Hypoxia-Selective Antitumor Agents. 14. Synthesis and Hypoxic Cell Cytotoxicity of Regioisomers of the Hypoxia-Selective Cytotoxin 5-[*N*,*N*-Bis(2-chloroethyl)amino]-2,4-dinitrobenzamide

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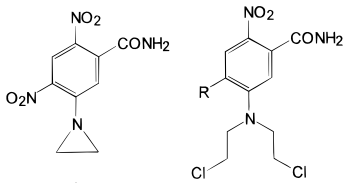
A series of regioisomers of the novel hypoxia-selective cytotoxin (HSC) 5-[*N*,*N*-bis(2-chloroethyl)-amino]-2,4-dinitrobenzamide (**2a**) have been prepared by displacement of the chloro group from methyl chlorodinitrobenzoates or the corresponding carboxamides with diethanolamine, followed by dimesylation and mesylate displacement with LiCl. The compounds fall into two classes, where the two nitro groups have either a *meta* or an *ortho* (or *para*) disposition to each other. The four *meta* derivatives had one-electron reduction potentials in the range −340 to −375 mV, similar to that of the known isomer **2a**, while the other isomers had much higher values (−262 to −285 mV). The *meta* derivatives were much less cytotoxic to AA8 cells under aerobic conditions (IC_{50}s from 75 to 470 μM) than were the other compounds (IC_{50}s from 1.6 to 20 μM). However, the ratios of IC_{50}s of the compounds in repair-proficient (AA8) and repair-deficient (UV4) cell lines varied, indicating differing contributions of DNA alkylation to aerobic toxicity between the isomers, with no clear relationship between this and nitro group disposition. The hypoxic selectivities of the (dimethylamino)ethylcarboxamide analogues for each isomer were determined by clonogenic assay against both AA8 and UV4 cells. With one exception, the *meta* derivatives showed excellent hypoxic selectivities (ca. 45−115-fold) against UV4 cells, while the *ortho* or *para* isomers had little selectivity (ca. 2−7-fold). A possible reason may be that the latter compounds, with higher reduction potentials, undergo rapid bioreduction even under aerobic conditions. None showed hypoxic selectivities greater than 2−3-fold against AA8 cells. The 3-[*N*,*N*-bis(2-chloroethyl)amino]-2,6-dinitrobenzamide isomer (**5b**), which showed the highest hypoxic selectivity for UV4 cells in this series, was active against both hypoxic and aerobic cells in KHT tumors in mice at well-tolerated doses, and showed superior *in vivo* activity to the previously studied 2,4-dinitro isomer **2b**.

Introduction

Bioreductive drugs which can undergo selective metabolic reduction in the absence of oxygen to generate cytotoxic agents (hypoxia-selective cytotoxins, HSCs) are of particular interest as potential anticancer drugs because of increasing evidence of the existence of severely hypoxic cells in human solid tumors.[1−4] Although examples of three classes of bioreductive drug (nitro compounds, quinones and aromatic *N*-oxides) are currently in clinical trial as hypoxia-selective cytotoxins, there are still relatively few examples of such compounds with activity against hypoxic cells in murine tumors, and the available examples appear to have limited therapeutic ratios. One possible improvement of this general concept is to have bioreductive drugs on activation release cytotoxins with sufficiently long half-lives that they can diffuse several cell diameters and thereby kill relatively well-oxygenated cells surrounding chronically hypoxic regions.[5]

We have suggested[5−7] that activated aromatic nitrogen mustards possess the required stability for such diffusion and that appropriately deactivated bioreductive prodrug forms of these can be made, since the cytotoxicity of aromatic nitrogen mustards is very dependent on the electronic properties of the substituents on the aromatic ring.[8,9] An example of this approach to HSCs is the nitro-deactivated mustard 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (**2a**; SN 23862). We have shown[10] that **2a** undergoes efficient cellular bioreduction of the 4-nitro function to an amino group under hypoxia, thus activating the mustard which is in a resonant position by electron release. While **2a** has an IC_{50} of 1600 μM against AA8 cells in culture (4 h exposure), the 4-amino derivative **45** has an IC_{50} under similar conditions of 180 μM. The hydroxylamino derivative (**44**) can be prepared by radiolytic or chemical reduction of **2a**, but its instability precluded an exact determination of its cytotoxicity.[10] We have also shown[7] that **2a** is an effective HSC in culture, being 60-fold more potent against anoxic than aerobic Chinese hamster UV4 cells. Unlike its aziridinyl analogue (**1**; CB 1954),[11] **2a** is not a substrate for the two-electron reductase DT-diaphorase,[12] which can activate nitroaromatics in an oxygen-insensitive manner.



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2a : R = NO_2
44 : R = NHOH
45 : R = NH_2

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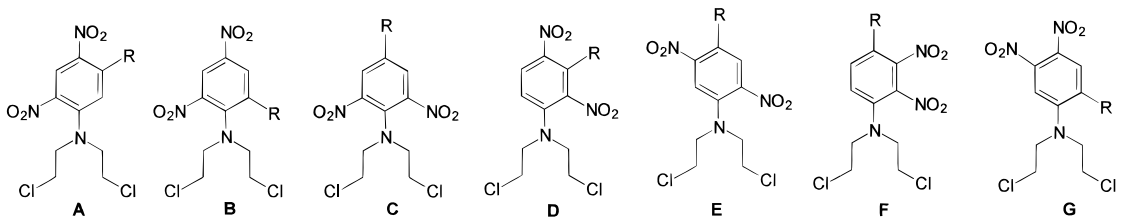
Table 1. Structural and Physicochemical Properties of Dinitrocarboxamide Mustards



no.	type	R	mp (°C)	sol (mM)[a]	$E(1)$ (mV)[b]
2a	A	$CONH_2$	ref 11	0.17	
2b	A	$CONH(CH_2)_2NMe_2$	ref 11	>44	−371
2c	A	$CONHCH_2CH(OH)CH_2OH$	ref 11	11	
3a	B	$CONH_2$	123−124	0.16	
3b	B	$CONH(CH_2)_2NMe_2$	115−118	0.32	−341
3c	B	$CONHCH_2CH(OH)CH_2OH$	nc[c]	6.25	
4a	C	$CONH_2$	113−116	0.14	
4b	C	$CONH(CH_2)_2NMe_2$·HCl	145−148	29.7	−375
4c	C	$CONHCH_2CH(OH)CH_2OH$	nc	0.91	
5a	D	$CONH_2$	141−141.5	0.19	
5b	D	$CONH(CH_2)_2NMe_2$·HCl	180−182	>45	−343
5c	D	$CONHCH_2CH(OH)CH_2OH$	nc	12	
6a	E	$CONH_2$	153	0.08	
6b	E	$CONH(CH_2)_2NMe_2$·HCl	90 dec	>48	−263
6c	E	$CONHCH_2CH(OH)CH_2OH$	nc	10	
7a	F	$CONH_2$	138	0.16	
7b	F	$CONH(CH_2)_2NMe_2$·HCl	60 dec	>47	−268
7c	F	$CONHCH_2CH(OH)CH_2OH$	nc	1.40	
8a	G	$CONH_2$	193−195	0.04	
8b	G	$CONH(CH_2)_2NMe_2$·HCl	88−92	45	−286
8c	G	$CONHCH_2CH(OH)CH_2OH$	nc	7.9	

[a] Solubility (mM) in water at 20 °C, measured spectrophotometrically. [b] One-electron reduction potential (mV), measured by pulse radiolysis in aqueous solutions containing 2-propanol (0.1 M) buffered at pH 7.0 (1 mM phosphate), by measuring the equilibrium constant for electron transfer between the radical anions of the compounds and benzyl viologen (see ref 18). Standard error is ± 10 mV in each case. [c] Noncrystalline.

Studies with analogues of **2a** have shown[12] that the use of alternate mustard leaving groups, and modification or substitution of the carboxamide moiety, can be used to influence potency, in vitro hypoxic selectivity, and solubility. With regard to these properties, the best compound in the series overall was the (dimethylamino)ethyl derivative **2b**, which DNA elution studies show is activated under hypoxia to a DNA cross-linking agent.[12] This analogue has some activity *in vivo* against both aerobic and hypoxic cells in KHT tumors,[12] although it is not yet clear whether this reflects efficient killing of aerobic cells as a result of diffusion of reduced metabolites from hypoxic regions, or whether its cytotoxicity in tumors is independent of hypoxia. The dinitrobenzamide mustards are also of interest as prodrugs for ADEPT (antibody-directed enzyme prodrug therapy),[13] using a nitroreductase (NR2) from *Escherichia coli* B[14] as the enzyme. Both **2a** and **2c** (but not the cationic (dimethylamino)ethyl derivative **2b**) are efficiently activated by this enzyme,[15] with the more than 10-fold better solubility of **2c** giving it an advantage in this application.

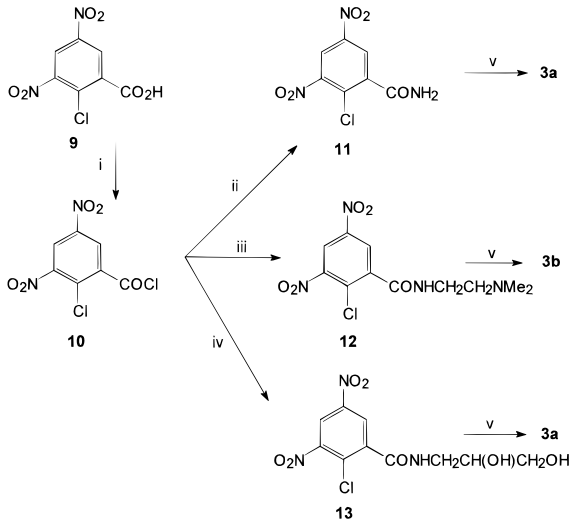
Following these results, and the structure−activity relationship (SAR) studies[12,15] which indicated the importance of the dinitrobenzamide motif, we now report the synthesis and biological evaluation of a series of six regioisomers of **2a**. Because of the positive results obtained previously with the soluble cationic and diol side chain analogues **2b** and **2c**, as discussed above, these analogues were prepared in addition to the parent benzamides.

Chemistry

The dinitrobenzamide mustards of Table 1 were prepared from the corresponding chloro dinitro acids or esters, the majority of which were known compounds. The chloro groups of the amides **11**−**13** of 2-chloro-3,5-dinitrobenzoic acid (**9**) were sufficiently reactive to undergo efficient displacement by bis(2-chloroethyl)amine at 50 °C, giving the required compounds **3a**−**3c** directly (Scheme 1). The carboxamide mustards **4a**−**4c**, **5a**−**5c**, **6a**−**6c**, and **7a**−**7c** were obtained from the corresponding methyl chlorodinitrobenzoates (**14**, **22**, **28**, and **33**, respectively) by displacement of the chlorides by diethanolamine at 20−50 °C to give the diols (**15**, **23**, **29**, and **34**), followed by dimesylation and mesylate displacement with LiCl in DMF at 100−120 °C (Schemes 2−5). The resulting mustard esters (**16**, **24**, **30**, and **35**) were cleanly hydrolyzed to the corresponding acids (**17**, **26**, **31**, and **36**) by 3 N KOH in *p*-dioxane at room temperature. These were converted to the acid chlorides using $SOCl_2$, followed by reaction of these with the appropriate amines to give the desired compounds.

