Selective Cytotoxicity of a System L Specific Amino Acid Nitrogen Mustard†

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The synthesis and characterization of DL-2-amino-7-bis[(2-chloroethyl)amino]-1,2,3,4-tetrahydro-2-naphthoic acid (6) and DL-2-amino-5-bis[(2-chloroethyl)amino]-1,2,3,4-tetrahydro-2-napthoic acid (7) were accomplished. The correct assignment of the site of attachment of the bis(2-chloroethyl)amino side chain was ascertained by selective proton decoupling of the ^{13}C NMR spectra performed on the corresponding nitrospirohydantoin precursors **2** and **3**, which were obtained from the nitration of β-tetralone hydantoin. The two target compounds **6** and **7** were designed as tumor-specific agents capable of being selectively transported into tumor cells by the leucine-preferring transport system (system L). Inhibition analysis of the initial rate of transport of the system L specific substrate 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) by **6** and **7** indicated that the 7-substituted isomer **6** was an extremely potent competitive inhibitor of that transport system in murine L1210 leukemic cells (K_i = 0.2 μM). Evaluation of the selectivity of this compound indicated that it possessed enhanced in vitro antitumor activity and reduced myelosuppressive activity when compared to its prototype amino acid nitrogen mustard, L-phenylalanine mustard (L-PAM). In addition to being more selectively toxic to tumor cells, this compound differs from L-PAM in having a 2-3-fold shorter half-life ($t_{1/2}$).

The phenylalanine mustards, a group of antitumor agents of the alkylating agent class, were synthesized[1-3] in the 1950s and found to possess a broad range of antitumor activity against both experimental[4] and human[5] neoplasms. These antitumor agents incorporated into their structure the physiological amino acid carrier phenylalanine and the cytotoxic bis(2-chloroethyl)amino group. Although effective as antitumor agents, the myelosuppression produced by this group of antineoplastic compounds is dose-limiting.

As part of an effort of this laboratory to develop more selective antitumor agents, studies were initiated to gain insight into the mechanism by which the phenylalanine mustards enter cells. These studies with murine L1210 leukemia cells[6] indicated that the transport of the L isomer, L-phenylalanine mustard (L-PAM), is mediated equally by two separate amino acid transport systems. One carrier system corresponds to system L, the classical sodium-independent leucine-preferring transport system originally described by Oxender and Christensen.[7] The model synthetic substrate for system L is 2-aminobicyclo-[2.2.1]heptane-2-carboxylic acid (BCH).[8,9] The remaining 50% of L-PAM transport is mediated by a second amino acid transport system that is monovalent cation-dependent and exhibits its highest affinity for leucine.[10] Identical carrier systems have been identified by Goldenberg and associates for L-PAM transport in LPC-1 plasmacytoma cells[11] and L5178Y lymphoblasts.[12]

The fact that marrow preparations are heterogeneous and contain only one undifferentiated progenitor cell for every 1000 nucleated cells makes it unfeasible to utilize standard transport methodology to identify amino acid carrier systems in such cells. The approach of this laboratory was to utilize amino acids whose mode of entry is restricted to specific amino acid transport systems in conjunction with the cytotoxicity produced by L-PAM to discern the mode of entry of L-PAM into progenitor cells. The failure of BCH to reduce L-PAM cytotoxicity to murine bone marrow progenitor cells (CFU-C)[13] indicated that these cells lack system L or have a reduced affinity for BCH. In an attempt to exploit this marked qualitative difference between tumor and normal host cells, a series of cyclic amino acids were examined[14] to determine which were transported with highest affinity by system L into tumor cells with the objective being that they could be utilized to selectively introduce the cytotoxic bis(2-chloroethyl)amino group into the tumor cell. These results indicated that the aromatic bicyclic amino acid 2-amino-1,2,3,-tetrahydro-2-naphthoic acid was a potent competitive inhibitor of system L (K_i = 5 μM).[14]

Taken together these observations prompted the synthesis of the nitrogen mustard derivatives of 2-amino-1,2,3,4-tetrahydro-2-naphthoic acid. The present paper describes (a) the synthesis of these compounds with special emphasis in determining the exact location of the bis(2-chloroethyl)amino substituent on the molecule, (b) their evaluation as competitive inhibitors of system L, and (c) the investigation of the most potent of these inhibitors of system L for antitumor and myelosuppressive activities as well as for alkylating potency and stability.

Chemistry

The general procedure for the preparation of the hydantoin and amino acid nitrogen mustards employed in this work has already been described by Mauger and Ross.[15] However, at an early stage of the synthesis, these

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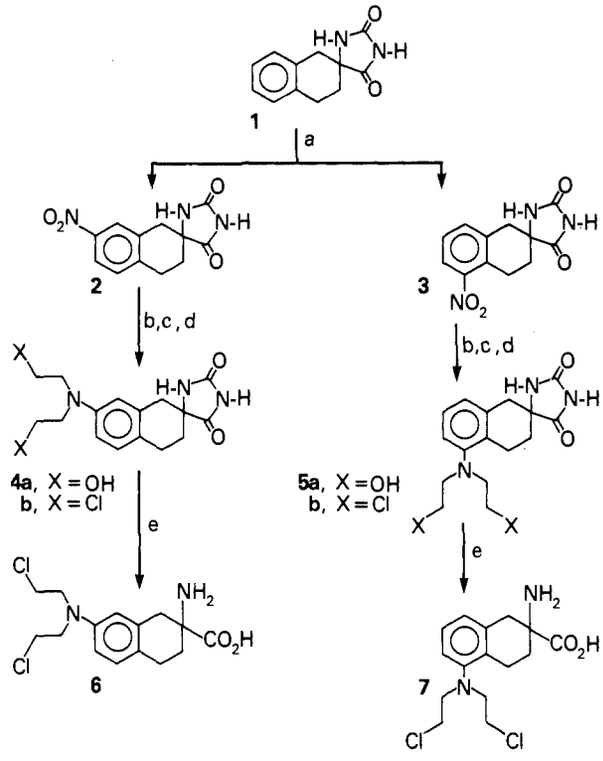
Scheme I



