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Leaving group effects in reductively triggered fragmentation of 4-nitrobenzyl carbamates †

Bridget M. Sykes,[b] Michael P. Hay,*[a] Dubravka Bohinc-Herceg,[b] Nuala A. Helsby,[a] Charmian J. O'Connor[b] and William A. Denny[a]

[a] *Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences and* [b] *Department of Chemistry, The University of Auckland, Private Bag 92019, Auckland, New Zealand*

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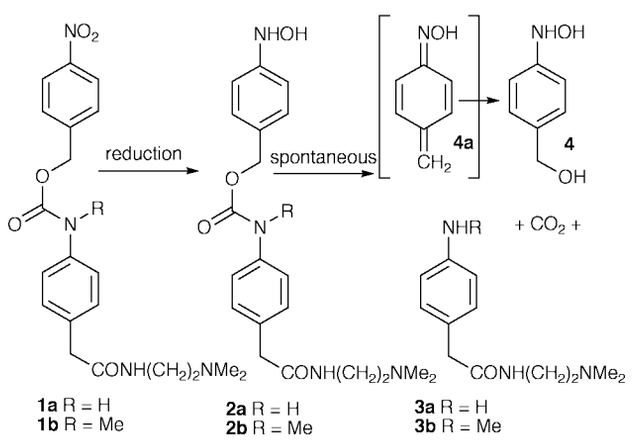
The rates and extent of release of a series of substituted anilines from 4-nitrobenzyl carbamates, following nitro group reduction by radiolytic, enzymic and chemical methods, are reported. The yield of released anilines decreased over the pH range 4–7, but was independent of the basicity of the leaving aniline. Detailed studies of the fragmentation of one example identified the 4-hydroxylamine as the key intermediate. At pH greater than 5 the released aniline **3b** condenses with a reactive 4-iminoquinomethane intermediate **4a** to give amine **26**, thus depleting the measurable amount of aniline **3b** released. At pH less than 5 the release of amine proceeds to completion. The efficiency of reductively triggered release of anilines **7** varied with small changes in the leaving group, but this was not uniformly related to aniline basicity. The competing reaction of the released aniline **3b** to form amine **26** lowers the efficiency of release of **3b**. This reaction occurs at the relatively high concentrations (50 μM) used in the study and indicates the released effector amine should be toxic at concentrations considerably lower than 50 μM. This highlights the need for prodrugs of very potent cytotoxic effectors to be used in tumour-directed nitroreductase enzyme-prodrug therapy.

Introduction

Many recent designs for bioreductive prodrugs have employed 4-nitrobenzyl carbamate triggers,[1,2] particularly in conjunction with the *E. coli* B nitroreductase enzyme (NTR)[3] in ADEPT[4] and GDEPT[5] protocols. This enzyme reduces 4-nitrobenzyl carbamates to the corresponding hydroxylamines[6] that are known to fragment to release amine-based effectors (Scheme 1).[7] However, there have been relatively few



Scheme 1 Reductive fragmentation of nitrobenzyl carbamates **1**.

comparative studies of important properties such as the rate of fragmentation following reduction, and the degree of release of the intact amine effector. One report,[8] on the acid-catalysed fragmentation of substituted benzyl carbamates, showed that fragmentation of the protonated carbamate was insensitive to changes on the carbamate nitrogen.

We recently reported[9] a study of the rates of fragmentation of substituted 4-{*N*-[4-(hydroxyamino)benzyloxycarbonyl]-amino}phenylacetamides (*e.g.*, **2**), which were generated from the corresponding nitro compounds (*e.g.*, **1**) by radiolytic reduction. Electron-donating substituents on the nitrobenzyl ring accelerated fragmentation (measured as the half-life of the hydroxyamino compounds), consistent with stabilisation of the developing positive charge on the benzylic carbon. This work also showed that the nature of the leaving amine had an effect on the rate of fragmentation (**2a** fragmented about twice as rapidly as **2b**), and the degree of release of the effector amine **3** (from various benzyl ring-substituted analogues of **2**) varied from 24 to 73%.[9] Other work[1] has also shown variations in rates of release, depending on the nature of the leaving amine, although these studies used the *E. coli* NTR enzyme, and enzyme or substrate variations cannot be ruled out.

Incomplete release of effector amines (*e.g.*, **3**) from hydroxylamines such as **2** places a serious caveat upon the use of nitrobenzyl carbamates in NTR enzyme prodrug therapy. Thus, it is of considerable importance to examine the effect of the leaving amine upon the rate and extent of fragmentation of nitrobenzyl carbamates. In the present paper, we investigate in more detail the mechanism of fragmentation of **2b** following reduction by a number of methods, and compare the fragmentation rates of a series of analogues **6**, derived from **5**, bearing aniline leaving groups of varying pK_a (Scheme 2).

Results and discussion

Synthesis of carbamates 5

A series of nitrobenzyl carbamates **5** bearing various substituents (H, Me, OMe, SO_2Me) on the leaving amine was prepared. Methyl 3-nitrophenylacetate **10a** was available commercially.

† Details of the syntheses of compounds **10**–**24** are available as supplementary data. For direct electronic access see http://www.rsc.org/suppdata/p1/b0/b000135j/, otherwise available from BLDSC (SUPPL. NO. 57699, 20 pp.) or the RSC Library. See Instructions for Authors available *via* the RSC web page (http://www.rsc.org/authors).

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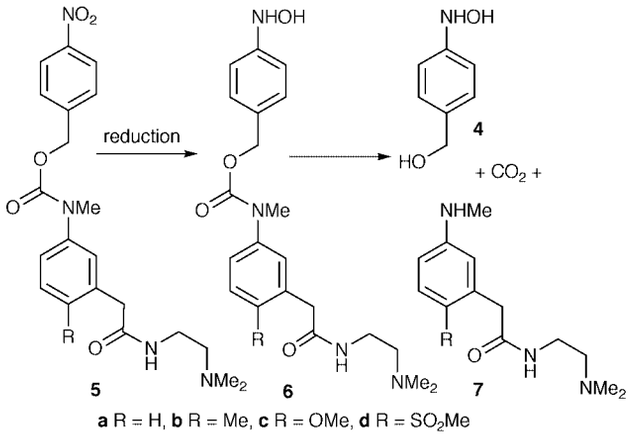
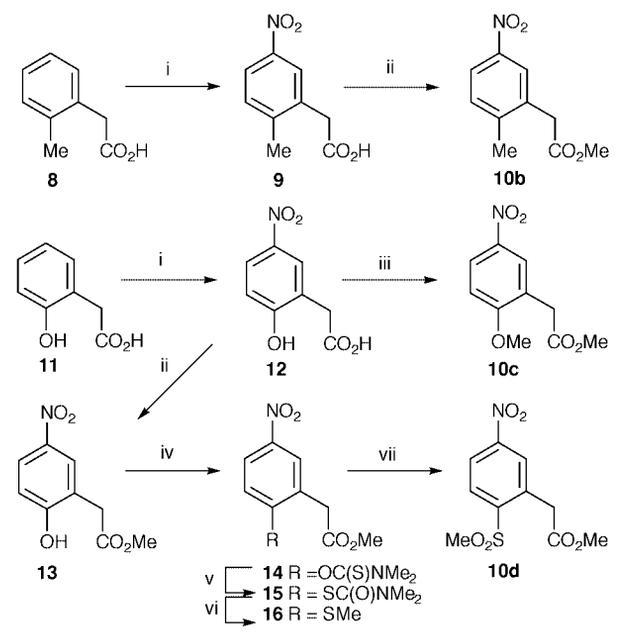


Scheme 2 Reductive fragmentation of nitrobenzyl carbamates **5**.