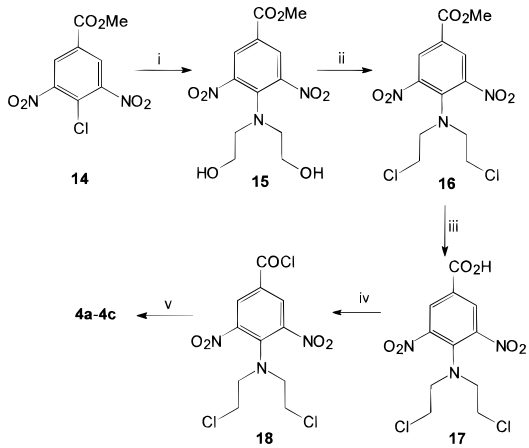
The hitherto unreported 3-chloro-2,6-dinitrobenzoic acid (**21**) was obtained as a minor product from nitration of 3-chlorobenzoic acid. Thus while nitration of 3-chlorobenzoic acid (**19**) with fuming HNO_3/concentrated H_2SO_4 at 120 °C gave predominantly the known 5-chloro-2,4-dinitro acid[16,17] **20** in high yield, reaction at 140 °C gave a mixture of **20** together with ca. 30% of the isomeric 3-chloro-2,6-dinitrobenzoic acid (**21**) (Scheme 3). The mixture could be readily separated by fractional

Scheme 1[a]



[a] (i) $SOCl_2$/reflux/5 h; (ii) concentrated NH_4OH/20 °C; (iii) $H_2N(CH_2)_2NMe_2$/0 °C/15 min; (iv) $H_2NCH_2CH(OH)CH_2OH$/0 °C/15 min; (v) $HN(CH_2CH_2Cl)_2$·HCl/Et_3N/*p*-dioxane/50 °C/18 h.

Scheme 2[a]



[a] (i) $NH(CH_2CH_2OH)_2$/*p*-dioxane/50 °C/5 h; (ii) MsCl/Et_3N/CH_2Cl_2/0 °C/15 min, then LiCl/DMF/120 °C/5 min; (iii) KOH/*p*-dioxane/H_2O/20 °C/3 h; (iv) $SOCl_2$/reflux/5 h; (v) RNH_2.

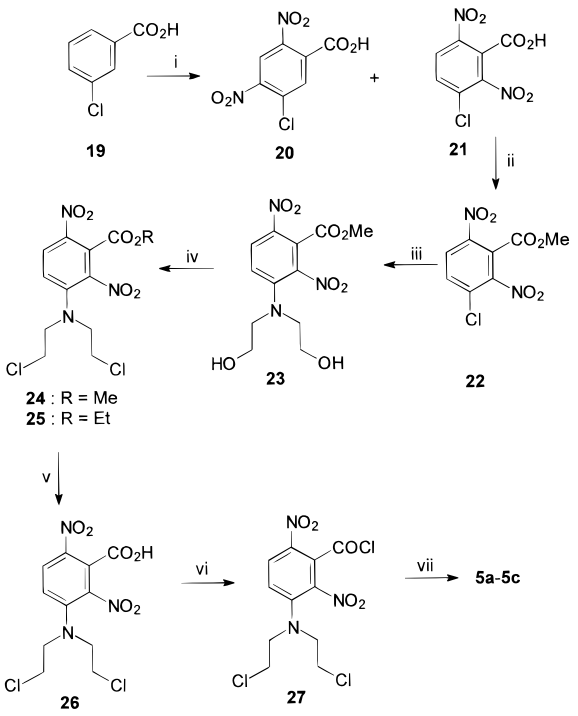
crystallization, providing the acid **21** in 9% yield. The regiochemistry of the nitro groups in **21** was confirmed by X-ray crystallographic analysis of a derivative, ethyl 3-[*N*,*N*-bis(2-chloroethyl)amino]-2,6-dinitrobenzoate (**25**) (see Supporting Information).

In the case of the 4,5-dinitro carboxamides **8a**−**c**, reaction of methyl 2-chloro-4,5-dinitrobenzoate with diethanolamine resulted in preferential displacement of the 5-nitro group. However, a similar reaction on the primary carboxamide (**40**) gave a mixture resulting from displacement of both nitro groups and chloride, from which the desired **41** was isolated in 65% yield (Scheme 6). The derived mustard **8a** was hydrolyzed slowly but cleanly by 90% H_2SO_4 at 70 °C to give the acid **42**, from which the carboxamides **8b** and **8c** were obtained as above, via the acid chloride **43**.

Results and Discussion

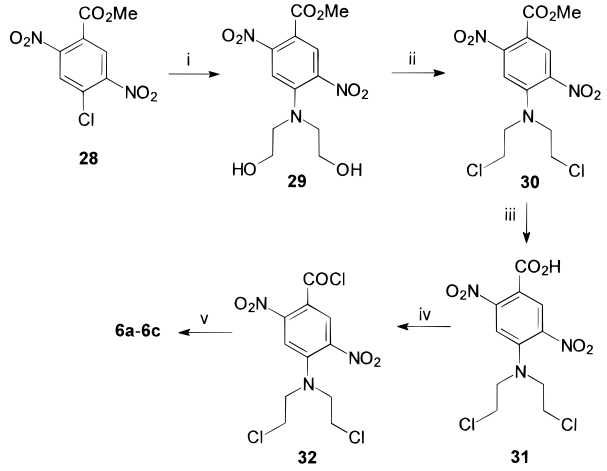
Because both theoretical[6] and experimental[9] studies suggested that the highest differential electron release (and thus the largest hypoxic selectivities) in mononitro aromatic mustards occurs when there is an *ortho* or

Scheme 3[a]



[a] (i) Concentrated H_2SO_4/fuming HNO_3 (*d* 1.42)/140 °C/6 h; (ii) MeOH/concentrated H_2SO_4/reflux/8 h; (iii) $NH(CH_2CH_2OH)_2$/*p*-dioxane/50 °C/5 h; (iv) MsCl/Et_3N/CH_2Cl_2/0 °C/15 min, then LiCl/DMF/120 °C/5 min; (v) KOH/*p*-dioxane/H_2O/20 °C/18 h; (vi) $SOCl_2$/reflux/5 h; (vii) RNH_2.

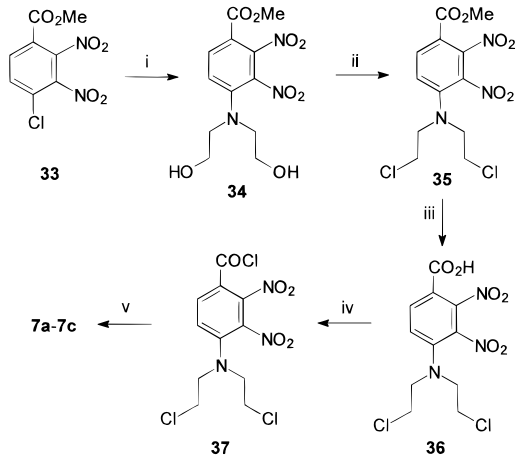
Scheme 4[a]



[a] (i) $NH(CH_2CH_2OH)_2$/*p*-dioxane/20 °C/48 h; (ii) MsCl/Et_3N/CH_2Cl_2/0 °C/15 min, then LiCl/DMF/100 °C/15 min; (iii) KOH/*p*-dioxane/MeOH/H_2O/20 °C/30 min; (iv) $SOCl_2$/1,2-dichloroethane/reflux/6 h; (v) RNH_2.

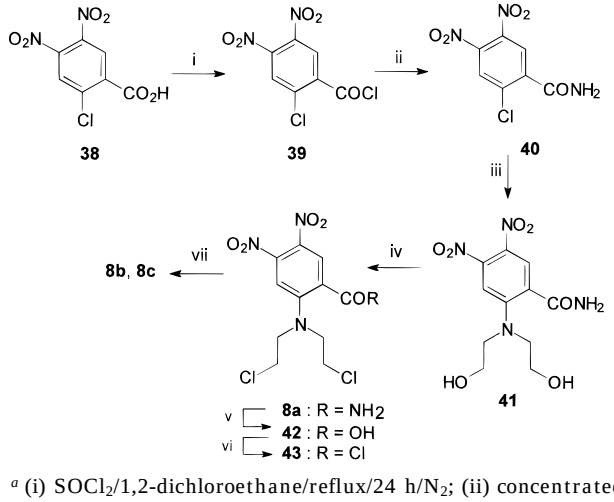
para relationship between the mustard and nitro group, this study was restricted to isomers having at least one of the nitro groups in such a position. Seven of these proved to be synthetically accessible and were examined, including all three where both nitro groups are *ortho* or *para* to the mustard. The structures of the compounds and their physicochemical properties are given in Table 1. The solubility of the compounds in water were determined by spectrophotometry. Solubilities in tissue culture medium (αMEM) containing 5% fetal calf serum were also measured and were similar to those in water in most cases. The exceptions were some of the cationic derivatives, which were less soluble in the chloride ion-containing culture medium as the

Scheme 5[a]



[a] (i) $NH(CH_2CH_2OH)_2$/*p*-dioxane/20 °C/72 h; (ii) MsCl/pyridine/20 °C/10 min, then LiCl/DMF/100 °C/15 min; (iii) KOH/MeOH/H_2O/20 °C/1 h; (iv) $SOCl_2$/1,2-dichloroethane/reflux/5 h; (v) RNH_2.

Scheme 6[a]



[a] (i) $SOCl_2$/1,2-dichloroethane/reflux/24 h/N_2; (ii) concentrated NH_4OH/Et_2O/0 °C/15 min; (iii) $NH(CH_2CH_2OH)_2$/*p*-dioxane/20 °C/18 h; (iv) MsCl/pyridine/20 °C/15 min, then LiCl/DMF/130 °C/15 min; (v) 90% H_2SO_4/70 °C/5 days; (vi) $SOCl_2$/1,2-dichloroethane/reflux/6 h; (vii) RNH_2.

hydrochloride salts, presumably due to common ion effects (e.g., **4b**; 29.7 mM in water, 1.8 mM in culture medium). The parent compound **2a** has only moderate solubility in water (0.17 mM), and most of the corresponding isomers showed very similar values, with two (**6a** and **8a**) being even less soluble. The cationic (dimethylamino)ethyl derivatives were much more soluble than the parent compounds, with the exception of **3b.** The carboxamide diols generally had intermediate solubilities, but showed large and unpredictable variations between isomers (from 0.9 mM for **4c** to 12 mM for **5c**).

One-electron reduction potentials [$E(1)$] for the more soluble (dimethylamino)ethyl derivatives of each regioisomer were measured by pulse radiolysis, using a modified Dynaray 4 (4 MeV) linear accelerator (200 ns pulse length and a custom-built optical radical detection system). Potentials were determined in aqueous solutions containing 2-propanol (0.1 M) buffered at pH 7.0 (1 mM phosphate) by measuring the equilibrium constant[18] for electron transfer between the radical anions of the compounds and benzyl viologen as reference standard. Data were obtained at three or more different concentrations, and are presented in Table 1 (see Supporting Information for additional details). The main determinant of $E(1)$ appeared to be the relative disposition of the two nitro groups. The four derivatives where these are *meta* to each other (**2b**−**5b**) had $E(1)$ values in the range −340 to −375 mV, while those compounds with *ortho*- or *para*-disposed nitro groups (**6b**−**8b**) had much higher values (−260 to −285 mV). Beyond this, the relative disposition of the other groups on the ring seemed to have a much lesser effect.

