In the Search for New Anticancer Drugs, XXI. Spin Labeled Nitrosoureas

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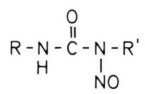
The spin labeled nitrosoureas **7a–e** and **12** were synthesized and evaluated *in vivo* for their anticancer activities against the murine lymphocytic leukemia P388. Compounds **7a–c**, **7e** and **12** possessed activities ranging from 31 to 542 percent increase in life span (% ILS), whereas compound **7d** was marginal (% ILS = 21). All CD_2F_1 male mice treated with the most active compounds (**7a** and **12**) at 35 mg/kg for 9 days were alive after 30 days, whereas all mice treated with the clinical drug CCNU (**1c**) succumbed. Compounds **7a–e** and **12** were further evaluated for their antineoplastic activity against lymphoid leukemia L1210. Compounds **7a** and **12** exhibited, on day 60, a % ILS of 713 and 620, respectively. The lipophilicities of compounds **7a–e** and **12** were determined using the EPR and UV methods. Compounds **7a** and **12** which differ from CCNU and MeCCNU by the replacement of the cyclohexyl and methylcyclohexyl groups with six and five membered nitroxyl radical moieties were more hydrophilic than the clinical drugs.

Introduction

Alkylnitrosoureas of the general formula **1a** have been known for sometime [1–4] to be highly carcinogenic and mutagenic. Although these properties have been thoroughly studied over the past two decades [1–8], none of the compounds found a practical application in the medicine. In contrast, the related 2-chloroethylnitrosoureas (CENUs), such as, N,N′-bis(2-chloroethyl)-N-nitrosourea (BCNU, **1b**), N′-cyclohexyl-N-(2-chloroethyl)-N-nitrosourea (CCNU, **1c**) and N′-(trans-4-methylcyclohexyl)-N-(2-chloroethyl)-N-nitrosourea (MeCCNU, **1d**) have been extensively used clinically for the treatment of a wide variety of human and animal neoplasms [3–11]. Their structure-activity relationship has been investigated [6, 9–11].



1a: R = H, R′ = alkyl, e.g., CH_3, C_2H_5.

1b: $R = R' = CH_2CH_2Cl$

1c: R =  , $R' = CH_2CH_2Cl$

1d: R =  , $R' = CH_2CH_2Cl$

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It appears, that in contrast to other anticancer agents, such as, anthracyclines, bleomycin, mitomycin C and streptonigrin which require activation prior to their interactions with the cell targets, the CENUs react in the cell without activation [12–17], although they are subject to oxidative metabolism at the alkyl group [18, 19]. Thus, they decompose spontaneously under physiological conditions to give electrophiles, such as 2-chloroethyldiazohydroxide or 2-chloroethyl cation [20–24] which undergo alkylation and/or interstrand cross-linking of the DNA and biological proteins. The other decomposition products, the alkyl isocyanates [25, 26] lead to the carbamoylation of amino groups in the biological macromolecules. Extensive studies on the mechanism of action of these agents, including the use of ^{15}N and ^{13}C labeled CENUs, led to a proposal involving three different pathways [27]: 1. formation of alkyl isocyanates and chloroethyl diazohydroxide, 2. formation of 2(alkylimino)-3-nitrosooxazolidine, and 3. formation of N-acyloxadiazolium species. Because of their nonspecificity, like other alkylating antineoplastic agents, such as, TEPA (**2a**) and Thio-TEPA (**2b**), the CENUs exhibit a wide range of cytotoxic effects [1].

In the search for more active and/or less toxic derivatives of **2a** and **2b**, the replacement of one of the aziridine groups with the nitroxyl radical and its reduced forms, led to the discovery of a TEPA derivative **3a** [28] which has a higher therapeutic index (26.5) than that (2.75) of the clinically used drug **2b**.

2a: Y = O
2b: Y = S

3a: X = NHC(O)NH, Y=O, R=Ȯ
3b: X = NHC(O)NH, Y=O, R=OH
3c: X = NHC(O)NH, Y=O, R=H

It was shown that while the nitroxyl moiety imparts a benefical influence on the antineoplastic properties of a drug [28], the nitroxyl radical by itself has no anticancer activity [29], is relatively non-toxic [29], is not carcinogenic or mutagenic [30], exhibits no synergistic effect [31], and has little effect on the cell growth and on the cell cycle kinetics [32]. This phenomenon can be explained by our hypothesis that the nitroxyl radical is a carrier moiety which facilitates the transport of the drug through the biological membranes on its way to the cellular DNA.

In support of this hypothesis, it was found that neutral or weakly acidic or basic nitroxyls are [33] rapidly permeating through cell membranes, and that a plausible correlation of the antineoplastic activity of compounds **2**, **3** and analogs with their lipophilic properties can be established [34, 35].

