

Reductive Chemistry of the Novel Hypoxia-Selective Cytotoxin 5-[*N,N*-Bis(2-chloroethyl)amino]-2,4-dinitrobenzamide

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5-[*N,N*-Bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (**1**; SN 23862) is a novel bioreductive drug whose selective toxicity for hypoxic cells appears due to oxygen-inhibited enzymatic reduction of one of the nitro groups to the corresponding amine or hydroxylamine. Radiolytic reduction of **1** using up to four reducing equivalents in 1 N sodium formate was shown to proceed via electron addition to the 4-nitro group, thereby identifying this substituent as the most electron-affinic site in the molecule. The initially-formed 4-hydroxylamine and its *N*-hydroxytetrahydroquinoxaline half-mustard cyclization product (formed by intramolecular reaction with one arm of the adjacent mustard group) are reduced to the corresponding 4-amines upon further addition of electrons, although reduction of the 2-nitro group leading to 2,4-diamino products begins after addition of only six electron equivalents. Radiolytic reduction of the structurally similar 5-(aziridin-1-yl)-2,4-dinitrobenzamide (**2**; CB 1954) with six electron equivalents also occurs at the 4-nitro group to give the 4-hydroxylamine and 4-amine. The product mixture from reduction of **2** is less complex, largely because the corresponding 4-hydroxylamine and 4-amine are stable. The major reduction products of **1** were chemically synthesized by unequivocal routes to provide authentic samples for identification of the products of radiolytic reduction and to allow determination of their cytotoxicities. The 2- and 4-amino derivatives of **1** are significantly more cytotoxic than the parent drug, although the toxicity of the 4-amine is moderated by its facile conversion to the corresponding less toxic tetrahydroquinoxaline half-mustard. Although the 2- and 4-hydroxylamino derivatives were prepared by chemical reduction of **1**, their toxicity could not be evaluated because of their instability. The 4-hydroxylamine reacts intramolecularly with the 5-mustard group somewhat more rapidly than does the 4-amine, while the 2-hydroxylamine is converted into a 2,2'-azoxy dimer following aerial oxidation to the 2-nitroso derivative. The fully reduced 2,4-diamino derivative of **1** is 10-fold more cytotoxic again than the 2-amine and, surprisingly, does not undergo spontaneous intramolecular alkylation. This elucidation of the reduction chemistry of **1** will facilitate further investigations of the toxic products generated from this compound both by hypoxic tumor cells and by ADEPT enzymes.

Introduction

Acute and transient hypoxia resulting from inefficient vascularization is frequently found within solid tumors.¹ Although hypoxia severely limits the efficacy of radiotherapy² and some forms of chemotherapy,³ it could, in principle, be turned into an advantage through the development of nontoxic prodrugs capable of activation only in oxygen-deficient tissue. Cellular reduction of aromatic nitro compounds is mediated by a range of nitroreductases which convert the nitro group, in a series of electron transfer steps, to the hydroxylamine and ultimately the amine (Scheme 1).⁴ For one-electron nitroreductases, the first species formed is the nitro radical anion, which in the presence of oxygen is efficiently back-oxidized to the starting nitro compound. Such scavenging of the nitro radical anion is suppressed in hypoxic tissue, leading to net reduction of the nitro group. Such a nitro-to-amine conversion can greatly activate a suitably placed nitrogen mustard on the same ring,⁴ since the cytotoxicity of these is directly related to the electron density on the nitrogen atom of the mustard.⁵

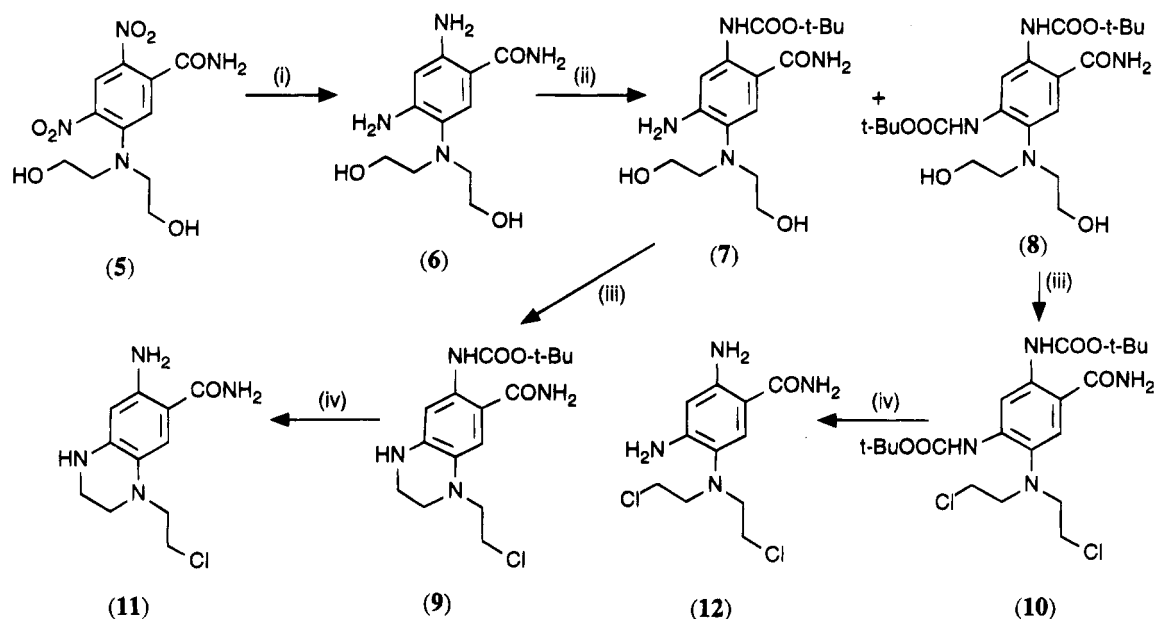
On the basis of this rationale, we recently evaluated a series of 2,4-dinitrobenzamide mustards for their hypoxia-selective cytotoxicity and found that several members of the class showed significant differential toxicity in a variety of cell lines.^{6,7} One of the more selective compounds, 5-[*N,N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (**1**; SN 23862), was 60-fold more cytotoxic to hypoxic UV4 cells than to the same cells under aerobic conditions *in vitro*. Separate DNA elution studies have shown⁷ that a more soluble analogue of **1** is activated under hypoxia to form a DNA cross-linking agent.

The mustard **1** is structurally similar to the aziridinyl-2,4-dinitrobenzamide (**2**; CB 1954), which has been shown to have exceptional activity against the rat Walker 256 carcinosarcoma^{8,9} due to efficient reduction by the obligate two-electron nitroreductase DT diaphorase present in these cells. This enzyme efficiently reduces **2** to the 4-hydroxylamine (**3**),¹⁰ which is metabolically converted to the corresponding *O*-acetate. This then behaves as a bifunctional electrophile capable of cross-linking DNA.¹¹ However, **2** has failed to show useful activity in most other experimental tumors and, despite similar structure and reduction potential to **1**, has much lower hypoxia-selective cytotoxicity than the

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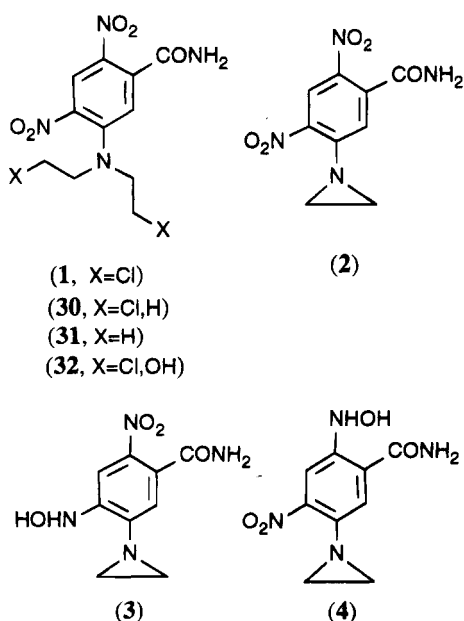
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Scheme 1. Intermediates in the Reduction of an Aromatic Nitro Group**Scheme 2^a**

^a (i) $\text{H}_2/\text{Pd}-\text{C}$; (ii) $(t\text{-BuOCO})_2\text{O}/65^\circ\text{C}$; (iii) $\text{MsCl}/\text{Et}_3\text{N}$, then $\text{LiCl}/\text{DMF}/120^\circ\text{C}$; (iv) concentrated HCl/MeOH (1:1)/ 50°C .

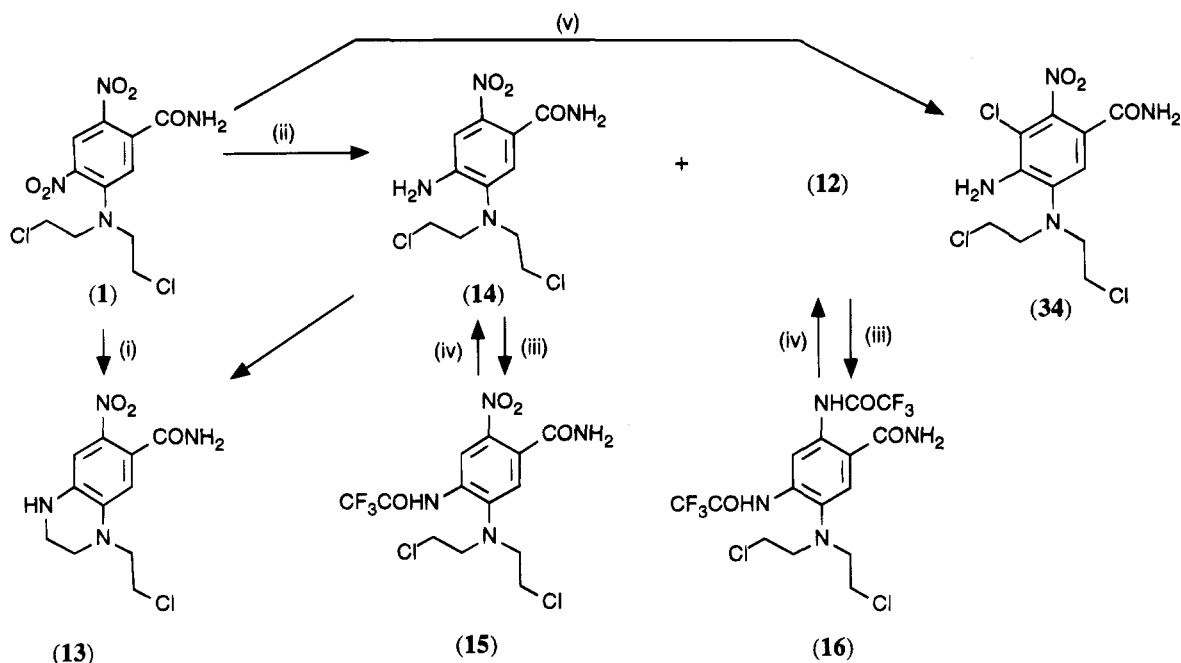
latter compound.⁶ Both **1** and **2** are good substrates for the *Escherichia coli* B nitroreductase, which is under investigation for use in Antibody Directed Enzyme Prodrug Therapy (ADEPT).¹² This enzyme is known to reduce **2** at either of the two nitro groups to produce a mixture of the corresponding 2- and 4-hydroxylamines **4** and **3**, of which the 2-hydroxylamine is significantly less toxic.¹³ In contrast, preliminary studies suggest that this enzyme reduces **1** to only a single product, tentatively identified as the 2-hydroxylamine **26**.¹⁴



In conjunction with continuing evaluation of **1** and its analogues as both hypoxia-selective cytotoxins^{6,7} and

prodrugs for use in ADEPT,¹⁴ we are examining aspects of its reductive chemistry. An understanding of these processes is important in optimizing rates of enzymatic reduction of these compounds. We report here an investigation of the reduction chemistry of **1** and **2** using radiolysis of solutions in sodium formate. Authentic samples of many of the reduction products of potential biological significance (the hydroxylamines and amines) were synthesized by unequivocal routes, and where possible, the cytotoxicities of these compounds have been determined.

