N-Substituted 2-(2,6-Dinitrophenylamino)propanamides: Novel Prodrugs That Release a Primary Amine via Nitroreduction and Intramolecular Cyclization

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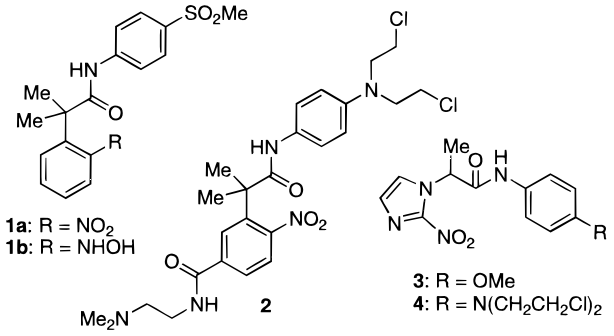
A series of *N*-dinitrophenylamino acid amides [(4-CONHZ-2,6-diNO_2Ph)N(R)C(X,Y)CONHPhOMe] were prepared as potential bioreductive prodrugs and reduced radiolytically to study their rates of subsequent intramolecular cyclization. Compounds bearing a free NH group (R = H) underwent rapid cyclization in neutral aqueous buffers ($t_{1/2}$ < 1 min) following 4-electron reduction, with the generation of a *N*-hydroxydihydroquinoxalinone and concomitant release of 4-methoxyaniline. Amine release from analogous *N*-methyl analogues (R = Me) was relatively slow. These results are consistent with intramolecular cyclization of a monohydroxylamine intermediate. The high rates of cyclization/extrusion by these very electron-deficient hydroxylamines suggest that the process is greatly accelerated by the presence of an H-bonding "conformational lock" between the anilino NH group and the adjacent *o*-nitro group (Kirk and Cohen, 1972). Changes in the phenylcarboxamide side chain or in C-methylation in the linking chain had little effect on the rate of cyclization. The model compounds had 1-electron reduction potentials in the range appropriate for cellular reduction (−373 mV for a measured example) and appeared suitable for development as prodrugs that release amine-based effectors following enzymic or radiolytic reduction. Prodrug examples containing 4-aminoaniline mustard and 5-amino-1-(chloromethyl)benz[*e*]indoline alkylating units were evaluated but were not activated efficiently by cellular nitroreductases. However, cell killing by the radiation-induced reduction of the latter prodrug was demonstrated.

The efficacy of most anticancer drugs is limited by the damage they cause to populations of normal cycling cells in the host. One approach to improving the utility of such drugs is to develop less toxic prodrug forms capable of selective release of the parent cytotoxins in tumors. A number of possible tumor-selective reductive mechanisms for the activation of prodrugs are known. These include oxygen-inhibited reduction by endogenous enzymes[1−4] or therapeutic radiation[5] and selective reduction by introduced exogenous enzymes in antibody- and gene-directed enzyme-prodrug therapy (ADEPT/GDEPT) strategies.[6−8] We have proposed a general conceptual approach to the design of such prodrugs, which divides the structure into three domains (trigger, linker, and effector), allowing the independent optimization of each.[9] The trigger units control the selectivity of activation, by undergoing reduction only in tumor cells.

We have recently reported the reductive release of amines from nitroarylamide precursors, via amino[10,11] and hydroxylamino[12] amide cyclization−extrusion reactions. Model compounds (e.g., **1a**) were shown to release amines, via the hydroxylamine intermediate **1b**, with suitable rapidity ($t_{1/2}$ < 1 min under physiological-type conditions) following radiolytic reduction. However, the related analogue **2** containing a cytotoxic effector (nitrogen mustard) did not show hypoxia-selective cytotoxicity in tumor cells in culture (W. R. Wilson, unpublished data). A likely reason is that **2** still has a 1-electron reduction potential (−517 mV; R. F. Anderson, unpublished data) too low for rapid reduction by endogenous enzymes. To raise the reduction potential sufficiently in this design would require the use of additional electron-withdrawing substituents on the nitrophenyl ring, which in turn would slow the intramolecular cyclization−extrusion process.[10] Nitroimidazole analogues do have suitable reduction potentials, but model compounds (e.g., **3**) do not undergo intramolecular cyclization following chemical reduction, and mustard-containing analogues (e.g., **4**) were not hypoxia-selective in cell culture.[13] The different geometry of the 5-membered ring is the most likely reason, since previous work[10,14,15] had shown that the ground-state conformation of the molecule has a very large influence on rates of cyclization.



In the present study we evaluate a new class of reductively activated prodrugs based on a related

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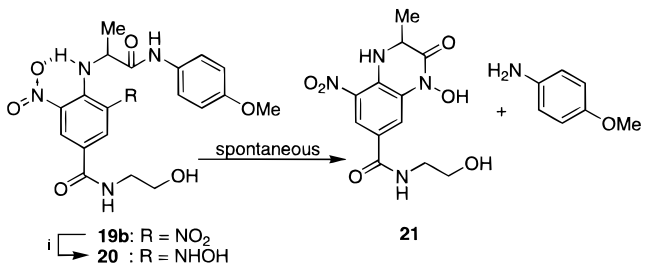
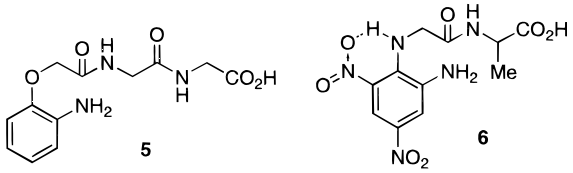
Scheme 1[a]



[a] (i) e^- (aq).

N-(2,6-dinitrophenyl)amino trigger. These were designed to both possess appropriate reduction potentials and to undergo rapid subsequent cyclization−extrusion. The second (symmetric) nitro group not only raises the reduction potential but also, through H-bonded locking, correctly positions the initial hydroxylamine or amine for fast cyclization−extrusion (Scheme 1). This approach has been used previously for the sequential cleavage of peptide bonds.[16−18] Coupling of an amino acid or peptide with 3,5-dinitro-2-fluoroaniline to give a 3,5-dinitroanilide derivative (e.g., **6**)[16] results in immediate cyclization, via attack on the neighboring acid or amide function by the amino group, to give a dihydroquinoxalinone. The surprisingly facile cyclization of the very electron-deficient **6** under alkaline conditions (preventing its isolation at pH 8) has been attributed[17] to conformational restriction of the side chain via H-bonding to the *o*-nitro group. An analogue (**5**),[17] more electron-rich but not so constrained, was much more stable ($t_{1/2}$ 5.8 h at pH 4.96 and >18 h at pH > 5).



Model compounds **19a**−**19e** were made to explore the geometry of the linker group between the trigger and model effector units and the importance of the H-bonding lock, while **16** and **17** compare different solubilizing functions. Analogues **26** and **32**, containing different types of cytotoxic effectors, were evaluated in cells to determine their utility as prodrugs.

Synthetic Chemistry

Schemes 2 and 3 outline the synthetic routes to the compounds. Diethyl cyanophosphonate (DECP)-induced coupling of the known *N*-BOC amino acids **7a**−**7c** with 4-methoxyaniline gave the intermediates **8a**−**8c**, which were deblocked to provide the amines **9a**−**9c**. The *N*-methylamine **9d** was prepared from **9b** via the trifluoroacetyl intermediate **10** and subsequent *N*-methyl derivative **11**. Amine **9e** was prepared from *N*-CBZ-sarcosine (**12**) via amide **13**. Reactions of amines **9a**−**9e** with 4-chloro-*N*-(2-hydroxyethyl)-3,5-dinitrobenzamide (**18**) (obtained from acid **14**) gave the target model prodrugs **19a**−**19e**. Condensation of **9b** with **14** to give **15**, followed by amide formation, gave the analogues **16** and **17**.

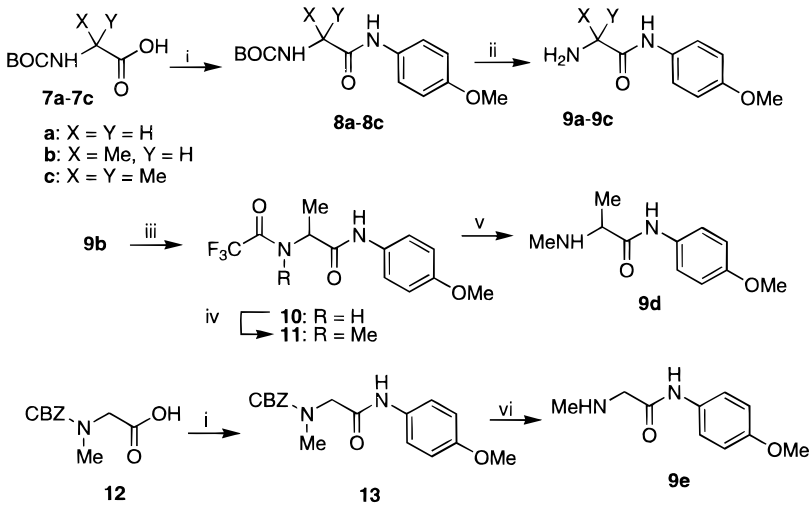
The mustard prodrug **26** was prepared as shown in Scheme 4. DECP-promoted reaction of 4-aminoaniline mustard[19] **22** with *N*-BOC-alanine (**7b**) gave **23**, which after alanine deprotection was reacted with 4-chloro-3,5-dinitrobenzoic acid (**14**). CDI-assisted amide formation from the resulting acid **25** gave **26** in good overall yield (40% from **22**).

The *seco*-cyclopropylindoline prodrug **32** was prepared as shown in Scheme 5. Reaction of **14** with alanine ester **27** gave the substituted alanine **28**, which was in turn converted to the amide **29**. Cleavage of the 4-methoxybenzyl ester group with anisole/TFA gave the acid **30**, which was coupled to 5-amino-*seco*-CBI-TMI[20] (**31**) to give the target compound.

