New Mustard Prodrugs for Antibody-Directed Enzyme Prodrug Therapy: Alternatives to the Amide Link

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Antibody-directed enzyme prodrug therapy (ADEPT) is a two-step approach for the treatment of cancer which seeks to generate a potent cytotoxic agent selectively at a tumor site. In this work described the cytotoxic agent is generated by the action of an enzyme CPG2 on a relatively nontoxic prodrug. The prodrug **1** currently on clinical trial is a benzamide and is cleaved by CPG2 to a benzoic acid mustard drug **1a**. We have synthesized a series of new prodrugs **3**−**8** where the benzamide link has been replaced by, for example, carbamate or ureido. Some of these alternative links have been shown to be good substrates for CPG2 and therefore new candidates for ADEPT. The active drugs **3a** and **4a** derived from the best of these prodrugs are potent cytotoxic agents (1−2 μM) some 100 times more than **1a**. The prodrugs **3** and **4** are some 100−200-fold less cytotoxic, in a proliferating cell assay, than their corresponding active drugs **3a** and **4a**.

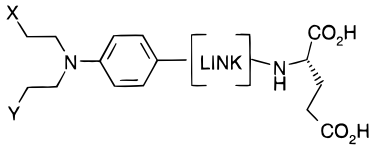
Introduction

Antibody-directed enzyme prodrug therapy[1,2] (ADEPT) is a two-step approach for the treatment of cancer which seeks to generate a potent cytotoxic agent selectively at the tumor site or its metastases. In the first step, a tumor selective antibody chemically linked to an enzyme (conjugate) is administered and allowed to localize at the tumor site. After a suitable time, to allow clearance of the antibody−enzyme conjugate from other tissues, this is followed, in the second step, by the administration of a relatively nontoxic prodrug. Since the conjugate will have cleared from the blood and other tissues the prodrug will be enzymatically converted to active drug only at the tumor site and the usual nonspecific toxicity associated with cytotoxic agents will be minimized. Potentially therefore one of the major advantages of ADEPT is the ability to deliver, to the tumor, a highly potent cytotoxic drug, e.g. an alkylating agent, in concentrations that cannot be achieved by conventional chemotherapy. Clinical studies to assess the potential of ADEPT in patients with advanced colorectal cancer are ongoing.[3−5] To maximize the benefit of ADEPT therapy it is beneficial to (a) have high tumor to normal tissue ratio of the conjugate, (b) ensure the prodrug to drug conversion is maximal at tumor site, and (c) generate a potent drug that will act locally and not diffuse away to normal tissue. Nitrogen mustard drugs are good candidates for ADEPT since their cytotoxicity is dose related and have the advantage that the active drug is non cell cycle specific so that total cell kill in a solid tumor will be maximized.

The ADEPT system currently under clinical investigation[4,8,15] is based on a conjugate of the bacterial carboxypeptididase enzyme (CPG2) and the F(ab′)$_2$ fragment of murine antibody A5B7. With this system, the amide bond of the benzoylglutamate of prodrug **1** is cleaved to generate the benzoic acid mustard drug **1a** and glutamic acid (Figure 1). The benzoic acid mustard drug of type **1a** is, however, a weak cytotoxic drug (IC_{50} = 80 μM). This necessitates high doses of prodrug in therapy studies, and furthermore the benzoic acid based drug **1a** has a long chemical half-life ($t_{1/2}$) which increases the risk of drug generated at the tumor escaping to the periphery and enhancing toxicity.[6] These considerations lead us to search for alternative systems which would be cleaved by CPG2 to release more potent cytotoxic drugs with shorter chemical half-lives. We initially chose our targets to be release of the phenol and aniline mustards **3a** and **4a.** Other groups in the ADEPT area have also chosen to release these compounds as the active drug moiety.[7]

This paper describes our investigations into modifications to the amide moiety of **1** and **2** and the design of novel prodrugs (**3**−8) (Table 1). Some of these prodrugs are transformed by the action of CPG2 into potent cytotoxic agents which are thus attractive for a CPG2-based ADEPT system (Figure 2).

Table 1. Structure, Biological Data, Enzyme Kinetics, and Chemical Reactivity of Prodrugs



compd	X	Y	link	K_m (μM)	k_{cat} (s^{-1})	$t_{1/2}$ (min)	k_{cat}/K_m	IC_{50} (μM)
1	Cl	$MeSO_3$	CO	3.4	583	46	171.5	>500
2	Cl	Cl	CO	3.4	700	660	206	>500
3	Cl	Cl	OCO	1.0	49	31	49	200
4	Cl	Cl	NHCO	3.0	7.5	16.9	2.5	200
5	Cl	Cl	CH_2CO	16	15	33	1.0	>500
6	Cl	Cl	SCO	50	4	32.2	0.08	100
7	Cl	Cl	OCS	NS[a]				
8	Cl	Cl	CH_2CS	NS[a]				

[a] NS, not a substrate.