Chemical Synthesis of Potential Amino- and Hydroxylamino-Reduction Products of 1. The fully reduced 2,4-diaminobenzamide mustard **12** was obtained from the dinitrodiol **5**⁶ (Scheme 2). Catalytic reduction over palladium provided the corresponding diamine **6**, which reacted with di-*tert*-butyl dicarbonate to give firstly the mono-Boc-protected product **7** and then the bis-Boc derivative **8** upon prolonged reaction. Conversion of the diol **8** to the corresponding mustard **10** was achieved by O-mesylation followed by mesylate displacement with chloride. Similar treatment of the mono-Boc derivative **7** gave the tetrahydroquinoxaline **9** as the result of intramolecular alkylation of one arm of the initially-formed mustard at the adjacent amino group. Deprotection of the Boc-amines **9** and **10** proceeded cleanly with concentrated HCl in methanol to give **11** and **12** as the corresponding hydrochloride salts. The free base of the 2,4-diaminomustard **12** was surprisingly stable with respect to formation of the ring-closed tetrahydroquinoxaline **11** (compare the instability of the 2-nitro analogue **14**, see later). Aqueous solutions of both diamines **11** and **12** at pH 7 gradually decomposed at room temperature over several days to more polar products, resulting in part from hydrolysis of the

Scheme 3^a

^a (i) Na₂S/MeOH; (ii) SnCl₂/concentrated HCl, 20 °C; (iii) (CF₃CO)₂O/CF₃COOH; (iv) concentrated HCl/MeOH (1:1)/50 °C; (v) SnCl₂/concentrated HCl, 65 °C.

mustard groups, but **11** could not be detected by HPLC during the decomposition of **12**.

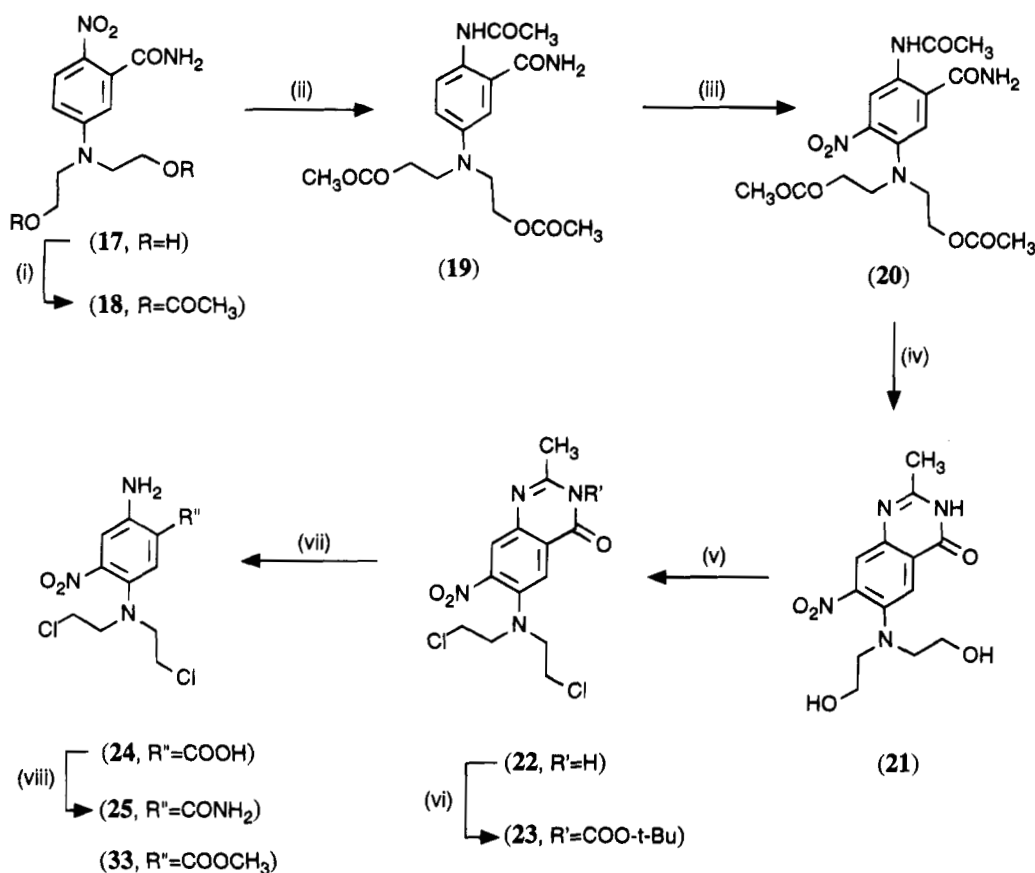
The 4-amino carboxamide mustard **14** was obtained by selective 4-nitro group reduction of **1** (Scheme 3). Treatment of **1** with Na₂S in aqueous methanol at room temperature gave predominantly the tetrahydroquinoxaline (**13**), resulting from intramolecular alkylation of the initially-formed **14**. The aziridine **2** is also selectively reduced at the 4-position by Na₂S to give the corresponding 4-amine, which is stable but can be converted into a tetrahydroquinoxaline by acid treatment.¹⁵ When **1** was reduced with 3.2 mol of SnCl₂ in concentrated HCl, the 4-amino derivative **14** was obtained as its hydrochloride salt. The neutralized mother liquor resulting from workup deposited pure crystals of the tetrahydroquinoxaline **13** upon standing. The trifluoroacetamide derivative **15** was obtained by rapid workup of the SnCl₂ reaction, followed by treatment with trifluoroacetic anhydride in trifluoroacetic acid. Small quantities of the bis-trifluoroacetamide **16** were also obtained, indicating some formation of the diamine **12**. The presence of **12** in the crude product from SnCl₂ reduction was directly confirmed by HPLC analysis, which also revealed the absence of any products arising from monoreduction of the 2-nitro group of **1** (there was a similar absence of such products in the Na₂S reduction). Removal of the trifluoroacetamide group of **15** proceeded in concentrated HCl to provide pure **14** as the hydrochloride salt. Solutions of **14** were stable at pH < ca. 3; however, neutralization resulted in clean conversion to the quinoxaline **13** with a measured half-life of ca. 1 h at 20 °C, pH 7.0 (HPLC and UV analyses).

Attempts to prepare the 2-amino derivative **25** by selective reduction of **1** with sodium dithionite (conditions reported¹⁵ to form the corresponding 2-amine from **2**, albeit in low yield) were unsuccessful. Authentic samples of the 2-amino mustard **25** were obtained from the nitro diol **17**⁶ (Scheme 4). O-acetylation (to improve solubility) followed by catalytic reduction and N-acety-

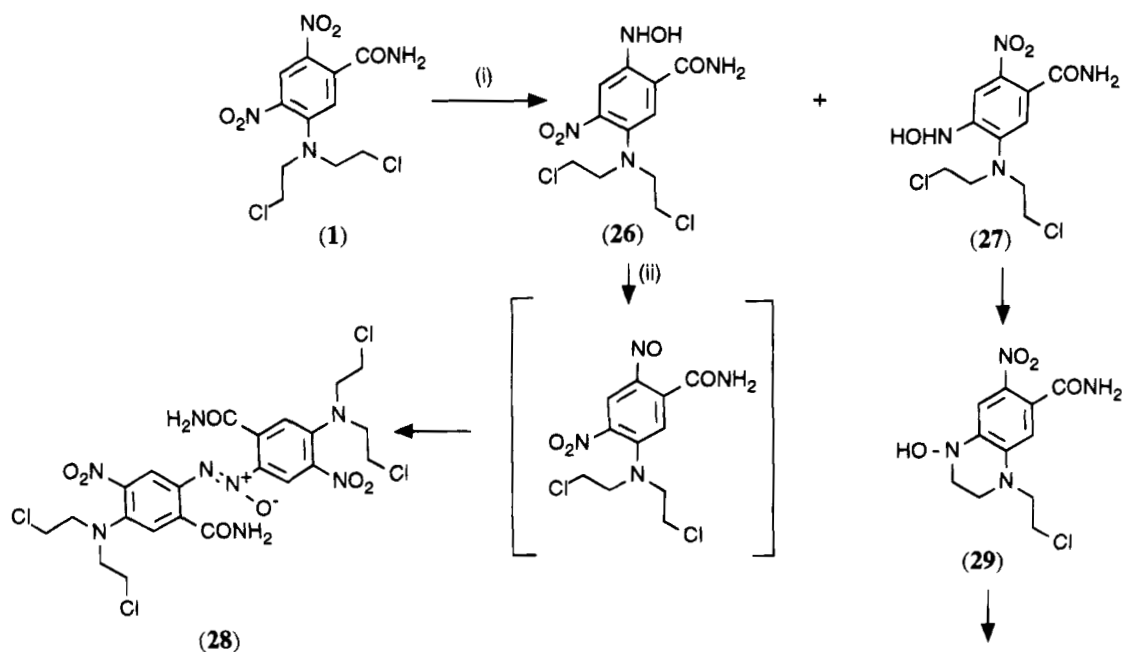
lation provided the triacetate **19** which reacted with acetyl nitrate at 0 °C to give the 4-nitro derivative **20** as the sole product. Attempts to hydrolyze the O-acetates of **20** with cesium carbonate at room temperature to give the corresponding diol were accompanied by concomitant quinoxalinone formation, giving **21** in high yield. Such reactions of O-acetamidobenzamides are well-precedented,^{16,17} although the mild conditions required in the present case are surprising. The chloro mustard **22** was readily obtained from **21** by mesylation and chloride displacement and proved to be inert to treatment with either acid or base. However, the corresponding N-Boc derivative **23** formed readily from **22** and reacted with 2 N NaOH followed by methanolic HCl to give the 4-nitroanthranilic acid **24** in excellent yield. The required benzamide **25** was obtained by treatment of an ammonia-saturated solution of **24** with diethyl cyanophosphonate. Aqueous solutions of the 2-aminobenzamide mustard **25** were stable at 20 °C, pH 7.0 (HPLC analysis).

Preparation of the 2- and 4-hydroxylamines **26** and **27** of **1** (Scheme 5) was complicated by their instabilities, which were greater than for the corresponding hydroxylamines derived from **2**. Treatment of **1** with 2 mol of zinc dust in aqueous ammonium chloride (conditions similar to those used for reduction of **2** to its 2- and 4-hydroxylamines)¹⁰ gave several products by HPLC analysis (Figure 1), as well as starting material. The mixture was separated into four fractions by column chromatography on silica. The first fraction eluted consisted of a mixture of peaks A and B, followed by pure peak C (starting material), followed by a mixture containing peaks D–F, and finally pure peak A. The mobility of peak A on silica was very dependent on the eluting solvent, with the compound often appearing more polar than expected from its HPLC retention time.

Column chromatography of an aged sample of a mixture of peaks A and B eventually provided a pure sample of peak A. It was evident from its ¹H and ¹³C

Scheme 4^a

^a (i) (CH₃CO)₂O/pyridine; (ii) H₂/Pd-C, then (CH₃CO)₂O/pyridine; (iii) CH₃COONO₂/(CH₃CO)₂O/0 °C; (iv) CsCO₃/MeOH; (v) MsCl/Et₃N, then LiCl/DMF/120 °C; (vi) (*t*-BuOCO)₂O/DMAP/Et₃N; (vii) 2 N NaOH, then concentrated HCl/MeOH/60 °C; (viii) NH₃(g)/(EtO)₂P(O)CN.

Scheme 5^a

^a (i) Zn dust/NH₄Cl; (ii) O₂.

