Significant Differences in Biological Parameters between Prodrugs Cleavable by Carboxypeptidase G2 That Generate 3,5-Difluoro-phenol and -aniline Nitrogen Mustards in Gene-Directed Enzyme Prodrug Therapy Systems

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Nine new nitrogen mustard compounds derived from 2,6-difluoro-4-hydroxy- (**3a**−**e**) and 2,6-difluoro-4-amino- (**4a**−**d**) aniline were synthesized as potential prodrugs. They were designed to be activated to their corresponding 3,5-difluorophenol and -aniline (4)-nitrogen mustards by the enzyme carboxypeptidase G2 (CPG2) in gene-directed enzyme prodrug therapy (GDEPT) models. The compounds were tested for cytotoxicity in the MDA MB-361 breast adenocarcinoma. The cell line was engineered to express stably either CPG2 tethered to the cell surface stCPG2-(Q)3 or β-galactosidase (β-Gal) as control. The cytotoxicity differentials were calculated between CPG 2-expressing and -nonexpressing cells and yielded different results for the two series of prodrugs despite their structural similarities. While the phenol compounds are ineffective as prodrugs, their aniline counterparts exhibit outstanding activity in the tumor cell lines expressing CPG2. {3,5-Difluoro-4-[bis(2-chloroethyl)amino]phenyl}carbamoyl-L-glutamic acid gave a differential of >227 in MDA MB361 cells as compared with 19 exhibited by 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid, **1a**, which has been in clinical trials.

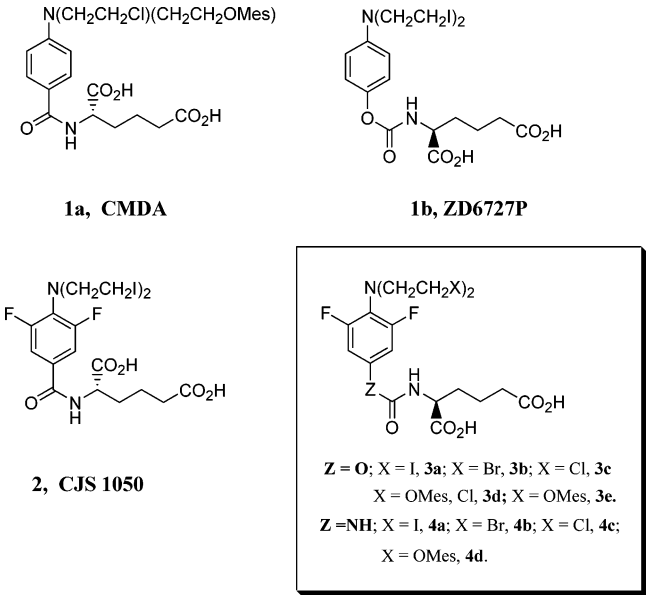
Introduction

In order to increase the selectivity of anticancer agents, targeting strategies such as antibody-directed enzyme prodrug therapy (ADEPT)[1] and gene-directed enzyme prodrug therapy (GDEPT)[2−6] have been developed. These strategies do not require highly specific anticancer drugs, as they both utilize prodrugs, which release highly cytotoxic drugs at the tumor after activation by an exogenous enzyme expressed in tumor cells.[7−14] The targeting of the genes for the exogenous enzyme is achieved by using viral or nonviral delivery vectors. Several enzyme/prodrug systems have been investigated, and a number of clinical trials using suicide gene therapy are ongoing.[6]

A GDEPT system based on the enzyme carboxypeptidase G2 (CPG2, glutamate carboxypeptidase EC 3.4.17.11) from *Pseudomonas aeruginosa* type RS16 has been developed.[15] CPG2 catalyzes the scission of amidic,[16] urethanic, or ureidic[17,18] linkages between an aromatic nucleus and L-glutamic acid. The enzyme has been expressed both intracellularly[19] and extracellularly[20] in a series of tumor cell lines. The extracellularly expressed enzyme obviates the need for the prodrug to enter the tumor cell for activation. This also improves the bystander effect.[20]

Rationale of the Design. 4-[(2-Mesyloxyethyl)(2-chloroethyl)amino]benzoyl-L-glutamic acid (CMDA, **1a**) and 4-{[bis(2-iodoethyl)amino]phenyl}oxycarbonyl-L-glutamic acid (ZD2767P, **1b**) have both been used in ADEPT clinical trials. They are substrates for CPG2 (K_M = 2.0−3.4 μM and k_{cat} = 29.5−583 s^{-1}).[11,21] Cleavage of CMDA by CPG2 releases 4-[bis(2-mesyloxyethyl)(2-chloroethyl)amino]benzoic acid as the drug.

Scheme 1



Further work indicated that two prodrugs related to CMDA: 3-fluoro-4-[bis(2-chloroethyl)amino]benzoyl-L-glutamic acid[22] and 3,5-difluoro-4-[bis(2-iodoethyl)amino]benzoyl-L-glutamic acid, **2**[23] were superior to CMDA in terms of cytotoxic differential, potency, and the bystander effect in vitro and in vivo in a number of CPG2/GDEPT systems. The reasons for these improvements are unclear.

To further investigate the 3,5-difluorinated analogues of ZD2767P, **3a**−**e** and the 3,5 difluorinated analogues

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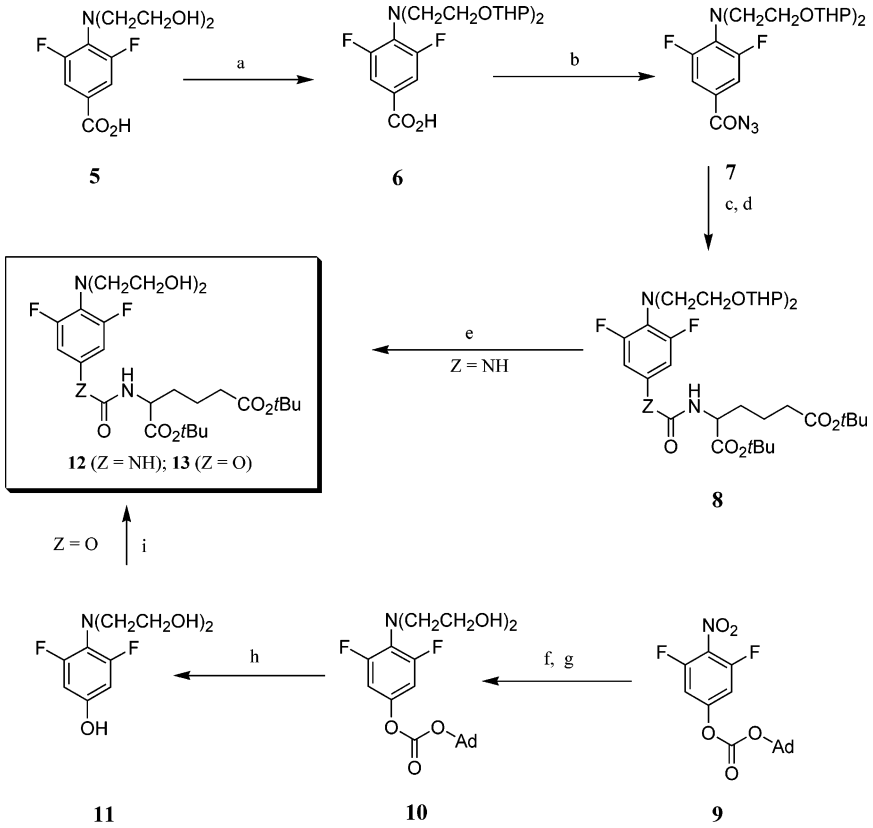
Scheme 2[a]



Reagents and conditions: (a) 3,4-Dihydro-2*H*-pyran, PPTS; (b) imidazolyl carbonate, DCM followed by NaN_3 soln; (c) boiling toluene; (d) di-*tert*-butyl-L-glutamate, THF, rt; (e) PPTS, EtOH, 65 °C; (f) HCO_2NH_4, Pd/C, EtOH; (g) ethylene oxide, AcOH; rt; (h) CF_3CO_2H, rt; (i) di-*tert*-butyl-4-nitrophenyloxycarbonyl-L-glutamate, Amberlyst 27; Ad = adamantanyl.

of the corresponding {4-[bis(2-chloroethyl)amino] phenyl}-carbamoyl-L-glutamic acid, **4a**−**d**, both series have been synthesized (see Scheme 3).

