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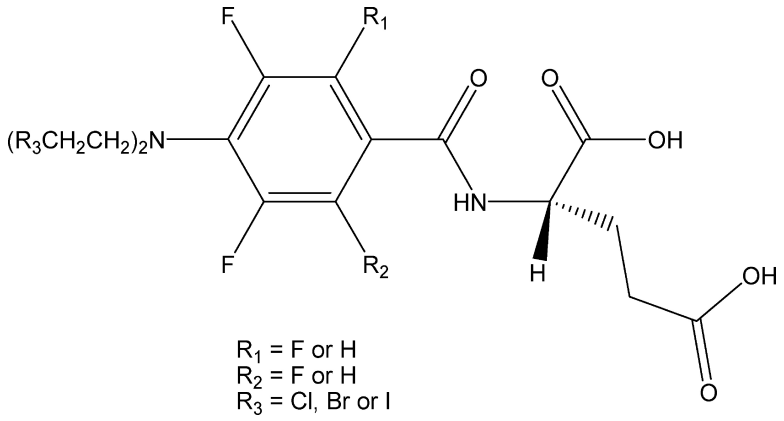

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Novel Fluorinated Prodrugs for Activation by Carboxypeptidase G2 Showing Good in Vivo Antitumor Activity in Gene-Directed Enzyme Prodrug Therapy

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Sixteen novel polyfluorinated benzoic acid mustards have been synthesized for use in gene-directed enzyme prodrug therapy (GDEPT). Eight of these were benzoic acid L-glutamate mustards for evaluation as prodrugs and the other eight were the active drugs formed by the action of the bacterial enzyme carboxypeptidase G2 (CPG2). All of the di- and trifluorinated prodrugs were efficiently cleaved by the enzyme. In contrast, the tetrafluorinated prodrugs were found to be competitive inhibitors of CPG2, the first such inhibitors to have been described. The di- and trifluorinated prodrugs were differentially cytotoxic to human breast carcinoma cells (MDA MB 361) expressing CPG2, compared to control cells that did not express the enzyme. The difluorinated prodrug {4-[bis(2-bromoethyl)amino]-3,5-difluorobenzoyl}-L-glutamic acid and its iodoethylamino analogue were effective substrates for the enzyme and showed excellent therapeutic activity in CPG2-expressing MDA MB 361 xenografts, either curing or greatly inhibiting tumor growth and extending the life of the animals.

Introduction

Gene-directed enzyme prodrug therapy (GDEPT) is a two-step approach to the therapy of cancer.[1] The foreign gene for a prodrug-activating enzyme is selectively introduced into the tumor by a vector, such as a virus or liposome, by either local or systemic delivery. Time is allowed for the enzyme to be expressed in the tumor, and then a nontoxic prodrug is administered. This prodrug is converted by the enzyme within the tumor to a toxic drug. This localized activation reduces the normal tissue toxicity associated with conventional cytotoxic chemotherapy. A number of enzyme/prodrug systems have been evaluated for GDEPT.[2] In our laboratory, we have concentrated on the enzyme carboxypeptidase G2 (CPG2, glutamate carboxypeptidase, EC 3.4.17.11) derived from *Pseudomonas RS16*. This enzyme catalyses the cleavage of amide bonds between a carboxyl-, phenol-, or aniline-substituted aromatic ring and L-glutamic acid, an activity unknown in any mammalian enzyme. Our GDEPT system has significant benefits in that prodrugs can be designed that release several different types of effector molecule, including aromatic mustards and antibiotics. Moreover, the active cytotoxic compound is released from the prodrug in a single step without requiring subsequent processing by host enzymes that may be lacking in tumor cells. The benzoic acid mustard-derived prodrug (2-chloroethyl)-(2-(methylsulfonyloxyethyl)amino)aminobenzyl-L-glutamic acid (**1**) is a substrate for CPG2 that releases the cytotoxic alkylating agent 4-(2-chloroethyl)(2-(methylsulfonyloxyethyl)amino)benzoic acid (**1a**) (Scheme 1). Prodrug **1** has been shown to be effective both in vitro and in vivo against tumor cells expressing CPG2, especially when the enzyme had been engineered to be expressed externally, tethered to the outer membrane of the cell.[3] When CPG2 was expressed in the cytoplasm, the efficacy was much reduced (Table 1). This has been shown to be due to poor transport of the prodrug through the cell membrane.[3] We have synthesized novel benzoic acid prodrugs (**21**−**28**) for use in GDEPT with di-, tri-, and tetrafluorinated benzene rings. These prodrugs are designed to be more lipophilic than **1** with the aim of improving intracellular access and efficacy. The substitution of a single fluorine atom at the 3-position of the aromatic ring has been shown previously by our group to improve efficacy in ADEPT (antibody-directed enzyme prodrug therapy).[4] The prodrugs were tested for their chemical half-life, as substrates of CPG2, and for cytotoxicity against a human breast carcinoma cell line (MDA MB 361) expressing CPG2, either cytoplasmically or tethered to the outer surface of the cell membrane. Prodrugs active in the latter assay were tested for therapeutic efficacy in xenografts of the same cell line, growing in nude mice.

Scheme 1. Hydrolysis of Prodrug 1 by Carboxypeptidase G2

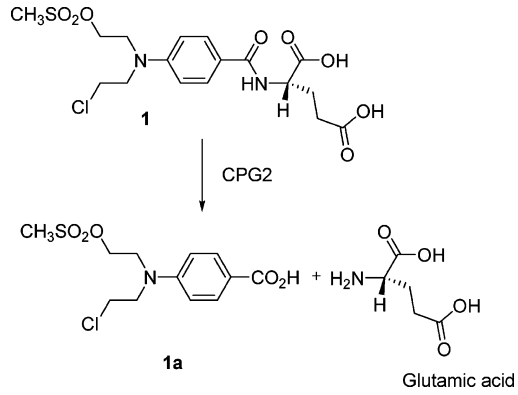

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Table 1. Cytotoxicity of Prodrugs and Drugs to the Three MDA MB361 Cell Lines[b]

	IC_{50}, μM			differential[a]	
no.	stCPG2(Q)3	CPG2*	LacZ	stCPG2(Q)3	CPG2*
			prodrugs		
1	165[3] (±13)	1880[3] (±128)	3130[3] (±234)	19	1.7
23	26 (18−38)	42 (25−69)	>4000	>150	>95
25	14 (7−29)	53 (26−109)	>4000	>280	>75
28	26 (16−42)	39 (22−66)	3300 (2040−5330)	127	86
22	46 (30−70)	73 (35−153)	>4000	>87	>55
24	50 (31−82)	82 (28−239)	>4000	>80	>49
27	173 (104−288)	671 (384−1180)	>4000	>23	> 6
21	>4000	>4000	>4000	N/A	N/A
26	>4000	>4000	>4000	N/A	N/A
			drugs		
48	25.9 (9.5−71)	22.0 (12−41)	23.8 (11−54)	1	1
50	21.1 (13−35)	20.6 (8.1−52)	23.4 (10−53)	1	1
53	12.7 (7.5−22)	11.9 (7−20)	6.0 (4.2−8.6)	0.5	1
47	57.8 (31−107)	57.9 (30−115)	47.6 (25−90)	1	1
49	30.0 (19−47)	35.1 (20−62)	44.9 (19−104)	1.5	1
52	24.7 (18−35)	34.4 (21−44)	29.6 (20−44)	1	1
46	378 (232−616)	533 (338−841)	447 (296−678)	1	1
51	60.5 (35−104)	66.7 (24−193)	57.7 (31−107)	1	1

[a] The differential is defined as (IC_{50} of the LacZ cell line)/(IC_{50} of the CPG2-expressing cell line). [b] The bracketed values indicate the 95% confidence intervals or the standard error of the mean.