NMR spectra that this product consisted of a dimer of the starting material, with one of the aromatic rings markedly more electron-deficient than the other, and it was identified as the 2,2'-azoxy compound 28. The FAB mass spectrum showed a weak molecular ion

cluster centred at *m/z* 655, consistent with a formula of C₂₂H₂₅Cl₄N₈O₇ and an expected¹⁸ [M + H - O]⁺ fragment at *m/z* 639. Addition of NaCl to the matrix gave a cluster at 681–675 amu corresponding to [M + Na]⁺, for which satisfactory high-resolution data were

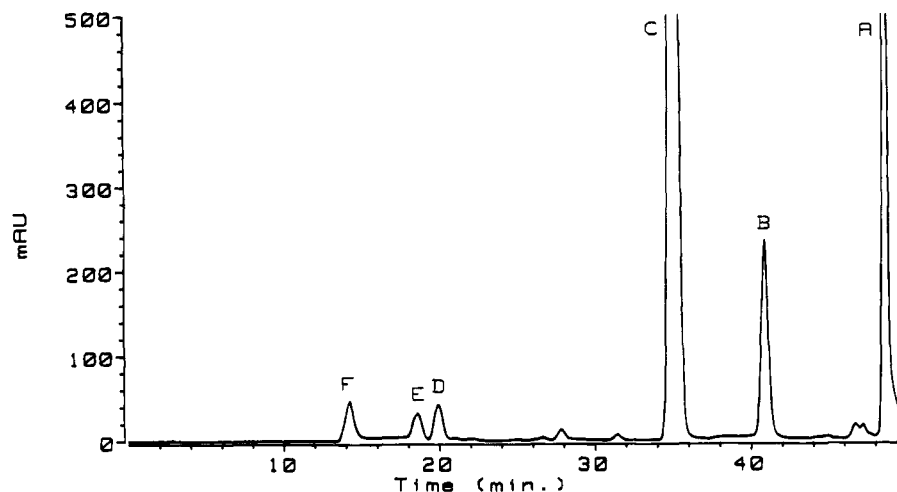


Figure 1. HPLC chromatogram of the crude product from reduction of **1** with zinc dust; UV detection is at 298 nm; see the Experimental Section for HPLC conditions.

obtained. The azoxy product **28** was reduced cleanly by CH_3SNa in refluxing propan-2-ol¹⁹ to the 2-amino compound **25**, confirming that the dimer was linked via the 2,2' (and not the 4,4' or 4,2' positions). This product (**28**) is presumably formed by facile aerial oxidation of the 2-hydroxylamine **26** to the corresponding 2-nitroso compound, followed by reaction of the two.²⁰ Knox¹² has recently reported a similar oxidation of solutions of the 4-hydroxylamine **3** derived from **2** to the 4-nitroso derivative but did not observe formation of the corresponding 4,4'-azoxy compound.

Peak B, with a UV spectrum similar to the authentic 2-amino derivative **25** of **1**, readily converted into peak A on standing in solution and is tentatively identified as the 2-hydroxylamine derivative **26**. Its apparent less polar character than **1** (peak C of Figure 1), also observed for the 2-amine **25**, probably results from intramolecular hydrogen bonding of the hydroxylamino (or amino) group with the adjacent carboxamide. This phenomenon is also observed for the 2-hydroxylamino and 2-amino derivatives of **2** (R. J. Knox, private communication). ReInjection of fractions containing pure peak B collected during the HPLC runs confirmed that conversion to peak A was occurring and that this process was slower for those fractions which had been purged with nitrogen prior to HPLC analysis.

The column chromatography fraction containing peaks D–F could not be purified further. All three peaks had UV spectra similar to those of the authentic 4-amines **13** and **14**, and are proposed to result from formation and further reactions of the 4-hydroxylamine **27**. Solutions of pure peak D collected following HPLC separation partially converted into peaks E and F upon standing (HPLC analysis). Treatment of the tetrahydroquinoxaline **13** with a carefully controlled amount of dimethyldioxirane in acetone (conditions reported to convert secondary amines into the corresponding hydroxylamines)²¹ gave a product mixture initially containing mainly peak F together with some peak E. This mixture converted into mostly peak E upon attempted purification on silica. If an excess of dimethyldioxirane was used, a complex mixture of products was obtained, consistent with the recent report that hydroxylamines are readily further oxidized to nitrones and hydroxamic acids by dimethyldioxirane.²² Taken together these results indicate an order of reaction: peak D → peak F

→ peak E and reasonably establish the identity of peak F as the N-hydroxytetrahydroquinoxaline **29**. Peak D would then be the 4-hydroxylamine precursor (**27**) of **29** and peak E a degradation product of peak F, possibly resulting from dehydration. It is unlikely that either of peaks E or F derived from the 4-hydroxylamine **27** (peak D) correspond to the 4-nitroso derivative, given their very similar UV/visible spectra and the large differences between the spectra of the 4-hydroxylamino and 4-nitroso derivatives of CB 1954 reported by Knox.¹²

Radiochemical Reduction of 5-[N,N-Bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (1). Radiolytic methods provide a particularly "clean" way of studying chemical reductions, since γ -irradiation of aqueous solutions (in the presence of a scavenger of hydroxyl radicals, such as formate) generates short-lived, powerful, one-electron reductants (e.g., e^-_{aq} and $\text{CO}_2^{\cdot-}$) in a dose-dependent manner with a precisely-defined stoichiometry.²³ Deoxygenated saturated aqueous solutions of **1** were radiochemically reduced at pH 7 with six electron equivalents, and the resulting mixture was analyzed by HPLC using diode array absorption detection. A representative chromatogram is shown in Figure 2, in this case using ^3H -labeled **1**. In similar experiments, fractions corresponding to the seven main peaks were concentrated and analyzed by mass spectrometry. Material collected from peaks H–K gave clean high-resolution molecular ions, from which structures could be clearly deduced. These structural assignments were all subsequently confirmed by direct HPLC and mass spectral comparisons with the authentic synthetic standards prepared above. Peak H corresponds to the tetrahydroquinoxaline **13**, and peak I to its nitro reduction product **11**. Peak J is the 4-amino mustard **14**, while peak K is the 2,4-diamino mustard **12**. Peaks D, G, and F did not give clean mass spectra, using a variety of ionization modes. However, peaks F and D correspond exactly in retention times and UV/visible absorption spectra to peaks F and D of the HPLC profile of the zinc reduction of **1** (Figure 1), where they were tentatively identified as the hydroxylamines **29** and **27**, respectively. Peak G remains unidentified; its UV/visible spectrum is almost superimposable upon that of peaks D and F, suggesting that it is also derived from the 4-hydroxylamine (possibly by mustard hydrolysis). Trace amounts of two additional products eluting at ca.

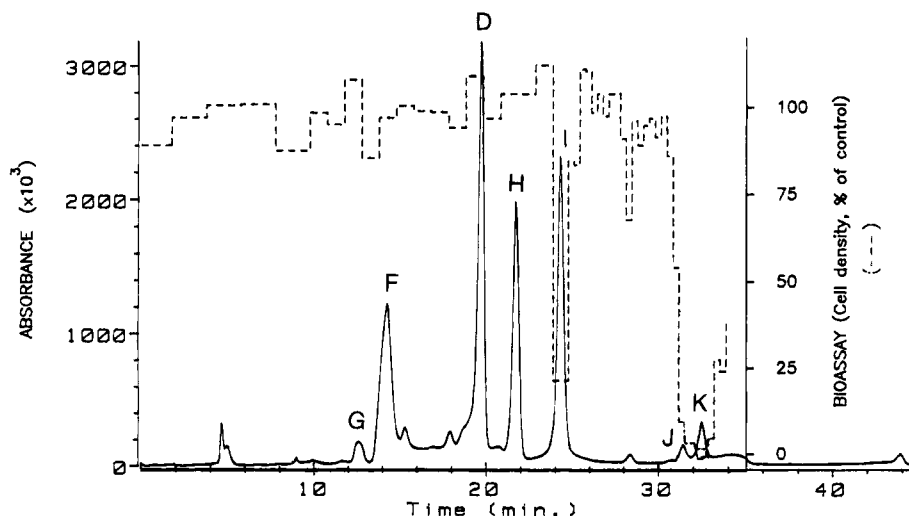


Figure 2. HPLC chromatogram of the product of radiochemical reduction of [^3H]-1 with six electron equivalents. UV detection is at 298 nm; see the Experimental Section for HPLC conditions. The bioactivity of various HPLC fractions is shown overlaid on the HPLC profile.

Table 1. HPLC Retention Times, Structural Assignments, and Characteristic UV/Visible Spectral Data for the Six-Electron Radiochemical Reduction Products of 1, Together with Values for Potential Products Not Observed in the Radiolyses

peak ^a	retention time (min) ^b	structural assignment	λ_{max} (nm) ^d
G	13.0	unknown	230, 296, 437
F	14.2	29 ^c	226, 291, 432
D	19.0	27 ^c	227, 288, 430
H	21.2	13	230, 295, 448
I	23.8	11	239, 264, 296, 352
J	30.1	14	250, 382
K	31.4	12	236, 280, 328
	35.9	1	227 (sh), 377
	41.6	26	211, 255, 347 br
	42.1	25	211, 255, 347 br
	48.8	28	245, 413
	28.2	32 ^c	239, 278, 326
	11.0	6	228, 269, 325

^a Peak designations are those of Figure 2. ^b HPLC retention time using the methanol–water gradient given in the Experimental Section. ^c Tentative assignments; see discussion. ^d Maxima determined from diode array detection of eluate: solvent is the methanol–water mix at the time of elution.

31 and 33 min from the six-electron reduction were isolated and characterized by mass spectral analysis and comparison with authentic samples as the mono- and bis-dechlorinated compounds **30** and **31**, resulting from homolytic C–Cl bond cleavage. Table 1 summarizes the HPLC retention times, UV/visible wavelength maxima, and structural assignments of the products of six-electron reduction of **1**, together with values for potential reduction products not actually observed in the radiochemical reductions.

The molar yield of each reduction product was determined by reducing [^3H]-**1** (which had been labeled in the aromatic ring) in the same manner and collecting HPLC fractions for off-line scintillation counting. The peak area/mole (at 298 and 430 nm) was calculated for each product, assuming the same molar specific activity as the starting material. This was used to determine the stoichiometry of loss of **1** and formation of reduction products from absorbance HPLC of radiochemical reductions using radiation doses up to 12 electron equivalents (Figure 3). Steady loss of **1** occurred over the addition of two to six electrons, with none remaining

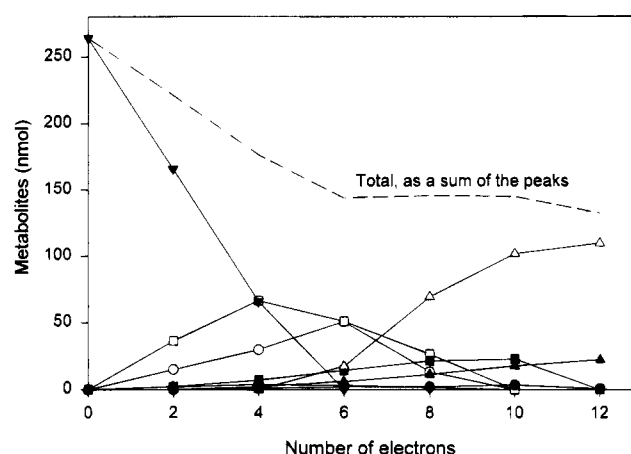


Figure 3. Distribution of the major radiolytic reduction products of **1** with numbers of added electrons. Peak designations are those in Figure 2. Symbols are: $\circ$, peak F (**29**); $\square$, peak D (**27**); $\blacksquare$, peak H (**13**); $\triangle$, peak I (**11**); $\bullet$, peak J (**14**); $\blacktriangle$, peak K (**12**); $\blacktriangledown$, SN 23862 (**1**).