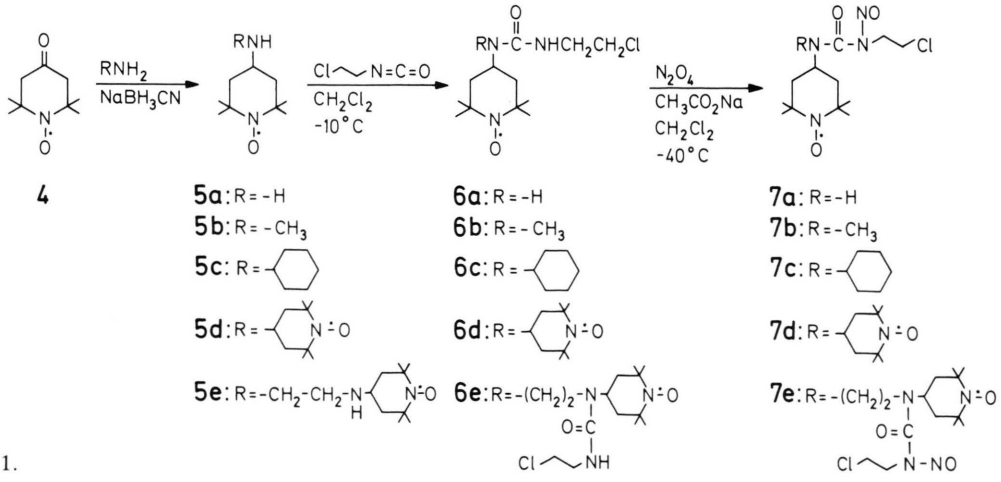
It is known that BCNU (**1b**), CCNU (**1c**) and MeCCNU (**1d**) rapidly enter the cerebrospinal fluid and thus can be used to control the meningeal tumors [36, 37]. Further, the nitroxyl radicals are known to cross the blood brain barrier [38] at the site of the diseased tissue. Hence, we assumed that a combination of the chloroethylnitrosourea and the nitroxyl moiety could lead to a more viable drug which could be followed pharmacokinetically by ESR in animal models, and diagnostically during therapy by NMR imaging [39]. Thus, a replacement of the cyclohexyl moiety in CCNU with nitroxyl radical moieties containing the five and six membered rings led to the development of nitroxyl labeled drugs **7a** and **12**.

In order to establish that the tautomeric proton on the N′ position in **7a** and **12** is essential for the level of antineoplastic activities of these drugs, several nitrosoureas (**7b-d**) were prepared by replacing the tautomeric proton with methyl, cyclohexyl, and nitroxyl moieties. In addition, the bifunctional "non-tautomeric" chloronitroso derivative **7e** was also synthesized, since it was reported [8] that some of this type of bifunctional chloroethyl nitrosoureas exhibit significant activity against Walker carcinoma 256 in rats. In 1985 we reported some preliminary results [40, 41] concerning the anticancer activity of these compounds. At that stage no correlation of the anticancer activity with lipophilicity and no experimental details were described. After completion of our work several studies came [42–44] to our attention. One of these studies [42] was essentially in agreement with our results concerning the high levels of activity of compounds **7a** and **12**, whereas the other study [44] could not confirm the activity of **7a**. In addition, these studies [42–44] are substantially different in rationale of design, interpretation and in detail of approach.

Results and Discussion

Chemistry

Compounds **5a–e** were prepared by the reductive amination of **4** with the corresponding amines such as



4

5a: R = -H
5b: R = -CH_3
5c: R =
5d: R =
5e: R = -CH_2-CH_2-N(H)-

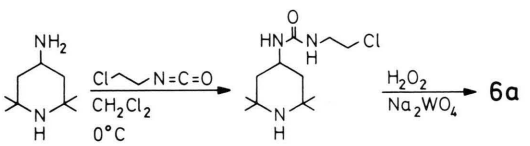
6a: R = -H
6b: R = -CH_3
6c: R =
6d: R =
6e: R = -$(CH_2)_2$-N(C(=O)-NH-CH_2CH_2Cl)-

7a: R = -H
7b: R = -CH_3
7c: R =
7d: R =
7e: R = -$(CH_2)_2$-N(C(=O)-N(NO)-CH_2CH_2Cl)-

Scheme 1.

methylamine, cyclohexylamine or ethylene diamine, in the presence of sodium cyanoborohydride. The condensation of the compounds **5a–e** with 2-chloroethyl isocyanate led to the introduction of the chloroethyl ureido moiety to give the corresponding compounds **6a–e**. Nitrosation of these spin labeled urea derivatives **6a–e** with dinitrogen tetraoxide resulted in the formation of the corresponding nitrosourea compounds **7a–e** (Scheme 1) in 61–82% yields.

The compound **6a** was also obtained by a different method *via* the condensation of the 4-amino-2,2,6,6-tetramethylpiperidine (**8**) with 2-chloroethyl isocyanate affording the 1-(2-chloroethyl)-3-(2,2,6,6-tetramethylpiperidine-4-yl)-urea (**9**) in a 89% yield. The subsequent oxidation of **9** using a 30% aqueous hydrogen peroxide solution in the presence of sodium tungstate gave the spin labeled urea derivative **6a** (Scheme 2). This compound (**6a**) was identical, in all respects, to the compound **6a** prepared by the preceeding method (Scheme 1).



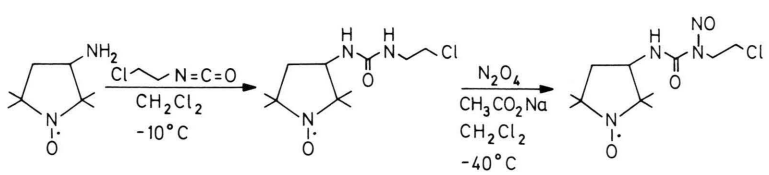
Scheme 2.