Reduction Chemistry of Model Prodrugs

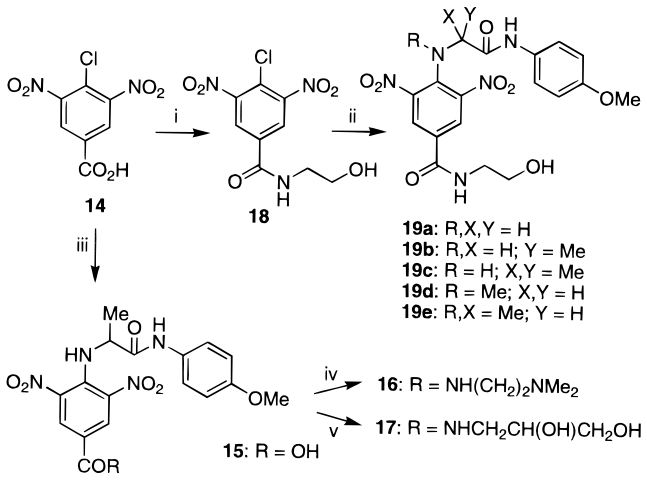
Anaerobic, aqueous solutions (pH 7.4, 0.01 M phosphate buffer, 0.5 M propan-2-ol) of **19a**−**19c**, **16**, and **17** were irradiated for times corresponding to the introduction of 1−6 molar equiv of reducing species (i.e., $e^-_{(aq)}$ and $(CH_3)_2C{\cdot}OH$). The concentration of starting material was 21 μM, with the exception of **19a**, whose low aqueous solubility allowed solutions of only 3 μM

Scheme 2[a]



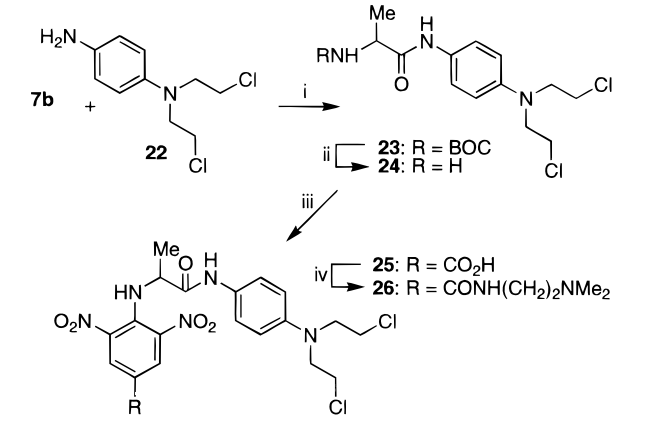
[a] (i) 4-Methoxyaniline/DECP/1-methylimidazole/DMF/0 °C, then 20 °C/2 h; (ii) TFA/20 °C/1 h; (iii) TFAA/pyridine/CH_2Cl_2/0 °C; (iv) NaH/DMF/20 °C/2 h, then MeI/20 °C/6 h; (v) Cs_2CO_3/MeOH/water/reflux/30 min; (vi) Pd/C/H_2/EtOH/20 °C/h/60 psi.

Scheme 3[a]



[a] (i) $SOCl_2$/DMF, then $H_2N(CH_2)_2OH$/Me_2CO/water/0 °C, then HCl; (ii) **9a**−**9e**/THF/(^{i}Pr)$_2$NEt/20 °C/8 h; (iii) **9b**/THF/(^{i}Pr)$_2$NEt/20 °C/12 h, then 50 °C/30 min; (iv) CDI/DMF/0 °C, then $H_2N(CH_2)_2NMe_2$/0 °C; (v) CDI/DMF/20 °C, then $H_2NCH_2CH(OH)CH_2OH$/0 °C.

Scheme 4[a]

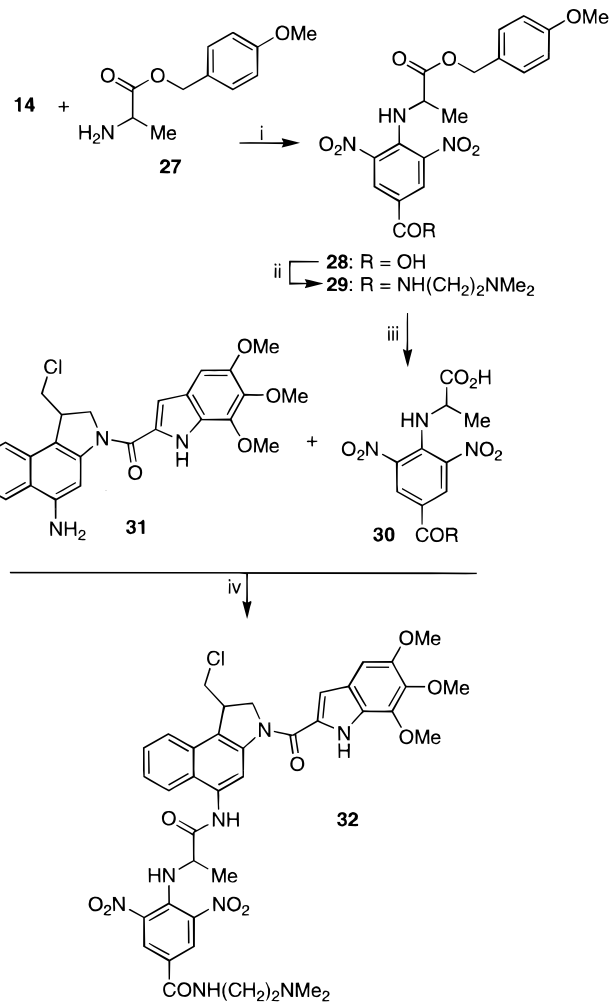


[a] (i) DECP/DMF/0 °C, then (^{i}Pr)$_2$NEt/20 °C/1.5 h; (ii) TFA/20 °C/2 h; (iii) 4-chloro-3,5-dinitrobenzoic acid (**14**)/(^{i}Pr)$_2$NEt/20 °C/12 h; (iv) CDI/DMF/25 °C/1 h, then $H_2N(CH_2)_2NMe_2$/25 °C/5 min.

in buffer containing 1 M 2-propanol, 1 M acetone. Radiolytic reductions of **19a** were therefore carried out in steps of 2 stoichiometric equiv (the addition of 1 reducing equiv to a 3 μM solution required an irradiation time of only 7.5 s).

Figure 1A depicts the changes in the composition of radiolysis solutions of **19b** as the stoichiometry of reduction was varied and typifies the radiolytic reduction chemistry of **19a**−**19c**, **16**, and **17**. The nitro compound **19b** was consumed with 4-fold stoichiometry, consistent with either monoreduction of a nitro group to the hydroxylamine or reduction of both nitro groups to the dinitroso compound. The two major products formed in parallel were 4-methoxyaniline (by comparison of retention time and spectrum with an authentic sample) and *N*-hydroxydihydroquinoxalinone (**21**) (identified by mass spectrometry of the mixture of products formed after 4-fold reduction). The latter arises from cyclization of the initially formed monohydroxylamine (**20**) (Scheme 1). 4-Methoxyaniline was likewise formed from reduced **19a**, **19c**, **16,** and **17** along with a coproduct, assumed to be the corresponding *N*-hydroxydihydroquinoxalinone on the basis of similar retention

Scheme 5[a]



[a] (i) (^{i}Pr)$_2$NEt/THF/25 °C/30 h; (ii) CDI/DMF/20 °C/1 h, then $H_2N(CH_2)_2NMe_2$; (iii) anisole/TFA/20 °C/15 min; (iv) EDCI HCl/DMA/20 °C/4 h.

time and spectrum. An exception was **16**, for which no corresponding *N*-hydroxydihydroquinoxalinone coproduct was detected at pH 7.4.

The yield of 4-methoxyaniline was not altered by irradiating solutions of **19 a**−**19c**, **16**, and **17** with >4 reducing equiv, but the *N*-hydroxydihydroquinoxalinones were consumed (e.g., irradiation of **19b** with up to 14 reducing equiv gave two new unidentified products which maximized at 8-fold stoichiometry; data not shown). The absorbance spectrum of solutions of **19b** and **19c** did not change with time up to 120 min after reduction with 4-fold stoichiometry (irradiation time 4 min), and the concentrations of released 4-methoxyaniline also showed no increase when HPLC analysis of irradiated solutions was delayed. Thus release of 4-methoxyaniline from the *N*-hydroxydihydroquinoxalinone intermediates is too fast to detect kinetically at these low dose rates, and the half-life for this reaction is estimated at <1 min.

The *N*-methyl analogues **19d** and **19e** at pH 7 were too hydrolytically unstable for their reduction to be studied at pH 7, but this was done at pH 4 (together with their desmethyl counterparts **19a** and **19b**). Figure 1B shows the changes in composition of **19e** (**19d** was similar, and **19a** and **19b** gave similar results to those

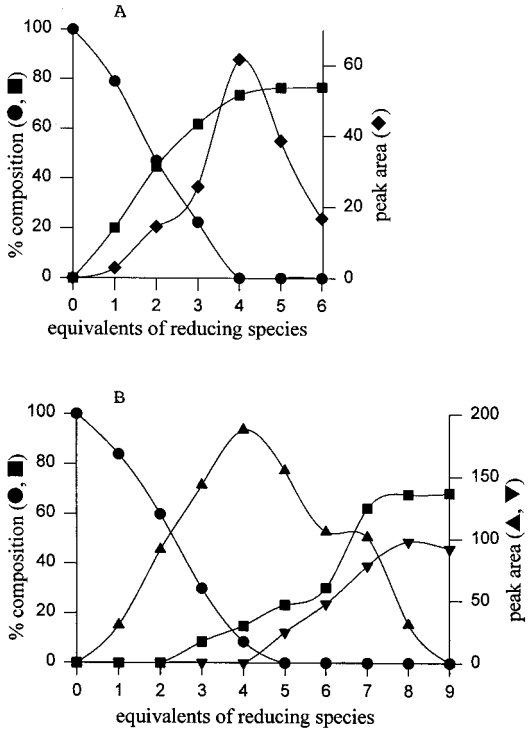


Figure 1. Changes in solution composition with changing stoichiometry of radiolytic reduction. A: Changes to **19b**, 21 μM at pH 7.4, 0.01 M phosphate, room temperature. B: Changes to **19e**, 25 μM at pH 4.0, 0.01 M formate, room temperature. Symbols refer to model prodrug (●), 4-methoxyaniline (■), ring-closed *N*-hydroxydihydroquinoxalinone (**21**) (◆), the monohydroxylamino reduction product (t_R = 4.4 min) (▼), the dihydroxylamino reduction product (t_R = 3.0 min) (▲).

at pH 7.4; Figure 1A). All were reduced with 4-fold stoichiometry at pH 4.0, but product formation differed between the *N*-methyl and desmethyl counterparts. Both **19d** (retention time 6.4 min) and **19e** were converted to a more polar product that maximized at 4-fold stoichiometry, and this was then converted to a second product at 8-fold stoichiometry. These patterns are consistent with sequential reduction of both nitro groups to the hydroxylamines.