[a]HNO_3, [b]H_2/Pd/C, [c]△/HOAc, [d]$POCl_3$, [e]HCl

workers were unable to separate and identify the mixture of the two mononitro derivatives obtained from β-tetralone hydantoin. Despite their success in the separation of one of the isomers at the bis(2-hydroxyethyl)amino stage and its conversion to the final target amino acid mustard, the assigned location of the substituent for this isomer was still uncertain. On the basis of the comparative rates of hydrolysis of the final product with a group of standard tetrahydronaphthalene nitrogen mustards, the bis(2-chloroethyl)amino side chain was postulated to be at either the 5- or 8-position.[15] Even the successful separation of the isomers at the nitro stage and their chemical oxidation to a nitrophthalic acid product, as suggested by these authors, would not have solved the issue of substituent position.

In repeating this synthetic sequence (Scheme I) we were able to separate small amounts (ca. 200 mg) of both nitro-substituted isomers, which we then studied by proton and carbon NMR spectroscopy to determine the exact position of the nitro group in the molecule (vide infra). It was easier, nevertheless, to continue the synthesis and perform the separation of isomers at the bis(2-hydroxyethyl)amino stage as described by Mauger and Ross, or even at the bis(2-chloroethyl)amino stage, prior to the hydrolysis of the hydantoin ring, as detailed in the Experimental Section of this paper.

The less water soluble bis(2-hydroxyethyl)amino isomer, mp 187–190 °C (which corresponded to the material isolated by Mauger and Ross having a melting point of 185–187 °C), had to be either the 5- or the 8-substituted isomer on the basis of its ^1H NMR spectrum that showed a pattern consistent with a 1,2,3-trisubstituted benzene (Table I, compound **5a**).[16] This agreed well with the two

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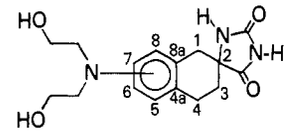
Table I. ^1H NMR Resonances of Aromatic Protons in Compounds **4a** and **5a**[a-c]



compd	H-5	H-6	H-7	H-8
5a (5-isomer)		6.77 (dd, $J_{6,7}$ = 6.3, $J_{1,8}$ = 2.0)	7.04 (m)	7.04 (m)
4a (7-isomer)	6.86 (d, $J_{5,6}$ = 8.4)	6.47 (dd, $J_{6,5}$ = 8.4, $J_{6,8}$ = 2.0)		6.33 (d, $J_{8,6}$ = 2.0)

[a] s = singlet, d = doublet, dd = doublet of doublets, m = multiplet. Chemical shifts are in parts per million and J values in Hertz. [b] All spectra were measured at 200 MHz in Me_2SO-d_6. [c] The correct numbering system for these spiro compounds requires that the naphthalene atoms be identified with primes (see Experimental Section). For the sake of simplicity the primes have been eliminated from this table and from the main body of the paper.

alternative substitution sites suggested by the same authors, which was based, as mentioned before, on the rate of hydrolysis of the final nitrogen mustard product.[15] However, since our ^{13}C NMR spectral studies performed on the precursor nitro-substituted isomers **2** and **3** demonstrated that these compounds carried the nitro substituent exclusively at the 5- or the 7-position, the correct structure for the less water soluble bis(2-hydroxyethyl)-amino isomer had to be **5a** and the products derived from it had to correspond to the 5-substituted derivatives. The more water soluble bis(2-hydroxyethyl)amino isomer, which was isolated from the mother solution (mp 190–192 °C), had a ^1H NMR spectrum consistent with a 1,2,4-trisubstituted benzene derivative (Table I, compound **4a**)[16] and therefore had to correspond to the 7-substituted isomer.

The remaining steps were performed essentially in the same manner (Mauger and Ross) for both of the isomeric 5- and 7-bis(2-hydroxyethyl)amino compounds to give the two rather unstable amino acid mustards **6** and **7** (Scheme I). These compounds were readily hydrolyzed in aqueous solution to one arm mustards as confirmed by GC–MS studies. For this reason the final products were not manipulated or purified any further and they were biologically evaluated as such. According to GC–MS studies the purity for both isomers was ca. 90%. Reversed-phase HPLC for the biologically important 7-isomer revealed a somewhat lower purity (ca. 74%) possibly due to partial hydrolysis in the aqueous phase of the system (30-min gradient from water to 0.5 M KH_2PO_4). All the other intermediates prior to the final amino acid mustards **6** and **7** were analytically pure according to spectral and combustion analysis.

