Glutathione-S-transferase Activates Novel Alkylating Agents

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Alkylating agents which are activated by glutathione-S-transferases (GSTs) have been designed and synthesized. The model compound γ-glutamyl-α-amino-β-[(2-ethyl *N,N,N′,N′*-tetraethylphosphorodiamidate)sulfonyl]propionylglycine (**1**) and the nitrogen mustards γ-glutamyl-α-amino-β-[[2-ethyl *N,N,N′,N′*-tetrakis (2-chloroethyl)phosphorodiamidate]sulfonyl]propionylglycine (**2**) and γ-glutamyl-α-amino-β-[[2-ethyl-*N,N,N′,N′*-tetrakis(2-chloroethyl)phosphorodiamidate]sulfonyl]-propionyl-(*R*)-(−)-phenylglycine (**3**) were prepared via multistep chemical synthesis. The compounds were tested with recombinant human A1-1, M1a-1a and P1-1 GSTs. HPLC studies showed that the compounds were differentially and catalytically cleaved by biologically relevant concentrations of the GSTs. Mass spectral studies of the cleavage mixture of **2** showed that M1a-1a GST liberated the cytotoxic phosphate moiety needed for efficacy as an alkylating agent. Cell culture studies with MCF-7 breast cancer cells showed that **1** was not toxic at 200 μM, while **2** and **3** showed IC_{50}s of 40.6 and 37.5 μM, respectively, for the same cell line. MCF-7 cells transfected to overexpress P1-1 GST showed enhanced sensitivity with **2** and **3**, with IC_{50}s of 20.9 and 9.5 μM, respectively. This result correlates well with the rates of cleavage of **2** and **3** by P1-1 GST observed *in vitro* and demonstrates that higher levels of cellular P1-1 GST will give increased sensitivity to these drugs.

Introduction

Glutathione-S-transferase (GST) levels have frequently been shown to be elevated in many tumors relative to surrounding healthy tissue.[1] Further, the isozyme distribution in many cancers is usually different from that found in normal tissues.[2] GSTs constitute 1–4% of all soluble cytosolic proteins in the human body, and this magnitude of expression makes GSTs a relevant therapeutic target. Recent analytical work has shown that P1-1 is elevated in lung, colon, and stomach cancers,[3] compared to matched normal tissues, with the average levels increasing by about 100%. A P1-1 GST activated alkylating agent should therefore be effective against these tumors.

We have designed compounds from which a toxic phosphorodiamidate species is released by the action of GSTs. The design principle involves the use of proton-extracting groups at the GST active site to initiate a cleavage reaction, liberating the toxic species. A review by Mannervik *et al.* suggested the presence of a histidine residue close to the glutathione-binding site which removed the sulfhydryl proton from the cosubstrate glutathione (γ-glutamylcysteinylglycine, GSH) generating a nucleophilic sulfide anion which would be more reactive with electrophiles than GSH in the absence of enzyme.[4] Later crystallographic work with M1a-1a[5] and P1-1 GSTs[6] showed that the proton-abstracting enzyme moiety was actually a tyrosine hydroxyl. In the enzyme-catalyzed cleavage of **1**, **2**, and **3**, this proton-abstracting enzyme moiety is presumed to deprotonate the carbon α to the sulfone, which can then undergo a β-elimination to give the species shown in Figure 1.

In the case of **2** and **3**, the liberated phosphate moiety should be highly cytotoxic, by cross-linking cellular DNA in the same manner as several currently used bifunctional alkylating agents.[7] In the design of **2** and **3**, we chose the tetrakis(chloroethyl)phosporodiamidate moiety because

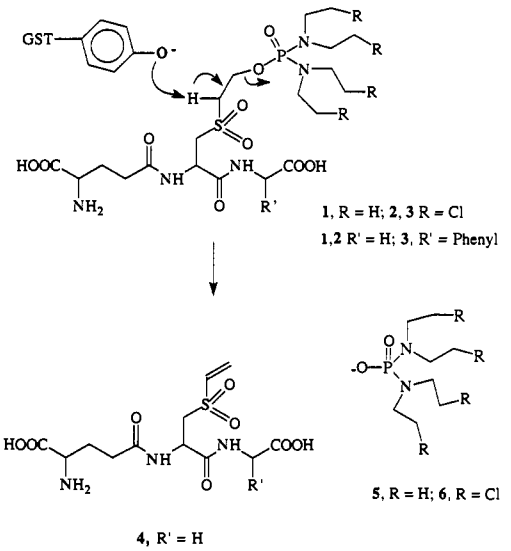

Figure 1. GST-mediated cleavage mechanism.

of ease of synthesis and isolation. A phosphorodiamidate structure with one bis(chloroethyl)amine and NH_2 as the other pendant group would impart chirality to the phosphorus, which might complicate enzyme reactivity as well as isolation and characterization of the compounds. Studies done by Borch and Valante[8] showed that the tetrakis phosphate moiety liberated from their compounds, which is the same species liberated by **2** and **3**, was more cytotoxic than the phosphorodiamidate with one bis-(chloroethyl)amine group.

Results and Discussion

A solution of **1** was treated with catalytic amounts of recombinant human A1-1, M1a-1a, and P1-1 GSTs.[9] Cleavage was monitored by measuring the disappearance of **1** by reverse-phase HPLC. From this analysis, it appears that M1a-1a cleaves **1** about 4 times faster than background and P1-1 cleaves it about twice as fast. A1-1 did not accelerate the decomposition of **1** above background,

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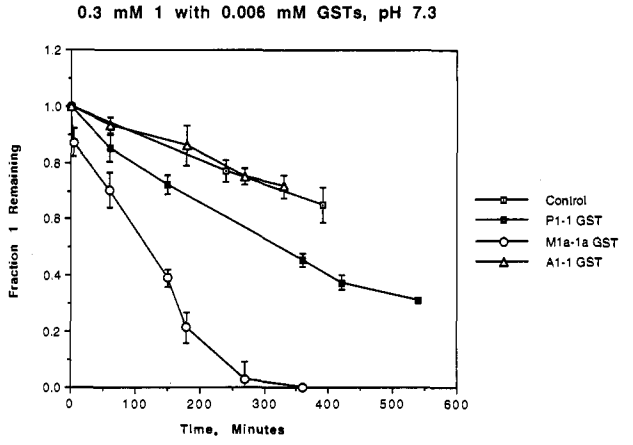

Figure 2. Differential cleavage of 1 by GSTs.