The N-(2-chloroethyl)-N′-(2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-yl)-urea (**11**) was prepared by the condensation of 2-chloroethyl isocyanate with 3-amino-2,2,5,5-tetramethylpyrrolidine-1-oxyl (**10**). Nitrosation of **11** with dinitrogen tetraoxide resulted in the formation of the N-(2-chloroethyl)-N′-(2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-yl)-N-nitrosourea (**12**) in a 71% yield. The synthetic sequence is delineated in Scheme 3.

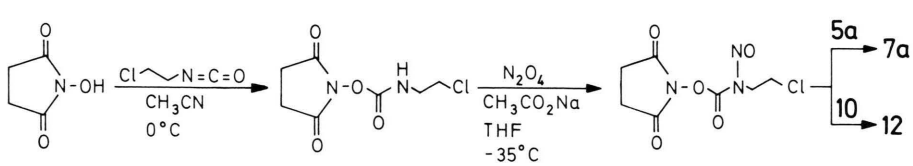
In order to ascertain the position of the nitroso group in the nitrosourea derivatives **7a** and **12**, a regio-selective method was used to transfer the chloroethyl moiety containing the nitroso group to the appropriate amine **5a** or **10** to give the corresponding nitrosoureas **7a** or **12**, respectively (Scheme 4). The regio-selective transfer reagent N′-hydroxysuccinimide-N-(2-chloroethyl)-N-nitrosocarbamate (**15**) was prepared by modifying the literature method [45]. The compounds **7a** and **12** prepared either by the conventional path (Scheme 1 and Scheme 3), or by the regio-selective route (Scheme 4) were identical. Thus, the nitroso group was attached at the N position of the urea derivatives **7a** and **12**. Compounds **7a** and **12** were also synthesized by a different approach using 2-chloroethylnitrosocarbamoyl azide [43].

Biological

Compounds **1c**, **7a–e** and **12** were tested *in vivo* against the murine lymphocytic leukemia P388 in CD_2F_1 male mice according to the National Cancer Institute protocol [46]. Compounds **7a** and **12** at a dose of 35 mg/kg/day possessed outstanding activity with a %ILS values of 542 and 514, respectively. In



Scheme 3.



Scheme 4.

the case of **7a** all the mice (6/6) were alive after 60 days, and in the case of **12** five mice (5/6) were alive after 60 days, whereas the %ILS of the clinical drug CCNU (**1c**) at a dose of 16 mg/kg/day was only 182 and all mice succumbed within 30 days. Even at a lower dose of 10 mg/kg compounds **7a** and **12** exhibited %ILS values of 298 and 282, respectively. Further, a lower chronic toxicity was observed in mice treated either with **7a** or **12** at 35 mg/kg/day than in mice treated with CCNU (**1c**) at 16 mg/kg/day. Compound **7b** possessed %ILS values of 54, 130, and 329 at 30, 60 and 90 mg/kg/day doses, respectively. Compounds **7c**, **7d**, and **7e** have shown a %ILS value of 31, 21, and 53, respectively. All these spin labeled CENUs (**7a–e** and **12**) exhibited distinct activities ranging from low for **7d** (borderline case) to a very high activity for **7a** as evidenced by the percent increase in life span values (%ILS, Table I). Compound **7a** was exceptional, possessing the highest activity over the tested range from 10 mg to 35 mg/kg/day.

The LD_{50} for compound **7a** (123 mg/kg) was about twice as high as that (56 mg/kg) for the clinically used drug CCNU. The LD_{50} for compounds **7b** and **7c** was 353 and 337 mg/kg, respectively. Compounds **7b–e** which are devoid of a tautomeric proton on the N′ position exhibited a substantial lowering of activities as compared to the tautomeric spin labeled nitrosoureas. On the basis of this result, it seems that a tautomeric proton decisively contributes to the activity of the spin labeled nitrosoureas. The compounds **1c**, **7a–e** and **12** were then tested against the lymphoid leukemia L1210. The compounds **7a** and **12** elicited at the optimum dose of 60 mg/kg/day a %ILS of 713 and 612, respectively. The clinical drug CCNU at a dose of 25 mg/kg/day has a %ILS of 646. The median survival times for **7a** and **12** were 61/7.5 and 54/7.5, respectively, whereas for CCNU it was 56/7.5. The therapeutic index for compound **7a** was about eight times and that for compound **12** was about five times higher than that for the clinical drug CCNU (Table II).

Table I. Anticancer activity of spin labeled nitrosoureas against P388 lymphocytic leukemia in CD_2F_1 male mice.