Carbon NMR Spectral Studies

Absolute assignment of the site of nitration was accomplished by selective proton decoupling of the ^{13}C NMR. The faster moving isomer of the two nitro compounds collected after preparative HPLC showed a distinct 1,2,3 aromatic substitution pattern in its proton NMR spectrum, indicating either 5- or 8-nitration. The fully coupled ^{13}C NMR spectrum showed the nonprotonated aromatic carbons at 148.9, 136.0, and 129.6 ppm (see Experimental Section). The nitrated carbon resonance position (148.9 ppm) could be assigned on the basis of its coupling pattern (doublet of doublets) since in either the 5- or the 8-nitro isomer, this carbon would show a ^2J (absolute value ~ 3 Hz)[17] and a ^3J (~ 9 Hz)[17] coupling to the neighboring

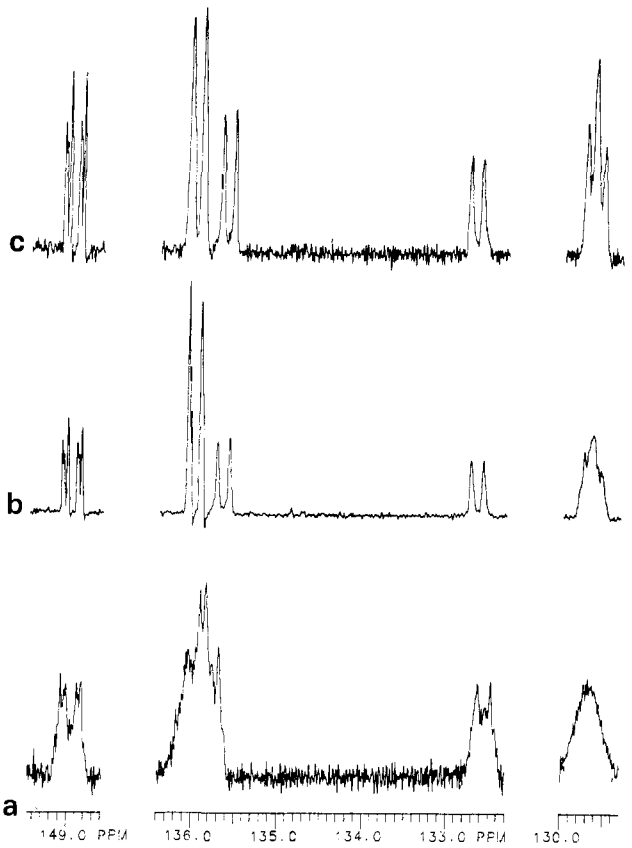


Figure 1. Selectively proton decoupled ^{13}C NMR spectra of **3**. (a) Fully coupled spectrum. (b) H-1 and H-4 decoupled spectrum. (c) H-1, H-3, and H-4 decoupled spectrum. The doublet of doublets ($^1J_{residual}$ = 147 Hz and $^3J_{residual}$ = 7 Hz) centered at 134.1 ppm is due to C-8.

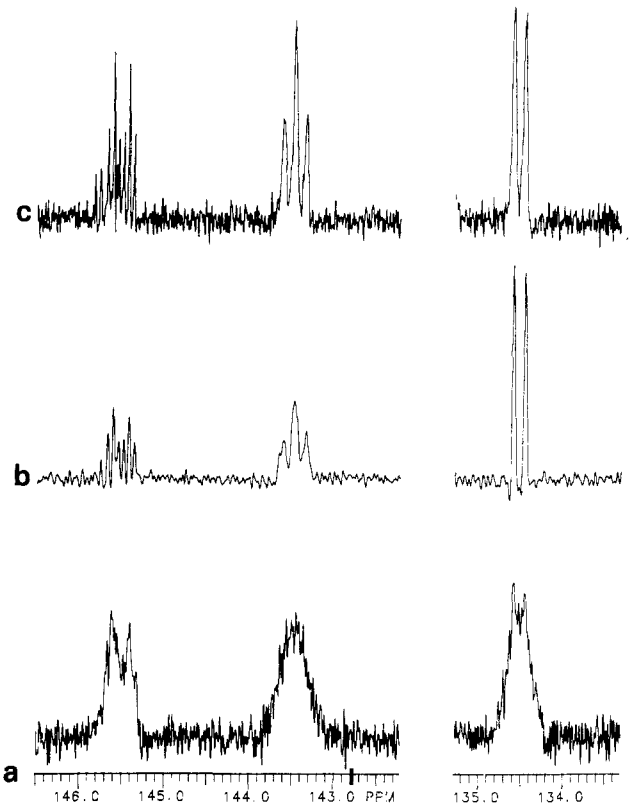


Figure 2. Selectively proton decoupled ^{13}C NMR spectra of **2**. (a) Fully coupled spectrum. (b) H-1 and H-4 decoupled spectrum. (c) H-1, H-3, and H-4 decoupled spectrum. The small peaks downfield of the doublet of triplets at 145.8 ppm are due to slight impurities in the sample.

aromatic protons (Figure 1a). At this point, assignment of the other two nonprotonated carbon resonances (C-4a and C-8a) could be achieved by determining which showed coupling to the methylene protons at C-3 and was therefore C-4a. Coupling of C-8a to these methylene protons (^4J) would be very small and therefore undetectable at the resolution of this experiment. Selective decoupling of the H-1 and H-4 protons by irradiation of the overlapping resonances with a decoupling power of 0.01 W simplified the resonances at 136.0 ppm to a sharp doublet and the resonances at 129.6 ppm to a broad triplet (Figure 1b). Irradiation at a position midway between the overlapping H-1 and H-4 resonances and the H-3 resonance with a decoupling power of 0.02 W resulted in complete decoupling of the aliphatic protons and further simplified the resonance at 129.6 ppm to a sharper triplet (Figure 1c). The resonance at 129.6 ppm is therefore coupled to the H-3 protons and is C-4a. That C-4a is a triplet with a residual coupling constant of the magnitude of an aromatic ^3J ($\sim$ 5.2 Hz)[17] indicates that it is coupled through three bonds to two aromatic protons. These aromatic protons must be H-6 and H-8 and the nitro gorup must therefore be at the 5-position (compound **3**). The C-8a resonance at 136.0 ppm is simplified to a doublet due to residual coupling with H-7 (^3J); coupling with H-8 (^2J) is expected to be small ($\sim$ 1 Hz)[17] and is not resolved. X-ray crystallographic analysis performed on **5a** confirmed the position of the side chain at C-5 in accordance with the NMR experiments.[18]