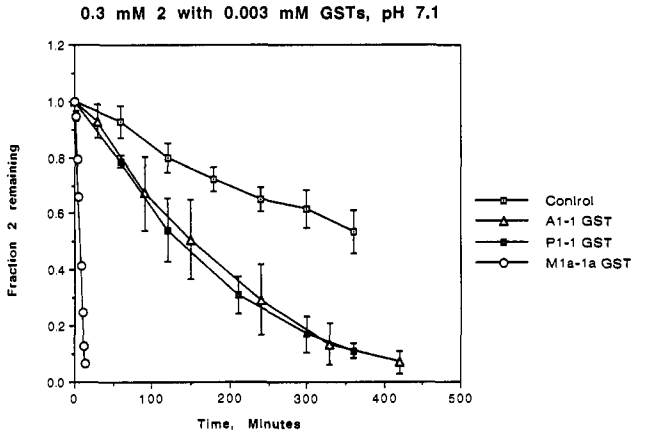

Figure 3. Differential cleavage of 2 by GSTs.

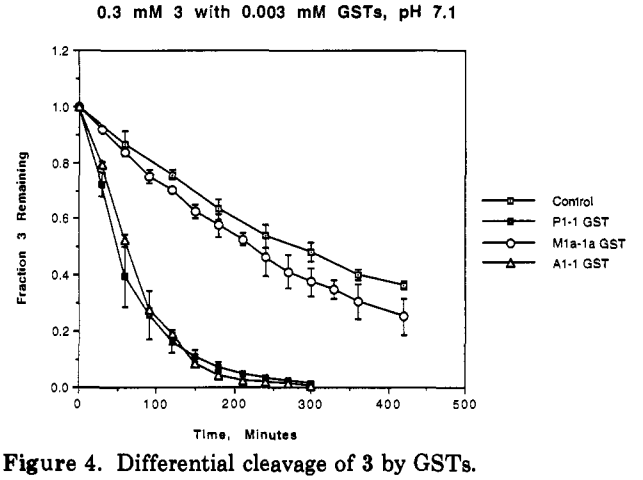

Figure 4. Differential cleavage of 3 by GSTs.

within experimental error. See Figure 2. For compound 2, the selectivity factor was somewhat different with the compound overall being cleaved faster at the same GST concentrations. The catalysis was slowed by using half as much GST as before. The cleavage of 2 was accelerated more than 20 times background by M1a-1a, while A1-1 and P1-1 decomposed the compound about 3 times faster. See Figure 3. For 3, the specificity was very different, with P1-1 and A1-1 accelerating the cleavage roughly 6 times faster than background, while negligable acceleration was seen with M1a-1a. See Figure 4. The background decomposition rates of 2 and 3 were similar, with 3 decomposing only slightly faster than 2.

While 2 was based on glutathione, 3 contained a substitution previously shown by us to make *S*-function-

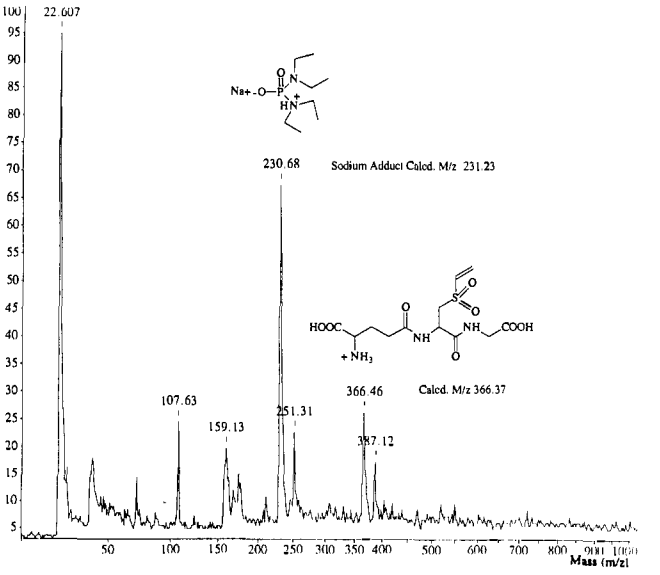

Figure 5. Mass spectrum of 1 cleaved by M1a-1a GST.

alized GSH analogs more inhibitory with P1-1.[10] In that work, several compounds were found to be moderately potent yet highly selective competative[11] inhibitors to various GST isozymes, with three examples of enhanced inhibition of P1-1 by C-terminal (*R*)-(−)-phenylglycine-containing analogs. In the present study, a substitution of C-terminal (*R*)-(−)-phenylglycine for glycine improved the specificity for P1-1 and diminished the preference for M1a-1a, in accordance with the earlier work.

To probe whether other proteins besides GSTs could catalyze cleavage, ovalbumin and human serum albumin (HSA) were used in place of GST. Experiments with ovalbumin and 1 showed no additional decomposition above that due to buffer alone. Ovalbumin was selected because of similar molecular weight to GSTs. HSA was also tested with 3, in place of GSTs, and no additional cleavage was observed. HSA was used because it will be present in human patients which may potentially receive 3 or a structurally similar drug.

To study the cleavage reaction, 1 and 2 were decomposed chemically and with GSTs, and the products were analyzed by mass spectrometry. For 1, peaks of mass attributable to vinyl sulfone 4 and tetraethylphosphorodiamidate (5) were seen, and these were generated about 4 times faster in the presence of a catalytic amount of M1a-1a than without enzyme. See Figure 5. Mass spectral studies of the decomposition of 2 with M1a-1a GST gave similar results, with clear signals present attributable to aziridinium and diaziridinium derivatives of 6, as well as the vinyl sulfone 4. See Figure 6. Interestingly, when 2 was decomposed by heating in solution, a different mass spectrum of products was obtained, and no 4 or derivatives of 6 were seen. This supports the notion that the desired cleavage of the compounds must be initiated by proton abstraction α to the sulfone. Several hundred milligrams of 1 was treated with sodium bicarbonate, and one of the byproducts was isolated by preparative HPLC. An NMR spectrum of the material corresponded to 5, isolated as its diethylammonium salt. Large-scale isolation of the decomposition products of 2 was not undertaken, due to the anticipated highly toxic nature of the byproducts. Support for the proposed catalytic role of the GSTs in cleaving 2 was seen when the concentration of enzyme was reduced; half as much enzyme resulted in half-lives of about twice