The aerobic cytotoxicities of the compounds were determined (as IC_{50} values) in two Chinese hamster lines (AA8 and UV4) and the murine mammary carcinoma EMT6, using a growth inhibition microassay.[19] The UV4 cell line is a repair-defective mutant of AA8 which is hypersensitive to alkylating agents whose cytotoxicity is due to bulky DNA adducts or cross-links.[20] IC_{50} values for the compounds following 18 h exposures in air are given in Table 2. Within each series, the parent benzamides and the (dimethylamino)ethyl derivatives had broadly similar IC_{50} values, with the diol derivatives generally being significantly less cytotoxic. Between series, the compounds fell into two well-defined classes. For the parent benzamides, those compounds (**2a**−**5a**) with *meta*-disposed nitro groups (and lower reduction potentials) were less cytotoxic (IC_{50}s from 73 to 470 μM), while those (**6a**−**8a**) with *ortho*/*para*-disposed nitro groups (and higher reduction potentials) were much more potent (IC_{50}s from 1.6 to 20 μM). However, this pattern did not hold for the ratios of their aerobic cytotoxicities in the repair-proficient and -deficient cell lines, with cationic derivatives from both classes (compounds **3b** and **6b**) showing the highest hypersensitivity factors [HF = IC_{50}(AA8)/IC_{50}(UV4)] of about 13-fold. Overall, there were no obvious relationships between HF values and either the structures of the compounds or their absolute aerobic cytotoxicities.

The hypoxic selectivities of the (dimethylamino)ethyl derivatives were determined by clonogenic assay of stirred plateau-phase cultures of both AA8 and UV4 cells, continuously gassed with 5% CO_2 in air or N_2, as described previously.[21−23] The cytotoxic potency in this assay was determined as C_{10}, the concentration of drug required for 10% cell survival after a 1 h exposure. Against the more sensitive UV4 line, the compounds fell into the same two groups as in the growth inhibition assay, with the *m*-dinitro derivatives having much higher aerobic C_{10}s and high hypoxic selectivities (45−115 fold), with the exception of **4b**, which was nonselective for unknown reasons. In contrast, the *ortho*/*para* isomers had much higher aerobic potencies but lower selectivities (2−7-fold). Illustrative data are shown in Figure 1 for one *m*-dinitro compound of high selectivity (**5b**) and one *o*-dinitro compound of low selectivity (**7b**). The hypoxic selectivities of both were much lower in the repair-competent AA8 line, with **7b** being slightly more toxic under aerobic than hypoxic conditions (Figure 1). The weaker hypoxic selectivity in AA8 cells was a general finding (Table 2). Although there was no clear correlation between the hypoxic selectivity in the two cell lines, all compounds giving an air/N_2 ratio of <5 in the UV4 line were devoid of hypoxic selectivity in AA8 cells.

Table 2. Biological Activities of the Dinitro Carboxamide Mustards of Table 1

	aerobic growth inhibition			Clonogenic assay			
		hypersensitivity factor[b]		hypoxic C_{10} (μM)[c]		air/N_2 ratio[d]	
no.	IC_{50}[a] (μM) AA8	UV4	EMT6	AA8	UV4	AA8	UV4
2a	470 ± 54	6.4 ± 0.3	1.1		3400[e]		42[e]
2b	220 ± 33	3.1 ± 0.6	6.7 ± 4.0	1300	400	3.1	44
2c	1930 ± 140	3.4 ± 0.7	2.3 ± 0.3		3600[e]		8.6[e]
3a	73 ± 27	4.3 ± 0.5	0.7				
3b	150 ± 8	13 ± 1	2.5 ± 0.7	>1500[f]	1100[f]	>2.35[f]	68[f]
3c	500 ± 66	4.9 ± 1.0	1.8 ± 0.2				
4a	97 ± 16	1.5 ± 0.1	1.3 ± 0.3				
4b	62 ± 14	1.8 ± 0.1	2.1 ± 0.4	270	250	0.96	1.04
4c	320 ± 70	0.9 ± 0.1	2.3 ± 1.0				
5a	>244[g]	>2.6[g]					
5b	426 ± 27	2.7 ± 0.3	3.0 ± 0.2	2200	2200	2.2	116
5c	652 ± 13	2.7 ± 0.1	1.7				
6a	11 ± 1	2.8 ± 0.1	1.9 ± 0.3				
6b	12 ± 3	13 ± 2	5.8 ± 1.5	120	12	3.2	7.5
6c	81 ± 26	12 ± 0.4	3.5 ± 1.3				
7a	20 ± 4	1.0 ± 0.1	3.1 ± 1.7				
7b	13 ± 5	1.0 ± 0.2	1.2	140	160	0.82	4.0
7c	108 ± 13	2.3 ± 0.1	3.0 ± 0.15				
8a	1.6 ± 0.2	0.88 ± 0.05	0.43 ± 0.05				
8b	4.0 ± 0.1	1.4	3.0	44	18	0.98	1.8
8c	73 ± 2	1.5 ± 0.1	1.9				

[a] IC_{50} determined against aerobic AA8 cells as described in the text, using an exposure time of 18 h. Values are means ± SEM. Values without SEM are for a single determination only. [b] Hypersensitivity factor = IC_{50} (AA8)/IC_{50} (named cell line). Values are intraexperiment means ± SEM. [c] C_{10}: the drug concentration (μM) required to reduce cell survival to 10% of controls under hypoxic conditions, using the indicated cell line at 10^6/mL in the clonogenic assay (see text). [d] Ratio of C_{10} values in air and N_2 [C_{10} (air)/C_{10} (nitrogen)]. [e] Estimated from data in ref 12. [f] Inactive using 1 h exposure at the solubility limit. The estimates of C_{10} are from experiments using up to 8 h exposure; equivalent C_{10} values (for 1 h exposure) were calculated assuming that concentration × time for 10% survival is constant. [g] Inactive against AA8 cells at the solubility limit.

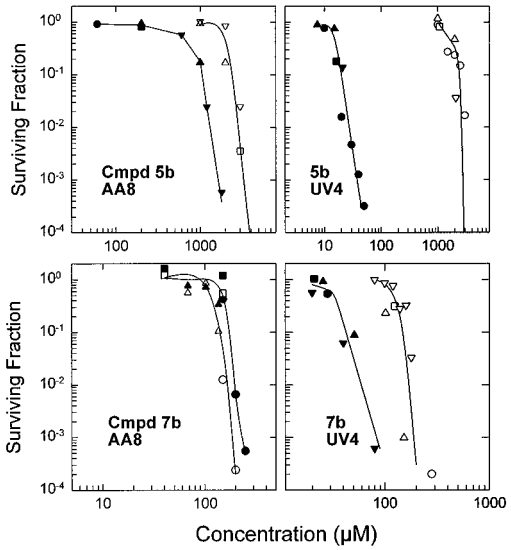


Figure 1. Survival curves for 1 h exposure of early plateau-phase AA8 and UV4 cells to compounds **5b** and **7b** under aerobic (open symbols) or hypoxic (filled symbols) conditions. Different symbol shapes refer to separate experiments.

The *in vitro* biological data can be rationalized by proposing at least two mechanisms of toxicity in the dinitrobenzamide mustards. One, due to net nitro-reduction (which is suppressed in aerobic cells), forms activated nitrogen mustards which are responsible for hypoxia-selective toxicity. The greater hypoxic selectivity in UV4 cells then reflects failure to repair the resulting DNA alkylation lesions. The second mechanism of toxicity is unrelated to DNA alkylation and is presumably due to redox cycling of the prodrug. This mechanism is relatively important in cells resistant to alkylating agents, such as AA8, and is enhanced in oxygenated cells, thereby counteracting the ability of oxygen to inhibit cytotoxicity.

The major factor determining differences in hypoxic selectivity between the isomeric dinitrobenzamides appears to be reduction potential, rather than the generation of species of different absolute cytotoxicity following reduction. Compounds in the first group, with *meta*-disposed nitro groups and relatively low reduction potentials, presumably undergo less reductive metabolism under aerobic conditions, leading to relatively low aerobic cytotoxicities and high hypoxic selectivities. The higher potencies and lower selectivities of the isomers in the second group, with *ortho*- or *para*-disposed nitro groups and higher reduction potentials, are then likely to be due to more extensive reduction, even under aerobic conditions.

The cationic derivative **2b** was previously evaluated against the KHT tumor *in vivo* in conjunction with irradiation, using an excision assay.[12] This compound caused substantial tumor cell killing, but gave significant effects only at doses above the maximum tolerated dose (MTD) for long-term survival of the host. It proved equally proficient at killing cells in the absence of radiation, indicating activity against oxic as well as hypoxic cells. Of the isomers studied here, **5b** showed the highest level of hypoxic selectivity against UV4 cells *in vitro* and was therefore evaluated against the KHT tumor *in vivo*. The MTD of **5b** was 450 μmol/kg (cf. 240 for **2b**), consistent with the lower toxicity of **5b** against aerobic cells in culture (Table 2). When **5b** was given either before or after radiation (15 Gy) at a dose corresponding to 75% of the MTD, significant additional cell killing was observed (Figure 2). Activity was also seen without radiation (Figure 2), indicating that this drug (like **2b** at supratoxic doses) kills both aerobic and hypoxic tumor cells. At an equivalent fraction of the MTD, compound **2b** showed only marginal activity either with or without radiation (Figure 2). The supe-

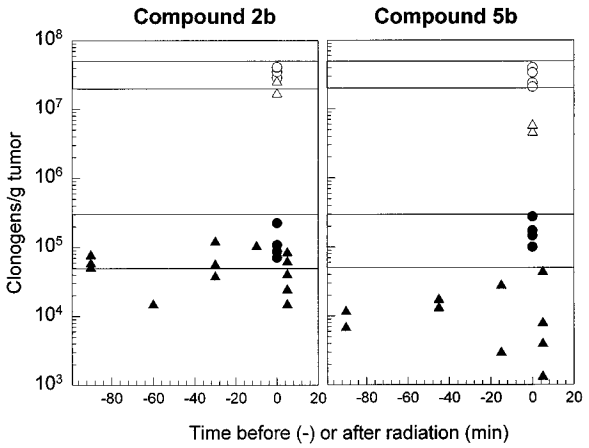


Figure 2. Activity of compounds **2b** and **5b** against the KHT tumor as determined by excision and clonogenic assay 18 h after treatment. Open symbols: unirradiated. Filled symbols: 15 Gy whole-body γ irradiation. Circles: no drug treatment. Triangles: ip drug at 180 (**2b**) or 338 (**5b**) μmol/kg. The drug doses correspond to 75% of the MTD (as determined in non-tumor-bearing mice using a 28-day observation period). Each point represents two or three pooled tumors from separate mice. Multiple points at each dose are from separate experiments. The horizontal lines show the 95% confidence intervals for untreated tumors (top band) and tumors treated with radiation only at 15 Gy (lower band) based on historical data.

riority of **5b** over **2b** was confirmed in dose−response experiments in which the drugs were administered 5 min after irradiation (data not shown).