4-Methoxyaniline was observed in irradiated solutions of **19d** and **19e**, but yields immediately after irradiation were low after 4-fold reduction stoichiometry and appeared to increase further with dose (Figure 1B). However, the detection of hydroxylamino intermediates in the case of the *N*-methyl compounds suggested that this apparent increase at higher doses might reflect slow cyclization kinetics of the latter. The spectra of solutions of **19d** and **19e** reduced with 4- and 8-fold stoichiometry changed with time following irradiation and passed through isosbestic points. The observed pseudo-first-order rate coefficients for reaction of the 4- and 8-fold reduced forms were measured, and values of the corresponding reaction half-lifes ($t_{1/2} = \ln 2/k_{obs}$) are presented in Table 1. The product solutions eventually showed essentially 100% release of 4-methoxyaniline and loss of the hydroxylamino intermediates (Table 1).

Biological Studies

In Vitro Biological Evaluation: Cytotoxicity and Activation by Radiation. To study the usefulness of 2-(2,6-dinitrophenylamino)propanamides as prodrugs,

Table 1. Comparison of the Observed Half-Life for and Percent of 4-Methoxyaniline Released from 4-fold Reduced **19a**−**19c**, **16**, and **17** (pH 7.4, 10 mM phosphate buffer, 0.5 M 2-propanol, rt) and **19a**, **19b**, **19d**, and **19e** (pH 4.0, 10 mM formate buffer, 0.5 M 2-propanol, rt)

	4-fold reduced		8-fold reduced	
no.	$t_{1/2}$ (min)	released 4-methoxyaniline	$t_{1/2}$ (min)	released 4-methoxyaniline
		pH 7.4		
19a	<1	69%		*a*
19b	<1	77%		*a*
19c	<1	90%		*a*
16	<1	89%		*a*
17	<1	92%		*a*
		pH 4.0		
19a	<1	83%		*a*
19b	<1	97%		*a*
19d	7.2 ± 5	80 ± 10%	4 ± 1	100 ± 2%
19e	12 ± 1	80 ± 20%	15 ± 1	94 ± 2%

[a] Percent release of 4-methoxyaniline was not significantly different from that released by 4-fold reduction, and no evidence was found for formation of a cyclizable, di-NHOH form of the prodrug.

analogues containing cytotoxic primary amine-based effectors were prepared (**26**, **32**). The trigger investigated was based on the rapidly cyclizing **19b**, but with a basic 4-side chain to improve aqueous solubility. The cytotoxicities of the prodrugs and effectors, as determined by growth inhibition (IC_{50} assays) and plating efficiency (clonogenic assays), are summarized in Table 2. The aniline mustard **22** was 33-fold more toxic (IC_{50} assay) than the corresponding prodrug **26** against the UV4 cell line, which is defective in DNA cross-link repair.[21] This indicates that the toxicity of the effector is masked effectively in the prodrug form. However, the repair-competent cell lines (AA8, EMT6, SKOV3) showed similar sensitivity to **22** and **26.** The lack of hypersensitivity of UV4 to **26,** relative to **22**, clearly demonstrates that the toxicity of the prodrug in repair-competent cells is not due to release of **22.**

A much more potent cytotoxic amine effector was therefore investigated, in an attempt to avoid the toxicity of the prodrug form seen in the nitrogen mustard example. The DNA minor groove alkylator 5-amino-*seco*-CBI-TMI[20] (**31)** had IC_{50} values ≤ 1 nM against all four cell lines, and the corresponding prodrug **32** was much less toxic (87−206-fold) in all lines. The cytotoxicity of these compounds against aerobic UV4 cells in clonogenic assays confirmed these conclusions, demonstrating the high potency of **31** relative to **32.** Under N_2 the potency of **32** (but not **31**) was increased 1.8-fold; this selectivity for hypoxia was statistically significant and was greater than that for the corresponding mustard prodrug **26** (which showed a differential no greater than its amine effector **22**). However, the increase in activity under hypoxia is minor relative to the large change theoretically possible (140-fold if all the **32** were converted rapidly to **31**), suggesting either that **32** is a poor substrate for reduction by reductases in these cells or that reduction intermediates such as the nitroso or hydroxylamine are intercepted before intramolecular cyclization occurs.

Although not activated efficiently by enzymatic reduction in hypoxic cells, the extreme potency of **31** suggested that it might be possible to reduce enough of the prodrug **32** with ionizing radiation to achieve

Table 2. Cytotoxicity Data for Prodrugs **26** and **32** and Their Precursors **22** and **31**

	growth inhibition (IC_{50}, nM)[a]				UV4 clonogenic assay	
no.	AA8	UV4	EMT6	SKOV3	C_{10} (nM)[b]	air/N_2[c]
22	5700 ± 1100	90 ± 30	1200 ± 100	2400 ± 300	220	1.4
26	10500 ± 900	3000 ± 600	1300 ± 200	1300 ± 200	2100	1.3
31	0.46 ± 0.05	0.29 ± 0.2	0.27 ± 0.03	1.0 ± 0.1	3.4 ± 0.4[d]	1.1 ± 0.1[d]
32	95 ± 6	53 ± 1	41 ± 8	87 ± 2	490 ± 20[d]	1.8 ± 0.3[d]

[a] IC_{50}, concentration of drug to reduce cell numbers to 50% of controls in a growth inhibition assay (4-h aerobic drug exposure). See text for description of cell lines. Values are mean ± SEM for 3−5 experiments. [b] C_{10}, drug concentration to reduce plating efficiency of UV4 cells to 10% of controls (1-h exposure) under aerobic conditions (20% O_2). [c] C_{10} in 20% O_2/C_{10} in N_2. [d] Values are mean ± SEM for 3 experiments.

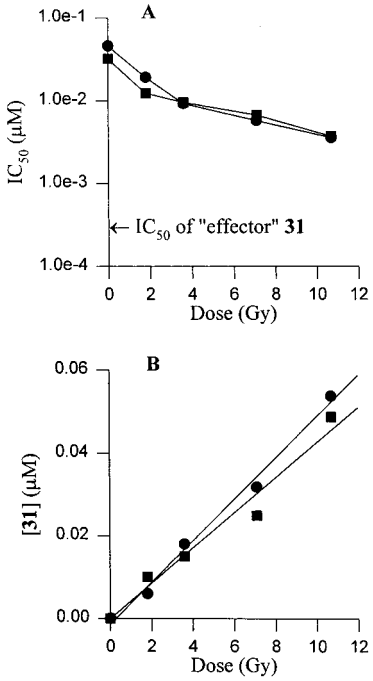


Figure 2. A: Increased cytotoxic activity of the prodrug **32** (1 μM) following γ-irradiation in duplicate (●, ◆) in anoxic culture medium containing 5% FCS. The IC_{50} of irradiated solutions was determined by bioassay against the UV4 cell line. B: Yield of effector **31** in irradiated solutions, calculated from the measured IC_{50} values[26] assuming the cytotoxic effects of **31** and **32** are independent and additive.

significant activation at clinically relevant radiation doses in biological systems. Irradiation of **32** (1 μM, close to its solubility limit of 1.3 μM) in anoxic culture medium containing 5% fetal calf serum at doses up to 10.7 Gy resulted in a significant activation as determined by bioassay against UV4 cells (Figure 2). No increase in cytotoxicity was observed upon radiolysis of the corresponding mustard prodrug **26** (50 μM, doses up to 200 Gy, data not shown), as expected given the low potency of the resulting effector.

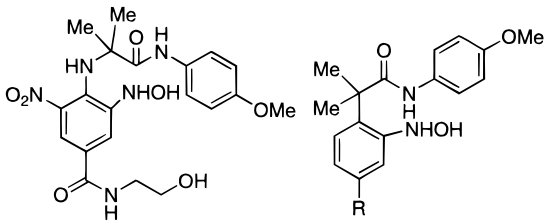
In Vivo Biological Evaluation: Toxicity and Antitumor Activity. Animals treated ip with **31** at doses in the range 5.6−10 μmol/kg died within 5−18 days; at doses lower than this, benign masses developed within the peritoneum after several weeks, resulting in distension of the abdomen, bowel obstruction, with sporadic deaths after about 4 weeks. The intraperitoneal masses involved the omentum and intestines, with histological evidence of fatty necrosis. The highest nontoxic dose was 1.78 μmol/kg. The 2-(2,6-dinitrophenylamino)propanamide prodrug **32** showed no acute toxicity at doses up to at least 178 μmol/kg, but delayed toxicity was again seen with development of abdominal masses at doses as low as 13.3 μmol/kg. The maximum nontoxic dose of **32** was approximately 7.5 μmol/kg. Compound **32** was tested against the RIF-1 tumor at 133 μmol/kg (i.e., well above the dose which caused delayed toxicity) by excising tumors for clonogenic assay 18 h after treatment. The compound showed no activity as a single agent, and no increase in cell killing above that for radiation only (15 Gy) was seen when **32** was administered either 30 min before or 5 min after irradiation.

