Hypoxia-Selective Antitumor Agents. 11. Chlorambucil *N*-Oxide: A Reappraisal of Its Synthesis, Stability, and Selective Toxicity for Hypoxic Cells

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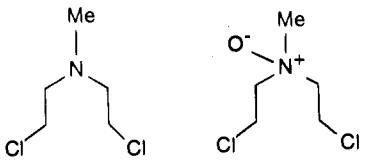
The potential hypoxia-selective cytotoxin 4-[4′-[*N*,*N*-bis(2″-chloroethyl)amino]phenyl]butanoic acid *N*-oxide (chlorambucil *N*-oxide, **4**) was synthesized and characterized as its hydrochloride salt. This compound was shown to be unstable, decomposing in some organic solvents to the hydroxylamine 4-[4′-[*N*-(2″-chloroethoxy)-*N*-(2‴-chloroethyl)amino]phenyl]butanoic acid (**11**) by a mechanism previously demonstrated for aliphatic mustard *N*-oxides and under aqueous conditions to a more complex mixture, of which the predominant components were the monochloroethyl derivative **7** and formaldehyde. Comparison of NMR spectra showed that a recent published synthesis of **4** in fact resulted in the rearrangement product **11**, indicating that recent reported investigations of the hypoxia-selective cytotoxicity and metabolism of chlorambucil *N*-oxide have examined this rearrangement product rather than **4**. In a clonogenic assay, **4** was less cytotoxic against AA8 cells than was chlorambucil, but the effect of oxygen on cytotoxicity was no greater than for chlorambucil itself.

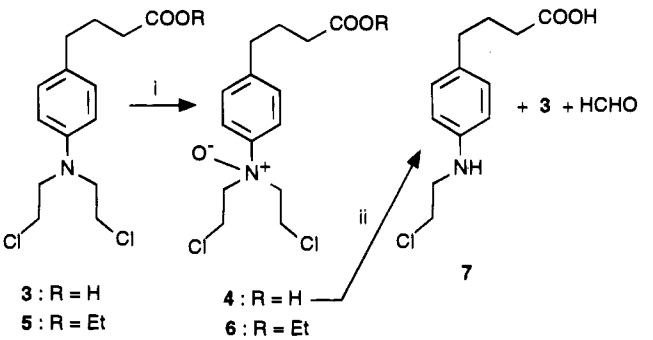
There is continuing interest in the development of drugs (hypoxia-selective cytotoxins, HSCs) which show selective toxicity toward the hypoxic cells[1] which can limit the sensitivity of solid tumors to radiotherapy.[2] We have suggested the utility of nitrogen mustards as "effector units" in HSCs[3] and have discussed several prodrug forms of these which can be selectively activated under hypoxic conditions (cobalt complexes,[4] quaternary salts,[5] and dinitro aromatic compounds[6]). Another potential chemistry for hypoxia-selective prodrugs of nitrogen mustards is by *N*-oxidation because this significantly decreases the electron density on the nitrogen (lowering pK_a by about 5 units[7]). Molecular oxygen can inhibit the metabolic reduction of tertiary amine *N*-oxides,[8,9] and the *N*-oxide derivative of mechlorethamine (**1**), nitromin (**2**), has been reported to have modest hypoxia selectivity (*ca.* 4-fold) in cell culture due to bioreductive release of the mustard.[10]



1 **2**

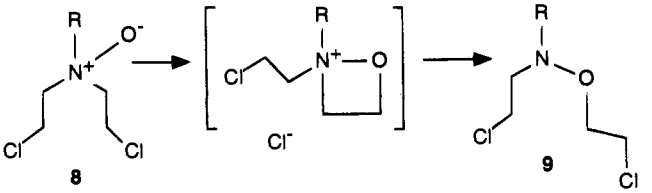
In contrast, recent reports[11–13] on the aniline mustard chlorambucil *N*-oxide (**4**) suggest this compound has essentially no hypoxic selectivity in cell culture, although modest selectivity was observed on addition of rat liver microsomes and NADPH.[13] The reported synthesis of **4** and its ethyl ester **6**, by oxidation of the parent mustards (**3** and **5**) with peracetic acid (Scheme 1), has a number of surprising features, including the low polarity of the products, their unusual ^{1}H NMR spectra, and the use of aqueous base in the workup (at least in the case of **6**).[11] The synthesis of **4** had earlier been reported[14,15] to give a crystalline hydrochloride salt

Scheme 1[a]



[a] (i) Peracetic acid, 0 °C, 4 h, then aqueous $NaHCO_3$ (ref 11), or peracetic acid, 20 °C, 2 h, then HCl (this work); (ii) H_2O, OH^- (ref 15).

Scheme 2



8 **9**

which decomposed in aqueous alkali (Scheme 1), with the half-mustard **7** being the major product.[15] In contrast, Sakurai and Izumi[16] and Owari[17] report that aliphatic mustard *N*-oxides **8** rearrange in aqueous solution *via* a cyclic intermediate, to give trisubstituted hydroxylamines **9** as products (Scheme 2). The rearrangement was found to be general for a range of *N*-oxides of aliphatic mustards, but aniline mustards were not studied. In view of the uncertainty surrounding both the synthesis and stability of chlorambucil *N*-oxide, and the bearing this may have on its use as a bioreductive agent, we report here a reinvestigation of its synthesis and properties.