This paper describes the synthesis and the physicochemical and biological properties of the new fluorinated potential prodrugs.

Results and Discussions

Chemistry. Nine new potential prodrugs (compounds **3a**−**e** and **4a**−**d**) derived from 2,6-difluoro-4-amino- and 2,6-difluoro-4-hydroxyaniline nitrogen mustards have been synthesized.

3,5-Difluoro-4-[bis(2-hydroxyethyl)amino]benzoic acid, **5**,[23] was used as the starting material for the aniline series. The compound was protected as 4-bis(2-tetrahydropyranyloxyethyl)aminobenzoic acid, **6**, and converted into the corresponding azide (using diimidazolyl carbonate and NaN_3). The intermediate was purified by preparative HPLC and submitted to the Curtius rearrangement (in boiling toluene). The isocyanate formed was coupled to the di-*tert*-butyl L-glutamate to obtain the di-*tert*-butyl {4-[bis(2-tetrahydropyranyloxyethyl)-amino]phenyl}carbamoyl-L-glutamate, **8**, which after deprotection gave intermediate **12** (Z = NH) (see Scheme 2).

The starting material for the corresponding *p*-hydroxyaniline series was 4-nitro-3,5-difluorophenyloxycarbonyl-adamantane, **9**.[24] After reduction and hydroxyethylation (see Scheme 2), the intermediate **10** was obtained in satisfactory yield and was deprotected by exposure to TFA at room temperature.

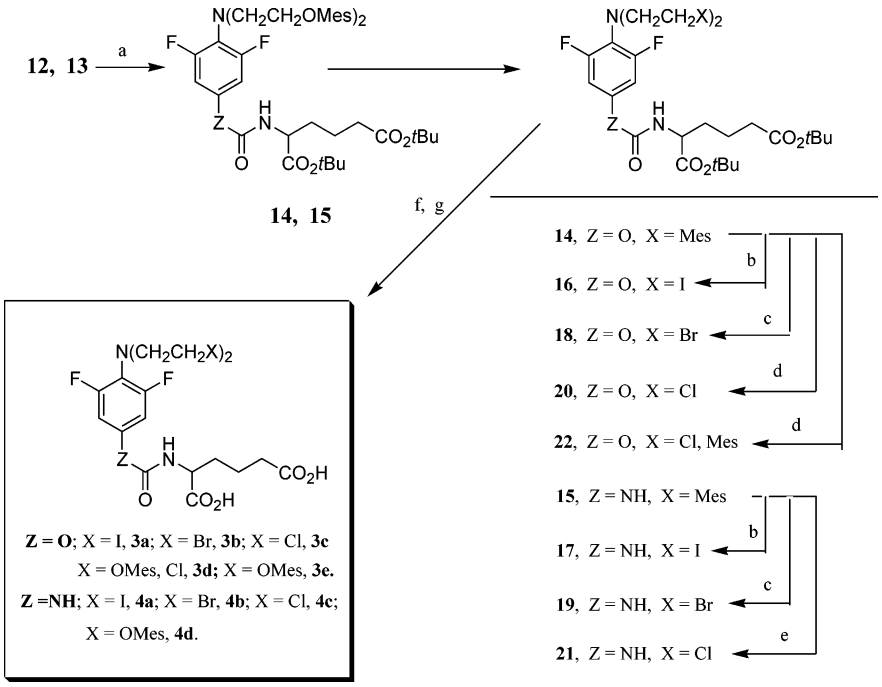
Following purification, the 4-[bis(2-hydroxyethyl)-amino]phenol, **11**, underwent transesterification with di-*tert*-butyl 4-nitrophenyloxycarbonyl-L-glutamate in the presence of the strong base ion exchanger, Amberlyst 27, to afford the key intermediate, **13** (Z = O). The intermediates **12** and **13** were converted into their alkylating analogues in the usual way (see Scheme 3).[25,26] The final deprotection, with formic acid of the protected prodrugs **14**−**22**, led to the desired nitrogen mustard prodrugs, **3a**−**e** and **4a**−**d**.

To obtain large amount of aniline prodrugs for in vivo assessment, a shorter synthetic access route was developed (Scheme 4) via the nitration of 2,6-difluro-4-bis(2-hydroxyethyl)aniline, **23**. This intermediate was converted to the corresponding amine, **25**, which was converted to the intermediate, **12** (Z = NH).

Physicochemical and Kinetic Data. The kinetics and the chemical half-lives of the prodrugs **3c** and **4c** were determined by HPLC (see Experimental Section). In contrast with {3,5-difluoro-4-[bis(2-chloroethyl)amino]-phenyl}oxycarbonyloxy-L-glutamic acid, **3c**, which is not a subtrate for CPG2, {3,5-difluoro-4-[bis(2-chloroethyl)-amino]phenyl}carbamoyl-L-glutamic acid, **4c**, proved to be a good substrate for the enzyme (K_M = 7.7 μM and k_{cat} = 29.0 s^{-1}). The chemical half-lives of prodrugs **3a**, **3c**, **4a**−**c** are summarized in Table 1.

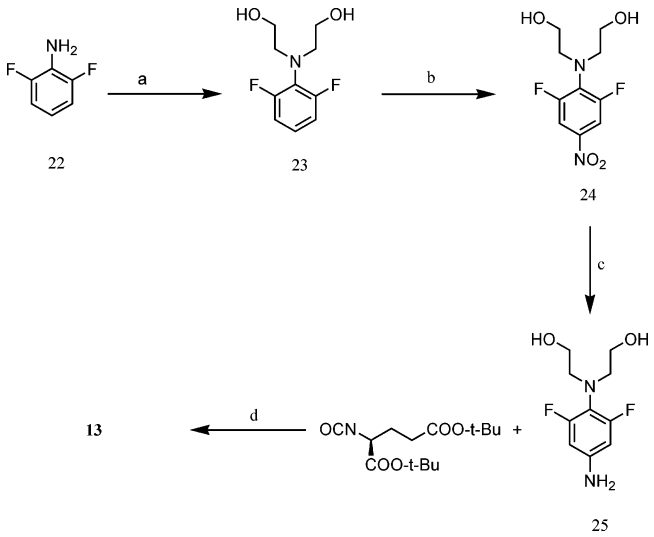
Cytotoxicity Assays. The prodrugs were tested for cytotoxicity in the line MDA MB 361 breast adenocarcinoma. The cell line was engineered to stably express either CPG2 tethered on the cell surface stCPG2-$(Q)_3$ or β-galactosidase (β-Gal) as the control. For compara-

Scheme 3[a]



[a] Reagents and conditions: (a) Mes_2O, DMAP, DCM, rt; (b) NaI, Me_2CO, reflux; (c) LiBr, THF, reflux; (d) NaCl, CH_3CN, 60 °C; (e) LiCl, DMA, rt; (f) TFA; (g) HCO_2H.

Scheme 4[a]



[a] Reagents and conditions: (a) AcOH, ethylene oxide; (b) fuming HNO_3, DCM; (c) H_2, Pd/C, THF; (d) NEt_3, THF.

tive purposes with benzoic acid mustard prodrugs, the MDA MB 361 cell line was treated with CMDA (the clinical ADEPT candidate). The degree of activation, defined as the ratio of the IC_{50} of the prodrug in the β-Gal- and CPG2-expressing cell lines, was calculated for the new compounds and compared to CMDA (see Table 1). The phenol prodrugs were inactive against MDA MB 361 stCPG2(Q)3 cells, compared with 19-fold activation for CMDA in these cells. The aniline prodrugs were much more effective than CMDA in CPG2-expressing cells. {3,5-Difluoro-4[bis(2-chloroethyl)amino]-phenyl}carbamoyl-L-glutamic acid, **4c**, exhibited a very high differential of >227-fold in this system. The compound was chosen as a candidate for further development.