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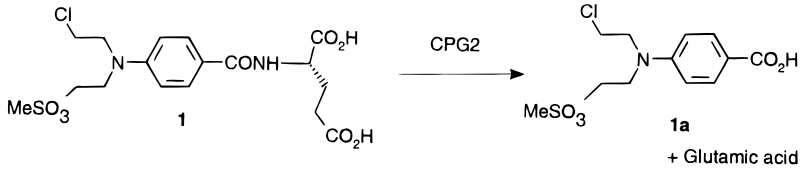


Figure 1.

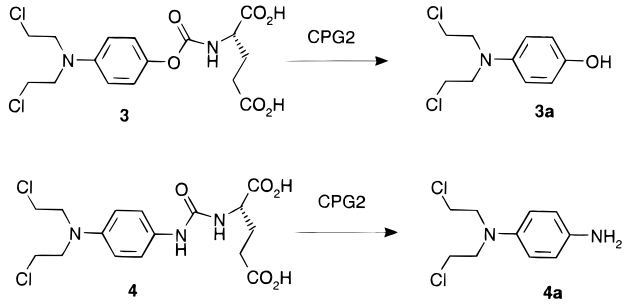
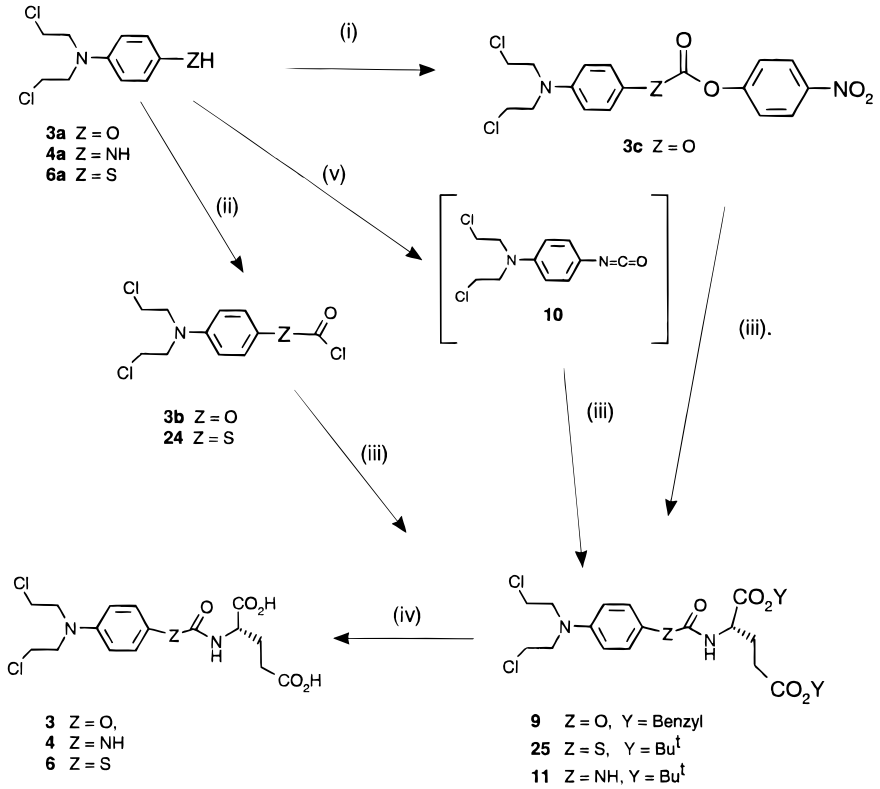


Figure 2.