Conclusions

The 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamides (**2a**−**c**) have confirmed activities as hypoxia-selective cytotoxins[7,12] and as prodrugs for ADEPT.[15] Because SAR studies[12] suggested that significant further improvements in these properties were not likely to be achieved by alteration of side chains, we have studied six additional regioisomers of these dinitrobenzamide mustards, including the two further isomers where both nitro groups are in resonant (*ortho* or *para*) positions with respect to the mustard. The results show that the reduction potentials for addition of the first electron are dominated by the positioning of the nitro groups with respect to each other; those isomers with *ortho*- or *para*-disposed nitro groups have higher reduction potentials than those where the two nitro groups are *meta*-positioned. The compounds fall into the same broad groups with respect to biological activity, with the former showing higher absolute cytotoxicities but lower hypoxic selectivities. These results are consistent with the compounds of higher reduction potential undergoing more rapid bioactivation by reduction under aerobic conditions.

The results suggest that a necessary (but not sufficient, given the data for **4**) requirement for significant hypoxic selectivity in the UV4 assay for the dinitrobenzamide mustard derivatives is positioning the two nitro groups *meta* to each other. The importance (if any) of the positioning of the nitro groups with respect to the mustard is less clear. However, the compounds showed much lower hypoxic selectivity against repair-competent AA8 cells, with no clear superiority for the *meta* analogues.

In the parent series, where studies with **2a** have shown[10] that metabolism in AA8 cells occurs exclusively at the 4-nitro group *ortho* to the mustard, the resulting 4-amino (and presumed 4-hydroxylamino) metabolites undergo quite rapid intramolecular cyclization ($t_{1/2}$ ca. 30 min at 37 °C for the amine, more rapidly for the hydroxylamine) to the much less cytotoxic monofunctional tetrahydroquinoxalines.[10] It is possible that this rapid deactivation detracts from the cytotoxicity of the reduction products. The only compound clearly superior to **2b** in the UV4 clonogenic assay was the 3-[*N*,*N*-bis(2-chloroethyl)amino]-2,6-dinitrobenzamide (**5b**), which also possesses a nitro group *ortho* to the mustard, but nothing is yet known about the hypoxic metabolism of this regioisomer. Compound **5b** showed activity superior to **2b** against both the hypoxic and aerobic subfraction of cells in KHT tumors *in vivo* when the two compounds were compared at equivalent toxicity. However, it is not yet clear whether this activity is due to hypoxia-selective bioreductive activation in tumors.

Experimental Section

Chemistry. Analyses were carried out in the Microchemical Laboratory, University of Otago, Dunedin, NZ. Melting points were determined on an Electrothermal 9200 melting point apparatus. NMR spectra were obtained on Bruker AC-200 or AM-400 spectrometers, and are referenced to Me_4Si for organic solutions and 3-(trimethylsilyl)propanesulfonic acid, sodium salt for D_2O solutions. Thin-layer chromatography was carried out on aluminum-backed silica gel plates (Merck 60 F_{254}). Flash column chromatography was carried out on Merck silica gel (230−400 mesh). Petroleum ether refers to the fraction boiling at 40−60 °C. Benzamides containing the 2,3-dihydroxypropyl side chain were all viscous oils or foams which retained trace amounts of EtOAc tenaciously, making successful combustion analysis difficult. Satisfactory high-resolution mass spectral data were obtained for these compounds using desorption electron impact ionization at 70 eV, or chemical ionization using NH_3 as carrier gas. All final products were judged to be >98% pure by reverse-phase HPLC analysis with diode array detection.

2-[*N*,*N*-Bis(2-chloroethyl)amino]-3,5-dinitrobenzamide (3a). 2-Chloro-3,5-dinitrobenzoic acid (**9**) (10.0 g of 75%, 0.03 mol) was treated with $SOCl_2$ (10 mL) containing 1 drop of DMF under reflux for 5 h. Evaporation of reagent followed by azeotroping with benzene gave the crude acid chloride **10**, which was dissolved in Me_2CO (30 mL) and treated with concentrated NH_4OH (10 mL) to give a quantitative yield of 2-chloro-3,5-dinitrobenzamide (**11**). A solution of **11** (1.00 g, 4.07 mmol) and Et_3N (1.42 g, 10 mmol) in *p*-dioxane (30 mL) was treated with *N*,*N*-bis(2-chloroethyl)amine hydrochloride (1.45 g, 8.14 mmol) at 50 °C for 18 h. The mixture was poured into water and extracted with EtOAc to give an oil, which was chromatographed on silica gel. Elution with EtOAc/petroleum ether (1:1) gave 2-[*N*,*N*-bis(2-chloroethyl)amino]-3,5-dinitrobenzamide (**3a**) (1.15 g, 80%): mp ($CHCl_3$/petroleum ether) 123−124 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.70 (d, J = 2.7 Hz, 1 H, H-4), 8.49 (d, J = 2.7 Hz, 1 H, H-6), 7.68, 7.35 (2 × br, 2H, $CONH_2$), 3.80, 3.61 (2 × t, J = 6.9 Hz, 8 H, NCH_2CH_2Cl); ^{13}C NMR δ 167.94 ($CONH_2$), 147.27 (C-2), 147.20, 142.84 (C-3,5), 137.70 (C-1), 128.40, 123.24 (C-4,6), 55.84 (CH_2N), 42.28 (CH_2Cl). Anal. ($C_{11}H_{12}Cl_2N_4O_5$) C, H, N, Cl.

Treatment of the crude acid chloride **10** (prepared as above from 10.0 g of acid **9** of 75% purity, 0.03 mol) in Et_2O (80 mL) with a solution of *N*,*N*-dimethylethylenediamine (4.9 mL, 0.06 mol) in water (15 mL) at 0 °C for 15 min, followed by workup in EtOAc, gave an oil which was chromatographed on silica gel. Elution with EtOAc/MeOH (9:1) gave *N*-[2-(*N*,*N*-dimethylamino)ethyl]-2-chloro-3,5-dinitrobenzamide (**12**) as a viscous oil (5.6 g, 59%). The hydrochloride salt crystallised from MeOH/isopropyl ether: mp 220−222 °C; ^{1}H NMR (D_2O) δ 8.99

(d, J = 2.3 Hz, 1 H, ArH-4), 8.74 (d, J = 2.3 Hz, 1 H, H-6), 3.89 (t, J = 6.3 Hz, 2 H, $CH_2N^+Me_2$), 3.51 (t, J = 6.3 Hz, 2 H, $CONHCH_2$), 3.04 (s, 6 H, N^+Me_2); ^{13}C NMR δ 169.32 (CONH), 150.88 (C-2), 148.70, 141,02 (C-3,5), 133.23 (C-1), 129.45, 125.27 (C-4,6), 58.64 ($CH_2N^+Me_2$), 45.88 (N^+Me_2), 37.94 ($CONHCH_2$). Anal. ($C_{11}H_{13}ClN_4O_5$·HCl) C, H, N, Cl. Reaction of **12** with bis(2-chloroethyl)amine hydrochloride and Et_3N as above, followed by chromatography on silica gel and elution with EtOAc/MeOH (9:1), gave *N*-[2-(*N*,*N*-dimethylamino)-ethyl]-2-[*N*,*N*-bis(2-chloroethyl)amino]-3,5-dinitrobenzamide (**3b**) (56% yield): mp (EtOAc/petroleum ether) 115−118 °C; ^{1}H NMR ($CDCl_3$) δ 8.63 (s, 2 H, H-4,6), 7.41 (br, 1 H, CONH), 3.74 (t, J = 6.3 Hz, 4 H, CH_2Cl), 3.59 (t, J = 5.8 Hz, 2 H, $CONHCH_2$), 3.55 (t, J = 6.3 Hz, 4 H, NCH_2CH_2Cl), 2.56 (t, J = 5.8 Hz, 2 H, CH_2NMe_2), 2.27 (s, 6 H, NMe_2); ^{13}C NMR δ 164.70 (CO), 145.93 (C-2), 144.86, 141.94 (C-3,5), 135.68 (C-1), 129.63 (C-4), 123.16 (C-6), 57.51 ($CONHCH_2$), 54.82 (NCH_2CH_2Cl), 45.12 (NMe_2), 41.39 (NCH_2CH_2Cl), 37.67 ($CONHCH_2$). Anal. ($C_{15}H_{21}Cl_2N_5O_5$) C, H, N, Cl. The hydrochloride salt was a hygroscopic foam.

Treatment of the crude acid chloride **10** in Me_2CO with a solution of 3-aminopropane-1,2-diol (2 equiv) in water, as above, followed by chromatography of the product on silica gel and elution with EtOAc, gave *N*-(2,3-dihydroxypropyl)-2-chloro-3,5-dinitrobenzamide (**13**) (40% yield) as a colorless gum: ^{1}H NMR [$(CD_3)_2SO$] δ 8.97 (d, J = 2.6 Hz, 1 H, H-4), 8.80 (t, J = 5.6 Hz, 1 H, CONH), 8.55 (d, J = 2.6 Hz, 1 H, H-6), 4.00 (br s, 2 H, OH), 3.70−3.63 (m, 1 H, $CHOH$), 3.50−3.42 (m, 1 H, $CONHCHH$), 3.41−3.37 (m, 2 H, CH_2OH), 3.22−3.15 (m, 1 H, $CONHCHH$); ^{13}C NMR δ 163.19 (CONH), 148.48, 145.89, 140.44 128.49 (4s), 126.10 (C-4), 120.55 (C-6), 70.01 (CHOH), 63.79 (CH_2OH), 42.93 ($CONHCH_2$); CIMS found [M + H]$^+$ 322.0253, 320.0274; $C_{10}H_{11}Cl_2N_3O_7$ requires 322.0256, 320.0285.

Reaction of **13** with bis(2-chloroethyl)amine hydrochloride and Et_3N as above, followed by chromatography on silica gel and elution with EtOAc, gave *N*-(2,3-dihydroxypropyl)-2-[*N*,*N*-bis(2-chloroethyl)amino]-3,5-dinitrobenzamide (**3c**) as a hygroscopic, yellow foam (65% yield): ^{1}H NMR [$(CD_3)_2SO$] δ 8.73 (d, J = 2.8 Hz, 1 H, H-4), 8.70 (t, J = 5.5 Hz, 1 H, CONH), 8.35 (d, J = 2.8 Hz, 1 H, H-6), 4.91, 4.65 (2 × br s, 2 H, OH), 3.72 (t, J = 7.5 Hz, 4 H, CH_2Cl), 3.66 (br s, 1 H, $CHOH$), 3.43 (t, J = 7.5 Hz, 4 H, CH_2N), 3.41−3.31 (m, 3 H, $CONHCHH$ and CH_2OH), 3.21−3.12 (m, 1 H, $CONHCHH$); ^{13}C NMR δ 165.41 (CONH), 145.79, 145.30, 140.92, 136.34 (4s), 127.54 (C-4), 122.11 (C-6), 69.86 (CHOH), 63.88 (CH_2OH), 54.07 (CH_2N), 42.98 ($CONHCH_2$), 41.52 (CH_2Cl). CIMS found [M + H]$^+$ 429.0587, 427.0612, 425.0637; $C_{14}H_{19}Cl_2N_4O_7$ requires 429.0572, 427.0601, 425.0631.