after the six-electron reduction. The amount of 4-hydroxylamine **27** peaked after the addition of four electrons, and its cyclization product **29** after six electrons. An estimate of the rate of cyclization of the 4-hydroxylamine **27** to give **29** was obtained by treating **1** with six electrons, then cooling the solution on ice, and analyzing the stability of the major reduction products with time (Figure 4). The decay of the 4-hydroxylamine **27** is closely matched by the build up of the ring-closed product **29**; kinetic analysis of the data in Figure 4 provides a half-life of 131 min for loss of hydroxylamine **27** and 138 min for production of **29**. These values compare with a half-life of ca. 1 h at 20 °C measured for cyclization of the corresponding 4-amine **14** (see earlier) and suggest that at equivalent temperatures the hydroxylamine **27** would cyclize somewhat faster than the amine **14**, an observation consistent with the greater nucleophilicity (in general) of hydroxylamines compared with their corresponding amines.²⁴ The apparent maximum production of **29** after addition of six electron equivalents (and later than that of the 4-hydroxylamine **27**) is presumably due to the time delay in its formation by ring closure, rather than from a requirement for six-electron reduction. At no stage

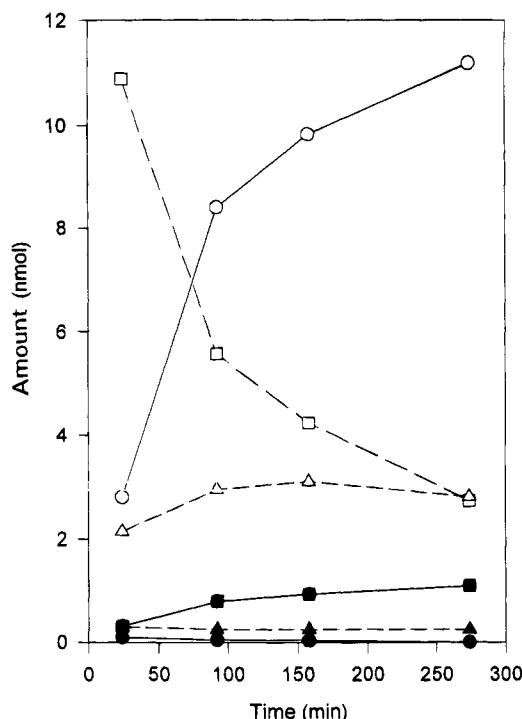


Figure 4. Variation of the major reduction products of **1** with time following six-electron reduction. The radiolysis solution was stored at 0 °C before HPLC analysis over 5 h. Peak designations are the same as those in Figure 2 (symbols as for Figure 3).

did substantial quantities of the six-electron reduction products **13** and **14** build up over the 2–12 electron reductions. Instead, there was a steady build up of 12-electron reduction products (the ring-closed diamine **11** and to a lesser extent the 2,4-diamine **12**) during the addition of 6–12 electrons. The total molar amount of material contained in the seven main peaks of the HPLC chromatogram dropped steadily over the two to six electron range (to ca. 57% of the total injected) and then stabilized. This is due primarily to the formation of many minor products during the two- to six-electron reductions which are not included in Figure 3, followed by a more direct interconversion of the major reduction products between 6 and 12 electrons. Consistent with this, the total amount of radioactivity collected from *all* HPLC fractions in the six-electron reduction of ³H-labeled **1** was 97% of that applied to the column, of which ca. 63% was contained in the seven main peaks featured in Figure 3.

It is clear from these results that the 4-nitro group of **1** is the one initially reduced and must therefore be the most electron-affinic site in the molecule. No products derived from selective monoreduction of the 2-nitro group of **1** were detected in the radiolyses. Progressive addition of electrons gives initially the 4-hydroxylamine **27**, which is capable of cyclization to the moderately stable **29**. The total amount of hydroxylamines **27** and **29** falls rapidly after the addition of six electrons, presumably as a result of reduction to their respective amines **14** and **13**. Further quantities of the tetrahydroquinoxaline **13** must then result from cyclization of the 4-amine **14**. Since **12** does not convert to **11** over the time course of the experiments (see earlier), the latter compound must result from direct reduction of **13**. Most of the initial products of the four- and six-electron reductions (e.g., **27** and **14**) will be later

converted to the tetrahydroquinoxalines **29** and **13**, which explains the low levels of **14** detected at any stage during the reductions, and the greater amount of **11** compared with **12** found in the final product of the 12-electron reduction. Scheme 6 summarizes the sequence of reductions occurring over the 2–12 electron equivalent reduction of **1**.

The build up of the 12-electron reduction product **11**, beginning after the addition of only six reducing equivalents, and without appreciable accumulation of its theoretical six-electron precursor **13** is surprising and suggests that **13** is reduced to **11** at a rate comparable to its formation by reduction of the hydroxylamines **27** and/or **29**. There are no 12-electron reduction products observed from six-electron reduction of the related aziridine **2** (see later), suggesting that the above results stem from an unexpectedly high electron affinity for the tetrahydroquinoxaline **13**.

Radiochemical Reduction of 5-Aziridin-1-yl-2,4-dinitrobenzamide (2). An aqueous solution of **2** was reduced with six electron equivalents, and the products were analyzed by HPLC as above (Figure 5). There was a clean conversion to one major product (peak L), identified as the 4-hydroxylamine **3** by direct comparison with an authentic sample.¹⁷ A small peak running at 10.9 min (peak M) corresponded to an authentic sample of the 4-amine.¹² This result is similar to that seen in the six-electron reduction of **1**. In both cases there is no starting material present after six-electron reduction, and both reductions result in product mixtures containing predominantly compounds at the 4-hydroxylamine oxidation state and no products resulting from monoreduction of the 2-nitro group. However, in the case of **1** there are also significant quantities of the 12-electron reduction product **11** and its six-electron precursor **13** at this stage. The more complex product mixture formed in by the reduction of **1** obviously results from the ability of several of its initially-formed reduction products to undergo intramolecular cyclization over the timescale of the radiolyses and HPLC analyses, thereby providing intermediates with increased electron affinity.

Cytotoxicities of the Reduction Products of **1**.

A preliminary estimate of the cytotoxicities of the major radiolytic reduction products of **1** was obtained by bioassay of fractions obtained following radiolytic reduction of the ³H-labeled compound with six electron equivalents and subsequent HPLC separation. The amount of material in each fraction was quantified radiochemically, and suitable dilutions of the eluate were evaluated in a growth inhibition assay over 4 days, using UV4 cells (Figure 2). High cytotoxicity is associated with the peaks eluting at 30–34 min and is centered at peak K, corresponding to the 2,4-diamine **12** and to a lesser extent with peak I (corresponding to the ring-closed diamine **11**). The tetrahydroquinoxaline **13** (peak H), and the hydroxylamines **27** and **29** (peaks D and F) do not appear to be appreciably toxic, although much of the 4-hydroxylamine **27** would have been converted into the ring-closed **29** during the course of the drug exposure.

There is a low level of cytotoxicity associated with a minor peak running at 28 min. This product has a similar absorption spectrum to that of the 2,4-diamino diol **6** (*t_R* = 11 min) and is also seen in aged, aqueous

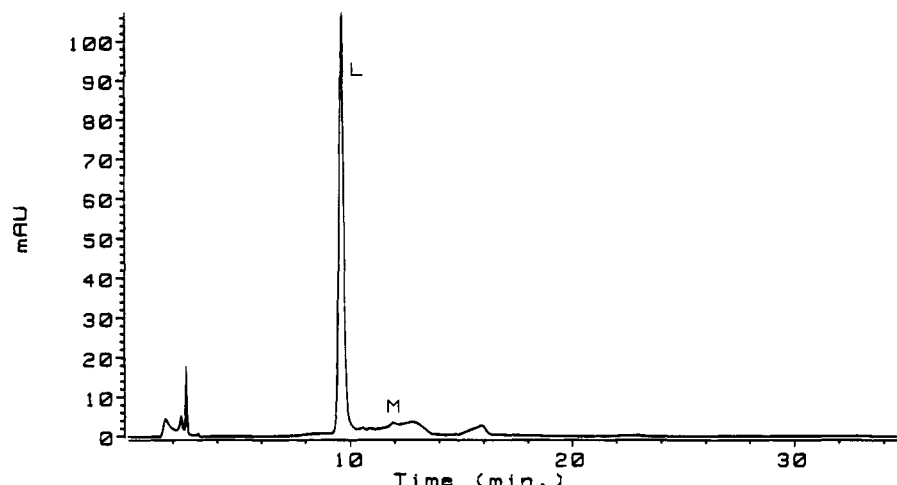
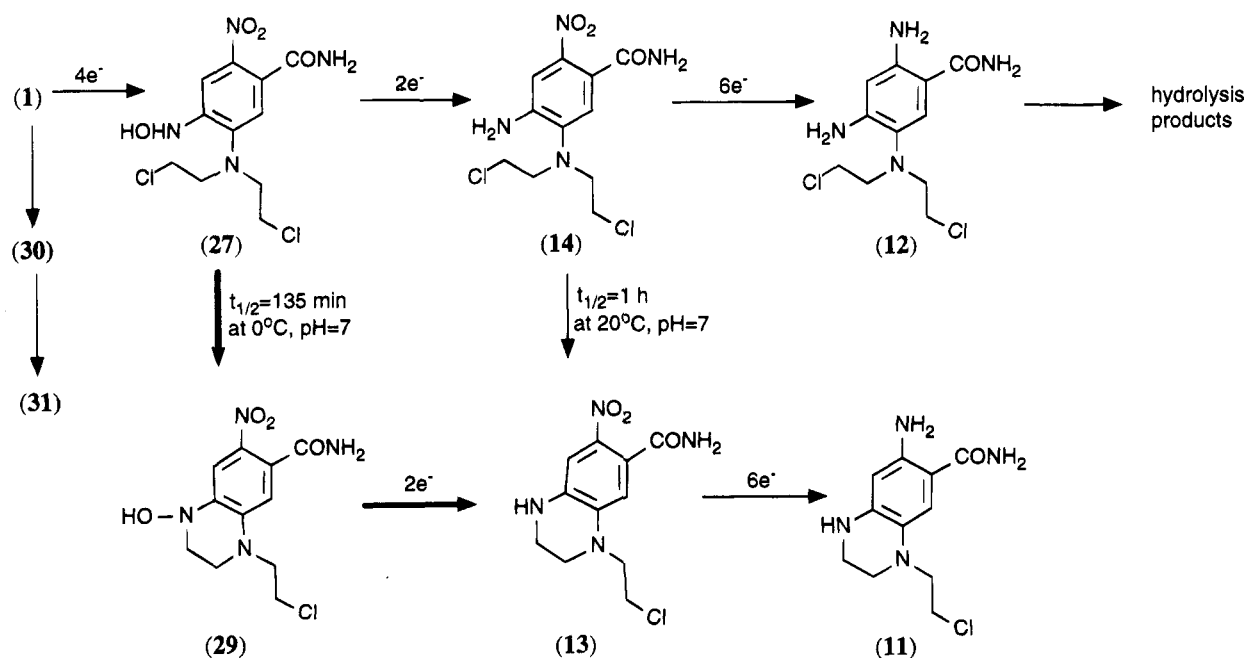


Figure 5. HPLC chromatogram of the product of radiochemical reduction of **2** with six electron equivalents; UV detection is at 298 nm.

Scheme 6. Sequence of Product Formation from Radiolytic Reduction of **1** with Increasing Numbers of Electrons (2–12)



solutions of the 2,4-diamino mustard **12**. This material is most likely the chloro alcohol **32**, resulting from partial hydrolysis of **12**. The order of cytotoxic potency, determined from the observed activity relative to the amount of material in each peak, is **12** $\gg$ **32** $>$ **11**.