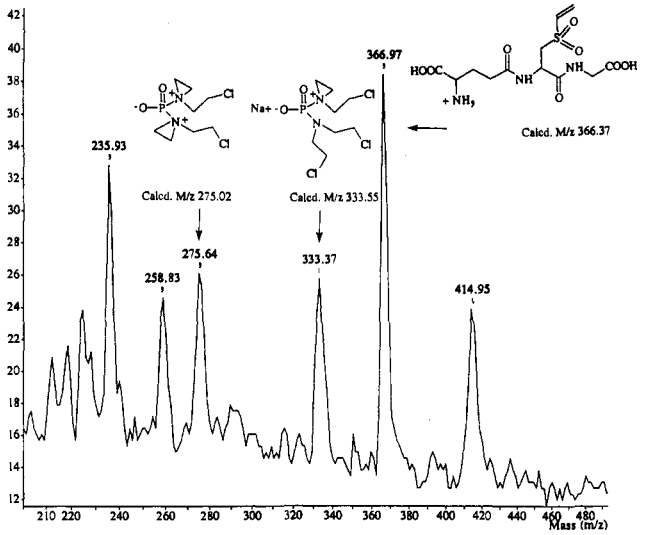

Figure 6. Mass spectrum of **2** cleaved by M1a-1a GST.

Table 1. Toxicity Data for Human Cancer Cell Lines with **2** and **3**

cell line	treatment time, min	IC_{50}, μM **2**	IC_{50}, μM **3**
HT-29	60	84.7[a]	170[a]
HT-29	120	68.9[a]	80.3[a]
MCF-7 *neo*	120	40.2 ± 11.2[c]	37.5 ± 2.1[b]
MCF-7 *pi*	120	20.9 ± 6.9[c]	9.5 ± 4.5[b]

[a] Single experiment. [b] Mean ± SD of two experiments. [c] Mean ± SD of at least five experiments.

as long for **2** when A1-1 and P1-1 GSTs were used. The cleavage of **2** by M1a-1a was too rapid to accurately measure at the higher GST concentration. Treatment of a solution of **2** and M1a-1a GST with *S*-hexylglutathione, known to be an inhibitor for M1a-1a GST,[11] showed a decrease in the rate of cleavage (data not shown), further supporting the catalytic involvement of GSTs in the cleavage reaction.

Three human cancer cell lines were used for toxicity studies with **1**, **2**, and **3**. HT-29 human colon carcinoma cells contain predominantly P1-1[12] GST. Analysis of MCF-7 cell lines transfected with a P1-1-containing vector (*pi*)[13,14] showed a 12.6-fold increase in the amount of P1-1 compared to control (*neo*) vector containing MCF-7 cells. HT-29 and MCF-7 cells were treated with varying concentrations of **2** and **3** for the times indicated in Table 1. On the basis of the IC_{50} values obtained, three conclusions can be drawn. The first is that longer exposure times with HT-29 cells give higher toxicity, which is consistant with a time course for activation of **2** and **3**. The second is that the MCF-7 *pi*-transfected cells having overexpressed P1-1 GSTs are more sensitive to **2** and **3** than MCF-7 *neo*. Finally, while the MCF-7 *neo* cells express negligable amounts of P1-1[14] and were equally affected by both **2** and **3**, the increase in sensitivity shown by the MCF-7 *pi* to **3** as compared to **2** correlates well with the increase in cleavage rates seen with P1-1 GST *in vitro*. This strongly supports the premise that increased P1-1 GST levels will give increased toxicicity for these drugs. All three cell lines were treated with **1** for 2 h, and very little toxicity (>90% survival) was seen at 200 μM concentration of compound. This demonstrates that there is little toxicity of **1** or its byproducts **4** or **5** and that toxicity is due to the liberation of the chlorinated species **6**.

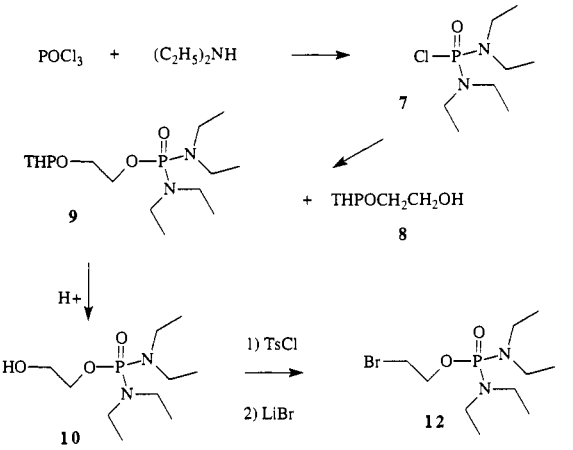

Figure 7. First Synthesis of **12**.

In summary, several studies were done to show that the cleavage has occurred in a manner consistant with the liberation of the cytotoxic agent **6**. The importance of **6** was verified by the cell studies with **1**, which cannot form **6** upon cleavage, and is therefore non-toxic. This also shows that vinyl sulfone **4** has much less toxicity in these systems than **6**. The work done with the two MCF-7 cell lines shows that **3**, which is more readily cleaved by P1-1, is twice as toxic to the P1-1 overexpressing cells as is **2**, and this correlates well with the different rates of cleavage seen in the *in vitro* studies with enzyme. Since the background decomposition is about the same, this can only be due to increased cellular GST levels, since the MCF-7 *neo*, with negligable GST levels, is effected similarly by both **2** and **3**.

Synthesis. **1** was made by alkylating GSH with 2-bromoethyl *N,N,N′,N′*-tetraethylphosphorodiamidate (**12**) and oxidizing the sulfide adduct **13** with hydrogen peroxide and peracetic acid. Compound **12** was initially made in a 5-step procedure: Adding 2 equiv of diethylamine to phosphorus oxychloride gave *N,N,N′,N′*-tetraethylphosphorodiamidic chloride (**7**), following the procedure of Borch *et al.*[8] Compound **7** was then alkylated with monotetrahydropyranyl ethyleneglycol (**8**)[15] to give 2-(tetrahydropyranyloxy)ethyl *N,N,N′,N′*-tetraethylphosphorodiamidate (**9**). This compound was treated with acid to remove the tetrahydropyran (THP)-protecting group to furnish 2-hydroxyethyl *N,N,N′,N′*-tetraethylphosphorodiamidate (**10**). This material was then converted into the bromide **12** in two steps which involved conversion of **10** into the tosylate **11** followed by reaction with lithium bromide. See Figure 7. It was later discovered that **12** could be made in a one-pot procedure by first adding 2-bromoethanol to phosphorus oxychloride and then adding 2 equiv of diethylamine. The procedure was also successfully used in the synthesis of 2-bromoethyl *N,N,N′,N′*-tetrakis(2-chloroethyl)phosphorodiamidate (**14**). Alkylation of glutathione with **14** followed by oxidation of the resulting sulfide **15** furnished **2**. For **3**, previously reported γ-glutamyl-(*S*-benzylcysteinyl)-(*R*)-(−)-phenylglycine (**16**)[10] was debenzylated to give γ-glutamylcysteinyl-(*R*)-(−)-phenylglycine (**17**). This compound was alkylated with **14**, and oxidation of the resulting sulfide **18** gave **3**. See Figure 8. The absolute configuration of the C-terminal amino acid of **3** was confirmed by aqueous acid digestion and analysis with Marphys reagent and HPLC.[10]