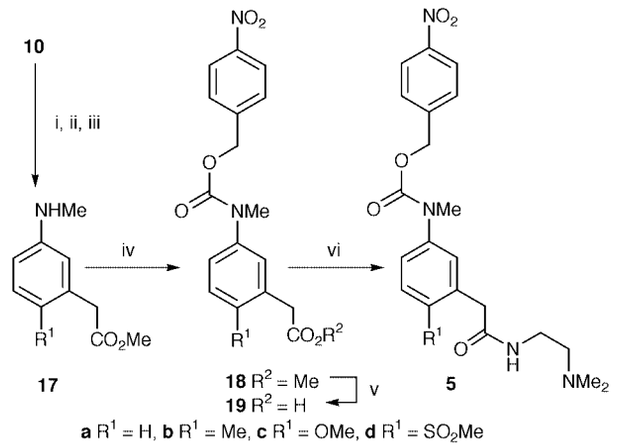


Scheme 3 Preparation of nitrophenylacetates **13**. *Reagents and conditions*: i, HNO_3–H_2SO_4; ii, $SOCl_2$, DMF, MeOH; iii, Me_2SO_4, NaOH; iv, $Me_2NC(S)Cl$, Et_3N, DCM; v, 1,2-dichlorobenzene, reflux; vi, KOH, MeOH, then Me_2SO_4; vii, MCPBA, DCM.

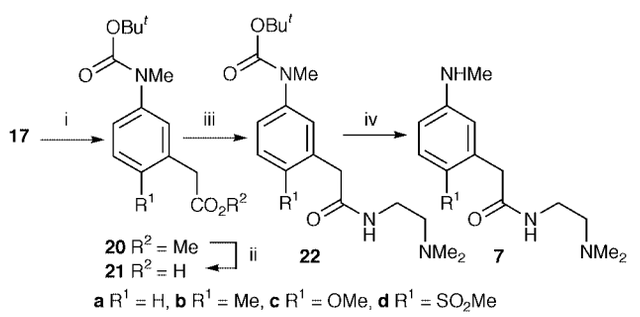
Nitration and esterification of 2-methylphenylacetic acid **8** gave the ester **10b** (Scheme 3). Similarly, 2-hydroxyphenylacetic acid **11** was nitrated and methylated to give the 2-methoxy-5-nitrophenylacetate **10c**. Esterification of phenylacetic acid **12** gave hydroxyphenylacetate **13**, which was converted to the thiophenylacetate using the Newman–Kwart rearrangement.[10] Thus, reaction of **13** with dimethylthiocarbamoyl chloride gave the *O*-aryl dimethylthiocarbamate **14** which underwent O- to S-rearrangement at 170 °C in 1,2-dichlorobenzene to give the *S*-aryl dimethylthiocarbamate **15**. Carbamate **15** was cleaved under basic conditions, methylated with dimethyl sulfate, and oxidised to give sulfone **10d**.

The nitrophenylacetates **10a**–**d** were hydrogenated with Pd/C and were reductively methylated to give the *N*-methylaminophenyl acetates **17a**–**d** (Scheme 4). Reaction of the *N*-methylaminophenyl acetates **17a**–**d** with 4-nitrobenzyl chloroformate gave the nitrobenzyl carbamates **18a**–**d** which were hydrolysed under basic conditions to give the acids **19a**–**d**. Coupling of the acids **19a**–**d** to (*N*,*N*-dimethylamino)ethylamine using 1,1′-carbonyldiimidazole (CDI) gave the nitrobenzyl carbamates **5a**–**d**.

Authentic samples of the released aminoacetamides **7a**–**d** were prepared from **17a**–**d** in a similar manner (Scheme 5). Thus, the aminophenylacetates **17a**–**d** were protected using



Scheme 4 Preparation of nitrobenzyl carbamates **5**. *Reagents and conditions*: i, H_2, Pd/C, EtOH; ii, acetic formic anhydride; iii, BH_3·DMS, THF; iv, NO_2PhCH_2OCOCl, THF; v, NaOH, MeOH; vi, CDI, DMF, then $NH_2CH_2CH_2NMe_2$.



Scheme 5 Preparation of aminoacetamides **3**. *Reagents and conditions*: i, $(Bu^tOCO)_2O$, THF; ii, NaOH, MeOH; iii, CDI, DMF, then $NH_2CH_2CH_2NMe_2$; iv, HCl, MeOH.

di-*tert*-butyl dicarbonate to give **20a**–**d**. The acetates **20a**–**d** were hydrolysed to the acids **21a**–**d** and coupled to (*N*,*N*-dimethylamino)ethylamine using CDI to give acetamides **22a**–**d** which were deprotected under acidic conditions to give the aminoacetamide dihydrochlorides **7a**–**d**.

Effect of different reductants on the fragmentation of hydroxylamines

HPLC analysis of solutions of prodrug **1b**, immediately after exposure to differing stoichiometric equivalents of radiolytically generated reductants, showed that four equivalents of reductant were required to completely consume all the compound at both pH 3.0 and 7.4 (Fig. 1). The data obtained for **1b** were typical of the results obtained for compounds **5a**–**d**. Solutions of **1b** were also reduced by other reductants, and Figs. 2b, 3a and 3b present HPLC kinetic data comparing the composition of a solution of **1b** reduced by 4-fold radiolytic reduction, the *E. coli* nitroreductase enzyme, and chemical reduction by Zn–ammonium acetate, respectively.

LCMS analysis of reaction solutions from the chemical reduction (Fig. 3b) yielded a molecular weight (*m*/*z*) of 401 for the transient reduction product, consistent with formation of the hydroxylamine **2b**. The same product was also found in the enzymic and radiolytic reduction reactions, although the starting nitro compound **1b** was completely consumed in radiolytically activated solutions. Samples of radiolytically-activated **1b** were collected as soon as possible after 4-fold reduction. Time zero on the *x*-axis of the radiolytic reduction plot (Figs. 2a and 2b) is the time at which irradiation, not data collection, was begun.

The presence of a transient intermediate **26** was detected in all the reduction reactions, but in amounts too small to be quantified in the chemical reduction (Fig. 3b) and is not shown.

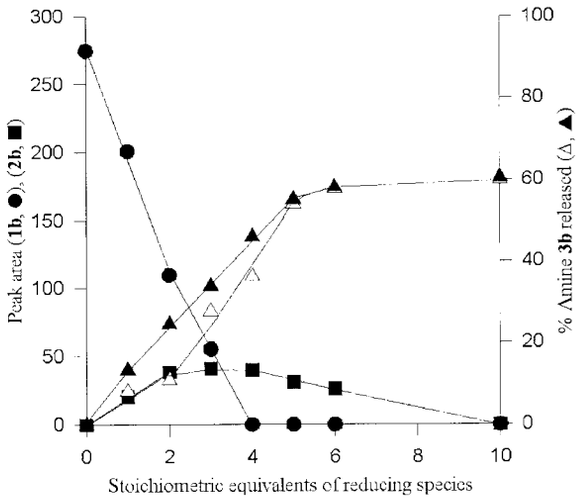
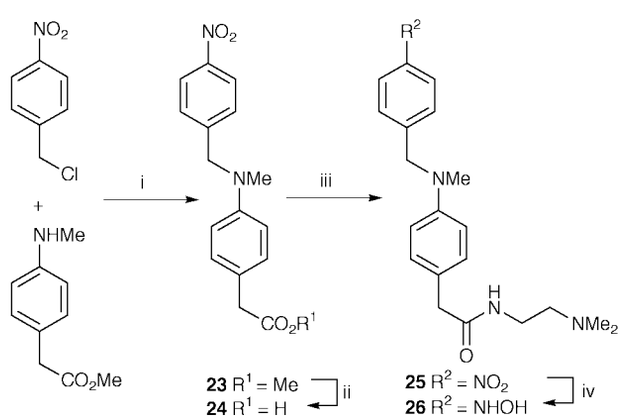


Fig. 1 Composition of solutions of **1b** after radiolytic reduction with varying molar equivalents of reducing species ($e^-_{(aq)}$ and $(CH_3)_2C^{\bullet}OH$). Left-hand ordinate plots peak areas of **1b** (●), and the corresponding hydroxylamine **2b** (■), measured immediately after radiolysis. Right-hand ordinate plots release of aminoacetamide **3b**, measured immediately (△) and 5 h after (▲) radiolytic reduction. $[\mathbf{1}]_0 = 50$ μM, pH 7.4, 10 mM phosphate, 4% (v/v) propan-2-ol, 18 °C. Data points were determined in triplicate and were reproducible to within ± 0.6%.