Chemistry

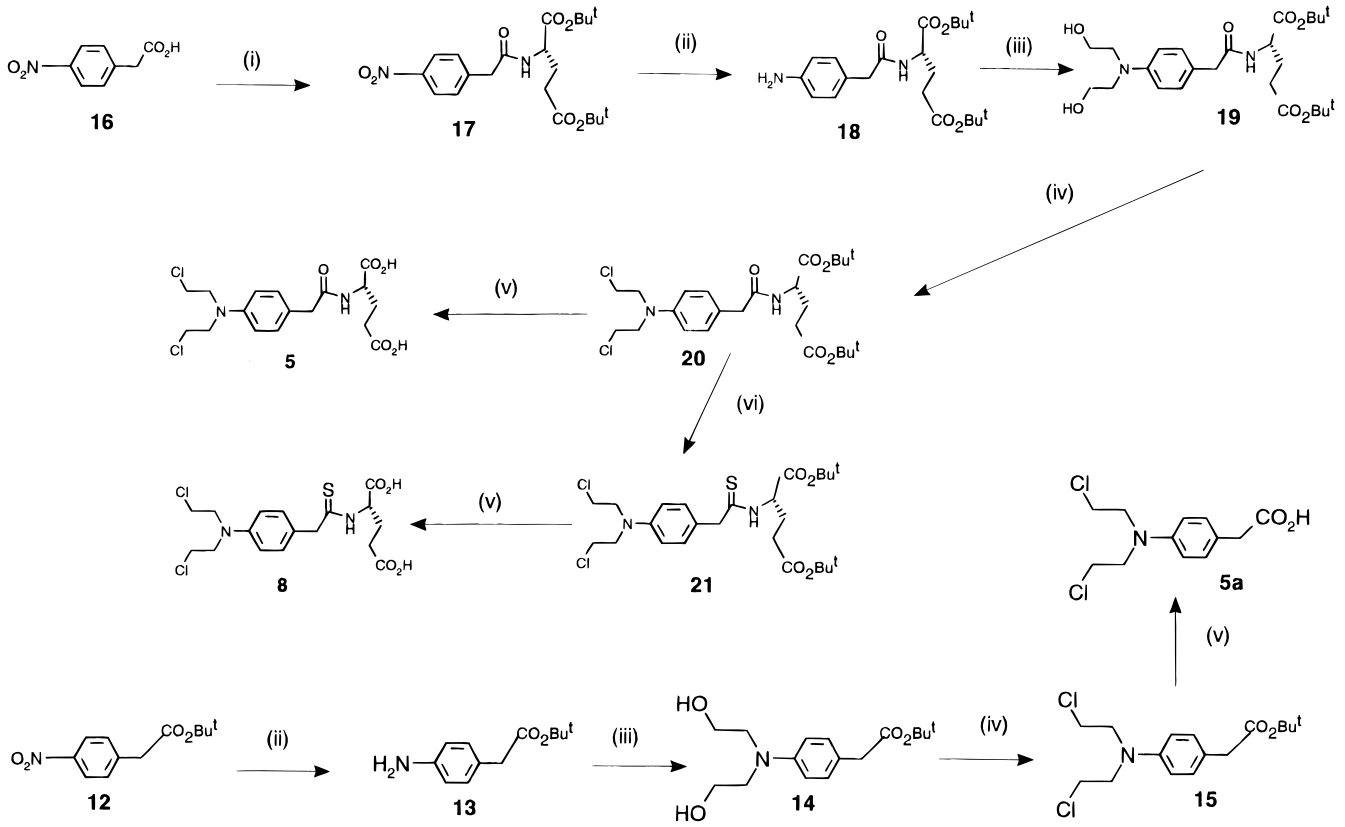
The prodrugs **1** and **2** are known compounds.[8,16] The prodrug **3** was made as shown in Scheme 1 from the known phenol mustard **3a**[9] by reaction with either phosgene or 4-nitrophenyl chloroformate to give the intermediate chloroformate **3b** or 4-nitrophenyl carbonate **3c**, respectively, which was then reacted with dibenzyl glutamate to give **9**. This dibenzyl ester was hydrogenated in the presence of 30% palladium on carbon to give **3**. The prodrug **4** was made from the amino mustard **4a**[9] by treatment with triphosgene in chloroform to give the intermediate isocyanate **10** which was not isolated but reacted with di-*tert*-butyl glutamate to give **11** as an oil. This oil was treated with hydrogen chloride gas in ether to give **4** as its hydrochloride salt (Scheme 1). The phenylacetic acid drug **5a** was made from 4-nitrophenylacetic acid *tert*-butyl ester[10] **12** by first reducing the nitro group using 30% palladium on carbon/hydrogen and then reacting the resulting aniline **13** with ethylene oxide in 1:1 acetic acid/water to give the diol **14**. This diol was converted to the dichloro mustard using methanesulfonyl chloride in pyridine to give **15**. On treatment with trifluoroacetic acid the phenylacetic acid drug **5a** was generated (Scheme 2). The corresponding prodrug **5** was prepared as shown in Scheme 2. A DCCI-mediated coupling of 4-nitrophenylacetic acid **16** with di-*tert*-butyl glutamate acid gave a good yield of **17** as an oil. This was reduced with hydrogen and 30% palladium on carbon to give the amine **18**. Treatment with ethylene oxide gave the diol **19** which was converted to the dichloro compound **20** with methanesulfonyl chloride in pyridine. Treatment of **20** with trifluoroacetic acid gave **5** as its trifluoroacetate salt which failed to crystallize. Compound **20** reacted with Lawesson's reagent to give the thioamide **21** which after deprotection with trifluoroacetic acid gave **8** (Scheme 2). Compound **7** was prepared by the route shown in Scheme 3 from the phenol drug **3a** by treatment with pentafluorophenyl chlorothioformate in chloroform in the presence of triethylamine to give the thiocarbonate **22** which was reacted with di-*tert*-butyl

Scheme 1[a]



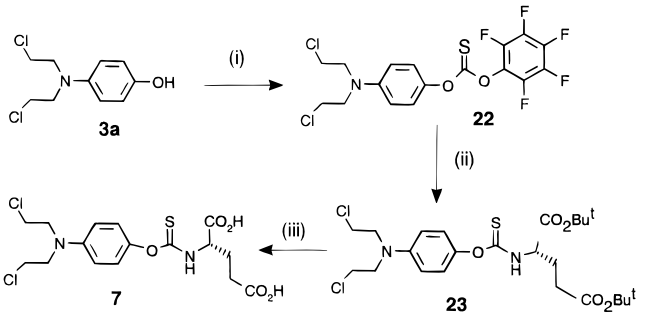
[a] (i) 4-Nitrophenyl chloroformate; (ii) $NaHCO_3$/phosgene; (iii) Z = dibenzyl glutamate, Z = S, Z = NH, di-*tert*-butyl glutamate; (iv) Z = O, H_2, 30% Pd/C; Z = *S*-formic acid; Z = NH, HCl, ether; (v) triphosgene, $CHCl_3$.