The cytotoxicities of many of the synthetic standards were determined in a growth inhibition assay against aerobic cultures of AA8 and UV4 cells using a 4 h drug exposure (Table 2). The UV4 mutant subline of AA8 is defective in the repair of DNA interstrand cross-links, and therefore DNA cross-linking agents show increased cytotoxicity to UV4 cells compared with the parent line.²⁵ Such agents typically display large hypersensitivity factors [HF; ratio IC_{50} (AA8)/ IC_{50} (UV4)] (8–200-fold) to DNA alkylating agents.²⁵ The parent mustard **1** shows low cytotoxicity in both cell lines, and the HF value of close to unity suggests that cell killing by this compound under aerobic conditions does not result from DNA alkylation, as must also be the case for the diol **6**. Reduction of the 2-nitro group of **1** to give the 2-amine **25** results in a 160-fold increase in cytotoxicity in the

Table 2. Cytotoxicity of **1** and Selected Reduction Products (4 h Exposure)

compd	$IC_{50}(\mu M)$		HF ^a
	AA8	UV4	
1	1600 ± 50^b	960 ± 60	1.6 ± 0.1
6	2300 ± 900	3000 ± 900	0.73 ± 0.10
11	6.9 ± 0.8	0.87 ± 0.10	8.0 ± 0.4
12	1.0 ± 0.1	0.029 ± 0.005	36 ± 3
13	730 ± 40	49 ± 1	15 ± 1
14	180 ± 10	12 ± 2	18 ± 6
25	9.8 ± 0.2	0.22 ± 0.01	46 ± 1

^a Hypersensitivity factor: ratio IC_{50} (AA8)/ IC_{50} (UV4). Values are intraexperiment ratios. ^b Errors are $\pm$ SEM.

AA8 line and a 4400-fold increase in the UV4 line. The corresponding HF of ca. 46 for **25** is consistent with the mechanism of cytotoxicity now being DNA alkylation. Monoreduction of the 4-nitro group to give the unstable 4-amine **14** leads to an 83-fold increase in potency in the UV4 line and an 8-fold increase in AA8 line (although these results must represent minimum values for cytotoxicity, since **14** will substantially convert to

the less toxic **13** during the drug exposure). This presumably also explains the moderate hypersensitivity factor observed.

Reduction of both nitro groups of **1** to give the diamine **12** leads to an increase in cytotoxicity by a further order of magnitude, consistent with the observations from bioassay of the radiolysis mixture (see above), and as expected, this mustard shows a high HF value. There is an approximately 10-fold loss in potency resulting from conversion of the mustard group of either **14** or **12** into the corresponding tetrahydroquinoxaline half mustards **13** and **11**, together with a reduction in their HF values, although the 2-amino half mustard **11** still retains appreciable cytotoxicity, consistent with the results of the bioassay shown in Figure 2.

Conclusions

The unequivocal synthesis of many of the observed products of the radiolytic reduction of **1** has allowed an understanding of this process (Scheme 6). Initial reduction always occurs via the 4-nitro group to give the 4-hydroxylamine **27**, which then forms the 6-nitroquinoxaline **13** by two pathways: ring closure and reduction of the *N*-hydroxytetrahydroquinoxaline **29** or reduction to the amine **14** followed by cyclization. Reduction of the remaining nitro groups of both **13** and **14** can then occur to give **11** and **12**, respectively; once formed, **12** appears not to cyclize to **11**. Overall, the radiolytic reduction of **1** is more complex than that of the corresponding aziridine **2** because of the above ability of the 4-amine and hydroxylamine products to undergo cyclization.

The (synthetic) 2-amino derivative **25** appears to be more cytotoxic than the isomeric 4-amino compound **14**, but the latter does undergo ring closure to the much less potent tetrahydroquinoxaline **13** sufficiently rapidly for this to be a factor ($t_{1/2} = 1$ h at 20 °C; equivalent to ca. 30 min at 37 °C). The fact that the 2,4-diamino derivative **12** does not deactivate in this manner probably contributes to its greater cytotoxicity.

The tetrahydroquinoxaline **13** has been identified as the major stable metabolite of **1** in hypoxic AA8 cultures.²⁶ However, the possibility that small amounts of more cytotoxic species such as **25** and **12** are also generated by hypoxic metabolism is currently under investigation. The understanding of the reduction chemistry of **1** provided by the present study, and the availability of authentic samples of its potential reductive metabolites, will facilitate the further investigation of the toxic products generated from this compound both by hypoxic tumor cells and by ADEPT enzymes.

Experimental Section

Chemistry. Melting points are uncorrected. Column chromatography was carried out by the method of Still²⁷ on 230–400 mesh silica gel. Petroleum ether refers to the fraction boiling at 40–60 °C. Tetrahydrofuran was distilled from a blue solution containing sodium/benzophenone immediately before use. ¹H and ¹³C NMR spectra were recorded at 400 and 100 MHz, respectively, while positive-ion desorption electron-impact mass spectra (*m/z*) were obtained using an ionization potential of 70 eV. The FAB mass spectrum of the azoxy compound **28** was obtained from a 3-nitrobenzyl alcohol matrix. The abundances listed for halogen-containing mass fragments refer to the most intense peak of the isotope cluster. Only major bands in the FTIR spectra ($\nu_{\max}$) are listed. Spectrophotometric determinations of cyclization kinetics were

obtained using a Hewlett-Packard model 8452A diode array spectrophotometer and were analyzed using the supplied kinetics software. Compounds judged to be identical by HPLC analysis had identical retention times and UV/visible spectra.

5-[*N,N*-Bis(2-hydroxyethyl)amino]-2,4-diaminobenzamide (6**) and Its *N*-Boc Derivatives.** A solution of 5-[*N,N*-bis(2-hydroxyethyl)amino]-2,4-dinitrobenzamide (**5**)⁶ (1.00 g, 3.18 mmol) in 1:1 MeOH/EtOAc (60 mL) containing 5% Pd–C (0.10 g) was shaken under an atmosphere of H₂ at 60 psi for 2 h. After removal of the catalyst by filtration through Celite, the solution was concentrated under reduced pressure to give 5-[*N,N*-bis(2-hydroxyethyl)amino]-2,4-diaminobenzamide (**6**) (0.80 g, 99%) as an unstable oil which rapidly colorized and was used directly: mass found M^+ 254.1382, C₁₁H₁₈N₄O₃ requires M^+ 254.1379; IR $\nu_{\max}$ (film/cm⁻¹) 3345 br, 1626, 1541, 1400, 1275, 1061; ¹H NMR [(CD₃)₂SO] δ 7.31 (s, 1 H, H-6), 6.37, 5.40 (2 $\times$ br, each 1 H, CONH₂), 5.82 (s, 1 H, H-3), 4.43 (br, 2 H, OH), 3.44 (br, 4 H, NH₂), 3.34 (br t, *J* = 6.0 Hz, 4 H, CH₂OH), 2.89 (t, *J* = 6.0 Hz, 4 H, CH₂N); ¹³C NMR δ 171.15 (CONH₂), 149.62, 149.14 (C-2,5), 126.09 (C-4), 124.27 (C-6), 102.53 (C-1), 98.67 (C-3), 58.87, 57.65 (NCH₂CH₂OH); MS *m/z* (rel int) 254 (M^+ , 65), 223 (100), 177 (32).

A solution of **6** (1.00 g, 3.93 mmol) and di-*tert*-butyl dicarbonate (1.28 g, 5.89 mmol) in THF (15 mL) was warmed under N₂ at 50 °C for 30 h. The solution was concentrated under reduced pressure onto silica gel and chromatographed. Elution with EtOAc gave unreacted di-*tert*-butyl dicarbonate (0.05 g), while MeOH/EtOAc (1:19) gave a 85:15 mixture (¹H NMR analysis) of the mono- and di-*N*-(*tert*-butoxycarbonyl)amine derivatives **7** and **8** as a foam (1.21 g). Crystallization first from EtOAc at –30 °C and then from Me₂CO gave 4-amino-5-[*N,N*-bis(2-hydroxyethyl)amino]-2-[*N*-(*tert*-butoxycarbonyl)amino]benzamide (**7**) (0.67 g, 48%): mp 192–194 °C; IR $\nu_{\max}$ (KBr/cm⁻¹) 3482, 3258, 1707, 1660, 1539, 1474, 1406, 1395, 1238, 1157, 1069, 1041, 1020; ¹H NMR [(CD₃)₂SO] δ 8.67 (s, 1 H, NHCO-*t*-Bu), 7.55, 6.82 (2 $\times$ br, 2 H, CONH₂), 7.49, 7.33 (2 s, each 1 H, H-3,6), 6.63 (s, 2 H, NH₂), 4.55 (t, *J* = 4.7 Hz, 2 H, OH), 3.27 (dd, *J* = 6.0, 4.7 Hz, 4 H, CH₂O), 2.90 (d, *J* = 6.0 Hz, 4 H, CH₂N), 1.45 (s, 9 H, C-CH₃); ¹³C NMR δ 170.70 (CONH₂), 152.15 (NHCOO), 148.87 (C-5), 140.14 (C-2), 127.16 (C-4), 124.23 (C-6), 107.08 (C-1), 103.50 (C-3), 79.06 (C(CH₃)₃), 58.51, 58.12 (NCH₂CH₂OH), 27.95 (C(CH₃)₃); MS *m/z* (rel int) 354 (M^+ , 46), 323 (17), 298 (20), 267 (100), 223 (37), 206 (21), 177 (22). Anal. (C₁₆H₂₆N₄O₅·0.25H₂O) C, H, N.

Repetition of the above reaction for a total of 72 h in 1:1 THF/dioxane with the further addition of 1 equiv of di-*tert*-butyl dicarbonate at 24 h and again at 48 h gave, after chromatography on silica and elution with MeOH/EtOAc (1:19), 5-[*N,N*-bis(2-hydroxyethyl)amino]-2,4-[*N,N'*-bis(*tert*-butoxycarbonyl)amino]benzamide (**8**) (87%) as a gum: mp ca. 40 °C; mass found M^+ 454.2427, C₂₁H₃₄N₄O₇ requires M^+ 454.2427; IR $\nu_{\max}$ (KBr/cm⁻¹) 3447, 3349, 1728, 1716, 1657, 1589, 1532, 1483, 1393, 1369, 1159, 1053; ¹H NMR [(CD₃)₂SO] δ 11.33 (s, 1 H, 2-NHCOO), 8.91 (s, 1 H, 4-NHCOO), 8.90 (s, 1 H, H-6), 8.13, 7.47 (2 $\times$ br, each 1 H, CONH₂), 7.72 (s, 1 H, H-3), 4.64 (d, *J* = 5.0 Hz, 2 H, OH), 3.32 (dd, *J* = 5.8, 5.0 Hz, 4 H, CH₂O), 2.97 (t, *J* = 5.8 Hz, 4 H, CH₂N), 1.48 (s, 18 H, C(CH₃)₃); ¹³C NMR δ 170.44 (CONH₂), 152.07, 151.95 (NHCOO), 140.07, 138.46 (C-2,5), 132.39 (C-4), 124.07 (C-6), 111.44 (C-1), 106.88 (C-3), 79.41, 79.25 (C(CH₃)₃), 58.42, 57.34 (NCH₂CH₂OH), 27.93 (C(CH₃)₃); MS *m/z* (rel int) 454 (M^+ , 2), 410 (5), 354 (32), 267 (56), 254 (54), 223 (100), 206 (30), 177 (39).

