In the Search for New Anticancer Drugs. 28. Synthesis and Evaluation of Highly Active Aminoxyl Labeled Amino Acid Derivatives Containing the [*N'*-(2-Chloroethyl)-*N'*-nitrosoamino]carbonyl Group

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Abstract □ The aminoxyl (nitroxyl) labeled (2-chloroethyl)nitrosocarbamoyl (CNC) derivatives of amino acids, i.e., *N*-[[*N'*-(2-chloroethyl)-*N'*-nitrosoamino]carbonyl]-A-(1-oxy-2,2,6,6-tetramethylpiperidin-4-yl)amides, A = glycyl (**10a**), A = L-alanyl (**10b**), A = L-valyl (**10c**), A = L-phenylalanyl (**10d**), were synthesized and evaluated *in vitro* for their anticancer activities against the murine lymphocytic leukemia P388. Compounds **10a–d** possessed activities ranging from 242 to 456% increase in life span (% ILS). All CDF_1 male mice treated with the highly active compounds **10b** and **10c** at 12 mg/kg/day for 9 days were alive after 30 days. Compounds **10a–d** were then tested *in vivo* against the murine lymphoid leukemia L1210. Compounds **10a–d** exhibited, on day 60, a % ILS of 496, 663, 663, and 581, respectively. All CDF_1 male mice treated with the highly active compounds **10b** and **10c** at 12 mg/kg/day for 9 days were alive after 60 days. The lipophilicities of compounds **10a–d** were determined using the UV method. The % ILS parameters obtained against the P388 and L1210 tumor lines were correlated with the corresponding lipophilicities, and a trend was generally observed toward an increase in cytotoxicity with a concomitant decrease in hydrophobicity.

Introduction

A large number[1–8] of alkylnitrosoureas and (2-chloroethyl)-nitrosoureas, containing the [*N'*-(2-chloroethyl)-*N'*-nitrosoamino]carbonyl (CNC) moiety, have been synthesized and biologically evaluated over the past three decades because of their inherent mutagenicity, carcinogenicity, and anticancer activity. It is assumed[4–6,8,9] that *in vitro* the nitroso- and (chloroethyl)nitrosoureas fragment, probably without biological activation, to give a variety of species which act as intracellular alkylating agents of DNA and other biological macromolecules. The structure–activity relationship of these and related compounds has been extensively studied[1–4,7,10–14] *in vitro* and *in vivo* against a variety of human and animal tumors. A comparatively very small number of these compounds, i.e., carmustine (BCNU, **1a**), lomustine (CCNU, **1b**), semustine (MeCCNU, **1c**), streptozotocin (**1d**), chlorozotocin (**1e**), and cymerin (MCNU, **1f**), have been used[13,15,16] in clinical oncology (Chart 1). The (2-chloroethyl)nitrosoureas **1a–c** exhibit a wide range of toxic effects, including a severe cumulative myelosuppression.[15,16] It was found[13,15,16] that the bone marrow toxicity can be either greatly reduced or eliminated in drugs, such as **1d–f** which contain various carbohydrate moieties.

In the last decade a number[2,17–25] of amino acid and peptide derivatives containing the CNC moiety have been synthesized and evaluated *in vivo* for anticancer activity. The rationale for this approach was that the L-amino acids are actively transported into mammalian tissue[17] and that some peptides accumulate in cancerous cells and, hence, may serve as carriers[17,26] of the CNC moiety. The amino acid amide derivatives[17,19] containing the CNC group (**1g**) possessed greatly increased anticancer activity, as compared to the corresponding acid[21] and ester analogs.[19]

L-Amino acid congeners of BCNU (**1a**) containing both the (2-chloroethyl)amino group at the C-terminus, and the CNC group at the N-terminus (**2**, $n = 1$) have been synthesized and shown[23–25] *in vivo* to have a high activity against the lymphocytic leukemia P388 and a low activity against the lymphoid leukemia L1210, although, in a preceding study,[24] a high activity for these compounds were reported for the L1210 tumor. The dipeptide analogs (**2**, $n = 2$) were found[25] to be less active than the amino acid derivatives **2**, $n = 1$, against both cancer lines.

On the basis of our hypothesis[12,27] that less hydrophobic congeners of **1a–c** would result in more active and less toxic drugs, the aminoxyl-labeled derivatives (SLCNU, **1h**, and analogue **1i**) were found[10–12] to be less toxic and more active than the clinical drugs **1b** and **1c**, as evidenced by a combination of the LD_{50}, therapeutic indices, and % ILS parameters. In an analogous approach, the replacement of either the methyl group in **1d** or the 2-chloroethyl group in **1e**, respectively, with the five- and six-membered aminoxyl moieties led, predictably,[14,27] to the more hydrophobic compounds **1j** and **1k** with a concominant increase in cytoxicities against P388 and L1210. It is believed[10,11,27,28] that while the aminoxyl moiety has a beneficial influence on the anticancer activity of a drug, the aminoxyl moiety by itself exhibits no anticancer activity even at very high doses, is relatively nontoxic, has little effect on the cell growth and on the cell cycle kinetics, and has no synergistic effect with the alkylating moieties of drugs. Most likely,[27,28] the aminoxyl moiety serves as a transport vehicle through cell membranes.

On the basis of these past experiences, it was assumed that a substitution of the highly hydrophobic (2-chloroethyl)amino group at the C-terminus in the amino acid derivatives **2**, $n = 1$,[25] with the less hydrophobic aminoxyl moiety should result in less hydrophobic compounds with a concomitant improvement in anticancer activities. These expectations were largely fulfilled in the present study.