Scheme 2[a]



[a] (i) Di-*tert*-butyl glutamate, DCCI, DMF; (ii) H_2, 30% Pd/C; (iii) ethylene oxide; (iv) $MeSO_2Cl$, pyridine; (v) CF_3CO_2H, CH_2Cl_2; (vi) Lawesson's reagent, CH_2Cl_2.

Scheme 3[a]



[a] (i) Pentafluorophenyl chlorothioformate; Et_3N; $CHCl_3$; (ii) di-*tert*-butyl glutamate; Et_3N; (iii) formic acid.

glutamate in chloroform to give **23**. Treatment of **23** with formic acid at room temperature gave **7**. Prodrug **6** was prepared from the known[11] thiophenol mustard **6a** by reaction with phosgene in pyridine to give the intermediate thiochloroformate **24** which was reacted immediately with di-*tert*-butylglutamic acid to give **25** as an oil. This oil on treatment with formic acid followed by evaporation gave **6 (**Scheme 1).

Results and Discussion

The weak growth inhibition (IC_{50}'s in Table 1) properties of the dichloro-**2a** or monomesylbenzoic acid mustard drugs **1a** is likely to be due to both the deactivating effect of the 4-carboxylic acid on the mustard reactivity and poor cell penetration due to the carboxyl function which is ionized at physiological pH. In an attempt to improve both of these factors we turned our attention to more potent alternative mustard drugs. It is known[12] that the cell-killing ability of an aromatic mustard is related to its chemical reactivity. Hydroxy and amino groups in the para position of an aromatic nitrogen mustard are reported[12] to increase the chemical reactivity and thus the cytotoxicity. In the search for a CPG2-based ADEPT system which would deliver a more potent drug, we examined alternative linkages to the amide bond between the aromatic nitrogen mustard and the glutamate moiety. To deliver such a phenol or amino drug required the corresponding prodrug to embody a carbamate **3** or urea **4** moiety. These compounds were synthesized and found to be surprisingly good substrates for CPG2 with K_m and k_{cat} values as shown in Table 1. From this data it can be seen that on replacement of the benzamide link by a carbamate or urea the K_m remains almost the same but there has been a significant drop in the k_{cat} value. These results show that the compounds **3** and **4** have a similar binding affinity as **1** and **2** for CPG2 but the enzyme cleaves the carbamate and urea link less efficiently than the amide bond (k_{cat}/K_m for **3** and **4** are 49 and 2.5, respectively, whereas **2** is 206). When compounds **3** and **4** were hydrolyzed by the enzyme CPG2 to the phenol and aniline mustard drugs **3a** and **4a**, respectively, the scissile bond is assumed (by analogy with other carboxypeptidases[13]) to be the carbonyl nitrogen bond thus yielding an unstable carbonic or carbamic acid which will lose carbon dioxide to give the phenol or aniline drug. The plasma stability of these carbamate and urea links has been examined by incubation of **3** and **4** in mouse plasma at 37 °C for 3 h. During this period no breakdown to the active drugs **3a** and **4a** could be detected. Although the aniline and phenol drugs **4a** and

Table 2. Reactivity, Biological Data, and Chemical Reactivity and Cytotoxicity Ratios of Drugs and Prodrugs

compd	chemical reactivity (min)	IC_{50} (μM)	compd	ratio of chemical reactivity	ratio of cytoxicity of drugs to prodrugs
1a	17	80	1:1a	2.7	>6.25
2a	274	>100	2:2a	2.4	>5
3a	4.5	1.0	3:3a	6.9	200
4a	2.8	1.8	4:4a	6.7	111
5a	19	19	5:5a	1.7	>26
6a	5.0	100	6:6a	6.4	1

3a are just 3.8−6 times more chemically reactive than monomesyl benzoic **1a** they are some 40−80 times more potent in a cell-killing test (Table 2). The results in Table 1 show the negative consequences of changing the linkage from carbamate or urea to phenylacetamide **5** or thiocarbamate **6**. The isomeric thiocarbamate **7** and the thiophenylacetamide **8** were found not to be substrates for CPG2 under the conditions of the test. The phenylacetic acid prodrug **5** was not considered as a linkage for further development because of its enzyme kinetics and the low reactivity and cytotoxicity of the derived drug **5a**. The three sulfur-containing analogues **6, 7,** and **8** all have poor enzyme kinetics against CPG2 and are therefore not candidates for ADEPT.

The phenol and aniline mustard drugs **3a** and **4a** show a marked increase in chemical reactivity over the benzoic acid drug **2a** and a substantial increase in cytotoxicity over the corresponding drugs **1a** and **2a** (Table 1). The prodrugs **3** and **4** are 50−100 times less cytotoxic than their corresponding drugs.

Interestingly the ratio of the chemical reactivities of the prodrug to drug which can be considered as a "deactivation factor" varies for the two series. In the series where there is a heteroatom between the amide bond and the aromatic ring (as in compounds **3**, **4**, and **6**) this deactivation factor is 2−3-fold larger than in the series where the amide is directly attached (**1** and **2**) or when there is a methylene insertion (**5**, Table 2).