The identity of **26** was determined by LCMS and confirmed by synthesis. The intermediate gave a molecular ion of 357; that is 44 mass units less than the MH^+ for **2b**, thus the structure was tentatively assigned as **26** [$MH^+ - CO_2$]. Confirmation of the structure of **26** was made by synthesis of the corresponding nitrobenzylamine **25** and subsequent zinc reduction to the hydroxylamine **26** (Scheme 6). LCMS analysis of the synthetic

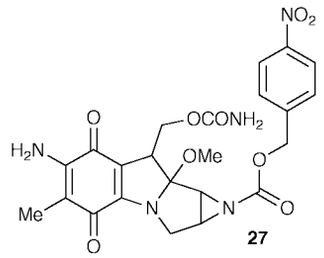


Scheme 6 Preparation of hydroxylamine **26**. *Reagents and conditions*: i, K_2CO_3, DMF; ii, NaOH, MeOH; iii, CDI, DMF, then $NH_2CH_2CH_2NMe_2$; iv, Zn, NH_4OAc, MeOH, H_2O.

26 gave a peak that coeluted with the intermediate from an NTR incubation of **1b** and also gave a molecular ion of 357 [MH^+]. Complete activation (no starting material left) of **1b** by either radiolytic, enzymatic, or chemical means did not result in total release of the aminoacetamide effector **3b**. Yields of **3b** were 49, 53, and 60% from radiolytic, enzymic and chemical activation, respectively. Thus, the difference in the extent of release of effectors between our previous work with **1b** and its analogues,[9] and work[1] with nitrobenzyl carbamates of mitomycin C (**27**) and actinomycin D is probably not due to inherent differences in the mechanisms of radiolytic and enzymic activation.

Effect of changing pH on the fragmentation of hydroxylamines

The range (24–76%) of amine effector release from 4-nitrobenzyl carbamate prodrugs prompted us to examine



effector properties that might affect this. The amine mitomycin C, released from prodrug **27**,[1] has a pK_a of 7.6.[11] This is significantly higher than the pK_a (4.6 ± 0.2) of the aminoacetamide **3b**, released from prodrug **1b**. The reductively triggered fragmentation of **1b** was therefore investigated at pH 3, as an initial probe into the role of protonation of the leaving amine on the efficiency of its release from nitrobenzyl carbamate prodrugs.

The same initial products are formed after 4-fold radiolytic reduction of **1b** at both pH 3.0 and 7.4 (Figs. 2a and 2b). Complete consumption of starting material **1b** had occurred before the first sample was taken. The major initial product is the hydroxylamine **2b,** with 40% of the coupled aminoacetamide **3b** having already been released. The same pattern was observed for the other prodrugs (**5a**–**d**) studied (data not shown).

The hydroxylamine **2b** disappears at similar rates at both pH 3.0 and 7.4 ($t_{\frac{1}{2}} = 9.4$ and 10.7 min, respectively), but significant differences are observed in the products that form in coincidence with this loss. At pH 3, loss of **2b** coincides with continued first-order release of the aminoacetamide **3b** ($t_{\frac{1}{2}} = 12.0$ min) (Fig. 2c), with a final yield of **3b** of 92%. Small quantities of the expected co-product, 4-(hydroxyamino)benzyl alcohol **4** were also observed. This species is unstable and its generation did not conform to first-order kinetics. At pH 7.4, complete loss of **2b** did not result in continued first-order release of **3b** (Fig. 2d); the amount of **3b** increased from 41% initially to only 49% 3 h after radiolytic activation. Instead, first-order loss of **2b** was accompanied by formation of hydroxylamine **26** ($t_{\frac{1}{2}} = 21$ min). 4-(Hydroxyamino)benzyl alcohol **4** was detected in the reaction at pH 7.4, and **26** was not observed in the reaction at pH 3.

Radiolytic reduction of **1b** was examined over the pH range 3–8 (Fig. 4). The extent of release of **3b**, at $t = 0$ and $t = 5$ h (stored under anaerobic conditions, left-hand ordinate), and production of **26** ($t = 5$ h) (right-hand ordinate) was monitored after complete reduction of **1b** with 4 stoichiometric reducing equivalents. Formation of **26** occurred only at pH > 5. The pK_a of the expelled aniline **3b** was determined spectrophotometrically to be 4.6 ± 0.2. There was *ca.* 50% more release of **3b** from **2b** at pH values more acidic than the pK_a.

Fragmentation of weakly basic carbamic acids indicates acid catalysis.[12,13] However, whether the increased release of **3b** from **2b** is due to acid catalysis, or reduced condensation of the released unprotonated aniline **3b** with reactive precursors of **4** is unclear.

To further study this possible effect, we prepared a small series of prodrugs **5a**–**d**, containing aminoacetamide-based effectors **7a**–**d** bearing 4-substituents of widely differing electron-donating properties, and with pK_a values ranging from 0.12 to 5.9 (Table 1). Compounds **5a**–**d** were radiolytically reduced in buffers of pH 3–8 in experiments analogous to that carried out on **1b** (Fig. 4). HPLC analysis showed that no prodrug remained in solutions exposed to 4 equivalents of reductant over this pH range. The extent of release of the aminoacetamides **7a**–**d** was measured both immediately after, and 5 h after, radiolytic activation. The pK_as of the amine effectors were determined spectrophotometrically (Table 1).

Compounds **5a**–**d** responded similarly to **1b**. At pH 7, aminoacetamide release was significantly less than 100% (from 15% for **5a** to 58% for **1b**), and did not continue to an appreciable extent after reductive activation. In contrast, at pH 3 the