Chemistry

Oxidation of the ethyl ester **5** using the method described by Mann and Shervington[11] (excess peracetic

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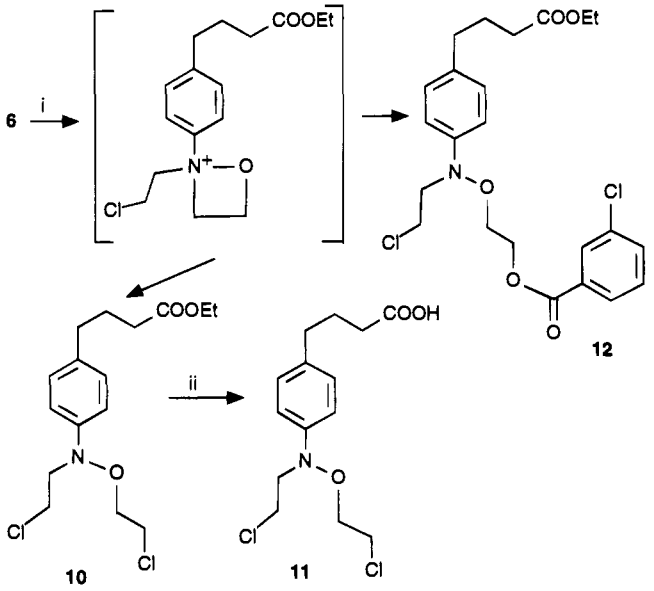
Table 1. ^{13}C NMR Data for Hydroxylamine Derivatives **10** and **11**

δ_C, ppm ($CDCl_3$)			
a	**10**	*b*	**11**
14.2	14.3		
26.5	26.6	26.2	26.2
33.6	33.7	33.2	33.3
34.4	34.4	34.2	34.2
	40.6		40.6
41.9	41.9	41.9	41.9
60.2	60.3		
	61.9		61.8
72.8	72.9	72.9	72.8
118.2	118.3	118.6	118.2
129.0	129.1	129.0	129.0
137.0	137.2	136.8	136.8
148.7	148.7	148.8	148.7
173.4	173.5	179.7	179.8

[a] Data attributed in ref 11 to the *N*-oxide **6**. [b] Data attributed in ref 11 to the *N*-oxide **4**.

Scheme 3[a]



[a] (i) mCPBA, CH_2Cl_2; (ii) LiOH, aqueous EtOH.

acid, 0 °C, 4 h) resulted in incomplete reaction. While the major product (*ca.* 70%) appeared identical to that described as the corresponding *N*-oxide **6**, it could not be separated from unreacted starting material, even by HPLC, while more vigorous oxidizing conditions (20 °C, 6 h) resulted in overoxidation to several byproducts of similar chromatographic mobility. However, oxidation of **5** with 3-chloroperbenzoic acid, followed by workup with aqueous bicarbonate, consistently gave a 55–70% yield of this product in a pure state. It had a ^1H NMR spectrum identical to that reported[11] as the *N*-oxide **6**, but the ^{13}C NMR spectrum contained two additional resonances (Table 1) and is more consistent with the hydroxylamine structure **10**. This suggests that the intramolecular rearrangement reported for aliphatic mustard *N*-oxides[16,17] (Scheme 2) also applies to aniline mustards (Scheme 3). The hydroxylamine structure is also more consistent with the observed ^1H NMR spectrum, with the set of four triplets in the range δ 4.0–3.5 arising from the two distinct chloroethyl side chains. The mass spectrum of **10** further supports a hydroxylamine structure, as the major fragmentation patterns result from loss of CH_2Cl (30%) and CH_2CH_2Cl (100%), not loss of OH (<1%) or H_2O as reported for alkyl *N*-oxides.[18] The intermediacy of a cyclic oxazetidinium species would also explain the isolation of what appeared to be **12** (as judged by ^1H and ^{13}C NMR) as a byproduct of the 3-chloroperbenzoic acid oxidation (Scheme 3). Trapping of this type of intermediate by nucleophiles such as bromide or benzoate has previously been described.[17]

Similar behavior was observed for the oxidation of chlorambucil (**3**), with the reported[11] conditions giving a major product identical to that described but in the presence of byproducts that were difficult to separate. The major product was more conveniently obtained by hydrolysis of the ester **10** and again had NMR spectra supporting its structure as the hydroxylamine **11** (Table 1). In contrast, authentic chlorambucil *N*-oxide (**4**) was prepared as the hydrochloride salt by a modification of the reported procedure,[11] in which chlorambucil was oxidized with peracetic acid and the *N*-oxide extracted directly with aqueous HCl. Although the hydrochloride salt could not be obtained crystalline, NMR analysis showed this material to be pure. The hydrochloride salt of **4** was stable for at least a week at room temperature in DCl (20% in D_2O) (**4**·HCl), and for at least 3 days in CD_3OD but underwent smooth rearrangement in $(CD_3)_2SO$ to the hydroxylamine **11** (Figure 1). This rearrangement was initially rapid, with some **11** appearing in the time taken to acquire the first spectrum, but the gradual release of HCl led to a decrease in the rate of reaction.

The rate of rearrangement of **4** under neutral aqueous conditions was investigated by UV spectroscopy. In phosphate buffer (pH 7.4)/MeOH (1:1) at 20 °C, a smooth transition was observed to a spectrum very similar to that of **11** (λ_{max} 244 nm), with first-order kinetics (k = 1.05×10^{-4} s^{-1}; $t_{1/2}$ = 110 min). However, on a preparative scale under the same conditions, a complex mixture of products was isolated, with the expected **11** accounting for only 7% of the material. The remaining products were not consistent with trapping of an oxazetidinium intermediate by the other nucleophiles present (H_2O, MeOH, phosphate), with the major product isolated (38%) being chlorambucil half-mustard (**7**). The hydroxylamine **11** was not an intermediate in the formation of **7**, being recovered from this solvent system in >95% yield, even in the presence of added trimethylamine *N*-oxide.

To confirm the solvent dependency of the course of rearrangement, the decomposition of **4** in deuterated phosphate buffer/MeOH was followed by ^1H NMR. In marked contrast to the behavior in anhydrous $(CD_3)_2SO$, the hydroxylamine **11** was not a detectable intermediate. Instead a rearrangement to the half-mustard **7** was observed (Figure 2), with this product accounting for over 90% of the material after 8 h. By plotting integral values from the aromatic region of the spectra, it was found that **4** disappeared in a first-order process with $t_{1/2}$ = 120 min, in good agreement with the half-life found by UV spectroscopy. In tissue culture medium (phenol red-free minimal essential medium containing 5% FCS, pH 7.4, 37 °C), following the reaction by UV spectroscopy, a first-order kinetic growth of absorbance at 244 nm ($t_{1/2}$ *ca.* 30 min) was seen. HPLC of the reaction mixture at 90 min showed **7** as the major product (33%), while **11** was not detected.