Conclusion

We have shown that a limited range of new prodrugs with improved properties for ADEPT can be prepared. The amide link in **1** can be replaced by a carbamate or a urea moiety as in compounds **3** and **4**, and these compounds retain recognition and turnover by CPG2. Other links between the aniline mustard and the glutamate portion were either unacceptably poor substrates for CPG2 (**5** and **6**) or were not substrates at all (**7** and **8**). The active drugs generated from the prodrugs **3** and **4** were markedly (~100-fold) more potent than the benzoic acid drug **1a**.

Experimental Section

Melting points were determined on a Buchi melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AC250 or Bruker AM200 instrument with TMS as an internal standard. Mass spectrum were determined on a VG-ZAB instrument. All organic extracts were dried over $MgSO_4$, and evaporations were carried out under reduced pressure. All microanalyses were within 0.4% of theory except where stated. All chromatography was done with Merck 9385 silica gell using the "flash chromatography" technique.

Cytotoxicity Assay. The colorectal tumor cell line, LoVo, was incubated with prodrug, or drug in 96-well (2500 cells/well) microtitre plates for 1 h. The cells were then washed and incubated for a further 3 days at 37 °C. TCA was then added and the amount of cellular protein adhering to the plates was assessed by addition of SRB dye. Cytotoxic potency of the compounds is expressed as the concentration required to inhibit cell growth by 50% (IC_{50}).[14]

CPG2 Enzyme Assay. The K_m and V_{max} of the prodrugs for CPG2 were determined based on the CPG2 assay method for methotrexate.[15] The absorbances of prodrug and corresponding drug were scanned from 200 to 350 nm using a spectrophotometer (Perkin-Elmer Lambda 2), and the wavelength was selected where the absorbance difference (due to release of glutamate from the prodrug) between prodrug and drug was maximal. The K_m and V_{max} were then determined by measuring the initial rate of conversion of prodrug at this wavelength using a range of prodrug concentrations (1−100 μM) and CPG2 enzyme concentrations (0.05−1 Unit). k_{cat} was calculated from the V_{max} by dividing by the amount of CPG2 in the reaction mixture.

Chemical Hydrolysis. The rates of hydrolysis, i.e. loss of the first chloro or mesyl group, of the prodrugs **1**−**8** and their corresponding drugs **1a**−**6a** were measured by HPLC analysis in phosphate buffer (pH 7.4) at 37 °C. Reaction products were separated on a 25 cm S5ODS1 column using acetonitrile/water/0.1% TFA as eluant. The $t_{1/2}$ was calculated using a kinetic program developed within Zeneca Pharmaceuticals Plc.

***N*-[[4-[*N*,*N*-Bis(2-chloroethyl)amino]phenoxy]carbonyl]-L-glutamic Acid (3).** A solution of dibenzyl *N*-[[4-[*N*,*N*-bis-(2-chloroethyl)amino]phenoxy]carbonyl]-L-glutamate (6 g, 10.2 mmol) in ethyl acetate (100 mL) was hydrogenated over 30% palladium on carbon (0.6 g) for 2 h. When the theoretical amount of hydrogen had been taken up, the catalyst was removed by filtration and the filtrate evaporated to dryness. The residue was taken up into hot ether (25 mL) and hexane added until cloudy. On cooling **3** was obtained as a white crystalline solid: 3.4 g, (79%); mp 87−89 °C. Anal. ($C_{16}H_{29}Cl_2N_2O_6$) C, H, N.

The title compound was also prepared using the above method but obtained in a different polymorphic form, not soluble in ether, with a melting point of 128−130 °C.

Dibenzyl *N*-[[4-[*N*,*N*-Bis(2-chloroethyl)amino]phenoxy]-carbonyl]-L-glutamate (9). Chloroformate Method. A suspension of 4-[*N*,*N*-bis(2-chloroethyl)amino]phenol hydrochloride **3a** (0.5 g, 2 mmol) in chloroform (10 mL) was shaken twice with 50% saturated sodium bicarbonate solution. The chloroform phase was dried and was added to a mixture of 1.9 M phosgene in toluene (6 mL) and quinoline (0.25 mL, 2 mmol) over 5 min at 15−20 °C. The yellow solution was allowed to stir at ambient temperature for 1 h, washed twice with water, dried, and evaporated to an oil. To a solution of this oil in chloroform (10 mL) was added, in one portion, L-glutamic acid dibenzyl ester tosylate (1 g, 2 mmol) followed by triethylamine (0.28 mL, 2 mmol). The mixture was stirred at ambient temperature for 1 h, washed with water (2 × 5 mL), and evaporated to dryness. The residue was chromatographed, eluting with 2.5% ethyl acetate in dichloromethane to give **9** as a white solid: 0.62 g (77%); mp 85−7 °C; NMR ($CDCl_3$) δ 7.35 (s, 5H), 7.30 (s, 5H), 6.98 (d, J = 13.6 Hz, 2H), 6.62 (d, J = 13.6 Hz, 2H), 5.68 (1, 1H), 5.19 (s, 2H), 5.11 (s, 2H), 4.5 (m, 1H), 3.65 (m, 8H), 2.47 (m, 2H), 2.04 (m, 2H). Anal. ($C_{30}H_{32}Cl_2N_2O_6 \cdot H_2O$) C, H, N.

