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Self-Immolative Nitrogen Mustard Prodrugs for Suicide Gene Therapy

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Four new potential self-immolative prodrugs derived from phenol and aniline nitrogen mustards, four model compounds derived from their corresponding fluoroethyl analogues and two new self-immolative linkers were designed and synthesized for use in the suicide gene therapy termed GDEPT (gene-directed enzyme prodrug therapy). The self-immolative prodrugs were designed to be activated by the enzyme carboxypeptidase G2 (CPG2) releasing an active drug by a 1,6-elimination mechanism via an unstable intermediate. Thus, *N*-[(4-{[4-(bis{2-chloroethyl}amino)phenoxycarbonyloxy]methyl}phenyl)carbamoyl]-L-glutamic acid (**23**), *N*-[(4-{[4-(bis{2-chloroethyl}amino)phenoxycarbonyloxy]methyl}phenoxy)carbonyl]-L-glutamic acid (**30**), *N*-[(4-{[*N*-(4-{bis[2-chloroethyl]amino}phenyl)carbamoyloxy]methyl}phenoxy)carbonyl]-L-glutamic acid (**37**), and *N*-[(4-{[*N*-(4-{bis[2-chloroethyl]amino}phenyl)carbamoyloxy]methyl}phenyl)-carbamoyl]-L-glutamic acid (**40**) were synthesized. They are bifunctional alkylating agents in which the activating effects of the phenolic hydroxyl or amino functions are masked through an oxycarbonyl or a carbamoyl bond to a benzylic spacer which is itself linked to a glutamic acid by an oxycarbonyl or a carbamoyl bond. The corresponding fluoroethyl compounds **25**, **32**, **42**, and **44** were also synthesized. The rationale was to obtain model compounds with greatly reduced alkylating abilities that would be much less reactive with nucleophiles compared to the corresponding chloroethyl derivatives. This enabled studies of these model compounds as substrates for CPG2, without incurring the rapid and complicated decomposition pathways of the chloroethyl derivatives. The prodrugs were designed to be activated to their corresponding phenol and aniline nitrogen mustard drugs by CPG2 for use in GDEPT. The synthesis of the analogous novel parent drugs (**21b, 51**) is also described. A colorectal cell line was engineered to express CPG2 tethered to the outer cell surface. The phenylenediamine compounds were found to behave as prodrugs, yielding IC_{50} prodrug/IC_{50} drug ratios between 20- and 33-fold (for **37** and **40**) and differentials of 12−14-fold between CPG2-expressing and control LacZ-expressing clones. The drugs released are up to 70-fold more potent than 4-[(2-chloroethyl)-(2-mesyloxyethyl)amino]benzoic acid that results from the prodrug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid (CMDA) which has been used previously for GDEPT. These data demonstrate the viability of this strategy and indicate that self-immolative prodrugs can be synthesized to release potent mustard drugs selectively by cells expressing CPG2 tethered to the cell surface in GDEPT.

Introduction

The major objective in cancer treatment is to kill tumor cells selectively without harming normal cells.[1] A strategy for achieving selectivity is called suicide gene therapy. One approach aims to deliver toxin genes to the cancer cells.[2] An alternative approach has been named gene-directed enzyme prodrug therapy (GDEPT)[3] or virally-directed enzyme prodrug therapy (VDEPT).[4] These therapies consist of tumor-specific conversion of prodrugs to active drugs following the delivery of genes for exogenous enzymes.[5]

Self-immolative prodrugs have been proposed for activation by tumor enzymes.[6−8] A self-immolative prodrug can be defined as generating an unstable intermediate which, following the activation process, will extrude the active drug in a number of subsequent steps.[6,8] A cascade of events are required. First, the activation process, which is enzymatic, culminates in the extrusion process that generates the drug. The site of activation will usually be separated from the site of extrusion. The advantage of self-immolation is that it increases the diversity of prodrugs which can be activated by a certain enzyme.

Self-immolative mustard prodrugs for activation by *Escherichia coli* nitroreductase in a GDEPT system have previously been synthesized and evaluated in tumor cell lines.[9] An alternative candidate for GDEPT is carboxypeptidase G2 (CPG2) from *Pseudomonas* sp.[10] since it has no mammalian homologue.[11] This enzyme catalyzes the scission of an amidic,[11,12] urethanic, or ureidic[13,14] bond between an aromatic nucleus and L-glutamic acid.

The enzyme CPG2 has recently been mutated and expressed tethered to the outer cell membrane in the human breast carcinoma cell line MDA MB 361.[15] The expression of CPG2 tethered to the surface of the cell overcomes the need for a prodrug to penetrate the tumor cell membrane. Incubation of the transfected lines with the prodrug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]-

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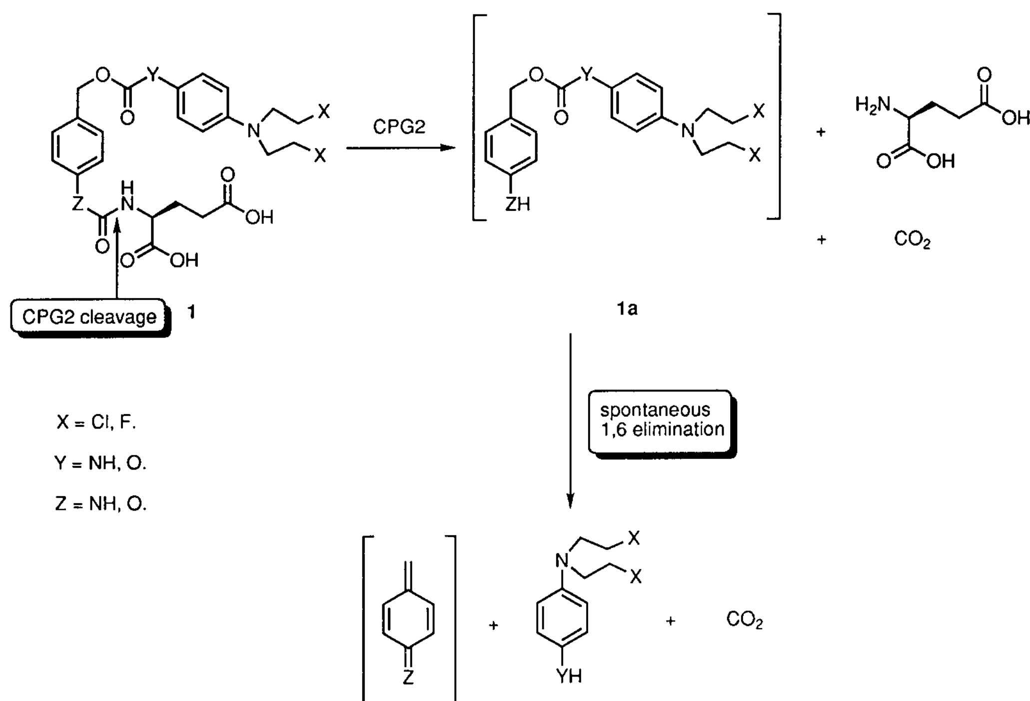
Scheme 1



benzoyl-L-glutamic acid (CMDA) leads to an increased sensitivity.[15] The prodrugs described herein were designed to release more potent active drugs than that cleaved from CMDA.

This report describes the first example of self-immolative prodrugs for activation by CPG2. It also describes the construction of the colorectal cell line LS174T made to express CPG2 tethered to the cell surface (LS174T-stCPG2(Q)3). Self-immolative prodrugs depicted as **1** in Scheme 1 were designed to be activated by CPG2 to generate the unstable intermediates **1a**, further releasing the active drug by a 1,6-elimination mechanism.

Rationale

For the synthesis of the novel nitrogen mustards prodrugs of general formula **1** (see Scheme 1), two self-immolative linkers: (4-hydroxymethylphenyl)carbamoyl-L-glutamic acid **12** and (4-hydroxymethylphenyl)-oxycarbonyl-L-glutamic acid **20** were designed, synthesized, and studied. The ureidic and carbamic linkages in **12** and **20**, respectively, between glutamic acid and the aromatic nucleus have a dual purpose. They provide a substrate cleavable by CPG2 and release an amino or hydroxy group required for the 1,6-elimination.

Aniline and phenol nitrogen mustards were chosen since the drugs are potent[16] and it is possible to reduce greatly their chemical reactivity through acylation. They were transformed to the corresponding self-immolative prodrugs **23**, **30**, **37**, and **40** by coupling with **12** or **20** through an oxycarbonyl or carbamoyl bond. The carbonate and carbamate bonds deactivate the phenol and aniline mustard, respectively by acylation, also acting as leaving groups in the 1,6-elimination, thus generating the drug via an irreversible process. The mechanism of activation of the prodrugs by CPG2 is presented in Scheme 1 and the physicochemical and kinetic data are in the Physicochemical and Kinetic Data section.

The corresponding fluorine mustard analogues **25**, **32**, **42**, and **44** were also synthesized. The rationale was to obtain compounds with greatly reduced alkylating abilities that would not react with nucleophiles as quickly as the corresponding chloroethyl derivatives. This enabled studies of these compounds as substrates for CPG2, without incurring the rapid and complicated decomposition pathways of the chloroethyl derivatives.

A broad panel of potential prodrugs with carbonate, carbamate, and ureido linkages were considered in order to find the optimal structural features responsible for the stability, substrate specificity for CPG2, and efficacy in cell lines transfected with the *stCPG2(Q)3* gene, as well as for further QSAR and optimization studies.

Chemistry

Two series of protected linkers (i.e., di-*tert*-butyl and diallyl L-glutamyl esters) were made since different deprotection strategies were needed to obtain the final prodrugs.

The starting material for **12**, 4-nitrobenzyl alcohol, was protected as *tert*-butyldiphenylsilyl ether **2a**[17,18] and reduced by hydrogen transfer (Pd/C (10%) and ammonium formate in EtOH) to the corresponding amine **3a**. The amine after conversion to isocyanate **4** (with triphosgene at 70 °C, in toluene) was coupled with di-*tert*-butyl L-glutamate, **5a**, in THF, at room temperature and in basic medium, leading to the protected linker **7a**. An alternative route to compound **7a** is the direct coupling of the amine **3a** with the di-*tert*-butyl L-glutamyl isocyanate **6a** under the conditions described above. Compound **6a** was obtained from di-*tert*-butyl L-glutamate, **5a**, by treatment with triphosgene and triethylamine at −78 °C in toluene. By exploiting this route, a one-pot procedure was devised to obtain compound **7a** directly, in good yield, starting from di-*tert*-butyl L-glutamate and amine **3a**. During this condensation the pyroglutamate analogue **10**, of compound **7a**, was formed as byproduct and was further deprotected with tetra-*n*-butylammonium fluoride in THF at room

Scheme 2[a]

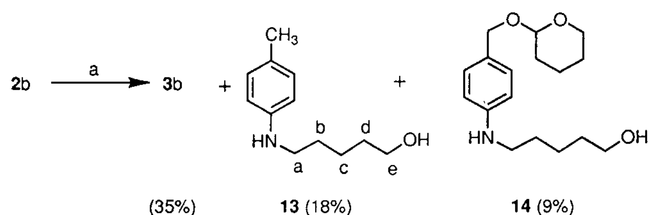
[a] (a) TBDPSCl, imidazole, DMF (THF) or 3,4-dihydropyran, PPTS, CH_2Cl_2, rt; (b) Pd/C (10%), HCO_2NH_4, EtOH; (c) $(Cl_3CO)_2CO$, NEt_3, toluene, 70 °C; (d) $(Cl_3CO)_2CO$, NEt_3, toluene, −78 °C; (e) THF, NEt_3, rt; (f) Bu_4NF, THF, rt or AcOH, THF, H_2O; (g) 4-nitrophenyl chloroformate, CH_3CN or THF, NEt_3, rt; (h) $Pd(Ph_3P)_4$, pyrrolidine, rt.

temperature to **11** (see Scheme 2). A similar deprotection afforded the linker **8a** which was purified by column chromatography and activated as 4-nitrophenyl carbonate **9a** (see Scheme 2).

The diallyl analogue **7c** was obtained using a different protection strategy, since the diallyl ester was unstable to tetra-*n*-butylammonium fluoride even at room temperature. The corresponding *O*-2′-tetrahydropyranyl ether **7b** was prepared by the sequence shown in Scheme 3. When the protected nitro derivative **2b** was reduced by hydrogen transfer to the amine **3b**, a mixture of compounds **3b**, **13**, and **14** resulted that were separated and purified by chromatography so that a yield of only 35−40% of the desired amine **3b** was obtained (see Scheme 3).