4-[*N*,*N*-Bis(2-chloroethyl)amino]-3,5-dinitrobenzamide (4a). A solution of methyl 4-chloro-3,5-dinitrobenzoate (**14**) (10.0 g, 0.036 mol) and diethanolamine (7.66 g, 0.073 mol) in *p*-dioxane (60 mL) was stirred at 50 °C for 5 h. Volatiles were removed under reduced pressure, and the residue was adsorbed directly onto silica gel and chromatographed. Elution with EtOAc/petroleum ether (1:10) gave foreruns, while elution with EtOAc/petroleum ether (3:2) gave methyl 4-[*N*,*N*-bis(2-hydroxyethyl)amino]-3,5-dinitrobenzoate (**15**) (9.61 g, 80%): mp ($CHCl_3$/petroleum ether) 101−103 °C; ^{1}H NMR ($CDCl_3$) δ 8.52 (s, 2 H, ArH-2,6), 3.97 (s, 3 H, $COOCH_3$), 3.76 (d × t, J = 6.5, 4.7 Hz, 4 H, CH_2OH), 3.26 (t, J = 4.7 Hz, 4 H, NCH_2), 2.76 (t, J = 6.5 Hz, 2 H, OH); ^{13}C NMR δ 163.14 ($COOCH_3$), 145.50 (C-4), 142.54 (C-3,5), 131.19 (C-2,6), 123.59 (C-1), 59.16 (CH_2OH)1, 54.58 (CH_2N), 53.14 (OCH_3). Anal. ($C_{12}H_{15}N_3O_8$) C, H, N.

MsCl (3.93 mL, 0.049 mol) was added dropwise at 0 °C to a solution of **15** (7.30 g, 0.022 mol) and Et_3N (7.72 mL, 0.055 mol) in CH_2Cl_2 (100 mL). After 15 min, the solution was washed several times with water and worked up to give the crude dimesylate, which was treated with LiCl (15.0 g) in DMF (80 mL) at 120 °C for 5 min. Workup and chromatography on silica gel, eluting with EtOAc/petroleum ether (1:4), gave methyl 4-[*N*,*N*-bis(2-chloroethyl)amino]-3,5-dinitrobenzoate (**16**) (6.21 g, 77%): mp ($CHCl_3$/petroleum ether) 93−95 °C; ^{1}NMR ($CDCl_3$) δ 8.54 (s, 2 H, H-2,6), 3.99 (s, 3 H, $COOCH_3$), 3.63 (t, J = 6.9 Hz, 4 H, CH_2Cl), 3.43 (t, J = 6.9 Hz, 4 H, CH_2N); ^{13}C NMR δ 162.90 ($COOCH_3$), 147.83 (C-4), 140.53 (C-3,5), 129.85 (C-2,6), 126.70 (C-1), 55.47 (CH_2N), 53.23 (OCH_3), 41.18 (CH_2Cl). Anal. ($C_{12}H_{13}Cl_2N_3O_6$) C, H, N, Cl. Hydrolysis of **16** with excess 3 N aqueous KOH in *p*-dioxane at 20 °C for 3 h gave a quantitative yield of 4-[*N*,*N*-bis(2-chloroethyl)amino]-3,5-dinitrobenzoic acid (**17**): mp (EtOAc/petroleum ether) 164−166 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.61 (s, 2 H, H-2,6), 3.78 (t, J = 6.9 Hz, 4 H, CH_2Cl), 3.52 (t, J = 6.9 Hz, 4 H, CH_2N) (COOH not visible); ^{13}C NMR δ 164.12 (COOH), 149.12 (C-4), 141.40 (C-3,5), 130.70 (C-2,6), 128.19 (C-1), 56.14 (CH_2N), 42.46 (CH_2Cl). Anal ($C_{11}H_{11}Cl_2N_3O_6$) C, H, N, Cl.

A solution of **17** (2.00 g, 5.46 mmol) in 1,2-dichloroethane (40 mL) containing DMF (1 drop) was treated with $SOCl_2$ (10 mL) as above to give the crude acid chloride **18**, which was dissolved in dry Et_2O (100 mL), cooled to 5 °C, and treated dropwise with concentrated NH_4OH (20 mL). After 10 min the solution was worked up and the residue was chromatographed on silica. Elution with EtOAc/petroleum ether (3:7) gave foreruns, while EtOAc/petroleum ether (7:3) gave 4-[*N*,*N*-bis(2-chloroethyl)amino]-3,5-dinitrobenzamide (**4a**) (80% overall yield), mp (EtOAc/petroleum ether) 113−116 °C; ^{1}H NMR [$(CD_3)_2CO$] δ 8.61 (s, 2 H, H-2,6), 7.98, 7.24 (2 × br, 2 H, $CONH_2$), 3.75 (t, J = 7.0 Hz, 4 H, CH_2Cl), 3.50 (t, J = 7.0 Hz, 4 H, CH_2N); ^{13}C NMR δ 164.91 ($CONH_2$), 149.32 (C-4), 140.07 (C-3,5), 132.18 (C-1), 128.91 (C-2,6), 56.37 (CH_2N), 42.55 (CH_2Cl). Anal. ($C_{11}H_{12}Cl_2N_4O_5$) C, H, N, Cl.

Similar reaction of crude **18** in CH_2Cl_2 with neat *N*,*N*-dimethylethylenediamine (2 equiv) and chromatography of the product on silica gel, eluting with EtOAc/MeOH (9:1), gave *N*-[2-(*N*,*N*-dimethylamino)ethyl]-4-[*N*,*N*-bis(2-chloroethyl)-amino]-3,5-dinitrobenzamide (**4b**) (62% yield) as a yellow oil. The hydrochloride salt crystallized from MeOH/isopropyl ether: mp 135−140 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 10.65 (br, 1 H, HCl), 9.49 (t, J = 5.4 Hz, 1 H, CONH), 8.75 (s, 2 H, Ar-H2,6), 3.71 (t, J = 6.9 Hz, 4 H, NCH_2CH_2Cl), 3.44−3.28 (m, 8 H, NCH_2CH_2Cl and $CH_2CH_2N^+Me_2$), 2.83 (s, 6 H, N^+Me_2); ^{13}C NMR δ 162.63 (CO), 147.43 (C-3,5), 138.75 (C-4), 130.22 (C-1), 128.31 (C-2,6), 55.44 ($CH_2N^+Me_2$), 54.75 (NCH_2CH_2Cl), 42.22 (N^+Me_2), 42.18 (NCH_2CH_2Cl), 34.75 ($CONHCH_2$). Anal. ($C_{15}H_{21}Cl_2N_5O_5$·HCl) C, H, N, Cl.

Similar reaction of crude **18** in Me_2CO with 3-aminopropane-1,2-diol (2 equiv) in water at 0 °C as above, followed by chromatography on silica gel, eluting with EtOAc, gave *N*-(2,3-dihydroxypropyl)-4-[*N*,*N*-bis(2-chloroethyl)amino]-3,5-dinitrobenzamide (**4c**) (66% yield) as a yellow oil: ^{1}H NMR [$(CD_3)_2SO$] δ 8.84 (t, J = 5.5 Hz, 1 H, CONH), 8.63 (s, 2 H, H-2,6) 4.85 (d, J = 5.0 Hz, 1 H, $CHOH$), 4.59 (t, J = 5.7 Hz, 1 H, CH_2OH), 3.70 (t, J = 6.7 Hz, 4 H, CH_2Cl), 3.50−3.35 (m, 8 H, CH_2N and $CHHCHOHCH_2OH$), 3.20 (m, 1 H, CHH); ^{13}C NMR δ 162.41 (CONH), 147.50 (s), 138.60 (s), 130.89 (s), 128.17 (C-2,6), 70.11 (CHOH), 63.87 (CH_2OH), 54.85 (CH_2N), 43.33 ($CONHCH_2$), 42.21 (CH_2Cl). EIMS found M$^+$ 428.0507, 426.0533, 424.0552; $C_{14}H_{18}Cl_2N_4O_7$ requires 428.0493, 426.0523, 424.0552).

3-[*N*,*N*-Bis(2-chloroethyl)amino]-2,6-dinitrobenzamide (5a). A solution of 3-chlorobenzoic acid (**19**) (60 g, 0.38 mol) in concentrated H_2S0_4 (600 mL) was treated portionwise with fuming nitric acid (d 1.42; 150 mL), and the solution was warmed gradually with stirring to 140 °C in an open flask and held at this temperature for 6 h. After cooling overnight, ice−water (4 L) was added cautiously and the mixture was kept at 5 °C for 3 h. The precipitated product was removed by filtration, washed with water (4 × 200 mL), and crystallized from aqueous EtOH to give 5-chloro-2,4-dinitrobenzoic acid (**20**) (62 g, 66%): mp 180−183 °C (lit.[16] mp 182−183 °C). The original filtrate and washings were combined and allowed to stand for several hours, depositing crystals of pure 3-chloro-2,6-dinitrobenzoic acid (**21**) (8.4 g, 9%): mp (H_2O) 162−163.5 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 10.50 (br, 1 H, COOH), 8.82 (d, J = 8.8 Hz, 1 H, H-5), 8.62 (d, J = 8.8 Hz, 1 H, H-4); ^{13}C NMR δ 162.03 (COOH), 146.34 (C-2), 145.57 (C-6), 133.69 (C-5), 130.01 (C-1), 128.26 (C-4), 125.19 (C-3). Anal. ($C_7H_3ClN_2O_6$) C, H, N, Cl.

The methyl ester **22**, prepared from **21** using MeOH/concentrated H_2SO_4 (reflux, 8 h), crystallized from MeOH as needles: mp 110−112 °C; ^{1}H NMR ($CDCl_3$) δ 8.22 (d, J = 8.9

Hz, 1H, H-5), 7.82 (d, J = 8.9 Hz, 1H, H-4), 3.96 (s, 3H, $COOCH_3$); ^{13}C NMR δ 161.30 ($COOCH_3$), 145.22, 143.74 (C-2,6), 133.20 (C-5), 132.31 (s), 127.06 (C-4), 125.28 (s), 54.61 ($COOCH_3$). Anal. ($C_8H_5ClN_2O_6$) C, H, N. The ester **22** was converted into the diol **23** with diethanolamine in *p*-dioxane at 50 °C, as above (83% yield), and this was reacted with MsCl/LiCl as above to give methyl 3-[*N*,*N*-bis(2-chloroethyl)amino]-2,6-dinitrobenzoate (**24**) (67% yield): mp (EtOAc/petroleum ether) 118−119.5 °C; ^1H NMR ($CDCl_3$) δ 8.30 (d, J = 9.1 Hz, H-5), 8.00 (d, J = 9.1 Hz, H-4), 3.95 (s, 3H, $COOCH_3$), 3.88−3.75 (m, 8H, NCH_2CH_2Cl); ^{13}C NMR δ 163.10 ($COOCH_3$), 148.21 (C-3), 140.98, 138.71 (C-2,6), 128.76, 124.58 (C-4,5), 127.34 (C-1), 54.77 ($COOCH_3$), 54.37 (CH_2N), 42.16 (CH_2Cl). Anal. ($C_{12}H_{13}Cl_2N_3O_6$) C, H, N.