Compound	Dose [mg/kg/d]	[mmol/kg/d]	LD_{50}[a] ip [mg/kg]	5 Day weight change[b] [%]	Cures[c] survival total	ILS[d] [%]	Partition coefficient[e] P		log P	
							ESR	UV	ESR	UV
CCNU (**1c**)	16	0.068	56[f]	− 9.2	0/6	182[g]		355		2.55[h]
MeCCNU (**1d**)						145[i]		1778		3.25[j]
7a	10	0.033	123	+ 9.8	2/6	298	40	38	1.60	1.58
	20	0.065		+ 4.4	6/6	542				
	35	0.114		− 5.1	6/6	542				
12	10	0.034		+ 8.4	2/6	282	44	47	1.64	1.67
	35	0.119		− 4.6	5/6	514				
7b	30	0.094	353	+ 2.5	0/6	54				
	60	0.187		+ 2.5	0/6	130	52	50	1.72	1.70
	90	0.28		−11.2	1/6	329				
7c	75	0.19	337	+ 4.7	0/6	31	199	162	2.30	2.21
7d	60	0.13		− 3.4	0/6	21	43	36	1.63	1.56
7e	30	0.047		+ 2.7	0/6	32	224	209	2.35	2.32
	60	0.094		− 2.6	0/6	53				

[a] LD_{50} was determined by using logarithmically spaced single injections in accordance with the Weil's method [54].
[b] The average percentage weight change on day 5 was taken as a measure of drug toxicity.
[c] Cures mean survival after 60 days.
[d] Results obtained on day 60.
[e] The partition coefficients $P = \frac{[\text{compound in l-octanol}]}{[\text{compound in water}]}$ were measured by both ESR and UV techniques, according to literature methods [47, 48].
[f] Ref. [55].
[g] In ref. [56], the ILS (%) for CCNU is 172 (NCI protocol followed).
[h] In ref. [57], 2.83.
[i] Ref. [56] (NCI protocol followed).
[j] In ref. [57], 3.30.

In order to establish a measurable and predictable parameter for the correlation of activities of drugs **1c**, **1d**, **7a–e** and **12** with the presumed permeation through cell membranes, an attempt was made to relate the lipophilicities of drugs **1c**, **1d**, **7a–e** and **12** to their structural features and to the levels of their anticancer activities. For this purpose, the partition coefficients for these drugs were determined in the *n*-octanol/water solvent system by using ESR and/or UV methodologies [47, 48]. The results are summarized in Table I. The lower the p (or $\log p$) values, the higher is the hydrophilicity of a compound. Although all compounds (**1c**, **1d**, **7a–e**, **12**) were water soluble their partition coefficients varied over a wide range.

As expected, there was a good correlation of activity-lipophilicity relationship among compounds **1c**, **1d**, **7a**, and **12** containing a tautomeric proton, whereas no plausible correlation could be established in the case of compounds **7b–e** containing no tautomeric proton. However, all compounds (**1c**, **1d**, **7a**, and **12**) with a tautomeric proton were substantially more active, than all compounds (**7b–e**) devoid of a tautomeric proton. In the series **1c**, **1d**, **7a**, and **12**, the clinical drug MeCCNU (**1d**) was the most hydrophobic and possessed concomitantly the lowest activity. CCNU was less hydrophobic than **1d** and exhibited a higher activity than **1d**. Both compounds **7a** and **12** were *substantially* more hydrophilic than **1c** and **1d**, and at the same time more active, whereby compound **7a** was the most hydrophilic and also the most active drug in this series (Table I). This result is essentially in agreement with a prediction based on a different series of "tautomeric" nitrosoureas [6, 11] that the most hydrophilic neutral congener should exhibit the highest activity.

Experimental Section

Materials

All reagents were of the finest quality commercially available. Solvents were dried by standard procedures [49]. Compounds **4** and **8** [50] and compounds **5a**, **5b**, and **5d** [51, 52] were prepared modifying the literature methods. N′-hydroxysuccinimide-N-(2-chloroethyl)-N-nitrosocarbamate (**15**) was prepared according to the literature method [45]. Compounds **5c** and **5e** were prepared analogous to the literature methods [51, 52] by the reductive amination of **4** using cyclohexylamine and ethylenediamine, respectively.

Analytical procedures

All melting points were obtained with a Thomas-Hoover capillary melting point apparatus, Model 6406-K, using a calibrated thermometer. Mass spectra were recorded on a Hewlett Packard Mass Spectrometer, Model 5985 GS, using a direct insertion probe, a source pressure of 2×10^{-7} torr, and methane as a reactant gas for chemical ionization. IR spectra were recorded on a Perkin-Elmer spectrophotometer, Model 735B. Microanalyses were performed either on a F&M Scientific Corporation carbon, hydrogen, nitrogen analyzer, Model 185 or on a Perkin-Elmer 240C Elemental Analyzer. The ERP spectra of 4.0×10^{-5} M solutions of the nitroxyl radicals in methylene chloride were obtained on a Varian E-115 EPR spectrometer. Column chromatography was performed either using the flash chromatography technique [53] on silica gel 60 (Fluka) finer than 230 mesh, or by conventional column chromatography on alumina (Basic, Brockmann activity I, 80–200 mesh, Fisher Scientific Co.). TLC analyses were performed either on silica gel 60 F_{254} or on aluminum oxide 60 F_{254}, neutral (type E) precoated sheets (EM Reagents), layer thickness 0.2 mm with visualization using UV light and/or iodine chamber. Purity of the compounds **7a–e** and **12** was checked in a solvent system composed of methylene chloride and methanol (9:1, v/v). Partition coefficients (P) were obtained by following the literature methodologies [47, 48] using UV spectrophotometry and EPR spectroscopy. For measuring the partition coefficients, 1-octanol and water layers were presaturated with each other prior to use. Thus, the areas of the double integrals of the first derivative curves of the EPR spectra of the corresponding spin labeled nitrosoureas (3 ml, 5 mM solutions) were computed. The areas of the initial octanol solutions and the separated water solutions were used to compute the concentrations of the spin labeled compounds in octanol and in the water layer. The partition coefficients

$$P = \frac{\text{[compound in 1-octanol]}}{\text{[compound in water]}}$$

and their logarithmic values so obtained were compared with their corresponding values obtained by using UV methodology [48]. All these values are shown in Table I.