Results and Discussion

Chemistry—The syntheses of the target compounds **10a–d** containing the aminoxyl (nitroxyl) moiety at the C-terminus, and the (2-chloroethyl)nitroso group at the N-terminus of the amino acids glycine, L-alanine, L-valine, and L-phenylalanine were achieved as delineated in Scheme 1. Thus, the reaction of the Fmoc-protected amino acid derivatives **3a–d** with the *N*-hydroxysuccinimide (**4**) in the presence of dicyclohexylcarbodiimide (DCC) gave the corresponding activated *N*-succinimidyl esters **5a–d** in crystalline form (Table 1). The transfer reaction of the activated esters **5a–d** at 0 °C with equivalent amounts of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (**6**) in tetrahydrofuran resulted in the aminoxyl-labeled Fmoc derivatives **7a–d** (Table 2). During this reaction a deblocking of the Fmoc moiety occurred to some extent, as detected by thin-layer chromatography. Hence, a strict adher-

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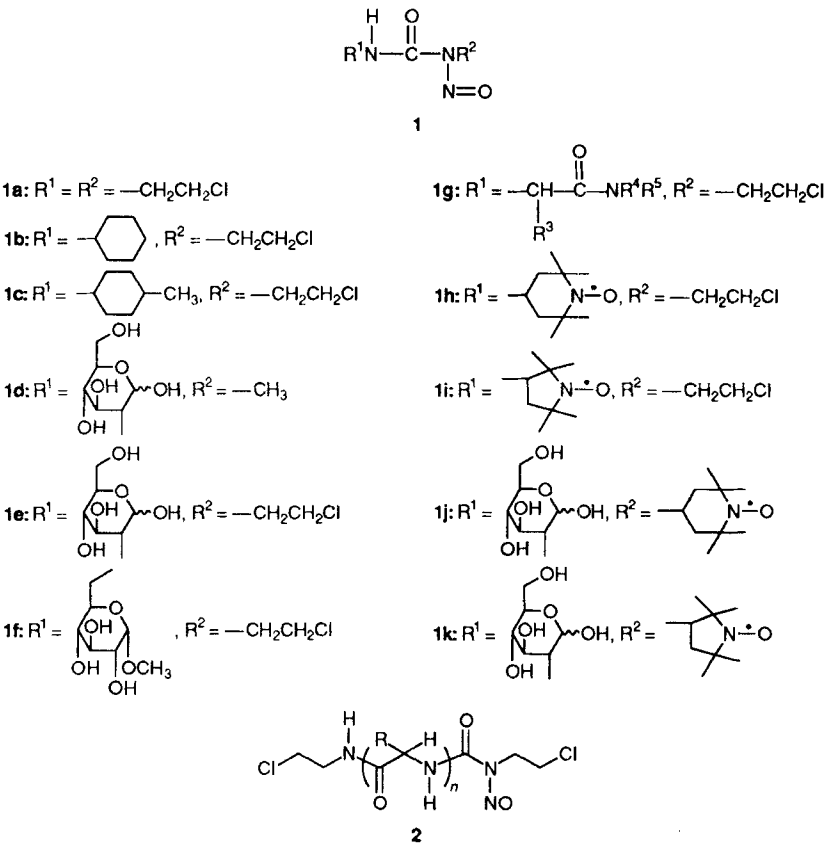


Chart 1

Table 1—Analytical Data of *N*-Hydroxysuccinimide Derivatives 5a–d

Compd	Yield (%)	Mp (dec) (°C)	Lit.[37] Mp (dec) (°C)	IR[a] ν_{max} (cm^{-1})	$[\alpha]^{24}_D$ (deg)	lit.[37] $[\alpha]^{25}_D$ (deg)
5a	73	174–177	175–180	1785[b], 1740[c]	—	—
5b	95	100–102	103	1785[b], 1740[c]	−23.0[d]	−25.0
5c	95	75–78	—	1785[b], 1740[c]	−37.6[e]	—
5d	95	155–159	157–159	1785[b], 1740[c]	−54.0[f]	−52.2

[a] KBr pellet. [b] N–C=O. [c] O–C=O. [d] c = 5% in chloroform. [e] c = 0.25% in methanol. [f] c = 1% in acetic acid.

Table 2—Analytical Data of Protected Amino Acid Amides of 4-Amino-2,2,6,6-tetramethylpiperidine-1-oxyl 7a–d

Compd	Yield (%)	Mp (dec) (°C)	Molecular Formula	MS (*m/e*)[a]	IR[b] ν_{max} (cm^{-1})	$[\alpha]^{24}_D$[c] (deg)
7a[d]	72	116–120	$C_{26}H_{32}N_3O_4$ (450.559)	178 (M^+ − 272, 100), 165 (M^+ − 285, 39), 152 (M^+ − 298, 40), 140 (M^+ − 310, 41), 124 (M^+ − 326, 53), 110 (M^+ − 340, 25)	1720[e], 1670[f], 1530[f], 1340[g]	—
7b[d]	74	98–102	$C_{27}H_{34}N_3O_4$ (464.586)	465 (M^+ + 1, 0.4), 464 (M^+, 0.1), 179 (M^+ − 285, 43), 178 (M^+ − 286, 100), 140 (M^+ − 324, 68), 124 (M^+ − 340, 64)	1720[e], 1670[f], 1530[f], 1330[g]	−16.8
7c[d]	83	99–103	$C_{29}H_{38}N_3O_4$ (492.640)	179 (M^+ − 313, 46), 178 (M^+ − 314, 100), 165 (M^+ − 327, 75), 140 (M^+ − 352, 36), 124 (M^+ − 368, 59)	1715[e], 1655[f], 1545[f], 1345[g]	−20
7d[d]	82	152–156	$C_{33}H_{38}N_3O_4$ (540.684)	541 (M^+ + 1, 1), 178 (M^+ − 362, 90), 165 (M^+ − 375, 30), 140 (M^+ − 400, 89), 124 (M^+ − 416, 94), 120 (M^+ − 420, 100)	1720[e], 1660[f], 1540[f], 1340[g]	−3.2