Results and Discussion

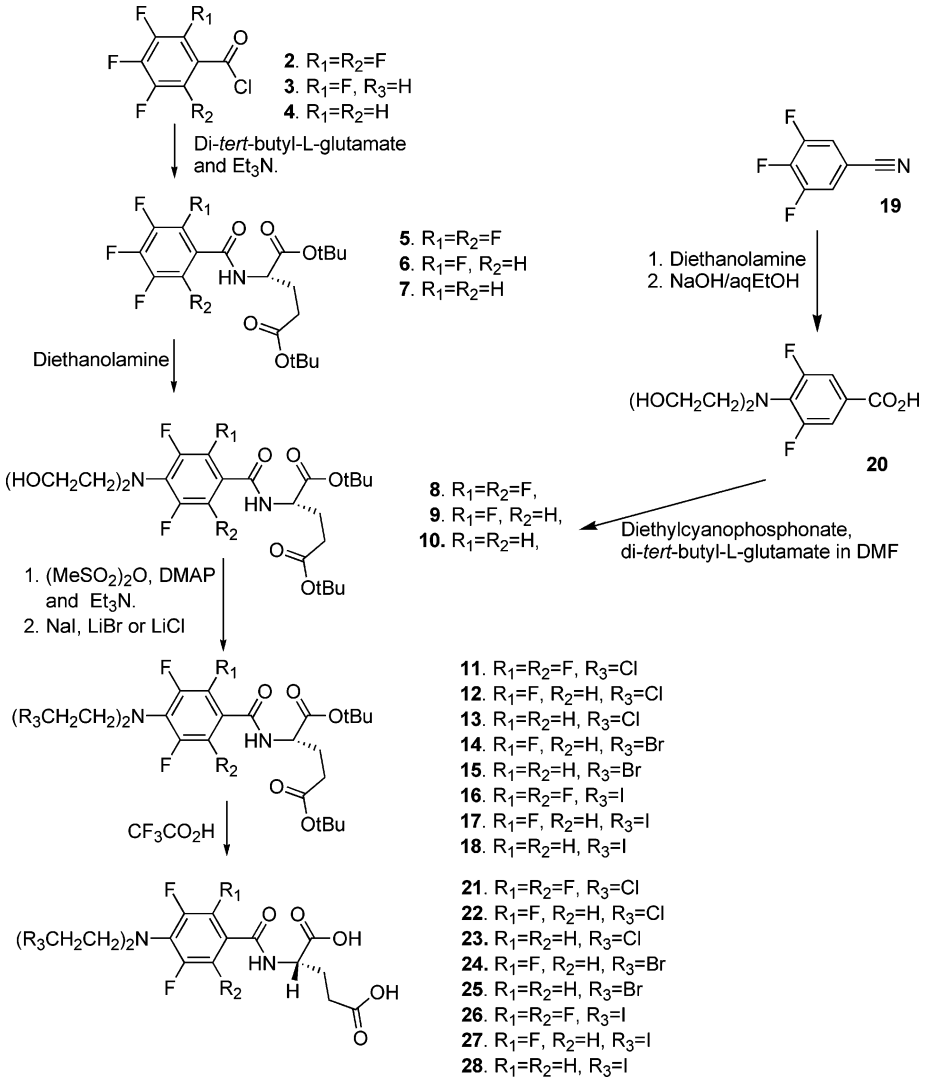
Chemistry. Our initial strategy for the synthesis of prodrugs (**21**−**28**) was to replace the *p*-fluorine of a protected polyfluorinated benzoyl glutamate with diethanolamine, followed by formation of the mustard functionality and deprotection (Scheme 2). Reaction of the polyfluorinated benzoyl chlorides (**2**−**4**) with di-*tert*-butyl-L-glutamate gave the amides (**5**−**7**) in 88−98% yield. Substitution of the *p*-fluorine of pentafluorobenzoyl-L-glutamate (**5**) by diethanolamine in DMA gave **8** in 82% yield. With 2,3,4,5-tetrafluorobenzoylglutamate (**6**), substitution gave **9** in only 31% yield. With 3,4,5-trifluorobenzoylglutamate (**7**), none of the desired product **10** was obtained; even when the solution was refluxed, only polar products were formed, probably due to cleavage of the *tert*-butyl protecting groups under the relatively harsh conditions. We reasoned that a strongly electron withdrawing group would greatly increase the reactivity of the *p*-fluorine to nucleophilic substitution. The cyano group was chosen because of the relative ease of its hydrolysis. Substitution of 3,4,5-trifluorobenzonitrile **19** with diethanolamine to form 4-(bis(2-hydroxyethyl)amino)-3,5-difluorobenzonitrile readily occurred, even at 50 °C. The cyano group was then hydrolyzed to the carboxylic acid in 87% yield by sodium hydroxide in aqueous ethanol, followed by acidic workup to form 4-(bis(2-hydroxyethyl)amino)-3,5-difluorobenzoic acid (**20**). The benzoic acid **20** was then activated with diethyl cyanophosphonate in DMF. Reaction with di-*tert*-butyl-L-glutamate formed the amide bond to give the desired di-*tert*-butyl-L-glutamate **10** in high yield. Protection of the alcoholic hydroxy groups was found to be unnecessary.

In previous mustard syntheses,[5] the mustard functionality was introduced by reaction of the hydroxyl of the hydroxyethyl group with methane sulfonyl chloride to form a bis-mesyl compound, followed by nucleophilic substitution with halide. This method works well in the case of chloro mustards, but for bromo and iodo mustards it is necessary to chromatographically purify the bis-mesyl compounds in order to eliminate the possibility of contamination by chloride. We decided to synthesize the bis-mesyl compounds using methane sulfonic anhydride instead of the chloride. Following substitution of the mesyl group by halide, we obtained excellent yields of the bis-chloro, bis-bromo, and bis-iodo mustards (**11**−**18**) without the need for intermediate chromatography. In the final step the *tert*-butyl group was removed by trifluoroacetic acid at room temperature to give the desired prodrugs (**21**−**28**) as white crystalline solids in yields of 80−100%. A preliminary report of the synthesis of **28** has been published.[6]

The corresponding drugs (**46**−**53**) were made according to Scheme 3. The commercially available polyfluorinated benzoyl chlorides (**29**−**31**) were reacted with lithium *tert*-butoxide giving the *tert*-butyl esters (**32**−**34**). The *p*-fluorine was then substituted with diethanolamine to give the bis-hydroxyethylamino compounds (**35**−**37**). The ester group is sufficiently electron withdrawing to allow substitution of the *p*-fluorine, even in the case of the 3,4,5-trifluorobenzoyl ester **34**. The bis(hydroxyethyl)amino compounds were then converted to the bis-bromo, bis-chloro, and bis-iodo mustard esters (**38**−**45**) as described above for the prodrugs. Acidic hydrolysis with trifluoroacetic acid gave the drugs (**46**−**53**) in 73−94% yield as white crystalline solids.

Biology and Biochemistry. We tested the eight new prodrugs (**21**−**28**) in vitro for cytotoxicity against three variants of the human breast carcinoma cell line MDA MB 361 that had been engineered to express CPG2 either internally in the cytoplasm (CPG2*), externally tethered to the outer cell membrane (stCPG2(Q)3), or that had been engineered to express an irrelevant enzyme (β-galactosidase) as a control (LacZ). All the difluoro- and trifluorobenzoyl compounds (**22**−**25**, **27**−**28**) were selectively cytotoxic to both the CPG2*- and stCPG2(Q)3-expressing cell lines, while the tetrafluorobenzoyl prodrugs (**21**, **26**) showed no toxicity to any of the cell lines even at 4 mM (Table 1). The compounds most selective toward cells expressing CPG2 were the three difluorobenzoyl compounds (**23**, **25**, **28**), with differentials between the IC_{50} values in control β-gal-expressing cells and in CPG2-expressing cells of >150×, >280×, and 127× for stCPG2(Q)3-expressing cells and

Scheme 2



>95×, >75×, and 86× for CPG2*-expressing cells, respectively. The bis(chloroethyl)amino- and bis(bromoethyl)aminotrifluorobenzoyl compounds (**22**, **24**) showed cytotoxicity differentials of >87× and >80× for stCPG2(Q)3-expressing cells and >55× and > 49× for CPG2*-expressing cells, respectively. The bis(iodoethyl)aminotrifluorobenzoyl prodrug **27** was less cytotoxic toward both types of CPG2-expressing cell, showing cytotoxicity differentials between control β-gal-expressing cells and stCPG2(Q)3-expressing cells or CPG2*-expressing cells of >23× and >6×, respectively. All the new compounds showed larger cytotoxicity differentials between control cells and both CPG2*- and stCPG2(Q)3-expressing cells than the literature compound **1**. To determine the reasons for the differences, we examined the kinetics of the cleavage of these compounds by CPG2 and their chemical half-lives.

The bis(chloroethyl)-, bis(bromoethyl)- and bis(iodoethyl)amino difluoro prodrugs (**23**, **25**, **28**) and the bis(chloroethyl)- and bis(bromoethyl)aminotrifluorobenzoyl prodrugs (**22**, **24**) had K_m values <3 μM and K_{cat} values >700 and are thus better substrates than the literature compound **1** (Table 2). The bis(iodoethyl)-amino trifluorobenzoyl prodrug (**27**) has a K_m of 2.8 μM but a lower K_{cat} of 220. The poorer rate of turnover probably explains the smaller cytotoxicity differential between control and CPG2-expressing cells exhibited by this compound. The two tetrafluorobenzoyl compounds (**21**, **26**) were not cleaved at all by CPG2, which explains their lack of enhanced activity in CPG2-expressing cells.

As expected, all the drugs tested (**46**−**53**) were cytotoxic but showed no cytotoxicity differential between the CPG2-expressing and β-gal-expressing lines (Table 1). The cytotoxicity of the five prodrugs that are good substrates (**22**−**25**, **28**) toward the cell line expressing CPG2 externally (stCPG2(Q)3) was comparable to that of their corresponding drug, implying that complete activation had occurred. In contrast, the cytotoxicity of these same prodrugs toward the cell line expressing CPG2 internally (CPG2*) was slightly lower, suggesting that there is still, to some degree, a barrier to their reaching the enzyme. Nevertheless, they are superior in this respect to compound **1**. The half-lives of all the drugs and prodrugs, presumed to reflect the chemical hydrolysis of the halogen in the mustard moiety, were measured in the same buffer as that used for the enzyme kinetics (Table 2). For a given extent of ring fluorination, the comparative rate of hydrolysis between different mustard halogens was bromo > iodo ≫ chloro. For a given mustard halogen series, the comparative rate of hydrolysis between different degrees of ring fluorination was difluoro > trifluoro > tetrafluoro.