Similar strategy allowed the structural assignment of the second compound as the 7-nitro isomer (compound **2**). In the aliphatic proton decoupled spectrum (Figure 2c) the nitrated carbon (C-7) at 145.5 ppm appears as a doublet of triplets due to coupling through two bonds to H-6 and H-8 and through three bonds to H-5. C-4a, at 143.5 ppm, identified by its coupling to the methylene protons at C-3, is coupled to two aromatic protons (H-6 and H-8) through three bonds and appears as a triplet. C-8a (134.6 ppm) is coupled to only one aromatic proton (H-5) through three bonds and is therefore a doublet.

Biological Results and Discussion

Compound **6** is an extremely potent competitive inhibitor of system L in murine L1210 leukemia cells (Figure 3). Inhibition analysis of the initial rate of transport of BCH indicated that the K_i is approximately 0.2 μM, 500-fold lower than that of L-phenylalanine mustard (K_i = 110 μM). This value indicates that the compound is a 25–50-fold more effective inhibitor of system L than BCH and approximately 40-fold more potent than 2-amino-1,2,3,4-tetrahydro-2-naphthoic acid.[14] Compound **7**, on the other hand, was a weaker inhibitor of system L with a K_i of 15.2 μM (data not shown).

L-Phenylalanine mustard, the prototype amino acid nitrogen mustard, is 2.5 times more cytotoxic to bone marrow progenitor cells than to murine L1210 leukemia cells (Figure 4). The LD_{90} concentration of L-PAM for murine CFU-C was 14 μM as compared to 35 μM for murine L1210 leukemia cells. Compound **6**, on the other

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(18) A complete crystal structure analysis for compound **5a** was performed by Dr. Cynthia S. Day from Crystalytics Co., Lincoln, NE. The detailed results from this work will be published elsewhere.

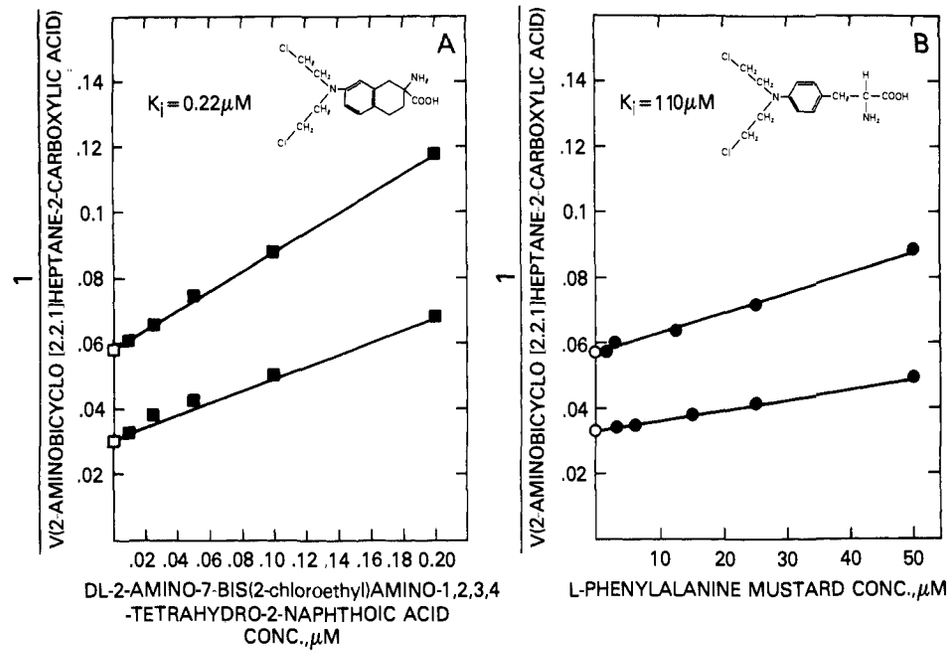

Figure 3. Competitive inhibition of the initial velocity of transport of 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid by L-PAM and compound **6**. Murine L1210 leukemia cells were prepared and transport studies performed exactly as described in the Experimental Section. Initial velocity (v) is expressed as picomoles (10^6 cells)$^{-1}$ min^{-1}. Panel A: Compound **6**. $K_i = 0.22 \pm 0.02$ μM [mean ± SE ($n = 3$)]. Panel B: L-PAM. $K_i = 111.6 \pm 7.7$ μM [mean ± SE ($n = 4$)].