This amine was coupled with the diallyl L-glutamyl isocyanate **6b**, and the tetrahydropyranyl group was removed using AcOH in aqueous THF to give the linker **8a**, which was purified by column chromatography and activated as 4-nitrophenyl carbonate **9b** (see Scheme 2). The deprotected **12** was prepared from **8b** by the removal of the allylic groups with Pd(0) and pyrrolidine.

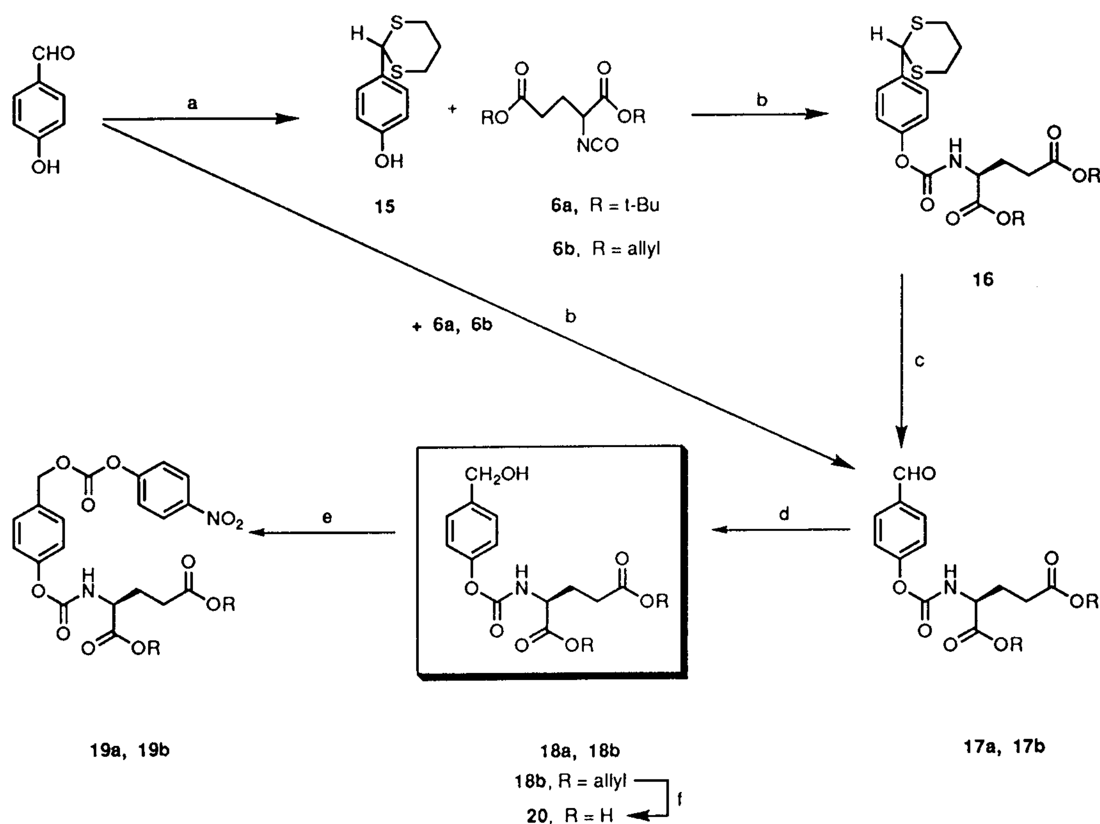
Scheme 3[a]



[a] (a) HCO_2NH_4, Pd/C, EtOH.

For the synthesis of the second self-immolative linker **20** (see Scheme 4), 4-hydroxybenzaldehyde was pro-

Scheme 4[a]



[a] (a) $HS(CH_2)_3SH$, $BF_3\cdot Et_2O$, $CHCl_3$, 20 °C; (b) NEt_3, toluene or $CHCl_3$, 20 °C; (c) $Hg(ClO_4)_2$, THF, 20 °C; (d) $NaBH_3CN$; (e) 4-nitrophenyl chloroformate, THF, NEt_3; (f) $Pd(Ph_3P)_4$, pyrrolidine, rt.

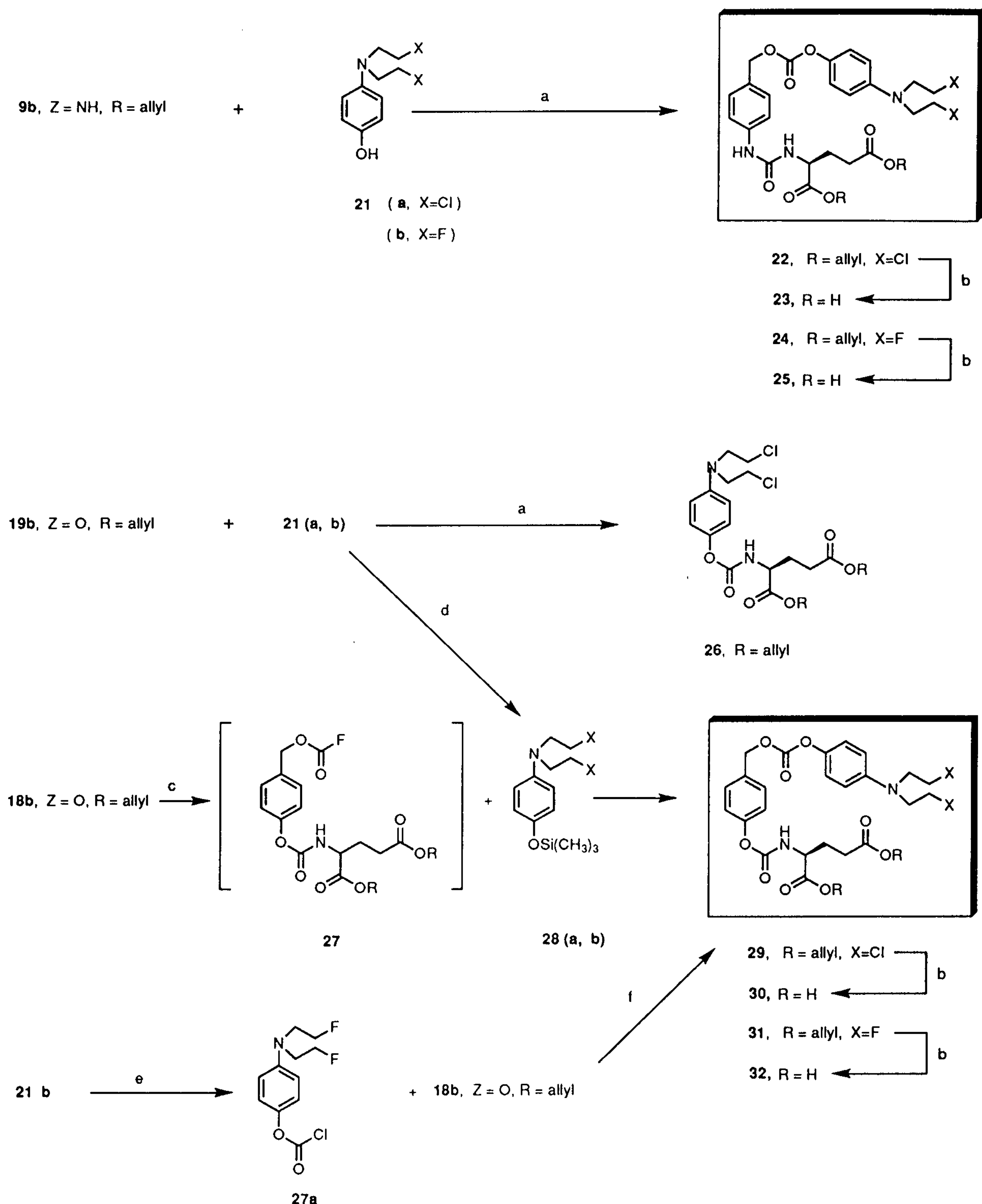
tected with 1,3-propanedithiol and boron trifluoride etherate, at room temperature, to give **15**[19] in very good yield. Reacting **15** with di-*tert*-butyl L-glutamyl isocyanate, **6a**, in toluene (NEt_3) and di-*tert*-butyl 4-[2′-(1′,3′-dithianyl)]phenoxycarbonyl-L-glutamate gave **16**. The deprotection of this intermediate with $Hg(ClO_4)_2$ in THF at room temperature led to the corresponding aldehyde **17a**. Subsequently, it was found that the aldehydes **17a**,**b** could be prepared by the direct coupling of the unprotected 4-hydroxybenzaldehyde with the isocyanates **6a**,**b**. The reduction of the aldehydes **17a**,**b** with sodium cyanoborohydride yielded the desired (4-hydroxymethylphenoxy)carbonyl-L-glutamates **18a**,**b**. Both linkers **18a**,**b** were activated as 4-nitrophenyl carbonates **19a**,**b**. The deprotection to **20** was achieved as described above for linker **12**.

The syntheses of aniline and phenol nitrogen mustard prodrugs **23**, **30**, **37**, and **40** and the corresponding fluorine mustard analogues **25**, **32**, **42**, and **44** require a new approach since the obvious route via the chloroformates of linkers **8a**,**b** and **18a**,**b** was not available. The failure of the direct phosgenation of some similar acylated benzylic derivatives has been reported previously.[20,21] Only one example of successful phosgenation of a similar 4-substituted benzylic alcohol has been claimed in a patent.[22] Our attempts to obtain chloroformates from the linkers using phosgene, diphosgene, or triphosgene under a variety of experimental conditions were also unsuccessful. The chloroformates are formed but are unstable above −40 °C[21] and difficult to handle. For this reason they were converted at −78 °C to the corresponding fluoroformates. Another difficulty lay with the deprotection procedures required to obtain the final prodrugs. The sensitivity of both carbamates and especially carbonates to basic and acidic media is widely recognized.[23]

The compounds fall into two categories: the phenol-derived and the aniline-derived self-immolative nitrogen mustards. Different procedures were employed to obtain the corresponding prodrugs **23**, **25**, **30**, and **32** from 4-bis(2-chloroethyl)- or 4-bis(2-fluoroethyl)aminophenol (**21a**,**b**) by condensation with the linkers **8a**,**b** and **18a**,**b**. Coupling **21a**,**b** with **9b** in the presence of the strongly basic anion exchanger Amberlyst 27 in acetonitrile resulted in the protected prodrugs **22** (X = Cl) and **24** (X = F). The removal of allylic groups, leading to the corresponding prodrugs **23** (X = Cl) and **25** (X = F), was achieved with Pd(0) and morpholine as allyl scavanger.

The same procedure failed when used with the activated linker **19b** leading unexpectedly to nitrogen mustard **26** (see Scheme 5). Therefore, a new procedure was designed taking advantage of the fluorine affinity for silicon.[24] Accordingly, linker **18b** was converted into the corresponding fluoroformate by reaction with phosgene, KF·HF, and 18-crown-6 ether at −78 °C in CH_2Cl_2 and coupled, at room temperature, in a one-pot procedure with the silylated nitrogen mustards **28a**,**b**. However, the coupling with the fluorine nitrogen mustard proceeded with very low yield (5%). Therefore the derivative **21b** was converted with phosgene to the corresponding chloroformate **27a** and coupled with linker **18b** to give compound **31** (X = F). The protected nitrogen mustard prodrugs **29** (X = Cl) and **31** (X = F) were deprotected to **30** (X = Cl) and **32** (X = F) with Pd(0) and morpholine (see Scheme 5).