Scheme 3

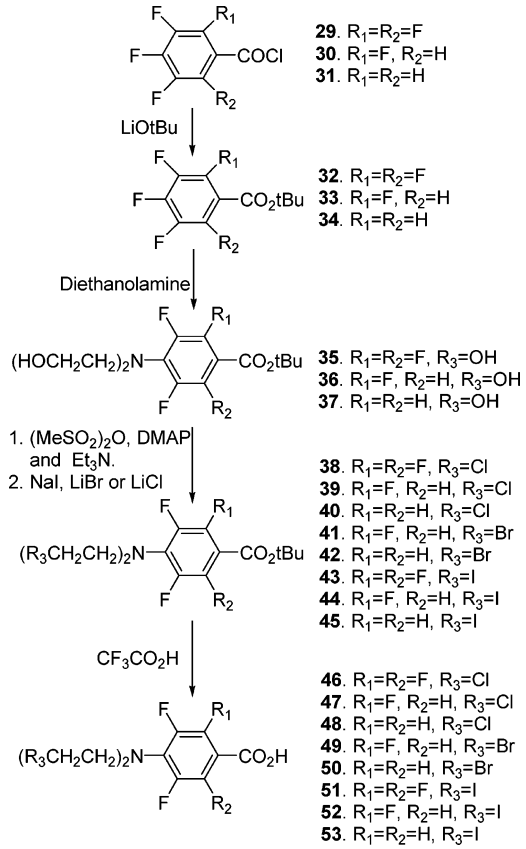


Table 2. Half-Lives of Prodrugs and Drugs and Enzyme Kinetic Properties of Prodrugs

prodrug	K_m, μM	K_{cat}, S^{-1}	$T_{1/2}$, min	drug	$T_{1/2}$, min
1	3.4[1]	583[1]	59	1a	30
23	1.71	732	294	48	91
25	1.18	827	5.5	50	2.5
28	1.74	1150	9.0	53	4.2
22	2.6	1150	1300	47	360
24	1.1	825	22.9	49	8.9
27	2.8	220	37.3	52	13.4
21	N/A	0	4940	46	1365
26	N/A	0	120	51	35

We tested both tetrafluorobenzoyl prodrugs (**21**, **26**) that were not substrates of CPG2 to see whether they were inhibitors, and this proved to be the case, both being competitive with methotrexate, the conventional unit-definition substrate for this enzyme. The bis(chloroethyl)amino compound **21** was the more potent inhibitor with a K_i of 127 μM, compared to 276 μM for the bis(iodoethyl)amino compound, **26**. This is the first reported example of an inhibitor of CPG2 found to be competitive with methotrexate. The only inhibitors of CPG2 described in the literature[7] are more potent but are noncompetitive with respect to methotrexate. The new compounds (**21**, **26**) therefore have the potential to be good models for designing a stable analogue to use as a probe of the CPG2 substrate binding site.

The six active compounds were tested for therapeutic efficacy against MDA MB 361 xenografts growing in nude mice (Figure 1). The bis(chloroethyl)amino- and bis(iodoethyl)aminotrifluorobenzoyl prodrugs (**22**, **27**) gave no delay in the growth of the tumors relative to the untreated controls, probably because the former

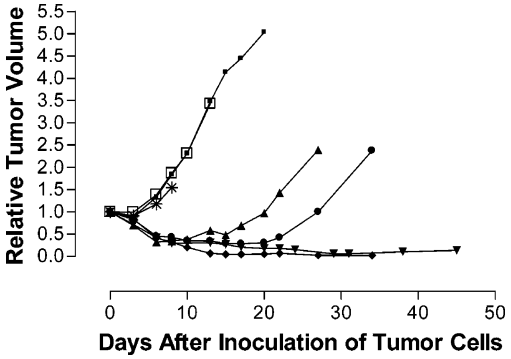


Figure 1. Human breast carcinoma MDA MB 361 (stCPG2-(Q)3) xenografts were treated with **22** (□), **23** (●), **24**(▲), **25** (◆), **27** (■), **28** (▼), or nothing (*) according to the regime detailed in Experimental Section. Relative tumor volumes are plotted against time from establishment of tumors, and the lines in each group are continued until the first death has occurred. Two mice were tumor free for >150 days in the group treated with prodrug **28**.

gives a drug with a very long half-life (360 min) and the latter is a poor substrate. The bis(chloroethyl)aminodifluoro- and bis(bromoethyl)aminotrifluorobenzoyl compounds (**23**, **24**), both effective substrates but yielding drugs with relatively long half-lives (90 and 9 min), gave a substantial delay in the growth of the tumors. The bis(bromoethyl)amino- and bis(iodoethyl)aminodifluorobenzoyl prodrugs (**25**, **28**), both excellent substrates for CPG2 and yielding drugs with short half-lives (<5min), gave a sustained delay in the growth of the tumors, and two mice treated with **28** were cured of their tumors. The latter compound had no therapeutic effect on a non-CPG2-expressing tumor (data not shown).

Conclusions

We have synthesized eight novel polyfluorinated prodrugs (**21**−**28**), along with their corresponding drugs (**46**−**53**), for use in GDEPT. We have shown that our fluorinated prodrugs are much more active than the literature compound **1** toward cells in which CPG2 has been engineered to be expressed in the cytoplasm and speculate that this reflects improved access to the enzyme. We have shown that several of our compounds have a lower K_m and higher K_{cat} than compound **1**. Within our series we find unsurprisingly that compounds of less favorable kinetic properties are less active. We also find that the tetrafluorobenzoyl prodrugs (**21**, **26**) were not substrates but were competitive inhibitors, whereas the trifluorobenzoyl prodrugs (**22**, **24**, **27**) were moderate or good substrates. The only difference between these two groups is the 6-fluorine atom on the aromatic ring. Modeling of these compounds in the active site of CPG2 will allow the design of more potent inhibitors and substrates with improved kinetic properties. These compounds could also serve as probes for engineering improvements in CPG2. Two of the difluorinated prodrugs (**25**, **28**) demonstrated good selectivity in vitro and were excellent substrates for CPG2. They have been shown to have excellent therapeutic activity in a xenograft tumor model and will make very good candidates for GDEPT.

Experimental Section

Chemistry. Preparative HPLC was performed on an Axxial Chromatospac Prep 10 using Merck Kieselgel 60 (0.015−0.040)

(Art. 15111). Melting points were determined on a Kofler hotstage (Reichert Thermovar) melting point apparatus and are uncorrected. EI, FAB, and LDI spectra were performed by the School of Pharmacy, University of London. Electrospray mass spectrum were carried out on a Waters/Micromass LCT system. NMR spectra were determined in Me_2SO-d_6 on a Bruker AC250 spectrometer at 25 °C.

Di-*tert*-butyl Pentafluorobenzoyl-L-glutamate (5). To an ice-cold solution of di-*tert*-butyl-L-glutamate hydrochloride (4.7 g, 16 mmol) and Et_3N (4.4 mL, 32 mmol) in dry CH_2Cl_2 (30 mL) was added pentafluorobenzoyl chloride (2.4 mL, 16 mmol) in dry CH_2Cl_2 (30 mL) over 5 min. After 1 h the ice was removed and the next day CH_2Cl_2 (140 mL) was added. The solution was washed with water, dried ($MgSO_4$), and concentrated to dryness. The residue crystallized from *n*-hexane as white crystals (6.36 g, 88%): mp 68−69 °C; NMR δ_H 1.40 + 1.43 (18H, 2s, tBu), 1.8−2.0 (2H, ddt, *CH*$_2$CH), 2.32 (2H, t, CH_2CO_2, J = 8 Hz), 4.35 (1H, ddd, CH), 9.24 (1H, d, NH, J = 8 Hz); δ_F −141.4 (2F, dd, F2, $J_{2,3}$ = 22 and 7 Hz), −152.3 (1F, t, F4, $J_{3,4}$ = 22 Hz), −160.9 (2F, ddd, F3, $J_{2,3+3,4}$ =22 Hz, $J_{2,3}$= 7 Hz); MS (FAB) *m/z* 454 (M^+ + 1), 398 (M^+ − tBu +2). Anal. ($C_{20}H_{24}F_5NO_5$) C, H, N, F.

Di-*tert*-butyl {4-[Bis(2-hydroxyethyl)amino]-2,3,5,6-tetrafluorobenzoyl}-L-glutamate (8). Ester **5** (3.63 g, 8 mmol) and diethanolamine (1.5 mL, 16 mmol) were heated in DMA (30 mL) at 85 °C for 6 days. The solvent was removed in vacuo and the residue partitioned between CH_2Cl_2 and water. The product was purified by preparative HPLC with CH_2Cl_2/EtOH (19:1) as eluent to give 2.62 g (61%) of white crystals: mp 65−67.5 °C; NMR δ_H 1.40 +1.42 (18H, 2s, t-Bu), 1.8 +2.0 (2H, 2m, *CH*$_2$CH), 2.31 (2H, dt, 2H, CH_2CO_2, J_{CH2CH2} = 8 Hz, J_{CHCH2} = 2.5 Hz), 3.33 (4H, t, CH_2N, J = 5.5 Hz), 3.50 (4H, q, CH_2O, J = 6 Hz), 4.30 (1H, octet, CH, J_{CHCH2} = 5 Hz), 4.54 (2H, t, OH, J = 5 Hz), 9.03(1H, d, NH, J = 7.5 Hz); δ_F −144.2 (2F, q, F2, $J_{2,3}$ = 23 Hz, $J_{2,5}$ = 8 Hz), −148.5 (2F, q, F3, $J_{2,3}$ = 23 Hz, $J_{3,6}$ = 8 Hz); MS (FAB) *m/z* 561 (M + Na^+), 449 (M − (CH_2-$CH_2OH)_2$ + 1), 280 (M − glutamate). Anal. ($C_{24}H_{34}F_4N_2O_7$) C, H, N, F.