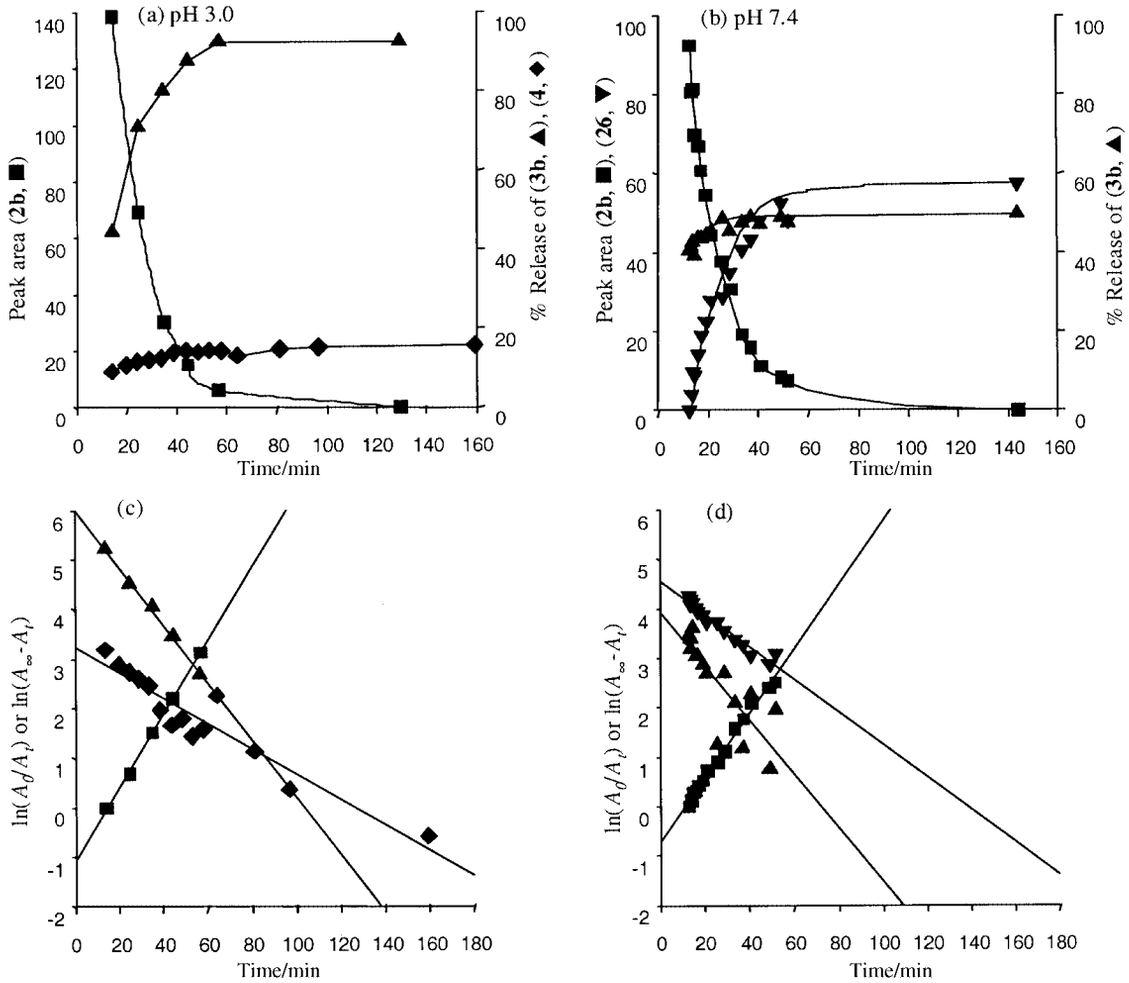


Fig. 2 Composition of solutions of **1b** reductively activated by 4-fold radiolysis against time at pH 3.0 (a) and pH 7.4 (b). Left-hand ordinate plots peak areas of hydroxylamine **2b** (■), and **26** (▼). Right-hand ordinate plots release of the aminoacetamide **3b** (▲) and **4** (◆). $[\mathbf{1b}]_0 = 50$ μM, 4% (v/v) propan-2-ol, anaerobic conditions, 18 °C: (a) pH 3.0, 10 mM formate buffer and 0.1 M NaCl; (b) pH 7.4, 10 mM phosphate buffer. First order plots (c) and (d) derived from (a) and (b), respectively. Data points were determined in triplicate and were reproducible to within ± 0.6%.

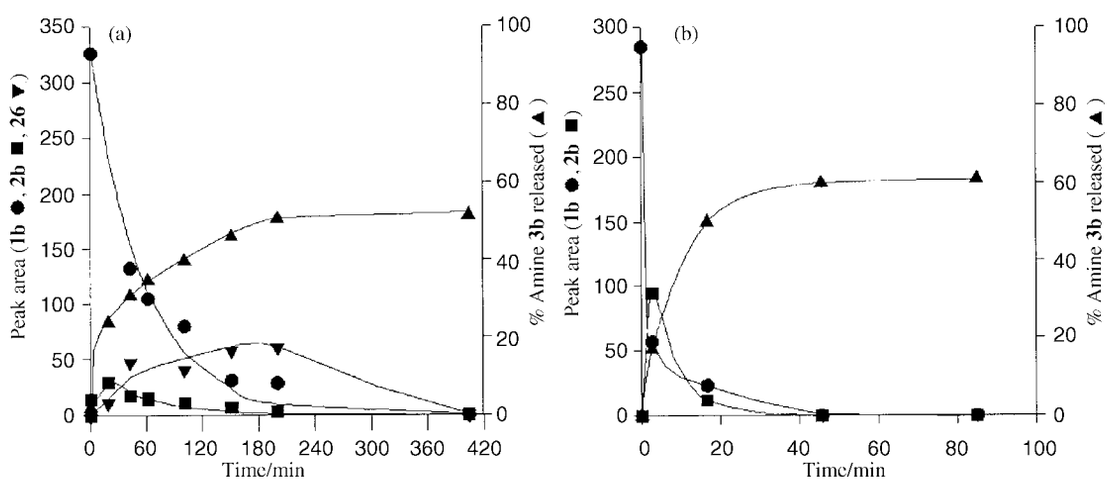


Fig. 3 Composition of solutions of **1b** reductively activated by (a) NTR and (b) $Zn–NH_4CH_3COO$, against time. Left-hand ordinate plots peak areas of **1b** (●), hydroxylamine **2b** (■), and **26** (▼). Right-hand ordinate plots release of the aminoacetamide **3b** (▲). $[\mathbf{1b}]_0 = 50$ μM, 18 °C: (a) [NTR] = 7 μg cm^{-3}, [NADH] = 0.5 mM, pH 7.4, 10 mM phosphate buffer; (b) Zn (5 mg), $[NH_4CH_3COO] = 50$ mM, 20 cm^3. Data in plots (a) and (b) were all collected and analysed in real-time. Data points were determined in triplicate and were reproducible to within ± 0.6%.

hydroxylamines **6a–d** continued to fragment after radiolytic reduction, eventually releasing the coupled aminoacetamides in up to 97% yield. There was no discernible relationship between the pK_a of the leaving aniline and the extent of its release at either pH 3 or 7; the greatest variation in effector release was observed for prodrugs having effectors of most similar pK_a. A comparison of pH profiles (analogous to Fig. 4 for **1b**; data not shown) also did not reveal any discernible trend between the pH below which amine release proceeded significantly after reductive activation, and the basicity of the leaving amine. This transition occurred in the pH range 5–6 for all the prodrugs. These observations suggest that the degree of amine release from 4-(hydroxyamino)benzyl carbamates is not related to the pK_a of the leaving amine, but rather that the further reaction of

Table 1 pK_a Values of released aminoacetamides **7a–d**

Compound	R^1	σ	pK_a	Amine release pH 7.4 (%)		Amine release pH 3 (%)	
				t_o	t_∞	t_o	t_∞
3b	—	—	4.6 ± 0.2	58	64	58	97
7a	H	0	4.7 ± 0.2	15	17	27	61
7b	Me	−0.14	4.8 ± 0.2	35	37	41	89
7c	OMe	−0.28	5.9 ± 0.1	47	47	61	92
7d	SO_2Me	0.73	0.12	53	54	75	96

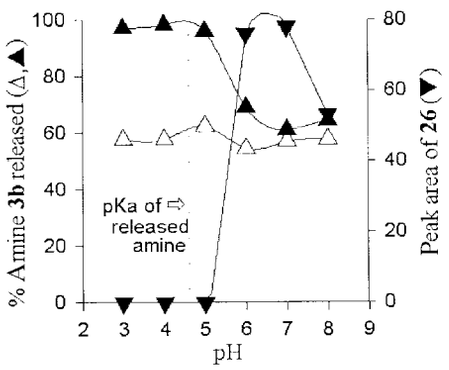


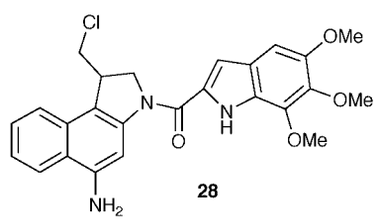
Fig. 4 Release of **3b** and **26** against pH. Left ordinate shows the extent of release of aminoacetamide **3b** from 4-fold radiolytically activated **1b** over the range pH 3–8, measured immediately (△) and 5 h after (▲) radiolytic activation of **1b**. Right ordinate gives peak area of **26**, (▼) measured 5 h after radiolysis. $[\mathbf{1b}]_0$ = 50 μM, 4% v/v propan-2-ol, 0.01 M buffer component, 0.1 M NaCl, 18 °C. Data points were determined in triplicate and were reproducible to within ± 0.6%.

weakly basic amines at pH 7 may diminish the total production of the amine.