Table 1. Prodrugs: Half-Lives and Cytotoxicity Differential between Expressing and Nonexpressing Cell Lines

		MDA-MB361		
	half-life	IC_{50} (μM)		degree of activation
compound	$t_{1/2}$ min	β-Gal	Q3	(fold)
CMDA	59	3125	165	19
3a	6.2	2.2	2.4	0.9
3b	-	3.6	4.0	0.9
3c	45.0	-	-	-
3d	-	19.9	22.5	0.9
3e	-	151.9	439.1	0.3
4a	2.2	805	15.9	50.6
4b	0.58	>1000	51	19.6
4c	31.0	>2000	8.8	>227
4d	-	>2000	>2000	1

In Vivo Antitumor Activity. Compound **4c** showed good antitumor activity in vivo compared to untreated control tumors grown of the MDA MB 361 engineered to express stably stCPG2(Q)3 in CD1 *nu/nu* mice (see Experimental Section and Figure 1).

Discussion

Previous data have shown that mono-[22] and difluoro-analogues of CMDA[23] exhibit improved cytotoxic and antitumor activity. To compare this series with the corresponding phenyloxycarbonyl- and phenylcarbamoyl-nitrogen mustards the new derivatives **3a**−**e** and **4a**−**d** have now been synthesized. These prodrugs release nitrogen mustard drugs after cleavage by CPG2. The drugs are more cytotoxic than the nitrogen mustards derived from 3,5-difluoro-4-aminobenzoic acid.

The use of I and Br instead of Cl as leaving groups in nitrogen mustards series generally leads to drugs with shorter half-lives and increased potency (IC_{50} = 0.5−2.7 μM).[11,27] As a general observation, carbamoyl-prodrugs are more effective than their oxycarbonyl-prodrug counterparts. This occurs despite the improved

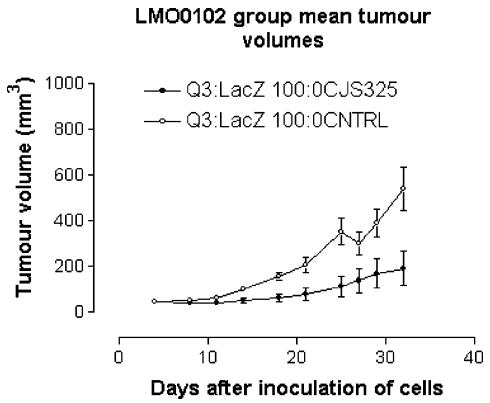


Figure 1. Tumor growth in vivo in CD1 nu/nu mice, following treatment with prodrug **4c** versus control group. All tumors 100% stCPG(Q)3-expressing MDA MB 361 cells. Controls injected with 5% DMSO in water. Points shown are mean tumor volumes over the whole group.

kinetics (k_{cat} and K_M) of the oxycarbonyl linker itself.[11,26] The differential obtained in tumor cells expressing stCPG2(Q)3 appears to be dependent on optimal chemical reactivity of the prodrugs and their corresponding drugs, despite the relatively low k_{cat} for the new prodrugs. This is in good agreement with previous observations of the in vitro and in vivo behavior of the benzoic acid mustard prodrugs in CPG2-based GDEPT systems.[23]

A suprising difference in activity is demonstrated between phenols (**3a**−**e**) and aniline (**4a**−**d**) compounds. As shown in Table 1, the phenol prodrugs are not substrates for CPG2. The reason for this behavior is unclear since the similar nonfluorinated prodrug ZD 2767P is a reasonable substrate for the enzyme and the corresponding difluorinated nitrogen mustard **2** is an excellent substrate for CPG2.[23] A difference in the positioning of the difluoro-prodrugs in the active site of CPG2 may explain these results.

Conclusions

Nine new potential prodrugs belonging to the 3,5-difluorophenol- and 3,5-difluoroaniline-nitrogen mustard series are synthesized and studied. Compounds **3a**−**e** are shown not to act as substrates for CPG2, whereas **4a**−**d** are excellent prodrugs for CPG2, showing activity in tumor cell lines engineered to express stCPG2(Q)3. The {3,5-difluoro-4[bis(2-chloroethyl)amino]-phenyl}carbamoyl-L-glutamic acid, **4c**, is the most effective prodrug on MDA MB361 cell line expressing stCPG2(Q)3 in vitro, and further studies in vivo show that it is significant at reducing the rate of tumor growth compared to control (see Figure 1).

Experimental Section

All starting materials, reagents, and anhydrous solvents (i.e., THF packed under N_2) were purchased from Aldrich, unless otherwise stated. Kieselgel 60 (0.043−0.060) was used in gravity columns (Art 9385 and 15111, Merck). TLC was performed on precoated sheets of Kieselgel 60 F_{254} (Art 5735, Merck). Melting points were determinated on a Kofler hotstage (Reichert Thermovar) melting point apparatus and are uncorrected. Low resolution EI and FAB mass spectra were performed on a VG-2AB−SE double focusing magnetic sector mass spectrometer (Fisons Instruments, Warrington, Manchester, UK), operating at a resolution of 1000 amu. High-resolution accurate mass spectra were determined on the same system, but with a resolution set to 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 Bruker AC250 spectrometer (250 MHz) 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, UK) and are within 0.4% of theory except when stated. The chemical stability of the prodrugs and their propensity to behave as substrate for CPG2 were determined by HPLC. Preparative HPLC purifications were performed on an Axial Chromatospac Prep 10 (Jobin-Yvon) using Merck Kieselgel 60 (0.015−0.040) (Art. 15,111).

3,5-Difluoro-4-[bis(2-hydroxyethyl)amino]benzoic Acid (5). A solution of 3,4,5-trifluorobenzonitrile (5.0 g, 0.032 mmol) and diethanolamine (9.0 mL, 0.94 mmol) in DMA (40 mL) was heated a 100 °C in the CEM Discover series microwave for 1 h at 30 W power. After solvent removal, the residue was redissolved in AcOEt (100 mL), extracted with water (2 × 100 mL), dried, and evaporated under vacuum. After purification by preparative HPLC (DCM:EtOH, 95:5), 4-[bis(2-hydroxyethyl)amino]benzonitrile was obtained (4.15 g, 53%) as a white solid (48 h reaction time and 35% yield reported by standard heating).[23] The hydrolysis of this compound by reflux in NaOH/ethanol/water solution for 3 h led to the corresponding 3,5-difluorobenzoic acid **5** (mp 147−149 °C).[23]

3,5-Difluoro-4-[bis(2-tetrahydropyranyloxyethyl)amino]benzoic Acid (6). To a stirred solution of 4-[bis(2-hydroxyethyl)amino]-3,5-difluorobenzoic acid (1.83 g, 7.0 mmol) and 3,4-dihydro-2*H*-pyran (1.60 mL, 1.47 g, 17.50 mmol) in DCM (100 mL) was added pyridinium-*p*-toluenesulfonate (1 g, 4.0 mmol), and the reaction mixture was refluxed for 3 h. The mixture was stirred for an additional 18 h at room temperature, washed with water (2 × 100 mL), dried ($MgSO_4$), and evaporated under vacuum. The product resulted as an oil (2.86 g, 95.2%) and was purified by HPLC using cyclohexane:AcOEt 1:1 as eluent. A solid resulted: mp 70−2 °C. ν_{max}/cm^{-1} (film): 3081 (OH), 2944, 2872 (CH_2), 1721 (CO, acid); ^{1}H NMR, δ_H 1.28−1.55 (m, 12H, $H_{3'}$+$H_{4'}$+$H_{5'}$), 3.32−3.45 (m, 8H, N(CH_2-CH_2OTHP)$_2$), 3.57−3.71 (m, 4H, $H_{6'}$), 4.49 (s, 2H, $H_{2'}$), 7.45 (d, 2H, $H_{arom2+6}$, J_{H-F} = 10.36 Hz); MS (FAB), *m*/*z*: 452 (M^+ + 23, 100); acc mass: ($C_{21}H_{29}F_2NO_6$Na) calcd 452.1861, found 452.1850. Anal. ($C_{21}H_{29}F_2NO_6$) C, H, N.