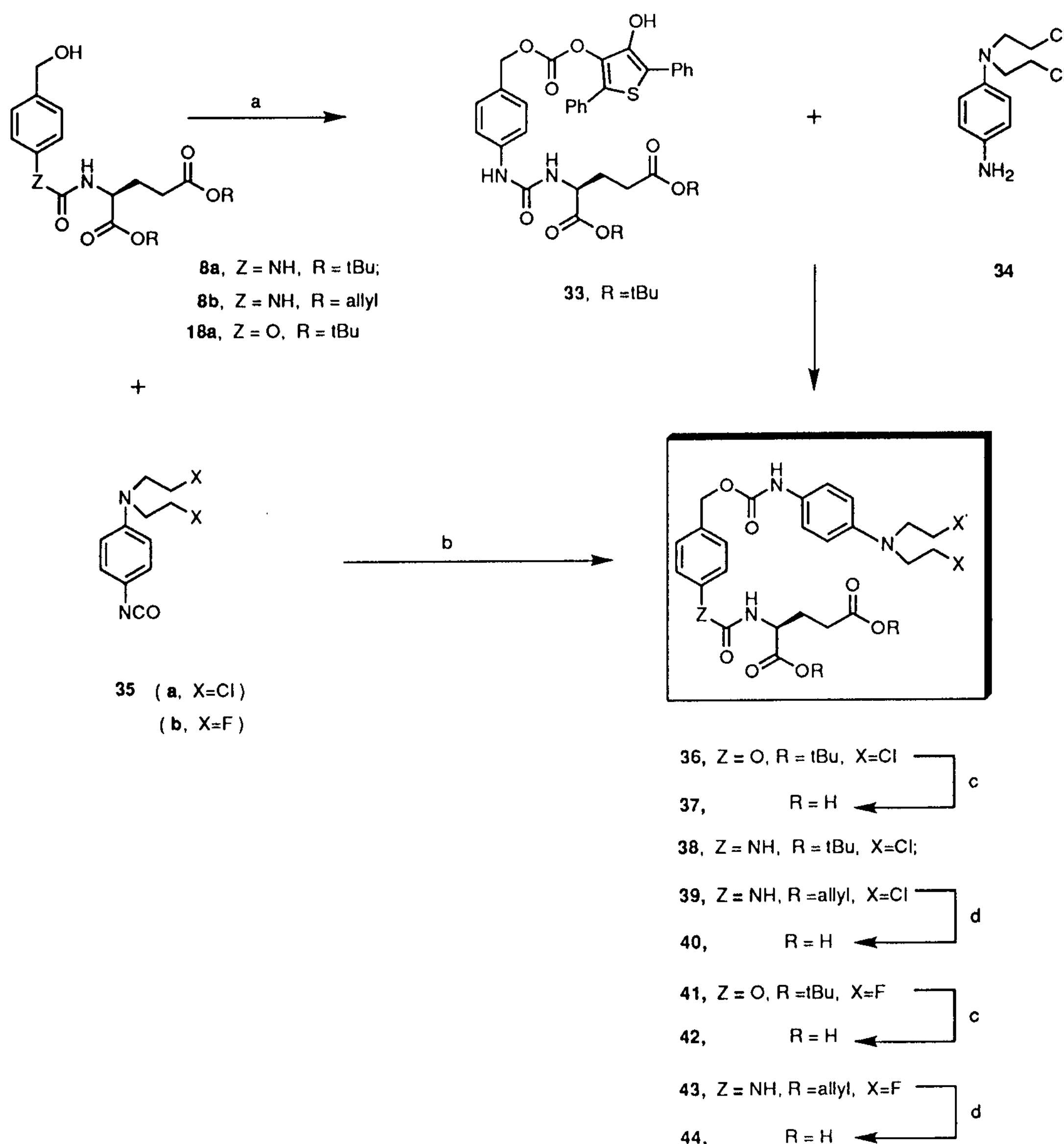
Scheme 5[a]



[a] (a) Amberlyst 27, CH_3CN; (b) $Pd(Ph_3)_4$, morpholine; (c) $COCl_2$, KF•HF, 18-crown-6, −78 °C; (d) Me_3SiCN; (e) $COCl_2$, NEt_3, CH_2Cl_2; (f) NEt_3, THF.

The aniline-derived self-immolative nitrogen mustards **37**, **40**, **42**, and **44** were obtained by a different route. In a first attempt the linker **8a** was activated by treatment with 4,6-diphenylthieno[3,4-*d*][1,3]dioxol-2-one 5,5-dioxide[25,26] to give the corresponding carbonate **33**. This intermediate is stable only at low temperature (−78 °C) and could not be isolated. However, the protected prodrug **38** was obtained in a one-pot procedure, using the nitrogen mustard **34**, in 10% yield (see Scheme 6). A more convenient procedure uses the 4-bis(2-chloroethyl)- or 4-bis(2-fluoroethyl)aminophenyl isocyanates (**35a**,**b**) (prepared from the corresponding aniline nitrogen mustards **34** (X = Cl) and **51** (X = F) with triphosgene) which were reacted with linkers **8a**,**b** and **18a** in the presence of dibutyltin dilaurate to give the protected prodrugs **36**, **39**, **41**, and **43**, respectively. The protected prodrugs **36** and **41** were deprotected to **37** and **42** with formic acid. The final deprotection of **38**

Scheme 6[a]



[a] (a) 4,6-Diphenylthieno[3,4-*d*][1,3]dioxol-2-one 5,5-dioxide; (b) dibutyltin dilaurate, toluene; (c) HCO_2H; (d) $Pd(Ph_3P)_4$, pyrrolidine.

with formic acid failed. Therefore, the self-immolative prodrugs **40** and **44** were obtained by deprotection with Pd(0) and pyrrolidine of the corresponding diallyl L-glutamates **39** and **43** (see Scheme 6).

The corresponding active drugs **21** (**a**, X = Cl; **b**, X = F), **34** (X = Cl), and **51** (X = F) were obtained according to Scheme 7. The starting materials were 4-fluoronitrobenzene for the aniline series and 4-amino-*O*-benzylphenol for the phenol series. The fluorinated nitrogen mustards were obtained by the nucleophilic displacement of the mesyl group (after mesylation of **46** and **48**) with KF and 18-crown-6 ether, for both series.

Physicochemical and Kinetic Data

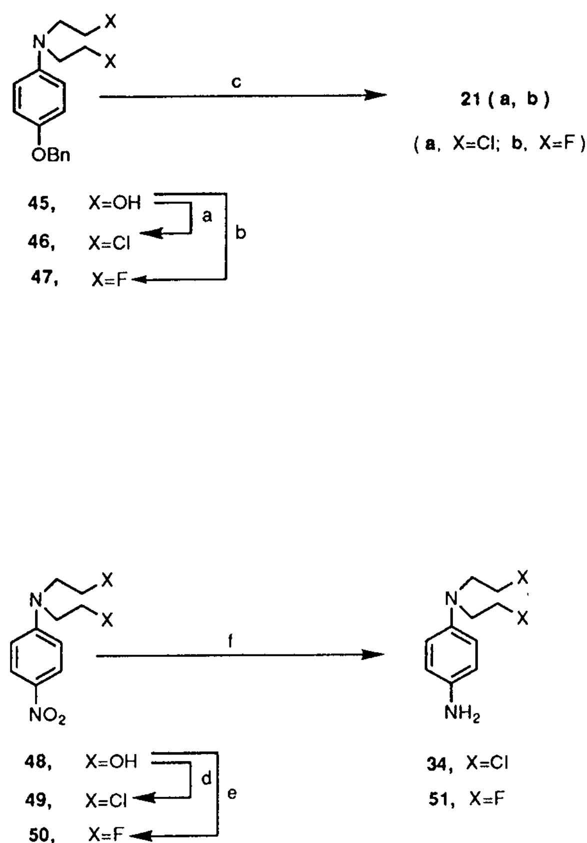
The chemical half-lives of the candidate prodrugs and parent drugs were determined by HPLC. The results are shown in Table 1. The K_M with CPG2 was determined for each of the novel potential prodrugs that had a $t_{1/2}$ greater than several minutes and for the deprotected linkers. The kinetic data are shown in Table 1.

The aqueous stability of the compounds (pH 7.4; 37 °C) varied considerably. The least stable prodrugs (**23**, **25**) showed little difference between the bischloro (**23**) and bisfluoro (**25**) congeners, implying that their instability lies elsewhere in the molecule. By contrast, and as expected, the bisfluoro compounds **42** and **44** were much more stable than their bischloro counterparts **37** and **40**. However, the relative stability of **30** as compared with **23** is surprising. It proved possible to determine the K_M's of the compounds **12**, **20**, **40**, **42**, and **44**. All were similar, in the <5 μM range. Prodrug **37** was shown to be a very poor substrate which required large amounts of enzyme (data not shown); thus full kinetic data could not be obtained. The k_{cat} for the isolated linker moieties **12** and **20** were determined.

Biological Evaluation

Cytotoxicity Assays. The synthesized prodrugs and some of the corresponding drugs were tested for cytotoxicity on the colon carcinoma cell line LS174T-stCPG2-(Q)3 made to express the surface-tethered CPG2. The plasmid pMCEF stCPG2(Q)3[15] encodes a CPG2 molecule that is expressed on the outer cell surface. It bears three asparagine-to-glutamine [(Q)3] mutations that

Scheme 7[a]



[a] (a) Mesyl chloride, Py, 70 °C; (b) mesyl anhydride, then KF, 18-crown-6, MeCN; (c) TFA, pentamethylbenzene; (d) $SOCl_2$, CH_2Cl_2; (e) mesyl anhydride, then F^-, $CHCl_3$; (f) H_2, Pd/C.

Table 1. Kinetic and Cytotoxicity (LS174T-stCPG2(Q)3) Data for Phenol and Aniline Self-Immolative Prodrugs and Their Bisfluoro Counterparts

compd	linker (L), prodrug (P), or drug (D)[a]	$t_{1/2}$, min	k_{cat},[b] s^{-1}	$t_{1/2}$, prodrug/drug	IC_{50}, μM
12	L1	602	+++[c]	NA	NA
20	L2	stable	+++[d]	NA	NA
23 (Cl)	P	2.9	+	0.3	0.48
25 (F)	P	2.4	+	NA	264.1
30 (Cl)	P	143	+	13.7	0.42
32 (F)	P	stable	+	NA	128.0
37 (Cl)	P	51	+	8.1	0.62 ± 0.21
40 (Cl)	P	48	++	7.6	0.46 ± 0.10
42 (F)	P	stable	++	NA	256.7
44 (F)	P	528	++	0.4	183.5
21a (Cl)	D	10.4	NA	NA	3.78 ± 1.83
34 (Cl)	D	6.3	NA	NA	0.34 ± 0.04
21b (F)	D	stable	NA	NA	322.9
51 (F)	D	1314	NA	NA	121.3
52 (Cl)	D	41	NA	NA	27.78 ± 1.27
CMDA	P	984	+++	17	24.74 ± 3.04

[a] This column describes the status of the compounds, being a prodrug (P), a drug (D), or a linker (L). [b] For +++, $k_{cat} > 50$ s^{-1}; for ++, $k_{cat} = 50-10$ s^{-1}; for +, $k_{cat} < 10$ s^{-1}; for all, $K_M < 5$ μM. [c] $k_{cat} = 65.4$ s^{-1} and $k_{cat}/K_M = 21.1$ s^{-1} μM. [d] $k_{cat} = 140.0$ s^{-1} and $k_{cat}/K_M = 83.3$ s^{-1} μM; NA, not applicable.

were otherwise inappropriately glycosylated. Two sublines were engineered and cloned. The control was transfected with the *LacZ* gene (LacZ), while the test line stCPG2(Q)3 expresses CPG2 tethered on the outer cell surface.[15] The LS174T-stCPG2(Q)3 cells were treated with the known prodrug CMDA for comparison with the synthesized prodrugs and the corresponding drugs. Expression of stCPG2(Q)3 in the LS174T results in an increased sensitivity to the treatment with CMDA compared with the control LS174T-LacZ-expressing cells. The IC_{50} of the assayed compounds against the stCPG2(Q)3-expressing cell clone, are presented in Table 1.

The phenol compounds (**25** and **30**) do not act as prodrugs, since they are as toxic as the corresponding drug in stCPG2(Q)3-expressing and LacZ-expressing cells. By contrast, the phenylenediamine compounds are successful prodrugs. Good IC_{50} prodrug/IC_{50} drug ratios of 20-fold (for **37**) and 33-fold (for **40**) and differentials of 12−14-fold between CPG2-expressing and control LacZ-expressing clones are achieved (see Figure 1A,B). The data that support these figures derive from the IC_{50} of the prodrug **37** in the control LS174T-LacZ line of 11.5 μM and the IC_{50} of the drug **34** in the test line of 0.34 μM resulting in a IC_{50} differential (11.5/0.34) of 33-fold. The result for the IC_{50} of the prodrug **40** in the control LS174T-LacZ line was 7 μM and the IC_{50} of the drug of 0.34 μM in the test line resulting in a IC_{50} differential (7/0.34) of 20-fold. The drugs released are between 7- and 70-fold more potent than the 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoic acid (**52**) released by CMDA (see Figure 1C).

These data demonstrate the viability of this strategy and indicate that self-immolative prodrugs can be synthesized to release potent mustard drugs selectively by CPG2 tethered to the cell surface.

Summary

Four new potential self-immolative prodrugs derived from phenol and aniline nitrogen mustards, four model compounds derived from their corresponding fluorinated analogues, and two new self-immolative linkers, all substrates for CPG2, were designed and synthesized.