Discussion

The 2-(2,6-dinitrophenylamino)propanamide prodrugs were designed to exploit a proposed[17] H-bonding "lock" to accelerate the kinetics of reductively induced cyclization. Examples **19a**−**19c** explored the geometry of the linker group between the trigger and model effector units. Examples **19d** and **19e**, with an *N*-methyl group preventing any H-bonding to the ring nitro group, were prepared for direct comparison with the corresponding "lockable" NH derivatives **19a** and **19b**, and **16** and **17** were made to examine the effects of different solubilizing side chains.

The results show that radiolytic reduction under physiological conditions (aqueous solution at pH 7) results in initial reduction of only one nitro group to the hydroxylamine (4-fold stoichiometry). The most likely alternative possibility (reduction of both nitro groups to a noncyclizable dinitroso species) is incompatible with the release of the ring-closed *N*-hydroxydihydroquinoxalinone species and 4-methoxyaniline. Cyclization of the monohydroxylamines from **19b**, **19c**, **16**, and **17** is rapid, going to completion during the irradiation. Given a time of 56 s for reduction of one-fourth of **19b** (addition of 1 reducing equiv), and a total time of approximately 4 min from commencement of the reduction to HPLC assay, the $t_{1/2}$ for cyclization appears to be <1 min (pH 7.4 and 4.0, room temperature). This is a much faster release rate than observed for any monohydroxylamino aryl amide system[12] (e.g., **33** and **34**) (Figure 3).

N-Methylation of the proposed H-donor site slowed cyclization significantly, although this could not be quantified due to the very rapid cyclization of the "locked" analogues **19a** and **19b** (Table 1). However, a significant role for H-bonding is suggested by previous work.[10−14] For the "locked" compounds (**19a**−**19c**), quantitative evaluation of the effects of C-substituents in the linker chain was not possible, since all cyclized with $t_{1/2}$ values < 1 min. However, a comparison of the slower-reacting "unlocked" compounds **19d** and **19e** suggests that C-methylation does slow the cyclization reaction slightly, via both the mono- and dihydroxyl-



hydroxylamine	half-life ($t_{1/2}$) for cyclisation (min)
ex **19c**	< 1
33	13.5
34	6.8

Figure 3. Comparison of the half-life for rate of cyclization (pH 7.4, room temperature) of the hydroxylamine intermediates derived from **19c** with two previously investigated[12] model prodrugs.

amino intermediates (Table 1). Changes to the carboxamide side chain increased the solubility significantly (relative solubilities **17** < **19b** < **16)** without affecting rates of reductive cyclization, as expected.

The kinetic investigation shows that NH analogues possessing an H-bonding "lock" capability cyclize rapidly following reduction to the monohydroxylamine. As **19b** has a 1-electron reduction potential of −373 ± 10 mV (measured by pulse radiolysis, R. F. Anderson, unpublished data), these compounds should be efficiently reduced by cellular nitroreductases, suggesting this as a suitable system for the development of hypoxia-activated prodrugs bearing amine-based effectors.[9] To evaluate the utility of this approach, analogues **26** and **32** were prepared as prodrugs of 4-aminoaniline mustard[19] (**22**) and 5-amino-*seco*-CBI-TMI[20−23] (**31**), respectively. Previous studies of both effectors have shown that such prodrugs should be markedly less cytotoxic than the corresponding amine effectors. The results listed in Table 2 show that while the mustard **22** was not consistently deactivated as expected, the indoline **31** was.

Despite this, and the fact that the prodrug **32** has a high enough reduction potential to expect rapid cellular reduction, it showed little hypoxic selectivity in cell culture. Previous studies[24] have shown that the rates of reduction of *ortho*-substituted nitro compounds is lower than predicted from their 1-electron reduction potentials, but it is not known if this is a factor here. It is also possible that intermediates of bioreduction such as the nitroso or hydroxylamine are intercepted before intramolecular cyclization occurs. However, Figure 2 shows that **32** is significantly activated by ionizing radiation at low radiation doses. The IC_{50} data correspond to a yield of effector [G(**31**) = 4.5 ± 0.3 nmol J^{-1}],[25] which is only 1.6% of the G value for $e^-_{(aq)}$, or 6.4% of the theoretical yield if all $e^-_{(aq)}$ were captured and 4-fold stoichiometry of activation was required. This low yield is not surprising, given the low concentration of **32** (1 μM) which must compromise its ability to compete for $e^-_{(aq)}$. However, the result demonstrates that even inefficient radiolytic activation might be exploited to activate prodrugs if a sufficiently potent effector is released. In this instance, a clinically relevant dose of 2 Gy is calculated to release 9 nM effector, 9 times the IC_{50} of **31** against the least sensitive tumor cell line tested (SKOV3).

Despite these promising characteristics in vitro, **32** lacks significant activity against hypoxic cells in RIF-1 tumors (as demonstrated by failure to kill additional cells when combined with ionizing radiation), even at prodrug doses high enough to eventually cause chronic toxicity to the host. These experiments were designed to detect either bioreductive activation by endogenous enzymes in hypoxic regions (increased killing when drug is administered before or after radiation) or radiolytic activation (increased killing when drug is administered before radiation only). Many requirements must be met simultaneously for activity against hypoxic cells in vivo. The lack of tumor activity of **32** could reflect unfavorable pharmacokinetics of the prodrug, inefficient radiolytic and enzymatic activation in tissue, alternate pathways for removal of the hydroxylamine in competition with cyclization, or lack of activity of the released effector in the tumor microenvironment. Nevertheless, the *N*-(2,6-dinitrophenyl)amino trigger system described here provides a novel and well-defined chemistry for reductive activation of prodrugs and may provide therapeutically useful agents if these issues can be addressed.

Experimental Section

Synthetic Chemistry. Analyses were performed by the Microchemical Laboratory, University of Otago, Dunedin. Melting points were determined on an Electrothermal model 9200 digital melting point apparatus and are as read. NMR spectra were measured on a Bruker AM-200 or AM-400 spectrometer and referenced to Me_4Si.

Preparation of Protected Amines. 2-[(*tert*-Butyloxycarbonyl)amino]-*N*-(4-methoxyphenyl)-2-methylpropanamide (8c). A stirred solution of 2-[(*tert*-butyloxycarbonyl)amino]-2-methylpropanoic acid (**7c**) (3.50 g, 17.2 mmol), 4-methoxyaniline (2.33 g, 18.9 mmol), and 1-methylimidazole (1.70 g, 20.7 mmol) in DMF (15 mL) was treated dropwise at 0 °C with diethyl cyanophosphonate (93%, 3.32 g, 18.9 mmol). The mixture was stirred at 20 °C for 24 h and then diluted with excess aqueous Na_2CO_3. The resulting solid was recrystallized twice from MeOH/H_2O to give **8c** (3.89 g, 73%): mp 201 °C; ^{1}H NMR ($CDCl_3$) δ 8.11 (br s, 1 H, CON*H*Ph), 7.42 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.85 (d, J = 9.0 Hz, 2 H, H-3′,5′), 4.91 (br s, 1 H, OCONH), 3.90 (s, 3 H, OCH_3), 1.56 (s, 6 H, $C(CH_3)_2$), 1.44 (s, 9 H, $C(CH_3)_3$). Anal. ($C_{16}H_{24}N_2O_4$) C, H, N.

[(*tert*-Butyloxycarbonyl)amino]-*N*-(4-methoxyphenyl)-acetamide (8a). Reaction of *N*-(*tert*-butyloxycarbonyl)glycine (**7a**) and 4-methoxyaniline as above gave **8a** (82%): mp (MeOH/H_2O) 143−144 °C; ^{1}H NMR ($CDCl_3$) δ 8.10 (br s, 1 H, CON*H*Ph), 7.40 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.85 (d, J = 9.0 Hz, 2 H, H-3′,5′), 5.33 (br s, 1 H, N*H*CH_2), 3.92 (d, J = 5.9 Hz, 2 H, CH_2), 3.79 (s, 3 H, OCH_3), 1.48 (s, 9 H, $C(CH_3)_3$). Anal. ($C_{14}H_{20}N_2O_4$) C, H, N.

2-[(*tert*-Butyloxycarbonyl)amino]-*N*-(4-methoxyphenyl)-propanamide (8b). Reaction of *N*-(*tert*-butyloxycarbonyl)-alanine (**7b**) and 4-methoxyaniline as above gave **8b** (77%): mp (MeOH/H_2O) 139−140 °C; ^{1}H NMR ($CDCl_3$) δ 8.34 (br s, 1 H, CON*H*Ph), 7.41 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.84 (d, J = 9.0 Hz, 2 H, H-3′,5′), 5.16 (br s, 1 H, OCONH), 4.31 (br s, 1 H, CHCO), 3.78 (s, 3 H, OCH_3), 1.46 (s, 9 H, $C(CH_3)_3$), 1.42 (d, J = 7.0 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{15}H_{22}N_2O_4$) C, H, N.

***N*-(4-Methoxyphenyl)-2-(trifluoroacetylamino)propanamide (10).** Reaction of trifluoroacetic anhydride with **9b** in CH_2Cl_2 containing pyridine gave **10** (78%): mp (MeOH/H_2O) 190−191 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 10.01 (s, 1 H, CON*H*Ph), 9.70 (s, 1 H, CF_3CONH), 7.49 (d, J = 9.1 Hz, 2 H, H-2′,6′), 6.89 (d, J = 9.1 Hz, 2 H, H-3′,5′), 4.46 (br s, 1 H, CH), 3.72 (s, 3 H, OCH_3), 1.40 (d, J = 7.2 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{12}H_{13}F_3N_2O_3$) C, H, N.