Di-*tert*-butyl {4-[Bis(2-iodoethyl)amino]-2,3,5,6-tetrafluorobenzoyl}-L-glutamate (16). To an ice-cold solution of ester **8** (0.94 g, 1.75 mmol), Et_3N (1.23 mL, 8.75 mmol), and DMAP (43 mg, 0.35 mmol) in dry CH_2Cl_2 (30 mL) was added methanesulfonic anhydride (1.23 g, 7 mmol) in dry CH_2Cl_2 (10 mL). After 30 min the ice was removed. The next day CH_2Cl_2 (30 mL) was added, the solution washed with 10% aqueous citric acid (70 mL), the solvent evaporated, and the residue dried in vacuo (P_2O_5). This was dissolved in acetone (40 mL), NaI (3.65 g) was added, and the solution was refluxed for 5 h. The solvent was evaporated, the residue partitioned between CH_2Cl_2 and water, the CH_2Cl_2 removed in vacuo, and the residue purified by preparative HPLC eluting with a gradient from CH_2Cl_2 to 10% EtOAc/CH_2Cl_2. The product crystallized from CH_2Cl_2/hexane to give 0.94 g (71%) of white crystals: mp 115−117 °C; NMR δ_H 1.40 + 1.42 (18H, 2s, t-Bu), 1.8 +2.0 (2H, 2m, *CH*$_2$CH), 2.34 (2H, t, CH_2CO_2, J = 8 Hz), 3.33 (4H, t, CH_2I, J = 7 Hz), 3.60 (4H, t, CH_2N, J = 7 Hz), 4.3 (1H, sextet, CH, J_{CHCH2} = 5 and 9 Hz, J_{CHNH} = 8 Hz), 9.07 (1H, d, NH, J = 8 Hz); δ_F −143.2 (2F, dd, F2, $J_{2,3}$ = 23 Hz, $J_{2,5}$ = 8.5 Hz), −147.4 (2F, dd, F3, $J_{2,3}$ = 23 Hz, $J_{3,6}$ = 9 Hz); MS *m/z* (FAB) 781 (M + Na^+), 759 (M^+ + 1). Anal. ($C_{24}H_{32}F_4I_2N_2O_5$) C, H, N, F, I.

{4-[Bis(2-iodoethyl)amino]-2,3,5,6-tetrafluorobenzoyl}-L-glutamic Acid (26). Ester **16** (0.76 g, 1 mmol) was stirred in trifluoroacetic acid (20 mL) for 50 min. The solvent was evaporated off followed by five evaporations with EtOAc. The residue was redissolved in EtOAc and filtered and then toluene was added. The solution was concentrated until crystals began to appear. After cooling at −30 °C, the white crystals were filtered off (0.58 g, 82%): mp 142−144 °C; NMR δ_H 1.85 + 2.0 (2H, 2m, *CH*$_2$CH), 2.33 (2H, t, CH_2CO_2, J = 7.5 Hz), 3.33 (4H, t, CH_2I), 3.60 (4H, t, CH_2N, J = 7 Hz), 4.40 (1H, m, CH), 9.05 (1H, bs, NH); δ_F −142.9 (2F, dd, F2, $J_{2,3}$ = 22 Hz, $J_{2,5}$ = 8 Hz), −147.4 (2F, dd, F3, $J_{2,3}$ = 22 Hz, $J_{3,6}$ = 8 Hz); MS *m/z* (FAB) 669 (M + Na^+), 647 (M^+ + 1). Anal. ($C_{16}H_{16}F_4I_2N_2O_5$) C, H, N, F, I.

Di-*tert*-butyl {4-[Bis(2-chloroethyl)amino]-2,3,5,6-tetrafluorobenzoyl}-L-glutamate (11). Hydroxy compound **8** was reacted with methane sulfonic anhydride, as in **16**, and then stirred with LiCl in DMF to give, after preparative HPLC with CH_2Cl_2, **11** as white crystals: mp 73−74 °C; NMR δ_H 1.40 + 1.42 (18H, 2s, t-Bu), 1.8 + 2.0 (2H, 2m, *CH*$_2$CH), 2.31 (2H, t, CH_2CO_2, J = 8 Hz), 3.62 (4H, t, CH_2N, J = 6 Hz), 3.73 (4H, t, CH_2Cl, J = 6 Hz), 4.3 (1H, m, CH), 9.08 (1H, d, NH, J = 7.5 Hz); δ_F −143.3 (2F, dd, F2, $J_{2,3}$ = 24 Hz, $J_{2,5}$ = 8 Hz), −147.7 (2F, dd, F3, $J_{2,3}$ = 24 Hz, $J_{3,6}$ = 8 Hz); MS *m/z* (FAB) 597 (M + Na^+). Anal. ($C_{24}H_{32}Cl_2F_4N_2O_5$) C, H, N, Cl, F.

{4-[Bis(2-chloroethyl)amino]-2,3,5,6-tetrafluorobenzoyl}-L-glutamic Acid (21). Ester **11** (0.188 g, 0.33 mmoL) was stirred in trifluoroacetic acid (5 mL) for 55 min and worked up as in **26** to give **21** (0.146 g, 96%) as white crystals: mp 114−118 °C; NMR δ_H 1.8 + 2.0 (2H, 2m, *CH*$_2$CH), 2.33 (2H, t, CH_2CO_2, J = 7.5 Hz), 3.61 (4H, t, CH_2N, J = 6 Hz), 3.73 (4H, t, CH_2Cl, J = 6 Hz), 4.40 (1H, ddd, CH, J_{CH2CH2} = 9 Hz and 5.5 Hz), 9.03 (1H, d, NH, J = 8 Hz), 12.5 (2H, bs, CO_2H); δ_F −143.2 (2F, dd, F2, $J_{2,3}$ = 23 Hz, $J_{2,5}$ = 8.5 Hz), −147.8 (2F, dd, F3, $J_{2,3}$ = 23 Hz, $J_{3,6}$ = 8.5 Hz); MS *m/z* (FAB) 485 (M + Na^+). Anal. ($C_{16}H_{16}Cl_2F_4N_2O_5$) C, H, N, Cl, F.

Di-*tert*-butyl 2,3,4,5-Tetrafluorobenzoyl-L-glutamate (6). 2,3,4,5-Tetrafluorobenzoyl chloride (**3**) (10 g, 47 mmol) was reacted with di-*tert*-butyl-L-glutamate (13.3 g, 50 mmol), as in **5**, to give **6** (21.3 g, 98%) as an oil: NMR δ_H 1.40 + 1.42 (18H, 2s, t-Bu), 1.8 + 2.0 (2H, 2m, *CH*$_2$CH), 2.33 (2H, t, CH_2CO_2, J = 7.5 Hz), 4.3 (1H, m, CH), 7.55 (1H, m, H6), 8.80 (1H, d, NH, J = 7.5 Hz); δ_F −138.6 and − 139.0 (2F, 2m, F2 + 5), −152.5 (1F, m, F4), −154.9 (1F, t, F3, J = 22 Hz); MS *m/z* (electrospray) 324 (M − 2tBu + 3), acc mass ($C_{12}H_{10}NF_4O_5$) calcd 324.0495, found 324.0508.

Di-*tert*-butyl {4-[Bis(2-hydroxyethyl)amino]-2,3,5,-trifluorobenzoyl}-L-glutamate (9). Amide **6** (21.3 g, 49 mmol) was reacted with diethanolamine (15 mL, 0.15 mol), as in **8**, to give **9** (7.94 g, 31%) as an oil: NMR δ_H 1.39 + 1.42 (18H, 2s, t-Bu), 2.0 (2H, m, *CH*$_2$CH), 2.32 (2H, t, CH_2CO_2, J = 7.5 Hz), 3.33 (4H, t, CH_2N, J = 6 Hz), 3.48 (4H, q, CH_2O, J = 5.5 Hz), 4.30 (1H, m, CH), 4.52 (2H, t, OH, J = 5.5 Hz), 7.18 (1H, ddd, H6, $J_{H,F2}$ = 6 Hz, $J_{H,F3}$ = 1.5 Hz, $J_{H,F5}$ = 13 Hz), 8.49 (1H, d, NH, J = 7.5 Hz); δ_F −122.8 (1F, q, F5, $J_{F5,H6}$ =13 Hz), −142.1 (2F, m, F2 + 3).