Table II. Anticancer activity of spin labeled nitrosoureas against L1210 lymphoid leukemia in CD_2F_1 male mice.

Compound	ILS_{30}[a] [mg/kg/d]	Optimum dose[b] [mg/kg/d]	[mmol/kg/d]	5 Day weight change[c] [%]	Median[d] survival T/C days	ILS_{max}[d] [%]	Cures[e] survival total	Therapeutic index[f]
CCNU (**1c**)	4.4	25	0.106	−11.10	56/7.5	646	4/6	5.7[g]
7a	1.5	60	0.196	− 8.8	61/7.5	713	6/6	40
12	2.4	60	0.205	− 9.4	54/7.5	620	3/6	25
7b		126	0.394	− 3.20	16.33/7.5	117	0/6	
7c		120	0.310	− 5.51	7.33/7.5	−3	0/6	
7d		120	0.261	+ 0.75	7.66/7.5	2	0/6	
7e		120	0.188	− 2.00	13.83/7.5	84	0/6	

[a] Daily dose eliciting 30% increase in life span over the control. %ILS = $[(T-C)/C] \times 100$.
[b] Daily dose providing the maximum increase in life span.
[c] The average percentage weight change on day 5 was taken as a measure of drug toxicity.
[d] Results obtained on day 60.
[e] Cures mean survival after 60 days.
[f] Therapeutic Index = optimum dose/ILS_{30}.
[g] In ref. [55], the therapeutic index is 5.1.

Mice

Male mice CD_2F_1 (for testing; average weight 18–20 g) and DBA/2 (for tumor propagation [46]) 6–7 weeks old were supplied by Harlan Sprague-Dawley, Inc., Indianapolis, IN. Mice were fed Rodent Laboratory Chow 5001 (Ralston Purina Co.) and water *ad libitum*.

Drugs

Compounds were administered in 0.85% aqueous sodium chloride solution (Sigma Chemical Company). No solubility problems were experienced with the test doses (Tables I and II).

Biological evaluations

Compounds **1c**, **7a–e** and **12** were evaluated *in vivo* against the lymphocytic leukemia P388 and lymphoid leukemia L1210 in mice following the protocol of the National Cancer Institute [46]. The CD_2F_1 male mice of 18–20 g weight, in groups of six, were inoculated i.p. either with 10^6 cells of P388 tumor, or with 10^5 cells of L1210 tumor on day zero of the experiment. The compounds **7a–e** and **12** were injected i.p. at doses listed in Table I for 9 successive days starting from day one. The animals were then observed according to the protocol [46] for 30 days and for 60 days, keeping a record of deaths and survivors. The anticancer activity was evaluated by comparing the mean survival time of the treated mice with that of the control animals, *i.e.*, by the T/C method where T represents the mean survival time of the treated group and C the mean survival time of the tumor bearing control group. The percent of increase in life span (%ILS) parameter was calculated by the formula $[(T\text{-}C)/C] \times 100$. The LD_{50} values were determined for the compounds **7a–c** using Weil's method [54]. For the calculation of the LD_{50} four logarithmically spaced doses were injected i.p. into four groups of four Swiss male mice. The observation period for the determinations of the LD_{50} was 30 days. The results of $\%T/C$ and %ILS and LD_{50} values are summarized in Tables I and II for P388 and for L1210 mice, respectively.

*Preparation of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (**5a**) and bis(2,2,6,6-tetramethylpiperidine-1-oxyl-4-yl)-amine (**5d**)*

To a stirred solution of **4** (3.00 g, 17.6 mmol) in methanol (100 ml) was added ammonium acetate (15.00 g, 195.0 mmol) and sodium cyanoborohydride (0.75 g, 120.0 mmol) at 24 °C. The reaction mixture was stirred for 24 h at 24 °C. The solvent was removed on a rotating evaporator at 40 °C/20 torr. The resulting oily residue was dissolved in water (20 ml) and saturated with sodium chloride and then extracted with chloroform (4 × 20 ml). The combined organic extracts were washed with a 5% aqueous citric acid solution (2 × 10 ml). The separated organic layer was dried over anhydrous magnesium sulfate and the solid material was separated

by filtration and washed with anhydrous ether. The combined filtrate and washings were concentrated on a rotating evaporator at 40 °C/20 torr. The red oily material, on the basis of TLC analysis (silica gel, *t*-butylmethyl ether and methanol, 9:1, v/v), contained two major fractions **5a** (R_f = 0.1) and **5d** (R_f = 0.6) and one minor impurity. The red oily material was purified by column chromatography on basic alumina using first *t*-butylmethyl ether to afford 1.74 g (58%) of **5d** and then *t*-butylmethyl ether and methanol (90:10, v/v) to afford 1.14 g (38%) of **5a**. Further purification of **5a** by Kugelrohr distillation (75–80 °C/0.02 torr) yielded 1.05 g (34%) of pure product **5a**, a red crystalline solid, m.p. 33–34 °C (lit. [50, 51] liquid). Compound **5d** was further purified by flash chromatography on silica gel using *t*-butylmethyl ether and methanol (90:10, v/v) as eluant. The concentration of the combined fractions containing the product on a rotating evaporator at 25 °C/20 torr, gave 1.65 g (55%) of pure product **5d** as pink crystalline material, m.p. 160–161 °C (lit. [51] m.p. 158–162 °C).