Dibenzyl *N*-[[4-[*N*,*N*-Bis(2-chloroethyl)amino]phenoxy]-carbonyl]-L-glutamate (9). 4-Nitrophenyl Chloroformate Method. 4-Nitrophenyl chloroformate (3.3 g,16 mmol) was added slowly to a solution of 4-[*N*,*N*-bis(2-chloroethyl)amino]-phenol hydrochloride **3a** (4.4 g, 16 mmol) and triethylamine (4.6 mL, 32 mmol) in chloroform (50 mL). The solution was allowed to stand at ambient temperature for 4 h and then evaporated to an oil. The oil was chromatographed, eluting with hexane/ethyl acetate (3:1) to give the intermediate carbonate **3c** as an oil: 5.5 g (86%), NMR (DMSO-d_6) δ 8.36 (d, J = 9.15 Hz, 2H), 7.68 (d, J = 9.19 Hz, 2H), 7.21 (d, J = 9.15 Hz, 2H), 6.81 (d, J = 9.19 Hz, 2H), 3.73 (s, 8H). This oil (5.5 g, 14 mmol) was dissolved in chloroform (40 mL) and triethylamine (4.0 mL, 28 mmol) and L-glutamic acid dibenzyl ester tosylate (13.75 g, 28 mmol) were added. The reaction

mixture was heated at 60 °C for 4 h and evaporated to dryness and the residue chromatographed using 3% ethyl acetate/chloroform as eluant to give **9**, 6.2 g (77%).

***N*-[4-[*N*,*N*-Bis(2-chloroethyl)amino]phenyl]carbamoyl]-L-glutamic Acid Hydrochloride (4).** A saturated solution of hydrogen chloride in ether (120 mL) was added to a solution of **11** (4.4 g, 8.5 mmol) in ethyl acetate (20 mL). After 1 h at ambient temperature the mixture was evaporated to a solid. This solid was triturated with ether to obtain **4** as a gray solid: 3.5 g (93%); mp 148−150 °C. Anal. ($C_{16}H_{21}Cl_2N_3O_5$·HCl·Et_2O·0.5EtOAc) C, H, N.

Di-*tert*-butyl Bis[[4-[(2-chloroethyl)amino]phenyl]carbamoyl]-L-glutamate (11). To a solution of triphosgene (200 mg, 0.67 mmol) in chloroform (10 mL) at 0−5 °C was added **4a** (339 mg, 2 mmol) followed by triethylamine (0.83 mL, 6 mmol). After 15 min at room temperature a solution of di-*tert*-butyl L-glutamate (0.31 g, 2.4 mmol) in chloroform (5 mL) was added. The mixture was allowed to stand at ambient temperature for 18 h, washed with water, dried, and evaporated to dryness. The residue was chromatographed and elution with hexane/ethyl acetate (3:1) gave **11** as an oil: 0.44 g (32%), NMR (DMSO-d_6) δ 7.2 (d, J = 9.9 Hz, 2H), 6.65 (d, J = 9.9 Hz, 2H), 4.1 (m, 1H), 3.66 (s, 8H), 1.7−2.2 (m, 4H), 1.38 (s, 9H), 1.42 (s, 9H).

***N*-[[4-[*N*,*N*-Bis(2-chloroethyl)amino]phenyl]acetyl]-L-glutamic Acid (5).** 1-Hydroxybenzotriazole (4.05 g, 30 mmol) was added to a solution of 4-nitrophenylacetic acid (**16**) (5.4 g, 30 mmol) in dimethylformamide (75 mL). Di-*tert*-butyl L-glutamate (7.77 g, 30 mmol) and then dicyclohexylcarbodiimide (6.2 g, 30 mmol) were added to the mixture. The mixture was then stirred for 18 h at ambient temperature. The mixture was filtered, and the filtrate was washed with saturated sodium bicarbonate solution, water, 0.5 M hydrochloric acid, dried, and evaporated to dryness. The residue was chromatographed and elution with hexane/ethyl acetate (2:1) gave **17** as an oil: 9.7 g (77%); NMR (DMSO-d_6) δ 8.2 (d, J = 9.9 Hz, 2H), 7.45 (d, J = 9.9 Hz, 2H), 6.45 (d, 1H), 4.45 (m, 1H), 3.15 (s, 2H), 2.1 (m, 4H), 1.4 (d, 9H), 1.35 (s, 9H).