***N*-(4-Methoxyphenyl)-2-[*N*-(trifluoroacetyl)methylamino]propanamide (11).** A solution of **10** (1.20 g, 4.13 mmol) in DMF (6 mL) was treated with NaH (0.173 g, 4.33 mmol, 60% dispersion in mineral oil), and the mixture was stirred at 20 °C for 2 h. Iodomethane (0.615 g, 4.33 mmol) was then added, and the mixture was stirred for a further 8 h at 20 °C and then poured into 0.1 N aqueous AcOH. Prolonged cooling gave a solid which was collected and chromatographed on silica gel. Elution with CH_2Cl_2 removed the mineral oil, and elution with CH_2Cl_2/EtOAc (19:1) gave material which was recrystallized from MeOH/H_2O to give **11** (0.72 g, 57%): mp 116−117 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.97 (s, 1 H, CON*H*Ph), 7.48 (d, J = 9.1 Hz, 2 H, H-2′,6′), 6.89 (d, J = 9.1 Hz, 2 H, H-3′,5′), 4.93 (q, J = 7.2 Hz, 1 H, CHCO), 3.72 (s, 3 H, OCH_3), 3.11 (s, 3 H, NCH_3), 1.44 (d, J = 7.2 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{13}H_{15}F_3N_2O_3$) C, H, N.

[*N*-(Benzyloxycarbonyl)methylamino]-*N*-(4-methoxyphenyl)acetamide (13). Reaction of *N*-(benzyloxycarbonyl)-sarcosine (**12**) and 4-methoxyaniline following the procedure for the preparation of **8c** gave **13** (69%): mp (MeOH/H_2O) 135 °C; ^{1}H NMR ($CDCl_3$) δ 8.01 (br s, 1 H, CON*H*Ph), 7.41−7.26 (m, 7 H, ArH, H-2′,6′), 6.84 (d, J = 9.1 Hz, 2 H, H-3′,5′), 5.19 (s, 2 H, CH_2O), 4.03 (s, 2 H, CH_2CO), 3.79 (s, 3 H, OCH_3), 3.08 (s, 3 H, NCH_3). Anal. ($C_{18}H_{20}N_2O_4$) C, H, N.

Preparation of Amines 9a−9e. 2-Amino-*N*-(4-methoxyphenyl)-2-methylpropanamide (9c). A solution of **8c** (3.60 g, 11.7 mmol) in CF_3COOH (20 mL) was stirred at 20 °C for 1 h and then concentrated under reduced pressure below 30 °C. The residue was shaken with saturated aqueous Na_2CO_3 and then evaporated to dryness under reduced pressure. The resulting solid was extracted with hot EtOAc, and the filtered extract was washed with saturated aqueous NaCl, dried, and concentrated under reduced pressure. The resulting oil was chromatographed on alumina 90, eluting with CH_2Cl_2, and the resulting product was recrystallized from petroleum ether to give **9c** (2.03 g, 84%): mp 51 °C; ^{1}H NMR ($CDCl_3$) δ 9.43 (br s, 1 H, CONH), 7.52 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.86 (d, J = 9.0 Hz, 2 H, H-3′,5′), 3.79 (s, 3 H, OCH_3), 1.45 (s, 6 H, $C(CH_3)_2$). Anal. ($C_{11}H_{16}N_2O_2$) C, H, N.

Amino-*N*-(4-methoxyphenyl)acetamide (9a). Similar treatment of **8a** gave **9a** (89%): mp (EtOAc/^{i}Pr$_2$O) 90−91 °C; ^{1}H NMR ($CDCl_3$) δ 9.24 (br s, 1 H, CONH), 7.50 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.87 (d, J = 9.0 Hz, 2 H, H-3′,5′), 3.79 (s, 3 H, OCH_3), 3.45 (s, 2 H, CH_2). Anal. ($C_9H_{12}N_2O_2$) C, H, N.

2-Amino-*N*-(4-methoxyphenyl)propanamide (9b). Similar treatment of **8b**, followed by purification of the product by chromatography on alumina 90, eluting with CH_2Cl_2/EtOAc (1:1), gave **9b** (85%): mp 64−65 °C; ^{1}H NMR ($CDCl_3$) δ 9.33 (br s, 1 H, CONH), 7.50 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.86 (d, J = 9.0 Hz, 2 H, H-3′,5′), 3.79 (s, 3 H, OCH_3), 3.61 (q, J = 7.0 Hz, 1 H, CH), 1.42 (d, J = 7.0 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{10}H_{14}N_2O_2$) C, H, N.

***N*-(4-Methoxyphenyl)-2-(methylamino)propanamide (9d).** A mixture of **11** (0.62 g, 2.04 mmol) and Cs_2CO_3 (1.63 g) in 50% aqueous MeOH (5 mL) was heated under reflux for 30 min and then evaporated to dryness under reduced pressure. The resulting residue was extracted with EtOAc (2 × 25 mL), and the combined extract was filtered and evaporated. Recrystallization of the product from petroleum ether gave **9d** (0.36 g, 86%): mp 77 °C; ^{1}H NMR ($CDCl_3$) δ 9.15 (br s, 1 H, CONH), 7.51 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.87 (d, J = 9.0 Hz, 2 H, H-3′,5′), 3.79 (s, 3 H, OCH_3), 3.15 (q, J = 6.9 Hz, 1 H, CH), 2.46 (s, 3 H, NCH_3), 1.38 (d, J = 7.0 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{11}H_{16}N_2O_2$) C, H, N.

***N*-(Methoxyphenyl)(methylamino)acetamide (9e).** A suspension of **13** in EtOH was hydrogenated over 5% Pd/C at 60 psi for 6 h to give **9e** (96%): mp (^{i}Pr$_2$O/petroleum ether) 53−54 °C; ^{1}H NMR ($CDCl_3$) δ 9.13 (br s, 1 H, CONH), 7.50 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.87 (d, J = 9.0 Hz, 2 H, H-3′,5′), 3.79 (s, 3 H, OCH_3), 3.34 (s, 2 H, CH_2), 2.50 (s, 3 H, NCH_3). Anal. ($C_{10}H_{14}N_2O_2$) C, H, N.

Preparation of (2,6-Dinitrophenyl)amino Acid Derivatives. 2-[(4-Carboxy-2,6-dinitrophenyl)amino]-*N*-(4-methoxyphenyl)propanamide (15). A mixture of 4-chloro-3,5-dinitrobenzoic acid (**14**) (2.12 g, 8.6 mmol), amine **9b** (2.00 g, 10.3 mmol), and diisopropylethylamine (2.34 g, 18.1 mmol) in THF (20 mL) was stirred at 20 °C for 12 h and then heated at 50 °C for 30 min. The mixture was concentrated under reduced pressure, and the residue was extracted with warm dilute aqueous ammonia. The filtrate was acidified with concentrated HCl, and the resulting solid was recrystallized twice from EtOH/H_2O to give **15** (2.97 g, 85%): mp 219−221 °C dec; ^{1}H NMR [$(CD_3)_2SO$] δ 13.60 (br s, 1 H, COOH), 10.22 (s, 1 H, CON*H*Ph), 9.30 (d, J = 7.5 Hz, 1 H, PhN*H*CH_2), 8.66 (s, 2 H, H-3,5), 7.47 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.90 (d, J = 9.0 Hz, 2 H, H-3′,5′), 4.15 (quint, J = 6.8 Hz, 1 H, C*H*CH_3), 3.73 (s, 3 H, OCH_3), 1.37 (d, J = 6.7 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{17}H_{16}N_4O_8$) C, H, N.

2-[[4-[*N*-[2-(Dimethylamino)ethyl]carbamoyl]-2,6-dinitrophenyl]amino]-*N*-(4-methoxyphenyl)propanamide (16). A stirred solution of acid **15** (1.20 g, 2.97 mmol) in DMF (8 mL) was treated with 1,1′-carbonyldiimidazole (0.577 g, 3.56 mmol), at 20 °C for 30 min, then cooled to 0 °C, and treated with *N*,*N*-dimethylethylenediamine (0.367 g, 4.16 mmol). The mixture was stirred for a further 15 min at 20 °C and then diluted with dilute $KHCO_3$. The precipitated solid was dissolved in dilute aqueous AcOH, and the filtered solution was basified with dilute $KHCO_3$. Crystallization of the precipitate from aqueous MeOH followed by EtOAc/petroleum ether gave **16** (0.98 g, 70%): mp 163−164 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 10.21 (s, 1 H. CON*H*Ph), 9.10 (d, J = 7.5 Hz, 1 H, PhN*H*CH), 8.82−8.73 (m, 3 H, PhCON*H*, H-3,5), 7.46 (d, J = 8.9 Hz, 2 H, H-2′,6′), 6.90 (d, J = 9.0 Hz, 2 H, H-3′,5′), 4.13 (quint, J = 6.8 Hz, 1 H, C*H*CH_3), 3.72 (s, 3 H, OCH_3), ca. 3.4 (under H_2O, CH_2), 2.42 (t, J = 6.6 Hz, 2 H, CH_2), 2.19 (s, 6 H, $N(CH_3)_3$), 1.37 (d, J = 6.7 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{21}H_{26}N_6O_7$) C, H, N.

2-[[4-[*N*-(2,3-Dihydroxypropyl)carbamoyl]-2,6-dinitrophenyl]amino]-*N*-(4-methoxyphenyl)propanamide (17). A stirred solution of acid **15** (1.60 g, 3.96 mmol) in DMF (10 mL) was treated with 1,1′-carbonyldiimidazole (0.707 g, 4.36 mmol) at 20 °C for 30 min, then cooled to −5 °C, and treated in one portion with 3-amino-1,2-propanediol (0.469 g, 5.15 mmol). The mixture was concentrated under reduced pressure below 50 °C, and the residue was shaken with dilute $KHCO_3$. The resulting solid was crystallized from aqueous MeOH followed by MeOH/EtOAc/^{i}Pr$_2$O to give **17** (1.21 g, 64%): mp 203 °C dec; ^{1}H NMR [$(CD_3)_2SO$] δ 10.21 (s, 1 H. CON*H*Ph), 9.09 (d, J = 7.4 Hz, 1 H, PhN*H*CH), 8.84−8.75 (m, 3 H, PhCON*H*, H-3,5), 7.46 (d, J = 8.8 Hz, 2 H, H-2′,6′), 6.90 (d, J = 8.8 Hz, 2 H, H-3′,5′), 4.85 (d, J = 4.9 Hz, 1 H, CHO*H*), 4.59 (t, J = 5.7 Hz, 1 H, CH_2O*H*), 4.13 (quint, J = 6.5 Hz, 1 H, C*H*CH_3), 3.72 (s, 3 H, OCH_3), 3.68−3.60 (m, 1 H, C*H*OH), 3.46−3.30 (m, 2 H, CH_2), 3.24−3.13 (m, 2 H, CH_2), 1.37 (d, J = 6.6 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{20}H_{23}N_5O_9$) C, H, N.

