molecular ion). The main product (t_R = ∼17 min) was isolated (650 mg, 66%) and characterized as the expected vinyl sulfone−DTC adduct **22**: a white powder; mp 156−158 °C; ^{1}H-NMR (400 MHz, DMSO-d_6/D_2O) δ 1.11−1.21

(m, 6H, $N(CH_2CH_3)_2$), 1.90−2.04 (m, 2H, CH_2CH_2CH), 2.25−2.36 (m, 2H, CH_2CH_2CH), 3.14−3.91 (m, 11H, CH_2CH_2CH + $CH_2SO_2CH_2CH_2S$ + $N(CH_2CH_3)_2$), 4.93 (dd, 1H, J = 4.0 and 9.0 Hz, SO_2CH_2CH), 5.24 (s, 1H, C_6H_5CH), 7.28−7.43 (m, 5H, C_6H_5CH); HRMS calcd for $C_{23}H_{34}N_4O_8S_3 + H^+$ 591.1617, found 591.1619. Anal. ($C_{23}H_{34}N_4O_8S_3 \cdot 0.5TFA \cdot H_2O$) C, H, N, S.

Chemical Decomposition of 3: Condition B/Control Experiment (Scheme 6). Diethyldithiocarbamic acid sodium salt (225 mg, 1 mmol) was dissolved in 4 mL of 3:1 acetonitrile/water, and the solution pH was adjusted to 7.3 with 1 N HCl. An 82 mg (0.1 mmol) sample of **3** was added to the above solution, and the pH of the resulting mixture was adjusted back to 7.3 with 1 N sodium carbonate, and the mixture was stirred at room temperature for 1 h when HPLC analysis of the mixture indicated only 10−15% decomposition of **3**. When the mixture was stirred further for 24 h, only 25−30% decomposition of **3** was observed with the formation of a small amount of vinyl sulfone−DTC adduct **22** which was confirmed by coelution with a pure sample of **22** (data not shown).

GST-Catalyzed Decomposition of 3: Condition C in Scheme 6/Experiment c in Figure 6 (with Both DTC and GST P1-1 Isozyme). Standards of 0.003 M solution of **3** and 0.0015 M solution of an internal standard peptide DQQN-AFYEILHOPN-NH_2 were prepared. Also prepared were buffer 1, 200 mM sodium phosphate at pH 7.2, and buffer 2, 200 mM sodium phosphate and 0.009 M diethyldithiocarbamic acid at pH 7.2. The reaction was initiated by mixing 0.2 mL of 0.003 M solution of **3**, 0.04 mL of 0.0015 M solution of internal standard, and 0.05 mL of 9.6×10^{-6} M solution of GST P1-1 in 1.71 mL of buffer 2, and the reaction progress was monitored by HPLC at 1 h intervals for 6 h. Progressive decomposition of **3** (t_R = ∼20 min) and formation of vinyl sulfone−DTC adduct **22** at t_R ∼17 min was identified by coelution with a pure sample of **22**. Also shown in the Figure 6 is the relative formation of DTC adduct **22** in the experiment with GST P1-1 isozyme.

GST-Catalyzed Decomposition of 3: Experiment d (Figure 6) (without DTC and with GST P1-1 Isozyme).[11] The reaction was initiated by mixing 0.2 mL of 0.003 M solution of **3**, 0.04 mL of 0.0015 M solution of internal standard, and 0.05 mL of 9.6×10^{-6} M solution of GST P1-1 in 1.71 mL of buffer 1 (see the above experiment), and the reaction progress was monitored by HPLC at 1 h intervals for 6 h. The rate of decomposition of **3** was almost close to that observed in the above experiment.

Control Experiments: Experiment a in Figure 6 (with DTC and without GST P1-1). The same amounts of reactants and conditions as described in experiment c, but without GST P1-1 isozyme, were used. The reaction progress was monitored by HPLC at 1 h intervals for 6 h. Also shown in the Figure 6 is the relative formation of DTC adduct **22** in the experiment without GST P1-1 isozyme.

Control Experiments: Experiment b in Figure 6 (without DTC and without GST P1-1). The same amounts of reactants and conditions as described for experiment d, but without GST P1-1 isozyme, were used. The reaction progress was monitored by HPLC at 1 h intervals for 6 h.

Molecular Modeling. A computer model of **3** at the active site of the GST P1-1 isozyme (Figure 1) was generated using the Insight II software (Biosym Technologies, Inc., San Diego, CA). The crystal structure of GST P1-1 complexed with hexylglutathione was used as the starting point.[13] The Builder facility of the Insight software was utilized to modify the structure of the substrate into that of **3**. Manual modification of the torsion angles were carried out on the modified hexyl portion, continuously checking in order to avoid steric clashes. Schematic two-dimensional representations of the site were then generated, respecting all the information contained in the model.

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