Previous reports[15,19] on the rearrangment of mustard *N*-oxides have claimed that oxidative cleavage occurs

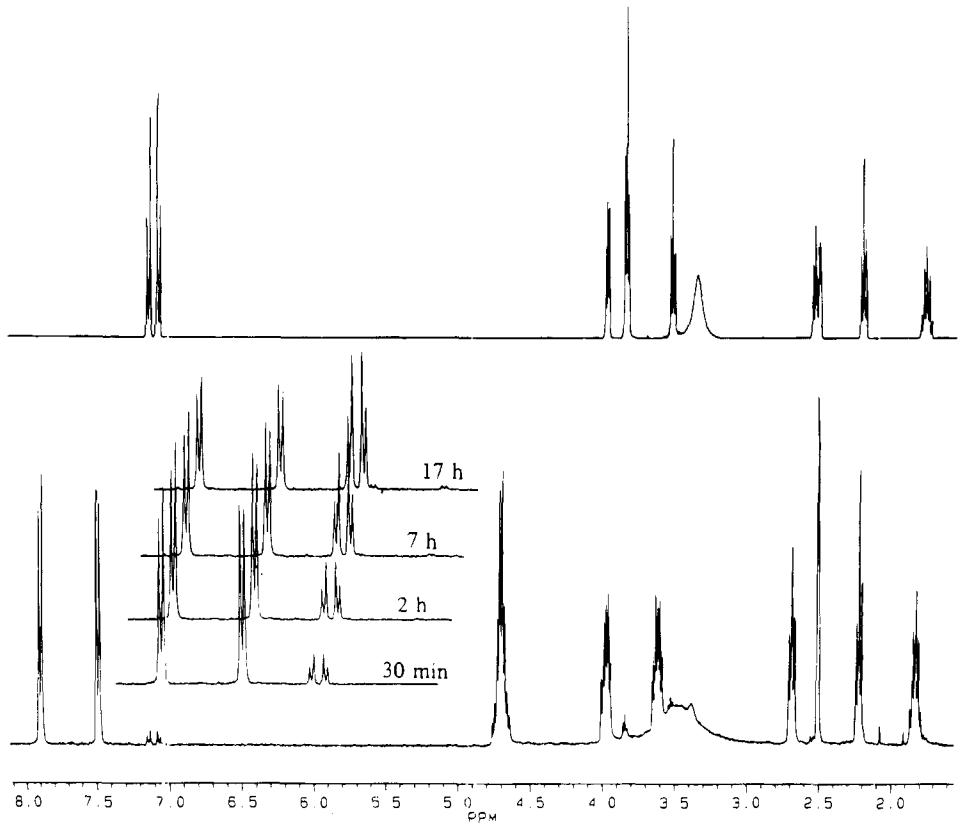


Figure 1. ^{1}H NMR spectra (400 MHz in $(CD_3)_2SO$) for **4** (bottom trace) and **11** (top trace) at zero time. Insert is an expansion of the aromatic region, showing evolution of trace of **4** with time.

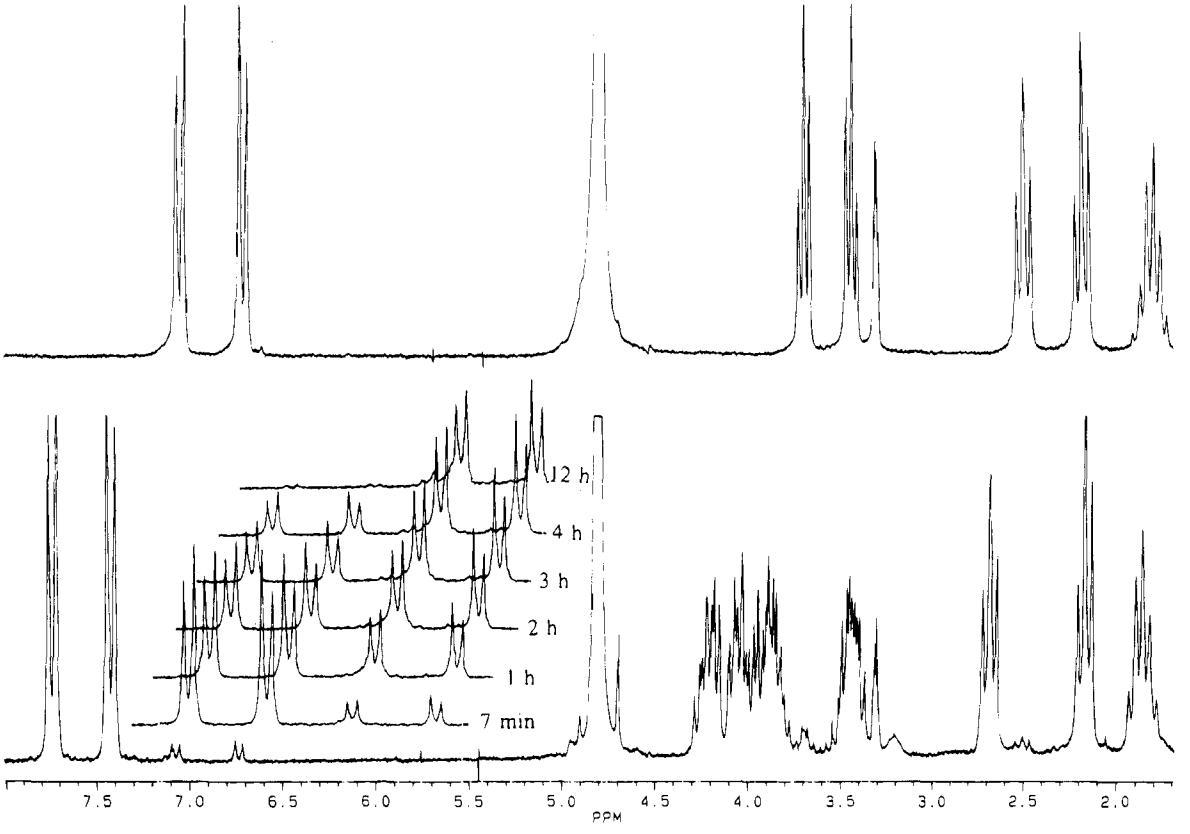
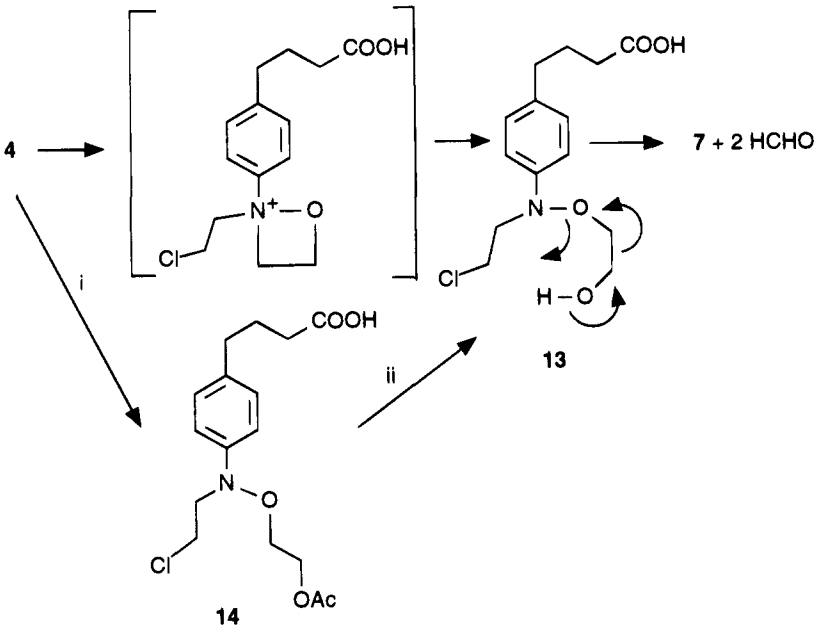