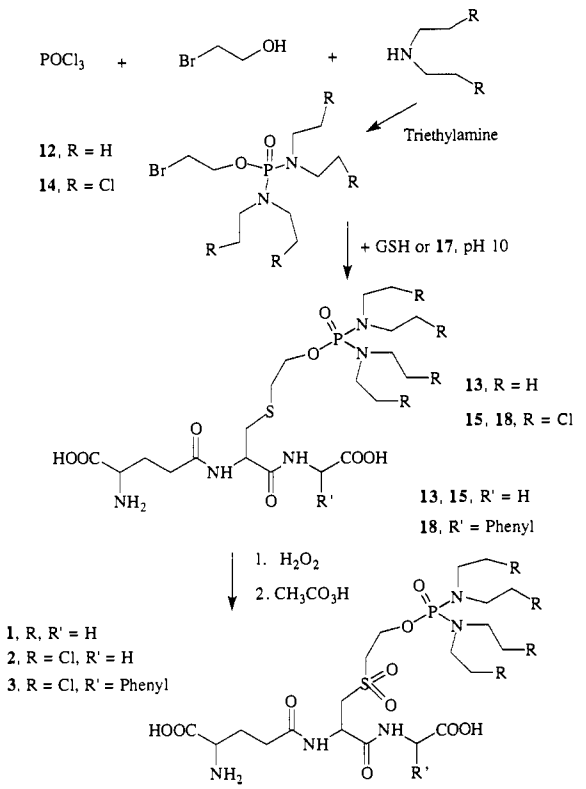

Figure 8. Synthesis of 1, 2, and 3.

Conclusion

Our results demonstrate that 2 and 3 meet basic criteria which may yield, on further development, effective chemotherapeutic agents for the treatment of cancers containing elevated levels of P1-1 GST. The increase in sensitivity shown by the MCF-7 *pi* over MCF-7 *neo* with 3 compared to 2 offers powerful pharmacological evidence that increased cellular P1-1 GST levels will give increased toxicity with these drugs. For cancers containing elevated levels of P1-1 GST, this should translate into a wider therapeutic index in the clinic, with either enhanced efficacy at subtoxic doses or comparable efficacy to existing alkylating agent therapies with less severe side effects. Other compounds containing the reactive nitrogen mustard moiety have been prepared, which show a different GST-promoted cleavage specficity than 2 or 3, some having strictly A1-1 or P1-1 selectivity. Cell culture and animal studies with the new compounds are in progress, and the results will be reported in detail later. We are also examining other biological systems for the delivery of active compounds into cells on the basis of this triggering mechanism.

Experimental Section

Materials. Methylene chloride, acetic acid, toluene, methanol, acetone, potassium hydrogen phosphate, sodium sulfate, ammonium acetate, and concentrated hydrochloric acid were reagent grade from J. T. Baker; ethyl acetate (EtOAc), petroleum ether (pet. ether), ethyl ether, 1-butanol, ethanol, and acetonitrile were Omnisolve grade from VWR. Tetrahydrofuran (THF), dimethyl sulfide, sodium hydroxide, sodium carbonate, sodium bicarbonate, dihydropyran, *p*-toluenesulfonic acid and chloride, lithium bromide, triethylamine (TEA), bis(2-chloroethyl)amine hydrochloride, diethylamine, hydrogen peroxide, peracetic acid, phosphorus oxychloride, and magnesium sulfate were from Aldrich. Glutathione was obtained from Sigma.

General Methods. Unless otherwise noted, mass spectra and elemental analysis were performed by the U.C. Berkeley Chemistry Department Analysis services. NMR was performed at Athena Neurosciences on a Varian Gemini 300 spectrometer. HPLC purification and analysis used a Ranin Rabbit HP solvent delivery system with a LDC Milton Roy detector, set at 225 nm. TLC plates were EM #5534 F 254 aluminum backed, and bulk silica for chromatography columns was J.T. Baker FC silica, 40 μm.

Testing of 1, 2, and 3 with GST Enzymes and HPLC Analysis. Solutions of 1, 2, and 3 were prepared in 0.2 M phosphate buffer, and a solution of GST was added. The final concentration was 0.3 mM of the test substrates and 0.006 mM GSTs for 1 and 0.003 mM GTSs for 2 and 3. The pH was 7.3 for 1 and 7.1 for 2 and 3; 0.006 mM ovalbumin (purified chicken egg, Sigma) or 0.003 mM HSA (Sigma) was used with the other conditions the same as with as the GST tests for 1 and 3, respectively. The samples were incubated at 37 °C, aliquots of the reaction mixture were removed at intervals, and the catalytic decomposition was quenched by the addition of acetic acid. The amount of cleavage was assayed by HPLC. The column was a J. T. Baker C_{18}, 250-× 4.6-mm, with 5-μm particles. The A buffer was 0.05 M NH_4OAc, pH 5.5, and 5% CH_3CN. The B buffer was 70% CH_3CN in A. The gradient was 20–50% B over 15 min to 100% B in 3 min and then back to 20% over 6 min; 10-μL was injected. The flow rate was 1 mL/min. Overnight HPLC runs utilizing an autosampler were used to generate the data. The rates of decomposition were calculated by measuring the rate of disappearance of the parent compound versus an internal standard, which was 0.06 mM of peptide DQQNAFYEIL-HOPN-NH_2 (O is ornithine). 1, 2, and 3 eluted at 10–12 min, and the internal standard eluted at 7–8 min. For the inhibition study, 0.06 mM *S*-hexylglutathione was used with 0.4 mM 2. All other conditions were the same as those reported above for 2. The inhibited rate was about 5 times slower than the rate without inhibitor.