The maximum yield of aminoacetamide **3b** at pH 7.4 coincides with complete 4-fold reduction of the starting material (Fig. 1), and this yield nears completeness at pH 3–5. No 4-aminobenzyl alcohol was detected in any of the reduced solutions of **1b**. Reduction of **1b** by pulse radiolysis show that the nitro radical anion of **1b** does not fragment.[14] These observations support the view that amine release does occur from the hydroxylamine **2b**, the major product of steady-state radiolysis at pH 3–8.

Changes in pH may alter products through changes in ionisation, catalysis, and reaction mechanism. The initial products of radiolytic reduction are the same at pH 3 and pH 7.4. The level of protonation of the hydroxylamine **2b** or **6** (pK_a *N*-phenylhydroxylamine = 3.2[15]) will change substantially over the pH range 3–5, but little thereafter. However, protonation of the hydroxylamine **2b** or **6** at the lower pH does not appear to increase aminoacetamide **3b** or **7** yield by speeding up the rate of fragmentation (either by catalysis or changing mechanism), rather the lower pH inhibits the loss of **3b** or **7**. Aminoacetamide **3b** or **7** release is not greatly different at pH 3–8 when measured initially after radiolysis; approximately 12 minutes after radiolysis was begun (Fig. 4 and Table 1). Fig. 2 also shows that 44 and 42% of **3b** were released from **1b** immediately after reduction, at pH 3 and 7.4, respectively. These percentage releases correspond to a maximum half-life for reductive fragmentation of 16 minutes at both pH values (which includes the finite rate of reduction). At pH 3 aminoacetamide **3b** continued to be released in a first-order manner with a half-life measured as 12 minutes. Thus, release of **3b** was not significantly faster at different pH values; the different yields of effector arose because at low pH amine release continued after radiolytic reduction.

We have established that the efficiency of effector release is similar across different reduction methods, and occurs predominantly from the hydroxylamine **2b**. At pH values greater than 5, the amine **3b** condenses with a reactive precursor of **4** which limit production of amine **3b**. The efficiency of reductively triggered release of amines **7** varied with small changes in the leaving group, but this efficiency is not obviously related to amine basicity. We propose that the effector domain of the prodrug impacts on the efficiency of effector release, not through fragmentation or leaving ability, but through a competing reaction of the effector **3b** or **7** with a precursor of **4**. This may be an artefact of the relatively high concentration (50 μM) at which the experiments were carried out. It does indicate that, if such nitrobenzyl carbamate prodrugs are to be used successfully in conjunction with the NTR enzyme prodrug therapy, the released effector amine should be toxic at concentrations considerably lower than 50 μM. Indeed, our studies using the NTR enzyme prodrug system have involved highly cytotoxic amine effectors, *e.g.*, the CBI-TMI amine **28** which has an IC_{50} in the low nanomolar range.[16] The concentration of prodrug **28** *in vivo* would be expected to be considerably lower than 50 μM and a condensation reaction is unlikely to compete effectively for the released amine effector.



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Experimental

Analyses were carried out in the Microchemical Laboratory, University of Otago, Dunedin, NZ. Melting points were determined on an Electrothermal 2300 Melting Point Apparatus. IR spectra were recorded on a Midac FT-IR as KBr discs, unless otherwise stated. NMR spectra were obtained on a Bruker AM-400 spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C spectra. Spectra were obtained in deuteriochloroform unless otherwise specified, and are referenced to Me_4Si. Chemical shifts and coupling constants were recorded in units of ppm and Hz, respectively. Mass spectra were determined on a VG-70SE mass spectrometer using an ionizing potential of 70 eV at a nominal resolution of 1000. High resolution spectra were obtained at nominal resolutions of 3000, 5000, or 10000 as appropriate. All spectra were obtained as electron impact (EI) using perfluorokerosine (PFK) as the reference unless otherwise stated. LCMS spectra were obtained using an HP 1100 series binary pump, vacuum degasser, autosampler, thermostatted column compartment and diode array detector with an Altima C8 5 μM 150 × 3.2 mm column eluting with formate buffer (pH 4.5)–80% acetonitrile (70 : 30). An Atmospheric Pressure Chemical Ionisation (APCI) interface was used with an HP1100 series mass spectrometer. Solutions in organic solvents were dried with anhydrous sodium sulfate, unless otherwise noted. Solvents were evaporated under reduced pressure on a rotary evaporator. Thin-layer chromatography was carried out on aluminium-backed silica gel plates (Merck 60 F_{254}) with

visualisation of components by UV light (254 nm) or exposure to I_2. Column chromatography was carried out on silica gel (Merck 230–400 mesh), and solvent gradients were of increasing polarity. DCM refers to dichloromethane; THF refers to tetrahydrofuran dried over sodium benzophenone ketyl; DMF refers to dry dimethyl formamide, EtOAc refers to ethyl acetate; ether refers to diethyl ether; MeOH refers to methanol; EtOH refers to ethanol. All solvents were freshly distilled. Compounds in the syntheses of **5a–d** and **7a–d** are named as phenylacetic acid derivatives in order to provide a consistent numbering scheme throughout. Preparation of compounds **10–24** are described in the Electronic Supplementary Information.

Coupling of acids 19 with (*N*,*N*-dimethylamino)ethylamine

***N*-[2-(*N*,*N*-Dimethylamino)ethyl]-3-[*N*-methyl-*N*-(4-nitrobenzyloxycarbonyl)amino]phenylacetamide 5a.** A solution of **19a** (0.76 g, 2.2 mmol) and CDI (0.54 g, 3.3 mmol) in DMF (10 cm^3) was heated at 50 °C for 10 min. (*N*,*N*-Dimethylamino)-ethylamine (0.48 cm^3, 4.4 mmol) was added dropwise and the solution stirred at 20 °C for 4 h. The solution was poured into water (150 cm^3) and extracted with EtOAc (3 × 50 cm^3). The combined organic extracts were washed with 0.1 M NaOH solution (50 cm^3), water (50 cm^3), brine (50 cm^3), dried, and the solvent evaporated to give *acetamide* **5a** (0.88 g, 96%) as a colourless solid, mp (EtOAc) 115–116.5 °C (Found: C, 60.8; H, 6.0; N, 13.6. $C_{21}H_{26}N_4O_5$ requires C, 60.65; H, 6.3; N, 13.5%); ν_{max}/cm^{-1} 3287, 1707, 1647, 1512, 1350, and 1153; δ_H [$(CD_3)_2SO$] 2.12 (6 H, s, $N(CH_3)_2$), 2.27 (2 H, t, *J* 6.6, CH_2), 3.14 (2 H, dt, *J* 6.6 and 6.4, CH_2N), 3.26 (3 H, s, NCH_3), 3.44 (2 H, s, CH_2), 5.24 (2 H, s, CH_2O), 7.12–7.31 (4 H, m, 2-H, 4-H, 5-H, 6-H), 7.56 (2 H, d, *J* 8.7, 2′-H, 6′-H), 8.00 (1 H, br t, *J* 6.4, CONH), and 8.21 (2 H, d, *J* 8.7, 3′-H, 5′-H); δ_C [$(CD_3)_2SO$] 36.8, 37.5, 41.9, 45.0 (2), 58.1, 65.3, 123.5 (2), 123.7, 126.3, 126.8, 127.9 (2), 128.5, 137.3, 142.6, 144.5, 146.8, 154.2, and 169.6.