Figure 2. ^{1}H NMR spectra (200 MHz in 1:1 CD_3OD:deuterated phosphate buffer (0.1M, pD 7.0)) for **4** (bottom trace) and **7** (top trace) at zero time. Insert is an expansion of the aromatic region, showing evolution of trace of **4** with time.

between the two carbons of one chloroethyl side chain, producing formaldehyde as a byproduct. Generation of formaldehyde was confirmed when a solution of **4** aged in buffer/MeOH was treated with (2,4-dinitrophenyl)-hydrazine reagent, yielding the hydrazone of formaldehyde (*ca.* 1–2 mol/mol of **4**) but not those of chloroacetaldehyde nor acetaldehyde. One plausible mechanism for this unusual reaction (Scheme 4), involving trapping

Scheme 4



[a] (i)NaOAc, Ag_2CO_3, aqueous THF; (ii) LiOH, aqueous THF.

Table 2. Cytotoxicity of Chlorambucil *N*-oxide (**4**) and Related Compounds

	IC_{50}[a] (μM; air)		AA8 CT_{10}	
no.	AA8	UV4	(μM h; air)	(air/N_2)[b]
3	38 ± 1 (3)[c]	1.2 ± 0.2 (3)	85.2 ± 7.6 (3)	1.28 ± 0.17 (3)
4	46 ± 2 (3)	4.9 ± 1.2 (4)	960 ± 0 (2)	1.7 ± 0 (2)
7	>250	90	2080 ± 80 (2)	1.26 ± 0.17 (2)
11	456 ± 36	176	>2400	
HCHO	179 ± 9 (3)	138 ± 21 (3)	2000 (1)	0.61 (1)

[a] IC_{50} values (μM) for 4 h drug exposures under aerobic conditions. [b] Air/N_2 ratios are the means of ratios of T_{10} values under aerobic and hypoxic conditions at the same drug concentration. [c] Values in parentheses are numbers of determinations. Errors are ranges (for two determinations) or standard errors.

of the oxazetidinium intermediate by water and rearrangement of the resulting alcohol, was discounted when it was found that the alcohol **13**, prepared by the route shown in Scheme 4, was completely stable in the phosphate buffer/MeOH mixture. The mechanism of dechloroethylation remains unclear.

Biological Assays

The biological activities of **3**, **4**, **7**, **11**, and formaldehyde are recorded in Table 2. IC_{50} values were determined in both repair-proficient (AA8) and repair-deficient (UV4) cells in a growth inhibition assay, while selectivity toward hypoxic cells was measured in stirred suspensions of AA8 cells using a clonogenic assay.[20,21] In the latter assay, the concentration × time to reduce the surviving fraction to 10% (CT_{10}) was used as an inverse measure of cytotoxic potency, while the ratio of T_{10} values under aerobic and hypoxic conditions at the same drug concentration was used as a measure of hypoxic selectivity. The large IC_{50} ratios between AA8 and UV4 cells for both **3** (32-fold) and **4** (10-fold) suggest a similar mode of cytotoxicity (*via* DNA alkylation) for both compounds; in contrast formaldehyde has a ratio of essentially unity. In the clonogenic assay (Table 2 and Figure 3), the *N*-oxide **4** was about 12-fold less cytotoxic than chlorambucil (**3**) under aerobic conditions (CT_{10} 960 versus 85 μM h) but the half-mustard **7** was even less cytotoxic (CT_{10} 2080 μM h). The *N*-oxide **4** showed a hypoxic selectivity of 1.7-fold, which was only slightly greater than that of the non-*N*-oxide compounds **3** and **7**. The hydroxylamine **11** was not cytotoxic under