Mass Spectral Analysis of 1 and 2 Decomposition. A solution of 10 mg of 1 in 1 mL of water was prepared. Dilute aqueous ammonia was added to raise the pH to 7.5, and the reaction mixture was analyzed at 0, 24, and 120 h with a Finnigan MALDI TOF mass spectrometer using 53 mM α-cyano-4-hydroxycinnamic acid (Aldrich) in methanol as the matrix. After a few hours, peaks assignable to 5 (MH^+ 208, MNa^+ 233) and 4 (MH^+ 366) were seen, and these got larger with time, while the peak assigned to 1 diminished. After 120 h, 4 and 5 were quite prominant and 1 had become much smaller. When 0.06 mM M1a GST was used to accelerate the decomposition of 5 mM 1 at pH 7.3, 4 and 5 appeared about 4 times as fast as without GST and 1 was completely gone after 72 h. For 2, 10 mg was dissolved in 1 mL of water, and the solution was made 0.01 M in Na_2CO_3. A mass spectrum after 90 min showed that 2 was almost gone and aziridinium species derived from 6 were clearly visible. 2 (0.3 mM) was treated with 0.003 mM M1a GST (final concentration) at pH 7.3, and a mass specrtrum after 16 h showed the two aziridinium derivatives of 6, as well as the vinyl sulfone 4. In all studies, 0.3 μL of the analyte was spotted upon already dried matrix on a steel sample slide and dried a few minutes before insertion into the spectrometer.

Large-Scale Chemical Decomposition of 1. To a solution of 1 , 0.7 g in 100 mL of water, was added saturated $NaHCO_3$ until the pH reached 9. The pH was maintained by adding more base as necessary. The solution was monitored by mass spectroscopy, and after 1 week, no more 1 was observed. The solution was brought to pH 4 with HOAc and purified by preparative HPLC. For this, a 1-in. diameter column packed with 5-μm C-18-functionalized silica (YMC Corp.) was first flushed with 300 mL of 90% CH_3CN and 10% H_2O which was 0.2% HOAc followed by 300 mL of 0.2% HOAc in H_2O. The solution containing decomposed 1 was loaded and eluted with 0.2% HOAc in H_2O at 4 mL/min for 30 min. Early fractions contained impure 4, and a late fraction contained 5 as its diethylammonium salt. ^1H NMR (300 mHz, $CDCl_3$): δ 3.0–2.7 (q, 8H), 3.7–3.55 (q, 4H), 1.25–1.15 (t, 6H), 1.05–0.95 (t, 12 H). MS *m/z* (Finnigan MALDI TOF): 209 (MH^+).

Cell Biology. HT-29 cells were grown in Eagles minimal essential medium with 2 mM L-glutamine and 10% fetal bovine serum. MCF-7 cells were grown in a 1:1 mixture of RPMI 1640 and F-10 Nutrient medium with 2 mM L-glutamine and 10% fetal bovine serum. Approximately 2 × 10^5 cells were exposed

to varying concentrations of the drug for the times shown in Table 1. Treatment of cells with 1 was for 2 h under the described conditions. Final drug concentrations were in 2-fold increments from 200 μm to less than 1 μM. Cells were then diluted to 7.5 × 10^3 cells/mL in serum containing medium, and four replicates of each sample were plated in 200-μL volumes in 96-well plates. After 5 or 6 days in culture, the number of surviving cells/well was quantified by a methylene blue uptake assay.[16] In order to determine the IC_{50} values, two independent experiments were done for 3 with both MCF-7 cell lines, while five experiments were done for 2 with MCF-7 *neo* and six experiments were performed for 2 in MCF-7 *pi*. For the HT-29 studies, only single experiments were done.

Chloro-*N,N,N',N'*-Tetraethylphosphorodiamidic Chloride, 7. Diethylamine, 33.72 mL (360 mmol), was added dropwise with magnetic stirring to a cooled (0–5 °C) solution of freshly distilled phosphorus oxychloride, 15.2 mL (160 mmol), in 1 L of CH_2Cl_2 to which triethylamine, 50 mL (360 mmol), had been previously added dropwise. The solution was allowed to warm to room temperature and magnetically stirred for 3 days. The reaction was quenched by cooling to 0 °C and adding 10% KH_2PO_4 in water, 200 mL. The mixture was warmed to room temperature. The lower organic layer was separated, and the aqueous layer was extracted with 100 mL of CH_2CL_2. The combined organic phases were washed with 500 mL of saturated brine and dried over Na_2SO_4. The solution was filtered and reduced to an oil under vacuum. Some solid material was removed from the oil by additional filtration. TLC analysis (developed with hexane:EtOAc 1:1 and visualized with iodine vapor) showed that two products were present, the major product, R_f 0.3, was isolated by column chromatography eluted isocratically with hexane:EtOAc 4:1. Column fractions containing the pure lower R_f compound were pooled and evaporated to give 12 g (33% yield) of a pale yellow oil. ^{1}H NMR (300 mHz, $CDCl_3$): δ 3.3–3.05 (q, J = 5.3 Hz, 4H), 1.2–1.05 (t, J = 5.3 Hz, 6H). MS m/z (rel. intensity): 227.1 (MH^+, 100), 191.2 (11), 154.1 (38), 136.1 (28). m/z calcd for $C_8H_{21}N_2OPCl$: 227.108005. Found: 227.107970.

Monotetrahydropyranyl Ethylene Glycol, 8.[15] Dihydropyran, 18.2 mL (200 mmol), was added dropwise to a cold (0 °C) mixture of ethyleneglycol, 55.9 mL (1 mol), in 500 mL of CH_2Cl_2 containing 3.8 g (20 mmol) of dissolved *p*-toluenesulfonic acid monohydrate over 2 h. The mixture was allowed to stir for an additional 2 h at this temperature and then allowed to warm to room temperature. Stirring continued for 2 days, after which the mixture was poured into a separatory funnel and allowed to separate. The lower organic layer was removed and saved, and the upper layer was reextracted with 100 mL of CH_2Cl_2. The combined CH_2Cl_2 layers were washed six times with 100-mL portions of saturated NaCl and dried over Na_2SO_4. The solution was filtered and reduced, and the residue was purified by chromatography. The column was eluted isocratically with 3:2 EtOAc:hexane which contained 1% triethylamine. Fractions which contained pure product, R_f 0.5, were pooled and evaporated to give 9.7 g (33% yield) of a colorless oil. ^{1}H NMR (300 mHz, $CDCl_3$): δ 5.6–5.5 (m, 1H), 4.0–3.9 (m, 1H), 3.85–3.65 (m, 4H), 3.6–3.5 (m, 1H), 1.9–1.7 (m, 2H), 1.65–1.5 (m, 4H). m/z calcd for $C_7H_{15}O_3$: 147.102120. Found: 147.102050.