*Preparation of 4-alkyl substituted amino-2,2,6,6-tetramethylpiperidine-1-oxyls (**5b** and **5c**)*

To a solution of **4** (2.50 g, 14.0 mmol) in methanol (25 ml) was added the corresponding alkylamine (16.0 mmol and sodium cyanoborohydride (0.62 g, 9.8 mmol) at 24 °C. The reaction mixture was stirred for 24 h at 24 °C and then heated under reflux for 4 h. The solvent was removed on a rotating evaporator at 40 °C/20 torr. The resulting oily residue was dissolved in water (20 ml) and saturated with sodium chloride and then extracted with chloroform (4 × 20 ml). The combined organic extracts were washed with a 5% aqueous citric acid solution (2 × 10 ml). The separated organic layer was dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated on a rotating evaporator at 25 °C/20 torr. The crude oily residue thus obtained was purified by flash column chromatography on silica gel using chloroform and methanol (90:10, v/v) as eluant. The concentration of the combined fractions containing the product on a rotating evaporator at 25 °C/20 torr, gave either pure **5b** (lit. [51], liquid) or pure **5c**. The yield and analytical data for **5c** are presented in Table III.

*Preparation of N,N'-bis(2,2,6,6-tetramethylpiperidine-1-oxyl-4-yl)-ethylenediamine (**5e**)*

Using the preceeding procedure similar to that described for **5b** and **5c**, the reaction mixture was stirred for 54 h at 24 °C. The crude product was purified by chromatography on basic alumina using chloroform and methanol (9:1, v/v) as eluant. Removal of the solvent on a rotating evaporator at 24 °C/20 torr gave pure product **5e**. The yield and analytical data are presented in Table III.

*Preparation of N-(2-chloroethyl)-N'-substituted ureas (**6a–e** and **11**). A general procedure*

A solution of 2-chloroethyl isocyanate (0.8 ml, 10.0 mmol) in methylene chloride (5 ml) was added dropwise, over a period of 10–15 min, to a stirred solution of either **5** or **10** (9.0 mmol) in methylene

Table III. Physical properties of 4-substituted amino-2,2,6,6-tetramethylpiperidine-1-oxyls.

Compound	Yield [%]	m.p. [°C]	Molecular formula[a]	MS[b] *m/e*	IR[c] ν_{max} [cm^{-1}]
5c	69	153–154	$C_{15}H_{29}N_2O$[d] (253.40)	255 (M^+ + 2, 22) 254 (M^+ + 1, 100) 253 (M^+, 27)	3350, 1635, 1530, 1470
5e	54	112–113	$C_{20}H_{40}N_4O_2$[e] (368.55)	370 (M^+ + 2, 83) 369 (M^+ + 1, 100) 368 (M^+, 42) 296 (M^+ − 72, 47)	3260, 1620, 1540, 1490

[a] The microanalyses were in satisfactory agreement with the calculated values (C, H, and N) within ±0.4%.
[b] Relative percent intensities of the peaks.
[c] Dispersed in Nujol mull.
[d] ESR: 3 lines a_N = 15 G.
[e] ESR: 5 lines, however, intensities are not 1:2:3:2:1, more complex exchange interactions.

Table IV. Physical properties of 1-(2-chloroethyl)-3-(2,2,6,6-tetramethylpiperidinyl-1-oxyl-4-yl)-3-substituted ureas.

Compound	Yield [%]	m.p. [°C]	Molecular formula[a]	MS[b] *m/e*	IR[c] ν_{max} [cm^{-1}]
6a	92	84– 86	$C_{12}H_{23}N_3O_2Cl$[d] (276.77)	278 ($M^+ + 2$, 37) 277 ($M^+ + 1$, 72) 276 (M^+, 26) 241 ($M^+ - 35$, 100)	3420, 2960, 1710, 1535, 1472
6b	75	95– 97	$C_{13}H_{25}N_3O_2Cl$[d] (305.77)	307 ($M^+ + 2$, 48) 306 ($M^+ + 1$, 59) 305 (M^+, 100) 291 ($M^+ - 14$, 63) 276 ($M^+ - 29$, 61)	3220, 1715, 1525, 1455
6c	83	156–157	$C_{18}H_{33}N_3O_2Cl$[d] (358.93)	360 ($M^+ + 2$, 54) 359 ($M^+ + 1$, 93) 358 (M^+, 66) 344 ($M^+ - 14$, 45) 323 ($M^+ - 35$, 100)	3300, 1620, 1510, 1420
6d	84	184–185	$C_{21}H_{39}N_4O_3Cl$[d] (431.01)	433 ($M^+ + 2$, 33) 432 ($M^+ + 1$, 67) 431 (M^+, 100) 395 ($M^+ - 36$, 42)	3400, 1650, 1500
6e	82	109–112	$C_{26}H_{48}N_6O_4Cl_2$[e] (579.6)	581 ($M^+ + 2$, 20) 580 ($M^+ + 1$, 41) 579 (M^+, 100) 544 ($M^+ - 35$, 62)	3360, 3210, 2940, 1710, 1680, 1560, 1520, 1490
11	89	113–114	$C_{11}H_{21}N_3O_2Cl$[e] (263.74)	265 ($M^+ + 2$, 34) 264 ($M^+ + 1$,71) 263 (M^+, 32) 228 ($M^+ - 35$, 100)	3320, 3210, 2970, 1720, 1560, 1480