Di-*tert*-butyl {4-[Bis(2-iodoethyl)amino]-2,3,5,-trifluorobenzoyl}-L-glutamate (17). Hydroxy compound **9** (2.74 g, 5.25 mmol) was reacted with methane sulfonic anhydride and then sodium iodide, as in **16**, to give 2.65 g (68%) of **17**: NMR δ_H 1.39 + 1.42 (18H, 2s, t-Bu), 1.85 + 2.0 (2H, 2m, *CH*$_2$CH), 2.32 (2H, t, CH_2CO), 3.3 (4H, t, CH_2I), 3.59 (4H, t, CH_2N, J = 7 Hz), 4.3 (1H, m, CH), 7.25 (1H, dd, H6, $J_{F5,H6}$ = 12 Hz, $J_{F2,H6}$ = 6 Hz), 8.57 (1H, d, NH, J = 7.5 Hz); δ_F −122.5 (1F, t, F5, J = 10 Hz), −141.3 (2F, m, F2 + 3); MS *m/z* (FAB) 763 (M + Na^+), 741 (M^+ + 1).

{4-[Bis(2-chloroethyl)amino]-2,3,5,-trifluorobenzoyl}-L-glutamic Acid (22). Hydroxy compound **9** (1.39 g, 2.65 mmol) was reacted with methane sulfonic anhydride and then lithium chloride (0.5 g, 11.8 mmol) in DMA (25 mL), as in **11**, to give **12** (1.37 g). Amide **12** (1.27 g, 2.28 mmol) was stirred in trifluoroacetic acid (30 mL) for 1 h and worked up as in **26** to give **22** (1.02 g, 98%) as white crystals: mp 133−138 °C; NMR δ_H 1.9 + 2.05 (2H, 2m, *CH*$_2$CH), 2.34 (2H, t, CH_2CO, J = 7.5 Hz), 3.60 (4H, CH_2N, J = 6 Hz), 3.71 (4H, t, CH_2Cl, J = 6 Hz), 4.4 (1H, m, CH), 7.25 (1H, m, H6), 8.56 (1H, d, NH, J = 8 Hz), 12.5 (2H, bs, CO_2H); δ_F −122.7 (1F, t, F5, $J_{F5,H6}$ =13.5 Hz), −141.3 (1F, m, F2), −141.5 (1F, d, F3, $J_{2,3}$ = 19 Hz); MS *m/z* (FAB) 467 (M + Na^+), 445 (M^+ + 1). Anal. $C_{16}H_{17}Cl_2F_3N_2O_5$ C, H, N, Cl, F.

{4-[Bis(2-bromoethyl)amino]-2,3,5,-trifluorobenzoyl}-L-glutamic Acid (24). Hydroxy compound **9** (1.39 g, 2.65 mmol) was reacted with methane sulfonic anhydride and then lithium bromide (0.87 g, 10 mmol) in acetone (25 mL), as in **16**, to give **14** (1.33 g). Amide **14** (1.25 g, 1.93 mmol) was stirred

in trifluoroacetic acid (30 mL) for 1 h and worked up as in **26** to give **24** (1.03 g, 99%) as white crystals: mp 141−144 °C; NMR δ_H 1.9 + 2.05 (2H, m, CH_2CH), 2.35 (2H, t, CH_2CO_2, J = 7.5 Hz), 3.58 (4H, t, CH_2N, J = 5.5 Hz), 3.66 (4H, t, CH_2Br, J = 5.5 Hz), 4.4 (1H, m, CH), 7.25 (1H, dd, H6, $J_{H6,F5}$ = 12 Hz, $J_{H6,F3}$ = 5 Hz), 8.56 (1H, d, NH, J = 7.5 Hz), 12.5 (2H, bs, CO_2H); δ_F −122.4 (1F, t, F5, $J_{F5,H6}$ = 14 Hz), −141.1 (2F, m, F2+3); MS *m/z* (FAB) 557 (M + Na^+), 535 (M^+ + 1). Anal. $C_{16}H_{17}Br_2F_3N_2O_5$ C, H, N, Br, F.

{**4-[Bis(2-iodoethyl)amino]-2,3,5,-trifluorobenzoyl**}-L-**glutamic Acid (27).** Amide **17** (0.67 g, 0.91 mmol) was stirred in trifluoroacetic acid (17 mL) for 1 h and worked up as in **26** to give **27** (0.53 g, 93%) as white crystals: mp 111−114 °C; NMR δ_H 1.9 + 2.05 (2H, 2m, CH_2CH), 2.35 (2H, t, CH_2CO_2, J = 7.5 Hz), 3.31 (4H, t, CH_2I, J = 7 Hz), 3.59 (4H, t, CH_2N, J = 7 Hz), 4.4 (1H, m, CH), 7.28 (1H, dd, H6, $J_{H,F5}$ = 11 Hz, $J_{H,F3}$ = 2.5 Hz), 8.54 (1H, d, NH, J = 7.5 Hz), 12.4 (2H, bs, CO_2H; δ_F −122.4 (1F, q, F5, $J_{F,H6}$ = 13 Hz), −141.1 (2F, d, F2 + 3); MS *m/z* (FAB) 651 (M + Na^+), 629 (M^++1). Anal. $C_{16}H_{17}F_3$-$I_2N_2O_5$ C, H, N, F, I.

Di-*tert*-butyl 3,4,5-Trifluorobenzoyl-L-glutamate (7). 3,4,5-Trifluorobenzoyl chloride **4** (10 g, 51.4 mmol) was reacted with *tert*-butyl-L-glutamate (14.8 g, 50 mmol), as in **5**, to give **7** (19.9 g, 95%) as white crystals: mp 49−54 °C; NMR δ_H 1.39 + 1.41 (18H, 2s, t-Bu), 1.9 + 2.0 (2H, 2m,CH_2CH), 2.34 (2H, t, CH_2CO_2, J = 7 Hz), 4.3(1H, m, CH), 7.84 (2H, q, H2, J = 7 and 9 Hz), 8.75 (1H, d, NH, J = 7.5 Hz); δ_F −133.7 (2F, dd, F3, J = 8 and 22 Hz), −156.3 (1F, tt, F4, J = 7 and 22 Hz); MS *m/z* (FAB) 440 (M + Na^+), 418 (M^++1). Anal. $C_{20}H_{26}F_3$-NO_5 C, H, N, F.

Di-*tert*-butyl {**4-[Bis(2-chloroethyl)amino]-3,5-difluorobenzoyl**}-L-**glutamate (13).** Di-*tert*-butyl-L-glutamate hydrochloride (1.28 g, 4.25 mmol), triethylamine (1.2 mL, 8.5 mmol), and hydroxy compound **20** (1.1 g, 4.25 mmol) were dissolved in dry DMF (65 mL), diethylcyanophosphonate (0.7 mL, 4.7 mmol) was added and the solution was stirred under argon for 3 days. The solvent was then evaporated and the residue partitioned between EtOAc and water to give the bis hydroxy compound **8**. This was then dissolved in dry CH_2Cl_2 (80 mL), and DMAP (105 mg, 0.85 mmol) and triethylamine (3.25 mL, 23 mmol) were added. The solution was cooled in ice and methane sulfonic anhydride (2.95 g, 17 mmol) was added in dry CH_2Cl_2 (25 mL). After 16 h CH_2Cl_2 (70 mL) was added and the solution was extracted with 10% citric acid. After evaporation the product was dissolved in DMA (60 mL) containing LiCl (1.45 g, 34 mmol) and heated for 1 h at 90 °C. The solvent was evaporated, and the residue was dissolved in CH_2Cl_2, extracted with H_2O, and purified by preparative HPLC eluting with CH_2Cl_2 to give 0.55 g of pure oil: NMR δ_H 1.39 + 1.41 (18H, 2s, t-Bu), 1.81 +2.01 (2H, 2m, CH_2CH), 2.33 (2H, t, CH_2CO_2, J = 8 Hz), 3.58 (4H, t, CH_2N, J = 7 Hz), 3.67 (4H, t, CH_2I, J = 7 Hz), 4.3 (1H, sextet, CH, J_{CHCH2} = 5 and 9 Hz, J_{CHNH} = 8 Hz), 7.5 (2H, d H_{H2+H6}, J_{HF} =10 Hz), 8.6 (1H, d, NH, J = 8 Hz).

Di-*tert*-butyl {**4-[Bis(2-bromoethyl)amino]-3,5-difluorobenzoyl**}-L-**glutamate (15).** Hydroxy compound **20** (1.1 g, 4.25 mmol) was reacted as in **13** above, using LiBr in acetone instead of LiCl/DMF, to yield 0.87 g of oil: NMR δ_H 1.39 + 1.42 (18H, 2s, t-Bu), 1.87 +2.03 (2H, 2m, CH_2CH), 2.33 (2H, t, CH_2CO_2, J = 8 Hz), 3.58 (4H, t, CH_2N, J = 7 Hz), 3.64 (4H, t, CH_2I, J = 7 Hz), 4.3 (1H, sextet, CH, J_{CHCH2} = 5 and 9 Hz, J_{CHNH} = 8 Hz), 7.2 (1H, dd H, J = 4.5 and 12.5 Hz,), 8.6 (1H, d, NH, J = 8 Hz).