2-(Tetrahydropyranyloxy)ethyl *N,N,N',N'*-Tetraethylphosphorodiamidate, 9. A solution of 8, 4.4 g (30 mmol), in 25 mL of THF which had been freshly collected from a sodium/benzophenone still was added dropwise to a stirred solution at 0 °C of sodium hydride, 0.72 g (30 mmol), in 50 mL of the same dry THF. To this was added a solution of 7, 5.67 g (25 mmol), in 25 mL of THF over 10 min, and this was magnetically stirred for 18 h at room temperature. The reaction was quenched with dropwise addition of 100 mL of water and the mixture extracted twice with 250-mL portions of EtOAc. The combined organic extracts were washed twice with 100-mL portions of brine and dried over Na_2SO_4. The solution was filtered and evaporated to give 8.3 g (98% yield) of an oil which was used without further purification. ^{1}H NMR (300 mHz, $CDCl_3$): δ 4.65 (m, 1H), 4.1–4.0 (m, 2H), 3.9–3.8 (m, 2H), 3.7–3.6 (q, J = 5.6 Hz, 1H), 3.55–3.45 (m, 1H), 3.1–3.0 (dq, J = 4.35, 16.3 Hz, 8H), 1.85–1.5 (m, 6H), 1.15–1.05 (t, J =16.3 Hz, 12 H). MS m/z (rel. intensity): 337.2 (MH^+, 28), 253.1 (100), 235.1 (12), 180.1 (22). m/z calcd for $C_{15}H_{34}N_2O_4P$: 337.225621. Found: 337.225830.

2-Hydroxyethyl *N,N,N',N'*-Tetraethylphosphorodiamidate, 10. To a solution of 9, 8.3 g (24.7 mmol), in 200 mL of methanol was added *p*-toluenesulfonic acid, 90 mg. The mixture was heated to reflux for 8 h and cooled, and 3 mL of TEA was added. The material was reduced and purified by chromatography, using 86 g of silica eluted with EtOAc. Fractions containing the product (R_f 0.5, by TLC, EtOAc:MeOH 9:1) were pooled and evaporated to give 4.5 g (72% yield) of a colorless oil. ^{1}H NMR (300 mHz, $CDCl_3$): δ 4.1–4.0 (dt, J = 12.7, 2.3 Hz, 2H), 3.8 (t, J = 2.3 Hz, 2H), 3.7–3.6 (br s, 1H (disappears with D_2O)), 3.2–3.0 (symmetrical 11-peak resonance, J = 3.4 Hz, 8H), 1.2 –1.1 (t, J = 7.0, 12 H). MS m/z (rel. intensity): 253.1 (MH^+, 100), 180.1 (11). m/z calcd for $C_{10}H_{26}N_2O_3P$: 253.168106. Found: 253.168400.

2-[(*p*-Tolylsulfonyl)oxy]ethyl *N,N,N',N'*-Tetraethylphosphorodiamidate, 11. A solution of *p*-toluene sulfonyl chloride, 4.5 g (23 mmol), in 10 mL of THF was added dropwise to a stirred solution of 10, 4.5 g (17.9 mmol), and NaOH, 1.6 g (40 mmol), in 12 mL of THF and 8 mL of water at 0 °C over a period of 2 h. The mixture was allowed to warm to room temperature and stirred overnight. The mixture was poured into 35 mL of water and extracted with two 50-mL portions of toluene, and the combined organic phases were dried over Na_2SO_4 and concentrated to 7.32 g (99% yield) of a viscous oil. TLC (9:1 EtOAc:MeOH) showed one spot: Rf 0.7, UV active. ^{1}H NMR (300 mHz, $CDCl_3$): δ 7.8 (d, J = 8.3 Hz, 2H), 7.35 (d, J = 8.3 Hz, 2H), 4.2 (m, 2H), 4.1–4.0 (m, 2H), 3.1–2.9 (m, 8H), 2.45 (s, 3H), 1.15–1.0 (t, J = 7.1 Hz, 12 H). MS m/z (rel. intensity): 407.1 (MH^+, 100), 334.0 (24), 253.1 (49), 199.0 (20). m/z calcd for $C_{17}H_{32}N_2O_5PS$: 407.176958. Found: 407.177550.

2-Bromoethyl *N,N,N',N'*-Tetraethylphosphorodiamidate, 12. To a solution of 11, 7.2 g (17.8 mmol), in 125 mL of acetone was added lithium bromide, 3.04 g (35 mmol). The mixture was refluxed for 7 h and allowed to cool to room temperature. The mixture was filtered, and the residual solids were washed with 25 mL of acetone. The filtrate and washes were stripped to a gummy solid. This material was triturated with 100 mL CH_2Cl_2 and filtered, and the liquid was reduced to an oil. ^{1}H NMR (400 mHz, $CDCl_3$): δ 4.25 (m, 2H), 3.6 (t, J = 5.5 Hz, 2H), 3.1–3.0 (m, 8H), 1.05 (t, J = 7 Hz, 12H). MS m/z (rel. intensity): 323.1 (96), 321.1 (MH^+, 100), 241.2 (26), 191.2 (14). The compound was also made as follows: Triethylamine, 14 mL (100 mM), was slowly added over 5 min to a magnetically stirred solution of freshly distilled phosphorus oxychloride, 9.3 mL (100 mM), in 450 mL of CH_2Cl_2 chilled to 0 °C under argon. Next, a solution of 2-bromoethanol, 7.09 mL (100 mM), in 50 mL of CH_2Cl_2 was added slowly over 90 min. The mixture was stirred overnight and allowed to warm to room temperature. The solution was re-chilled to 0 °C, and more triethylamine, 28 mL (200 mM), was added over 5 min followed by addition of diethylamine, 22.7 mL (220 mM), over 30 min. The mixture was again stirred overnight and allowed to warm to room temperature. The solution was warmed to 35 °C and allowed to stand for 2 days, resulting in a muddy brown suspension. Analysis of a small portion of the mixture by mass spectroscopy confirmed the presence of the desired product, and the suspension was filtered free of the solids and concentrated to a brown oil. Silica, 300 g, was used to prepare a 30-× 5.5-cm column in EtOAc, and the crude product was dissolved in 50 mL of EtOAc and eluted through the column under a slight N_2 pressure. Fractions containing pure material, R_f 0.5, were pooled and concentrated to give 4.7 g (15% yield) of 12 which was identical, by mass and NMR spectra, to that obtained above. Anal. ($C_{10}H_{24}N_2O_2PBr$) C, H, N.