4-Chloro-*N*-(2-hydroxyethyl)-3,5-dinitrobenzamide (18). A mixture of **14** (4.66 g, 18.9 mmol), $SOCl_2$ (25 mL), and DMF (0.2 mL) was heated under reflux for 30 min; then excess reagent was removed under reduced pressure. Traces of $SOCl_2$ were removed by azeotroping with benzene, to give a crude acid chloride, which was dissolved in Me_2CO (35 mL). The solution was cooled to 0 °C and added in one portion to a stirred solution of 2-aminoethanol (2.53 g, 41.4 mmol) in water (45 mL) at 0 °C. The mixture was stirred vigorously for a further 30 s and then immediately treated with concentrated HCl (1 mL). Dilution with water (400 mL) gave a yellow solid, which was stirred as a suspension in 10% aqueous $KHCO_3$ (50 mL) and then collected and recrystallized sequentially from EtOAc and MeOH/H_2O/trace concentrated HCl to give **18** (4.06 g, 74%): mp 167−168 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.02 (t, J = 5.3 Hz, 1 CONH), 8.80 (s, 2 H, H-2,6), 4.80 (t, J = 5.7 Hz, 1 H, OH), 3.54 (q, J = 5.8 Hz, 2 H, CH_2), 3.37 (q, J = 5.8 Hz, 2 H, CH_2). Anal. ($C_9H_8ClN_3O_6$) C, H, N.

2-[[2,6-Dinitro-4-[*N*-(2-hydroxyethyl)carbamoyl]phenyl]amino]-*N*-(4-methoxyphenyl)propanamide (19b). A mixture of **18** (1.00 g, 3.45 mmol) and amine **9b** (1.01 g, 5.20 mmol) in THF (12 mL) was treated with diisopropylethylamine (0.45 g, 3.48 mmol). The reaction was stirred at 20 °C for 8 h and then diluted with 0.5 N HCl. The precipitated solid was

recrystallized twice from MeOH/H_2O to give **19b** (1.36 g, 88%): mp 183−184 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 10.20 (s, 1 H, CON*H*Ph), 9.09 (d, J = 7.5 Hz, 1 H, PhN*H*CH), 8.85−8.76 (m, 3 H, H-3,5, PhCON*H*), 7.46 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.90 (d, J = 9.0 Hz, 2 H, H-3′,5′), 4.76 (br s, 1 H, OH), 4.13 (quint, J = 7.1 Hz, 1 H, C*H*CH_3), 3.73 (s, 3 H, OCH_3), 3.52 (t, J = 6.0 Hz, 2 H, CH_2), 3.38−3.30 (m, 2 H, CH_2), 1.37 (d, J = 6.7 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{19}H_{21}N_5O_8$) C, H, N.

[[2,6-Dinitro-4-[*N*-(2-hydroxyethyl)carbamoyl]phenyl]-amino]-*N*-(4-methoxyphenyl)acetamide (19a). Reaction of **18** with amine **9a** by the above procedure gave **19a** (91%): mp (DMF/MeOH) 238−239 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 10.22 (s, 1 H, CON*H*Ph), 9.37 (s, 1 H, PhN*H*CH_2), 8.80 (s, 3 H, H-3,5, PhCON*H*), 7.47 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.90 (d, J = 9.0 Hz, 2 H, H-3′,5′), 4.76 (t, J = 5.6 Hz, 1 H, OH), 3.85 (d, J = 3.8 Hz, 2 H, PhNHC*H*$_2$), 3.72 (s, 3 H, OCH_3), 3.52 (q, J = 5.8 Hz, 2 H, CH_2), 3.38−3.27 (m, 2 H, CH_2). Anal. ($C_{18}H_{19}N_5O_8$) C, H, N.

2-[[2,6-Dinitro-4-[*N*-(2-hydroxyethyl)carbamoyl]phenyl]-amino]-*N*-(4-methoxyphenyl)-2-methylpropanamide (19c). Reaction of **18** with amine **9c** (for 60 h) by the above procedure gave **19c** (35%): mp (MeOH/H_2O) 199−200 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.64 (s, 1 H, CON*H*Ph), 8.85 (t, J = 5.4 Hz, 1 H, PhCON*H*), 8.78 (s, 2 H, H-3,5), 8.11 (s, 1 H, N*H*C$(CH_3)_2$), 7.52 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.91 (d, J = 9.0 Hz, 2 H, H-3′,5′), 4.77 (t, J = 5.7 Hz, 1 H, OH), 3.74 (s, 3 H, OCH_3), 3.52 (q, J = 5.9 Hz, 2 H, CH_2), 3.40−3.30 (m, 2 H, CH_2), 1.45 (s, 6 H, $C(CH_3)_2$. Anal. ($C_{20}H_{23}N_5O_8$) C, H, N.

[*N*-[2,6-Dinitro-4-[*N*-(2-hydroxyethyl)carbamoyl]phenyl]methylamino]-*N*-(4-methoxyphenyl)acetamide (19d). Reaction of **18** with amine **9e** by the above procedure for 2 h gave a crude product which was purified by chromatography on silica gel. Elution with CH_2Cl_2/EtOAc (1:3) followed by two recrystallizations from MeOH/H_2O containing a trace of AcOH (the compound is extremely base-sensitive, giving deep-purple solutions at pH > 7) gave **19d** (63%): mp 98−101 °C; ^{1}H NMR [$(CD_3)_2SO$, 200 MHz] δ 9.68 (s, 1 H, CON*H*Ph), 8.84 (t, J = 5.4 Hz, 1 H, PhCON*H*), 8.59 (s, 2 H, H-3,5), 7.46 (d, J = 9.1 Hz, 2 H, H-2′,6′), 6.89 (d, J = 9.1 Hz, 2 H, H-3′,5′), 4.77 (t, J = 5.6 Hz, 1 H, OH), 3.72 (s, 5 H, OCH_3, CH_2CO), 3.53 (q, J = 5.6 Hz, 2 H, CH_2), 3.40−3.30 (m, 2 H, CH_2), 2.89 (s, 3 H, NCH_3). Anal. ($C_{19}H_{21}N_5O_8$·H_2O) C, H, N.

2-[*N*-[2,6-Dinitro-4-[*N*-(2-hydroxyethyl)carbamoyl]phenyl]methylamino]-*N*-(4-methoxyphenyl)propanamide (19e). Reaction of **18** with amine **9d** by the above procedure for 48 h gave a crude product which was purified by chromatography on silica gel. Elution with EtOAc followed by recrystallization from MeOH/H_2O containing a trace of AcOH (the compound is extremely base-sensitive, giving deep-purple solutions at pH > 7) gave **19e** (74%): mp 192−193 °C; ^{1}H NMR [$(CD_3)_2SO$, 200 MHz] δ 9.70 (s, 1 H, CON*H*Ph), 8.83 (t, J = 5.5 Hz, 1 H, PhCON*H*), 8.85 (s, 2 H, H-3,5), 7.42 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.86 (d, J = 9.1 Hz, 2 H, H-3′,5′), 4.75 (br s, 1 H, OH), 3.87 (q, J = 6 7 Hz, 1 H, C*H*CH_3), 3.71 (s, 3 H, OCH_3), 3.58−3.47 (m, 2 H, CH_2), 3.41−3.38 (m, 2 H, CH_2), 2.81 (s, 3 H, NCH_3), 1.27 (d, J = 6.7 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{20}H_{23}N_5O_8$) C, H, N.

***N*^{1}-Hydroxy-7-[*N*-(2-hydroxyethyl)carbamoyl]-3-methyl-5-nitro-3,4-dihydroquinoxalin-2(1*H*)-one (21) (Cyclization Product of 19b)**. Radiolytic reductions revealed that ring-closed (dihydroquinoxaline) products underwent subsequent reductions when excess (>4-fold stoichiometry) reducing equivalents were added. This propensity to undergo further reductive reactions thwarted attempts to prepare them by chemical reduction, and a sample had to be obtained by purification of a radiolysis reaction. A stirred solution of **19b** (22 μM), 2-propanol (1 M), and acetone (0.3 M) in Milli-Q was evacuated and irradiated for 4.5 h (^{60}Co, 0.01 Gy s^{-1}). The resultant solution was assayed by HPLC to verify formation of 4-methoxyaniline and a cyclized product. Solvents were then removed by rotary evaporation under reduced pressure. The mass spectrum of the resultant product mixture (FAB, sample solubilized in 2-nitrobenzyl alcohol) gave an MH$^+$ peak at 311, corresponding to the *N*-hydroxydihydroquinoxalinone **21** (calcd for $C_{12}H_{15}N_4O_6$, 311.0992; found, 311.0987).