An identical series of reactions beginning with ethyl 3-chloro-2,6-dinitrobenzoate gave ethyl 3-[*N*,*N*-bis(2-chloroethyl)amino]-2,6-dinitrobenzoate (**25**): mp (EtOAc/petroleum ether) 84−87 °C; ^1H NMR [$(CD_3)_2CO$] δ 8.32 (d, J = 9.4 Hz, 1 H, H-5), 7.79 (d, J = 9.4 Hz, 1 H, H-4) 4.39 (q, J = 7.1 Hz, 2 H, OCH_2CH_3), 3.85−3.77 (m, 8 H, NCH_2CH_2Cl), 1.34 (t, J = 7.1 Hz, 3 H, OCH_2CH_3); ^{13}C NMR δ 163.24 (COOEt), 148.30 (C-3), 141.04, 138.80 (C-2,6), 128.84, 124.65 (C-4,5), 127.39 (C-1), 63.94 (OCH_2CH_3), 54.39 (NCH_2), 42.00 (CH_2Cl) 13.89 (OCH_2CH_3). Anal. ($C_{13}H_{15}Cl_2N_3O_6$) C, H, N, Cl. An X-ray crystallographic determination was carried out on **25** (see below) for confirmation of structure.

Hydrolysis of **24** or **25** in excess 3 N KOH/*p*-dioxane at 20 °C for 18 h gave 3-[*N*,*N*-bis(2-chloroethyl)amino]-2,6-dinitrobenzoic acid (**26**) which was converted to the crude acid chloride **27** with $SOCl_2$ as above. A solution of **27** in Et_2O at 0 °C was treated with excess concentrated NH_4OH as described above, and the product was chromatographed on silica gel, EtOAc/petroleum ether (1:1), eluting 3-[*N*,*N*-bis(2-chloroethyl)-amino]-2,6-dinitrobenzamide (**5a**) (56% overall yield): mp (EtOAc/petroleum ether) 140−141.5 °C; ^1H NMR [$(CD_3)_2CO$] δ 8.26 (d, J = 9.3 Hz, 1 H, H-5), 7.71 (d, J = 9.3 Hz, 1 H, H-4), 7.63, 7.23 (2 × br, 2 H, $CONH_2$), 3.75 (s, 8 H, NCH_2CH_2Cl); ^{13}C NMR δ 163.99 ($CONH_2$), 147.38 (C-3), 142.92, 139.76 (C-2,6), 130.48 (C-1), 128.22, 124.23 (C-4,5), 54.74 (NCH_2), 42.00 (CH_2Cl). Anal. ($C_{11}H_{12}Cl_2N_4O_5$) C, H, N, Cl.

Reaction of the crude acid chloride **27** in CH_2Cl_2 with *N*,*N*-dimethylethylenediamine (2 equiv) as described above, followed by chromatography of the product on silica gel and elution with EtOAc/MeOH (10:1), gave *N*-[2-(*N*,*N*-dimethylamino)ethyl]-3-[*N*,*N*-bis(2-chloroethyl)amino]-2,6-dinitrobenzamide (**5b**) as a yellow oil (58% yield). The hydrochloride salt crystallized from MeOH/isopropyl ether: mp 180−182 °C; ^1H NMR (D_2O) δ 8.37 (d, J = 9.6 Hz, 1 H, H-5), 7.63 (d, J = 9.6 Hz, 1 H, H-4), 3.80−3.71 (m, 10 H, NCH_2CH_2Cl and $CH_2N^+Me_2$), 3.44 (t, J = 6.4 Hz, 2 H, $CONHCH_2$), 3.00 (s, 6 H, N^+Me_2); ^{13}C NMR δ 168.75 (CONH), 150.91 (C-3), 141.55, 138,72 (C-2,6), 131.61 (C-4), 130.45 (C-1), 125.85 (C-5), 58.31 ($CH_2N^+Me_2$), 55.74 (NCH_2CH_2Cl), 45.97 (N^+Me_2), 44.16 (NCH_2CH_2Cl), 37.91 ($CONHCH_2$). Anal. ($C_{15}H_{21}Cl_2N_5O_5$·HCl) C, H, N, Cl.

Reaction of the crude acid chloride **27** in Me_2CO with 3-aminopropane-1,2-diol (2 equiv) in water at 0 °C as described above, followed by chromatography of the product on silica gel and elution with EtOAc, gave *N*-(2,3-dihydroxypropyl)-3-*N*,*N*-bis(2-chloroethyl)amino]-2,6-dinitrobenzamide (**5c**) (62% yield) as a yellow foam: ^1H NMR [$(CD_3)_2SO$] δ 8.72 (t, J = 5.8 Hz, 1H, CONH), 8.32 (d, J = 9.3 Hz, 1H, H-5), 7.68 (d, J = 9.3 Hz, 1H, H-4), 4.84, 4.58 (2 × br, 2H, OH), 3.83 (t, J = 6.0 Hz, 4H, CH_2Cl), 3.68 (t, J = 6.0 Hz, 4H, CH_2N), 3.65 (m, 1H, C*H*OH), 3.43 (m, 1H, CONHC*H*H), 3.39 (m, 2H, CH_2OH), 3.14 (m, 1H, CONHCH*H*); ^{13}C NMR δ 167.71 (CONH), 147.16 (C-3), 142.17, 138.61 (C-2,6), 131.27, 130.48 (C-1,4), 125.47 (C-5), 70.09 (CHOH), 63.78 (CH_2OH), 52.55 (CH_2N), 42.80 ($CONHCH_2$), 41.75 (CH_2Cl); CIMS found $[M + H]^+$ 429.0584, 427.0602, 425.0630; $C_{14}H_{19}Cl_2N_4O_7$ requires 429.0572, 427.0601, 425.0631.

4-[*N*,*N*-Bis(2-chloroethyl)amino]-2,5-dinitrobenzamide (6a). A mixture of methyl 4-chloro-2,5-dinitrobenzoate (**28**)[24] (0.60 g, 2.30 mmol) and diethanolamine (0.48 g, 4.60 mmol) in *p*-dioxane (25 mL) was stirred at room temperature for 48 h and then adsorbed directly onto silica gel by concentration and chromatographed. Elution with EtOAc gave methyl 4-[*N*,*N*-bis(2-hydroxyethyl)amino]-2,5-dinitrobenzoate (**29**) (0.75 g, 99%): mp (EtOAc/petroleum ether) 102 °C; ^1H NMR [$(CD_3)_2SO$] δ 8.25 (s, 1 H, H-6), 7.86 (s, 1 H, H-3), 4.79 (t, J = 5.3 Hz, 2 H, OH), 3.80 (s, 3 H, OCH_3), 3.56 (dt, J = 5.5, 5.3 Hz, 4 H, CH_2OH), 3.40 (t, J = 5.5 Hz, 4 H, NCH_2); ^{13}C NMR δ 162.13 ($COOCH_3$), 151.86 (s), 147.54 (s), 147.54 (s), 137.68 (s), 130.10 (C-6), 115.21 (C-3), 109.15 (s), 58.14 (CH_2OH), 54.15 (CH_2N), 52.80 ($COOCH_3$); mass spectrum m/z (rel int) 329 (M^+, 7), 298 (100), 282 (5), 268 (20), 254 (18), 251 (9), 222 (19). Anal. ($C_{12}H_{15}N_3O_8$) C, H, N.

A stirred solution of **29** (14.47 g, 0.044 mol) and Et_3N (15.31 mL, 0.110 mol) in CH_2Cl_2 (270 mL) was treated dropwise with MsCl (7.79 mL, 0.097 mol) at 0 °C. After a further 15 min at 0 °C, the solution was washed with 1 N Na_2CO_3 and worked up to give the crude dimesylate as an oil. This was dissolved in DMF (100 mL), LiCl (20.00 g, 0.471 mol) was added, and the mixture was stirred at 100 °C for 15 min. After removal of the DMF under reduced pressure, the residue was partitioned between EtOAc and water, and the organic portion was worked up to give an oil which was chromatographed on silica. Elution with EtOAc/petroleum ether (3:7) gave methyl 4-[*N*,*N*-bis(2-chloroethyl)amino]-2,5-dinitrobenzoate (**30**) (15.08 g, 86%): mp (EtOAc/petroleum ether) 108 °C; ^1H NMR ($CDCl_3$) δ 8.29 (s, 1 H, H-6), 7.49 (s, 1 H, H-3), 3.91 (s, 3 H, $COOCH_3$), 3.64 (br s, 8 H, NCH_2CH_2Cl); ^{13}C NMR δ 162.52 ($COOCH_3$), 151.85 (s), 146.30 (s), 129.91 (C-6), 116.97 (C-3), 116.23 (s), 53.70 (CH_2N), 53.31 ($COOCH_3$), 40.71 (CH_2Cl). Anal. ($C_{12}H_{13}Cl_2N_3O_6$) C, H, N, Cl.

A solution of **30** (13.10 g, 0.033 mol) and 2 N aqueous KOH (60 mL, 0.12 mol) in *p*-dioxane/MeOH (1:1; 200 mL) was stirred at 20 °C for 30 min, then acidified with concentrated HCl, and extracted with EtOAc. Workup of the organic portion gave 4-[*N*,*N*-bis(2-chloroethyl)amino]-2,5-dinitrobenzoic acid (**31**) (11.81 g, 93%): mp (EtOAc/petroleum ether) 188 °C; ^1H NMR [$(CD_3)_2SO$] δ 8.31 (s, 1 H, H-6), 8.00 (s, 1 H, H-3), 4.18 (br s, 1 H, COOH), 3.80 (t, J = 5.8 Hz, 4 H, CH_2Cl), 3.65 (t, J = 5.8 Hz, 4 H, CH_2N); ^{13}C NMR δ 162.99 (COOH), 152.26 (s), 146.95 (s), 139.92 (C-6), 116.24 (C-3), 113.92 (s), 52.56 (CH_2N), 41.85 (CH_2Cl). Anal. ($C_{11}H_{11}Cl_2N_3O_6$) C, H, N, Cl.

A mixture of **31** (2.00 g, 5.21 mmol) and $SOCl_2$ (10.00 mL) in 1,2-dichloroethane (150 mL) containing DMF (1 drop) was refluxed with protection from moisture for 6 h and then concentrated to dryness to give crude 4-[*N*,*N*-bis(2-chloroethyl)-amino]-2,5-dinitrobenzoyl chloride (**32**), which was used directly. Concentrated NH_4OH (4 mL) was added in one portion to a vigorously stirred solution of **32** (2.00 g, 4.78 mmol) in Me_2CO (30 mL) at 0 °C. After 10 min, the mixture was diluted with EtOAc, washed with 2 N $NaHCO_3$, and worked up to give an oil which was chromatographed on silica gel. Elution with EtOAc/petroleum ether (2:3) gave 4-[*N*,*N*-bis(2-chloroethyl)-amino]-2,5-dinitrobenzamide (**6a**) (1.38 g, 82%): mp (EtOAc/petroleum ether) 153 °C; ^1H NMR [$(CD_3)_2SO$] δ 8.22, 7.70 (2 × br, 2 H, $CONH_2$), 8.15 (s, 1 H, H-6), 7.97 (s, 1 H, H-3), 3.77 (t, J = 6.0 Hz, 4 H, CH_2Cl), 3.61 (t, J = 6.0 Hz, 4 H, CH_2N); ^{13}C NMR δ 164.40 ($CONH_2$), 151.05 (s), 145.04 (s), 140.74 (s), 127.11 (C-6), 121.17 (s), 117.63 (C-3), 52.64 (CH_2N), 41.88 (CH_2Cl). Anal. ($C_{11}H_{12}Cl_2N_4O_5$) C, H, N, Cl.