The phenylenediamine compounds were found to behave as prodrugs, yielding IC_{50} prodrug/IC_{50} drug ratios between 20- and 33-fold (for **37** and **40**) and differentials of 12−14-fold between CPG2-expressing and control LacZ-expressing clones. The drugs released are up to 70-fold more potent than 4-[(2-chloroethyl)-(2-mesyloxyethyl)amino]benzoic acid that results from the prodrug CMDA which has been used previously for GDEPT.

Experimental Section

All starting materials, reagents, and anhydrous solvents were purchased from Aldrich, unless otherwise stated. The di-*tert*-butyl L-glutamate was bought from Novabiochem and diallyl L-glutamate from Fluka. 4-Bis(2-hydroxyethyl)amino-nitrobenzene was obtained using the known procedure.[27]

Kieselgel 60 (0.043−0.060) was used in gravity columns (Art. 9385, Merck). TLC was performed on precoated sheets of Kieselgel 60 F254 (Art. 5735, Merck). Preparative HPLC was performed on an Axxial Chromatospac Prep 10 (Jobin-Yvon), using Merck Kieselgel 60 (0.015−0.040) (Art. 15,111). Reverse-phase HPLC was performed on a ThermoQuest system using a 5-mm C18300A column Jupiter Phenomenex. Melting points were determined on a Kofler hot-stage (Reichert Thermovar) melting point apparatus and are uncorrected. Low-resolution EI and FAB spectra were performed on a VG-2AB-SE double focusing magnetic sector mass spectrometer (Fisons Instruments, Warrington, U.K.), operating at a resolution of 1000. High-resolution accurate mass spectra were determined on the same system, but with a resolution set to

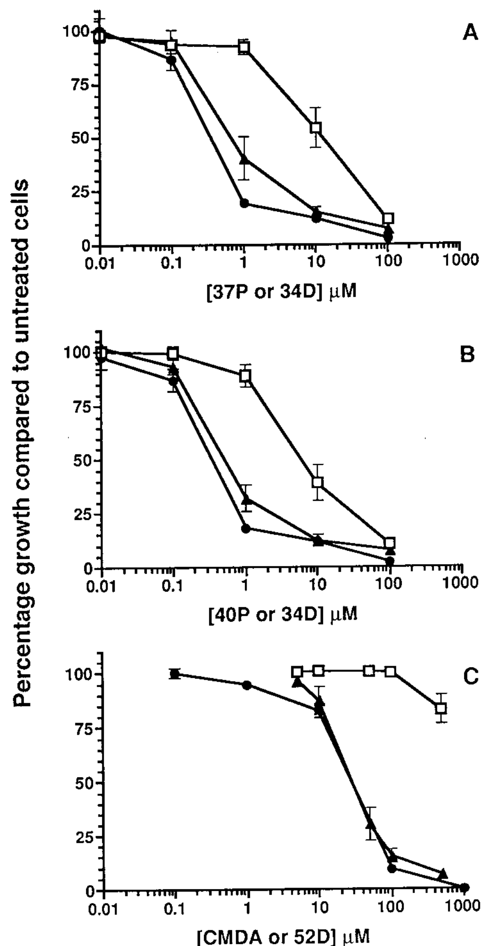


Figure 1. Sensitivity of LS174T cells expressing either control LacZ or stCPG2(Q)3 to challenge with prodrugs or drugs: (▲) cells expressing stCPG2(Q)3 in the presence of the prodrug; (●) cells expressing stCPG2(Q)3 in the presence of the drug; (□) cells expressing LacZ in the presence of the prodrug. Survival is determined by sulforhodamine assay, and the results are expressed as the proportion of cells surviving relative to similar untreated cells: A, incubation with prodrug **37** or drug **34** (mean of six separate experiments); B, incubation with prodrug **40** or drug **34** (mean of seven separate experiments); C, incubation with prodrug CMDA or drug **52** (mean of three separate experiments).

8000−10000. Masses are measured by peak matching the unknown with a mass of known composition. Reported spectra are by FAB unless otherwise stated. NMR spectra were determined in Me_2SO-d_6 on a 250-MHz spectrometer Brucker AC250 at 30 °C (303 K) unless otherwise stated. IR spectra (film) were recorded on a Perkin-Elmer 1720X FT-IR spectrometer. Elemental analyses were determined by Butterworth Laboratories Ltd. (Teddington, Middlesex, U.K.).

4-Nitrobenzyl *tert*-Butyldiphenylsilyl Ether (2a). To a stirred solution of 4-nitrobenzyl alcohol (1.00 g, 6.50 mmol) and imidazole (0.97 g, 14.10 mmol) in DMF (10.0 mL) was added *tert*-butyldiphenylchlorosilane (1.98 g, 1.87 mL, 7.20 mmol) over 10 min under nitrogen, at room temperature. The reaction mixture was stirred for an additional 5 h, diluted with 75 mL of Et_2O, washed with water (5 × 15 mL), dried ($MgSO_4$), and evaporated under vacuum. An oil, which crystallized on standing, was obtained. Recrystallization from aqueous 70% EtOH afforded 2.36 g (93%) of a white solid: mp 80−81 °C; ν_{max}/cm^{-1} (film) 2931, 2857 (CH_2), 1521, 1345 (NO_2); ^{1}H NMR δ_H 1.06 (s, 9H, t-Bu), 4.92 (s, 2H, $PhCH_2$), 7.42−7.46 (m, 5H, H_{arom}), 7.63−7.65 (m, 7H, Ph + $H_{arom2+6}$), 8.23 (d, 2H, $H_{arom3+5}$, J = 8.23 Hz); MS (EI) m/z 334 (M^+ − t-Bu, 100), 288 (M^+ − t-Bu − NO_2, 10), 256 (M^+ − t-Bu − Ph, 20), 199 (Ph_2SiOH^+, 100). Anal. ($C_{23}H_{25}NO_3Si$) C, H, N.

4-Nitrobenzyl 2′-Tetrahydropyranyl Ether (2b). To a stirred solution of 4-nitrobenzyl alcohol (1.53 g, 10.0 mmol) in CH_2Cl_2 (50 mL) were added 3,4-dihydropyran (1.36 mL, 1.26 g, 15.0 mmol) and pyridinium *p*-toluenesulfonate (0.25 g). After 1.5 h no starting material could be detected. The reaction mixture was evaporated to 4−5 mL, AcOEt (25 mL) added, and the solution washed with H_2O (3 × 50 mL) and brine, dried ($MgSO_4$), and evaporated under vacuum. The product **2b** (2.46 g, 99%) resulted as an oil: ^{1}H NMR δ_H 1.51−1.63 (m, 4H, $2H_{4'}$ + $2H_{5'}$), 1.66−1.74 (m, 2H, $2H_{3'}$), 3.50 (s, 1H, $H_{6'}$), 3.78 (m, 1H, $H_{6'}$), 4.62 (d, 1H, CH_2−Ph, J = 3.8 Hz), 4.72 (m, 1H, $H_{2'}$), 4.81 (d, 1H, CH_2−Ph), 7.62 (d, 2H, H_{arom}, J = 8.5 Hz), 8.22 (d, 2H, H_{arom}); MS m/z 260 (M^+ + 23, 15), 236 (M^+ − 1, 45), 136 (4-$NO_2C_6H_5CH_2^+$, 100).

4-Aminobenzyl *tert*-Butyldiphenylsilyl Ether (3a). To a stirred solution of **2a** (5.00 g, 12.80 mmol) in EtOH (100 mL) were added 10% Pd/C (1.50 g) and ammonium formate (4.60 g) in one portion at 20 °C. After 1.5 h no starting material could be detected. The catalyst was removed by filtration, the filtrate concentrated under vacuum, and the residue partitioned between EtOAc and H_2O. The organic layer was dried ($MgSO_4$) and concentrated to give **3a** (4.24 g, 91%) as an oil: ν_{max}/cm^{-1} (film) 3433, 3378 (NH_2), 2931, 2857 (CH_2); ^{1}H NMR δ_H 1.00 (s, 9H, t-Bu), 4.57 (s, 2H, $PhCH_2$), 4.98 (s broad, 2H, NH_2), 6.52 (d, 2H, $H_{arom3+5}$, J = 8.25 Hz), 6.96 (d, 2H, $H_{arom2+6}$), 7.42−7.46 (m, 5H, H_{arom}), 7.62−7.65 (m, 5H, H_{arom}); MS m/z 361 (M^+, 8), 304 (M^+ − t-Bu, 100), 199 (Ph_2SiOH^+, 100). Anal. ($C_{23}H_{27}NOSi$) C, H; N: calcd, 3.87; found, 4.33.

4-Aminobenzyl *O*-2′-Tetrahydropyranyl Ether (3b). The same procedure as for **3a**, starting from **2b** (8.53 g, 35.9 mmol), led to a three-component reaction mixture (6.40 g) after workup, which was separated by column chromatography (cyclohexane:AcOEt, 1:1). The desired compound **3b** eluted first as an oil (2.59 g, 38%): ν_{max}/cm^{-1} (film) 3447, 3360 (NH_2), 2943, 2870 (CH_2); ^{1}H NMR δ_H 1.42−1.53 (m, 4H, $2H_{4'}$ + $2H_{5'}$), 1.55−1.72 (m, 2H, $2H_{3'}$), 3.45 (s, 1H, $H_{6'}$), 3.79 (m, 1H, $H_{6'}$), 4.22 (d, 1H, $PhCH_2$, J = 11.1 Hz), 4.37 (d, 1H, $PhCH_2$), 4.60 (d, 1H, $H_{2'}$), 5.00 (s, 2H, NH_2), 6.52 (d, 2H, H_{arom}, J = 8.23 Hz), 6.97 (d, 2H, H_{arom}); MS (EI) m/z 207 (M^+, 100), 106 ($H_2NC_6H_4CH_2^+$, 100). Anal. ($C_{12}H_{17}NO_2$) C, H, N.

The fraction eluting second, as an oil (1.15 g, 18%), was the **4-*N*-(5′-hydroxypentyl)toluidine, 13**: ν_{max}/cm^{-1} (film) 3353 (NH-, OH, broad), 2934, 2861 (CH_2); ^{1}H NMR δ_H* 1.39−1.55 (m, 6H, CH_{2b+c+d}), 2.13 (s, 3H, $PhCH_3$), 2.93 (q, 2H, CH_{2a}, J = 6.60 Hz), 3.39 (q, 2H, CH_{2e}, J = 6.10 Hz), 4.31 (t, 1H, OH or NH, J = 5.19 Hz), 5.19 (t, 1H, NH or OH), 6.45 (d, 2H, H_{arom}, J = 8.15 Hz), 6.86 (d, 2H, H_{arom}); MS (EI) m/z 193 (M^+, 18), 120 ($CH_3C_6H_4NHCH_2^+$, 100) (*for numbering see formula **13**).

The third fraction, eluting as an oil (0.88 g, 9%), was ***N*-(5′-hydroxypentyl)aminobenzyl 2′-*O*-tetrahydropyranyl ether, 14**: ν_{max}/cm^{-1} (film) 3367 (NH, OH, broad), 2937, 2863 (CH_2); ^{1}H NMR δ_H* 1.36−1.59 (m, 12H, $2H_b$ + $2H_c$ + $2H_d$ + $2H_{3'}$ + $2H_{4'}$ + $2H_{5'}$), 2.97 (q, 2H, $2H_a$, J = 5.91 Hz), 3.40 (q, 2H, $2H_e$), 3.50 (m, 1H, $H_{6'}$), 3.80 (m, 1H, $H_{6'}$), 4.24 (d, 1H, $PhCH_2$, J = 11.10 Hz), 4.32 (t, 1H, OH or NH, J = 5.23 Hz), 4.48 (d, 1H, $PhCH_2$), 4.60 (d, 1H, $H_{2'}$), 5.50 (t, 1H, NH or OH), 6.51 (d, 2H, H_{arom}, J = 8.81 Hz), 7.02 (d, 2H, H_{arom}); MS (EI) m/z 293 (M^+, 52), 192 ($C_5H_{10}NHC_6H_4CH_2^+$, 100) (*for numbering see formula **13**).