γ-Glutamyl-α-amino-β-[(2-ethyl *N,N,N',N'*-tetraethylphosphorodiamidate)thio]propionylglycine, 13. Glutathione, 250 mg (0.8 mmol), was dissolved in 5 mL of water and brought to pH 12 with concentrated NaOH solution. This was added, with magnetic stirring, to a solution of 12, 300 mg (0.95 mmol), in 5 mL of ethanol. The mixture was stirred for 4 h and neutralized with 1 N HCl. A product TLC spot, R_f 0.66, (1:1:1:1 butanol:HOAc:EtOAc:water, visualized with ninhydrin spray) was present. The solution was concentrated to a solid and purified by HPLC: A 1-in. diameter column packed with 5-μm C-18- functionalized silica (YMC Corp.) was flushed with 90% CH_3CN in 0.2% HOAc–

water (buffer B) followed by equilibration with 300 mL of 0.2% HOAc–water (buffer A) at 12 mL/min flow rate. The crude product was dissolved in 20 mL of water and loaded onto the column, followed by 100 mL of buffer A. A gradient to 50% buffer B was run over 90 min, and fractions which appeared pure by TLC were pooled and lyophilized. The yield was 240 mg (55%) of a white powder, over 90% pure by analytical HPLC, mp 100–105 °C. ^{1}H NMR (400 mHz, $CDCl_3$): δ 4.3 (m, 1 H), 3.8 (m, 2H), 3.7 (s, 2H), 3.5 (m, 1H), 2.9–2.5 (m, 12 H), 2.2 (m, 2H), 1.9 (m, 2H), 0.8 (t, J = 7 Hz, 12 H). MS m/z (rel. intensity): 564.2 (MNa^+, 18), 542.2 (MH^+, 100), 306.1 (32), 209.2 (26), 177.1 (43). Anal. ($C_{20}H_{40}N_5O_8PS$·HOAc·1/2 HCl) C, N; H: calcd, 7.23; found, 6.58.

γ-Glutamyl-α-amino-β-[(2-ethyl *N,N,N',N'*-tetraethylphosphorodiamidate)sulfonyl]propionylglycine, 1. To a solution of **13**, 528 mg (0.97 mmol), in 10 mL of HOAc was added 30% H_2O_2, 400 μL (2 mmol). The mixture was magnetically stirred at room temperature for 2.5 h, and 32% peracetic acid, 210 μL, was added. The mixture was stirred for 3 days, and the reaction was quenched with 200 μL of dimethyl sulfide. The material was lyophilized to a white solid and purified by HPLC: The same column as for the purification of 13 was flushed with 90% CH_3CN in 0.2% HOAc–water (buffer B) followed by equilibration with 300 mL of 0.2% HOAc (buffer A) at 12 mL/min flow rate. The crude product was dissolved in 10 mL of HOAc and diluted with 150 mL of water before being loaded onto the column and followed by 100 mL of buffer A. A gradient to 100% buffer B was run over 60 min, and fractions which appeared pure by analytical HPLC were pooled and lyophilized. The yield was 350 mg (63%) of a white solid, mp 120–125 °C. ^{1}H NMR (400 mHz, $CDCl_3$): δ 4.9 (dd, J = 3.5, 9.1 Hz, 1H), 4.1 (dd, J = 5.3, 10 Hz, 2H), 3.7 (s, 2H), 3.69–3.65 (dd, J = 3.6, 14.6 Hz, 1H), 3.6 (t, J = 6.4 Hz, 1H), 3.5–3.45 (m, 3H), 2.8 (symmetrical 6-peak resonance, with J = 7 Hz, 8H), 2.3 (m, 2H), 1.9 (m, 2H), 0.9 (t, J = 7 Hz, 12 H). ^{13}C NMR (100 mHz, $CDCl_3$): δ 177.05, 176.01, 173.00, 60.79, 56.60, 56.26, 50.05, 44.25, 42.04, 41.99, 33.82, 28.30, 16.07. MS m/z (Finnigan MALDI TOF): 575 (MH^+), 596 (MNa+). Anal. ($C_{20}H_{40}O_{10}N_5PS$·$2H_2O$) C, H, N.

2-Bromoethyl *N,N,N',N'*-Tetrakis(2-chloroethyl)phosphorodiamidate, 14. Triethylamine, 14 mL (100 mM), was slowly added over 5 min to freshly distilled phosphorus oxychloride, 9.3 mL (100 mM), in 450 mL of CH_2Cl_2 chilled to 0 °C under argon in a 1000-mL 3-necked flask fitted with a mechanical stirrer. Next, a solution of 2-bromoethanol, 7.09 mL (100 mM), in 50 mL of CH_2Cl_2 was added slowly over 4 h. The mixture was stirred overnight and allowed to warm to room temperature. The solution was rechilled to 0 °C, and bis(2-chloroethyl)amine hydrochloride, 35.7 g (200 mM), was added as a solid. To this stirred suspension was added a solution of triethylamine, 61.33 mL (440 mM), in 100 mL of CH_2Cl_2 dropwise over 3 h. The mixture was allowed to warm to room temperature and stirred for 3 days. The suspension was suction filtered and the filtrate reduced *in vacuo* to a brown, viscous oil. This was mixed with 100 mL of EtOAc and filtered again to remove triethylamine hydrochloride. The solution was concentrated to about 50 mL in volume and subjected to chromatographic purification. A bed of silica, 38-× 5.5-cm was used, eluted with EtOAc:pet. ether 1:1; 16 g (35% yield) of a clear yellow oil was isolated, R_f 0.5. ^{1}H NMR (300 mHz, $CDCl_3$): δ 4.4–4.3 (m, 2H), 3.75–3.6 (t, J = 2.2 Hz, 8H), 3.6–3.5 (t, J = 5 Hz, 2H), 3.5–3.4 (m, 8H). MS m/z (Finnigan MALDI TOF): 454.2 (MH^+). Anal. ($C_{10}H_{20}O_2N_2PCl_4Br$) C, H, N.