Reaction of **32** in CH_2Cl_2 with neat *N*,*N*-dimethylethylenediamine (2 equiv) as above gave *N*-[2-(*N*,*N*-dimethylamino)-ethyl]-4-[*N*,*N*-bis(2-chloroethyl)amino]-2,5-dinitrobenzamide (**6b**) (64%) as a yellow oil. The hydrochloride salt crystallized from MeOH/Et_2O as a hygroscopic orange foam: mp 70 °C; ^1H NMR (D_2O) δ 8.20, 8.12 (2 × s, 2 H, H-3,6), 3.85−3.67 (m, 10 H, NCH_2CH_2Cl and $CH_2N^+Me_2$), 3.45 (t, J = 6.1 Hz, 2 H, $CONHCH_2$), 3.00 (s, 6H, $^+NMe_2$); ^{13}C NMR δ 168.04 (CONH), 149.58 (s), 146.92 (s), 141.85 (s), 128.28 (d), 120.94 (s), 119.96 (d), 56.41 ($CH_2N^+(CH_3)_2$), 53.23 (CH_2N), 43.50 ($CH_2N^+(CH_3)_2$), 42.05 (CH_2Cl), 35.55 ($CONHCH_2$). Anal. ($C_{15}H_{22}Cl_2N_5O_5$) C, H, N, Cl.

Reaction of **32** in Me_2CO with 3-aminopropane-1,2-diol (2 equiv) in water as above, followed by chromatography of the product on silica gel and elution with EtOAc, gave *N*-(2,3-dihydroxypropyl)-4-[*N*,*N*-bis(2-chloroethyl)amino]-2,5-dinitrobenzamide (**6c**) (83%) as an orange gum: ^1H NMR [$(CD_3)_2SO$] δ 8.76 (t, J = 5.5 Hz, 1 H, CONH), 8.15, 7.98 (2 × s, 2 H, H-3,6), 3.77 (t, J = 5.8 Hz, 4 H, CH_2Cl), 3.62 (t, J = 5.8

Hz, 4 H, CH_2N), 3.48−3.29 (m, 6 H, C*H*(O*H*)CH_2O*H* and CONHC*H*), 3.12 (m, 1 H, CONHC*H*); ^{13}C NMR δ 163.10 (CONH), 150.79 (s), 144.88 (s), 141.06 (s), 127.35 (C-6), 121.66 (s), 117.74 (C-3), 70.19 (CHOH), 63.81 (CH_2OH), 52.73 (CH_2N), 42.96 (CONH*C*H_2), 41.92 (CH_2Cl); CIMS found $[M + H]^+$ 429.0569, 427.0595, 425.0616; $C_{14}H_{19}Cl_2N_4O_7$ requires 429.0572, 427.0601, 425.0631.

4-[*N*,*N*-Bis(2-chloroethyl)amino]-2,3-dinitrobenzamide (7a). A mixture of methyl 4-chloro-2,3-dinitrobenzoate[24] (**33**) (3.15 g, 0.012 mol) and diethanolamine (2.54 g, 0.024 mol) in *p*-dioxane (120 mL) was stirred at 20 °C for 3 days, adsorbed onto silica gel by concentration under reduced pressure, and chromatographed by elution with MeOH/EtOAc (1:19) to give methyl 4-[*N*,*N*-bis(2-hydroxyethyl)amino]-2,3-dinitrobenzoate (**34**) (3.12 g, 78%): mp (EtOAc) 151−152 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 7.96 (d, J = 9.3 Hz, 1 H, H-6), 7.61 (d, J = 9.3 Hz, 1 H, H-5), 4.81 (t, J = 5.2 Hz, 2 H, OH), 3.81 (s, 3 H, $COOCH_3$), 3.56 (dd, J = 5.4, 5.2 Hz, 4 H, CH_2OH), 3.38 (d, J = 5.4 Hz, 4 H, CH_2N); ^{13}C NMR δ 161.96 (*C*OOCH_3), 147.48 (s), 145.51 (s), 132.92 (C-6), 131.30 (s), 122.34 (C-5), 109.53 (s), 58.13 (CH_2OH), 53.82 (CH_2N), 53.00 (COO*C*H_3). Anal. ($C_{12}H_{15}N_3O_8$) C, H, N.

MsCl (1.59 mL, 0.020 mol) was added dropwise to a solution of **34** (2.96 g, 8.99 mmol) in pyridine (30 mL) at 20 °C. After 10 min, the pyridine was removed under reduced pressure, and the residue was partitioned between EtOAc and water. Workup of the organic layer gave the crude dimesylate, which was dissolved in DMF (50 mL) containing LiCl (10 g) and warmed at 100 °C for 15 min. The DMF was removed under reduced pressure, the residue was partitioned between EtOAc and water, and the oily residue from the organic layer was chromatographed on silica gel. Elution with EtOAc/petroleum ether (1:1) gave methyl 4-[*N*,*N*-bis(2-chloroethyl)amino]-2,3-dinitrobenzoate (**35**) (3.05 g, 93%) as yellow needles: mp (EtOAc/petroleum ether) 90−92 °C; ^{1}H NMR ($CDCl_3$) δ 8.05 (d, J = 8.9 Hz, 1 H, H-6), 7.45 (d, J = 8.9 Hz, 1 H, H-5), 3.90 (s, 3 H, $COOCH_3$), 3.62 (br t, J = 3.1 Hz, 8 H NCH_2CH_2Cl); ^{13}C NMR δ 162.07 ($COOCH_3$), 146.09 (s), 144.12 (s), 133.49 (C-6), 131.20 (s), 124.61 (C-5), 116.51 (s), 54.18 (CH_2N), 53.35 ($COOCH_3$), 40.87 (CH_2Cl). Anal. ($C_{12}H_{13}Cl_2N_4O_5$) C, H, N, Cl.

A solution of **35** (3.00 g, 8.19 mmol) and 3 N KOH (20 mL) in MeOH (100 mL) was stirred at 20 °C for 1 h. MeOH was removed under reduced pressure, and the residue was acidified with 3 N HCl, diluted with water, and extracted with EtOAc to give 4-[*N*,*N*-bis(2-chloroethyl)amino]-2,3-dinitrobenzoic acid (**36**) (2.81 g, 90%) as yellow plates: mp (EtOAc/petroleum ether) 190 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.05 (d, J = 8.5 Hz, 1 H, H-6), 7.74 (d, J = 8.5 Hz, 1 H, H-5), 3.85 (br, COOH), 3.76, 3.62 (2 × br, 8 H, NCH_2CH_2Cl); ^{13}C NMR δ 162.97 (COOH), 146.30 (s), 144.37 (s), 134.32 (s), 133.82 (C-6), 124.87 (C-5), 115.43 (s), 52.70 (CH_2N), 41.76 (CH_2Cl). Anal. ($C_{11}H_{11}Cl_2N_3O_6$) C, H, N, Cl.

A solution of **36** (1.00 g, 2.84 mmol) and $SOCl_2$ (15 mL) in 1,2-dichloroethane (30 mL) containing DMF (1 drop) was heated under reflux for 5 h and then concentrated to dryness under reduced pressure. The crude acid chloride **37** was dissolved in Me_2CO (30 mL), and the solution was cooled to 0 °C and treated with concentrated NH_4OH (5 mL). After 10 min, the mixture was diluted with water, extracted with EtOAc, and worked up to give an oil which was chromatographed on silica gel. Elution with EtOAc/petroleum ether (1:5) gave foreruns, while EtOAc eluted 4-[*N*,*N*-bis(2-chloroethyl)amino]-2,3-dinitrobenzamide (**7a**) (0.87 g, 87%): mp (EtOAc/petroleum ether) 138 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.35 (br s, 1 H, CONH*H*), 7.87 (br s, 3 H, H-5,6, CON*H*H), 3.71 (t, J = 6.3 Hz, 4 H, CH_2Cl), 3.58 (t, J = 6.3 Hz, 4 H, CH_2N); ^{13}C NMR δ 164.32 ($CONH_2$), 144.54 (s), 142.79 (s), 136.75 (s), 131.59 (C-6), 127.01 (C-5), 123.51 (s), 53.32 (CH_2N), 41.83 (CH_2Cl). Anal. ($C_{11}H_{12}Cl_2N_4O_5$) C, H, N, Cl.

Reaction of **37** in CH_2Cl_2 with *N*,*N*-dimethylethylenediamine (2 equiv) as above gave *N*-[2-(*N*,*N*-dimethylamino)ethyl]-4-[*N*,*N*-bis(2-chloroethyl)amino]-2,3-dinitrobenzamide (**7b**) as a yellow oil (62% yield). The hydrochloride salt crystallized from MeOH/Et_2O as hygroscopic yellow cubes: mp 60 °C dec; ^{1}H NMR (D_2O) δ 7.84 (d, J = 8.7 Hz, 1 H, H-6), 7.69 (d, J = 8.7 Hz, 1 H, H-5), 3.75 (t, J = 6.0 Hz, 2 H, C*H*N^+Me_2), 3.56 (t, J = 5.1 Hz, 4 H, CH_2Cl), 3.51 (t, J = 5.1 Hz, 4 H, CH_2N), 3.40 (t, J = 6.0 Hz, 2 H, CONHCH_2), 2.95 (s, 6 H, N^+Me_2); ^{13}C NMR δ 167.96 (CONH), 148.05 (s), 145.25 (s), 140.23 (s), 133.89 (C-6), 130.34 (C-5), 125.14 (s), 58.51 ($CH_2N^+(CH_3)_2$), 56.71 (CH_2N), 45.71 ($N^+(CH_3)_2$), 44.12 (CH_2Cl), 37.73 (CONH*C*H_2). Anal. ($C_{15}H_{21}Cl_2N_5O_5$·HCl) C, H, N, Cl.

Reaction of **37** in Me_2CO with a solution of 3-aminopropane-1,2-diol (2 equiv) in water as above, followed by chromatography on silica gel and elution with MeOH/EtOAc (1:19), gave *N*-(2,3-dihydroxypropyl)-4-[*N*,*N*-bis(2-chloroethyl)amino]-2,3-dinitrobenzamide (**7c**) (63% yield) as a yellow oil: ^{1}H NMR [$(CD_3)_2SO$] δ 8.87 (t, J = 5.6 Hz, 1 H, CONH), 7.88 (d, J = 8.8 Hz, 1 H, H-6), 7.85 (d, J = 8.8 Hz, 1 H, H-5), 3.75 (m, 1 H, CHOH), 3.70 (t, J = 6.3 Hz, 4 H, CH_2Cl), 3.65 (br, 2 H, OH), 3.57 (t, J = 6.3 Hz, 4 H, CH_2N), 3.40−3.30 (m, 3 H, CONHC*H*H and C*H*$_2$OH), 3.14−3.08 (m, 1 H, CONHCH*H*); ^{13}C NMR δ 162.85 (CONH), 144.46 (s), 142.59 (s), 136.94 (s), 131.77 (C-6), 127.34 (C-5), 124.03 (s), 70.11 (CHOH), 63.80 (CH_2OH), 53.42 (CH_2N), 43.00 (CONH*C*H_2), 41.86 (CH_2Cl). Anal. ($C_{14}H_{18}Cl_2N_4O_7$) C, H, N, Cl.