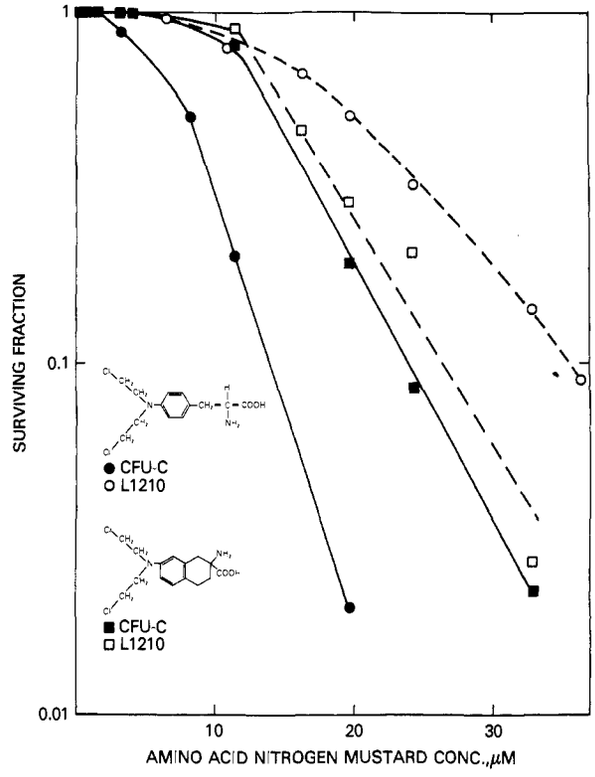

Figure 4. Antitumor and myelosuppressive activity of L-PAM and compound **6**. Antitumor and myelosuppressive activity was determined as described in the Experimental Section. The therapeutic index was calculated accordingly; L-PAM: LD_{90}-(CFU-C/L1210) = 14μM/35μM = 0.40. Compound **6**: LD_{90}-(CFU-C/L1210) = 23.8 μM/26.5 μM = 0.9.

hand, possesses both enhanced antitumor activity (LD_{90} = 26.5 μM) and reduced myelosuppressive activity (LD_{90} = 23.8 μM) (Figure 4). A comparison of the therapeutic indices for L-PAM and compound **6** indicated a 2–2.5-fold improvement for the latter amino acid nitrogen mustard.

Compound **6** and L-PAM possess identical alkylating capacity as determined by their reaction with γ-(4-nitrobenzyl)pyridine (Figure 5). However these two amino acid nitrogen mustard differ significantly in their rate of dechlorination. Compound **6** has a half-life ($t_{1/2}$) of approximately 40 min as compared to 120 min for L-PAM.

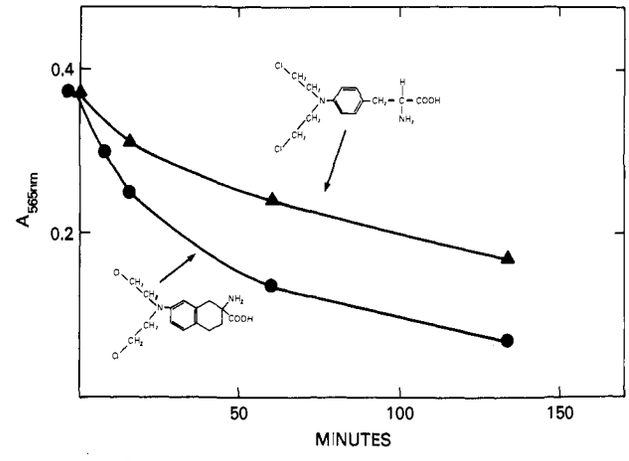

Figure 5. Comparative rate of dechlorination of L-PAM and compound **6**. Alkylation of γ-(4-nitrobenzyl)pyridine by L-PAM and compound **6** was performed as described in the Experimental Section.

The results presented here indicate that compound **6** is the most potent competitive inhibitor of system L, the sodium-independent leucine-preferring amino acid transport system described to date. It is 25–50 times more effective than BCH and has a 500-fold greater affinity for system L than L-PAM. In addition to differing from L-PAM in its affinity for system L, compound **6** exhibits both enhanced antitumor and reduced myelosuppressive activity, which results in a 2-fold improvement in the therapeutic index. Finally, the rate of dechlorination of compound **6** differs significantly from L-PAM, resulting in a 2–3-fold decrease in the $t_{1/2}$. This more rapid conversion to a noncytotoxic derivative may contribute to the observed increase in selectivity of compound **6**. This could occur provided the time required to achieve steady-state concentrations of the drug in tumor cells is sufficiently

short compared to its $t_{1/2}$. Residual drug would then be more rapidly dechlorinated, resulting in a reduction in host toxicity. However, we believe the increase in selectivity is primarily due to the avidity of compound **6** for system L. The high affinity of the drug for that carrier system would produce higher intracellular concentrations of the drug in tumor cells; conversely, with an altered or absent system L in progenitor cells, one might expect lower concentrations to be achieved in this sensitive host tissue, the result being a reduction in myelosuppression.

Experimental Section

General Procedures. All chemical reagents utilized for synthesis were purchased from Aldrich Chemical Co., Milwaukee, WI. Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. Proton NMR spectra were determined on Varian T-60 and XL-200 instruments. Chemical shifts are given as δ values with reference to Me_4Si. Elemental analyses were carried out by Galbraith Laboratories, Knoxville, TN. Coupled and selectively decoupled ^{13}C NMR studies were conducted on the Varian XL-200 instrument on saturated Me_2SO-d_6 samples. Chemical shifts were determined by reference to the Me_2SO-d_6 peak at 39.5 ppm relative to Me_4Si. The spectra were accumulated with a total time of 3.5 s between transmitter pulses and 30016 data points. The fully coupled spectra were enhanced by irradiation of the aliphatic proton region during the delay period. Spectra were processed with a resolution enhancement parameter that was manually adjusted for an optimum resolution to noise ratio.