***tert*-Butyldiphenylsilyl 4-Oxymethylphenyl Isocyanate (4).** To a stirred solution of **3a** (0.63 g, 1.70 mmol) and triethylamine (0.25 mL, 0.18 g, 1.80 mmol) in toluene (10 mL) heated at 70 °C was added triphosgene (0.18 g, 0.61 mmol) in one portion. The reaction was monitored by IR (ν_{NCO} 2275 cm^{-1}). After 5 h the reaction mixture was filtered and the filtrate evaporated under vacuum, giving **4** (0.65 g, 99%) as an oil which was used without further purification: ν_{max}/cm^{-1} (film) 2931, 2857 (CH_2), 2275 (NCO); ^{1}H NMR δ_H 1.03 (s, 9H, t-Bu), 4.76 (s, 2H, $PhCH_2$), 7.23 (d, 2H, $H_{arom3+5}$, J = 8.38 Hz), 7.35 (d, 2H, $H_{arom2+6}$), 7.37−7.48 (m, 5H, H_{arom}), 7.62−7.71 (m, 5H, H_{arom}); MS (EI) m/z 330 (M^+ − t-Bu, 52), 286 (M^+ − t-Bu-NCO, 48), 199 (Ph_2SiOH^+, 100).

Di-*tert*-butyl *N*-[(4-{[*tert*-Butyldiphenylsilyl]oxymethyl}-phenyl)carbamoyl]-L-glutamate (7a) and *tert*-Butyl 1-

[*N*-(4-{[*tert*-butyldiphenylsilyl]oxymethyl}phenyl)-carbamoyl]-5-oxopyrrolidine-2-carboxylate (10). Method A: To a solution containing 6.03 g (13.10 mmol) of isocyanate **4** and 3.9 mL (2.64 g, 26.2 mmol) of triethylamine in 60 mL of THF was added at room temperature, during 30 min and under stirring, a solution of di-*tert*-butyl L-glutamate hydrochloride (3.90 g, 13.10 mmol) in 20 mL of THF. After 3 h the reaction was complete. The precipitate was filtered and the solvent removed under vacuum, leaving an oil. The oil was redissolved in AcOEt (25 mL), washed with H_2O (25 mL), aqueous HCl (2%) (25 mL), aqueous Na_2CO_3 (2%) (25 mL), and brine (2 × 25 mL), dried, and evaporated again giving 7.53 g of an oil. The product was purified by column chromatography (AcOEt:cyclohexane, 2:1). The first eluting compound was **7a** (5.31 g, 63%) as a solid: mp 89−90 °C (hexane); ν_{max}/cm^{-1} (film) 3359 (NH), 2932, 2857 (CH_2), 1729 (C=O, ester), 1670 (C=O, urea), 1154 (C−O); ^{1}H NMR δ_H 1.03 (s, 9H, t-Bu), 1.40 (s, 9H, t-Bu-G), 1.43 (s, 9H, t-Bu-G), 1.68−2.00 (2m, 2H, CH_2(NH)-CH), 2.18−2.32 (m, 2H, CH_2CO_2), 4.08−4.12 (m, 1H, CH(NH)-CH_2), 4.68 (s, 2H, $PhCH_2$), 6.38 (d, 1H, NH-G, J = 8.12 Hz), 7.19 (d, 2H, $H_{arom3+5}$, J = 8.41 Hz), 7.32−7.47 (m, 7H, H_{arom} + $H_{arom2+6}$), 7.62−7.70 (m, 5H, H_{arom}), 8.54 (s, 1H, NH−Ph); MS (EI) m/z 590 (M^+ − t-Bu + 1, 2), 534 (M^+ − 2t-Bu, 5), 478 (M − 3t-Bu, 100), 199 (Ph_2SiOH^+, 100). Anal. ($C_{37}H_{50}N_2O_6Si$) C, H, N.

The compound eluting second was **10**, an oil (0.39 g, 5%), which was deprotected without further characterization.

Method B (one-pot synthesis of compound 7a): To a solution of di-*tert*-butyl L-glutamate hydrochloride (4.14 g, 14.0 mmol) and triphosgene (1.39 g, 4.67 mmol) in toluene, cooled at −78 °C, was added triethylamine (3.90 mL, 2.83 g, 28 mmol) in toluene (10 mL) dropwise over 30 min. The reaction ws allowed to reach room temperature and finished in 50 min (monitored by IR, ν_{NCO} 2253 cm^{-1}). To this mixture was added a solution containing 4-aminobenzyl *tert*-butyldiphenylsilyl ether (**3a**) (5.00 g, 13.8 mmol) and triethylamine (1.95 mL, 14.0 mmol) in 30 mL of toluene over 5−10 min. The reaction was monitored by IR (disappearance of the ν_{NCO} 2253 cm^{-1} peak) and was finished in 14−20 h. The reaction mixture was filtered, washed with H_2O (200 mL), aqueous HCl (1%) (200 mL), aqueous Na_2CO_3 (1%) (200 mL), and H_2O (2 × 200 mL), dried ($MgSO_4$), and evaporated under vacuum to give an oil (9.90 g). The product was deprotected without further purification.

Diprop-2-enyl *N*-[(4-{[2′-*O*-tetrahydropyranyl]oxy-methyl}phenyl)carbamoyl]-L-glutamate (7b) was obtained, starting from diallyl L-glutamate *p*-toluenesulfonate (1.08 g, 3.1 mmol) and **3b** (0.52 g, 2.5 mmol) by the one-pot procedure. An oil resulted which was separated by preparative HPLC using cyclohexane:AcOEt (1.5:1) as eluent (0.75 g, 65%): ν_{max}/cm^{-1} (film) 3362 (NH_2), 2944, 2871 (CH_2); ^{1}H NMR δ_H 1.47−1.50 (m, 4H, $2H_{4'}$ + $2H_{5'}$), 1.60−1.88 (m, 3H, $2H_{3'}$ + CH_2(NH)-CH), 1.88−2.05 (m, 1H, −CH_2CH(NH)−), 2.37−2.45 (m, 2H, CH_2CO_2), 3.45 (s, 1H, $H_{6'}$), 3.80 (m, 1H, $H_{6'}$), 4.20−4.31 (m, 1H, CH(NH)CH_2), 4.35 (d, 1H, $PhCH_2$, J = 11.55 Hz), 4.53−4.63 (m, 5H, $PhCH_2$ + CH_2O, allyl), 5.18−5.33 (m, 4H, CH_2= allyl), 5.80−6.00 (m, 2H, CH= allyl), 6.60 (d, 1H, NH-G, J = 8.03 Hz,), 7.20 (d, 2H, H_{arom}, J = 8.47 Hz), 7.35 (d, 2H, H_{arom}), 8.47 (s, 1H, NH−Ph); MS (EI) m/z 461 (M^+ + 1, 15). Mass ($C_{24}H_{32}N_2O_7Na$) calcd, 483.2107; found, 483.2120.

Diprop-2-enyl *N*-[(4-{[*tert*-butyldiphenylsilyl]oxy-methyl}phenyl)carbamoyl]-L-glutamate (7c) was synthesized by the same method starting from 5.0 g (13.8 mmol) of amine **3a**. An oil resulted which was purified by column chromatography (cyclohexane:AcOEt, 2:1) leading to **7c** (6.47 g, 47%): ν_{max}/cm^{-1} (film) 3382 (NH_2), 1739 (C=O, ester), 1651 (C=O, amide); ^{1}H NMR δ_H 1.03 (s, 9H, t-Bu), 1.85−2.11 (2m, 2H, CH_2(NH)CH), 2.46−2.52 (m, 2H, CH_2CO_2), 4.32−4.35 (m, 1H, CH(NH)CH_2), 4.56 (dd, 2H, CH_2= allyl, J = 4.62 Hz), 4.62 (dd, 2H, CH_2= allyl), 4.69 (s, 2H, $PhCH_2$), 5.17−5.38 (m, 4H, CH_2O allyl), 5.80−6.00 (m, 2H, CH= allyl), 6.60 (d, 1H, NH-G, J = 8.03 Hz), 7.20 (d, 2H, $H_{arom3+5}$, J = 8.49 Hz), 7.34−7.48 (m, 7H, H_{arom} + $H_{arom2+6}$), 7.63−7.72 (m, 5H, H_{arom}), 8.57 (s, 1H, NH−Ph).

Di-*tert*-butyl *N*-[(4-{Hydroxymethyl}phenyl)carbamoyl]-L-glutamate (8a). Compound **7a** (5.15 g, 8.0 mmol) was dissolved in THF (100 mL) and tetrabutylammonium fluoride solution in THF (1 M, 20.0 mL, 2.5 equiv) was added, in one portion, under stirring at room temperature. The reaction was finished in 3 h. The reaction mixture was evaporated under vacuum. The residue was dissolved in AcOEt (50 mL), washed with H_2O (3 × 100 mL), dried ($MgSO_4$), and evaporated again. A yellow oil (5.08 g) resulted which was purified by column chromatography (AcOEt:cyclohexane, 3:1) yielding an oil (1.88 g, 58%) which crystallized on standing: mp 103−4 °C (aqueous MeOH, 60%); ν_{max}/cm^{-1} (film) 3370 (broad, NH+OH), 2967 (CH_3), 2930, 2857 (CH_2), 1716 (C=O, ester), 1678 (C=O, amide), 1153 (C−O). Anal. ($C_{21}H_{32}N_2O_6$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

The fraction eluting second was ***tert*-butyl 1-[*N*-(4-{hydroxymethyl}phenyl)carbamoyl]-5-oxopyrrolidine-2-carboxylate (11)** (0.356 g, 13%): white solid, mp 157−9 °C; ν_{max}/cm^{-1} (film) 3354 (NH_2), 2977, 2932 (CH_2), 1719 (C=O, ester), 1676 (C=O, amide); ^{1}H NMR δ_H 1.41 (s, 9H, t-Bu), 1.90−2.08 (2m, 2H, CH_2(NH)CH), 2.37 (t, 2H, CH_2CO, J = 7.67 Hz), 4.23 (t, 1H, CH(N)CH_2), 4.52 (d, 2H, CH_2, J = 5.64 Hz), 5.23 (t, 1H, CH_2OH), 7.28 (d, 2H, $H_{arom3+5}$, J = 8.36 Hz), 7.39 (d, 2H, $H_{arom2+6}$), 8.44 (s, 1H, PhNH); MS (EI) m/z 334 (M^+, 16), 278 (M^+ − t-Bu, 82). Anal. ($C_{17}H_{22}N_2O_5$) C, H; N: calcd, 8.38; found, 7.92.

Diprop-2-enyl *N*-[(4-{Hydroxymethyl}phenyl)carbamoyl]-L-glutamate (8b). The intermediate **7b** (0.610 g, 1.3 mmol) was hydrolyzed at 45 °C in a mixture of AcOH:THF:H_2O (24.5 mL, 4:2:1) for 3.5 h. The reaction mixture was diluted with H_2O (50 mL) and extracted with ether (2 × 25 mL) and then with AcOEt (2 × 30 mL). The pooled organic layers were washed with H_2O (2 × 30 mL), dried ($MgSO_4$), and evaporated to dryness (with addition of toluene, 2 × 30 mL). An oil resulted which was purified by preparative HPLC (cyclohexane:AcOEt, 1:2), leading to **8b** (0.221 g, 45%): ν_{max}/cm^{-1} (film) 3354 (NH_2, OH, broad), 1737 (C=O, ester), 1659 (C=O, amide). Anal. ($C_{19}H_{24}N_2O_6$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Di-*tert*-butyl *N*-[(4-{[4-Nitrophenoxycarbonyloxy]-methyl}phenyl)carbamoyl]-L-glutamate (9a). To a stirred solution of **8a** (0.200 g, 0.49 mmol) in dry THF (10 mL) were added 4-nitrophenyl chloroformate (0.11 g, 0.5 mmol) and triethylamine (0.1 mL, 0.6 mmol) at room temperature. The reaction was complete after 1 h. The formed precipitate was filtered and the solution concentrated under vacuum. AcOEt (10 mL) was added; the solution was washed with brine (2 × 10 mL), dried ($MgSO_4$), and evaporated again, giving an oil which was purified by column chromatography (0.160 g, 57%) and repurified by preparative HPLC (0.140 g, 50%): mp 55−6 °C; ν_{max}/cm^{-1} (film) 3349 (NH_2), 2979, 2932 (CH_2), 1767 (C=O, carbonate), 1716 (C=O, ester), 1652 (C=O, amide), 1527, 1349 (NO_2). Mass ($C_{28}H_{36}N_3O_{10}$) calcd, 574.2401; found, 574.2420. Anal. ($C_{28}H_{35}N_3O_{10}$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Diprop-2-enyl *N*-[(4-{[4-Nitrophenoxycarbonyloxy]-methyl}phenyl)carbamoyl]-L-glutamate (9b). Starting from **8b** (0.190 g, 0.50 mmol), **9b**, obtained by the same procedure as **9a**, was purified by preparative HPLC to a solid (0.132 g, 48.6%): mp 106−7 °C; ν_{max}/cm^{-1} (film) 3356 (NH_2), 2933 (CH_2), 1766 (C=O, carbonate), 1738 (C=O, ester), 1660 (C=O, amide), 1525, 1346 (NO_2). Mass ($C_{26}H_{27}N_3O_{10}Na$) calcd, 564.1594; found, 564.1590. Anal. ($C_{26}H_{27}N_3O_{10}$) H, N; C: calcd, 57.67; found, 58.09. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