***N*-[4-[Bis(2-chloroethyl)amino]phenyl]-2-[[4-[*N*-[2-(dimethylamino)ethyl]carbamoyl]-2,6-dinitrophenyl]amino]propanamide (26) (Scheme 4).** A stirred mixture of *N*-(*tert*-butyloxycarbonyl)alanine (**7b**) (1.89 g, 10 mmol) and 4-[bis(2-chloroethyl)amino]aniline hydrochloride[19] (**22**) (2.70 g, 10 mmol) in DMF (10 mL) was treated at 0 °C with diethyl cyanophosphonate (1.84 g of 93%, 10.5 mmol) and then dropwise with diisopropylethylamine (2.71 g, 21 mmol). The mixture was stirred at 20 °C for 1.5 h and then diluted with 10% aqueous Na_2CO_3. The precipitated semisolid was dissolved in CH_2Cl_2, washed with water, and dried. The solution was concentrated under reduced pressure below 30 °C to ca. 20 mL and then diluted with petroleum ether to give *N*-[4-[bis-(2-chloroethyl)amino]phenyl]-2-(*tert*-butyloxycarbonylamino)-propanamide (**23**) (3.79 g, 94%): mp (CH_2Cl_2/^{i}Pr_2O) 61−63 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 9.62 (s, 1 H, CON*H*Ph) 7.42 (d, J = 9.0 Hz, 2 H, H-2′,6′), 6.96 (d, J = 7.3 Hz, 1 H, N*H*CH) 6.71 (d, J = 9.1 Hz, 2 H, H-3′,5′), 4.07 (quintet, J = 7.1 Hz, 1 H, NHC*H*) 3.69 (t, J = 3.2 Hz, 8 H, $N(CH_2CH_2Cl)_2$), 1.38 (s, 9 H, $C(CH_3)_3$), 1.23 (d, J = 7.1 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{18}H_{27}Cl_2N_3O_3$·$0.5C_6H_{14}O$) C, H, N.

A solution of **23** (3.39 g, 8.4 mmol) in trifluoroacetic acid (15 mL) was stirred at 20 °C for 2 h and then poured slowly into excess ice-cold 10% Na_2CO_3 solution. The resulting mixture was extracted with EtOAc, washed twice with water, and dried, and the organic layer was evaporated under reduced pressure below 30 °C to provide 2-amino-*N*-[4-[bis(2-chloroethyl)amino]phenyl]propanamide (**24**) (2.37 g, 93%) as an unstable colorless oil, which was used directly: ^{1}H NMR [$(CD_3)_2SO$] δ 9.56 (br s, 1 H, CONH), 7.46 (d, J = 9.1 Hz, 2 H, H-2′,6′), 6.70 (d, J = 9.1 Hz, 2 H, H-3′,5′), 3.69 (t, J = 3.7 Hz, 8 H, $N(CH_2CH_2Cl)_2$), 3.36 (q, J = 6.9 Hz, 1 H, CHCO), 1.19 (d, J = 6.9 Hz, 3 H, CH_3).

A mixture of **24** (2.19 g, 7.2 mmol) and 4-chloro-3,5-dinitrobenzoic acid (1.48 g, 6.00 mmol) in THF (12 mL) was treated with diisopropylethylamine (1.71 g, 13.2 mmol) and stirred at 20 °C for 12 h. The mixture was poured into excess in aqueous AcOH, and the precipitated semisolid was taken up in EtOAc. The organic solution was washed successively with 1 N aqueous AcOH and water, then dried, concentrated under reduced pressure below 30 °C to ca. 20 mL, and diluted with petroleum ether. The resulting solid was chromatographed on silica gel. EtOAc eluted *N*-[4-[bis(2-chloroethyl)-amino]phenyl]-2-[(4-carboxy-2,6-dinitrophenyl)amino]propanamide (**25**) (2.19 g, 71%), which crystallized from EtOAc/CH_2Cl_2/petroleum ether as green-brown prisms: mp 192−194 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 13.60 (br s, 1 H, CO_2H), 10.11 (s, 1 H, CONH), 9.31 (d, J = 6.9 Hz, 1 H, N*H*CH), 8.66 (s, 2 H, H-3,5), 7.39 (d, J = 9.1 Hz, 2 H, H-2′,6′), 6.73 (d, J = 9.1 Hz, 2 H, H-3′,5′), 4.14 (quintet, J = 6.8 Hz, 1 H, CHCO), 3.70 (s, 8 H, $N(CH_2CH_2Cl)_2$), 1.36 (d, J = 6.7 Hz, 3 H, CH_3). Anal. ($C_{20}H_{21}Cl_2N_5O_7$) C, H, N, Cl.

A stirred solution of **25** (0.86 g, 1.67 mmol) in DMF (4 mL) was treated with 1,1′-carbonyldiimidazole (0.30 g, 1.84 mmol) at 25 °C for 1 h, then cooled to 0 °C, and treated with *N*,*N*-dimethylethylenediamine (0.19 g, 2.16 mmol). The mixture was allowed to warm to 25 °C over a 5-min period and was then diluted with aqueous $KHCO_3$. The resulting solid was purified by chromatography on neutral Al_2O_3 (activity I). Elution with CH_2Cl_2 removed nonpolar impurities. Further elution with EtOAc/MeOH (20:1) gave an eluent which was concentrated under reduced pressure below 30 °C to ca. 10 mL and then diluted with petroleum ether to afford **26** (0.62 g, 62%): mp (EtOAc/petroleum ether) 95−98 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 10.10 (s, 1 H, CHCON*H*), 9.13 (d, J = 7.5 Hz, 1 H, N*H*CHCO), 8.78 (s, 2 H, H-3,5), 8.76 (t, J = 5.6 Hz, 1 H, N*H*CH_2), 7.38 (d, J = 9.1 Hz, 2 H, H-2′,6′), 6.73 (d, J = 9.1 Hz, 2 H, H-3′,5′), 4.11 (quintet, J = 6.8 Hz, 1 H, CHCO), 3.70 (s, 8 H, $N(CH_2CH_2Cl)_2$), 3.28−3.42 (m, 2 H, NHC*H*$_2$), 2.39 (t, J = 6.7 Hz, 2 H, C*H*$_2$N$(CH_3)_2$), 2.17 (s, 6 H, N$(CH_3)_2$), 1.35 (d, J = 6.6 Hz, 3 H, CH_3). Anal. ($C_{24}H_{31}Cl_2N_7O_6$) C, H, N, Cl. Treatment with

EtOAc/HCl (1:1 equiv) followed by addition of petroleum ether gave the hydrochloride salt.

***N*-[1-(Chloromethyl)-3-[(5,6,7-trimethoxyindol-2-yl)carbonyl]-1,2-dihydro-3*H*-benz[*e*]indol-5-yl]-2-[[4-[*N*-[2-(dimethylamino)ethyl]carbamoyl]-2,6-dinitrophenyl]amino]propanamide (32) (Scheme 5).** A mixture of **14** (0.59 g, 2.4 mmol), alanine 4-methoxybenzyl ester (**27**) (0.55 g, 2.6 mmol), and diisopropylethylamine (0.63 g, 4.9 mmol) in THF (10 mL) was stirred at 25 °C for 30 h and then concentrated under reduced pressure below 30 °C. The residue was shaken with dilute HCl, and the resulting yellow solid was twice recrystallized from EtOAc/$^{i}Pr_2O$ to give 4-methoxybenzyl 2-[(4-carboxy-2,6-dinitrophenyl)amino]propanoate (**28**) (0.51 g, 51%): mp 156.5−157 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 13.6 (v br s, 1 H, CO_2H), 8.60 (s, 2 H, H-3′,5′), 8.59 (d, J = 8.4 Hz, partially obscured, 1 H, NH), 7.25 (d, J = 8.7 Hz, 2 H, H-2″, 6″), 6.89 (d, J = 8.7 Hz, 2 H, H-3″,5″), 5.09 (d, J = 11.9 Hz, 1 H, Ph C*H*H), 5.02 (d, J = 11.9 Hz, 1 H, PhCH*H*), 4.05−3.95 (m, 1 H, C*H*CH_3), 3.75 (s, 3 H, OCH_3), 1.41 (d, J = 7.0 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{18}H_{17}N_3O_9$) C, H, N.

A mixture of **28** (252 mg, 0.60 mmol) and 1,1′-carbonyldiimidazole (117 mg, 0.72 mmol) in DMF (4 mL) was stirred at 20 °C for 1 h and then warmed to 50 °C for 5 min. The mixture was cooled to 0 °C and treated with *N*,*N*-dimethylethylenediamine (79 mg, 0.90 mmol). After being stirred at 20 °C for 5 min, the mixture was diluted with excess dilute $KHCO_3$, and the resulting oily precipitate was partitioned between CH_2Cl_2 and dilute $KHCO_3$. The organic layer was washed with NaCl solution, dried, and concentrated under reduced pressure. The residue was triturated with $^{i}Pr_2O$, providing a solid that was twice recrystallized from CH_2Cl_2/EtOAc to give 4-methoxybenzyl 2-[[4-[*N*-[2-(dimethylamino)ethyl]carbamoyl]-2,6-dinitrophenyl]amino]propanoate (**29**) (218 mg, 74%): mp 107−109 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 8.76 (t, J = 5.5 Hz, partially obscured, 1 H, CONH), 8.74 (s, 2 H, H-3′,5′), 8.45 (d, J = 8.4 Hz, 1 H, PhN*H*), 7.24 (d, J = 8.6 Hz, 2 H, H-2″,6″), 6.89 (d, J = 8.6 Hz, 2 H, H-3″,5″), 5.08 (d, J = 11.9 Hz, 1 H, Ph C*H*H), 5.02 (d, J = 11.9 Hz, 1 H, Ph CH*H*), 4.02 (m, 1 H, C*H*CH_3), 3.75 (s, 3 H, OCH_3), 3.37 (q, J = 6.3 Hz, 2 H, NHC*H*$_2$), 2.40 (t, J = 6.7 Hz, 2 H, $NHCH_2CH_2$), 2.18 (s, 6 H, $N(CH_3)_2$), 1.40 (d, J = 6.9 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{22}H_{27}N_5O_8$) C, H, N.