Microscale trimethylsilylation of compounds **6** and **7** was conducted at room temperature with a large excess of a 1:2 (v/v) solution of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and redistilled acetonitrile. Gas chromatography (GC) of these derivatives was accomplished with a Varian 2740 GC equiped with a flame-ionization detector and interfaced to a Spectra-Physics 4100 computing integrator. A 1.83 m × 2 mm i.d. glass column packed with 3% OV-17 on 100/120 mesh Gas-Chrom Q was operated with a temperature program from 220 to 280 °C at 4 °C/min after being held at the initial temperature for 2 min. Typical GC operating conditions employed an injector and detector temperature of 250 °C, a 30 mL/min flow rate for both helium carrier gas and hydrogen, and a 300 mL/min flow rate for air.

Electron-impact mass spectra were obtained on a Du Pont instrument 21-492B interfaced to a VG 2035 data sytem. Samples were introduced via a Varian 2740 GC (492) operated as previously described and interfaced to the mass spectrometer by a single-stage glass jet separator. Typical operating conditions were as follows: jet separator, 210 °C; transfer line, 230 °C; ion source, 245 °C; accelerating voltage, 1.6 kV; resolution, 1000; electron energy, 75 eV; ionizing current, 250 μA; scan speed, 2 s/decade.

DL-2-Aminobicyclo[2.2.1]heptane-2-carboxylic-^{14}C acid (4.78 mCi/mmol) was obtained from New England Nuclear, Boston, MA. RPMI (Roswell Park Memorial Institute) 1630 medium, McCoy's 5A medium and Dulbecco's phosphate buffered saline were obtained from GIBCO Laboratories, Chagrin Falls, OH. Salt-free bovine serum albumin was obtained from Miles Laboratories, Naperville, IL. Fetal calf serum was purchased from Advanced Biotechnologies, Inc., Silver Spring, MD. Versilube F-50 silicone oil was obtained from the General Electric Co., Waterford, NY.

DL-7′-Nitrospiro[imidazolidine-4,2′(1′*H*)-3′,4′-dihydronaphthalene]-2,5-dione (2) and DL-5′-Nitrospiro[imidazolidine-4,2′(1′*H*)-3′,4′-dihydronaphthalene]-2,5-dione (3). β-Tetralone hydantoin (20.0 g, 0.092 mol) was added during 4 h to stirred concentrated nitric acid (200 mL, d = 1.42) at room temperature. The reaction mixture was then stirred for an additional hour, and water (300 mL) was added slowly. A mixture of two mononitro compounds separated out as a granular solid (19.6 g, 81%), mp 240 °C, which corresponded exactly to the product observed by Mauger and Ross.[15] A small sample (0.4 g) of this mixture was separated on a Waters LC-500 preparative HPLC apparatus using two silica gel columns and eluting with ethyl acetate/hexane (1:1). The 7′-nitro isomer (mp 300 °C) as well as the 5′-nitro isomer (mp 260 °C dec) were characterized by ^1H and ^{13}C NMR studies (Figures 1 and 2).

Compound **2** (slower moving isomer): ^1H NMR (Me_2SO-d_6) δ 1.95 (m, 2 H, CH_2-3′), 3.10 (m, 4 H, CH_2-1′ and CH_2-4′), 7.35 (d, 1 H, J = 8.2 Hz, H-5′), 7.92 (m, 2 H, H-6′ and H-8′), 8.35 (s, 1 H, N_3-H), 10.70 (s, 1 H, N_1-H); ^{13}C NMR (Me_2SO-d_6) δ 25.2 (C-4′), 29.6 (C-3′), 36.7 (C-1′), 60.1 (C-2′), 120.7 (C-6′), 123.7 (C-8′), 129.7 (C-5′), 134.6 (C-8′a), 143.5 (C-4′a), 145.5 (C-7′), 156.2 (C-4), 177.7 (C-2).

Compound **3** (faster moving isomer): ^1H NMR δ 1.95 (m, 2 H, CH_2-3′), 3.10 (m, 4 H, CH_2-1′ and CH_2-4′), 7.40 (m, 2 H, H-7′ and H-8′), 7.78 (dd, 1 H, J = 7.8 Hz, J' = 1.6 Hz, H-6′), 8.30 (s, 1 H, N_3-H), 10.75 (s, 1 H, N_1-H); ^{13}C NMR (Me_2SO-d_6) δ 21.9 (C-4′), 29.3 (C-3′), 37.0 (C-1′), 59.7 (C-2′), 122.2 (C-6′), 126.5 (C-7′), 129.6 (C-4′a), 134.1 (C-8′), 136.0 (C-8′a), 148.9 (C-5′), 156.2 (C-4), 177.7 (C-2).

DL-5′-[Bis(2-hydroxyethyl)amino]spiro[imidazolidine-4,2′(1′*H*)-3′,4′-dihydronaphthalene]-2,5-dione (5a) and DL-7′-[Bis(2-hydroxyethyl)amino]spiro[imidazolidine-4,2′-(1′*H*)-3′,4′-dihydronaphthalene]-2,5-dione (4a). The mixture of nitro compounds (5.0 g, 0.019 mol) was dissolved in 70 mL of DMF and hydrogenated at 30 psi with 10% Pd on charcoal for 90 min. The filtered solution was evaporated to dryness and dissolved in a minimum amount of 1 N HCl. Concentrated ammonium hydroxide was then added to the solution and the precipitated product washed with water and dried to give 3.2 g (72%) of the monoamines, mp 300 °C. A mixture of amines (5 g), ethylene oxide (15 mL), acetic acid (25 mL), and water (25 mL) was stirred at room temperature for 17 h. The solid obtained after evaporation revealed on TLC analysis (silica gel, 10% 2-propanol in ethyl acetate) two close spots, ca. R_f 0.4, corresponding to the two isomers. The solid was recrystallized three times from water, giving 0.250 g (3.6%) of pure **5a**: mp 187–190 °C; NMR (Me_2SO-d_6) δ 1.77 (m, 2 H, CH_2-3′), 2.68 (m, 4 H, CH_2-1′ and CH_2-4′), 3.00 (m, 4 H, NCH_2CH_2OH), 3.40 (m, 4 H, NCH_2CH_2OH), 4.38 (t, 2 H, J = 5.3 Hz, O*H*, D_2O exchanged), 6.77 (dd, 1 H, J = 6.3 Hz, J' = 2.0 Hz, H-6′), 7.04 (m, 2 H, H-7′ and H-8′), 8.36 (s, 1 H, N_3-H), 10.66 (s, 1 H, N_1-H). Anal. ($C_{16}H_{21}N_3O_4$) C, H, N.