[(4-Hydroxymethylphenyl)carbamoyl]-L-glutamic Acid (12). In the stirred solution of **8b** (0.200 g, 0.53 mmol) in CH_2Cl_2 (15 mL) were added $Pd(PPh_3)_4$ (40 mg) and pyrrolidine (1 mL, 11.8 mmol). On addition of pyrrolidine, a precipitate started to form immediately. After 40 min of stirring, the solvent was removed and the precipitate washed with AcOEt and CH_2Cl_2. The residue was dissolved in methanol (10 mL) and passed through a weakly acid resin IRC50 ion-exchange column. After eluting with methanol (50 mL), the eluate was evaporated under vacuum to yield **12** (0.135 g, 86%) as a solid;

mp 73−6 °C. Mass ($C_{13}H_{17}N_2O_6$) calcd, 297.1087; found, 297.1070. Anal. ($C_{13}H_{16}N_2O_6$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Di-*tert*-butyl *N*-[(4-{1,3-Dithian-2-yl}phenyloxy)carbonyl]-L-glutamate (16). Compound **16** was prepared by the one-pot procedure (as described above for compound **7a**, method B) from di-*tert*-butyl L-glutamate hydrochloride (1.50 g, 5.0 mmol) and 4-[2′-(1′,3′-dithianyl)]phenol, **15** (1.10 g, 5.0 mmol). The reaction was left for 12 h. The solid formed was filtered; the solution was washed with aqueous NaOH (1%) (75 mL) and H_2O (2 × 75 mL), dried ($MgSO_4$), and evaporated under vacuum to yield a solid (2.30 g, 95.8%). Recrystallization from aqueous 50% EtOH afforded **16** (1.81 g, 72.7%): mp 137−8 °C; ν_{max}/cm^{-1} (film) 3347 (NH_2), 2977, 2945 (CH_2), 1728 (C=O, ester); ^{1}H NMR δ_H 1.41 (s, 9H, t-Bu), 1.42 (s, 9H, t-Bu), 1.62−1.90 (m, 2H, $2H_{5'}$), 1.90−2.32 (2m, 2H, CH_2(NH)CH), 2.30−2.40 (m, 2H, CH_2CO_2), 2.86−2.93 (m, 2H, $2H_{4'or6'}$), 3.09 (t, 2H, $2H_{6'or4'}$, J = 12.3 Hz), 5.41 (s, 1H, $H_{2'}$), 7.08 (d, 2H, $H_{arom3+5}$, J = 8.50 Hz), 7.43 (d, 2H, $H_{arom2+6}$), 8.12 (d, 1H, NH-G, J = 7.81 Hz); MS (EI) m/z 497 (M$^+$, 2), 451 (M$^+$ − t-Bu, 2), 359 (M$^+$ − 2t-Bu, 20). Anal. ($C_{24}H_{25}NO_6S_2$) C, N; H: calcd, 7.09; found, 6.68.

Di-*tert*-butyl *N*-[(4-Formylphenyloxy)carbonyl]-L-glutamate (17a). Procedure A: To a stirred solution of dithiane **16** (0.50 g, 1.0 mmol) in $CHCl_3$ (10 mL) and THF (5 mL) was added dropwise a solution of $Hg(ClO_4)_2 \cdot 3H_2O$ (0.91 g, 2.0 mmol) in THF (5 mL). The reaction was completed in 5 min. The precipitate was filtered and the solution washed with H_2O (2 × 25 mL), aqueous Na_2CO_3 (25 mL), and H_2O (2 × 25 mL), dried ($MgSO_4$), and evaporated under vacuum. The compound **17a** (0.33 g, 81%) was obtained as a clear oil.

Procedure B: To a solution of di-*tert*-butyl L-glutamyl isocyanate **6a** (prepared from di-*tert*-butyl L-glutamate hydrochloride (0.200 g, 0.67 mmol), triphosgene (0.067 g, 0.22 mmol), and triethylamine (0.190 mL, 1.35 mmol) according to the procedure described above) in $CHCl_3$ (15 mL) were added at room temperature 4-hydroxybenzaldehyde (0.082 g, 0.67 mmol) and triethylamine (0.190 mL, 1.35 mmol) in $CHCl_3$ (10 mL) over 10 min. The reaction was heated at reflux for 2 h, when the ν_{NCO} peak disappeared. The solvent was removed under vacuum and the compound purified by preparative HPLC (cyclohexane:AcOEt, 6:1). A clear oil resulted (0.197 g, 72%): ν_{max}/cm^{-1} (film) 3347 (NH_2), 2979, 2934 (CH_2), 1731 (C=O, ester), 1712 (C=O, aldehyde); ^{1}H NMR δ_H 1.41 (s, 9H, t-Bu), 1.43 (s, 9H, t-Bu), 1.80−2.02 (2m, 2H, CH_2(NH)CH), 2.32−2.39 (m, 2H, CH_2CO_2), 3.99−4.04 (m, 1H, −CH(NH)CH_2-), 7.34 (d, 2H, $H_{arom3+5}$, J = 8.39 Hz), 7.96 (d, 2H, $H_{arom2+6}$), 8.29 (d, 1H, NH-G, J = 7.72 Hz), 9.98 (s, 1H, CHO). Anal. ($C_{21}H_{29}NO_7$) C, H, N.

Diprop-2-enyl *N*-[(4-formylphenyloxy)carbonyl]-L-glutamate (17b) was obtained as a clear oil (2.90 g, 76%) using procedure B: ν_{max}/cm^{-1} (film) 3347 (NH_2), 2979, 2934 (CH_2), 1731 (C=O, ester), 1712 (C=O, aldehyde); ^{1}H NMR δ_H 1.93−2.16 (2m, 2H, CH_2(NH)CH), 2.53 (t, 2H, CH_2CO_2), 4.19−4.26 (m, 1H, CH(NH)CH_2), 4.57 (d, 2H, CH_2-allyl, J = 5.35 Hz), 4.64 (d, 2H, CH_2-allyl), 5.18−5.37 (m, 4H, CH_2=allyl, J = 5.35 Hz), 5.86−5.94 (m, 2H, CH=allyl), 7.35 (d, 2H, $H_{arom3+5}$, J = 8.50 Hz), 7.77 (d, 2H, $H_{arom2+6}$), 8.49 (d, 1H, NH-G, J = 7.77 Hz), 9.98 (s, 1H, CHO); MS m/z 398 (M$^+$ + 23, 16), 376 (M$^+$ + 1, 14). Anal. ($C_{19}H_{21}NO_7$) C, H; N: calcd, 3.73; found, 4.16.

Di-*tert*-butyl *N*-[(4-{Hydroxymethyl}phenoxy)carbonyl]-L-glutamate (18a). Compound **17a** (0.150 g, 0.36 mmol) dissolved in a mixture of H_2O:AcOH:CH_3OH (5.2 mL, 1:1:1) was reduced with sodium cyanoborohydride (0.027 g, 0.43 mmol) at room temperature. The reaction was complete after 1 h. AcOEt (20 mL) was added, the reaction mixture evaporated under vacuum, the residue dissolved in AcOEt (20 mL), and the solution washed with H_2O (20 mL) and brine (2 × 20 mL), dried ($MgSO_4$), and evaporated to dryness. Compound **18a** (0.127 g, 86%) was obtained as an oil. The final purification was achieved by preparative HPLC (cyclohexane:AcOEt, 2:1): ν_{max}/cm^{-1} (film) 3352 (NH_2, OH, broad), 2979, 2945 (CH_2), 1728 (C=O, ester), 1712 (C=O, aldehyde). Mass ($C_{21}H_{31}NO_7$-Na) calcd, 432.1998; found, 432.1990. Anal. ($C_{21}H_{31}NO_7$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Diprop-2-enyl *N*-[(4-{Hydroxymethyl}phenoxy)carbonyl]-L-glutamate (18b). Compound **18b** was obtained by a similar reduction procedure as a clear oil (0.233 g, 78%). Mass ($C_{19}H_{23}NO_7Na$) calcd, 400.1372; found, 400.1376. Anal. ($C_{28}H_{35}N_3O_{10}$) H, N; C: calcd, 60.47; found, 60.01. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Di-*tert*-butyl *N*-[(4-{[4-Nitrophenoxycarbonyloxy]methyl}phenoxy)carbonyl]-L-glutamate (19a). Activation of linker **18a** (1.0 g, 2.7 mmol) was achieved by the same procedure as for **9a**. After purification, an oil resulted (0.64 g, 41%): ν_{max}/cm^{-1} (film) 3367 (NH_2), 2980, 2933 (CH_2), 1767 (C=O, carbonate), 1728 (C=O, ester). Anal. ($C_{28}H_{34}N_2O_{11}$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Starting from **18b** (0.4 g, 1.06 mmol), **19b** was obtained by the same method as an oil (0.448 g, 75.5%). Mass ($C_{26}H_{26}N_2O_{11}$-Na) calcd, 565.1434; found, 565.1430. Anal. ($C_{26}H_{26}N_2O_{11}$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

[(4-Hydroxymethylphenyl)oxycarbonyl]-L-glutamic Acid (20). Starting from **18b** (150 mg, 0.4 mmol) in CH_2Cl_2 (12 mL) and using $Pd(PPh_3)_4$ (25 mg) and pyrrolidine (152 mL, 1.8 mmol), compound **20** was obtained by the procedure described above for compound **12**. However, the compound eluting from the IRC50 ion-exchange column, after evaporation under vacuum, led only to an impure **20** (103 mg). This product was purified by reverse-phase (C18) preparative HPLC using as mobile phase a gradient of 0.1% aqueous TFA/acetonitrile. After lyophilization **20** (36 mg, 30%) was obtained as a white powder: mp 135−7 °C. Mass ($C_{13}H_{15}NO_7Na$) calcd, 320.0746; found, 320.0730. Anal. ($C_{13}H_{15}NO_7$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Diprop-2-enyl *N*-[(4-{[4-(Bis{2-chloroethyl}amino)-phenoxy carbonyloxy]methyl}phenyl)carbamoyl]-L-glutamate (22). The solution containing the activated linker **9b** (0.200 g, 0.37 mmol), 4-bis(2-chloroethyl)aminophenol (**21a** (X = Cl)), (0.140 g, 0.60 mmol) dissolved in CH_3CN (10 mL), and a strongly basic resin-Amberlyst 27 HO$^-$ (0.13 g) was stirred for 20 h. The resin was filtered off, and a further 0.080 g of fresh resin was added. After 30 min of stirring, the second portion of resin was filtered off and the solvent evaporated. The product was purified by column chromatography (cyclohexane:AcOEt, 2:1) to afford **28** (82 mg, 32%) as a solid: mp 134−5 °C. Mass ($C_{30}H_{35}N_3O_8Cl_2Na$) calcd, 658.1699; found, 658.1690. Anal. ($C_{30}H_{35}N_3O_8Cl_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Diprop-2-enyl *N*-[(4-{[4-(Bis{2-fluoroethyl}amino)-phenoxycarbonyloxy]methyl}phenyl)carbamoyl]-L-glutamate (24). **24** (120 mg, 40%) was obtained as a solid, mp 129−31 °C, by a similar procedure as **22**. Mass ($C_{30}H_{36}N_3O_8F_2$) calcd, 604.2470; found, 604.2450. Anal. ($C_{30}H_{35}N_3O_8F_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[4-(Bis{2-chloroethyl}amino)phenoxycarbonyloxy]methyl}phenyl)carbamoyl]-L-glutamic Acid (23).** To a solution of **22** (0.200 g, 0.32 mmol) and $Pd(PPh_3)_4$ (25 mg, 0.022 mmol) in CH_2Cl_2 (9 mL) was added morpholine (107 mL, 1.23 mmol), and the mixture stirred for 2 h under argon at room temperature. The reaction mixture was diluted with AcOEt (a precipitate formed upon addition of AcOEt). The solvent was evaporated, and the insoluble residue was washed with AcOEt. It was dissolved in methanol (10 mL) and passed through a weakly acid resin, IRC50 ion-exchange column. After eluting with methanol (50 mL), the eluate was evaporated under vacuum to yield **23** (0.145 g, 81%) as a solid: mp 73−6 °C. Mass ($C_{24}H_{27}N_3O_8Cl_2Na$) calcd, 578.1073; found, 578.1070. Anal. ($C_{24}H_{27}N_3O_8Cl_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[4-(Bis{2-fluoroethyl}amino)phenoxycarbonyloxy]methyl}phenyl)carbamoyl]-L-glutamic acid (25)** was

obtained as a white solid (68 mg, 72%), mp 104−7 °C, by a procedure similar to **23**. Mass ($C_{24}H_{27}N_3O_8F_2Na$) calcd, 546.1664; found, 546.1680. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