3,5-Difluoro-4-[bis(2-tetrahydropyranyloxyethyl)amino]benzoyl Azide (7). To a solution containing the acid **6** (11.8 g, 27.5 mmol) in 200 mL of dry THF was added the imidazolyl carbonate (4.70 g, 29.0 mmol) under stirring and nitrogen at room temperature. After 1 h a solution of 5 M NaN_3 (12.5 g of NaN_3 in 30 mL of H_2O) was added, and the reaction mixture stirred for an additional 3 h. The reaction mixture was poured into 1.0 L of H_2O and extracted with ether (2 × 1 L), dried, and evaporated to an oily residue. This was redissolved in dry benzene (2 × 100 mL) and evaporated each time under vacuum. The final purification was carried out by HPLC (cyclohexane:AcOEt, 2:1) to give an oil (11.05 g, 88.4%). ν_{max}/cm^{-1} (film); 2943, 2871 (CH_2), 2146 (CO, azide); ^{1}H NMR, δ_H 1.33−1.55 (m, 12H, 4×$H_{3'}$+4×$H_{4'}$+4×$H_{5'}$), 3.32−3.44 (m, 8H, N(CH_2CH_2OTHP)$_2$), 3.60−3.70 (m, 4H, 4×$H_{6'}$), 4.47 (s, 2H, 2×$H_{2'}$), 7.45 (d, 2H, $H_{arom2+6}$, J_{H-F} = 10.36 Hz); MS (FAB), *m*/*z*: 455 (M^+ + 1, 55), 412 (M^+ − N_3, 45); acc mass: ($C_{21}H_{29}F_2N_4O_5$)calcd 455.2106, found 455.2120. Anal. ($C_{21}H_{28}F_2N_4O_5$) C, H.

Di-*tert*-butyl {3,5-Difluoro-4-[bis(2-tetrahydropyranyloxyethyl)amino]phenyl}carbamoyl-L-glutamate (8). The azide **7** (2.80 g, 6.53 mmol) was heated in 150 mL of dry toluene and submitted to condensation without further purification. The reaction completion was monitored by IR (ν = 2146 cm^{-1}). The solvent was evaporated under vacuum and the residue dissolved in 10 mL of THF. The clear solution obtained from di-*tert*-butyl-L-glutamate·HCl (1.70 g, 6.5 mmol) and NEt_3 (0.97 mL, 7.0 mmol) in 50 mL of THF, after filtration, was added dropwise (15 min) at room temperature with stirring to the solution containing the previously prepared

isocyanate. The reaction was finished in 30 min (disappearance of the $\nu = 2268\ cm^{-1}$). The solvent was evaporated, the residue dissolved in AcOEt (50 mL), washed with H_2O (2 × 50 mL), and evaporated under vacuum. A thick oil was obtained which after HPLC (cyclohexane:AcOEt 2.5:1) gave (4.22 g, 94.3%) of **8** (oil). ν_{max}/cm^{-1} (film); 2942, 2872 (CH_2), 1731 (CO, ester); ^1H NMR, δ_H 1.38−1.63 (m, 30H, t-Bu: 18H; 2×$H_{3'}$+2×$H_{4'}$+-2×$H_{5'}$: 12H), 1.71−1.96 (2 × m, 2H, CH_2CHNH), 2.18−2.33 (m, 2H, CH_2CO_2tBu), 3.20−3.30 (m, 8H, N($CH_2CH_2OTHP)_2$), 3.57−3.68 (m, 4H, $H_{6'}$), 4.10−4.16 (m, 1H, CH-G), 4.49 (s, 2H, $H_{2'}$), 6.57 (d, 1H, NH-G, J = 8.02 Hz), 7.04 (d, 2H, $H_{arom2+6}$, J_{H-F} = 11.23 Hz), 8.79 (s, 1H, NHPh); MS (FAB), *m/z*: 708 (M^+ + 23, 26), 685 (M, 25); acc mass: ($C_{34}H_{53}F_2N_3O_9$) calcd 685.3750, found 685.3733. Anal. ($C_{34}H_{53}F_2N_3O_9$) C, H, N.

Di-*tert*-butyl {3,5-Difluoro-4-[*N*,*N*-bis(2-hydroxyethyl)-amino]phenyl}carbamoyl-L-glutamate (13). Pyridinium-*p*-toluene sulfonate (0.17 g, 0.67 mmol) was added to a stirred solution of **8** (2.30 g, 3.35 mmol) in 100 mL of EtOH. The reaction mixture was heated for 3 h at 65 °C, the solvent evaporated, and the residue dissolved in AcOEt (100 mL). The solution was washed with H_2O (2 × 100 mL), dried ($MgSO_4$), and evaporated under vacuum giving an oil (1.53 g, 88.6%) which crystallizes on standing mp 109−110 °C. ^1H NMR, δ_H 1.18 (s, 18H, t-Bu), 1.65−1.99 (2×m, 2H, CH_2NH-G), 2.23−2.34 (m, 2H, CH_2CO_2H), 3.07 (t, 4H, NCH_2, J = 6.25 Hz), 3.37 (q, 4H, CH_2OH), 4.39 (t, 2H, OH, J = 5.37 Hz), 6.58 (d, 1H, NH-G, J = 8.04 Hz), 7.05 (d, 2H, $H_{arom2+6}$, J_m = 4.38 Hz, J_{H-F} = 15.62 Hz), 8.81 (s, 1H, NHPh); MS (FAB), *m/z*: 540 (M^+ + 23, 100), 518 (M^+ + 1, 40); acc mass: ($C_{24}H_{38}F_2N_3O_7$) calcd 518.2678, found 518.2664. Anal. ($C_{24}H_{38}F_2N_3O_7$) C, H, F, N.

4-Nitro-3,5-difluorophenyloxycarbonyladamantane (9). Compound **9** was prepared starting from 4-nitro-3,5-difluorophenol (10 g, 57.1 mmol).[24] After purification by column chromatography, a solid was obtained (14.90 g, 73.9%). Recrystallized from MeOH aq 95%, mp 74−75 °C. ν_{max}/cm^{-1} (film); 2916, 2856 (CH_2), 1766 (CO, carbonate) 1544, 1347 (NO_2); ^1H NMR, δ_H 1.64 (s, 6H, $CH_{2,\ adam}$), 2.12 (s, 6H, $CH_{2,\ adam}$), 2.20 (s, 3H, $CH_{,\ adam}$), 7.60 (dd, 2H, $H_{arom2+6}$, J_{H-F} = 11.23 Hz, J_m = 2.01 Hz); MS (FAB), *m/z*: 376 (M^+ + 23, 17), 354 (M^+ + 1, 52), 309 ($M^+ - CO_2$ + 1,100); acc mass: ($C_{17}H_{17}F_2NO_5Na$) calcd 376.0972, found 376.0950. Anal. ($C_{17}H_{17}F_2NO_5$) C, H, N.