{**4-[Bis(2-chloroethyl)amino]-3,5-difluorobenzoyl**}-L-**glutamic acid (23).** Ester **13** (1.11 g, 2.05 mmol) was stirred in TFA (28 mL) for 1 h and worked up as in **26** to give **23** (0.88 g, 100%) as white crystals: mp 114−115 °C; NMR δ_H 1.95 + 2.05 (2H, 2m, CH_2CH), 2.35 (2H, t, CH_2CO_2, J = 7.5 Hz), 3.57 (4H, t, CH_2N, J = 6 Hz), 3.68 (4H, t, CH_2Cl, J = 6 Hz), 4.4 (1H, m, CH), 7.60 (2H, d, 2 + 6-CH, J = 10 Hz), 8.61 (1H, d, NH, 7.5 Hz), 12.4 (2H, bs, CO_2H); δ_F −117.4 (2F, d, F3 + 5, J = 10 Hz); MS *m/z* (FAB) 449 (M + Na^+), 427 (M^+ + 1). Anal. $C_{16}H_{18}Cl_2F_2N_2O_5$ C, H, N, Cl, F.

{**4-[Bis(2-bromoethyl)amino]-3,5-difluorobenzoyl**}-L-**glutamic Acid (25).** Ester **15** (0.388 g, 0.62 mmol) was stirred in TFA (10 mL) for 1 h and worked up as in **26** to give **25** (0.317 g, 99%) as a white solid: NMR δ_H 1.95 + 2.05 (2H, 2m, CH_2CH), 2.35 (2H, t, CH_2CO_2, J = 7.5 Hz), 3.55 (4H, t, CH_2-CO_2, J = 5.5 Hz), 3.61 (4H, t, CH_2Br, J = 5.5 Hz), 4.4 (1H, m, CH), 7.60 (2H, d, 2 + 6-CH, J = 10 Hz), 8.62 (1H, d, NH, J = 7.5 Hz), 12.4 (2H, bs, CO_2H); δ_F −117.3 (2F, d, F3 + 5, J = 10 Hz); MS *m/z* (FAB) 539 (M + Na^+), 517 (M^+ + 1). Anal. $C_{16}H_{18}$-$Br_2F_2N_2O_5$ C, H, I, N, F.

***tert*-Butyl 3,4,5-Trifluorobenzoate (34).** To an ice-cold solution of 3,4,5-triflorobenzoyl chloride (5.0 g, 25.7 mmol) in dry THF (25 mL) was added lithium *tert*-butoxide (30 mL of 1 M solution in THF) over 45 min. One hour later, the ice was removed and after one further hour the solvent was evaporated and the residue partitioned between CH_2Cl_2 and water. Crystallization from pentane (5 mL) at −30 °C gave colorless crystals: mp 20−21 °C (4.63 g, 78%); NMR δ_H 1.54 (9H, s, t-Bu), 7.75 (2H, dd, 2+6CH, $J_{H-F3,5}$ = 8 Hz, $J_{H,F4}$ = 7 Hz); δ_F −133.2 (2F, dd, F3, J_{FF} = 21 Hz, J_{HF} = 8 Hz), −154.2(1F, tt, F4, J_{FF} =21 Hz, J_{HF} = 7 Hz); MS *m/z* (EI) 176 (M^+ − tBu + 1), 159 (M^+ − OtBu). Anal. ($C_{11}H_{11}F_3O_2$) C, H, F.

***tert*-Butyl 4-[bis(2-hydroxyethyl)amino]-3,5-difluorobenzoate (37).** Diethanolamine (3.9 g. 37 mmol) was added to ester **34** (4.33 g, 18.6 mmol), dissolved in DMA (50 mL), and heated at 90 °C for 3.5 days. The solvent was evaporated and the residue partitioned between CH_2Cl_2 and water, and the organic layer was concentrated and purified by preparative HPLC eluting with a gradient from CH_2Cl_2 to 5% EtOAc in CH_2Cl_2 to give white crystals (2.21 g, 37%): mp 58−66 °C; NMR δ_H 1.52 (9H, s t-Bu), 3.30 (4H, t, CH_2N, J = 6 Hz), 3.45 (4H, q, CH_2O, J_{CH2CH2} = 6 Hz, J_{CH2OH} = 5 Hz), 4.47 (2H, t, OH, J = 5 Hz), 7.40 (2H, d, 2+6-CH, J = 10 Hz); δ_F −117.4 (2F, d, J = 10 Hz); MS *m/z* (LDI) 340 (M + Na^+), 318 (M^+ + 1); Anal. ($C_{15}H_{21}F_2NO_4$) C, H, N, F.

***tert*-Butyl 4-(Bis(2-bromoethyl)amino)-3,5-difluorobenzoate (42).** To an ice-cold solution of ester **37** (0.43 g, 1.5 mmol), Et_3N (1.05 mL, 7.5 mmol), and DMAP (37 mg, 0.3 mmol) in dry CH_2Cl_2 (27.5 mL) was added methanesulfonic anhydride (1.05 g, 6 mmol) in dry CH_2Cl_2 (9 mL) over a few minutes. The ice was removed, the next day CH_2Cl_2 (25 mL) was added, the solution was washed with 10% citric acid, dried, and then evaporated to dryness. This was dissolved in acetone (25 mL), LiBr (1.30 g, 15 mmol) added, and the mixture refluxed for 5 h. The solvent was evaporated and the residue dissolved in CH_2Cl_2, washed with water, and evaporated to dryness. Preparative HPLC with CH_2Cl_2 as eluent gave a low-melting solid (0.40 g, 60%): mp 22−26 °C; NMR δ_H 1.52 (9H, s, t-Bu), 3.56 (4H, t, CH_2N, J = 5.5 Hz), 3.65 (4H, t, CH_2Br, J = 5.5 Hz), 7.48 (2H, d, 2+6CH, J = 9 Hz); δ_F −117.0 (2F, d, 3+5F, J = 9 Hz); MS *m/z* (FAB) 440/2/4 (M^+ + 1), 385/7/9 (M^+ − tBu + 1); Anal. ($C_{15}H_{19}Br_2F_2NO_2$) C, H, N, F.

4-(Bis(2-bromoethyl)amino)-3,5-difluorobenzoic Acid (50). Ester **42** (0.40 g, 0.91 mmol) was stirred in trifluoroacetic acid (12 mL) for 55 min. The solvent was evaporated and then EtOAc was added and evaporated five times. The residue was redissolved in EtOAc and filtered, toluene added, and the solution evaporated to dryness. It was redissolved in toluene, hexane was slowly added to give a mass of white crystals (0.30 g, 85%): mp 111−113.5 °C; NMR δ_H 3.56 (4H, t, CH_2N, J = 5.5 Hz), 3.63 (4H, t, CH_2Br, J = 5.5 Hz), 7.52 (2H, d, 2+6CH, J = 10 Hz), 13.2 (1H, bs, CO_2H); δ_F −117.0 (2F, d, 3+5F, J = 10 Hz); MS *m/z* (FAB) 388 (M^+ + 1), 345 (M^+ − CO_2 + 1), 307 (M^+ − Br). Anal. ($C_{11}H_{11}Br_2F_2NO_2$), C, H, N, Br, F.

***tert*-Butyl 4-(Bis(2-chloroethyl)amino)-3,5-difluorobenzoate (40).** Ester **37** (0.43 g, 1.5 mmoL) was reacted with methane sulfonic anhydride as in **42** and then reacted with LiCl (0.64 g, 15 mmol) in DMF (25 mL) at room temperature to give, after preparative HPLC with CH_2Cl_2, **40** (0.32 g, 61%) as white crystals: mp 59−60 °C; NMR δ_H 1.53 (9H, s, t-Bu), 3.59 (4H, t, CH_2N, J = 5.5 Hz), 3.69 (δH, t, CH_2Cl, J = 5.5 Hz), 7.48 (2H, d, 2+6CH, J = 10 Hz); δ_F −117.1 (2F, d, F3 + 5, J = 9.5 Hz); MS *m/z* (FAB) 354 (M^+ + 1). Anal. $C_{15}H_{19}Cl_2F_2$-NO_2 C, H, N, Cl, F.

4-(Bis(2-chloroethyl)amino)-3,5-difluorobenzoic Acid (48). Ester **40** (0.25 g, 0.71 mmoL) was stirred in trifluoroacetic acid (6 mL) for 55 min and worked up as in **50** to give **48** (0.177 g, 85%) as white crystals: mp78−79 °C; NMR δ_H 3.59 (4H, t, CH_2N, J = 5.5 Hz), 3.69 (4H, t, CH_2Cl, J = 5.5 Hz), 7.51 (2H, d, 2 + 6CH, J = 9.5 Hz), 13.3 (1H, bs, CO_2H); δ_F −117.1 (2F,d, 3 + 5F, J = 9.5 Hz); MS *m/z* (FAB) 298 (M^+ + 1). Anal. $C_{11}H_{11}NCl_2F_2O_2$ C, H, N, Cl, F.

4-(Bis(2-iodoethyl)amino)-3,5-difluorobenzoic Acid (53). Ester **45** (0.163 g, 0.30 mmoL) was stirred in TFA (5 mL) for 40 min and worked up as in **50** to give **53** (0.106 g, 73%) as white crystals: mp 123−125 °C; NMR δ_H 3.29 (4H, t, CH_2I, J = 7 Hz), 3.58 (4H, t, CH_2N, J = 7 Hz), 7.52 (2H, d, 2 + 6CH, J = 10 Hz); δ_F −117.0 (2F, d, 3 + 5F, J = 10 Hz); MS *m/z* (FAB) 482 (M^+ + 1), 481 M^+. Anal. $C_{11}H_{11}NF_2I_2O_2$ C, H, N, F, I.