4-[Bis(2-fluoroethyl)amino]phenyl Chloroformate (27a). Over a solution of **21b** (0.2 g, 1.0 mmol) in CH_2Cl_2 (8 mL) phosgene (20% in toluene, 3.5 mL, 7 mmol) was added. After 5 min of stirring, a solution of NEt_3 (0.14 mL, 1 mmol) in CH_2Cl_2 (8 mL) was added dropwise over 15 min. The solvent was evaporated, the residue taken up in AcOEt, the precipitate filtered off, and the filtrate evaporate to dryness to afford **27a** (0.184 g, 74.5%) as an oil: IR ν_{max}/cm^{-1} (film) 1780 (OCOCl), 1515, 1117; ^{1}H NMR ($CDCl_3$) δ_H 3.73 (2t, 4H, NCH_2, $J = 5.20$ Hz, $J_{H-F} = 23.63$ Hz), 4.61 (2t, 4H, CH_2F, $J = 5.22$ Hz, $J_{H-F} = 47.15$ Hz), 6.68 (d, 2H, $H_{arom2+6}$, $J = 9.26$ Hz), 7.08 (d, 2H, $H_{arom3+5}$).

4-Bis(2-chloroethyl)aminophenyl Trimethylsilyl Ether (28a). Phenol nitrogen mustard **21a** (X = Cl) (60 mg, 0.26 mmol) and trimethylsilyl cyanide (40 mL, 0.3 mmol) were stirred at room temperature without solvent for 30 min. Boiling hexane (10 mL) was added to the reaction mixture and the remaining solid filtered off. After evaporation, **28a** (70 mg, 88%) as an oil resulted: ^{1}H NMR δ_H 0.20 (s, 9H, $Si(CH_3)_3$), 3.55−3.70 (m, 8H, $N(CH_2CH_2Cl)_2$), 6.65 (d, 2H, $J = 9.30$ Hz, $H_{arom2+6}$), 6.73 (d, 2H, $H_{arom3+5}$); MS *m/z* 305 (M^+, 100), 270 ($M^+ - Cl$, 35). Mass ($C_{13}H_{21}NOCl_2Si$) calcd, 305.0769; found, 305.0765. Anal. ($C_{13}H_{21}NOCl_2Si$) C, H, N, Cl.

4-Bis(2-fluoroethyl)aminophenyl Trimethylsilyl Ether (28b). Method A: 28b (0.65 g, 80%) was synthesized according to the same procedure as **21b** but using excess trimethylsilyl cyanide and longer time periods: ^{1}H NMR δ_H 0.19 (s, 9H, $Si(CH_3)_3$), 3.61 (2t, 4H, NCH_2, $J = 5.20$ Hz, $J_{H-F} = 24.44$ Hz), 4.54 (2t, 4H, CH_2F, $J = 5.17$ Hz, $J_{H-F} = 47.55$ Hz), 6.68 (s, 4H, H_{arom}); MS *m/z* 273 (M^+, 100), 254 ($M^+ - F$, 23). Mass ($C_{13}H_{21}NOF_2Si$) calcd, 273.1360; found, 273.1345. Anal. ($C_{13}H_{21}NOF_2Si$) C, H, N.

Method B: Over a solution of **21b** (0.25 g, 1.25 mmol) in CH_2Cl_2 (3 mL) trimethylsilyl *N,N*-dimethylcarbamate (0.475 mL, 2.74 mmol) was added under argon. After 1.5 h, the solvent was evaporated, the residue taken in hexane, and the precipitate filtered off. The hexane was evaporated to dryness to afford **28b** (0.24 g, 70%) as an oil.

Diprop-2-enyl *N*-[(4-{[4-(Bis{2-chloroethyl}amino)phenoxycarbonyloxy]methyl}phenoxy)carbonyl]-L-glutamate (29). Over a solution of **18b** (0.375 g, 1.0 mmol) in CH_2Cl_2 (15 mL) cooled to −78 °C, phosgene (20% in toluene, 0.75 mL, 1.5 mmol), 18-crown-6 ether (0.255 g, 1.0 mmol), and KF·HF (0.3 g, 3.8 mmol) were added. After stirring for 12 h and allowing the mixture to warm to room temperature, the fluoroformate **27** was formed (ν_{OCOF} 1827 cm^{-1}). The suspension was cooled to 0 °C, and a solution of **28a** (0.41 g, 1.35 mmol) in CH_2Cl_2 (5 mL) was added in one portion. After 1 h stirring, the suspension was filtered and the filtrate evaporated to dryness. The residue was purified by preparative HPLC (cyclohexane: AcOEt, 3:1) to afford **29** (0.31 g, 49%) as an oil. Mass ($C_{30}H_{35}N_2O_9Cl_2$) calcd, 637.1710; found, 637.1726. Anal. ($C_{30}H_{34}N_2O_9Cl_2$) C, H, N, Cl. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[4-(Bis{2-chloroethyl}amino)phenoxycarbonyloxy]methyl}phenoxy)carbonyl]-L-glutamic Acid (30). 30** was obtained from **29** by the same method as **23** as a solid (0.105 g, 78.5%): mp 66−9 °C. Mass ($C_{24}H_{26}N_2O_9Cl_2Na$) calcd, 579.0913; found, 579.0903. Anal. ($C_{30}H_{35}N_3O_8Cl_2$) C, H; N: calcd, 5.04; found, 4.61. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Diprop-2-enyl *N*-[(4-{[4-(Bis{2-fluoroethyl}amino)phenoxycarbonyloxy]methyl}phenoxy)carbonyl]-L-glutamate (31). Method A: A solution of **18b** (0.4 g, 1.08 mmol) in THF (10 mL) and **27a** (prepared from 2.0 mmol of **21b**) was dissolved in THF (10 mL) and NEt_3 (0.25 mL, 1.8 mmol) added. The reaction mixture was stirred for 1.5 h and the solvent evaporated to dryness. The residue was purified by preparative HPLC (CH_2Cl_2:AcOEt, 39:1) to afford **31** (0.21 g, 35%) as an oil. Mass ($C_{30}H_{35}N_2O_9F_2$) calcd, 605.2311; found, 605.2330. Anal. ($C_{30}H_{34}N_2O_9F_2$): H, N; C: calcd, 59.60; found, 60.02.

Method B: Fluoroformate **27** (starting from 0.67 mmol of **18b**) was prepared as described for **29**. The suspension containing **27** was cooled at −78 °C, a solution of **28b** (0.12 g, 0.44 mmol) in CH_2Cl_2 (4 mL) added dropwise over 15 min, and the reaction mixture stirred for a further 30 min. The suspension was filtered, the filtrate evaporated to dryness, and the residue purified by preparative HPLC to afford **31** (0.018 g, 7%). ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[4-(Bis{2-fluoroethyl}amino)phenoxycarbonyloxy]methyl}phenoxy)carbonyl]-L-glutamic Acid (32). 32** (0.04 g, 42%) was obtained as an oil from **31** by the same method as for **23**. Mass ($C_{24}H_{26}N_2O_9F_2Na$) calcd, 547.1504, found, 547.1526. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Di-*tert*-butyl *N*-[(4-{[*N*-(4-{Bis[2-chloroethyl]amino}phenyl)carbamoyloxy]methyl}phenoxy)carbonyl]-L-glutamate (36). To a CH_2Cl_2 solution (3 mL) of **34** (0.147 g, 0.63 mmol) and triethylamine (180 mL, 1.25 mmol) was added triphosgene (63 mg, 0.21 mmol) in one portion. After 15 min stirring, the solvent was evaporated, the residue taken up in THF, and the insoluble salt filtered off. The THF was evaporated, and isocyanate **35a** (X = Cl) was obtained as an oil which was used immediately in the next reaction.

To the toluene solution (5 mL) of **18b** (60 mg, 0.15 mmol) was added isocyanate **35a** (X = Cl) (0.60 mmol) in toluene (5 mL) and dibutyltin dilaurate (10 mL). The reaction mixture was stirred for 12 h at room temperature, filtered, and evaporated to dryness. Purification was achieved by column chromatography (cyclohexane:AcOEt, 3:1), when product **36** (90 mg, 90%) was obtained as a solid: mp 44−7 °C; ν_{max}/cm^{-1} (film) 1728 (C=O, ester, carbamate). Mass ($C_{32}H_{43}N_3O_8Cl_2$) calcd, 667.2427; found, 667.2420. Anal. ($C_{32}H_{43}N_3O_8Cl_2$) C, H, N, Cl. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Di-*tert*-butyl *N*-[(4-{[*N*-(4-{bis[2-fluoroethyl]amino}phenyl)carbamoyloxy]methyl}phenoxy)carbonyl]-L-glutamate (41) was obtained in a similar manner from **18a** and isocyanate **35b** (X = F) as a solid (0.205 g, 69%): mp 39−41 °C. Isocyanate **35b** (X = F) was prepared from **51** by the method previously described for **35a**. Mass ($C_{32}H_{43}N_3O_8F_2$) calcd, 635.3018; found, 635.3040. Anal. ($C_{32}H_{43}N_3O_8F_2$) H, N; C: calcd, 60.46; found, 60.03. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[*N*-(4-{Bis[2-chloroethyl]amino}phenyl)carbamoyloxy]methyl}phenoxy)carbonyl]-L-glutamic Acid (37).** Di-*tert*-butyl ester **36** (0.250 g, 0.37 mmol) was dissolved in formic acid (10 mL) and stirred under argon at room temperature for 48 h. The formic acid was evaporated under vacuum (oil pump) and the residue reevaporated again five times with CH_2Cl_2 and toluene to yield **37** as a solid (0.185 g, 89%): mp 99−102 °C; ν_{max}/cm^{-1} (film) 1713 (C=O, acid). Mass ($C_{24}H_{27}N_3O_8Cl_2Na$) calcd, 578.1073; found, 578.1070. Anal. ($C_{24}H_{27}N_3O_8Cl_2$) C, H, Cl; N: calcd, 7.57; found, 6.01. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[*N*-(4-{Bis[2-fluoroethyl]amino}phenyl)carbamoyloxy]methyl}phenoxy)carbonyl]-L-glutamic acid (42)** was obtained identically from **41** as a solid (60 mg, 82%): mp 124−7 °C. Mass ($C_{24}H_{27}N_3O_8F_2$) calcd, 523.1766; found: 523.1780. Anal. ($C_{24}H_{27}N_3O_8F_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

Diprop-2-enyl *N*-[(4-{[*N*-(4-{Bis[2-chloroethyl]amino}phenyl)carbamoyloxy]methyl}phenyl)carbamoyl]-L-glutamate (39). The same procedure as for **36** was used starting from isocyanate **35a** (X = Cl) (0.54 mmol) in toluene (5 mL), linker **8b** (0.100 g, 0.27 mmol) dissolved in CH_2Cl_2 (5 mL), and dibutyltin dilaurate (10 mL). **39** was obtained as a solid. Further recrystallization from hexane:AcOEt (9:2) afforded **39** (0.110 mg, 64%) as a pure compound: mp 148−9 °C; ν_{max}/cm^{-1} (film) 1731 (C=O, ester), 1691 (C=O, carbamate), 1642 (C=O, urea). Mass ($C_{30}H_{36}N_4O_7Cl_2Na$) calcd, 657.1859;

found, 657.1850. Anal. ($C_{30}H_{36}N_4O_7Cl_2$) C, H, N, Cl. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