{3,5-Difluoro-4-[bis(2-hydroxyethyl)amino]phenyl}-oxycarbonyladamantane (10). 9 (14.34 g, 40.6 mmol) was submitted to reduction by hydrogen transfer using 2 g of Pd/C 10% and finely ground ammonium formate (18.9 g, 300 mmol) in 200 mL of EtOH under vigorous stirring. The amine obtained, after a conventional workup, as a red oil (13.8 g) was submitted without purification to hydroxyethylation. The compound was dissolved in 300 mL of AcOH, ethylene oxide (17.6 mL, 400 mM) was added at room temperature, and the reaction mixture was stirred for 48 h. The solvent was evaporated under vacuum and the oily residue washed with $NaHCO_3$ sol. 5% (3 × 100 mL) and water, dried ($MgSO_4$), and evaporated again. An oil resulted, which was purified by HPLC (AcOEt), and then a solid resulted: 6.91 g (41.3% with respect to **9**). Recrystallized from *n*-hexane:AcOEt, 20:7, mp 78−9 °C. ^1H NMR, δ_H 1.63 (s, 6H, $CH_{2,\ adam}$), 2.10 (s, 6H, $CH_{2,\ adam}$), 2.18 (s, 3H, $CH_{,\ adam}$), 3.14 (t, 4H, NCH_2, J = 6.27 Hz), 3.41 (q, 4H, CH_2OH), 4.42 (t, 2H, OH, J = 5.30 Hz), 7.02 (d, 2H, $H_{arom2+6}$, J_{H-F} = 9.86 Hz); MS (FAB), *m/z*: 412 (M^+ + 1, 8), 336 ($M^+ - CO_2$ + 1, 16); acc mass: ($C_{21}H_{28}F_2NO_5$) calcd 412.1936, found 412.1940. Anal. ($C_{21}H_{28}F_2NO_5$) C, H, F, N.

4-[Bis(2-hydroxyethyl)amino]-3,5-difluorophenol (11). Compound **10** (2.0 g, 4.9 mmol) was deprotected with TFA (150 mL) by stirring for 2 h at room temperature. The solvent was evaporated under vacuum, and then the residue was redissolved in 20 mL of ACOEt and evaporated again (five times). A clear oil (3.07 g) was obtained which was submitted to HPLC (AcOEt). After purification, an oil was obtained (0.86 g, 75.5%) ^1H NMR, δ_H 3.02 (t, 4H, NCH_2, J = 5.58 Hz), 3.35 (t, 4H, CH_2-OH), 4.39 (s, broad, 2H, OH), 6.39 (d, 2H, $H_{arom2+6}$, J_{H-F} = 9.86 Hz), 10.05 (s, broad, 1H, OH_{phenol}); MS (FAB), *m/z*: 256 (M^+ + 23, 100), 234 (M^+ + 1, 72); acc mass: ($C_{10}H_{14}F_2NO_3$) calcd 234.0942, found 234.0936. Anal. ($C_{10}H_{13}F_2NO_3$) C, H, N.

Di-*tert*-butyl {3,5-Difluoro-4-[bis(2-hydroxyethyl)amino]-phenyl}oxycarbonyl-L-glutamate (12). To a stirred solution containing the phenol **11** (1.50 g, 5.07 mmol) and di-*tert*-butyl 4-nitrophenyloxycarbonyl-L-glutamate (2.26 g, 5.3 mmol) in THF (60 mL) was added 5.0 g Amberlyst 27 (OH^-) at once. The reaction mixture was kept at room temperature for 3.5 h. The resin was filtered, the solvent evaporated under vacuum, and the residue redissoved in AcOEt (20 mL). The solution was washed (2 × 25 mL of H_2O), dried, and evaporated again, giving an oil. After purification by column chromatography (AcOEt), a solid resulted (1.28 g, 48.8%), mp 109−110 °C. ^1H NMR, δ_H 1.40 (s, 9H, t-Bu), 1.42 (s, 9H, t-Bu), 1.93−2.08 (m, 2H, CH_2CHNH), 2.33 (t, 2H, CH_2CO_2, J = 7.99 Hz), 3.13 (t, 4H, NCH_2, J = 6.37 Hz), 3.41 (q, 4H, CH_2OH, J = 6.12 Hz), 3.93−3.99 (m, 1H, C*H*NH), 4.44 (t, 2H, OH, J = 5.19 Hz), 6.87 (d, 2H, $H_{arom2+6}$, J_{H-F} = 9.80 Hz), 8.24 (d, 1H, NH-G, J = 7.80 Hz); MS (FAB), *m/z*: 540 (M^+ + 23, 98), 518 (M^+ + 1,100); acc mass: ($C_{24}H_{38}F_2N_3O_7$) calcd 518.2678, found 518.2656. Anal. ($C_{24}H_{38}F_2N_3O_7$) C, H, F, N.

Di-*tert*-butyl {3,5-Difluoro-4-[bis(2-mesyloxyethyl)-amino]phenyl}oxycarbonyl-L-glutamate (14). To a solution of compound **12** (0.330 g, 0.64 mmol) in 30 mL of DCM were added mesyl anhydride (0.334 g, 1.92 mmol), DMAP (0.033 g), and triethylamine (0.313 mL, 2.25 mmol) under stirring at room temperature. The reaction mixture was stirred for 2 h, and then the solution washed with citric acid 10% (30 mL), dried, and evaporated under vacuum. After HPLC purification (cyclohexane:AcOEt, 1:1), a clear oil was obtained (0.290 g, 67.2%). ^1H NMR, δ_H 1.40 (s, 9H, t-Bu), 1.42 (s, 9H, t-Bu), 1.72−2.05 (m, 2H, CH_2CHNH), 2.34 (t, 2H, CH_2CO_2, J = 8.24 Hz), 3.10 (s, 6H, CH_3SO_2), 3.45 (t, 4H, NCH_2, J = 5.35 Hz), 3.90−4.05 (m, 1H, C*H*NH), 4.20 (t, 4H, C$H_2OSO_2CH_3$, J = 6.12 Hz), 6.94 (d, 2H, $H_{arom2+6}$, J_{H-F} = 9.52 Hz), 8.26 (d, 1H, NH-G, J = 7.89 Hz); MS (FAB), *m/z*: 697 (M^+ + 23, 18), 675 (M^+ + 1, 22); acc mass: ($C_{26}H_{41}F_2N_2O_{12}S_2$) calcd 675.2042, found 675.2069. Anal. ($C_{26}H_{40}F_2N_2O_{12}S_2$) C, H, F, N, S.

Di-*tert*-butyl {3,5-difluoro-4-[bis(2-mesyloxyethyl)amino]phenyl}carbamoyl-L-glutamate (15) was prepared using the same procedure as for compound **14**. Starting from intermediate **13** (1.28 g, 2.47 mmol) and after purification by HPLC (cyclohexane:AcOEt, 1:1), a solid resulted (1.00 g, 59.8%), mp 61−63 °C. ^1H NMR, δ_H 1.39 (s, 9H, t-Bu), 1.42 (s, 9H, t-Bu), 1.72−2.08 (m, 2H, CH_2CHNH), 2.23−2.32 (m, 2H, CH_2CO_2), 3.11 (s, 6H, CH_3SO_2), 3.35−3.41 (m, 4H, NCH_2), 4.16 (t, 4H, C$H_2OSO_2CH_3$, J = 5.44 Hz), 6.61 (d, 1H, NH-G, J = 8.19 Hz), 7.10 (dd, 2H, $H_{arom2+6}$, J_{H-F} = 14.97 Hz, J_m = 3.77 Hz), 8.88 (s, 1H, NHPh); MS (FAB), *m/z*: 696 (M^+ + 23, 5), 674 (M^+ + 1, 14); acc mass: ($C_{26}H_{42}F_2N_3O_{11}S_2$) calcd 673.2151, found 673.2176. Anal. ($C_{26}H_{41}F_2N_3O_{11}S_2$) C, H, F, N.