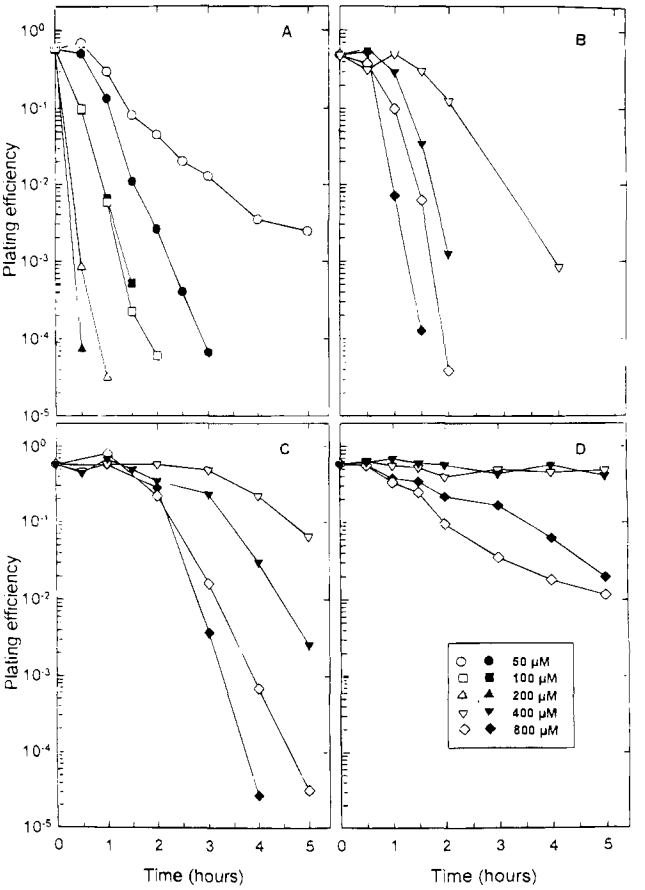


Figure 3. Cell killing by various concentrations of chlorambucil (**3**) (panel A), chlorambucil *N*-oxide (**4**) (panel B), the half-mustard **7** (panel C), and formaldehyde (panel D) of suspension cultures of late log phase AA8 cells (10^6 cells/mL) under aerobic (open symbols) or hypoxic (filled symbols) conditions. The plating efficiencies of non-drug-treated cells were constant over this time under either aerobic or hypoxic conditions (data not shown).

either aerobic or hypoxic conditions at concentrations up to 800 μM.

Discussion and Conclusions

The data presented above strongly suggest that the recently reported[11,13] syntheses of chlorambucil *N*-oxide (**4**) (quoted[13] as a stable compound of mp 46.1 °C) in fact describe the (stable) hydroxylamine rearrangement product **11** (mp 45.5–47.5 °C; see the Experimental Section). Earlier syntheses[14,15] of **4**, and several features of its instability, are likely to have been correctly reported. We have shown that **4** decomposes in some organic solvents to give the hydroxylamine **11**, as reported[16,17] for aliphatic mustard *N*-oxides. In contrast, in aqueous buffer **4** undergoes decomposition to give largely the half-mustard **7**. The mechanism by which one chloroethyl side chain is lost has not been determined but appears unrelated to the metabolic oxidative dechloroethylation of nitrogen mustards[22] because chloroacetaldehyde is not a detectable byproduct.

The half-life of **4** in culture medium at 37 °C is approximately 30 min, and the major product of decomposition under these conditions is the half-mustard **7**. Despite this, when evaluated in a clonogenic assay over a time course of several hours (Figure 3), **4** is significantly more cytotoxic than either of the major detected decomposition products, the half-mustard **7** and formaldehyde, and it appears unlikely that all of the observed toxicity of **4** is due to these products. This suggests that either **4** itself or some other unidentified byproduct contributes significantly to the observed cytotoxicity. In the clonogenic assay both chlorambucil (**3**) and the half-mustard **7** showed a slight selectivity (1.3-fold) for hypoxic cells, an unexplained effect noted previously for other aromatic nitrogen mustards.[23,24] However, the *N*-oxide **4** did not show any improvement in hypoxic selectivity (in contrast[10] to the behavior of nitromin (**2**)). It is not clear whether **4** itself lacks hypoxic selectivity or whether it is simply too unstable under the neutral, aqueous conditions of the assay. Taken together, these results suggest that the instability of *N*-oxides of aniline mustards places severe constraints on their development as HSCs.

Experimental Section

Chemistry. Analyses were carried out in the Microchemical Laboratory, University of Otago, Dunedin, NZ. Melting points were determined on an Electrothermal 2300 melting point apparatus. NMR spectra were obtained on Bruker AC-200 or AM-400 spectrometers and are referenced to Me_4Si, except for spectra in buffer which are referenced to $Me_3Si(CH_2)_3SO_3Na$. Thin-layer chromatography was carried out on aluminum-backed silica gel plates (Merck 60 F_{254}). Dry column chromatography was carried out on Merck silica gel (20–40 mesh).[25] Petroleum ether refers to the fraction boiling at 40–60 °C. Deuterated phosphate buffer (0.1 M, pD 7.0) was prepared from NaD_2PO_4 and NaOD in D_2O. For the kinetics experiment a solution of **4**·HCl (10 mg, 28 μmol) in CD_3OD (0.5 mL) was added to deuterated phosphate buffer (0.5 mL) containing NaOD (28 μmol). ^1H NMR spectra were recorded automatically at 15 min intervals over 12 h, and each spectrum was processed identically. A plot of log percent integral assignable to **4** (δ 7.85–7.30 from the aromatic region δ 7.90–6.60) versus time for the first 5 h gave a straight line (r^2 = 0.999) with $t_{1/2}$ = 120 min.