The benzyl ester **29** (1.75 g, 3.58 mmol) was treated with anisole (0.58 mL) followed by ice-cold CF_3CO_2H (15 mL). The resulting solution was stirred at 20 °C for 15 min and then concentrated under reduced pressure below 30 °C. The residue was triturated repeatedly with $^{i}Pr_2O$ to provide a semisolid which was dissolved in EtOAc. The solution was clarified by filtration, cooled to −5 °C, treated with dry HCl/EtOAc, and then diluted with $^{i}Pr_2O$. The precipitated yellow solid was collected and washed with EtOAc/$^{i}Pr_2O$ to give 2-[[4-[*N*-[2-(dimethylamino)ethyl]carbamoyl]-2,6-dinitrophenyl]amino]-propanoic acid hydrochloride (**30**) (1.34 g, 92%) as a hygroscopic solid, suitable for further use. A sample recrystallized from MeOH/EtOAc/$^{i}Pr_2O$ had mp 92−96 °C; ^{1}H NMR [$(CD_3)_2$-SO] δ 13.5 (v br s, 1 H, CO_2H), 10.35 (s, 1 H, N^+H), 9.23 (t, J = 5.5 Hz, 1 H, CONH), 8.83 (s, 2 H, H-3′,5′), 8.76 (d, J = 7.7 Hz, 1 H, Ph N*H*), 3.92 (quin, J = 7.1 Hz, 1 H, C*H*CH_3), 3.65 (q, J = 5.2 Hz, 2 H, CONHC*H*$_2$), 3.27 (q, J = 5.5 Hz, 2 H, C*H*$_2N^+H(CH_3)_2$), 2.81 (d, J = 4.5 Hz, 6 H, $N(CH_3)_2$), 1.36 (d, J = 6.8 Hz, 3 H, CHC*H*$_3$). Anal. ($C_{14}H_{20}ClN_5O_7\cdot H_2O$) C, H, N, Cl.

A mixture of **30** (172 mg, 0.42 mmol), 5-amino-1-(chloromethyl)-3-[(5,6,7-trimethoxyindol-2-yl)carbonyl]-1,2-dihydro-3*H*-benz[*e*]indole[20] **(31)** (165 mg, 0.35 mmol) and EDCI·HCl (201 mg, 1.05 mmol) in DMA (2 mL) was stirred at 20 °C for 4 h and then diluted with dilute $KHCO_3$. The precipitated solid was collected, washed with water, then extracted with 0.1 N AcOH, and filtered. The clarified solution was treated with solid $KHCO_3$ and extracted twice with EtOAc. The combined extracts were washed with water, dried (Na_2SO_4), concentrated to a small volume under reduced pressure below 30 °C, and then diluted with $^{i}Pr_2O$. The resulting product was purified by precipitation from a MeOH/EtOAc solution at 20 °C with $^{i}Pr_2O$ to give **32** (130 mg, 45%) as a light-sensitive solid: mp >200 °C; ^{1}H NMR [$(CD_3)_2SO$] δ 11.49 (s, 1 H, indole NH), 10.43 (s, 1 H, N*H*COCH), 9.08 (t, J = 7.6 Hz, 1 H, N*H*CH), 8.81 (s, 2 H, H-3‴,5‴), 8.78 (t, J = 5.7 Hz, 1 H, CON*H*CH_2), 8.59 (br s, 1 H, H-4′), 8.03−7.94 (m, 2 H, H-6′,9′), 7.60 (t, J = 7.6 Hz, 1 H, H-8′), 7.49 (t, J = 7.6 Hz, 1 H, H-7′), 7.11 (d, J = 1.7 Hz, 1 H, H-3″), 6.98 (s, 1 H, H-4″), 4.81 (t, J = 10.1 Hz, 1 H, H-2′), 4.54 (dd, J = 11.0, 1.8 Hz, 1 H, H-2′), 4.47−4.33 (m, 2 H, H-1′, C*H*HCl), 4.08 (dd, J = 11.1, 2.9 Hz, 1 H, CH*H*Cl), 3.99−3.96 (m, 1 H, C*H*CH_3), 3.93 (s, 3 H, OCH_3), 3.82 (s, 3 H, OCH_3), 3.80 (s, 3 H, OCH_3), 3.38 (q, J = 6.3 Hz, 2 H, NHC*H*$_2$), 2.42 (t, J = 6.5 Hz, 2 H, $NHCH_2CH_2$), 2.19 (s, 6 H, $N(CH_3)_2$), 1.55 (dd, J = 6.5, 1.7 Hz, 3 H, CHC*H*$_3$). Treatment of the free base with MeOH/EtOAc/HCl (1:2 equiv) followed by dilution with $^{i}Pr_2O$ gave the hydrochloride salt: mp 210−215 °C dec. Anal. ($C_{39}H_{41}$-$ClN_8O_{10}\cdot HCl\cdot 3H_2O$) C, H, N, Cl.

Radiolytic Reductions. These were carried out by ^{60}Co γ-radiation (ca. 0.7 Gy s^{-1}) in 10 mM sodium formate or phosphate buffers at pH range 4.0 or 7.4, respectively, under anaerobic conditions. Reducing conditions were realized by the addition of either 1 M 2-propanol and 1 M acetone or 0.5 M 2-propanol to the anaerobic buffer, to convert oxidizing $^{\cdot}OH$ radicals (formed on γ-radiolysis of water) to the reducing $(CH_3)_2C^{\cdot}OH$ radical. The 2-propanol/acetone mixed system was used for compounds **19a** and **17** to increase solubility. All radiolytic reductions, and studies of subsequent reactions, were carried out at room temperature. HPLC and spectrophotometry was carried out immediately on removing samples from the γ-source. Samples were introduced to the spectrophotometer by pouring the solution through a sidearm into a pre-attached quartz cuvette. The observed pseudo-first-order rate coefficients (k_{obs}) were determined using a modified Marquardt iterative curve-fitting procedure.

HPLC assays employed an Econosphere C-18 column (5-mm particle size, 250-mm × 4.6-mm i.d.), injecting 50 μL of sample through a 20-μL sample loop. Both isocratic and gradient elutions were used, with mobile phases comprising methanol and either phosphate buffer (pH 6.5, 0.01 M) or formate buffer (pH 4.0, 0.01 M) to assay radiolysis products formed at pH 7.4 and 4.0, respectively, at a flow rate of 0.8 mL min^{-1}. An HP1040M SeriesII diode array detector allowed the eluent to be monitored at several analytical wavelengths (internally blanked against 590 nm) as well as the collection of spectra (in the range 200−400 nm).

Cell Culture Growth Inhibition Assays. Cell cultures were initiated in 96-well plates in 50 μL of culture medium (αMEM with 5% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin) at 200 (AA8), 300 (UV4), 75 (EMT6), or 600 (SKOV3) cells/well and grown for 24 h before exposure to drugs. Drug stock solutions were prepared in DMSO and diluted into culture medium (final DMSO concentration < 1%), and the pH was adjusted to 7.4 if necessary under 5% CO_2. Cells were exposed under aerobic conditions for 4 h, drugs were removed by washing cultures three times with fresh medium, and cultures were incubated in 150 μL of medium for a further 4−5 days before staining with methylene blue.[26] IC_{50} values were calculated as the drug concentration providing 50% inhibition of growth relative to controls on the same plate.

Determination of Hypoxia-Selective Cytotoxicity in Vitro by Clonogenic Assay. UV4 cells were grown to early plateau phase (5 × 10^5 cell/mL) in spinner flasks and resuspended in fresh medium. Cell suspensions and drug solutions in growth medium were preequilibrated with magnetic stirring under 5% CO_2 in air or N_2 for 1 h prior to mixing to ensure essentially complete anoxia throughout the period of drug contact in hypoxic cultures. After exposure of cells to drug for 1 h at 10^6/mL with continuous gassing, clonogenic assays were performed as detailed elsewhere.[27] The ratios of the concentration for a surviving fraction of 10% (C_{10}) for the aerobic and hypoxic survival curves were used as the measure of hypoxic selectivity.

Determination of Cytotoxicity of Radiolytically Activated Prodrugs. Compounds were dissolved in culture medium containing 5% fetal bovine serum and deoxygenated

by stirring under a stream of 5% CO_2 in N_2. Solutions were irradiated (2.2 Gy min^{-1}, ^{60}Co) and immediately frozen for subsequent bioassay against UV4 cells using the above growth inhibition assay. The IC_{50} of authentic effector was determined in the same experiments. The concentration of cytotoxic effector, [E], was calculated from the change in apparent IC_{50} of the irradiated solution using the following relationship:

$$[E] = \frac{\left(\frac{IC_{50},E}{IC_{50}} \times [P]_0\right) - \left(\frac{IC_{50},E}{IC_{50},P} \times [P]_0\right)}{1 - \frac{IC_{50},E}{IC_{50},P}}$$

where IC_{50},E and IC_{50},P are the IC_{50} values of the pure effector and (unirradiated) prodrug, respectively, and IC_{50} is the IC_{50} value of the irradiated mixture, expressed relative to the initial concentration of the prodrug, $[P]_0$. This calculation assumes that the cytotoxicity of prodrug and effector is independent and additive. Full details of this testing methodology, including derivation of the above equation, will be reported in full elsewhere.[25]

In Vivo Testing. All studies used C_3H/HeN mice bred in this institution, and all experiments were approved under The University of Auckland Animal Ethics Committee. Compounds were formulated in dimethylacetamide, poly(ethylene glycol) (MW 400), and water (10/40/50, v/v/v) and administered ip at 0.01 mL/g of body weight. Toxicity was evaluated using 1.33-fold dose increments, with an observation time of 60 days. The maximum tolerated dose was defined at the highest dose causing no deaths or significant morbidity in a group of 6 animals. Evaluation of antitumor activity was performed essentially as described by Twentyman et al.[28] Briefly, RIF-1 tumors were grown im to 0.5 g (leg + tumor diameter 10 mm), and mice were treated with single doses of drug and/or radiation (15 Gy, ^{60}Co, 2 Gy min^{-1}), the latter by restraining unanesthetized mice in boxes and extending the leg into the radiation field using a clip to tether the ankle. Tumors were excised 18 h after the last treatment, dissociated enzymatically, and plated in vitro to determine the number of surviving clonogenic cells/tumor.

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