γ-Glutamyl-α-amino-β-[[2-ethyl *N,N,N',N'*-tetrakis(2-chloroethyl)phosphorodiamidate]thio]propionylglycine, 15. Glutathione, 4.5 g (15 mM), was dissolved in 75 mL of deionized water, and the pH was adjusted to between 9 and 10 by adding 1 N NaOH. To this stirred solution was added a solution of 14, 6.8 g (15 mM), in 100 mL of ethanol. The turbid mixture became clear after 2 min and was stirred overnight. TLC monitoring showed partial reaction, and the mixture was stirred another 3 days. The solution was then neutralized to pH 5–6 with 1 N HCl, and the ethanol was removed *in vacuo*. The solution was divided into three roughly equal portions and purified by preparative HPLC as for 13 to give 2.5 g (36% yield) of a white powder, mp 80 °C dec. ^{1}H NMR (300 mHz, DMSO-d_6): δ 8.8 (t, J = 1.5 Hz, 1H), 8.43 (d, J = 2.5 Hz, 1H), 4.5–4.4 (br s, 1H), 4.1–4.0 (dd, J = 2.2, 1.8 Hz, 2H), 3.8–3.6 (m, 11 H), 3.5–3.2 (br m, 12 H), 3.0–2.9 (dd, J = 1.3, 3.2 Hz, 1H), 2.9–2.8 (t, J = 1.9 Hz, 2H), 2.75–2.65 (dd, J = 3.2, 1.3 Hz, 1H), 2.4–2.3 (m, 2H), 2.0–1.85 (m, 2H). MS m/z (Finnigan MALDI TOF): 680.2 (MH^+). Anal. ($C_{20}H_{36}N_5O_8PSCl_4$·$3H_2O$) C, H, N.

γ-Glutamyl-α-amino-β-[[2-ethyl *N,N,N',N'*-tetrakis(2-chloroethyl)phosphorodiamidate]sulfonyl]propionylglycine, 2. To a solution of **15**, 1.5 g (2.2 mM), in 22 mL of HOAc was added 30% H_2O_2, 0.78 mL (4 mM). After 2 h, a mass spectrum of the mixture revealed complete conversion to the sulfoxide; 32% peracetic acid, 0.525 mL (2.5 mM), was added, and the mixture was magnetically stirred overnight. A mass spectrum of the mixture revealed almost total conversion of the material to the desired compound, and the mixture was lyophilized and purified by HPLC with the same method as for 1 to give 1.5 g (96% yield) of **2** as a white powder, mp 95 °C dec. ^{1}H NMR (300 mHz, D_2O): δ 5.2–5.1 (m, 1H), 4.6–4.6 (d, J = 1.4 Hz, 2H), 4.0 (s, 2H), 3.95–3.9 (m, 1H), 3.85–3.65 (m, 12H), 3.55–3.45 (m, 8H), 2.2–2.5 (m, 2H), 2.3–2.15 (m, 2H). MS m/z (Finnigan MALDI TOF) 712.7 (MH^+). Anal. ($C_{20}H_{36}N_5O_{10}PSCl_4$·$2H_2O$) C, H, N.

γ-Glutamylcysteinyl-(*R*)-(−)-phenylglycine, 17. About 50 mL of dry ammonia was condensed in a flask which had been cooled to −78 °C in a dry ice bath under argon. About 0.5 g of sodium metal was added, and the solution turned dark blue and was magnetically stirred for 10 min. **16**,[10] 240 mg (0.506 mM), was added, and the solution was stirred for 1 h. NH_4Cl solid was added until the solution became colorless, and the ammonia was allowed to evaporate. The flask and contents were subjected to high vacuum overnight. A small amount of the material was purified by preparative HPLC as for 1 (except that 2% TFA was used in the buffers instead of HOAc) to give a white powder, mp 68–73 °C. ^{1}H NMR (300 mHz, DMSO-d_6): δ 8.8 (d, 1H), 8.3 (d, 1H), 7.4–7.3 (m, 6H), 5.35 (d, 1H), 4.45 (q, 1H), 3.8 (t, 1H), 2.8–2.7 (m, 1H), 2.7–2.6 (m, 1H), 2.4–2.3 (m, 2H), 2.3–2.2 (m, 1H), 2.1–1.9 (m, 2H). MS m/z (rel. intensity): 406.3 (MNa^+, 100). Anal. ($C_{16}H_{21}N_3O_6S$·TFA) C, H, N.

γ-Glutamyl-α-amino-β-[[2-ethyl *N,N,N',N'*-tetrakis(2-chloroethyl)phosphorodiamidate]thio]propionyl-(*R*)-(−)-phenylglycine, 18. All of the crude material obtained above was dissolved in 7.5 mL of water under argon, and the solution was sparged with argon for 5 min. The pH of the solution was adjusted to 9–10 with 1 N NaOH, and a solution of 1.36 g (3 mM) of **14** dissolved in 7.5 mL of EtOH and 7.5 mL of CH_3CN was added. The mixture was stirred overnight and acidified to pH 5 with HOAc. The solution was evaporated and purified by HPLC as for **17**. The yield was 139 mg (36% yield from **16**), mp 57–80 °C. MS m/z: 780 (MNa^+), 799 (MK^+). Anal. ($C_{26}H_{40}N_5O_8PSCl_4$·TFA) C, H, N.

γ-Glutamyl-α-amino-β-[[2-ethyl *N,N,N',N'*-tetrakis(2-chloroethyl)phosphorodiamidate]sulfonyl]propionyl-(*R*)-(−)-phenylglycine, 3. To a solution of **18**, 125 mg (0.16 mM), dissolved in 1.7 mL of acetic acid was added 67 μL of 30% H_2O_2. The solution was wrapped in foil to exclude light, and magnetically stirred for 2 h. A mass spectrum of the crude showed that transformation to the sulfoxide had occurred; 42 μL of 32% peracetic acid was added, and the solution was stirred overnight. The solution was reduced to a solid and purified by HPLC as for 1 to give 87 mg (67% yield) of a white material, mp 82–90 °C. ^{1}H NMR (300 mHz, DMSO-d_6): δ 8.8–8.75 (m, 1H), 8.7 (m, 1H), 7.4–7.2 (m, 6H), 5.3–5.2 (d, 1H), 4.9 (m, 2H), 4.3–4.2 (m, 3H), 3.8–3.2 (m, 22H), 2.4–2.3 (m, 2H), 2.0–1.9 (m, 2H). MS m/z: 790 (MH^+), 812 (MNa^+), 830 (MK^+). Anal. ($C_{26}H_{40}N_5O_{10}PSCl_4$) C, H, N.

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