A solution of **17** (9.7 g) in ethyl acetate (200 mL) was hydrogenated over 30% Pd/C (900 mg). The mixture was then filtered through Celite, and the filtrate was evaporated to yield **18** as yellow oil (8.2 g, 90%) which was used without further purification: NMR (DMSO-d_6) δ 8.23 (d, J = 12.0 Hz, 1H), 6.92 (d, J = 12.3 Hz, 2H), 6.50 (d, J = 12.3 Hz, 2H), 4.86 (s, 2H), 4.15 (m, 1H), 3.24 (s, 2H), 2.22 (m, 2H), 1.8 (m, 2H), 1.4 (d, 18H). Ethylene oxide (7.1 g) was bubbled into a solution of **18** (8.2 g) in glacial acetic acid (40 mL) and water (40 mL). The mixture was then stirred at ambient temperature for 24 h. The solution was evaporated to dryness, redissolved in ether, washed with water, dried, and evaporated to yield **19** as an oil (6.3 g) which was used without further purification: NMR (DMSO-d_6) δ 8.16 (d, J = 12.3 Hz, 1H), 7.02 (d, J = 10.3 Hz, 2H), 6.58 (d, J = 10.3 Hz, 2H), 4.1 (m, 1H), 3.4 (m, 8H), 3.25 (s, 2H), 2.25 (m, 2H), 1.8 (m, 2H), 1.4 (s, 18H). Methanesulfonyl chloride (2.91 mL) was added dropwise to a solution of **19** (2.88 g) in pyridine (45 mL) under an argon atmosphere, maintaining the temperature below 25 °C. The solution was stirred at 80 °C for 1 h, cooled, poured into 10% citric acid (500 mL), and extracted with ether. The ether extract was washed with water, dried, and evaporated to a brown oil. This oil was chromatographed eluting with hexane/ethyl acetate (2:1) to give **20** as an oil: 1.3 g (42%); NMR (DMSO-d_6) δ 8.19 (d, J = 8.3 Hz, 1H), 7.10 (d, J = 9.9 Hz, 2H), 6.66 (d, J = 9.9 Hz, 2H), 4.10 (m, 1H), 3.69 (s, 8H), 3.32 (s, 2H), 2.21 (m, 2H), 1.9 (m, 2H), 1.37 (d, 18H). This oil was dissolved in dichloromethane (1.5 mL), and trifluoroacetic acid (1.5 mL) was added. The solution was stirred at ambient temperature for 2 h. The solution was then evaporated to give **5** as an oil (0.8 g): NMR (DMSO-d_6) δ 8.22 (d, J = 11.6 Hz, 1H), 7.10 (d, J = 11.6 Hz, 2H), 6.66 (d, J = 11.6 Hz, 2H), 4.19 (m, 1H), 3.69 (s, 8H), 3.32 (s, 2H), 2.24 (m, 2H), 1.9 (m)2H. Anal. ($C_{17}H_{22}Cl_2N_2O_5$·0.75H_2O·2.5TFA) C, H, N.

4-[Bis(2-chloroethyl)amino]phenylacetic Acid (5a). To a solution of 4-nitrophenylacetic acid *tert*-butyl ester[10] (**12**) (10 g, 42 mmol) in ethyl acetate (150 mL) was added 30% palladium on carbon (50% moist with water) (1.5 g). The mixture was stirred under an atmosphere of hydrogen until the theoretical amount of hydrogen had been taken up. The catalyst was removed by filtration through Celite, and the filtrate was evaporated to an oil **13** (8.5 g). This oil **13** was dissolved in 1:1 acetic acid/water (140 mL), and a slow stream of ethylene oxide was passed in until 12 g had been absorbed. The mixture was allowed to stand at ambient temperature for 48 h, and then evaporated to dryness. The residue was dissolved in ethyl acetate (150 mL), washed with water and saturated sodium bicarbonate solution, dried, and evaporated to a solid, **14** (2.9 g): NMR (DMSO-d_6) δ 7.1 (d, J = 8.3 Hz, 2H), 6.6 (d, J = 8.3 Hz, 2H), 3.4−3.7 (m, 8H), 3.35 (s, 2H), 1.4 (s, 9H). This solid was dissolved in pyridine (75 mL) and, under an argon atmosphere, was added dropwise methanesulfonyl chloride (4.85 mL). The temperature was kept at 20−25 °C during the addition, and the mixture was then heated to 80 °C for 1 h. After cooling the mixture was diluted with ethyl acetate (500 mL), washed with 10% citric acid solution (×2) and water, dried, and evaporated to an oil. This oil was chromatographed, eluting with 9:1 hexane/ethyl acetate gave **15** as an oil: 1.65 g; NMR (DMSO-d_6) δ 7.18 (d, J = 8.2 Hz, 2H), 6.68 (d, J =8.2 Hz, 2H), 3.71 (s, 8H), 3.42 (s, 2H), 1.39 (s, 9H). This oil (1 g) was dissolved in dichloromethane (45 mL) and trifluoroacetic acid (4.5 mL) added. The mixture was stirred at ambient temperature for 2 h and then evaporated to an oil **5a** which crystallized: 0.39 g (48%); mp 102−4 °C. Anal. ($C_{12}H_{15}Cl_2NO_2$) C, H, N.