[a] The microanalyses were in satisfactory agreement with the calculated values (C, H, and N) within ±0.4%.
[b] Relative percent intensities of the peaks.
[c] Dispersed in Nujol mull.
[d] ESR: 3 lines $a_N = 16-17$ G.
[e] ESR: 5 lines, however, intensities are not 1:2:3:2:1, more complex exchange interactions.

chloride (20 ml) at −10 °C. The reaction mixture was stirred for 4 h at −10 °C and for 16 h at 24 °C. The reaction mixture was washed with water (2 × 5 ml) and then dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated on a rotating evaporator at 24 °C/20 torr. The resulting residue was purified by flash chromatography on silica gel using methylene chloride and methanol (9:1, v/v) as eluant. The corresponding ureas were obtained in pure form. The yields and analytical data are presented in Table IV.

*Preparation of N-(2-chloroethyl)-N'-substituted-N-nitrosoureas (**7a–e** and **12**). A general procedure*

A mixture of either **6** or **11** (1.80 mmol) and anhydrous sodium acetate (0.9 g, 11.00 mmol) in methylene chloride (15 ml) was cooled to −40 °C. To this cooled and stirred mixture was added dropwise, over a period of 10–15 min, a solution of dinitrogen tetraoxide (0.2 g, 2.17 mmol) in dry carbon tetrachloride (5 ml). After the addition, the reaction mixture was stirred for 1 h at −40 °C. The mixture was then poured into ice water (20 ml) and the organic layer separated. The aqueous layer was extracted with methylene chloride (4 × 20 ml). The combined organic extracts were successively washed with a 5% aqueous sodium bicarbonate solution (2 × 10 ml) and water (2 × 5 ml). Then the organic layer was dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated on a rotating evaporator at 40 °C/20 torr. The resulting crude product was purified by flash column chromatography on silica gel using a mixture of methylene chloride and methanol (95:5, v/v) as eluant. The concentration of

Table V. Physical properties of 1-(2-chloroethyl)-3-substituted-1-nitrosoureas.

Compound	Yield [%]	m.p. [°C]	Molecular formula[a]	MS[b] *m/e*	IR[c] ν_{max} [cm^{-1}]
7a	82	80– 83 (dec.)	$C_{12}H_{22}N_4O_3Cl$[d] (305.78)	307 ($M^+ + 2$, 48) 306 ($M^+ + 1$, 59) 305 (M^+, 100) 276 ($M^+ - 29$, 62) 240 ($M^+ - 65$, 54) 183 ($M^+ - 122$, 92)	3220, 1716, 1525
7b	60	68– 70	$C_{13}H_{24}N_4O_3Cl$[d] (319.81)	321 ($M^+ + 2$, 36) 320 ($M^+ + 1$, 52) 319 (M^+, 62) 290 ($M^+ - 29$, 100) 255 ($M^+ - 64$, 64) 215 ($M^+ - 104$, 65) 156 ($M^+ - 163$, 84)	2960, 1690, 1460
7c	61	128–129 (dec.)	$C_{18}H_{32}N_4O_3Cl$[d] (387.93)	389 ($M^+ + 2$, 33) 388 ($M^+ + 1$, 20) 387 (M^+, 25) 358 ($M^+ - 29$, 25) 254 ($M^+ - 133$, 100) 224 ($M^+ - 163$, 43)	2970, 1670, 1460
7d	67	127–128	$C_{21}H_{38}N_5O_4Cl$[d] (460.01)	461 ($M^+ + 1$, 20) 460 (M^+, 22) 432 ($M^+ - 28$, 28) 355 ($M^+ - 105$, 70) 327 ($M^+ - 133$, 82) 253 ($M^+ - 207$, 100)	2960, 1690, 1470
7e	69	142–143 (dec.)	$C_{26}H_{46}N_8O_6Cl_2$[e] (637.61)	396 ($M^+ - 241$, 64) 395 ($M^+ - 242$, 100) 380 ($M^+ - 257$, 22) 309 ($M^+ - 328$, 29)	2950, 1690, 1460
12	71	110–112 (dec.)	$C_{11}H_{20}N_4O_3Cl$[d] (291.76)	293 ($M^+ + 2$, 50) 292 ($M^+ + 1$, 62) 291 (M^+, 100) 262 ($M^+ - 29$, 54) 226 ($M^+ - 65$, 48) 169 ($M^+ - 122$, 90)	3210, 2970, 1680, 1470

[a] The microanalyses were in satisfactory agreement with the calculated values (C, H, and N) within ±0.4%.
[b] Relative percent intensities of the peaks.
[c] Dispersed in Nujol mull.
[d] ESR: 3 lines, $a_N = 16-17$ G.
[e] ESR: 5 lines, however, intensities are not 1:2:3:2:1, more complex exchange interactions.

the combined fractions containing the product on a rotating evaporator at 25 °C/20 torr gave pure compounds **7a–e** or **12**. The yields and analytical data are presented in Table V.