[a] Relative percent intensities of the peaks; electron impact (70 eV). [b] KBr pellet. [c] c = 0.25% in methanol. [d] EPR: 3 lines a_N = 16 G (methylene chloride). [e] O–C=O. [f] CONH. [g] N–O

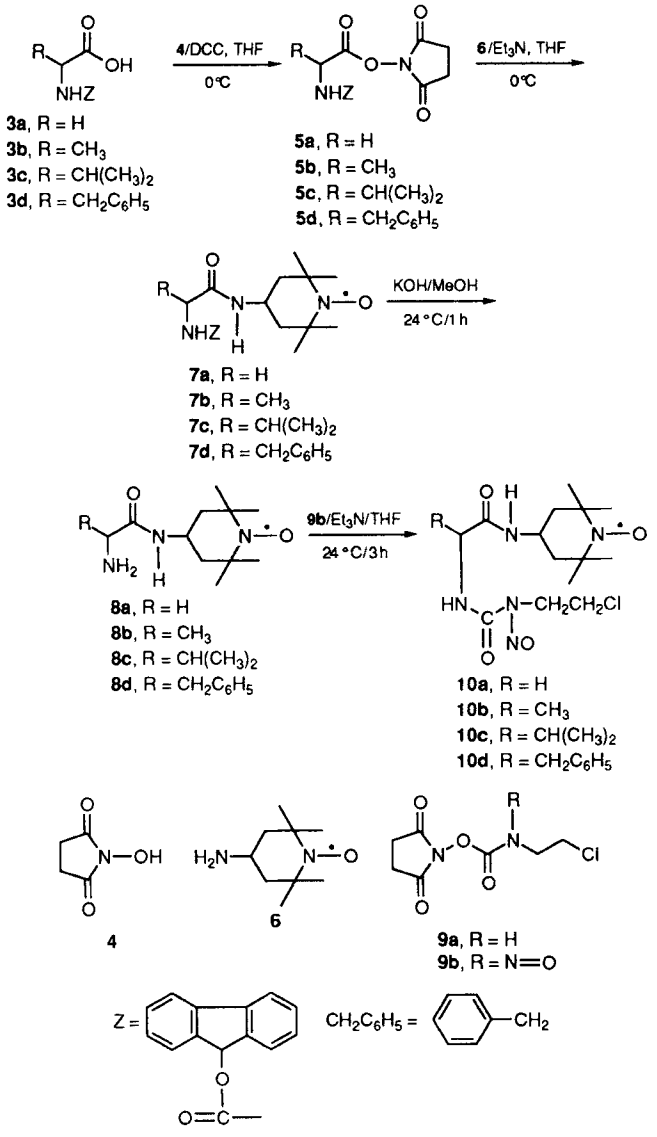
ence to experimental conditions is advisable in order to minimize this premature deblocking side reaction.

The complete removal of the Fmoc group in **7a–d** to give the amino acid derivatives **8a–d** (Table 3) was successfully accomplished with a 10% methanolic potassium hydroxide solution. This reagent was found to be more effective than other Fmoc deblocking agents, such as an excess of piperidine. The incorporation of the [*N'*-(2-chloroethyl)-*N'*-nitrosoamino]car-

Table 3—Analytical Data of Amino Acid Amides of 4-Amino-2,2,6,6-tetramethylpiperidine-1-oxyl 8a–d

Compd	Yield (%)	Mp (dec), (°C)	Molecular Formula	MS (*m/e*)[a]	IR[b] ν_{max} (cm^{-1})	$[\alpha]^{24}_D$[c] (deg)
8a[d]	65	89–93	$C_{11}H_{22}N_3O_2$ (228.316)	229 (M^+ + 1, 2), 228 (M^+, 7), 140 (M^+ – 88, 38), 125 (M^+ – 103, 100), 124 (M^+ – 104, 37), 110 (M^+ – 118, 36), 109 (M^+ – 118, 47)	3400[e], 3310[e], 1645[f], 1525[f], 1345[g]	—
8b[d]	76	97–100	$C_{12}H_{24}N_3O_2$ (242.343)	243 (M^+ + 1, 2), 242 (M^+, 7), 140 (M^+ – 102, 16), 139 (M^+ – 103, 100), 138 (M^+ – 104, 19), 124 (M^+ – 118, 60), 109 (M^+ – 133, 49)	3330[e], 3300[e], 1660[f], 1520[f], 1330[g]	+4.0
8c[d]	80	88–92	$C_{14}H_{28}N_3O_2$ (270.397)	271 (M^+ + 1, 2), 270 (M^+, 7), 167 (M^+ – 103, 87), 152 (M^+ – 118, 60), 140 (M^+ – 130, 41), 139 (M^+ – 131, 42), 124 (M^+ – 146, 100), 109 (M^+ – 161, 83)	3400[e], 3310[e], 1650[f], 1530[f], 1350[g]	+16.8
8d[d]	82	91–94	$C_{18}H_{28}N_3O_2$ (318.441)	319 (M^+ + 1, 0.4), 318 (M^+, 1), 124 (M^+ – 194, 8), 120 (M^+ – 198, 100), 119 (M^+ – 199, 15), 103 (M^+ – 215, 10)	3330[e], 3310[e], 1650[f], 1525[f], 1340[g]	+30.4

[a] Relative percent intensities of the peaks; electron impact (70 eV). [b] KBr pellet. [c] c = 0.25% in methanol. [d] EPR: 3 lines a_N = 16 G (methylene chloride). [e] NH_2. [f] CONH. [g] N–O.