***N*-[4-[*N*,*N*-Bis(2-chloroethyl)amino]phenoxy]thiocarbonyl]-L-glutamic Acid (7).** To a suspension of **3a** (0.8 g. 3 mmol) in chloroform (10 mL) cooled to −10 °C was added pentafluorophenyl chlorothioformate (0.53 mL, 3.3 mmol) followed dropwise by triethylamine (0.88 mL, 6.3 mmol). After stirring for 60 min at −10 °C the mixture was evaporated to an oil and chromatographed. Elution with hexane/ethyl acetate (4:1) gave **22** as a colorless oil: 1.04 g (75%); NMR (DMSO-d_6) δ 7.18 (d, J = 8.8 Hz, 2H), 6.83 (d, J = 8.8 Hz, 2H), 3.73 (s, 8H). This oil (0.46 g, 1 mmol) was dissolved in chloroform (10 mL), and L-glutamic acid di-*tert* butyl ester (0.69 g, 2.8 mmol) was added. Triethylamine (0.4 mL, 2.8 mmol) was added, and the mixture was heated at 50 °C for 4 h. After cooling the mixture was evaporated to dryness and the residue was chromatographed, eluting with 3% ethyl acetate in chloroform to give **23** as a colorless oil: 168 mg (31%); NMR ($CDCl_3$) δ 7.36 (d, J = 8.9 Hz, 2H), 6.98 (d, J = 8.9 Hz, 2H), 6.66 (d, 2H), 4.82 (m, 1H), 3.65 (m, 8H), 2.24 (m, 4H), 1.50 (d, 18H).. This oil (0.44 g 0.8 mmol) was dissolved in formic acid (25 mL) and kept at ambient temperature for 18 h. After evaporation to dryness the residue was chromatographed eluting with dichloromethane/ethyl acetate/formic acid (6:4:0.5) gave **7** as an oil: 160 mg (46%). NMR ($CDCl_3$) δ 7.22 (s, 1H), 6.97 (d, J = 9.0 Hz, 2H), 6.67 (d, J = 9.0 Hz, 2H), 5.04 (m, 1H), 3.66 (m, 8H), 2.56 (m, 4H). Anal. ($C_{16}H_{20}Cl_2N_2O_5S$·0.3EtOAc) C, H, N.

***N*-[4-[*N*,*N*-Bis(2-chloroethyl)amino]phenylsulfamyl]-L-glutamic Acid (6).** To a mixture of **6a** (0.5 g, 2 mmol) and pyridine (0.16 mL, 2 mmol) in anhydrous ether (10 mL) was added over 5 min a solution of phosgene in toluene (1.1 mL of 1.9 M). The mixture was stirred for 30 min at ambient temperature, and a white precipitate was removed by filtration. The filtrate was evaporated to dryness to give the crude thiochloroformate **24** and redissolved in chloroform (10 mL), and L-di-*tert*-butyl glutamate (0.49 g, 2 mmol) was added followed by triethylamine (0.3 mL, 2 mmol). The mixture was stirred at ambient temperature for 1 h and then evaporated to dryness. The residue was chromatographed elution with 3% ethyl acetate in chloroform gave **25** as an oil: 380 mg (36%); NMR ($CDCl_3$) δ 7.42 (d, J = 6.0 Hz, 2H), 6.7 (d, J = 6.0 Hz, 2H), 6.01 (d, 1H), 4.44 (m, 1H), 3.72 (m, 8H), 2.46 (m, 2H), 2.26 (m, 2H), 1.47 (s, 18H).

This oil **25** (0.2 g) was dissolved in dichloromethane (2 mL) at 0 °C, and trifluoroacetic acid (0.5 mL) was added. The mixture was stirred at 0 °C for 3 days, evaporated to dryness, and azeotroped twice with ethyl acetate to give **6** as a colorless oil: 130 mg (46%); NMR (DMSO-d_6) δ 8.36 (d, J = 10.2 Hz, 1H), 7.26 (d, J = 6.2 Hz, 2H), 6.78 (d, J = 6.2 Hz, 2H), 4.19

(m, 1H), 3.86 (s, 8H), 2.23 (m, 2H), 1.89 (m, 2H). Anal. ($C_{16}H_{20}Cl_2N_2O_5$·3TFA) C, H, N.

***N*-[[4-[*N*,*N*-Bis(2-chloroethyl)amino]phenyl]thioacetyl]-L-glutamic Acid (8).** To a solution of the dichloro-*tert*-butyl ester **20** (0.84 g, 1.6 mmol) in dichloromethane (30 mL) was added Lawesson's reagent (0.78 g, 1.6 mmol). The mixture was allowed to stir at ambient temperature for 18 h, evaporated to dryness, and chromatographed. Elution with hexane/ethyl acetate (4:1) gave **21** as an oil: 0.68 g (80%); NMR (DMSO-d_6) δ 10.2 (d, J = 9.9 Hz, 1H), 7.2 (d, J = 8.3 Hz, 2H), 6.7 (d, J = 8.3 Hz, 2H), 4.7 (m, 1H), 3.85 (s, 2H), 3.7 (s, 8H), 2.0−2.3 (m, 4H), 1.5 (s, 9H), 1.3 (s, 9H). This ester **21** (400 mg, 0.7 mmol) was dissolved in formic acid (30 mL) and allowed to stand at ambient temperature for 18 h. The solution was evaporated to an oil **8**: 200 mg (60%); NMR (DMSO-d_6) δ 10.2 (d, J = 10.1 Hz, 1H), 7.1 (d, J = 7.3 Hz, 2H), 6.6 (d, J = 7.3 Hz, 2H), 4.9 (m, 1H), 3.85 (m, 1H), 3.7 (s, 8H), 2.0−2.3 (m, 4H). Anal. ($C_{17}H_{22}Cl_2N_2O_4S$. 0.5EtOAc) C, H, N.

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