4-[4′-[*N*-(2″-Chloroethoxy)-*N*-(2‴-chloroethyl)amino]-phenyl]butanoic Acid (11). 3-Chloroperoxybenzoic acid (1.42 g, 80%, 6.6 mmol) was added in portions over 10 min to ethyl 4-[4′-[*N*,*N*-bis(2″-chloroethyl)amino]phenyl]butanoate (**5**)[26] (1.68 g, 5.1 mmol) in CH_2Cl_2 (30 mL), and the solution was stirred at 20 °C for 80 min. Saturated aqueous $NaHCO_3$ (50 mL) was added, and the mixture was stirred vigorously for 30 min. The organic layer was separated, combined with two further CH_2Cl_2 extracts, dried (Na_2SO_4), and evaporated. Dry column chromatography, eluting with petroleum ether/EtOAc (10:1), gave ethyl 4-[4′-[*N*-(2″-chloroethoxy)-*N*-(2‴-chloroethyl)-amino]phenyl]butanoate (**10**) as a colorless oil (1.03 g, 58%): ^1H NMR ($CDCl_3$) δ 7.15–7.05 (m, 4 H, ArH), 4.13 (q, J = 7.2 Hz, 2 H, OCH_2CH_3), 3.98 (t, J = 5.5 Hz, 2 H, CH_2), 3.77 (t, J = 6.4 Hz, 2 H, CH_2), 3.71 (t, J = 5.5 Hz, 2 H, CH_2), 3.48 (t, J = 6.4 Hz, 2 H, CH_2), 2.61 (t, J = 7.6 Hz, 2 H, $ArCH_2$), 2.32 (t, J = 7.5 Hz, 2 H, CH_2CO_2Et), 1.98–1.89 (m, 2 H, $CH_2CH_2CO_2Et$), 1.26 (t, J = 7.2 Hz, 3 H, OCH_2CH_3); ^{13}C NMR see Table 1; MS (EI, ^{35}Cl peaks) m/z 347 (55, M^+), 302 (20, M−OEt), 298 (30, $M-CH_2Cl$), 284 (100, $M-CH_2CH_2Cl$). Anal. ($C_{16}H_{23}Cl_2NO_3$) C, H, N.

A mixture of **10** (1.03 g, 3.0 mmol) and $LiOH·H_2O$ (0.25 g, 6.0 mmol) in EtOH (40 mL) and H_2O (15 mL) was stirred at 20 °C for 17 h. The EtOH was evaporated, and the aqueous phase was made slightly acidic with 2 N HCl and extracted with CH_2Cl_2 (3×). The organic extracts were dried (Na_2SO_4) and evaporated, and the residual oil was crystallized from petroleum ether (20 mL, −18 °C) to give 4-[4′-[*N*-(2″-chloroethoxy)-*N*-(2‴-chloroethyl)amino]phenyl]butanoic acid (**11**) as a white crystalline solid (0.80 g, 84%): mp 45.5–47.5 °C; ^1H NMR ($CDCl_3$) δ 9.3 (br s, 1 H, CO_2H), 7.17–7.03 (m, 4 H, ArH), 4.00 (t, J = 5.5 Hz, 2 H, CH_2), 3.76 (t, J = 6.3 Hz, 2 H, CH_2), 3.72 (t, J = 5.5 Hz, 2 H, CH_2), 3.48 (t, J = 6.3 Hz, 2 H, CH_2), 2.64 (t, J = 7.5 Hz, 2 H, $ArCH_2$), 2.38 (t, J = 7.4 Hz, 2 H, CH_2CO_2H), 2.05–1.85 (m, 2 H, $CH_2CH_2CO_2H$); ^{13}C NMR see Table 1; MS (EI, ^{35}Cl peaks) m/z 319 (35, M^+), 270 (35, $M-CH_2Cl$), 256 (100, $M-CH_2CH_2Cl$); HRMS calcd for $C_{14}H_{19}Cl_2NO_3$ 319.0742 (M^+), found 319.0739. Anal. ($C_{14}H_{19}Cl_2NO_3$) C, H, N, Cl.

4-[4′-[*N*,*N*-Bis(2″-chloroethyl)amino]phenyl]butanoic Acid *N*-Oxide Hydrochloride (4·HCl). Peracetic acid was prepared by adding H_2O_2 (1.5 mL of a 35% aqueous solution, 14 mmol) dropwise to Ac_2O (1.5 mL, 16 mmol) and stirring for 15 min, giving a clear homogeneous solution. To this was added dropwise a solution of 4-[4′-[*N*,*N*-bis-(2″-chloroethyl)-amino]phenyl]butanoic acid (**3**)[26] (1.00 g, 3.29 mmol) in CH_2Cl_2 (20 mL), and the mixture was stirred vigorously at 20 °C for 2 h. The pale yellow mixture was diluted with aqueous HCl (2 N, 20 mL) and stirred for a further 30 min. The aqueous layer was separated and washed repeatedly with CH_2Cl_2 until the extracts were colorless and the aqueous layer evaporated. The residue was dissolved in MeCN, dried (Na_2SO_4), and reduced in volume to *ca.* 4 mL. Et_2O (50 mL) was slowly added and the resulting suspension allowed to stand at 4 °C for 15 min. The supernatant was decanted and the oil evacuated to remove residual solvent, giving 4-[4′-[*N*,*N*-bis-(2″-chloroethyl)amino]phenyl]butanoic acid *N*-oxide hydrochloride (**4**·HCl) as a pale yellow oily foam (0.60 g, 51%): ^1H NMR ($(CD_3)_2SO$) δ 7.87 (d, J = 8.7 Hz, 2 H, ArH *ortho* to *N*-oxide), 7.49 (d, J = 8.7 Hz, 2 H, ArH *meta* to *N*-oxide), 4.75–4.60 (m, 4 H, $N(CH_2CH_2Cl)_2$), 4.00–3.92 (m, 2 H of $N(CH_2CH_2Cl)_2$), 3.66–3.58 (m, 2 H of $N(CH_2CH_2Cl)_2$), 2.69 (t, J = 7.6 Hz, 2 H, $ArCH_2$), 2.22 (t, J = 7.3 Hz, 2 H, CH_2CO_2H), 1.90–1.78 (m, 2 H, $CH_2CH_2CO_2H$); ^{13}C NMR δ 174.1 (CO_2H), 145.0, 141.0 (aromatic C-CH_2 and C-N), 130.0, 120.7 (aromatic CH), 69.6 (NCH_2), 35.3 (CH_2Cl), 33.6, 32.9 ($CH_2CH_2CH_2CO_2H$), 25.8 ($CH_2CH_2CO_2H$); MS (FAB, ^{35}Cl peaks) m/z 320 (100, M^+), 303 (55, M−OH), 254 (50, M−OH−CH_2Cl); HRMS calcd for $C_{14}H_{20}Cl_2NO_3$ 320.0820 (M^+), found 320.0827.