5-[*N,N*-Bis(2-chloroethyl)amino]-2,4-[*N,N'*-bis(*tert*-butoxycarbonyl)amino]benzamide (10**).** Methanesulfonyl chloride (MsCl) (0.37 mL, 4.84 mmol) was added dropwise to a solution of **8** (1.00 g, 2.20 mmol) and Et₃N (0.77 mL, 5.50 mmol) in dry THF (30 mL). After 15 min the solution was partitioned between EtOAc, and water and the organic portion was worked up to give an oil which was dissolved in DMF (50 mL). LiCl (5 g) was added, and the mixture was stirred at 120 °C for 15 min, concentrated under reduced pressure to remove DMF, and the residue partitioned between EtOAc and water. The organic portion was worked up to give an oil which was chromatographed on silica gel. Elution with EtOAc/petroleum ether (1:1) gave the 5-[*N,N*-bis(2-chloroethyl)amino]-2,4-[*N,N'*-bis(*tert*-butoxycarbonyl)amino]benzamide (**10**) (0.87

g, 47%): mp (EtOAc/petroleum ether) 128–130 °C; IR $\nu_{\max}$ (KBr/cm⁻¹) 3459, 3343, 3229, 1725, 1717, 1663, 1653, 1587, 1532, 1481, 1393, 1369, 1153; ¹H NMR (CDCl₃) δ 10.30 (s, 1 H, NHCOO), 9.14 (s, 1 H, NHCOO), 8.32 (s, 1 H, H-6), 7.33 (s, 1 H, H-3), 5.74 (br, 2 H, CONH₂), 3.45 (t, J = 6.2 Hz, 4 H, CH₂Cl), 3.28 (t, J = 6.2 Hz, 4 H, CH₂N), 1.52, 1.51 (2 $\times$ s, 18 H, C(CH₃)₃); ¹³C NMR δ 170.49 (CONH₂), 152.78, 152.27 (NHCOO), 142.21, 140.93 (C-2,5), 129.06 (C-4), 123.77 (C-6), 111.84 (C-1), 108.20 (C-3), 80.89, 80.49 (C(CH₃)₂), 57.80 (CH₂N), 41.75 (CH₂Cl), 28.31 (C(CH₃)₃); MS m/z (rel int) 494, 492, 490 (M⁺, 9), 394, 392, 390 (12), 336, 334 (20), 324 (25), 311 (50), 231 (55), 203 (32), 57 (100). Anal. (C₂₁H₃₂Cl₂N₄O₅) C, H, N.

6-[(*tert*-Butoxycarbonyl)amino]-1-(2-chloroethyl)-1,2,3,4-tetrahydroquinoxaline-7-carboxamide (9). Treatment of the diol 7 with MsCl and Et₃N followed by LiCl, as described above, gave an oil which was chromatographed on silica gel. Elution with EtOAc/petroleum ether (2:3) gave 6-[(*tert*-butoxycarbonyl)amino]-1-(2-chloroethyl)-1,2,3,4-tetrahydroquinoxaline-7-carboxamide (9) (91%): mp (EtOAc/petroleum ether) 152–154 °C; IR $\nu_{\max}$ (KBr/cm⁻¹) 3401, 3368, 3212, 1701, 1653, 1620, 1587, 1533, 1456, 1368, 1273, 1159; ¹H NMR (CDCl₃) δ 10.40 (s, 1 H, NHCOO), 7.54 (s, 1 H, H-6), 6.61 (s, 1 H, H-3), 5.63 (br, 2 H, CONH₂), 4.23 (s, 1 H, NH), 3.67 (t, J = 6.7 Hz, 2 H, CH₂Cl), 3.55 (t, J = 6.7 Hz, 2 H, CH₂N), 3.43, 3.32 (2 $\times$ m, 4 H, NHCH₂CH₂N), 1.49 (s, 9 H, C(CH₃)₃); ¹³C NMR δ 171.56 (CONH₂), 153.48 (NHCOO), 139.64 (C-5), 135.05 (C-2), 127.37 (C-4), 110.46 (C-6), 107.18 (C-1), 103.98 (C-3), 79.76 (C(CH₃)₃), 53.91 (CH₂N), 47.69 (CH₂N), 40.44, 40.33 (CH₂N and CH₂Cl), 28.40 (C(CH₃)₃); MS m/z (rel int) 356, 354 (M⁺, 25), 300, 298 (19), 281 (19), 256, 254 (62), 231 (55), 205 (72), 41 (100). Anal. (C₁₆H₂₃ClN₄O₃) C, H, N, Cl.

1-(2-Chloroethyl)-6-nitro-1,2,3,4-tetrahydroquinoxaline-7-carboxamide (13). (a) A solution of Na₂S₂O₈ (0.86 g, 3.59 mmol) in water (5 mL), was added to a solution of 15 (0.30 g, 0.85 mmol) in MeOH (15 mL) and the mixture was stirred at room temperature for 24 h. The red precipitate was filtered off, washed with water, dried, and dissolved in MeOH saturated with HCl (10 mL). Addition of EtOAc (10 mL) gave a red solid which was redissolved in water (10 mL). On standing, red needles of 1-(2-chloroethyl)-6-nitro-1,2,3,4-tetrahydroquinoxaline-7-carboxamide (13) (0.17 g, 70%) were deposited: mp >260 °C dec; IR $\nu_{\max}$ (KBr/cm⁻¹) 3383, 3175, 1655, 1580, 1539, 1491, 1310, 1290, 1233; ¹H NMR [(CD₃)₂SO] δ 7.69, 7.26 (2 $\times$ br s, 2 H, CONH₂), 7.12 (s, 1 H, H-6), 6.47 (s, 1 H, H-3), 6.27 (br s, 1 H, NH), 3.84 (t, J = 6.7 Hz, 2 H, CH₂Cl), 3.74 (t, J = 6.7 Hz, 2 H, CH₂N), 3.50 (t, J = 4.3 Hz, 2 H, CH₂N), 3.27 (t, J = 4.3 Hz, 2 H, CH₂N); ¹³C NMR δ 168.63 (CONH), 137.52, 135.35, 134.36 (C-2,4,5), 125.23 (C-1), 108.17, 107.14 (C-3,6), 51.75, 47.99 (CH₂N), 40.84 (CH₂Cl), 38.47 (CH₂N); MS m/z (rel int) 286, 284 (M⁺, 47), 235 (100), 146 (46), 89 (19), 45 (42). Anal. (C₁₁H₁₃ClN₄O₃) C, H, N.

(b) A solution of 15 (0.10 g, 0.24 mmol) in 1:1 MeOH/concentrated HCl (5 mL) was warmed at 70 °C for 45 min. The solution was basified with concentrated ammonia and allowed to stand at room temperature for 72 h, during which time red needles of 13 (0.061 g, 89%) were deposited.

5-[*N,N*-Bis(2-chloroethyl)amino]-2-nitro-4-(trifluoroacetamido)benzamide (15). A solution of 1 (1.83 g, 5.21 mmol) and SnCl₂·2H₂O (4.00 g, 17.72 mmol) in concentrated HCl (60 mL) was stirred at room temperature for 18 h and poured into a mixture of concentrated ammonia (100 mL) and crushed ice. The mixture was extracted rapidly with ice-cold CH₂Cl₂ (25 mL), and the extract was washed with cold water and dried (Na₂SO₄). Trifluoroacetic acid (2.5 mL, 32.4 mmol) and trifluoroacetic anhydride (2.5 mL, 17.7 mmol) were added, and the solution was gently heated under reflux for 30 min and then concentrated to dryness under reduced pressure, and the residue was chromatographed on silica gel. Elution with EtOAc gave 5-[*N,N*-bis(2-chloroethyl)amino]-2,4-bis(trifluoroacetamido)benzamide (16) (0.08 g, 3%): mp (CHCl₃/petroleum ether) 247–250 °C; IR $\nu_{\max}$ (KBr/cm⁻¹) 3439, 3356, 3231, 1653, 1605, 1543, 1398, 1375; ¹H NMR [(CD₃)₂CO] δ 13.77 (s, 1 H, 2-NHCOCF₃), 10.33 (s, 1 H, 4-NHCOCF₃), 9.71 (s, 1 H, H-6), 8.28 (s, 1 H, H-3), 8.12, 7.34 (2 $\times$ br, 2 H, CONH₂), 3.66 (t, J = 5.5 Hz, 4 H, CH₂Cl), 3.50 (t, J = 5.5 Hz, 4 H, CH₂N); ¹³C

NMR δ 171.18 (CONH₂), 155.54 (J 37.8, COCF₃), 139.46 (C-5), 138.55 (C-2), 135.81 (C-4), 127.17 (C-6), 117.56 (C-1), 116.80 (J 288.3, COCF₃), 116.60 (J 288.0, COCF₃), 58.30 (CH₂N), 42.95 (CH₂Cl); MS m/z (rel int) 486, 484, 482 (M⁺, 18), 468, 466, 462 (12), 450, 448, 446 (15), 435, 433 (96), 417, 415 (100), 397 (16), 379 (26), 354 (15), 69 (40), 63 (77). Anal. (C₁₅H₁₄Cl₂F₆N₄O₃) C, H, N.

Elution with MeOH/EtOAc (1:19) gave 5-[*N,N*-bis(2-chloroethyl)amino]-2-nitro-4-(trifluoroacetamido)benzamide (15) (0.43 g, 20%): mp (EtOAc/petroleum ether) 158–159 °C; IR $\nu_{\max}$ (KBr/cm⁻¹) 3397, 3252, 3187, 1719, 1680, 1541, 1165; ¹H NMR [(CD₃)₂CO] δ 10.22 (br, 1 H, NHCOCF₃), 8.87 (s, 1 H, H-6), 7.91 (s, 1 H, H-3), 7.69, 7.12 (2 $\times$ br, 2 H, CONH₂), 3.72 (t, J = 5.8 Hz, 4 H, CH₂Cl), 3.58 (t, J = 5.8 Hz, 4 H, CH₂N); ¹³C NMR δ 171.14 (CONH₂), 155.78 (J = 38.8 Hz, COCF₃), 146.15 (C-5), 144.47 (C-4), 136.06 (C-2), 131.27 (C-1), 127.02 (C-6), 116.93 (C-3), 116.50 (J = 288.0 Hz, COCF₃), 57.19 (CH₂N), 42.84 (CH₂Cl); MS m/z (rel int) 420, 418, 416 (M⁺, 8), 382, 380 (27), 369, 367 (100), 331 (71), 242 (30), 69 (18), 63 (36). Anal. (C₁₃H₁₃Cl₂F₃N₄O₄) C, H, N.

If the SnCl₂ reduction of 1 was carried out as above, but at elevated temperatures, significant quantities of the stable, ring-chlorinated reduction product, 4-amino-5-[*N,N*-bis(2-chloroethyl)amino]-3-chloro-2-nitrobenzamide (34), mp 154 °C, were isolated: ¹H NMR (CD₃CO) δ 7.42 (s, 1 H, H-6), 6.70, 6.00 (2 $\times$ br, 2 H, CONH₂), 5.52 (br s, 2 H, NH₂), 3.59 (t, J = 6.2 Hz, 4 H, CH₂Cl), 3.36 (t, J = 6.2 Hz, 4 H, CH₂N); ¹³C NMR δ 166.17 (CONH), 147.02, 136.29, 124.52 (C-6), 118.97, 116.49, 110.57, 56.02 (CH₂N), 43.16 (CH₂Cl); MS m/z (rel int) 358, 357, 356, 355, 354 (M⁺, 10), 322, 320, 318 (42), 307, 305, 303 (48), 271, 269 (100), 182, 180 (35), 36 (50). Anal. (C₁₁H₁₃Cl₃N₄O₃) C, H, N.

Deprotection of 9, 10, and 15 to the Corresponding Amines. A solution of the protected amine (50 mg) in MeOH/concentrated HCl (1:1) (15 mL) was warmed at 50 °C for 30 min and then concentrated to dryness under reduced pressure. The resulting crude hydrochloride salt was dissolved in a suitable volume of water, and the resulting solution was used for HPLC analyses and cytotoxicity evaluations.