Scheme 1

bonyl group into the N-terminus of aminoxyl-labeled amino acid derivatives **8a–d** was achieved by the reaction of **8a–d** with the transfer agent *N*-succinimidyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**9b**) in the presence of triethylamine to give the target compounds **10a–d** (Table 4). There were severe difficulties experienced in the purification of the final products **10a–d**. The crude products were repeatedly subjected to a combination of flash chromatography on either silica gel and/or neutral alumina with methylene chloride and a mixture of methylene chloride–methanol as the eluants, followed by crystallization from a mixture of ethyl acetate and petroleum ether. Neutral alumina was found to be, in general, more effective than silica gel for the purification by flash chromatography. Furthermore, compounds **10a–c** were also purified by preparative TLC. In spite of these multistep purification procedures, the microanalyses were often not entirely satisfactory because of a sensitivity of these compounds (**10a–d**) to heat and moisture. Nevertheless, the structures were ascertained by infrared, electron paramagnetic resonance, mass spectrometries and polarimetry (Tables 1–4).

The transfer reagent *N*-succinimidyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**9b**) was prepared by nitrosation of the *N*-succinimidyl *N*-(2-chloroethyl)carbamate (**9a**) with dinitrogen tetraoxide in accordance with the literature method.[25] Compound **9a** was synthesized by modifying the literature procedure,[25] whereby a shortening of the reported reaction time from 17 h to 10 min was achieved. According to this modification, it is important to adhere to the sequence of combining the reagents as described in the Experimental Section. Thus, the addition of 2-chloroethyl isocyanate to a solution of **4** in ethyl acetate was followed by the addition of triethylamine (TEA) in order to prevent the formation of the insoluble adduct between TEA and **4**, instead of the product.

Mass Spectrometry—The M^+ and (M^+ + 1) ions could not be detected in the mass spectra of the Fmoc-protected carbamate esters **5a–d**, probably because of a low stability of these molecules causing an elimination of the Fmoc $C_{14}H_{10}$ fragment of *m/z* 178. However, the M^+ and (M^+ + 1) ions could be detected in spectra of the Fmoc-protected aminoxyl-labeled amides **7b** and **7d** (Table 2). The $C_{14}H_{10}$ species at *m/z* 178 was the base peak in the spectra of all activated esters **5** and **7** except for **7d**. The spectra of the 2,2,6,6-tetramethylpiperidine-1-oxyl moiety was found, in general, to be in accord with the fragmentation pattern reported in the literature.[29–32] Thus, it was proposed that the aminoxyl moiety abstracts a hydrogen atom to give the (M^+ + 1) species which decomposes with the loss of the methyl group to form the corresponding (M^+ – 14) species and other fragments recorded in Tables 2–4. The mass spectra of the aminoxyl-labeled amides **8a–d** and related *N*-(2-chloroethyl)-*N*-nitrosoureido derivatives **10a–d** reveal that all structurally significant fragments are derived from the aminoxyl group. In all nitroso compounds **10a–d** the characteristic loss of nitroso fragment

Table 4—Analytical Data of Amino Acids 10a–d Containing (Chloroethyl)nitroso and 4-Amino-2,2,6,6-tetramethylpiperidine-1-oxyl Moieties

Compd	Yield (%)	Mp (dec) (°C)	Molecular Formula[a]	MS (*m/e*)[b]	IR[c] ν_{max} (cm^{-1})	$[\alpha]^{24}_D$[d] (deg)
10a[e]	72	72–75	$C_{14}H_{25}ClN_5O_4$ (362.838)	362 (M^+, 2), 168 (M^+ – 194, 45), 154 (M^+ – 208, 33), 139 (M^+ – 223, 24), 125 (M^+ – 237, 40), 124 (M+ – 238, 100), 109 (M^+ – 253, 70)	1720[f], 1660[g], 1525[g], 1495[h], 1350[i]	—
10b[e]	74	80–82	$C_{15}H_{27}ClN_5O_4$ (376.865)	376 (M^+, 2), 182 (M^+ – 194, 37), 154 (M^+ – 222, 36), 140 (M^+ – 236, 28), 139 (M^+ – 237, 57), 124 (M^+ – 252, 100), 109 (M^+ – 267, 66)	1725[f], 1665[g], 1525[g], 1490[h], 1350[i]	+32.0
10c[e]	81	150–153	$C_{17}H_{31}ClN_5O_4$ (404.919)	404 (M^+, 2), 210 (M^+ – 194, 27), 154 (M^+ – 250, 35), 140 (M^+ – 264, 23), 139 (M^+ – 265, 23), 124 (M^+ – 280, 100), 109 (M^+ – 295, 49)	1710[f], 1650[g], 1525[g], 1490[h], 1350[i]	+30.4
10d[e]	83	95–99	$C_{21}H_{31}ClN_5O_4$ (452.963)	452 (M^+, 1), 167 (M^+ – 285, 31), 154 (M^+ – 298, 45), 140 (M^+ – 312, 17), 139 (M^+ – 313, 20), 124 (M^+ – 328, 100), 109 (M^+ – 343, 48)	1720[f], 1670[g], 1530[g], 1500[h], 1345[i]	+12.0

[a] The microanalyses for **10c** were in satisfactory agreement with the calculated values (C, H, and N within ±0.4%). **10a**: calcd C, 46.34; H, 6.94; N, 19.30; found 46.46, 7.02, 18.76; **10b**: calcd C, 47.80; H, 7.22; N, 18.58; found 47.91, 6.98, 19.24; **10d**: calcd C, 55, 69; H, 6.90; N, 15.46; found 56.22, 7.08, 14.55. [b] Relative percent intensities of the peaks; electron impact (70 eV). [c] KBr pellet. [d] $c = 0.25\%$ in methanol. [e] EPR: 3 lines a_N = 16 G (methylene chloride). [f] N–C(O)–N. [g] CONH. [h] N–N=O. [i] N→O.