The remaining aqueous solution was evaporated and triturated with methanol, and the residual solid was recrystallized twice from water to give 0.3 g (4.3%) of pure **4a**: mp 190–192 °C; NMR (Me_2SO-d_6) δ 1.79 (m, 2 H, CH_2-3′), 2.70–3.20 (m, 4 H, CH_2-1′ and CH_2-4′), 3.31 (m, 4 H, NCH_2CH_2OH), 3.45 (m, 4 H, NCH_2CH_2OH), 4.67 (br t, 2 H, O*H*, D_2O exchanged), 6.33 (d, 1 H, J = 2.0 Hz, H-8′), 6.47 (dd, 1 H, J = 8.4 Hz, J' = 2.0 Hz, H-6′), 6.86 (d, 1 H, J = 8.4 Hz, H-5′), 8.23 (s, 1 H, N_3-H), 10.63 (s, 1 H, N_1-H). Anal. ($C_{16}H_{21}N_3O_4$) C, H, N.

DL-5′-[Bis(2-chloroethyl)amino]spiro[imidazolidine-4,2′-(1′*H*)-3′,4′-dihydronaphthalene]-2,5-dione (5b). Compound **5a** (1.0 g, 0.0031 mol) was stirred overnight with freshly distilled $POCl_3$ (10 mL, 0.1 mol) at room temperature. The reaction mixture was evaporated to a syrup and then coevaporated with benzene. The residue obtained was dissolved in concentrated HCl and evaporated again. The remaining semisolid was purified by silica gel chromatography with ethyl acetate to give 0.38 g (34%) of **5b** as a white solid: mp 180–184 °C; NMR (Me_2SO-d_6) δ 1.79 (m, 2 H, CH_2-3′), 2.70–3.30 (m, 4 H, CH_2-1′ and CH_2-4′), 3.32 (m, 4 H, NCH_2CH_2Cl), 3.53 (m, 4 H, NCH_2CH_2Cl), 6.88 (dd, 1 H, J = 3.1 Hz, J' = 1.4 Hz, H-6′), 7.10 (m, 2 H, H-7′ and H-8′), 8.39 (s, 1 H, N_3-H), 10.69 (s, 1 H, N_1-H). Anal. ($C_{16}H_{19}N_3Cl_2O_2$) C, H, N, Cl. This compound was also isolated from a reaction starting with a mixture of both bis(2-hydroxyethyl)amino compounds. After workup, column chromatography over silica gel with ethyl acetate–hexane (1:1) produced a faster moving isomer that corresponded exactly to this material.

DL-7′-[Bis(2-chloroethyl)amino]spiro[imidazolidine-4,2′-(1′*H*)-3′,4′-dihydronaphthalane]-2,5-dione (4b). In a reaction similar to that described above, compound **4a** was converted to **4b**, which was isolated as a white solid: mp 212 °C; NMR (Me_2SO-d_6) δ 1.70 (m, 2 H, CH_2-3′), 2.70–3.30 (m, 4 H, CH_2-1′ and CH_2-4′), 3.35–3.60 (m, 8 H, NCH_2CH_2Cl), 6.43 (d, 1 H, J = 2.0 Hz, H-8′), 6.56 (dd, 1 H, J = 8.6 Hz, J' = 2.0 Hz, H-6′), 6.93 (d, 1 H, J = 8.6 Hz, H-5′), 8.25 (s, 1 H, N_3-H), 10.64 (s, 1 H, N_1-H). Anal. ($C_{16}H_{19}N_3Cl_2O_2$) C, H, N, Cl. This compound was also isolated from a reaction starting with a mixture of both bis(2-hydroxyethyl)amino compounds. After workup, column chro-

matography over silica gel with ethyl acetate–hexane (1:1) produced a slower moving isomer that corresponded exactly to this material.

DL-**2-Amino-7-[bis(2-chloroethyl)amino]-1,2,3,4-tetrahydro-2-naphthoic Acid Hydrochloride Salt (6).** The hydantoin mustard **4b** (0.15 g) was dissolved in concentrated HCl and heated at 140 °C for 16 h in a sealed tube. The reaction mixture was evaporated in the cold and the residue dissolved in a small volume of 1 N HCl and extracted with ethyl acetate several times. The aqueous fraction was lyophilized to give 40 mg of the 7-isomer mustard **6** which was 87% pure by GC–MS when analyzed as a persilylated derivative: mass spectrum, m/z (relative intensity) 474 ($M^{\cdot+}$, 5.7), 459 (M – CH_3, 2.2), 431 (M – CH_3 – C_2H_4, 2.9), 402 (M – M_3Si + H, 3.7), 385 (M – Me_3SiNH_2, 1.9), 357 (M – Me_3SiO_2C, 100).