2-Acetamido-5-[*N,N*-bis(2-acetoxyethyl)amino]benzamide (19). Acetic anhydride (0.40 mL, 4.24 mmol) was added to a solution of 5-[*N,N*-bis(2-hydroxyethyl)amino]-2-nitrobenzamide (17)⁶ (0.50 g, 1.86 mmol) in pyridine (20 mL). After 15 min the solution was concentrated to dryness under reduced pressure, and the resulting crude diol diacetate (18) was dissolved in EtOAc (50 mL) containing 5% Pd–C (0.15 g) and shaken under an atmosphere of hydrogen at 60 psi for 3 h. Solvent was removed under reduced pressure from the filtered solution to give an oil which was dissolved in acetic anhydride (10 mL) and warmed at 60 °C for 15 min. Excess acetic anhydride was removed to give 2-acetamido-5-[*N,N*-bis(2-acetoxyethyl)amino]benzamide (19) (0.48 g, 71%): mp (MeOH/H₂O) 150–152 °C; IR $\nu_{\max}$ (KBr/cm⁻¹) 3370 br, 3204, 1734, 1665, 1651, 1532, 1379, 1269, 1227, 1049; ¹H NMR (CDCl₃) δ 11.11 (s, 1 H, NHCOCF₃), 8.48 (d, J = 9.2 Hz, 1 H, H-3), 7.29 (br, 1 H, CONH₂), 7.25 (d, J = 2.9 Hz, 1 H, H-6), 6.90 (dd, J = 9.2, 2.9 Hz, 1 H, H-4), 5.67 (br, 1 H, CONH₂), 4.21 (t, J = 6.9 Hz, 4 H, CH₂O), 3.56 (t, J = 6.9 Hz, 4 H, CH₂N), 2.17 (s, 3 H, NHCOCF₃), 2.07 (s, 6 H, OCOCH₃); ¹³C NMR δ 171.54, 171.46, 168.60 (CO), 142.23 (C-5), 131.39 (C-2), 123.03, 117.07, 110.84 (C-3,4,6), 119.50 (C-1), 60.78 (CH₂N), 49.88 (CH₂O), 25.22 (NHCOCF₃); MS m/z (rel int) 365 (M⁺, 13), 347 (10), 292 (14), 274 (18), 87 (100), 43 (33). Anal. (C₁₇H₂₃N₃O₆) C, H, N.

2-Acetamido-5-[*N,N*-bis(2-acetoxyethyl)amino]-4-nitrobenzamide (20). A solution of concentrated HNO₃ (0.22 mL, 3.40 mmol) in acetic anhydride (0.5 mL) was added dropwise over 10 min to a cooled (0 °C) solution of 19 (0.30 g, 0.85 mmol) in 1:1 THF/acetic anhydride (15 mL). The solution was stirred at room temperature for 1 h, poured into EtOAc, washed with water, and worked up to give an oil which was chromatographed on silica gel. Elution with EtOAc gave 2-acetamido-5-[*N,N*-bis(2-acetoxyethyl)amino]-4-nitrobenzamide (20) as an orange oil (0.32 g, 95%): IR $\nu_{\max}$ (film/cm⁻¹) 3428–3220 (br), 1738, 1674, 1593, 1532, 1369, 1244, 1044. ¹H NMR (CDCl₃) δ 11.05 (s, 1 H, NHCOCF₃), 8.97 (s, 1 H, H-6),

7.66 (s, 1 H, H-3), 7.43, 5.98 (2 × br, 2 H, CONH₂), 4.13 (t, *J* = 6.3 Hz, 4 H, CH₂O), 3.33 (t, *J* 6.3, 4 H, CH₂N), 2.20 (s, 3 H, NHCOCH₃), 2.01 (s, 6 H, OCOCH₃); ¹³C NMR δ 171.51 (OCOCH₃), 169.85, 169.07 (CO), 148.22 (C-5), 135.91, 135.74 (C-2,4), 124.28 (C-6), 121.85 (C-1), 117.10 (C-3), 61.43 (CH₂N), 52.39 (CH₂O), 25.10 (NHCOCH₃), 20.86 (OCOCH₃); MS *m/z* (rel int) 410 (M⁺, 2), 392 (0.3), 337 (8), 320 (5), 87 (100); mass found M⁺ 410.1431, C₁₇H₂₂N₄O₈ requires M⁺ 410.1438.

6-[*N,N*-Bis(2-hydroxyethyl)amino]-2-methyl-7-nitro-4-quinazolinone (21). A solution of CsCO₃ (0.50 g, 1.53 mmol) in water (2 mL) was added to a solution of **20** (0.30 g, 0.73 mmol) in MeOH (30 mL), and the mixture was stirred at room temperature for 1 h. After careful acidification with 3 N HCl, the solution was concentrated onto silica gel by evaporation under reduced pressure. Chromatography, eluting with MeOH/EtOAc (1:9), gave 6-[*N,N*-bis(2-hydroxyethyl)amino]-2-methyl-7-nitro-4-quinazolinone (**21**) (0.22 g, 97%): mp (EtOAc/petroleum ether) 217–219 °C; IR *v*_{max} (KBr/cm⁻¹) 3403 br, 3177, 1676, 1663, 1624, 1526, 1481, 1373, 1292, 1040; ¹H NMR [(CD₃)₂SO] δ 12.37 (br, 1 H, NH), 7.97, 7.88 (2 s, 2 H, H-5,8), 4.60 (br, 2 H, OH), 3.47 (t, *J* = 6.2 Hz, 4 H, CH₂O), 3.22 (t, *J* = 6.2 Hz, 4 H, CH₂N), 2.34 (s, 3 H, CH₃CN); ¹³C NMR δ 160.61 (CO), 154.07 (CH₃CN), 148.82 (C-6), 142.77 (C-7), 140.77 (C-8a), 123.24 (C-4a), 122.21 (C-5), 119.41 (C-8), 58.52 (CH₂N), 55.18 (CH₂O), 21.28 (CH₃CN); MS *m/z* (rel int) 308 (M⁺, 8), 277 (100), 233 (32), 188 (43). Anal. (C₁₃H₁₆N₄O₅) C, H, N.

6-[*N,N*-Bis(2-chloroethyl)amino]-2-methyl-7-nitro-4-quinazolinone (22). MsCl (0.052 mL, 0.64 mmol) was added to a solution of the diol **21** (0.10 g, 0.29 mmol) in pyridine (2 mL). After 15 min, the solution was concentrated to dryness under reduced pressure, and the residue was dissolved in DMF (5 mL) containing LiCl (1.00 g). After stirring at 120 °C for 15 min, the DMF was removed under reduced pressure, and the residue was partitioned between EtOAc and water. The organic extract was chromatographed on silica, eluting with EtOAc/petroleum ether (4:1) to give 6-[*N,N*-bis(2-chloroethyl)amino]-2-methyl-7-nitro-4-quinazolinone (**22**) (0.087 g, 82%): mp (CHCl₃/petroleum ether) 238 °C; IR *v*_{max} (KBr/cm⁻¹) 3462–3036, 1676, 1624, 1533, 1369, 1296; ¹H NMR (CDCl₃/(CD₃)₂SO) δ 2.15 (s, 1 H, NH), 8.15 (s, 1 H, H-5), 7.85 (s, 1 H, H-8), 3.54 (br s, 8 H, NCH₂CH₂Cl), 2.47 (s, 3 H, CH₃CN); ¹³C NMR δ 161.51 (CO), 155.43 (CH₃CN), 155.34 (C-6), 145.81 (C-7), 139.26 (C-8a), 123.79 (C-4a), 123.22, 123.11 (C-5,8), 56.08 (CH₂N), 41.22 (CH₂Cl), 21.91 (CH₃CN); MS *m/z* (rel int) 348, 346, 344 (M⁺, 10), 331, 329, 327 (5), 297, 295 (100), 233 (16), 187 (20). Anal. (C₁₃H₁₄Cl₂N₄O₃) C, H, N.

6-[*N,N*-Bis(2-chloroethyl)amino]-1-(*tert*-butoxycarbonyl)-2-methyl-7-nitro-4-quinazolinone (23). A solution of **22** (0.20 g, 0.55 mmol), di-*tert*-butyl dicarbonate (0.24 g, 1.10 mmol), 4-(dimethylamino)pyridine (0.056 g, 0.55 mmol), and Et₃N (0.077 mL, 0.55 mmol) in THF (10 mL) was stirred at room temperature for 3 h, then adsorbed directly onto silica gel by concentration under reduced pressure, and chromatographed. Elution with EtOAc gave 6-[*N,N*-bis(2-chloroethyl)amino]-1-(*tert*-butoxycarbonyl)-2-methyl-7-nitro-4-quinazolinone (**23**) (0.21 g, 82%) as a yellow oil: IR *v*_{max} (film/cm⁻¹) 1778, 1699, 1539, 1479, 1261, 1146; ¹H NMR (CDCl₃) δ 8.14 (s, 1 H, H-5), 7.87 (s, 1 H, H-8), 3.55 (s, 8 H, NCH₂CH₂Cl), 2.54 (s, 3 H, CH₃CN), 1.67 (s, 9 H, C(CH₃)₃); ¹³C NMR δ 158.84 (CO), 150.85, 150.68, 149.55 (*t*-BuOCO, CH₃CN and C-6), 143.31 (C-7), 140.26 (C-8a), 123.78, 122.90 (C-5,8), 123.25 (C-4a), 88.30 (C(CH₃)₃), 55.67 (CH₂N), 41.13 (CH₂Cl), 27.46 (C(CH₃)₃), 21.55 (CH₃CN); MS *m/z* (rel int) 448, 446, 444 (M⁺, 3), 375, 373, 371 (2), 348, 346, 344 (10), 297, 295 (100); mass found M⁺ 448.0901, 446.0927, 440.0956, C₁₈H₂₂Cl₂N₄O₅ requires M⁺ 448.0908, 446.0938, 440.0967.

5-[*N,N*-Bis(2-chloroethyl)amino]-4-nitroanthranilic Acid (24). A solution of **23** (0.21 g, 0.45 mmol) in MeOH/THF (1:1) (20 mL) was stirred at room temperature with 2 N NaOH (5 mL) for 18 h. The solution was carefully neutralized with 1 N acetic acid, extracted with EtOAc, and worked up to give an oil which was chromatographed on silica gel. Elution with MeOH/EtOAc (1:19) gave an orange oil which was dissolved in MeOH (10 mL) containing concentrated HCl (1 mL) and warmed at 60 °C for 15 min. Solvents were removed under

reduced pressure, the residue was dissolved in water and extracted with EtOAc, and the organic portion was separated and chromatographed on silica gel. Elution with EtOAc gave 5-[*N,N*-bis(2-chloroethyl)amino]-4-nitroanthranilic acid (**24**) (0.12 g, 83% overall): mp (benzene/petroleum ether) 160–162 °C; IR *v*_{max} (film/cm⁻¹) 3487, 3378, 1684, 1586, 1539, 1238; ¹H NMR (CDCl₃) δ 8.00 (s, 1 H, H-3), 6.88 (s, 1 H, H-6), 6.40 (br, 3 H, COOH and NH₂), 3.48, 3.42 (2 × m, 8 H, NCH₂CH₂Cl); ¹³C NMR δ (CDCl₃) 171.29 (COOH), 154.05 (C-5), 149.06 (C-4), 132.91 (C-3), 129.87 (C-2), 111.93 (C-1), 111.19 (C-6), 57.60 (CH₂N), 41.64 (CH₂Cl); MS *m/z* (rel int) 325, 323, 321 (M⁺, 20), 274, 272 (100). Anal. (C₁₁H₁₃Cl₂N₃O₄) C, H, N, Cl.

Reaction of **24** with an excess of ethereal diazomethane gave the methyl ester (**33**) (100%): mp (petroleum ether) 98–99.5 °C; IR *v*_{max} (KBr/cm⁻¹) 3482, 3370, 1701, 1539, 1246; ¹H NMR (CDCl₃) δ 7.92 (s, 1 H, H-6), 6.87 (s, 1 H, H-3), 5.96 (br s, 2 H, NH₂), 3.93 (s, 3 H, COOCH₃), 3.46 (t, *J* = 7.0 Hz, 4 H, CH₂N), 3.36 (t, *J* = 7.0 Hz, 4 H, CH₂Cl); ¹³C δ 166.68 (COOCH₃), 153.23 (C-5), 148.43 (C-4), 131.88 (C-6), 129.62 (C-2), 113.37 (C-1), 110.86 (C-3), 57.66 (CH₂N), 52.23 (COOCH₃), 41.61 (CH₂Cl); MS *m/z* (rel int) 339, 337, 335 (M⁺, 20), 288, 286 (100). Anal. (C₁₂H₁₅Cl₂N₃O₄) C, H, N, Cl.

