

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

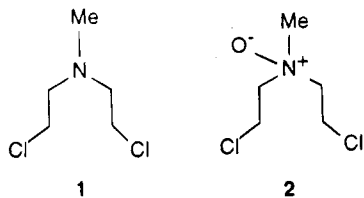
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The potential hypoxia-selective cytotoxin 4-[4'-[*N,N*-bis(2''-chloroethyl)amino]phenyl]butanoic acid *N*-oxide (chlorambucil *N*-oxide, **4**) was synthesized and characterized as its hydrochloride salt. This compound was shown to be unstable, decomposing 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 cells¹ which can limit the sensitivity of solid tumors to radiotherapy.² We have suggested the utility of nitrogen mustards as "effector units" in HSCs³ 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 compounds⁶). 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 units⁷). 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



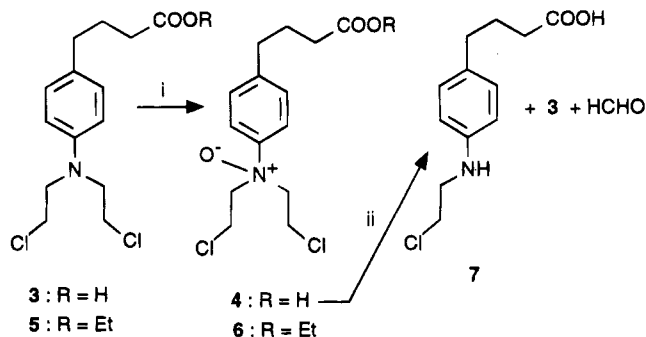
In contrast, recent reports¹¹⁻¹³ on the aniline mustard chlorambucil *N*-oxide (**4**) suggest this compound has essentially no hypoxic selectivity in cell culture, although modest selectivity was observed on addition of rat liver microsomes and NADPH.¹³ The reported synthesis of **4** and its ethyl 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 ¹H 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

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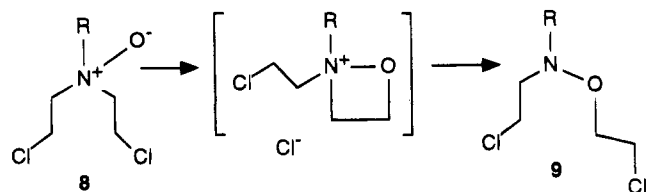
[⊗] Abstract published in *Advance ACS Abstracts*, February 15, 1995.

Scheme 1^a



^a (i) Peracetic acid, 0 °C, 4 h, then aqueous NaHCO₃ (ref 11), or peracetic acid, 20 °C, 2 h, then HCl (this work); (ii) H₂O, OH⁻ (ref 15).

Scheme 2



which decomposed in aqueous alkali (Scheme 1), with the half-mustard **7** being the major product.¹⁵ In contrast, Sakurai and Izumi¹⁶ and Owari¹⁷ 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.

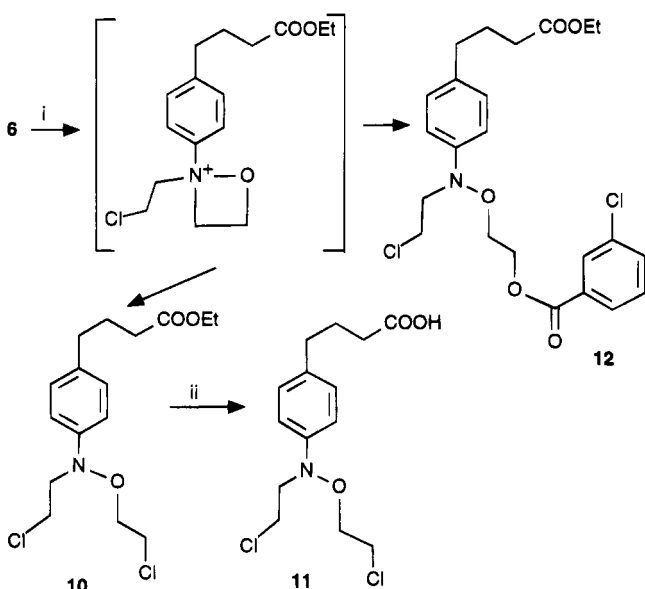
Chemistry

Oxidation of the ethyl ester **5** using the method described by Mann and Shervington¹¹ (excess peracetic