Di-*tert*-butyl {3,5-Difluoro-4-[bis(2-iodoethyl)amino]-phenyl}oxycarbonyl-L-glutamate (16). To a solution of compound **14** (0.200 g, 0.30 mmol) in 30 mL of acetone was added sodium iodide (0.674 g, 10 equiv) under stirring, and the solution was refluxed for 5 h. The solvent was evaporated, the residue redissolved in AcOEt (25 mL), washed (2 × 25 mL H_2O), dried and evaporated again. An oil resulted which was purified by HPLC (cyclohexane:AcOEt, 3:1) leading to a solid (0.146 g, 75.6%) mp 46−8 °C. ^1H NMR, δ_H 1.40 (s, 9H, t-Bu), 1.42 (s, 9H, t-Bu), 1.72−2.07 (m, 2H, CH_2CHNH), 2.33 (t, 2H, CH_2CO_2, J = 7.96 Hz), 3.22 (t, 4H, NCH_2), 3.44 (t, 4H, CH_2I, J = 7.16 Hz), 3.92−4.06 (m, 1H, C*H*NH), 6.94 (d, 2H, $H_{arom2+6}$, J_{H-F} = 9.69 Hz), 8.26 (d, 1H, NH-G, J = 7.77 Hz); MS (FAB), *m/z*: 739 (M^+ + 1, 18); acc mass: ($C_{24}H_{35}F_2I_2N_2O_6$) calcd 739.0553, found 739.0500. Anal. ($C_{24}H_{34}F_2I_2N_2O_6$) C, H, F, N.

Di-*tert*-butyl {3,5-Difluoro-4-[bis(2-iodoethyl)amino]-phenyl}carbamoyl-L-glutamate (17). The same procedure was used to obtain **17** (0.186 g, 67.6%) starting from the intermediate **15** (0.250 g, 0.37 mmol). After HPLC purification, a thick oil resulted. ^1H NMR, δ_H 1.40 (s, 9H, t-Bu), 1.42 (s, 9H, t-Bu), 1.69−1.99 (m, 2H, CH_2CHNH), 2.24−2.30 (m, 2H, CH_2CO_2), 3.17 (t, 4H, NCH_2), 3.38 (t, 4H, CH_2Br, J = 7.21 Hz), 4.10−4.17 (m, 1H, CHNH), 6.60 (d, 1H, NH-G, J = 7.97 Hz), 7.10 (d, 2H, $H_{arom2+6}$, J_{H-F} = 11.37 Hz), 8.87 (s, 1H, NHPh);

MS (FAB), *m/z*: 760 ($M^+ + 23$, 25), 737 (M^+, 100); acc mass: (C) calcd 737.0634, found 737.0598. Anal. ($C_{24}H_{35}Br_2F_2N_3O_5$) C, H, N, F,

Di-*tert*-butyl {3,5-Difluoro-4-[bis(2-bromoethyl)amino]-phenyl}oxycarbonyl-L-glutamate (18). To a solution of compound **14** (0.197 g, 0.30 mmol) in 25 mL of THF was added lithium bromide (0.260 g, 10 equiv) under stirring, and the solution was refluxed for 5 h. After a standard workup, an oil resulted (0.201 g) which was purified by HPLC (cyclohexane: AcOEt, 3:1) leading to a foam (0.122 g, 63.1%). ^{1}H NMR, δ_H 1.40 (s, 9H, t-Bu), 1.42 (s, 9H, t-Bu), 1.72−2.04 (m, 2H, CH_2-CHNH), 2.34 (t, 2H, CH_2CO$_2$, J = 8.24 Hz), 3.50 (s, 8H, N(CH$_2$-CH$_2$Br)$_2$), 3.92−4.06 (m, 1H, C*H*NH), 6.94 (d, 2H, H$_{arom2+6}$, J_{H-F} = 9.61 Hz), 8.26 (d, 1H, NH-G, J = 7.75 Hz); MS (FAB), *m/z*: 645 ($M^+ + 1$, 35); acc mass: ($C_{24}H_{35}Br_2F_2I_2N_2O_6$) calcd 643.0830, found 643.0801. Anal. ($C_{24}H_{35}Br_2F_2N_2O_6.0.1C_6H_{12}$) C, H, N.

Di-*tert*-butyl {3,5-Difluoro-4-[bis(2-bromoethyl)amino]-phenyl}carbamoyl-L-glutamate (19). Starting from the intermediate **15** (0.250 g, 0.37 mmol), the same procedure as for **18** was used to obtain **19** (0.180 g, 75.7%). After HPLC purification, a foam resulted. ^{1}H NMR, δ_H 1.39 (s, 9H, *t*-Bu), 1.41 (s, 9H, *t*-Bu), 1.71−1.98 (2m, 2H, CH_2CHNH), 2.21 (m, 2H, CH_2CO$_2$), 3.37 (d, 4H, CH$_2$N, J = 6.43), 3.56 (t, 4H, CH$_2$-Br), 4.09−4.15 (m, 1H, C*H*NH-G), 6.60 (d, 1H, NH-G, J = 8.03 Hz), 7.08 (dd, 2H, H$_{arom2+6}$, J_{H-F} = 11.29 Hz), 8.85 (s, 1H, NHPh); MS (FAB), *m/z*: 666 ($M^+ + 23$, 12), 643 ($M^+ + 1$, 56); acc mass: ($C_{24}H_{35}Br_2F_2N_3O_5$) calcd 641.0912, found 641.0900. Anal. ($C_{24}H_{35}Br_2F_2N_3O_5$) C, H, F,

Di-*tert*-butyl {3,5-Difluoro-4-[bis(2-chloroethyl)amino]-phenyl}oxycarbonyl-L-glutamate (20) and Di-*tert*-butyl {3,5-Difluoro-4-[(2-chloroethyl)(2-mesyloxyethyl)amino]-phenyl}oxycarbonyl-L-glutamate (22). To a solution of compound **14** (0.214 g, 0.31 mmol) in 25 mL of acetonitrile was added sodium chloride (0.175 g, 10 equiv) under stirring, and the solution was heated at 65 °C (bath) for 48 h. After solvent evaporation and workup, an oil resulted (0.196 g) which was separated and purified by HPLC (cyclohexane:AcOEt, 3:1). The first fraction was compound **20**, which was obtained as an oil (0.102 g, 59.5%). ^{1}H NMR, δ_H 1.40 (s, 9H, t-Bu), 1.42 (s, 9H, t-Bu), 1.79−2.04 (2×m, 2H, CH_2CHNH), 2.33 (t, 2H, CH_2CO$_2$, J = 7.88 Hz), 3.45 (t, 4H, NCH$_2$, J = 6.28 Hz), 3.61 (t, 4H, CH_2Cl), 3.93−4.02 (m, 1H, C*H*NH), 6.94 (d, 2H, H$_{arom2+6}$, J_{H-F} = 9.64 Hz), 8.27 (d, 1H, NH-G, J = 7.75 Hz); MS (FAB), *m/z*: 577 ($M^+ + 23$, 13), 555 ($M^+ + 1$, 45), 519 ($M^+ - Cl + 1$, 22); acc mass: ($C_{24}H_{35}Cl_2F_2N_2O_6$) calcd 555.1840, found 555.1842. Anal. ($C_{24}H_{34}Br_2F_2N_2O_6$) C, H, Cl, N.

The second fraction was compound **22**, resulting also as an oil (0.032 g, 16.8%). In another experiment a yield of 21.7% was achieved. ^{1}H NMR, δ_H 1.40 (s, 9H, t-Bu), 1.42 (s, 9H, t-Bu), 1.75−1.98 (2×m, 2H, CH_2CHNH), 2.34 (t, 2H, CH_2CO$_2$, J = 7.87 Hz), 3,31 (s, 3H, CH$_3$SO$_2$-), 3.41−3.45 (m, 4H, NCH$_2$), 3.61 (t, 2H, CH_2Cl, J = 6.27 Hz), 3.93−4.02 (m, 1H, C*H*NH), 4.20 (t, 2H, CH_2OSO$_2$CH$_3$, J = 5.30 Hz), 6.94 (d, 2H, H$_{arom2+6}$, J_{H-F} = 9.60 Hz), 8.26 (d, 1H, NH-G, J = 7.75 Hz); MS (FAB), *m/z*: 637 ($M^+ + 23$, 24), 615 ($M^+ + 1$, 35), 579 ($M^+ - Cl + 1$, 9), 519 ($M^+ - Mes + 1$, 12); acc mass: ($C_{25}H_{38}ClF_2N_2O_9S$) calcd 615.1955, found 615.1942. Anal. ($C_{25}H_{38}ClF_2N_2O_9S$) C, H, N, S.