***N*-[2-(*N*,*N*-Dimethylamino)ethyl]-2-methyl-5-[*N*-methyl-*N*-(4-nitrobenzyloxycarbonyl)amino]phenylacetamide 5b.** Similarly, reaction of **19b** as described above gave *acetamide* **5b** (94%) as white prisms, mp (EtOAc) 144–145 °C (Found: C, 61.4; H, 6.9; N, 13.1. $C_{22}H_{28}N_4O_5$ requires C, 61.7; H, 6.6; N, 13.1%); ν_{max}/cm^{-1} 3281, 1709, 1645, 1514, 1350, 1281, and 1154; δ_H 2.15 (6 H, s, $N(CH_3)_2$), 2.30 (3 H, s, CH_3), 2.35 (2 H, t, *J* 6.0, CH_2N), 3.28–3.33 (5 H, m, CH_2N, NCH_3), 3.55 (2 H, s, CH_2), 5.23 (2 H, s, CH_2O), 6.07 (1 H, s, CONH), 7.05–7.10 (2 H, m, 4-H, 6-H), 7.19 (1 H, d, *J* 8.0, 3-H), 7.42–7.46 (2 H, m, 2′-H, 6′-H), and 8.19 (2 H, d, *J* 8.2, 3′-H, 5′-H); δ_C 19.1, 36.8, 38.0, 41.4, 45.0 (2), 57.7, 65.8, 123.7 (2), 127.4, 127.9 (3), 131.0, 134.5, 135.4, 141.0, 144.0, 147.5, 155.0, and 170.1.

***N*-[2-(*N*,*N*-Dimethylamino)ethyl]-2-methoxy-5-[*N*-methyl-*N*-(4-nitrobenzyloxycarbonyl)amino]phenylacetamide 5c.** Similarly, reaction of **19c** as described above gave *acetamide* **5c** (88%) as white prisms, mp (EtOAc) 136–137 °C (Found: C, 59.0; H, 6.1; N, 12.7. $C_{22}H_{28}N_4O_6$ requires C, 59.4; H, 6.4; N, 12.6%); ν_{max}/cm^{-1} 3283, 1707, 1647, 1514, 1345, and 1290; δ_H [$(CD_3)_2SO$] 2.12 (6 H, s, $N(CH_3)_2$), 2.26 (2 H, t, *J* 6.4, CH_2N), 3.14 (2 H, dt, *J* 6.4 and 6.0, CH_2N), 3.21 (3 H, s, NCH_3), 3.39 (2 H, s, CH_2), 3.77 (3 H, s, OCH_3), 5.21 (2 H, s, CH_2O), 6.95 (1 H, d, *J* 8.3, 3-H), 7.17–7.20 (2 H, m, 4-H, 6-H), 7.53–7.57 (2 H, m, 2′-H, 6′-H), 7.77 (1 H, br s, CONH), and 8.21 (2 H, d, *J* 8.5, 3′-H, 5′-H); δ_C [$(CD_3)_2SO$] 36.6, 36.7, 38.8, 45.0 (2), 55.5, 58.1, 65.1, 110.7, 123.5 (2), 125.0, 125.2, 127.7 (2), 128.3, 135.1, 144.7, 146.8, 154.4, 155.4, and 169.6.

***N*-[2-(*N*,*N*-Dimethylamino)ethyl]-2-methylsulfonyl-5-[*N*-methyl-*N*-(4-nitrobenzyloxycarbonyl)amino]phenylacetamide 5d.** Similarly, reaction of **19d** as described above gave *acetamide* **5d** (90%) as white prisms, mp (EtOAc–light petroleum) 126–129.5 °C (Found: C, 53.2; H, 5.7; N, 11.4; S, 6.1. $C_{22}H_{28}N_4O_7S$ requires C, 53.6; H, 5.7; N, 11.4; S, 6.5%); ν_{max}/cm^{-1} 3312, 1716, 1650, 1526, 1334, and 1163; δ_H 2.23 (6 H, s, $N(CH_3)_2$), 2.42 (2 H, t, *J* 6.1, CH_2N), 3.13 (3 H, s, SO_2CH_3), 3.27–3.33 (2 H, m, CH_2N), 3.40 (3 H, s, NCH_3), 4.00 (2 H, s, CH_2), 5.30 (2 H, s, CH_2O), 6.71 (1 H, br s, CONH), 7.41 (1 H, dd, *J* 8.6, 2.1, 4-H), 7.49 (1 H, d, *J* 2.1, 6-H), 7.51 (2 H, d, *J* 8.8, 2′-H, 6′-H), 8.02 (1 H, d, *J* 8.6, 3-H), and 8.23 (2 H, d, *J* 8.8, 3′-H, 5′-H); δ_C 37.1, 37.2, 40.2, 45.0, 45.1 (2), 57.7, 66.4, 123.8, 123.9 (2), 128.3 (2), 128.6, 130.7, 135.3, 136.2, 143.1, 147.5, 147.7, 154.3, and 169.3; *m*/*z* (FAB^+) 493.1741 (MH^+, $C_{22}H_{29}N_4O_7S$ requires 493.1757); *m*/*z* (FAB^+) 493 (MH^+, 30%), 477 (5), and 448 (5).

Deprotection of acetamides 22

***N*-[2-(*N*,*N*-Dimethylamino)ethyl]-3-(*N*-methylamino)phenylacetamide dihydrochloride 7a.** A solution of HCl saturated MeOH (20 cm^3) was added to a stirred solution of **22a** (1.24 g, 3.7 mmol) in MeOH (50 cm^3) and the mixture stirred at 20 °C for 1 h. The solvent was evaporated to give *acetamide dihydrochloride* **7a** (1.21 g, 100%) as a hygroscopic foam (Found: C, 47.9; N, 13.1; Cl, 22.1. $C_{13}H_{21}N_3O{\cdot}2HCl{\cdot}H_2O$ requires C, 47.9; N, 12.9; Cl, 21.7%); ν_{max}(thin film)/cm^{-1} 3402, 1655, 1549, and 1473; δ_H [$(CD_3)_2SO$] 2.77 (6 H, d, *J* 4.6, $N(CH_3)_2$), 2.88 (3 H, s, NCH_3), 3.11–3.18 (2 H, m, CH_2N), 3.41–3.46 (2 H, m, CH_2N), 3.55 (2 H, s, CH_2), 3.67 (2 H, br s, NH_2Cl), 7.31–7.40 (4 H, m, 2-H, 4-H, 5-H, 6-H), 8.64 (1 H, t, *J* 5.5, CONH), and 10.52 (1 H, br s, NHCl); δ_C [$(CD_3)_2SO$] 34.0, 35.8, 41.8, 42.2 (2), 55.6, 119.9, 122.3, 129.0, 129.6, 137.9, 138.3, and 170.2.