DL-**2-Amino-5-[bis(2-chloroethyl)amino]-1,2,3,4-tetrahydro-2-naphthoic Acid Hydrochloride Salt (7).** Following a similar procedure, the 5-isomer mustard **7** was isolated as a lyophilized powder, which was 92% pure by GC–MS when analyzed as a persilylated derivative: mass spectrum, m/z (relative intensity) 474 ($M^{\cdot+}$, 1.0), 459 (M – CH_3, 2.3), 431 (M – CH_3 – C_2H_4, 2.4), 402 (M – Me_3Si + H, 1.7), 357 (M – Me_3SiO_2, 100).

Transport Studies. Murine L1210 leukemia cells were grown in RPMI 1630 medium containing 16% heat-inactivated fetal calf serum and passaged every 2–3 days when cell densities approached 1×10^6 cells/mL. For experimental studies, cells were harvested from growth medium and washed twice in transport medium composed of, in mM, $CaCl_2 \cdot 2H_2O$ (0.7), $MgCl \cdot 6H_2O$ (0.5), choline chloride (125), HEPES (25), and salt-free bovine serum albumin (0.1). The final pH of the transport medium was 7.4. Cells were then incubated with 1.5 or 3.0 μM 2-aminobicyclo[2.2.1]heptane-2-carboxylic-^{14}C acid (BCH) along with the indicated concentration of either L-phenylalanine mustard or DL-2-amino-7-bis[(2-chloroethyl)amino]-1,2,3,4-tetrahydro-2-naphthoic acid (**6**). The initial rate of transport of BCH was terminated at 40 s by centrifugation of 1×10^6 cells through Versilube F-50 silicone oil. The cell pellets were solubilized in 0.2 N NaOH, acidified, and counted in a Packard 460 C liquid scintillation counter.

Evaluation of Antitumor and Myelosuppressive Activity. Murine L1210 leukemia cells were grown as described above under transport studies. Cells were harvested and washed twice in fresh growth medium. Bone marrow cells were removed from femurs of male CDF_1 mice and washed twice in RPMI 1630 containing 16% heat-inactivated fetal calf serum. A cell suspension containing *both* 100 tumor cells/mL and 100 CFU-C/mL (1.0×10^5 nucleated cells/mL) was prepared and the cells coexposed for 45 min to the respective drug in RPMI 1630 containing 16% heat-inactivated fetal calf serum. The cells were then harvested, washed twice in McCoy's 5A medium supplemented with 10% fetal bovine serum, 20 units/mL penicillin, and 20 μg/mL streptomycin. Cell survival was assessed following 1 week of growth in the same medium at 37 °C in a humidified atmosphere of 5% CO_2.[19] Experimental points represent the mean of three separate platings. Pregnant mouse uterine extract was used as a source of colony-stimulating factor for the bone marrow. The presence of either cell type has no effect on the plating efficiency of the other.

Determination of Alkylating Potency and Half-Life ($t_{1/2}$). The reaction of L-phenylalanine mustard and DL-2-amino-7-bis-[(2-chloroethyl)amino]-1,2,3,4-tetrahydro-2-naphthoic acid (**6**) with γ-(4-nitrobenzyl)pyridine[20] was used to determine both alkylating potency and stability of the drugs in aqueous solution containing physiological concentrations of chloride ion (Dulbecco's phosphate buffered saline). The drugs were made up as 65 mM stock solutions in 75% ethyl alcohol containing equimolar hydrochloric acid and diluted 200-fold into Dulbecco's phosphate buffered saline to initiate the study. One-milliliter aliquots were removed at time = 0, 7.5, 15, 60, and 135 min, the pH adjusted to 4.8 with 0.1 M sodium acetate, and residual alkylating activity determined exactly as described by Truhaut et al.[21] following reaction with γ-(4-nitrobenzyl)pyridine. Experimental points represent the mean of three separate determinations.

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Registry No. 1, 6270-37-7; **2**, 106094-73-9; **3**, 106094-74-0; **4a**, 106094-75-1; **4a** (monoamine), 106094-81-9; **4b**, 106094-79-5; **5a**, 106094-76-2; **5a** (monoamine), 106094-82-0; **5b**, 106094-80-8; **6**, 106094-83-1; **6**·HCl, 106094-77-3; **7**, 106114-32-3; **7**·HCl, 106094-78-4; ethylene oxide, 75-21-8.

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2-[(3-Pyridinylmethyl)thio]pyrimidine Derivatives: New Bronchosecretolytic Agents

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2-[(3-Pyridinylmethyl)thio]pyrimidine derivatives (**1a–n**) promote the excretion of phenol red into the mouse trachea, indicating an increased tracheobronchial secretion. Furthermore, 2-[(3-pyridinylmethyl)thio]pyrimidine (**1a**) (tasuldine) produces greater excretion of phenol red into the mouse trachea after systemic administration than the known bronchosecretolytic ambroxol. Compound **1a** also reduces the viscosity of canine bronchial mucus. Compound **1a** has been selected for clinical investigations.

In some diseases of the respiratory tract, mucus secretion is disturbed and the abnormal secretion often aggrevates the illness.[1] Therefore, much effort has gone into designing forms of treatment that change the quantity or quality of tracheobronchial mucus. In general, expectorants can be devided in two groups.[2] The first, the so-called mucolytics such as acetylcysteine[1] and 2-mercaptoethanesulfonate,[1] is capable of reducing sputum viscosity after local application (e.g., inhalation) by breaking disulfide bridges that link strands in mucus glycoproteins. The second group, the so called broncho-

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