(M^+ – 30) was observed at *m/z* 346, 374, and 422 for **10b**, **10c**, and **10d**, respectively, except for **10a**.

The mass spectra for **8a–d** and **10a–d** molecular weight ions contained the M^+ and ($M^+ + 1$). However, the relative intensities of these ions were quite low. The fragment at *m/z* 124 was the base peak for all nitroso compounds. Another common fragmentation pattern seems to be the loss of the NH_2OH molecule from the ($M^+ + 1$) ion. Further, the fragments (M^+ – 107) and (M^+ – 108) can be interpreted as the loss of $ClCH_2CH_2N$–NO and $ClCH_2CH_2N$=NOH species, respectively.[32] These fragments arise either by the cleavage of the N–C bond or by a McLafferty type rearrangement of the *N*-(2-chloroethyl)-*N*-nitrosoureido group. All characteristic fragments in the mass spectra of compounds **7a–d**, **8a–d**, and **10a–d** are shown in Tables 2–4.

Infrared Spectrometry—In the infrared spectra the following important absorptions were identified: imide, N–C=O, at 1785 cm^{-1} in compounds **5a–d**; ester, O–C=O, at 1715–1740 cm^{-1} in compounds **5a–d** and **7a–d**; amide N–C=O, at 1520–1540 and 1645–1670 cm^{-1} in compounds **7a–d**, **8a–d**, and **10a–d**; urea N–C(O)–N, 1710–1725 cm^{-1} in compounds **10a–d**; N→O at 1330–1345 cm^{-1} in compounds **7a–d**, **8a–d**, and **10a–d**; NH_2 at 3300–3400 cm^{-1} in compound **8a–d**; and N–N=O at 1490–1500 cm^{-1} in compounds **10a–d**. The data are presented in Tables 1–4.

Biological Evaluations—Acute toxicity tests for **10a** and **10b** were obtained with Swiss male mice at 35, 70, 100, 200, 300, and 500 mg/kg doses and for **10c** and **10d** at 100, 125, 150, 200, 300, and 500 mg/kg doses. Compounds **10a** and **10b** possessed acute toxicity at 100 mg/kg doses, and **10c** and **10d** at 125 mg/kg doses. Compounds **10a–d** were evaluated *in vivo* against the murine lymphocytic leukemia P388 in CDF_1 male mice following exactly the National Cancer Institute Protocol,[33] and the results are shown in Table 5.

The compounds **10a–d** exhibited a high activity against the lymphocytic leukemia P388 tumor (Table 5). Compound **10c** had the highest activity with a %ILS value of 456 at a dose of 12 mg/kg/day, followed by **10b** (%ILS 447, 12 mg/kg/day), **10a** (%ILS 247, 12 mg/kg/day), and **10d** (%ILS 242, 15 mg/kg/day). Compounds **10a–d** were then evaluated *in vivo* against the lymphoid leukemia L1210 in accordance with the protocol of the National Cancer Institute.[33] The compounds **10a–d** exhibited a very high activity against the lymphoid leukemia L1210 (Table 6). Compounds **10b** and **10c** had the highest activity (%ILS 663, 12 mg/kg/day), followed by **10d** (%ILS 581, 15 mg/kg/day) and **10a** (%ILS, 496, 12 mg/kg/day).

In comparison, although the more hydrophobic L-alanine and L-phenylalanine derivatives **2**, $n = 1$,[25] were also highly active against the P388 leukemia with the %ILS values of 481 and 297, respectively, they were much less effective against the L1210 leukemia, eliciting only 56 and 48 %ILS, respectively. The amino acids chosen for the present investigation have a wide spread of lipophilicities,[34,35] ranging from the low hydrophobic glycine to the high hydrophobic L-phenylalanine, i.e., glycine < L-alanine < L-valine < L-phenylalanine. It was assumed that this sequence of lipophilicities would be retained in the series of anticancer drugs **10a–d** derived from these amino acids. Hence, a systematic, predictable correlation could be made between the lipophilicities and anticancer activities of **10a–d**.

This supposition was to some extent experimentally confirmed. Thus, the partition coefficient values (log *P*) followed the expected order, i.e., **10a** (1.23) < **10b** (1.28) < **10c** (2.09) < **10d** (2.30). However, the correlations of lipophilicities with anticancer activities *in vivo* of compounds **10a–d** were less ideal. Nevertheless, with the exception of the glycine congener **10a**, a trend was observed with both leukemias P388 and L1210, whereby an increase in activity was associated with a decrease in hydrophobicity. The unexpected lower anticancer activity of **10a** might be explained by two factors, i.e., the optimum dose was misjudged, and/or the toxicity of **10a** was causing a lowering of the number of survivors. A similar trend is discernible in an earlier study[17] with the highly active compounds **1g** against the L1210, although the protocol in this work was different from that used in the present investigation.

Finally, an attempt was made to fit the results of the present study into the appropriate (hydrophobic) part of the graph (Figure 1) shown[14,27] in earlier studies. However, from the earlier graph[14] was removed the entry for cymerin (**1f**) which was in error, i.e., log *P* should be –0.7 instead of 1.24, and a revised average value of **1c** was entered based on the present and past work.[12,25] It is felt that this graphical correlation upholds our general working hypothesis[27] in the quest for new anticancer drugs.