***N*-[2-(*N*,*N*-Dimethylamino)ethyl]-2-methyl-5-(*N*-methylamino)phenylacetamide dihydrochloride 7b.** Similarly, reaction of **22b** as described above gave *acetamide dihydrochloride* **7b** (100%) as a hygroscopic foam (Found: C, 49.6; N,12.3; Cl, 20.9. $C_{14}H_{23}N_3O{\cdot}2HCl{\cdot}H_2O$ requires C, 49.4; N, 12.4; Cl, 20.8%); ν_{max}(thin film)/cm^{-1} 3402, 1655, 1549, and 1473; δ_H [$(CD_3)_2SO$] 2.27 (3 H, s, CH_3), 2.77 (6 H, d, *J* 4.6, $N(CH_3)_2$), 2.86 (3 H, s, NCH_3), 3.12–3.19 (2 H, m, CH_2N), 3.42–3.47 (2 H, m, CH_2N), 3.57 (2 H, s, CH_2), 7.30 (1 H, d, *J* 8.2, 3-H), 7.34 (1 H, dd, *J* 8.2 and 2.0, 4-H), 7.42 (1 H, d, *J* 2.0, 6-H), 9.13 (1 H, t, *J* 5.4, CONH), 10.62 (1 H, br s, NHCl), and 11.35 (2 H, br s, NH_2Cl); δ_C [$(CD_3)_2SO$] 18.9, 34.0, 35.8, 38.8, 42.1 (2), 55.5, 120.4, 123.5, 130.9, 135.5, 136.4, 137.6, and 169.9; *m*/*z* 249.1845 (M^+, $C_{14}H_{23}N_3O$ requires 249.1841); *m*/*z* 249 (M^+, 10%), 178 (20) and 58 (100).

***N*-[2-(*N*,*N*-Dimethylamino)ethyl]-2-methoxy-5-(*N*-methylamino)phenylacetamide dihydrochloride 7c.** Similarly reaction of **22c** as described above gave *acetamide dihydrochloride* **7c** (85%) as a white solid, mp (MeOH) 168–170 °C (Found: C, 49.3; H, 7.5; N, 12.3; Cl, 20.9. $C_{14}H_{23}N_3O_2{\cdot}2HCl$ requires C, 49.7; H, 7.5; N, 12.4; Cl, 21.0%); ν_{max}/cm^{-1} 3350, 1657, 1505, and 1265; δ_H [$(CD_3)_2SO$] 2.77 (6 H, d, *J* 4.8, $N(CH_3)_2$), 2.84 (3 H, s, NCH_3), 3.13–3.19 (2 H, m, CH_2N), 3.42–3.50 (4 H, m, CH_2, CH_2N), 3.79 (3 H, s, OCH_3), 7.10 (1 H, d, *J* 8.7, 3-H), 7.46 (1 H, dd, *J* 8.7 and 2.7, 4-H), 7.49 (1 H, d, *J* 2.7, 6-H), 8.43 (1 H, t, *J* 5.4 Hz, CONH), 10.68 (1 H, br s, NHCl), and 11.41 (2 H, br s, NH_2Cl); δ_C [$(CD_3)_2SO$] 34.0, 36.3, 36.4, 42.2 (2), 55.6, 55.9, 111.5, 122.1, 124.6, 125.7, 128.9, 157.2, and 170.0; *m*/*z* 265.1796 (M^+, $C_{14}H_{23}N_3O_2$ requires 265.1790); *m*/*z* 265 (M^+, 10%), 194 (20), and 58 (100).

***N*-[2-(*N*,*N*-Dimethylamino)ethyl]-2-methylsulfonyl-5-(*N*-methylamino)phenylacetamide dihydrochloride 7d.** Similarly, reaction of **22d** as described above gave *acetamide dihydrochloride* **7d** (100%) as a foam, δ_H (CD_3OD) 2.93 (3 H, s, NCH_3), 2.94 (6 H, s, $N(CH_3)_2$), 3.14 (3 H, s, SO_2CH_3), 3.27–3.30 (2 H, m, CH_2N), 3.56–3.60 (2 H, m, CH_2N), 3.99 (2 H, s, CH_2), 7.87 (1 H, d, *J* 8.4, 3-H), and 7.93–7.97 (2 H, m, 4-H, 6-H)*; δ_C 32.2, 36.0, 41.5, 44.1 (2), 45.3, 59.1, 114.9, 120.7, 130.2, 133.2, 137.6,

151.7, and 174.7; *m/z* 313.1449 (M^+, $C_{14}H_{23}N_3O_3S$ requires 313.1460); *m/z* 313 (M^+, 1%), 243 (10), 71 (25), and 58 (100).* CONH, NHCl, and NH_2Cl exchangeable signals not observed.

Preparation of hydroxylamine 26

A solution of acid **24** (813 mg, 2.71 mmol) and CDI (658 mg, 4.06 mmol) in DMF (10 cm^3) was stirred at 50 °C for 10 min and (*N*,*N*-dimethylamino)ethylamine (0.59 cm^3, 5.41 mmol) was added dropwise. The solution was stirred at 20 °C for 16 h, the solvent evaporated and the residue partitioned between EtOAc (100 cm^3) and water (100 cm^3). The organic fraction was washed with 0.01 M NaOH solution (50 cm^3), water (50 cm^3), brine (25 cm^3), dried and the solvent evaporated to give *N-[2-(N,N-dimethylamino)ethyl]-4-[methyl(4-nitrobenzyl)amino]-phenylacetamide* **25** (0.92 g, 92%) as a colourless oil which was converted to the dihydrochloride salt which formed a hygroscopic gum (Found: C, 52.4; H, 6.7; N, 12.4; Cl, 15.3. $C_{20}H_{26}N_4O_3{\cdot}2HCl{\cdot}H_2O$ requires C, 52.1; H, 6.55; N, 12.15; Cl, 15.4%); δ_H (free base) [$(CD_3)_2SO$] 2.12 (6 H, s, $N(CH_3)_2$), 2.25 (2 H, t, *J* 6.7, CH_2N), 3.02 (3 H, s, NCH_3), 3.05–3.12 (2 H, m, CH_2N), 3.24 (2 H, s, CH_2), 4.67 (2 H, s, CH_2N), 6.63 (2 H, br d, *J* 8.7, 3-H, 5-H), 7.04 (2 H, d, *J* 8.7, 2-H, 6-H), 7.45 (2 H, d, *J* 8.8, 2′-H, 6′-H), 7.80 (1 H, br t, *J* 5.4, CONH), and 8.17 (2 H, br d, *J* 8.8, 3′-H, 5′-H); δ_C [$(CD_3)_2SO$] 36.7, 38.7, 41.3, 45.1 (2), 55.0, 58.1, 111.9 (2), 123.5 (2), 124.1, 127.8 (2), 129.5 (2), 146.3, 147.2, 147.8, and 170.5; *m/z* 370.1998 (M^+, $C_{20}H_{26}N_4O_3$ requires 370.2005); *m/z* 370 (M^+, 5%), 299 (8), 282 (8), 255 (5), and 58 (100).

Zn powder (35 mg, 0.54 mmol) was added to a mixture of nitrobenzylamine **25** (0.10 g, 0.27 mmol) in MeOH (5 cm^3) and aqueous NH_4OAc (23 mg, 0.30 mmol) in water (3 cm^3) and stirred at 20 °C for 2 h. The mixture was filtered, washed with MeOH (3 cm^3) and the solvent evaporated to give crude **26** which was used for LCMS analysis without further purification.

Preparation of 4-hydroxyaminobenzyl alcohol 4

Radiolytic reduction of 4-nitrobenzyl alcohol (2.5 mg, 16 μmol) in milli-Q water (5 cm^3) and propan-2-ol (4% v/v) with 4 stoichiometric equivalents of reductants (exposure time = 10.6 h, dose rate = 0.554 J L^{-1} s^{-1}) gave a solution that was immediately frozen and freeze-dried to give *alcohol* **4** as an unstable yellow solid, δ_H [$(CD_3)_2SO$] 4.37 (2 H, d, *J* 5.6, CH_2O), 4.92 (1 H, t, *J* 5.6, OH), 6.78 (2 H, br d, *J* 8.2, H-3, H-5), 7.10 (2 H, br d, *J* 8.2, H-2, H-6), 8.16–8.20 (2 H, m, NHOH); *m/z* 139 (M^+, 10%).