2-Amino-5-[*N,N*-bis(2-chloroethyl)amino]-4-nitrobenzamide (25). Diethyl cyanophosphonate (170 μL, 1.12 mmol) was added dropwise with stirring to a solution **24** (0.30 g, 1.07 mmol) in THF (20 mL) which had been saturated with NH₃ gas. After 30 min the solution was partitioned between EtOAc and water, and the organic portion was worked up and chromatographed on silica gel. Elution with EtOAc/petroleum ether (7:3) gave 2-amino-5-[*N,N*-bis(2-chloroethyl)amino]-4-nitrobenzamide (**25**) (0.25 g, 83%): mp (benzene/petroleum ether) 104 °C; IR *v*_{max} (KBr/cm⁻¹) 3482 br, 3370 br, 1659, 1528, 1373, 1267; ¹H NMR (CDCl₃) δ 7.50 (s, 1 H, H-6), 7.27 (s, 2 H, CONH₂), 6.95 (s, 1 H, H-3), 5.90 (br, 2 H, NH₂), 3.48 (t, *J* = 6.1 Hz, 4 H, CH₂N), 3.38 (t, *J* = 6.1 Hz, 4 H, CH₂Cl); ¹³C NMR δ 169.40 (CONH₂), 157.60 (C-5), 147.51 (C-4), 130.11 (C-6), 129.70 (C-2), 117.21 (C-1), 111.89 (C-3), 57.49 (CH₂N), 42.23 (CH₂Cl); MS *m/z* (rel int) 324, 322, 320 (M⁺, 23), 273, 271 (100). Anal. (C₁₁H₁₄Cl₂N₄O₃) C, H, N.

Zinc Reduction of 1. A vigorously stirred suspension of **1** (0.50 g, 1.42 mmol) in MeOH/THF (1:1) (10 mL) was treated sequentially with a solution of ammonium chloride (84 mg, 1.57 mmol) in water (10 mL), followed by Zn dust (0.19 g, 2.85 mmol). After 2.5 h the solution gave the HPLC profile shown in Figure 1 and was filtered through Celite and worked up in EtOAc to give an oil. This was chromatographed on silica gel. Elution with EtOAc/petroleum ether (3:2) gave a mixture of peaks A and B (Figure 1) (0.18 g), followed by starting material (**1**; peak C) (0.11 g). Elution with EtOAc gave an inseparable fraction containing peaks D–F (0.14 g), while MeOH/EtOAc (1:19) eluted pure azoxy compound (**28**; peak A) (0.03 g). The mixed fraction containing peaks A and B was dissolved in MeOH (10 mL) and stood at room temperature for 1 week, during which time the 2-hydroxylamine (**26**) (peak B; *R*_f 0.92 on silica gel, EtOAc eluant) converted into the azoxy compound (**28**) (peak A, *R*_f 0.30 on silica gel, EtOAc eluant). Rechromatography of this aged fraction on silica, eluting with MeOH/EtOAc (1:19), gave pure **28** (0.12 g, 32%): mp (EtOAc/petroleum ether at –30 °C) 204–206 °C (yellow powder); IR *v*_{max} (KBr/cm⁻¹) 3383, 3180, 1663, 1607, 1522, 1472, 1435, 1346; ¹H NMR [(CD₃)₂SO] δ 8.65 (s, 1 H, H-3), 8.30 (s, 1 H, H-6), 8.22, 7.97, 7.74, 7.71 (4 × br s, 4 H, 2 × CONH₂), 7.58, 7.51 (2 × s, 2 H, H-3'), 3.81 (t, *J* = 5.9 Hz, 4 H, CH₂Cl), 3.77 (t, *J* = 6.0 Hz, 4 H, CH₂Cl), 3.64 (t, *J* = 5.9 Hz, 4 H, CH₂N), 3.61 (t, *J* = 6.0 Hz, 4 H, CH₂N); ¹³C NMR δ 166.90, 166.18 (2 × CONH₂), 144.95, 142.98, 140.43, 139.30, 138.74, 137.77, 136.29, 132.90 (8 × s), 122.29, 122.24, 122.09, 119.66 (C-6, 6', 3, 3'), 52.94, 52.92 (CH₂N), 41.76, 41.69 (CH₂Cl); FAB *m/z* (rel int) 657, 656, 655, 654, 653, 652, 651 ([M + H]⁺, 0.5), 641, 640, 639, 638, 637, 636 ([M + H – O]⁺, 0.7), 460 (1), 329 (3), 307 (18), 289 (11), 176 (10), 154 (100), 136 (78), 107 (30), 89 (29), 77 (31), with NaCl added to the matrix a [M + Na]⁺ cluster appeared at 681–675 amu; mass found [M + Na]⁺ 679.0376, 677.0428, 675.0443, C₂₂H₂₄Cl₄N₈O₇Na requires 679.0361, 677.0390, 675.0420). Anal. (C₂₂H₂₄Cl₄N₈O₇) C, H, N.

Reaction of the Azoxy Mustard (28) with CH_3SNa . A solution of **28** (5.0 mg, 0.007 mmol) and CH_3SNa (2.0 mg, 0.028 mmol) in propan-2-ol (1 mL) was heated under reflux for 30 min, poured into water, and extracted with EtOAc. The extract was worked up and chromatographed on silica gel. Elution with petroleum ether gave foreruns, while EtOAc/petroleum ether (3:7) eluted **25** (1.9 mg, 42%) (HPLC behavior and ^1H NMR spectrum identical to that of authentic material).

Reaction of the Tetrahydroquinoxaline 13 with Dimethyldioxirane. A solution of dimethyldioxirane in Me_2CO (81 μL of ca. 0.09 M, 0.007 mmol), prepared by the "small scale" method,²⁸ was added dropwise at 0 °C to a solution of **13** (3.0 mg, 0.010 mmol) in Me_2CO (6.0 mL). After 10 min HPLC analysis of the resulting solution revealed the presence of starting material and two new products, corresponding to peaks E and F in the HPLC profile of the zinc reduction of **1** (Figure 1), in an approximate ratio of 2:1:7, respectively (calculated from integrated peak areas at 298 nm). The solution was adsorbed directly onto silica gel by concentration under reduced pressure and chromatographed. Elution with EtOAc/MeOH (9:1) gave an orange solid (2.2 mg) which HPLC analysis showed to be a mixture of peaks E and F from the zinc reduction, now in an approximate ratio of 7:3. Peak F was identified earlier as the ring-closed 4-hydroxylamine (**29**) and peak E as its decomposition product. No further attempts to purify this mixture were made. The use of an excess of dimethyldioxirane gave a multitude of products.

Mono- and Bis-Dechlorinated Benzamides 30 and 31. A mixture of 5-chloro-2,4-dinitrobenzamide²⁹ (0.5 g, 2.04 mmol) and diethylamine (0.63 mL, 6.10 mmol) in THF (20 mL) was stirred at 20 °C for 48 h. After workup in EtOAc the product was chromatographed on silica gel. Elution with EtOAc/petroleum ether (3:2) gave 5-(*N,N*-diethylamino)-2,4-dinitrobenzamide (**31**) (0.38 g, 66%): mp (CHCl_3 /petroleum ether) 166–168 °C; IR ν_{max} ($\text{KBr}/\text{cm}^{-1}$) 3464, 3385, 3165, 1670, 1606, 1584, 1518, 1360, 1323, 1265; ^1H NMR [$(\text{CD}_3)_2\text{SO}$] δ 8.48, (s, 1 H, H-3), 8.08, 7.76 (2 $\times$ br, 2 H, CONH_2), 7.15 (s, 1 H, H-6), 3.36 (q, J = 7.0 Hz, 4 H, NCH_2), 1.13 (t, J = 7.0 Hz, 6 H, CH_2CH_3); ^{13}C NMR δ 166.62 (CONH), 146.26 (C-5), 137.69, 135.82, 133.46 (C-1, 2, 4), 124.59 (C-3), 117.96 (C-6), 45.80 (NCH_2), 12.12 (CH_2CH_3); MS m/z (rel int) 282 (M^+ , 21), 267 (89), 265 (100), 239 (28), 207 (18), 193 (11), 178 (18). Anal. ($\text{C}_{11}\text{H}_{14}\text{N}_4\text{O}_5$) C, H, N.

The half-mustard **30** was prepared as previously described.⁷

Synthesis of Tritium-Labeled 1 and 2. These compounds (^3H -SN 23862 and ^3H -CB 1954), with specific activities of 70.7 and 70.2 mCi/mmol, respectively, were synthesized using published procedures^{6,30} from 5-chloro-2,4-dinitrobenzoic acid which had been randomly tritiated in the aromatic ring by exchange with [^3H] H_2O (Amersham).

Radiochemical Reductions. Radiochemical reductions were performed on 5 mL degassed saturated solutions of the nitro compounds, in 0.085 M phosphate buffer containing 0.1 M sodium formate at a pH of 7. These solutions were typically about 115 μM for **1** and 50 μM for **2** (determined spectrophotometrically) and were irradiated in glass using a ^{60}Co source, which supplied a dose rate of approximately 1.12 Gray s^{-1} (determined by the method of Fricke³¹). Under the above conditions, generation of each two-electron reducing equivalent took ca. 5.5 min for **1**.

HPLC analyses of the resulting solutions were carried out using a Waters 600 multisolvent delivery system, on a Waters 8 $\times$ 100 mm μ -Bondapak C-18 cartridge coupled to a Hewlett-Packard 1040A diode array detector. The mobile phase consisted of the following sequential methanol/water gradients at a flow rate of 1.5 mL min^{-1} : 0–12% methanol (2 min), 12–64% methanol (38 min), and 64–80% methanol (2 min). Initial radiolyses were carried out using [^3H]SN 23862 (specific activity 0.87 mCi/mmol) and additions of six-electron equivalents. The HPLC chromatogram was integrated by peak area at 298 and 430 nm, and 180 fractions of 20 s each were collected and counted by liquid scintillation. These results were used to calculate a peak area/nmol index for each peak at each wavelength, which was used to quantify subsequent radiolyses using nonlabeled **1**. For mass spectral analysis of the major reduction products, 2 mL of the crude radiolysis

solution from a six-electron reduction was separated by HPLC, with manual collection of the desired fractions. These were freeze-dried and stored at -80 °C until analysis by desorption electron-impact mass spectrometry.

Bioassay of the HPLC eluate from reduction of 1. A 10 mL saturated solution of labeled **1** (specific activity 0.11 mCi/mmol) was radiolytically reduced with six-electron equivalents and separated by HPLC using 5 $\times$ 2 mL injections. Various fraction sizes (depending on the distribution of peaks of interest) were collected, and the concentration of material in each fraction was determined by liquid scintillation counting. The fractions were diluted 100-fold into cultures of UV4 cells in 96-well plates, and after incubation for 4 days the cultures were stained and scored by absorbance, essentially as described.⁵ The cytotoxicities of the fractions of Figure 2 are given as a percentage of control wells which did not receive column eluate. Additional experiments (data not shown) indicate that fractions obtained when a blank sample was injected did not inhibit cell growth at this dilution.

Evaluation of Cytotoxicity in Vitro. A48 or UV4 cell cultures⁶ were initiated in 96-well microtiter trays to give 200 (A48) or 300 (UV4) cells in 0.05 mL per well. After growth in a CO_2 incubator for 24 h, drugs were added in culture medium, using serial 2-fold dilutions to provide duplicate cultures at five different concentrations for each of eight drugs (plus eight controls) per tray. Drugs (as HCl salts) were dissolved in water or DMSO immediately prior to addition to the cell culture. The unstable compounds **11**, **12**, and **14** were prepared immediately before use by deprotection of their *tert*-butoxycarbonyl or trifluoroacetamide precursors, as described above. After 4 h, drugs were removed by washing cultures three times with fresh medium, and the trays were incubated for a further 3 days. Cell density was then determined by staining with methylene blue as described previously,³² and the IC_{50} was calculated as the drug concentration providing 50% inhibition of growth relative to the controls.

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