Di-*tert*-butyl {3,5-Difluoro-4-[bis(2-chloroethyl)amino]-phenyl}carbamoyl-L-glutamate (21). A similar procedure starting from **15** (0.232 g. 0.34 mmol) was used to obtain compound **21**. Lithium chloride (0.085 g, 6 equiv) was added and the reaction carried out in DMA, by stirring 48 h at room temperature. An oil resulted which after purification on HPLC (cyclohexane:AcOEt, 3:1) gave (0.130 g, 67.6%). ^{1}H NMR, δ_H 1.39 (s, 9H, t-Bu), 1.41 (s, 9H, t-Bu), 1.72−1.98 (2×m, 2H, CH_2-CHNH), 2.23−2.30 (m, 2H, CH_2CO$_2$), 3.38 (t, 4H, NCH$_2$, J = 6.43 Hz), 3.57 (t, 4H, CH_2Cl), 4.04−4.16 (m, 1H, C*H*NH), 6.60 (d, 1H, NH-G, J = 7.96 Hz), 7.09 (d, 2H, H$_{arom2+6}$, J_{H-F} = 11.28 Hz), 8.86 (d, 1H, NHPh); MS (FAB), *m/z*: 576 ($M^+ + 23$, 20), 553 (M^+, 96), 518 ($M^+ - Cl$, 33); acc mass: ($C_{24}H_{35}Cl_2F_2N_3O_5$) calcd 553.1922, found 553.1906. Anal. ($C_{24}H_{35}Cl_2F_2N_3O_5$) C, H, N.

{3,5-Difluoro-4-[bis(2-iodoethyl)amino]phenyl}-oxycarbonyl-L-glutamic Acid (3a). Compound **16** (0.069 g, 0.09 mmol) was deprotected at room temperature by stirring 24 h, in 5.0 mL of formic acid. After evaporation of the solvent under vacuum, the residue was redissolved 3 × 5.0 mL of AcOEt and 3 × 5.0 mL of DCM and evaporated each time. A white solid resulted (0.052 g, 92.3%), mp 38−40 °C. ^{1}H NMR, δ_H 1.90−2.10 (2×m, 2H, CH_2CHNH), 2.36 (t, 2H, CH_2CO$_2$, J = 7.91 Hz), 3.21 (t, 4H, NCH$_2$, J = 7.20 Hz), 3.45 (t, 4H, CH_2I), 3.98−4.07 (m, 1H, C*H*NH), 6.95 (d, 2H, H$_{arom2+6}$, J_{H-F} = 9.72 Hz), 8.22 (d, 1H, NH-G, J = 7.95 Hz); MS (FAB), *m/z*: 649 ($M^+ + 23$, 38), 627 ($M^+ + 1$, 64), 499 ($M^+ - I + 1$, 52); acc mass: ($C_{16}H_{19}I_2F_2N_2O_6$) calcd 626.9301, found 626.9281. Anal. ($C_{16}H_{18}I_2F_2N_2O_6$) C, H, N.

Using a similar procedure, the following prodrugs were prepared:

{3,5-Difluoro-4-[bis(2-bromoethyl)amino]phenyl}-oxycarbonyl-L-glutamic acid (3b): except toluene 3 × 5.0 mL was used instead of the AcOEt in order to remove formic acid (0.041 g, 86.5%), mp 97−8 °C. ^{1}H NMR, δ_H 1.81−2.07 (2×m, 2H, CH_2CHNH), 2.36 (t, 2H, CH_2CO$_2$, J = 7.79 Hz), 3.50 (s, 8H, N(CH$_2$CH$_2$Br)$_2$), 4.00−4.07 (m, 1H, C*H*NH), 6.95 (d, 2H, H$_{arom2+6}$, J_{H-F} = 9.63 Hz), 8.24 (d, 1H, NH-G, J = 7.95 Hz); MS (FAB), *m/z*: 555 ($M^+ + 23$, 17), 533 ($M^+ + 1$, 62), 499 ($M^+ - I + 1$, 52); acc mass: ($C_{16}H_{19}Br_2F_2N_2O_6$) calcd 530.9578, found 530.9569. Anal. ($C_{16}H_{18}Br_2F_2N_2O_6$) C, H, N:calc 5.22, found 4.78.

{3,5-Difluoro-4-[bis(2-chloroethyl)amino]phenyl}-oxycarbonyl-L-glutamic acid (3c): 0.063 g, 98.0%, glassy solid. ^{1}H NMR, δ_H 1.82−2.10 (2×m, 2H, CH_2CHNH), 2.36 (t, 2H, CH_2CO$_2$, J = 7.37 Hz), 3.44 (d, 4H, NCH$_2$, J = 6.28 Hz), 3.61 (t, 2H, CH$_2$Cl), 4.00−4.12 (m, 1H, C*H*NH), 6.95 (d, 2H, H$_{arom2+6}$, J_{H-F} = 9.64 Hz), 8.25 (d, 1H, NH-G, J = 7.85 Hz); MS (FAB), *m/z*: 444 ($M^+ + 1$, 62); acc mass: ($C_{16}H_{19}Cl_2F_2N_2O_6$) calcd 443.0588, found 443.0588. Anal. ($C_{16}H_{18}Cl_2F_2N_2O_6$) C, H, N: calcd 6.32, found 5.80.

{3,5-Difluoro-4-[bis(2-iodoethyl)amino]phenyl}-carbamoyl-L-glutamic acid (4a): except toluene 3 × 5.0 mL was used instead of the AcOEt in order to remove formic acid (0.112 g, 94.7%), mp 78−80 °C. ^{1}H NMR, δ_H 1.75−2.05 (2×m, 2H, CH_2CHNH), 2.28 (t, 2H, CH_2CO$_2$, J = 7.98 Hz), 3.17 (t, 4H, NCH$_2$, J = 7.20 Hz), 3.38 (t, 4H, CH_2I), 4.04−4.23 (m, 1H, C*H*NH), 6.61 (d, 2H, NH-G, J = 7.93 Hz), 7.10 (d, 2H, H$_{arom2+6}$, J_{H-F} = 11.35 Hz), 8.91 (s, 1H, NH-G); MS (FAB), *m/z*: 626 ($M^+ + 1$, 64); acc mass: ($C_{16}H_{20}I_2F_2N_3O_5$) calcd 625.9461, found 625.9447. Anal. ($C_{16}H_{19}I_2F_2N_3O_5$) C, H, N.

{3,5-Difluoro-4-[bis(2-bromoxyethyl)amino]phenyl}-carbamoyl-L-glutamic acid (4b): 0.080 g, 93.7%, mp 81−3 °C. ^{1}H NMR, δ_H 1.75−2.08 (2×m, 2H, CH_2CHNH), 2.25−2.35 (m, 2H, CH_2CO$_2$), 3.44 (s, 8H, N(CH$_2$CH$_2$Br)$_2$), 4.11−4.21 (m, 1H, C*H*NH), 6.62 (d, 1H, NH-G, J = 7.95 Hz), 7.10 (d, 2H, H$_{arom2+6}$, J_{H-F} = 11.29 Hz), 8.1 (s, 1H, NHPh); MS (FAB), *m/z*: 532 ($M^+ + 1$, 50), 452 ($M^+ - Br + 1$, 12); acc mass: ($C_{16}H_{20}$-$Br_2F_2N_3O_5$) calcd 529.9738, found 529.9732. Anal. ($C_{16}H_{19}$-$Br_2F_2N_2O_6$) C, H, N.