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

δ_{C} , ppm (CDCl_3)			
<i>a</i>	10	<i>b</i>	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) *m*CPBA, 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 reported¹¹ 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 $\text{CH}_2\text{CH}_2\text{Cl}$ (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 reported¹¹ 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) ($4\cdot\text{HCl}$), and for at least 3 days in CD_3OD but underwent smooth rearrangement in $(\text{CD}_3)_2\text{SO}$ 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 ($k = 1.05 \times 10^{-4} \text{ s}^{-1}$; $t_{1/2} = 110 \text{ min}$). 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 $(\text{CD}_3)_2\text{SO}$, 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 $t_{1/2} = 120 \text{ min}$, 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 rearrangement of mustard *N*-oxides have claimed that oxidative cleavage occurs

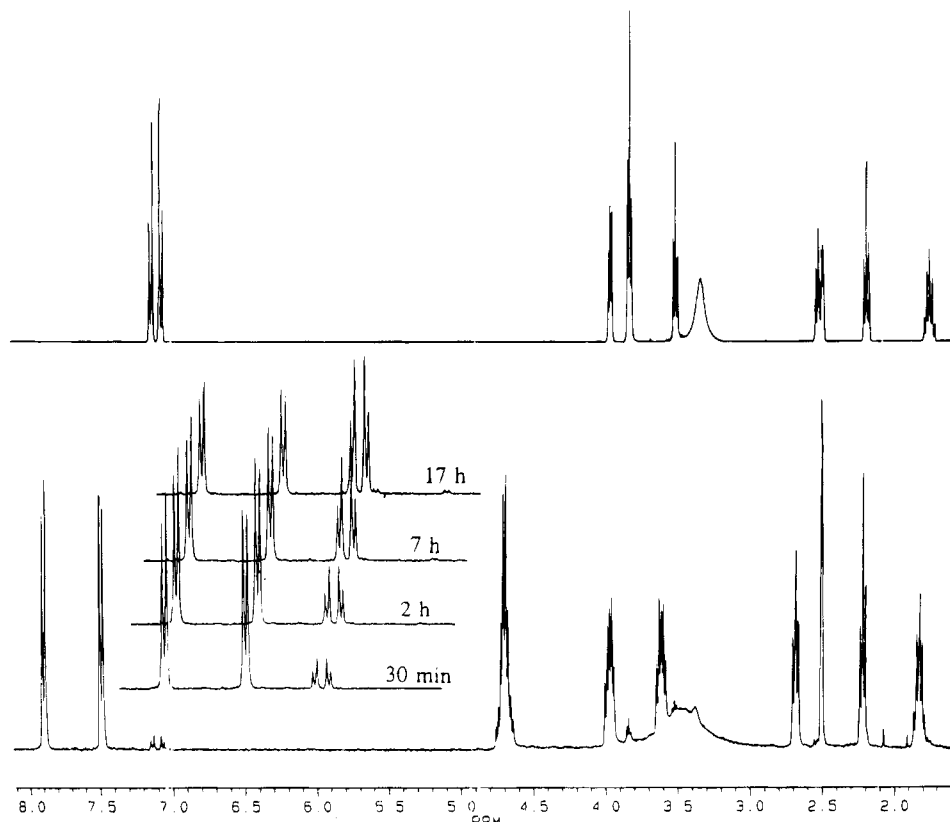


Figure 1. ^1H NMR spectra (400 MHz in $(\text{CD}_3)_2\text{SO}$) for **4** (bottom trace) and **11** (top trace) at zero time. Insert is an expansion of the aromatic region, showing evolution of trace of **4** with time.