2-[*N*,*N*-Bis(2-chloroethyl)amino]-4,5-dinitrobenzamide (8a). A mixture of 2-chloro-4,5-dinitrobenzoic acid[25] (**38**) (6.00 g, 0.024 mol) and $SOCl_2$ (15 mL) in 1,2-dichloroethane (60 mL) containing DMF (1 drop) was refluxed under nitrogen for 24 h and then concentrated to dryness. The residue of crude acid chloride **39** was dissolved in Et_2O (100 mL), cooled to 0 °C, and treated with concentrated NH_4OH (3.30 mL, 0.049 mol). The resulting precipitate was filtered off and recrystallized from MeOH to give 2-chloro-4,5-dinitrobenzamide (**40**) (4.89 g, 82%): mp 206−209 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.54 (s, 1 H, H-6), 8.40 (s, 1 H, H-3), 8.24, 8.08 (2 × br, 2 H, $CONH_2$); ^{13}NMR δ 169.84 (CONH), 147.11, 147.02, 145.20, 140.69 (4 × s), 132.02, 130.59 (C-3,6). Anal. ($C_7H_4ClN_3O_3$) C, H, N, Cl.

A mixture of **40** (3.70 g, 0.015 mol) and diethanolamine (3.33 g, 0.032 mol) in *p*-dioxane (100 mL) was stirred at 20 °C for 18 h, then adsorbed directly onto silica by concentration, and chromatographed. Elution with EtOAc gave foreruns, while EtOAc/MeOH (9:1) gave 2-[*N*,*N*-bis(2-hydroxyethyl)amino]-4,5-dinitrobenzamide (**41**) (3.10 g, 65%): mp (EtOAc/petroleum ether) 146−148 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.13, 7.84 (2 × br, 2 H, $CONH_2$), 7.82 (s, 1 H, H-6), 7.60 (s, 1 H, H-3), 4.87 (br, 2 H, OH), 3.60−3.20 (m, 8 H, NC*H*$_2$C*H*$_2$OH); ^{13}NMR δ 168.42 ($CONH_2$), 153.14, 145.82 (2 × s), 128.44 (C-6), 126.92, 125.55 (2 × s), 112.89 (C-3), 58.20 (CH_2OH), 54.45 (CH_2N). Anal. ($C_{11}H_{14}N_4O_7$) C, H, N.

The diol **41** was treated sequentially with MsCl/pyridine (20 °C, 15 min) and LiCl/DMF (130 °C, 15 min) as above, and the product was chromatographed on silica gel. Elution with EtOAc/petroleum ether (1:1) gave foreruns, while EtOAc/petroleum ether (2:1) gave 2-[*N*,*N*-bis(2-chloroethyl)amino]-4,5-dinitrobenzamide (**8a**) (1.08 g, 60%): mp (EtOAc/petroleum ether) 193−195 °C (yellow cubes); ^{1}H NMR [$(CD_3)_2SO$] δ 8.15, 7.94 (2 × br, 2 H, $CONH_2$), 8.06 (s, 1 H, H-6), 7.74 (s, 1 H, H-3), 3.84−3.80 (m, 8 H, CH_2CH_2Cl); ^{13}NMR δ 168.06 ($CONH_2$), 151.32, 145.55, 128.53 (3 × s), 128.08 (C-6), 126.63 (s), 112.51 (C-3), 53.05 (CH_2N), 41.15 (CH_2Cl); MS *m*/*z* (rel int) 354, 352, 350 (M^+, 1), 316,314 (19), 303, 301 (32), 285, 283 (38), 278 (54), 265 (31), 63 (90), 43 (100). Anal. ($C_{11}H_{12}Cl_2N_4O_5$) C, H, N.

A solution of **8a** (3.6 g, 0.010 mol) in 90% H_2SO_4 (100 mL) was warmed at 70 °C for 5 days, poured into crushed ice, and extracted with EtOAc. The organic layer was extracted with saturated aqueous $NaHCO_3$, and the aqueous extract was acidified with concentrated HCl, extracted into EtOAc, and worked up to give 2-[*N*,*N*-bis(2-chloroethyl)amino]-4,5-dinitrobenzoic acid (**42**) (2.78 g, 79%) as a gum, which was used directly. The neutral portion from the reaction was worked up to give starting material (**8a**) (0.80 g, 22%).

A solution of **42** (1.00 g, 2.83 mmol) and $SOCl_2$ (5 mL) in 1,2-dichloroethane (50 mL) containing DMF (1 drop) was heated under reflux for 6 h and then concentrated to dryness to give the crude acid chloride (**43**). This was dissolved in Me_2CO (20 mL) and treated with a solution of 3-aminopropane-1,2-diol (0.58 g, 6.42 mmol) in water (5 mL) cooled to 5 °C. After 15 min, the solution was partitioned between EtOAc and water, and the organic portion was worked up to give an oil

which was chromatographed on silica gel. EtOAc eluted *N*-(2,3-dihydroxypropyl)-2-[*N*,*N*-bis(2-chloroethyl)amino]-4,5-dinitrobenzamide (**8c**) (0.82 g, 68%) as a yellow oil: ^{1}H NMR [$(CD_3)_2SO$] δ 8.73 (t, J = 5.6 Hz, 1 H, CONH), 8.09 (s, 1 H, H-6), 7.72 (s, 1 H, H-3), 4.02 (br, 2 H, OH), 3.84−3.75 (m, 9 H, NCH_2CH_2Cl and CHOH), 3.42−3.35 (m, 3 H, CONHC*H*H and C*H*$_2$OH), 3.16 (m, 1 H, CONHCH*H*); ^{13}C NMR δ 166.39 (CONH), 151.21 (s), 145.62 (s), 128.70 (s), 128.42 (C-6), 125.32 (s), 112.35 (C-3), 69.91 (CHOH), 64.00 (CH_2OH), 53.12 (CH_2N), 43.05 (CONH*C*H$_2$) 39.07 (CH_2Cl); EIMS found M^+ 428.0492, 426.0523, 424.0532; $C_{14}H_{18}Cl_2N_4O_7$ requires 428.0493, 426.0523, 424.0552.

A solution of the crude acid chloride **43** (0.60 g, 1.70 mmol), prepared as above, in CH_2Cl_2 (20 mL) was reacted with *N*,*N*-dimethylethylenediamine (0.37 mL, 3.41 mmol) for 15 min. The solution was diluted with CH_2Cl_2, washed with water, and extracted with 3 N HCl. The extract was basified with concentrated NH_4OH, extracted with EtOAc and worked up to give an oil which was chromatographed on silica gel. Elution with MeOH/EtOAc (1:5) gave *N*-[2-(*N*,*N*-dimethylamino)ethyl]-2-[*N*,*N*-bis(2-chloroethyl)amino]-4,5-dinitrobenzamide (**8b**) (0.24 g, 33%) as a yellow oil. The hydrochloride salt crystallized from MeOH/Et_2O as a hygroscopic orange solid: mp 88−92 °C; ^{1}H NMR (D_2O) δ 8.27 (s, 1 H, H-6), 7.46 (s, 1 H, H-3), 7.28 (br, 1 H, CONH), 3.83−3.65 (m, 10 H, NCH_2CH_2Cl and C*H*$_2N^+Me_2$), 3.44 (br, 2 H, CONHC*H*$_2$), 2.99 (s, 6 H, N^+Me_2); ^{13}C NMR δ 170.77 (CONH), 155.50 (s), 148.43 (s), 133.06 (s), 131.67 (C-6), 128.68 (s), 116.90 (C-3), 58.45 (*C*H$_2N^+Me_2$), 55.80 (CH_2N), 45.74 (N^+Me_2), 43.96 (CH_2Cl), 37.75 (CONH*C*H$_2$); CIMS found $[M + H]^+$ 426.0944, 424.0959, 422.0999; $C_{15}H_{22}Cl_2N_5O_5$ requires 426.0939, 424.0968, 422.0998.

X-ray Crystallographic Determination of 25. Ethyl 3-[*N*,*N*-bis(2-chloroethyl)amino]-2,6-dinitrobenzoate (**25**) was crystallized from EtOAc/petroleum ether as yellow needles, mp 84−87 °C, space group $P2_1/n$; cell constants a = 13.007(2) Å, b = 7.654(4) Å, c = 16.770(3) Å, β = 98.87(1)°; z = 4; V = 1649.5(9) Å^3. Lattice constants and intensity data were measured using graphite monochromated Mo Ka radiation, λ = 0.710 69 Å, on a Nonius CAD-4 diffractometer. The data set consisted of 2581 unique reflections, of which 1956 were considered observed ($I > 3\sigma(I)$). The structure was solved using SHELXS and refined with SHELX-76.[26] All atoms including hydrogens were found from successive difference maps and allowed to refine freely. Anisotropic temperature factors for atoms other than H and isotropic temperature factors for H atoms were allowed. R and R_w were 0.0408 and 0.0436. The largest shift/esd values during the final refinement were less than 0.08, and maximum and minimum peaks in the final difference map were 0.27 and 0.32 e Å^{-3}, respectively. The nitro group at N3, the ester group, and the nitro group at N2 were rotated from the plane of the aromatic ring by 1.5, 86.4, and 48.5°, respectively.

Cell Culture Growth Inhibition Assay. Cell cultures were initiated in 96-well microtiter trays to give 200 (AA8), 300 (UV4), or 75 (EMT6) cells in 0.05 mL per well and grown for 24 h before drugs were added in culture medium (αMEM with 5% fetal bovine serum plus antibiotics).[12] After incubating under aerobic conditions for 18 h, drugs were removed by washing cultures three times with fresh medium. Cultures were incubated for a further 78 h before staining with methylene blue.[27] IC_{50}s were calculated as the drug concentration providing 50% inhibition of growth relative to the controls.

Determination of Hypoxia-Selective Cytotoxicity *in Vitro*. Cells were grown to early plateau phase in spinner flasks (AA8 cells to 10^6/mL and UV4 to 6×10^5/mL) and resuspended in fresh medium. Clonogenic assays were performed using magnetically stirred 10 mL cell suspensions (10^6/mL) after drug exposure for 1 h while continuous gassing with 5% CO2 in air or N2 as detailed elsewhere.[21,22] Both cell suspensions and drug solutions in growth medium were preequilibrated under the appropriate gas phase for 1 h prior to mixing to ensure essentially complete anoxia throughout the period of drug contact in hypoxic cultures. The ratios of the concentration for a surviving fraction of 10% (C_{10}) for the aerobic and hypoxic survival curves was used as the measure of hypoxic selectivity.

***In Vivo* Toxicity and Antitumor Activity.** Drugs were formulated in phosphate-buffered saline and injected ip at 0.01 mL/g body weight using 20−25 g male C3H/HeN mice. Toxicity was determined using groups of six non-tumor-bearing mice with 1.33-fold dose increments and an observation time of 28 days. The maximum tolerated dose (MTD) was defined as the highest dose which did not cause deaths or unacceptable morbidity. Antitumor activity was assessed using subcutaneous KHT tumors in the size range 0.5−1 g as determined by caliper measurements, with excision of tumors 18 h after treatment, pooling of tumors from two mice, and determination of clonogenic survivors by preparation of single cell suspensions and plating in agar using a modification[28] of the original method.[29] Drugs were combined with whole-body γ radiation (^{60}Co) at a dose of 15 Gy to test activity against hypoxic (radioresistant) tumor cells.

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Supporting Information Available: ORTEP diagram, X-ray bond angles, and bond lengths for compound **25** (10 pages); structure factors for compound **25** (14 pages). Ordering information is given on any current masthead page.

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