Experimental Section

Materials—All chemicals were of the finest quality commercially available. Solvents were dried by standard procedures. All Fmoc-protected amino acids were purchased either from Schweizerhall Inc., South Plainfield, NJ, or Novabiochem, USA, La Jolla, CA. The *N*-succinimidyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**9b**) was pre-

Table 5—Partition Coefficients and Anticancer Activities of 10a–d against P388 Lymphocytic Leukemia in CDF_1 Male Mice

Compd	Dose (mg/kg/day)[a]	Weight Change (%)[b] 5-Day	Weight Change (%)[b] 10-Day	T/C (%)[c]	T/C (%)[d]	ILS (%)[c]	ILS (%)[d]	Survivors Survivors/Total Day 30	Survivors Survivors/Total Day 60	UV Partition Coefficient[e] P	UV Partition Coefficient[e] log P
5-Fluorouracil	200[f]	−15.37	+8.62	156		56		0/6			
10a	12	−8.52	−12.90	269	347	169	247	3/6	1/6	16.81	1.23
	9	−4.25	−1.59	226		126		0/6			
	6	+1.73	+3.25	195		95		0/6			
	3	+4.68	+12.76	156		56		0/6			
10b	12	−8.19	−12.93	278	547	178	447	6/6	6/6	19.03	1.28
	9	−4.84	−4.88	300	342	200	242	5/6	0/6		
	6	−1.43	+0.69	251	261	151	161	1/6	0.6		
	3	−1.67	+0.57	175		75		0/6			
10c	15	−15.28	−25.36	278	476	178	376	6/6	4/6	124.10	2.09
	12	−6.51	−10.31	305	556	205	456	6/6	5/6		
	8	−3.85	−6.76	278	280	178	180	1/6	0/6		
	4	−0.50	+3.08	202		102		0/6			
10d	15	−3.45	−7.78	275	342	175	242	5/6	1/6	197.30	2.30
	12	−5.17	−8.42	264	270	164	170	1/6	0/6		
	8	−1.69	+0.16	215		115		0/6			
	4	+3.73	+8.57	156		56		0/6			

[a] All compounds were administered in 0.85% saline/cremophore EL/ethanol (v/v/v, 6:2:2).[21] [b] The average percentage weight changes on days 5 and 10 were taken as a measure of drug toxicity. [c] Results obtained on day 30. [d] Results obtained on day 60. [e] The partition coefficients P = [compound in 1-octanol]/[compound in water] were obtained by a UV technique, according to the literature method.[36] [f] 5-Fluorouracil in a single dose of 200 mg/kg in 0.85% saline solution (Sigma Chemical) was administered on day 1, in accordance with the protocol of the National Cancer Institute.[33]

Table 6—Anticancer Activities of 10a–d against L1210 Lymphoid Leukemia in CDF_1 Male Mice

Compd	Dose (mg/kg/day)[a]	Weight Change (%)[b] 5-Day	Weight Change (%)[b] 10-Day	T/C (%)[c]	T/C (%)[d]	ILS (%)[c]	ILS (%)[d]	Survivors Survivors/Total Day 30	Survivors Survivors/Total Day 60
5-Fluorouracil	200[e]	−7.65	−6.03	163		63		0/6	
10a	12	−1.14	−1.32	346	596	246	496	4/6	4/6
	9	−0.74	−0.62	217		117		0/6	
10b	12	−11.39	−15.51	388	763	288	663	6/6	6/6
	9	−2.47	−4.48	371	683	271	583	5/6	5/6
10c	15	−7.96	−20.48	352	665	252	565	5/6	5/6
	12	−5.71	−17.78	388	763	288	663	6/6	6/6
10d	15	−6.60	−6.66	369	681	269	581	5/6	5/6
	12	−2.45	−1.31	315	440	215	340	2/6	2/6

[a] All compounds were administered in 0.85% saline/cremophor EL/ethanol (v/v/v, 6:2:2).[21] [b] The average percentage weight changes on days 5 and 10 were taken as a measure of drug toxicity. [c] Results obtained on day 30. [d] Results obtained on day 60. [e] Fluorouracil in a single dose of 200 mg/kg in 0.85% saline solution (Sigma Chemical) was administered on day 1, in accordance with the protocol of the National Cancer Institute.[33]

pared by a modification of the method reported in literature.[12,25] Compound **6** was prepared from 4-amino-2,2,6,6-tetramethylpiperidine by the reaction sequence of acetylation, sodium tungstate-catalyzed peroxide[38] oxidation, and deacetylation according to the literature procedure.

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 electron impact, 70 eV (EI). IR spectra were recorded on a Mattson Instruments, Inc. Model IR-10400 spectrometer, and UV spectra on a Hewlett-Packard Model HP 8451A diode array spectrophotometer. Microanalyses were performed on a Perkin-Elmer elemental analyzer, Model 240C. The EPR spectra of 4.0×10^{-5} M solutions of the nitroxyl radicals in methylene chloride were obtained on a Varian E-115 EPR spectrometer. The specific optical rotations of compounds **5c**, **7b–d**, **8b–d**, and **10b–d** in methanol were obtained first on a Model SR-4 Polarimeter, Polyscience Group, Niles, IL, and later confirmed on a JASCO Model DIP-370 Digital Polarimeter, Japan Spectroscopic Co., Ltd., Tokyo, Japan. Column chromatography was performed either by conventional column chromatography or by the flash chromatography technique using silica gel 60 (Fluka) finer than 230 mesh, and alumina (neutral, Woelm activity IV and V, 80–200 mesh, Alupharm Chemicals, New Orleans, LA). All reactions, chromatographic procedures, and product purities were monitored by TLC. 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. Preparative TLC was performed on silica gel PLK5F precoated plates, 1000-μm thickness (Whatman, USA). The elution solvents for TLC were as follows: a mixture of ethyl acetate, cyclohexane, and acetic acid (v/v/v, 10:5:0.5) for the compounds **5b**, **5d**, **7b** and **7d**; chloroform and methanol (v/v, 9:1) for the compounds **5a**, **5c**, **7a**, and **7c**; chloroform and methanol (v/v, 3:1) for the compounds **8a–d** and **10a–d**. Partition coefficients (P) were obtained by following the literature methodology[36] using UV spectrophotometry. For measuring the partition coefficient 1-octanol and water layers were presaturated with each other prior to use. The partition coefficients P = [compound in 1-octanol]/[compound in water], and their logarithmic values are shown in Table 5.