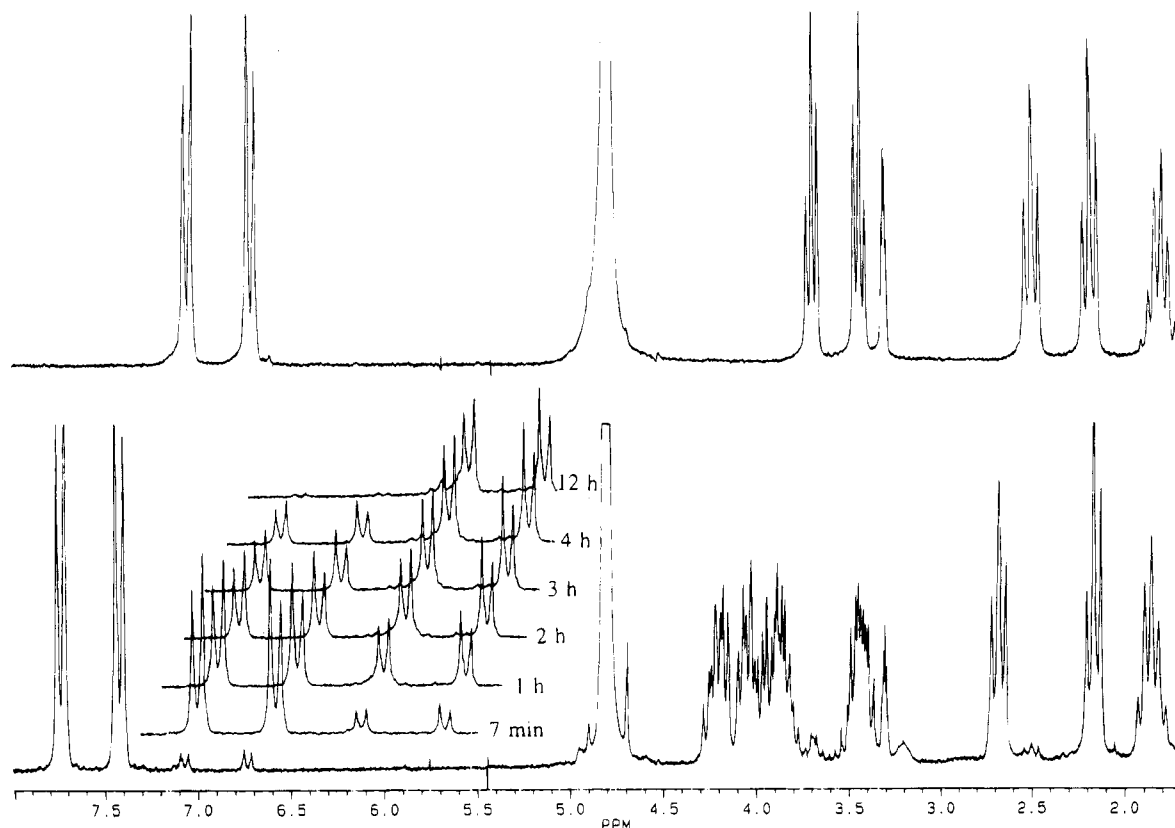
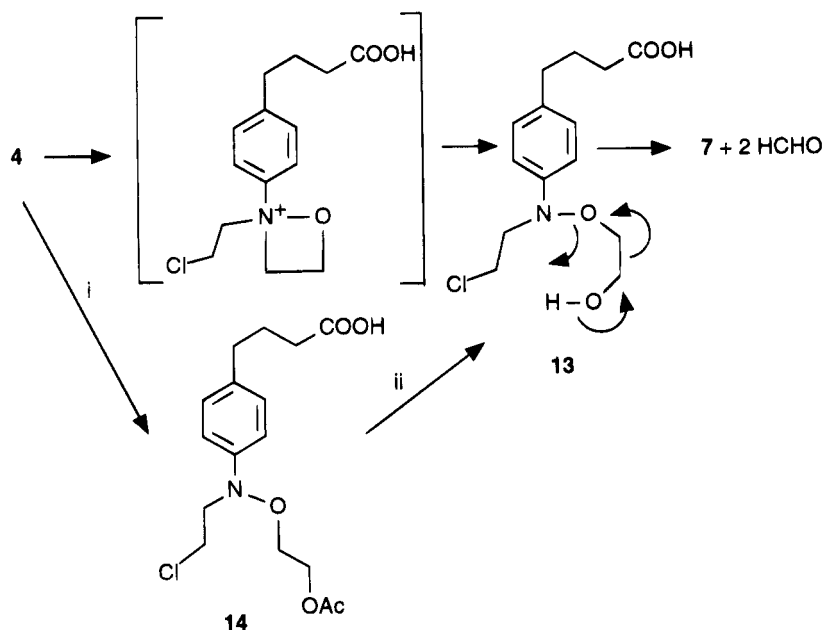


Figure 2. ^1H NMR spectra (200 MHz in 1:1 CD_3OD :deuterated phosphate buffer (0.1M, pD 7.0)) for **4** (bottom trace) and **7** (top trace) at zero time. Insert is an expansion of the aromatic region, showing evolution of trace of **4** with time.