***tert*-Butyl 2,3,4,5-Tetrafluorobenzoate (33).** 2,3,4,5-Tetrafluorobenzoyl chloride (10.6 g, 50 mmol) was reacted with lithium *tert*-butoxide (4.8 g, 60 mmol) as in **34** to give **33** (5.35 g, 43%) of pale yellow crystals that melted at room temperature: NMR δ_H 1.55 (9H, s, t-Bu), 7.77 (1H, m, H6, $J_{H,F5}$ = 8.5 Hz); δ_F −136.25 (1F, m, F2), −138.4 (1F, quintet, F5, J = 11 Hz), −148.9 (1F, m, F4), −154.0 (1F, t, F3, J = 21.5 Hz); MS *m/z* (EI) 177 (M^+ − OtBu), 149 (M^+ − CO_2tBu). Anal. $C_{11}H_{10}F_4O_2$ C, H, F.

***tert*-Butyl 4-(Bis(2-hydroxyethyl)amino)-2,3,5-trifluorobenzoate (36).** Ester **33** (3.75 g, 15 mmol) and diethanolamine (4.2 g, 40 mmol) were heated in DMA as in **37** to give pure **36** (2.5 g, 40%): NMR δ_H 1.52 (9H, s, t-Bu), 3.35 (4H, t, CH_2N, J = 5.5 Hz), 3.48 (4H, q, CH_2O, J = 5.5 Hz), 4.56 (2H, t, OH, J = 5 Hz), 7.32 (1H, ddd, H6, $J_{F5,H6}$ = 13 Hz, $J_{F2,H6}$ = 6.5 Hz, $J_{F3,H6}$ = 2 Hz); δ_F −122.8 (1F, dt, F5, $J_{F5,H6}$ = 13 Hz, $J_{F2,F5}$ = 12.5 Hz, $J_{F3,F5}$ = 7 Hz), −139.3 (1F, ddd, F2, $J_{F2,F3}$ = 20 Hz, $J_{F2,F5}$ = 12.5 Hz, $J_{F2,H6}$ = 6.5 Hz), −142.3 (1F, dd, F3, $J_{F2,F3}$ = 20 Hz, $J_{F3,F5}$ = 7 Hz).

***tert*-Butyl 4-(Bis(2-chloroethyl)amino)-2,3,5-trifluorobenzoate (39).** Hydroxy compound **36** (0.67 g, 2 mmol) was reacted with methane sulfonic anhydride as in **42** and then reacted with LiCl (0.85 g, 20 mmol) in DMF (33 mL) at room temperature to give, after preparative HPLC with CH_2Cl_2, **39** (0.68 g, 91%) as white crystals: mp 52−54 °C; NMR δ_H 1.53 (9H, s, t-Bu), 3.63 (4H, t, CH_2N, J = 5.5 Hz), 3.72 (4H, t, CH_2-Cl, J = 5.5 Hz), 7.42 (1H, q, H6, $J_{F5,H6}$ = 13 Hz, $J_{F2,H6}$ = 6.5 Hz); δ_F −122.7 (1F, q, F5, $J_{H2,F5}$ = 13 Hz, $J_{F5,H6}$ = 5 Hz), −138.8 (1F, m, F2, $J_{F2,F3}$ = 20 Hz, $J_{F2,F5}$ = 13 Hz), −141.3 (1F, q, F3, $J_{F2,F3}$ =20 Hz, $J_{F3,F5}$ = 5 Hz); MS *m/z* (FAB) 394 (M + Na^+), 371 (M^+). Anal. $C_{15}H_{18}Cl_2F_3NO_2$ C, H, N, Cl, F.

***tert*-Butyl 4-(bis(2-bromoethyl)amino)-2,3,5-trifluorobenzoate (41).** Hydroxy compound **36** (0.67 g, 2 mmol) was reacted with methane sulfonic anhydride as in **42** and then refluxed with LiBr (1.7 g, 20 mmol) in acetone (35 mL) to give, after preparative HPLC with CH_2Cl_2, **41** (0.93 g, 100%) as white crystals: mp 27−29 °C; NMR δ_H 1.52 (9H, s, t-Bu), 3.60 (4H, t, CH_2N, J = 5.5 Hz), 3.69 (4H, t, CH_2Br, J = 5.5 Hz), 7.42 (1H, m, H6); δ_F −122.7 (1F, sextet, F5, $J_{F2,F5}$ = 13 Hz, $J_{F3,F5}$ = 5 Hz), −138.7 (1F, octet, F2, $J_{F2,F3}$ = 20 Hz, $J_{F2,F5}$ = 13 Hz), −141.2 (1F, q, F3, $J_{F2,F3}$ = 20 Hz, $J_{F3,F5}$ = 5 Hz); MS *m/z* (FAB) 461 (M^+), 406 (M − tBu + 1), acc mass ($C_{11}H_{11}NBr_2F_3O_2$) calcd 403.9109, found 403.9142.

***tert*-Butyl 4-(Bis(2-iodoethyl)amino)-2,3,5-trifluorobenzoate (44).** Hydroxy compound **36** (0.67 g, 2 mmol) was reacted with methane sulfonic anhydride, as in **42**, and then refluxed with NaI (3.0 g, 20 mmol) in acetone (35 mL) to give, after preparative HPLC with CH_2Cl_2, **44** (0.86 g, 77%) as an oil: NMR δ_H 1.54 (9H, s, t-Bu), 3.35 (4H, m, CH_2I), 3.63 (4H, t, CH_2N, J = 7 Hz), 7.43 (1H, dd, H6, $J_{F5,H6}$ = 11.5 Hz, $J_{F2,H6}$ = 5.5 Hz); δ_F −123.1 (1F, dt, F5, $J_{F2,F5}$ = 13 Hz), −139.1 (1F, m, F2), −141.6 (1F, d, F3, $J_{F2,F3}$ = 21.5 Hz); MS *m/z* (electrospray) 555 (M+), 500 (M − tBu + 1), acc mass ($C_{15}H_{18}F_3I_2NO_2$) calcd 555.9457, found 555.9464.

4-(Bis(2-chloroethyl)amino)-2,3,5-trifluorobenzoic Acid (47). Ester **39** (0.64 g, 1.72 mmol) was stirred in TFA (15 mL) for 1 h and worked up as in **50** to give **47** (0.48 g, 88%) as white crystals: mp 81−83 °C; NMR δ_H 3.63 (4H, t, CH_2N, J = 5.5 Hz), 3.73 (4H, t, CH_2Cl, J = 5.5 Hz), 7.45 (1H, m, H6), 13.5 (1H, bs, CO_2H); δ_F −122.8 (1F, sextet, F5, $J_{2,5}$ = 13 Hz, $J_{3,5}$ = 5 Hz), −138.5 (1F, octet, F2, $J_{2,5}$ = 13 Hz, $J_{2,3}$ = 20 Hz), −144.5 (1F, q, F3, $J_{2,3}$ = 20 Hz, $J_{3,5}$ = 5 Hz); MS *m/z* (FAB) 338 (M + Na^+), 316 (M^+ + 1). Anal. $C_{11}H_{10}Cl_2F_3NO_2$ C, H, N, Cl, F.

4-(Bis(2-bromoethyl)amino)-2,3,5-trifluorobenzoic Acid (49). Ester **41** (0.88 g, 1.9 mmol) was stirred in TFA (15 mL) for 55 min and worked up as in **50** to give **49** (0.72 g, 94%) as white crystals: mp 89−90 °C; NMR δ_H 3.60 (4H, t, CH_2N, J = 5.5 Hz), 3.68 (4H, t, CH_2Br, J = 5.5 Hz), 7.45 (1H, m, H6), 13.5 (1H, bs, CO_2H); δ_F −122.8 (1F, sextet, F5, $J_{2,5}$ = 13.5 Hz, $J_{3,5}$ = 5 Hz), −138.5 (1F, octet, F2, $J_{2,3}$ = 20 Hz, $J_{2,5}$ = 13.5 Hz), −141.4 (1F, q, F3, $J_{2,3}$ = 20 Hz, $J_{3,5}$ = 5 Hz); MS *m/z* (FAB) 428 (M + Na^+), 406 (M^+ + 1). Anal. $C_{11}H_{10}Br_2F_3NO_2$ C, H, N, Br, F.

4-(Bis(2-iodoethyl)amino)-2,3,5-trifluorobenzoic Acid (52). Ester **44** (0.82 g, 1.48 mmoL) was stirred in TFA (20 mL) for 55 min and worked up as in **50** to give **52** (0.69 g, 93%) as white crystals: mp 81−83 °C; NMR δ_H 3.33 (4H, t, CH_2I, J = 7 Hz), 3.62 (4H, t, CH_2N, J = 7 Hz), 7.45 (1H, m, H6), 13.5 (1H, bs, CO_2H); δ_F −122.8 (1F, sextet, F5, $J_{2,5}$ = 13 Hz $J_{3,5}$ = 5 Hz), −138.4 (1F, octet, F2, $J_{2,3}$ = 20 Hz, $J_{2,5}$ = 13 Hz), −141.3 (1F, q, F3, $J_{2,3}$ = 20 Hz, $J_{3,5}$ = 5 Hz); MS *m/z* (FAB) 522 (M + Na^+), 500 (M^+ + 1). Anal. $C_{11}H_{10}F_3I_2NO_2$ C, H, N, F, I.