*Preparation of 1-(2-chloroethyl)-3-(2,2,6,6-tetramethylpiperidine-4-yl) urea (**9**)*

To a stirred solution of 4-amino 2,2,6,6,-tetramethylpiperidine (**8**, 0.59 g, 3.80 mmol) in methylene chloride (20 ml) was added dropwise, over a period of 10–15 min, a solution of 2-chloroethyl isocyanate (0.32 ml, 3.70 mmol) in methylene chloride (10 ml) at 0 °C. The reaction mixture was stirred for 16 h at 25 °C. The solvent was removed on a rotating evaporator at 25 °C/20 torr. Repeated recrystallization of the resulting residue from methylene chloride and benzene (1:1, v/v) gave 0.87 g (89%) of pure compound **9**, m.p. 230–232 °C.

*Preparation of 1-(2-chloroethyl)-3-(2,2,6,6,-tetramethylpiperidine-1-oxyl-4-yl) urea (**6a**)*

To a solution of **9** (0.30 g, 1.15 mmol) in deionized water (5 ml) was added sodium tungstate (0.03 g) and a 30% aqueous hydrogen peroxide solution (1 ml). The reaction mixture was left overnight at 25 °C and then extracted with chloroform (4 × 15 ml). The combined chloroform extracts were dried over anhydrous magnesium sulfate and filtered. The filtrate was evaporated on a rotating evaporator at 25 °C/20 torr. The resulting residue was purified by flash chromatography on silica gel using methylene chloride and methanol (95:5, v/v) as eluant. The concentration of the combined fractions on a rotating evaporator at 25 °C/20 torr gave 0.21 g (66%) of the pure product **6a**, m.p. 84–86 °C. This compound was identical in all respects to the compound **6a**, prepared by a different method (Scheme 1).

*Preparation of N'-hydroxysuccinimide-N-(2-chloroethyl)-carbamate (**14**)*

The literature procedure was modified as follows. To a stirred solution of N-hydroxysuccinimide (**13**, 2.0 g, 17.40 mmol) in acetonitrile (20 ml), 2-chloroethylisocyanate (1.40 ml, 17.40 mmol) in acetonitrile (10 ml) was added dropwise, over a period of 15–20 min, at 0 °C. After the addition, the reaction mixture was stirred for 1 h at 0 °C and then for 16 h at 25 °C. The reaction mixture was concentrated on a rotating evaporator at 40 °C/20 torr. Repeated recrystallizations of the resulting residue from methylene chloride and *t*-butylmethyl ether gave 3.3 g (87%) of pure compound **14**, m.p. 106–108 °C (lit. [45], 106–109 °C).

*Preparation of N'-hydroxysuccinimide-N-(2-chloroethyl)-N-nitrosocarbamate (**15**)*

To a stirred solution of **14** (2.2 g, 10.0 mmol) in dry tetrahydrofuran (20 ml) was added anhydrous sodium acetate (4.93 g, 61.0 mmol). The mixture was cooled to −35 °C. To this cooled and stirred mixture, a solution of dinitrogen tetraoxide (1.0 g, 10.90 mmol) in dry carbon tetrachloride (20 ml) was added dropwise over a period of 15–20 min. After the additions, the reaction mixture was stirred for 1 h at −35 °C and then for ½ h at 5 °C. To the reaction mixture was then added a mixture of methylene chloride (20 ml) and ice water (10 ml). The organic layer was separated and the aqueous layer was extracted with methylene chloride (4 × 20 ml). The combined organic extracts were washed with a 5% aqueous sodium bicarbonate solution (2 × 10 ml) and then with water (2 × 5 ml). The organic solution was dried over anhydrous magnesium sulfate and filtered. Concentration of the filtrate on a rotating evaporator at 40 °C/20 torr gave the crude product. Repeated recrystallizations of the product from ether and petroleum ether afforded 2.42 g (98%) of pure **15**, m.p. 102–104 °C (lit. [45], 104–106 °C).

*Preparation of **7a** and **12** via the transfer reagent **15***

A solution of, either **5a** or **10** (1.75 mmol) and **15** (1.80 mmol) in methylene chloride (20 ml) was stirred for 2 h at 5 °C and then for 15 h at 25 °C. The reaction mixture was washed with a saturated sodium chloride solution (2 × 5 ml) and then dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated on a rotating evaporator at 25 °C/20 torr. The resulting residue was purified by flash chromatography on silica gel using methylene chloride and methanol (95:5, v/v) as eluant. Concentration of the combined fractions on a rotating evaporator at 25 °C/20 torr afforded pure compound **7a** or **12**. These compounds were identical in all respects to the corresponding compounds **7a** or **12** prepared by a different method (Scheme 1 and Scheme 3).

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