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, Ag₂CO₃, aqueous THF; (ii) LiOH, aqueous THF.

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

no.	IC ₅₀ ^a (μM; air)		AA8 CT ₁₀	
	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 IC₅₀ values (μM) for 4 h drug exposures under aerobic conditions. ^b Air/N₂ ratios are the means of ratios of T₁₀ 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. IC₅₀ 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% (CT₁₀) was used as an inverse measure of cytotoxic potency, while the ratio of T₁₀ values under aerobic and hypoxic conditions at the same drug concentration was used as a measure of hypoxic selectivity. The large IC₅₀ 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 (CT₁₀ 960 versus 85 μM h) but the half-mustard **7** was even less cytotoxic (CT₁₀ 2080 μM h). The *N*-oxide **4**

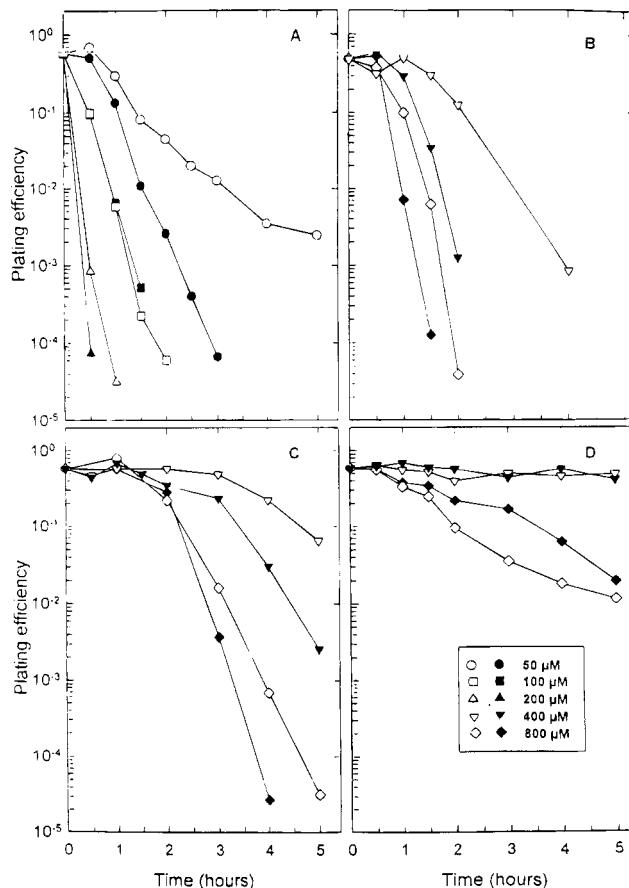


Figure 3. Cell killing by various concentrations of chlorambucil (**3**) (panel A), chlorambucil *N*-oxide (**4**) (panel B), the half-mustard **7** (panel C), and formaldehyde (panel D) of suspension cultures of late log phase AA8 cells (10⁶ cells/mL) under aerobic (open symbols) or hypoxic (filled symbols) conditions. The plating efficiencies of non-drug-treated cells were constant over this time under either aerobic or hypoxic conditions (data not shown).

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

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**) (quoted¹³ 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 mustards²² 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 contrast¹⁰ to the behavior of nitrovin (**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 Me₄Si, except for spectra in buffer which are referenced to Me₃Si-(CH₂)₃SO₃Na. Thin-layer chromatography was carried out on aluminum-backed silica gel plates (Merck 60 F₂₅₄). 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 NaD₂PO₄ and NaOD in D₂O. For the kinetics experiment a solution of **4**·HCl (10 mg, 28 μmol) in CD₃OD (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 CH₂Cl₂ (30 mL), and the solution was stirred at 20 °C for 80 min. Saturated aqueous NaHCO₃ (50 mL) was added, and the mixture was stirred vigorously for 30 min. The organic layer was separated, combined with two further CH₂Cl₂ extracts, dried (Na₂SO₄), 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 (CDCl₃) δ 7.15–7.05 (m, 4 H, ArH), 4.13 (q, $J = 7.2$ Hz, 2 H, OCH₂CH₃), 3.98 (t, $J = 5.5$ Hz, 2 H, CH₂), 3.77 (t, $J = 6.4$ Hz, 2 H, CH₂), 3.71 (t, $J = 5.5$ Hz, 2 H, CH₂), 3.48 (t, $J = 6.4$ Hz, 2 H, CH₂), 2.61 (t, $J = 7.6$ Hz, 2 H, ArCH₂), 2.32 (t, $J = 7.5$ Hz, 2 H, CH₂CO₂Et), 1.98–1.89 (m, 2 H, CH₂CH₂CO₂Et), 1.26 (t, $J = 7.2$ Hz, 3 H, OCH₂CH₃); ¹³C NMR see Table 1; MS (EI, ³⁵Cl peaks) m/z 347 (55, M⁺), 302 (20, M-OEt), 298 (30, M-CH₂Cl), 284 (100, M-CH₂CH₂Cl). Anal. (C₁₆H₂₃Cl₂-NO₃) C, H, N.