Mice—Male Swiss mice (for acute toxicity, average weight 18–21 g), DBA/2 mice (for tumor propagation)[33] 6–7 weeks old, and male CDF_1 mice (for testing, average weight 18–20 g) were supplied by Charles River Laboratories, Wilmington, MA. The tumor lines P388 and L1210 cells were obtained from the Frederick Cancer Research Facility, Frederick, MD. Mice were fed Rodent Laboratory Chow 5001 (Purina Mills, Inc., St. Louis, MO) and water *ad libitum*.

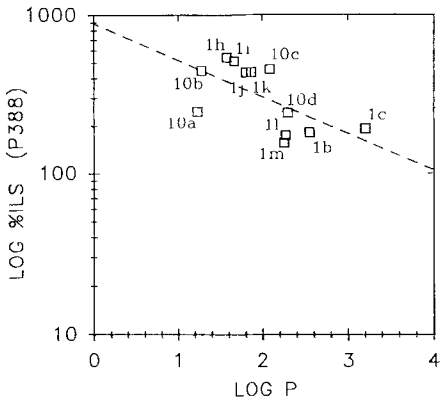


Figure 1—Anticancer activity–lipophilicity correlations of various nitrosoureas. Entries **1l** and **1m** are the acyl derivatives[14] of **1j** and **1k**, respectively. The dotted line is our averaging linear regression line which is used only to indicate a general trend.

Drugs—5-Fluorouracil (5FU) was administered in a 0.85% saline solution (Sigma Chemical Co.). Compounds **10a–d** were dissolved with sonication in a mixture of Cremophor EL–ethanol–0.85% saline (v/v/v, 2:2:6).[21] Cremophor EL is a product of BASF, Ludwigshafen, Germany, and was obtained from the Sigma Chemical Co. **All compounds, but, in particular, the CNC-containing compounds, are sensitive to moisture and elevated temperatures. These materials were always stored in a freezer at −20 °C. Shortly before microanalyses and animal experiments, the homogeneity of all compounds was determined by thin-layer chromatography on silica gel and neutral aluminas. Fresh solutions of each drug were prepared shortly before every animal experiment.**

Biological Evaluations—Acute toxicity tests for **10a** and **10b** were obtained with Swiss male mice at 35, 70, 100, 200, 300, and 500 mg/kg doses and for **10c** and **10d** at 100, 125, 150, 200, 300, and 500 mg/kg doses. Compounds were evaluated *in vivo* against the lymphocytic leukemia P388 and the lymphoid leukemia L1210 in mice following the protocol of the National Cancer Institute.[33] The CDF_1 male mice of 18–20-g weight, in groups of six, were inoculated ip either with 10^6 cells of P388 tumor, or with 10^5 cells of L1210 tumor on day zero of the experiment. The compounds **10a–d** were injected ip at doses listed in Tables 5 and 6 during 9 successive days starting on day one. The animals were then observed according to the protocol[33] for 30 and 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 increase in life span (%ILS) parameter was calculated by the formula $[(T - C)/C] \times 100$.

Syntheses—*Preparation of N′-Hydroxysuccinimide Esters of Fmoc-Amino Acids (**5a–d**): A General Procedure*—Dicyclohexylcarbodiimide (DCC, 2.06 g, 10 mmol) was added to a stirred solution of Fmoc-protected amino acids **3a–d** (10 mmol) and *N*-hydroxysuccinimide (**4**, 10 mmol) in dry tetrahydrofuran (THF, 30 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 4 h and then allowed to stand in a refrigerator for 0 °C for 16 h. The separated solid was collected by filtration and washed with THF (2 × 5 mL). The filtrate and washings were combined, and the solvent was removed in a rotating evaporator at 30 °C (20 Torr). The residue was dissolved in methylene chloride (10 mL), and the solution was filtered to remove some insoluble material. Removal of the solvent at 30 °C (20 Torr) from the filtrate produced a "foamy" solid which was crystallized from a mixture of methylene chloride and petroleum ether (bp 40–60 °C; v/v, 1:1). The yields and analytical data are presented in Table 1.