Attempts to crystallize this material from a number of solvents (Me_2CO, Me_2CO/Et_2O, MeCN/Et_2O) were unsuccessful. A sample was converted to the tosylate salt and crystallized from *i*-PrOH/Et_2O to give white hygroscopic needles, mp 100–105 °C dec (lit.[13] mp 117–118 °C (Me_2CO/Et_2O)). ^1H NMR analysis showed this sample contained much trapped solvent.

Decomposition of 4 in Phosphate Buffer/MeOH. A solution of **4** (251 mg, 0.70 mmol) in MeOH (20 mL) and phosphate buffer (0.1 M, pH 7.4, 20 mL) was stirred at 20 °C for 15 h. The mixture was extracted with CH_2Cl_2 (4×), and the extracts were dried (Na_2SO_4) and evaporated. The residue

was separated by dry column chromatography, eluting with petroleum ether/EtOAc (3:1), to give **11** (15 mg, 7%) identical (TLC, ^1H and ^{13}C NMR) to the material described above. Further elution with petroleum ether/EtOAc (1:1) gave **7** (65 mg, 38%) identical (TLC, ^1H and ^{13}C NMR) to authentic chlorambucil half-mustard,[27] mp 50–52.5 °C (petroleum ether) (lit.[27] mp 39–40 °C, for a sample containing $0.25H_2O$).

Decomposition of 4 in Culture Medium. A fresh stock solution of **4** (2 mM in MeOH) was diluted into phenol red-free Eagle's modified MEM containing 5% FCS and 10 mM HEPES, pH 7.4, to a final concentration of 40 μM. This was incubated at 37 °C in a water-jacketed Hewlett Packard 8452A diode array spectrophotometer, spectra were collected at intervals of 5 min, and the absorbance change at 244 nm was analyzed. After 1.5 h a sample of the reaction mixture was analyzed by HPLC with a pH 4.5 ammonium formate mobile phase as described[28] but with an initial MeCN concentration of 14% which was increased linearly to 80% MeCN between 2 and 12 min. This gave retention times of 14.3 min for **7** and 16.2 min for **3** and **11** (not resolved).

(2,4-Dinitrophenyl)hydrazine Trapping Experiments. (2,4-Dinitrophenyl)hydrazine (2,4-DNP) reagent (0.1 M in EtOH/phosphoric acid)[29] and authentic samples of the (2,4-dinitrophenyl)hydrazones of formaldehyde,[15] acetaldehyde,[30] and chloroacetaldehyde[30] were prepared by literature methods. A solution of **4**·HCl in buffer/methanol, at the same concentration as described above for the NMR experiment, was allowed to stand in a sealed flask at 20 °C for 5 h (sufficient to result in >80% decomposition of **4**). This mixture was then treated with 2,4-DNP reagent (4 equiv) and left at 20 °C for 17 h. The mixture was extracted with CH_2Cl_2 (3×), and the extracts were dried (Na_2SO_4), evaporated onto silica gel, and chromatographed on a short column of silica gel, collecting all fractions running faster than the excess 2,4-DNP. Analysis by ^1H NMR showed the presence of formaldehyde (2,4-dinitrophenyl)-hydrazone. Control experiments confirmed that both chloroacetaldehyde and acetaldehyde were converted to their hydrazones under these conditions, but these were not detected from the aged solution of **4**·HCl.

Clonogenic Assay for Hypoxia-Selective Cytotoxicity. These were performed using magnetically stirred 10 mL suspension cultures (late log phase AA8 cells, 10^6/mL), removing samples periodically during continuous gassing with 5% CO_2 in air or N_2 as detailed elsewhere.[20] The concentration x time for a surviving fraction of 10% (CT_{10}) was used as an inverse measure of cytotoxicity.

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Supplementary Material Available: Syntheses of **13** and **14** (2 pages). Ordering information is given on any current masthead page.