{3,5-Difluoro-4-[bis(2-chloroethyl)amino]phenyl}-carbamoyl-L-glutamic acid (4c): 0.079 g, 94.0%, mp 74−6 °C. ^{1}H NMR, δ_H 1.76−2.05 (2×m, 2H, CH_2CHNH), 2.28 (t, 2H, CH_2CO$_2$, J = 6.11 Hz), 3.38 (d, 4H, NCH$_2$, J = 6.44 Hz), 3.57 (t, 2H, CH$_2$Cl), 4.14−4.23 (m, 1H, C*H*NH), 6.62 (d, 1H, NH-G, J = 7.94 Hz), 7.10 (d, 2H, H$_{arom2+6}$, J_{H-F} = 11.32 Hz), 8.91 (s, 1H, NHPh); MS (FAB), *m/z*: 464 ($M^+ + 23$, 11), 442 ($M^+ + 1$, 100); acc mass: ($C_{16}H_{20}Cl_2F_2N_2O_6$) calcd 442.0748, found 442.0757. Anal. ($C_{16}H_{19}Cl_2F_2N_3O_5$) C, H, N: calcd 9.50, found 8.91.

2,6-Difluoro-[bis(2-hydroxyethyl)]aniline (23). 2,6-Difluoroaniline **22** (6.45 g, 50 mmol) was dissolved in AcOH (400 mL), and ethylene oxide (22.5 mL) was added. The reaction was stirred at room temperature for 3 days. The solvent was evaporated, first under water vacuum then under pump vacuum, to afford **2** (12.0 g) as an oil, still containing ethylene glycol monoacetate as an impurity. This mixture was used crude, without further purification, in the next step. ^{1}H NMR, δ_H, (ppm): 3.16 (t, 4H, N(CH_2CH$_2$OH)$_2$, J = 6.33 Hz), 3.37−

3.45 (m, 4H, $N(CH_2CH_2OH)_2$), 4.43 (t, 2H, OH, $J = 5.28$ Hz), 6.96−7.13 (m, 3H, $H_{arom3,4,5}$).

2,6-Difluoro-4-nitro-[bis(2-hydroxyethyl)]aniline (24). Crude 2,6-difluoro[bis(2-hydroxyethyl)]aniline **2** (12 g) from the previous step was cooled on an ice-bath, and a solution of fuming HNO_3 (18 mL) in DCM (100 mL) was added in portions, with stirring. After 15 min, the reaction was neutralized with solid Na_2CO_3 added in portions until CO_2 evolution stopped. The solid was filtered off, the filtrate was evaporated, and the residue was purified by column chromatography (eluent cyclohexane: ethyl acetate 1:1 then 1:2) to afford **3** (4.2 g, 32% over two steps) as a yellow solid. ^{1}H NMR, δ_H, (ppm): 3.41 (t, 4H, $N(CH_2CH_2OH)_2$, $J = 5.35$ Hz), 3.47−3.55 (m, 4H, $N(CH_2CH_2OH)_2$), 4.60 (t, 2H, OH, $J = 4.99$ Hz), 7.92 (d, 2H, $H_{arom3,5}$, $J_{H-F} = 10.47$ Hz). MS, *m/z*: 263 ($M^+ + H$, 100), 218 ($(M^+ + H - NO_2$, 97).

2,6-Difluoro-4-amino-[bis(2-hydroxyethyl)]aniline (25). 4-Nitro-2,6-difluoro-[bis(2-hydroxyethyl)]aniline **24** (3.9 g) was dissolved in THF (70 mL), palladium on carbon 10% catalyst (1 g) was added, and the reaction mixture was stirred under H_2 atmosphere for 4 h. The catalyst was filtered off and the solvent evaporated to afford a brown oil (3.4 g, 98%). ^{1}H NMR, δ_H, (ppm): 2.97 (t, 4H, $N(CH_2CH_2OH)_2$, $J = 6.62$ Hz), 3.29−3.37 (m, 4H, $N(CH_2CH_2OH)_2$), 4.30 (t, 2H, OH, $J = 5.38$ Hz), 5.45 (s, 2H, NH_2), 6.13 (d, 2H, $H_{arom3,5}$, $J_{H-F} = 11.36$ Hz). MS, *m/z*: 233 ($M^+ + H$, 100).

Aqueous Half-Life Determination. Compounds were prepared as 10 mM concentrates in DMSO 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 Synergi Polar RP phenyl phase column (150 × 4.6 mm, 4 μm, Phenomenex) and eluted isocratically (1 mL/min) with 0.1% TFA solution containing percentages of methanol (65−85%) that gave retention times of 3−4 min, and the elute was monitored at 250−275 nm. The amount of starting material remaining after various periods of incubation was determined either by repeat injection from a single vial (**3c**, **4c**) or by delayed injections from a new vial each time (all others). The results were expressed as fraction of starting materials as a function of time, and the half-life was determined by nonlinear regression to a one-phase exponential decay, constraining the maximum to 1 and the minimum to 0 (Graphpad Prism, Graphpad Software Inc., San Diego, CA).

Enzyme Kinetics. Reactions were set up containing CPG2 assay buffer (1 mL), CPG2 (50 mU) and prodrug (5−50 μM in steps of 5) from concentrates as above. The vials were incubated at 37 °C, and the amount of prodrug remaining in the mixture was determined by HPLC as above at 0, 5, 10, 15, and 20 min poststart. The rate of loss of compound in μM/min was determined by regression. The rate of chemical-only loss, calculated from the first derivative of the equation for exponential decay, was subtracted, and the kinetic parameters were derived from the nonlinear regression to the Michaelis−Menten equation (Graphpad Prism).

Cytotoxicity Determination. MDA MB 361 (5×10^5) cells were seeded into six-well tissue culture dishes, yielding similarly confluent monolayer at 48 h. Prodrugs were dissolved in DMSO at 100 fold the highest dose (**3a**−**e**: 10mM; **4a**,**b**: 100 mM; **4c**,**d**: 200 mM), immediately prior to treatment and administered in a two-stage protocol, as described previously. After exposure to prodrug, the cells were harvested, diluted, reseeded, and grown until the control wells reached confluence, and the extent of cell growth was assayed by sulforhodamine-B dye. The results were expressed as the percentage of control growth against log dose, and the IC_{50} was determined by nonlinear regression to a log dose−effect sigmoid, constraining the minimum to be positive (GraphPad Prism, GraphPad Software Inc., San Diego, CA).

In Vivo Antitumor Activity. Cells of the human breast tumor cell line MDA MB 361 were engineered for stable expression of stCPG2(Q)3 or for control β-galactosidase. Suspensions prepared in Dulbecco's PBS (Gibco) of 100% MDA MB 361stCPG2(Q)3. Cells (10^7/mouse) were inoculated subcutaneously on the right flank of female CD1 *nu/nu* mice (20−27 g) (Charles River UK). After 4 days, six mice per group were allocated by stratified distribution of tumor sizes. Compound **4c** was prepared at a concentration of 12 mg/mL in dimethyl sulfoxide/1.26% (w/v) sodium bicarbonate (1:19 by volume). Mice were dosed intraperitoneally with either 0.2 mL per 20 g bodyweight 5% (v/v) DMSO in water for injection of controls or compound **4c** at 360 mg/kg bodyweight three times over 24 h. Six courses of treatment, where one course is defined as 3 doses over 24 h, were given at weekly intervals. Tumor growth was assessed by caliper measurement in three dimensions, and volumes were calculated from the formula length × width × depth × 0.5236. Mice were culled when tumor size exceeded 2 cm in one dimension or 1.5 cm in two dimensions or when tumors became ulcerated.

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