The same procedure was used to obtain **di-*tert*-butyl *N*-[(4-{[*N*-(4-{bis[2-chloroethyl]amino}phenyl)carbamoyloxy]methyl}phenyl)carbamoyl]-L-glutamate (38)** (0.310 g, 95%), mp 68−71 °C, from **18a** and **35a**. Mass ($C_{32}H_{44}N_4O_7Cl_2Na$) calcd, 689.2485; found, 689.2480. Anal. ($C_{32}H_{44}N_4O_7Cl_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information. Also obtained was **diprop-2-enyl *N*-[(4-{[*N*-(4-{bis[2-fluoroethyl]amino}phenyl)carbamoyloxy]methyl}phenyl)carbamoyl]-L-glutamate (43)** (0.190 g, 75%), mp 152−4 °C, from **8b** and **35b**. Mass ($C_{30}H_{37}N_4O_7F_2$) calcd, 603.2630; found, 603.2650. Anal. ($C_{30}H_{36}N_4O_7F_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

***N*-[(4-{[*N*-(4-{Bis[2-chloroethyl]amino}phenyl)carbamoyloxy]methyl}phenyl)carbamoyl]-L-glutamic Acid (40).** Diallyl ester **39** (0.190 g, 0.3 mmol) dissolved in CH_2Cl_2 (5 mL) was stirred with $Pd(PPh_3)_4$ (15 mg) and pyrrolidine (120 mL, 1.45 mmol). The reaction mixture was kept under argon for 45 min and then diluted with AcOEt. The solvent was evaporated, and the insoluble residue was washed with AcOEt. It was dissolved in methanol (10 mL) and passed through an IRC50 ion-exchange column (weakly acid resin). After eluting with 50 mL of methanol, the eluate was evaporated under vacuum to yield **40** (0.100 g, 60%) as a solid: mp 127−9 °C. Mass ($C_{24}H_{28}N_4O_7Cl_2Na$) calcd, 577.1233; found, 577.1230. Anal. ($C_{24}H_{28}N_4O_7Cl_2$) H, N; C: calcd, 51.97; found, 51.54. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

The same procedure was used to obtain ***N*-[(4-{[*N*-(4-{bis[2-fluoroethyl]amino}phenyl)carbamoyloxy]methyl}phenyl)carbamoyl]-L-glutamic acid (44)** from **43** as a glassy solid (0.125 g, 80%): mp 104−7 °C. Mass ($C_{24}H_{29}N_4O_7F_2$) calcd, 523.2004; found, 523.2020. Anal. ($C_{24}H_{28}N_4O_7F_2$) C, H, N. ^{1}H NMR and low-resolution mass spectra are in Supporting Information.

4-[Bis(2-fluoroethyl)amino]-*O*-benzylphenol (47). 4-[Bis-(2′-hydroxyethyl)amino]-*O*-benzylphenol (1.72 g, 6.0 mmol), triethylamine (4.3 mL, 31.0 mmol), and 4-(dimethylamino)pyridine (0.120 g, 1.0 mmol) were dissolved in CH_2Cl_2 (40 mL) followed by methanesulfonic anhydride (4.3 g, 25.0 mmol) dissolved in CH_2Cl_2 (40 mL). After 1 h stirring the reaction mixture was extracted with aqueous citric acid 10% (100 mL) and the organic layer washed with water (100 mL), dried ($MgSO_4$), and evaporated to dryness. The residue was redissolved in acetonitrile (50 mL), KF (3.8 g, 65 mmol) and 18-crown-6 ether (0.5 g, 1.9 mmol) were added, and the mixture stirred at 60 °C for 48 h. The mixture was filtered and the filtrate evaporated to dryness. Purification was achieved by preparative HPLC (cyclohexane:CH_2Cl_2, 2:1) when **47** (1.0 g, 57%) was obtained as an oil: ^{1}H NMR δ_H 3.60 (2t, 4H, *N*-CH_2, J = 5.12 Hz, J_{H-F} = 22.5 Hz), 4.53 (2t, 4H, CH_2F, J_{H-F} = 47.5 Hz), 5.00 (s, 2H, $PhCH_2$-), 6.72 (d, 2H, $H_{arom2+6}$, J = 8.68 Hz), 6.87 (d, 2H, $H_{arom3+5}$), 7.30−7.45 (m, 5H, H_{arom}); MS *m/z* 291 (M^+, 100), 200 ($M^+ - PhCH_2$, 100). Mass ($C_{17}H_{19}F_2NO$) calcd, 291.1435; found, 291.1450. Anal. ($C_{17}H_{19}F_2NO$) C, H, N.

4-[Bis(2-fluoroethyl)amino]phenol (21b). **47** (1.0 g, 3.4 mmol) and pentamethylbenzene (3.8 g, 27.7 mmol) were dissolved in trifluoroacetic acid (25 mL) and stirred for 3 days at room temperature. TFA was then evaporated and the residue triturated with hexane. The insoluble oil was partitioned between acetonitrile (80 mL) and hexane (80 mL). The acetonitrile solution was separated, the solvent evaporated, and the residue taken in AcOEt (80 mL) and washed with aqueous $NaHCO_3$ (50 mL) and water (50 mL). The organic layer was dried ($MgSO_4$) and evaporated to dryness to yield a dark-yellow oil (0.670 g, 98%): ^{1}H NMR δ_H 3.54 (2t, 4H, *N*-CH_2, J = 5.25 Hz, J_{H-F} = 25.0 Hz), 4.51 (2t, 4H, CH_2F, J_{H-F} = 47.5 Hz), 6.64 (s, 4H, H_{arom}), 8.62 (s, 1H, OH); MS *m/z* 201 (M^+, 100), 182 ($M^+ - F$, 12). Mass ($C_{10}H_{13}F_2NO$) calcd, 201.0965; found, 201.0975. Anal. ($C_{10}H_{13}F_2NO$) C, H, N.

4-[Bis(2-fluoroethyl)amino]nitrobenzene (50). 4-[Bis-(2′-hydroxyethyl)amino]nitrobenzene (1.4 g, 6.2 mmol), triethylamine (4.3 mL, 31 mmol), and 4-(dimethylamino)pyridine (0.120 g, 1.0 mmol) were dissolved in CH_2Cl_2 (40 mL), and methanesulfonic anhydride (4.3 g, 25 mmol) dissolved in CH_2Cl_2 (40 mL) was added. After 1 h stirring the reaction mixture was extracted with aqueous citric acid 10% (100 mL) and the organic layer washed with water (100 mL), dried ($MgSO_4$), and evaporated to dryness. The residue was redissolved in chloroform (50 mL), Amberlyst 26 F^- was added, and the mixture was stirred at reflux for 72 h. The reaction mixture was filtered and the filtrate evaporated to dryness. Purification was achieved by preparative HPLC (cyclohexane:AcOEt, 3:1) when **50** was obtained as a solid. Recrystallization from hexane−diethyl ether−chloroform, 6:2:1, yielded **50** (0.290 g, 20%) as yellow crystals: ^{1}H NMR δ_H 3.87 (2t, 4H, *N*-CH_2, J = 5.1 Hz, J_{H-F} = 25.3 Hz), 4.64 (2t, 4H, CH_2F, J_{H-F} = 47.5 Hz), 6.92 (d, 2H, $H_{arom2+6}$, J = 9.48 Hz), 8.03 (d, 2H, $H_{arom3+5}$); MS *m/z* 231 (M^+ + 1, 100). Mass ($C_{10}H_{12}F_2N_2O_2$) calcd, 231.0945; found, 231.0955. Anal. ($C_{10}H_{12}F_2N_2O_2$) C, H, N.

4-[Bis(2-fluoroethyl)amino]aniline (51). **50** (0.260 g, 1.1 mmol) was dissolved in EtOH (12 mL); 10% Pd/C (0.120 g) and ammonium formate (0.500 g) were added. The suspension was stirred for 1.5 h at room temperature. The catalyst was filtered off and the filtrate evaporated to dryness. Purification was achieved by preparative HPLC (cyclohexane: AcOEt, 2:1) to yield **51** (0.190 g, 84%) as a dark-yellow oil: ^{1}H NMR δ_H 3.47 (2t, 4H, *N*-CH_2, J = 5.22 Hz, J_{H-F} = 25.0 Hz), 4.47 (2t, 4H, CH_2F, J_{F-H} = 47.5 Hz), 6.49 (d, 2H, $H_{arom2+6}$, J = 8.80 Hz), 6.60 (d, 2H, $H_{arom3+5}$); MS *m/z* 200 (M^+, 100). Mass ($C_{10}H_{14}N_2F_2$) calcd, 200.1115; found, 200.1130. Anal. ($C_{10}H_{14}N_2F_2$) C, H, N.

Chemical Stability Determination. Compounds were prepared as 10 mM concentrates in distilled water (**12**, **20**), DMSO (**23**, **25**, **37**, **40**, **42**, **44**), or MeOH (**30**) and diluted 100-fold in CPG2 assay buffer (100 mM Tris-HCl, pH 7.3; 260 μM $ZnCl_2$; 1 mL) to give 100 μM solutions. Aliquots (10 μL) were injected onto a Partisphere 5-μm C18 column (110 mm × 4.6 mm) and eluted isocratically (1 mL/min) with 10 mM ammonium acetate (pH 5.0) containing percentages of methanol chosen to produce a retention time of 3−4 min and monitored at the optimum wavelength (**12**, 10%, 246 nm; **20**, 10%, 213 nm; **23**, 63%, 255 nm; **25**, 50%, 250 nm; **30**, 65%, 258 nm; **32**, **37**, 58%, 268 nm; **40**, 58%, 253 nm; **42**, 45%, 267 nm; **44**, 43%, 251 nm). The amount of starting material remaining after various periods of incubation at 37 °C was determined either by repeat injections (7.5-min intervals) from a single vial (**12**, **20**, **30**, **32**, **37**, **40**, **42**, **44**) or by delayed injections (1-min intervals) from a new vial each time (**23**, **25**).

Kinetic Determinations. The optimum wavelength for spectrophotometric kinetic analysis was established from a differential spectrum comparing each parent compound with an equimolar mixture of its products (**12**, 250 nm; **20**, 228 nm; **40**, 280 nm; **42**, 267 nm; **44**, 251 nm). CPG2 (50−250 ng) was added to CPG2 assay buffer (100 mM Tris-HCl, pH 7.3; 260 μM $ZnCl_2$; 1 mL) containing 1−100 μM compound and the rate of change of absorbance at the chosen wavelength measured. In the case of the less stable **40**, compound was included in both reference and sample cuvettes to negate the contribution of purely chemical loss. Kinetic parameters were derived from Hanes−Woolfe plots.

Biological Methods: Cytotoxicity Assays. For this study, LS174T cells were constructed to express surface-tethered mutant CPG2 stably, by transfection of cells with the plasmid pMCEFstCPG2(Q)3[15] using lipofectamine, by the method previously described. The plasmid pMCEFstCPG2(Q)3 encodes for a CPG2 molecule that is expressed on the outer cell membrane. It bears three asparagine-to-glutamine [(Q)3] mutations that were otherwise inappropriately glycosylated. This plasmid encodes a mutated CPG2 molecule fused between the signal and the transmembrane domains of the c-erbB2 protein tyrosine kinase receptor under the transcriptional control of the EF-1a promoter and also encodes neomycin resistance for selection of stably expressing mammalian clones.

Single colonies were cloned by limiting dilution, and those expressing surface-tethered CPG2 were identified by their ability to degrade methotrexate[28] and by Western blot using a rabbit anti-CPG2 polyclonal serum. Control cells expressing β-galactosidase have already been described.[15]

The cytotoxicity of the compounds was assayed by a modification of the published procedure.[29] Cells (2×10^6) were seeded into 6-well plates, producing confluent monolayers in 48 h. Compounds were dissolved in DMSO at 10 mM (**30**) or 100 mM (**25**, **42**, **44**) immediately prior to treatment, diluted in full medium, and added to the wells. A similar concentration of drug solution was added after an incubation of 1 h, and the cells were incubated for an additional 20 h. The cells were harvested and reseeded in quadruplicate in 96-well plates at $\sim 2 \times 10^3$ cells/well and incubated until the control wells achieved confluence. The plates were fixed and stained with sulforhodamine-B, the extinction at 590 nm was read, the results are expressed as percentage of control growth, and the IC_{50} values were evaluated by interpolation.

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Supporting Information Available: Spectral data for the final compounds **12**, **20**, **23**, **25**, **30**, **32**, **37**, **40**, **42**, and **44**, the protected linkers **8a**,**b** and **18a**,**b**, the activated linkers **9a**,**b** and **19a**,**b**, the protected prodrugs **22**, **29**, **36**, **38**, and **39**, and the corresponding fluorinated analogues **24**, **31**, **41**, and **43** (16 pages). Ordering information is given on any current masthead page.

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