References

(1) Moulder, J. E.; Rockwell, S. Hypoxic fractions of solid tumors: experimental techniques, methods of analysis and a survey of existing data. *Int. J. Radiat. Oncol. Biol. Phys.* **1984**, *10*, 695–712.
(2) Wilson, W. R. Tumour hypoxia: challenges for cancer chemotherapy. In *Cancer Biology and Medicine;* Waring, M. J., Ponder, B. A. J., Eds.; Kluwer Academic Publishers: Lancaster, 1992; Vol. 3, pp 87–131.
(3) Denny, W. A.; Wilson, W. R. Bioreducible mustards: a paradigm for hypoxia-selective prodrugs of diffusible cytotoxins (HPDCs). *Cancer Met. Rev.* **1993**, *12*, 135–151.
(4) Ware, D. C.; Palmer, B. D.; Wilson, W. R.; Denny, W. A. Hypoxia-selective antitumor agents. 7. Metal complexes of aliphatic mustards are a new class of hypoxia-selective cytotoxins. Synthesis and evaluation of cobalt (III) complexes of bidentate mustards. *J. Med. Chem.* **1993**, *36*, 1839–1846.
(5) Tercel, M.; Wilson. W. R.; Denny, W. A. Nitrobenzyl mustard quaternary salts: a new class of hypoxia-selective cytotoxins showing high *in vitro* selectivity. *J. Med. Chem.* **1993**, *36*, 2578–2579.
(6) Palmer, B. D.; Wilson, W. R.; Cliffe, S.; Denny, W. A. Hypoxia-selective antitumor agents. 5. Synthesis of water-soluble nitroaniline mustards with selective cytotoxicity for hypoxic mammalian cells. *J. Med. Chem.* **1992**, *35*, 3214–3222.
(7) Perrin, D. D. *Dissociation constants of organic bases in aqueous solution;* Butterworths: London, 1965; Supplement 1972.
(8) Wilson, W. R.; van Zijl, P.; Denny, W. A. Bis-bioreductive agents as hypoxia-selective cytotoxins: Nitracrine N-oxide. *Int. J. Radiat. Oncol. Biol. Phys.* **1992**, *22*, 693–696.
(9) Patterson, L. H. Rationale for the use of aliphatic N-oxides of cytotoxic anthraquinones as prodrug DNA binding agents: a new class of bioreductive agent. *Cancer Met. Rev.* **1993**, *12*, 119–134.
(10) White, I. N. H.; Suzanger, M.; Mattocks, A. R.; Bailey, E.; Farmer, P. B.; Connors, T. A. Reduction of nitromin to nitrogen mustard: unscheduled DNA synthesis in aerobic or anaerobic rat hepatocytes, JB1, BL8 and Walker carcinoma cell lines. *Carcinogenesis* **1989**, *10*, 2113–2118.
(11) Mann, J.; Shervington, L. A. Synthesis of novel *N*- and *S*-mustards as potential pro-drugs activated by bioreductive processes. *J. Chem. Soc. Perkin Trans. 1* **1991**, 2961–2964.
(12) Kirkpatrick, D. L.; Campbell, K.; Zhu, L.; Schroeder, H. Bioreductive potential of *N*-oxide derivatives of alkylating agents. *Proceedings of the 8th International Conference on Chemical Modifiers of Cancer Treatment*, Kyoto, Japan, June 16–19, 1993.
(13) Kirkpatrick, D. L.; Schroeder, H. L.; Chandler, K. J. Synthesis and bioreductive potential of a mono *N*-oxide derivative of the alkylating agent chlorambucil. *Anti-Cancer Drugs* **1994**, *5*, 467–472.
(14) Degutene, A.; Rasteikene, L.; Degutis, Y. *p*-[Di(2-chloroethyl)-amino]phenylalkanoic acid *N*-oxides. *J. Org. Chem. USSR* **1971**, *7*, 1433–1435.
(15) Degutene, A.; Dzhyuvene, D.; Shukyalene, D.; Degutis, Y. Synthesis and spontaneous decomposition of the *N*-oxides of di-(2-chloroalkyl)aminophenylalkanoic Acids. *J. Org. Chem. USSR* **1986**, *22*, 1968–1972.
(16) Sakurai, Y.; Izumi, M. *N*-oxides of β-chloroethylamine derivatives. *Chem. Pharm. Bull.* **1953**, *1*, 297–301.
(17) Owari, S. Transformation reaction of nitrogen mustard *N*-oxides in aqueous solution. *Chem. Pharm. Bull.* **1953**, *1*, 353–357.
(18) Bild, N.; Hesse, M. Notiz uber die Massenspektren von N-Oxiden. (Note on the mass spectra of N-oxides.) *Helv. Chim. Acta* **1967**, *50*, 1885–1892.
(19) Degutis, Y.; Medekshene, G. Transformation of aryldi(2-chloroethyl)amine *N*-oxides in solution. *J. Org. Chem. USSR* **1972**, *8*, 791–794.
(20) Wilson, W. R.; Anderson, R. F.; Denny, W. A. Hypoxia-selective antitumor agents. 1. Relationships between structure, redox properties and hypoxia-selective cytotoxicity for 4-substituted derivatives of nitracrine. *J. Med. Chem.* **1989**, *32*, 23–30.
(21) Whillans, D. W.; Rauth, A. M. An experimental and analytical study of oxygen depletion in stirred cell suspensions. *Radiat. Res.* **1980**, *34*, 97–103.
(22) Shaw, I. C.; Graham, M. I.; McLean, A. E. M. 2-Chloroacetaldehyde, a metabolite of cyclophosphamide in the rat. *Xenobiotica* **1983**, *13*, 433–437.
(23) Palmer, B. D.; Wilson, W. R.; Pullen, S. M.; Denny, W. A. Hypoxia-selective antitumor agents. 3. Relationships between structure and cytotoxicity against cultured tumor cells for substituted N,N-bis(2-chloroethyl)anilines. *J. Med. Chem.* **1990**, *33*, 112–121.
(24) Chaplin, D. J.; Acker, B.; Olive, P. L. Potentiation of the tumor cytotoxicity of melphalan by vasodilating drugs. *Int. J. Radiat. Oncol. Biol. Phys.* **1989**, *16*, 1131–1135.
(25) Harwood, L. M. *Aldrichim. Acta* **1985**, *18*, 25.
(26) Everett, J. L.; Roberts, J. J.; Ross, W. C. J. Aryl-2-halogenoalkylamines. Part XII. Some carboxylic derivatives of *N,N*-di-2-chloroethylaniline. *J. Chem. Soc.* **1953**, 2386–2392.
(27) Ferguson, L. R.; Palmer, B. D.; Denny, W. A. Microbial mutagenicity of chlorambucil, its half-mustard and mitomycin C: a modified screening strategy for genetic toxicology of bis-alkylating anti-tumour drugs. *Anti-Cancer Drug Des.* **1988**, *3*, 67–76.
(28) Siim, B. G.; Atwell. G. J.; Wilson, W. R. Metabolic and radiolytic reduction of 5–nitroquinoline bioreductive drugs: relationship to selectivity for hypoxic cells. *Biochem. Pharmacol.*, in press.
(29) Fieser, L. F.; Fieser, M. *Reagents for organic synthesis;* John Wiley & Sons, Inc.: New York, 1967; Vol. 1, p 330.
(30) Hegarty, A. F.; Scott, F. L. The kinetics of *syn-anti* conversions of 2,4-dinitrophenylhydrazones. *J. Org. Chem.* **1968**, *33*, 753–762.

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