*Preparation of Fmoc-Amino Acid Amides of 4-Amino-2,2,6,6-tetramethylpiperidine-1-oxyl (**7a–d**): A General Procedure*—Triethylamine (TEA, 0.505 g, 0.7 mL, 5 mmol) was added at 0 °C to a stirred mixture of the corresponding *N*-succinimidyl derivatives **5a–d** (5 mmol) and 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (**6**, 0.855 g, 5 mmol) in THF (25 mL). The mixture was stirred for 3 h at 0 °C and then for 24 h at 24 °C. THF was removed on a rotating evaporator at 30 °C (20 Torr), and the red residue extracted with ethyl acetate. The ethyl acetate solution was successively washed with a 10% aqueous citric acid solution (2 × 25 mL), water (2 × 25 mL), a 10% aqueous sodium bicarbonate solution (2 × 25 mL), and water (2 × 25 mL). The ethyl acetate solution was dried over anhydrous magnesium sulfate and filtered. Concentration of the filtrate on a rotating evaporator at 30 °C (20 Torr) gave an oily liquid which was triturated with petroleum ether (bp 40–60 °C), whereby a pink solid material was formed. The solid was collected by filtration and washed with petroleum ether (bp 40–60 °C; 3 × 5 mL). Recrystallization of the solid from a mixture of ethyl acetate and cyclohexane (v/v, 1:2) gave the corresponding Fmoc-amino acid amides **7a–d**. The yields and analytical data are shown in Table 2.

*Preparation of Amino Acid Amides of 4-Amino-2,2,6,6-tetramethylpiperidine-1-oxyl (**8a–d**): A General Procedure*—A 10% solution of potassium hydroxide in methyl alcohol (5 mL) was added at 25 °C to a stirred solution of Fmoc-amino acid derivatives **7a–d** (2 mmol) in methyl alcohol (15 mL), and the reaction mixture was stirred for 1 h at 25 °C. The mixture was then poured into ice-cold water (30 mL) and the precipitate collected by filtration. The filtrate was extracted with methylene chloride (3 × 30 mL), and the combined organic extracts were dried over magnesium sulfate. The drying agent was removed by filtration, and the filtrate was concentrated on a rotating evaporator at 30 °C (20 Torr). Recrystallization of the resulting solid from a mixture of ethyl acetate and cyclohexane (v/v, 1:3) gave the amino acid amides **8a–d**. The yields and analytical data are presented in Table 3.

*Preparation of N-[(2-Chloroethyl)-N-nitrosocarbamoyl]amino Acid Amides of 4-Amino-2,2,6,6-tetramethylpiperidine-1-oxyl (**10a–d**): A General Procedure*—TEA (0.152 g, 0.21 mL, 1.5 mmol) was added at 24 °C to a stirred solution of the transfer agent **9b** (0.375 g, 1.5 mmol) and the corresponding amino acid amides **8a–d** (1.5 mmol) in THF (15 mL). The reaction mixture was stirred for 3 h at 24 °C, and then the solvent was removed on a rotating evaporator at 25 °C (20 Torr). The resulting residue was dissolved in ethyl acetate (20 mL), and the solution was extracted successively with water (2 × 25 mL), 10% aqueous citric acid (2 × 25 mL), and a saturated aqueous sodium chloride solution (2 × 25 mL). The organic layer was dried over anhydrous magnesium sulfate and filtered. Concentration of the filtrate on a rotating evaporator at 25 °C (20 Torr) gave the crude products. Purification of **10a**, **10b**, and **10d** by flash chromatography on alumina (neutral, activity IV and V) using methylene chloride followed by a mixture of methylene chloride and methanol (v/v, 9:1) as eluants and subsequent concentration of the combined fractions on a rotating evaporator at 24 °C (20 Torr) gave orange crystals. These compounds, as well as compound **10c**, were subsequently recrystallized from a mixture of ethyl acetate and petroleum ether (bp 40–60 °C; v/v, 1:3), resulting in crystalline products. The yields and analytical data are presented in Table 4.

*Preparation of N-Succinimidyl N-(2-Chloroethyl)carbamate (**9a**)*—2-Chloroethyl isocyanate (12.7 g, 10.3 mL, 120 mmol) was added at 0 °C in a dropwise manner over a period of 15–20 min to a stirred solution of *N*-hydroxysuccinimide (**4**, 11.5 g, 100 mmol) in ethyl acetate (100 mL) followed by an addition of TEA (15.15 g, 10.3 mL, 150 mmol) at 0 °C. The reaction mixture became clear and, then, within a few minutes a white solid started to precipitate. The reaction was completed within 10 min and the precipitate collected by filtration, washed with cold ethyl acetate (10 mL), and dried at 25 °C (0.1 Torr). There was obtained 18.8 g (85%) of the target compound **9a**, mp 104–107 °C (lit.[12,25] mp 106–108 °C). The purity control by TLC (silica gel, chloroform–methanol, v/v, 9:1) indicated a single spot.

*Preparation of N-Succinimidyl N-(2-Chloroethyl)-N-nitrosocarbamate (**9b**)*—Anhydrous sodium acetate (98.6 g, 120 mmol) was added to a stirred solution of **9a** (2.21 g, 10 mmol) in ethyl acetate (75 mL). The mixture was cooled to −35 °C with a mixture of dry ice and 2-propanol. A solution of dinitrogen tetraoxide (1.84 g, 20 mmol) in dry carbon tetrachloride (20 mL) was added in a dropwise manner to this cooled and stirred mixture over a period of 30 min. After the addition, the reaction mixture was stirred for 1 h at −35 °C, during which time the color of the mixture became blue, and then for 0.5 h at 5 °C, during which time the color changed from blue to green, and finally to yellow. The reaction mixture was poured into ice water (40 mL). The ethyl acetate layer was separated and successively washed with a saturated aqueous sodium bicarbonate solution (2 × 30 mL) and a saturated sodium chloride solution (2 × 30 mL). The organic layer was dried over anhydrous magnesium sulfate and filtered. Concentration of the filtrate on a rotating evaporator at 25 °C (20 Torr) gave the crude product. Repeated recrystallizations of the product from a mixture of methylene chloride and petroleum ether (bp 40–60 °C; v/v, 1:1) gave 2.05 g (82%) of **9b** as yellow needles, mp 101–104 °C dec (lit.[12,25] mp 102–104 °C dec).

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