A mixture of **10** (1.03 g, 3.0 mmol) and LiOH·H₂O (0.25 g, 6.0 mmol) in EtOH (40 mL) and H₂O (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 CH₂Cl₂ (3 \times). The organic extracts were dried (Na₂SO₄) 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 (CDCl₃) δ 9.3 (br s, 1 H, CO₂H), 7.17–7.03 (m, 4 H, ArH), 4.00 (t, $J = 5.5$ Hz, 2 H, CH₂), 3.76 (t, $J = 6.3$ Hz, 2 H, CH₂), 3.72 (t, $J = 5.5$ Hz, 2 H, CH₂), 3.48 (t, $J = 6.3$ Hz, 2 H, CH₂), 2.64 (t, $J = 7.5$ Hz, 2 H, ArCH₂), 2.38 (t, $J = 7.4$ Hz, 2 H, CH₂CO₂H), 2.05–1.85 (m, 2 H, CH₂CH₂CO₂H); ¹³C NMR see Table 1; MS (EI, ³⁵Cl peaks) m/z 319 (35, M⁺), 270 (35, M-CH₂Cl), 256 (100, M-CH₂CH₂Cl); HRMS calcd for C₁₄H₁₉-Cl₂NO₃ 319.0742 (M⁺), found 319.0739. Anal. (C₁₄H₁₉Cl₂NO₃) 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 H₂O₂ (1.5 mL of a 35% aqueous solution, 14 mmol) dropwise to Ac₂O (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 CH₂Cl₂ (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 CH₂Cl₂ until the extracts were colorless and the aqueous layer evaporated. The residue was dissolved in MeCN, dried (Na₂SO₄), and reduced in volume to ca. 4 mL. Et₂O (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 ((CD₃)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(CH₂CH₂Cl)₂), 4.00–3.92 (m, 2 H of N(CH₂CH₂-Cl)₂), 3.66–3.58 (m, 2 H of N(CH₂CH₂Cl)₂), 2.69 (t, $J = 7.6$ Hz, 2 H, ArCH₂), 2.22 (t, $J = 7.3$ Hz, 2 H, CH₂CO₂H), 1.90–1.78 (m, 2 H, CH₂CH₂CO₂H); ¹³C NMR δ 174.1 (CO₂H), 145.0, 141.0 (aromatic C-CH₂ and C-N), 130.0, 120.7 (aromatic CH), 69.6 (NCH₂), 35.3 (CH₂Cl), 33.6, 32.9 (CH₂CH₂CH₂CO₂H), 25.8 (CH₂CH₂CO₂H); MS (FAB, ³⁵Cl peaks) m/z 320 (100, M⁺), 303 (55, M-OH), 254 (50, M-OH-CH₂Cl); HRMS calcd for C₁₄H₂₀-Cl₂NO₃ 320.0820 (M⁺), found 320.0827.

Attempts to crystallize this material from a number of solvents (Me₂CO, Me₂CO/Et₂O, MeCN/Et₂O) were unsuccessful. A sample was converted to the tosylate salt and crystallized from *i*-PrOH/Et₂O to give white hygroscopic needles, mp 100–105 °C dec (lit.¹³ mp 117–118 °C (Me₂CO/Et₂O)). 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 CH₂Cl₂ (4 \times), and the extracts were dried (Na₂SO₄) 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 0.25H₂O).

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 described²⁸ 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 chloroacetaldehyde³⁰ 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 CH₂Cl₂ (3 \times), and the extracts were dried (Na₂SO₄), 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, 10⁶/mL), removing samples periodically during continuous gassing with 5% CO₂ in air or N₂ as detailed elsewhere.²⁰ The concentration \times time for a surviving fraction of 10% (CT₁₀) 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.

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