***tert*-Butyl 2,3,4,5,6-Pentafluorobenzoate (32).** Pentafluorobenzoyl chloride (11.5 g, 50 mmol) was reacted with lithium *tert*-butoxide (50 mmol) as in **34** to give, after distillation, **32** (9.8 g, 73%) as a solid: mp 9−11 °C; NMR δ_H 1.55 (9H, s, t-Bu); δ_F −140.9 (2F, dd, F2, $J_{2,3}$ = 27 Hz, $J_{2,4}$ = 8.5 Hz), −149.9 (1F, t, F4, $J_{3,4}$ = 22 Hz), −160.7 (2F, m, F3); MS *m/z* (EI) 268 (M^+), 195 (C_6H_5 CO). Anal. $C_{11}H_9F_5O_2$ C, H, F.

***tert*-Butyl 4-(Bis(2-hydroxyethyl)amino)-2,3,5,6-tetrafluorobenzoate (35).** Ester **32** (5.36 g, 20 mmol) and diethanolamine (4.2 g, 40 mmol) were heated in DMA as in **37** to give, after preparative HPLC with CH_2Cl_2/EtOAc (4:1) as eluent, pure **35** (3.68 g, 52%) as pale yellow crystals: mp 45−49 °C; NMR δ_H 1.52 (9H, s, t-Bu), 3.37 (4H, t, CH_2N, J = 5.5 Hz), 3.50 (4H, q, CH_2O, J_{CH2CH2} = 5.5 Hz, J_{CH2OH} = 5 Hz), 4.55 (2H, t, OH, J = 5 Hz); δ_F −143.0 (2F, dd, F2, $J_{2,3}$ = 20 Hz), −148.7 (2F, dd, F3, $J_{2,3}$ = 20 Hz); MS *m/z* (LDI) 354 (M^+). Anal. $C_{15}H_{19}F_4NO_4$ C, H, N, F.

***tert*-Butyl 4-(Bis(2-iodoethyl)amino)-2,3,5,6-tetrafluorobenzoate (43).** Hydroxy compound **35** (0.35 g, 1 mmol) was reacted with methane sulfonic anhydride, as in **42**, and then refluxed with NaI (1.5 g, 10 mmol) in acetone (25 mL) to give **43** (0.40 g, 70%) as white crystals: mp 48−49.5 °C; NMR δ_H 1.53 (9H, s, t-Bu), 3.34 (4H, t, CH_2N, J = 7 Hz), 3.62 (4H, CH_2I, J = 7 Hz); δ_F −142.3 (2F, dd, F2, $J_{2,3}$ = 22 Hz, $J_{2,5}$ = 8 Hz), −147.6 (2F, dd, F3, $J_{2,3}$ = 22 Hz, $J_{3,6}$ = 8 Hz); MS *m/z* (EI) 573 (M^+). Anal. $C_{15}H_{17}F_4I_2NO_2$ C, H, N, F, I.

4-(Bis(2-chloroethyl)amino)-2,3,5,6-tetrafluorobenzoic Acid (46). Ester **38** (0.365 g, 0.94 mmoL) was stirred in trifluoroacetic acid (10 mL) for 1 h and worked up as in **50** to give **46** (0.288 g, 92%) as white crystals: mp 98−100 °C; NMR δ_H 3.64 (4H, t, CH_2N, J = 6 Hz), 3.74 (4H, CH_2Cl, J = 6 Hz); δ_F −141.6 (2F, dd, F2, $J_{2,3}$ = 22 Hz, $J_{2,5}$ = 8 Hz), −148.0 (2F, dd, F3, $J_{2,3}$ = 22 Hz, $J_{3,6}$ = 8 Hz); MS *m/z* (FAB) 356 (M + Na^+), 334 (M^+ + 1). Anal. $C_{11}H_9Cl_2F_4NO_2$ C, H, N, Cl, F.

4-(Bis(2-iodoethyl)amino)-2,3,5,6-tetrafluorobenzoic Acid (51). Ester **43** (0.287 g, 0.50 mmoL) was stirred in trifluoroacetic acid (5 mL) for 40 min and worked up as in **50** to give **51** (0.22 g, 85%) as white crystals: mp 107−108 °C; NMR δ_H 3.34 (4H, t, CH_2N, J = 7 Hz), 3.62 (4H, CH_2I, J = 7 Hz); δ_F −141.5 (2F, dd, F2, $J_{2,3}$ = 21 Hz, $J_{2,5}$ = 7 Hz), −147.9 (2F, dd, F3, $J_{2,3}$ = 21 Hz, $J_{3,6}$ = 7 Hz). Anal. $C_{11}H_9F_4I_2NO_2$ C, H, N, F, I.

Biology. Determination of Aqueous Half-Life. 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 10 mM ammonium acetate (pH

5.0) containing percentages of methanol that gave retention times of 3−4 min. The eluate was monitored at the previously determined wavelength of maximum absorbance. The amount of starting material remaining after various periods of incubation was determined by repeat injections. The fraction of compound remaining was expressed 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 Substrate Kinetic Determinations. Initially, a differential spectrum between prodrug and drug (100 μM each) was used to determine the wavelength of greatest difference and the conversion factor for the amount of substrate lost per unit change in absorbance. Reactions (37 °C) were then set up containing CPG2 assay buffer (1 mL) and prodrug at concentrations from 0.2 to 50 μM in 25 steps using increments of 0.2 μM (0.2−1.0 μM), 1 μM (1−10 μM), 2 μM (10−20 μM), and 5 μM (20−50 μM), with or without 50 mU CPG2. The change in the previously established wavelength was monitored spectrophotometrically for 1 min, and the enzyme-specific change in the concentration of prodrug was determined after subtraction of the chemical-only change, and application of the conversion factor. The K_m and K_{cat} were determined by nonlinear regression to a Michalis−Menten plot (Graphpad Prism, Graphpad Software Inc., San Diego, CA).

Enzyme Inhibitor Kinetic Determinations. Repeat K_m determinations were performed as above, using methotrexate as substrate at concentrations of 2−100 μM in 17 steps using increments of 2 μM (2−10 μM), 5 μM (10−50 μM), and 10 μM (50−100 μM), plus 50 mU CPG2, in the presence of increasing concentrations of inhibitor (0−1000 μM). The observed K_m values were plotted against inhibitor concentration, and the K_i values were calculated as the positive value where the linear regression line crosses the ordinate.

Cell Lines and Culture Conditions. The generation of β-gal-, CPG2*-, and stCPG2(Q)3-expressing MDA MB 361 stable cell lines together with a detailed description of the subcellular localization of the enzymes has been described previously.[3,8] The cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere.

Cytotoxicity Determinations. The cytotoxicity of the compounds was determined in a two-step protocol as described before.[9] Cells (5×10^5 per well) were seeded into 6-well plates, producing confluent monolayers in 48 h. Compounds were initially dissolved in DMSO at 100-fold the highest dose (1−4 mM) and, immediately prior to treatment, prepared as a dilution series in full medium. The medium in the wells was removed and replaced with prodrug-containing medium (225 μL). After an incubation of 1 h, a similar concentration of freshly prepared drug solution (1275 μL) was added, and the cells were incubated for an additional 20 h. The cells were harvested and reseeded in 96-well plates at 2×10^3/well and incubated until the control wells achieved confluence (5 days). The plates were then fixed and stained with sulforhodamine-B, the extinction at 540 nm was read, the results were expressed as a percentage of control growth as a function of log dose, and the IC_{50} values were determined by nonlinear regression to a four-parameter logistic sigmoid, constraining the minimum to be positive (Graphpad Prism, Graphpad Software Inc., San Diego, CA).

In Vivo Therapy Studies. All experiments were conducted in accordance with United Kingdom Home Office regulations and United Kingdom Coordinating Committee on Cancer Research Guidelines.[10] MDA MB 361 human breast carcinoma cells that had been transfected with the CPG2 gene and were expressing the CPG2 enzyme displayed on the cell surface (stCPG2(Q)3) were grown as xenografts by implanting cells (10^7) subcutaneously in the right flank of nude (nu/nu) female BALB/C mice (20−22 g). After 4 days the tumor volumes were 124.3 ± 4.4 mm^3. At this point the mice were randomly distributed into control and treatment groups, and the prodrugs, freshly prepared in 1.26% sodium bicarbonate/5% DMSO, were administered to treatment groups of six mice as three intraperitoneal doses of 500 (**22**−**25**, **27**) or 200 mg/kg (**28**) over 24 h, defined as one course of treatment. All treated groups received one such course of treatment per week for 3 weeks followed by 1 week free of treatment and were redosed according to this schedule if palpable tumors remained. Tumor growth was assessed by periodic calliper measurement. The maximum bodyweight loss produced by these doses (<20%) was in accordance with United Kingdom Coordinating Committee on Cancer Research guidelines.[10] No other toxicity was observed.

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