Reduction of 1b with Zn–NH_4Cl

Freshly activated Zn powder (washed with conc. HCl, water, then EtOH) was added to a stirred solution of **1b** (100 μM) and ammonium formate (0.05 M) in 20 cm^3 of milli-Q water (pH of mixture = 6.7). Aliquots of the mixture were removed, filtered, and assayed by HPLC at the times noted.

Radiolytic reduction

Compounds were radiolytically reduced as 50 μM solutions in 5 cm^3 of buffer containing either 4% (v/v) propan-2-ol, or 5 mM CH_3CO_2Na to scavenge oxidizing $OH^{\bullet}$ radicals, at room temperature. Oxygen was removed by evacuating the radiolysis vessel. The dose rate of the ^{60}Co source was measured as 0.721 J L^{-1} s^{-1} using the NaCl modified Fricke dosimeter[17] and corrected for decay of ^{60}Co in subsequent irradiations. The time, *t*, required to form 1 mol. equivalent of reducing species in a 5 cm^3 solution of 50 μM nitrobenzyl carbamate **1b** or **5a–d**, was calculated from $t = n/(Gdv)$, where n = moles of starting material, G = radiation chemical yield [taken as 0.62 μmol J^{-1}, $G(e^-_{aq}) + G(H^{\bullet}) + G(OH^{\bullet})$], d = dose rate and v = the volume of radiolysis solution.

HPLC analysis

Samples (50 μL) were injected through a 25 μL loop onto either Econosphere C-18 (UV/Vis detection) (5 μM particle size, 250 × 4.6 mm id) or Altima C-8 (MS detection) (150 × 3.2 mm id) analytical columns. Elution was by both gradient and isocratic methods, using solvents comprised of either mixtures of MeOH, pH 6.5 10 mM phosphate buffer and 10 mM heptanesulfonic acid (UV/Vis detection, C-18 stationary phase) or MeCN and pH 4 10 mM ammonium formate. HP1040M Series II (UV/Vis) and HPMSD (MS) detectors permitted collection of UV/Vis and mass spectra of chromatographic peaks.

Kinetics and freeze–thaw quenching

HPLC collection of real-time kinetic data was limited by assay time, so samples were freeze–thaw quenched to obtain data for the faster reactions. Solutions of prodrugs (5 cm^3, 50 μM) were deoxygenated in radiolysis vessels by purging with nitrogen through a septum, then irradiated and returned to the nitrogen purge. Samples were syringed from the vessel, and immediately frozen in vials (submerged in liquid N_2) at noted times. Samples were later thawed by immersion in boiling water and injected immediately onto the HPLC. Real-time data were collected from the same reaction and checked for consistency with freeze–thaw quenched samples.

Reductions with *E. coli* nitroreductase (NTR)

The enzyme (15 μL of 100 μg cm^{-3} stock, 7 μg cm^{-3}) was added to **1b** (50 μM) and NADH (0.5 mM) in buffer (5 cm^3, pH 7.4 10 mM phosphate). Reactions were monitored by HPLC. The experiment measuring the release of 4-hydroxyaminobenzyl alcohol **4** was deoxygenated by purging with N_2 prior to addition of enzyme, and covered with a flow of N_2 whilst the reaction was monitored. Blank reactions were carried out under identical conditions without enzyme.

Radiolysis at varying pH

Solutions (50 μM) in buffers of pH values = 3.0, 4.0 (formate), 5.0, 6.0 (succinate), 7.0 and 8.0 (phosphate) (10 mM in given buffer species, 0.1 M NaCl) were reduced with 4-fold stoichiometry and assayed either immediately after radiolysis or after being left under anaerobic conditions for 5 h.

Determination of pK_a values

Solutions (50 μM) of the amines **7** were prepared in the same buffers, the UV/Vis spectrum recorded, and HPLC assayed. The pK_a was calculated[18] from the change in absorbance at 246 nm over the pH range 2–8, using the relationship $\log[(A_{AH} - A)/(A - A_{A^-})] = 0$ when pH = pK_a. The pK_a of **7d** was estimated spectrophotometrically in solutions of HCl over the pH range 1–3.

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References

1 A. B. Mauger, P. J. Burke, H. H. Somani, F. Friedlos and R. J. Knox, *J. Med. Chem.*, 1994, **37**, 3452.
2 (*a*) M. P. Hay, W. R. Wilson and W. A. Denny, *Bioorg. Med. Chem.*

Lett., 1995, **5**, 2829; (*b*) M. Tercel, W. R. Wilson and W. A. Denny, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 2741; (*c*) M. Lee, J. E. Simpson, Jr, S. Woo, C. Kaenzig, G. M. Anlezark, E. Eno-Amooquaye and P. J. Burke, *Bioorg. Med. Chem. Lett.*, 1997, **7**, 1065.
3 N. P. Michael, J. K. Brehm, G. M. Anlezark and N. P. Minton, *FEMS Microbiol. Lett.*, 1994, **124**, 195.
4 (*a*) M. P. Hay and W. A. Denny, *Drugs Future*, 1996, **21**, 917; (*b*) W. A. Denny, *Curr. Pharm. Des.*, 1996, **2**, 281; (*c*) R. G. Melton and R. F. Sherwood, *J. Natl. Cancer Inst.*, 1996, **88**, 153.
5 I. Niculescu-Duvaz, R. Spooner, R. Marais and C. J. Springer, *Bioconjugate Chem.*, 1998, **9**, 4.
6 (*a*) G. M. Anlezark, R. G. Melton, R. F. Sherwood, B. Coles, F. Friedlos and R. J. Knox, *Biochem. Pharmacol.*, 1992, **44**, 2289; (*b*) R. J. Knox, F. Friedlos, R. F. Sherwood, R. G. Melton and G. M. Anlezark, *Biochem. Pharmacol.*, 1992, **44**, 2297.
7 P. L. Carl, P. K. Charkravarty and J. A. Katzenellenbogen, *J. Med. Chem.*, 1981, **24**, 479.
8 K. Blaha and J. Rudinger, *Collect. Czech. Chem. Commun.*, 1965, **30**, 585.
9 M. P. Hay, B. M. Sykes, W. A. Denny and C. J. O'Connor, *J. Chem. Soc.*, *Perkin Trans. 1*, 1999, 2759.
10 (*a*) M. S. Newman and H. A. Karnes, *J. Org. Chem.*, 1966, **31**, 3980; (*b*) H. Kwart and E. R. Evans, *J. Org. Chem.*, 1966, **31**, 410.
11 C. R. Boruah and B. E. Skibo, *J. Org. Chem.*, 1995, **60**, 2232.
12 S. P. Ewing, D. Lockshon and W. P. Jencks, *J. Am. Chem. Soc.*, 1980, **109**, 3072.
13 S. L. Johnson and D. L. Morrison, *J. Am. Chem. Soc.*, 1972, **94**, 1323.
14 R. F. Anderson, unpublished work.
15 *Dictionary of Organic Compounds*, 6th edn., ed. J. I. G. Cadogan and P. H. Rhodes, Chapman and Hall, London, 1996, vol. 5, p. 5267.
16 M. P. Hay, B. M. Sykes, W. A. Denny and W. R. Wilson, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 2237.
17 H. Fricke and E. J. Hart, in *Radiation Dosimetry*, ed. F. H. Attix and W. C. Roesch, Academic Press, New York, 1966, Vol. II, p. 167.
18 A. Albert and E. P. Serjeant, in *The determination of ionization constants. A laboratory manual*, 3rd edn., Chapman and Hall, London, 1984.