Synthesis of 3'-CTNU[1] Radiolabelled with ^{14}C in the Alkylating Moiety and ^{3}H in the Carbamoylating Moiety

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SUMMARY

[6-^{3}H]3'-[3-(2-Chloroethyl)-3-nitrosoureido]-3'-deoxythymidine([^{3}H]3'-CTNU) radiolabelled with ^{3}H in the carbamoylating moiety has been synthesized in high yield by conversion of tritiated 3'-amino-3'-deoxythymidine([^{3}H]3'-ATdR) to [6-^{3}H]3'-[3-(2-chloroethylureido)]-3'-deoxythymidine ([^{3}H]3'-UTdR), followed by nitrosation with N_2O_3 gas. The title compound radiolabelled with ^{14}C in the alkylating moiety was prepared by conversion of [2-^{14}C]ethanolamine hydrochloride to [1-^{14}C]chloroethylamine hydrochloride, followed by reaction with phosgene to yield [1-^{14}C]chloroethyl isocyanate. This was converted to 3'-[3-([1-^{14}C]2-chloroethylureido)]-3'-deoxythymidine([^{14}C]3'-UTdR), and nitrosated to afford 3'-[3-([1-^{14}C]2-chloroethyl)-3-nitrosoureido]-3'-deoxythymidine ([^{14}C]3'-CTNU). HPLC methodology was developed for the purification of intermediate and final products.

Key words: Nitrosourea nucleosides, [^{3}H]- and [^{14}C]3'-[3-(2-chloroethyl)-3-nitrosoureido[-3'-deoxythymidine, [^{3}H]- and [^{14}C] 3'-[3-(2-chloroethylureido)]-3'-deoxythymidine, HPLC, nitrosation.

INTRODUCTION

The anticancer activity both *in vitro* and *in vivo* of certain 2-haloethyl nitrosourea derivatives is well documented (1,2);

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[1]The abbreviations used are: 3'-CTNU, 3'-[3-(2-chloroethyl)-3-nitrosoureido]-3'-deoxythymidine; 3'-UTdR, 3'-[3-(2-chloroethylureido)]-3'-deoxythymidine; 3'-ATdR, 3'-amino-3'-deoxythymidine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MeCCNU, N-(2-chloroethyl)-N'-(*trans*-4-methylcyclohexyl)-N-nitrosourea; PBS, phosphate buffered saline; TLC, Thin layer chromatography.

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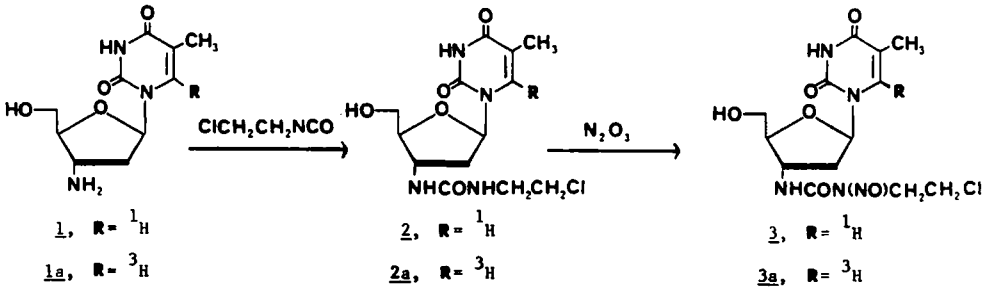
BCNU, CCNU and MeCCNU are currently in clinical use. Under physiological conditions decomposition yields alkylating and carbamoylating moieties (3), which are responsible for the alkylation of nucleic acids and proteins (4,5) and the carbamoylation of proteins (4,6). The nitrosourea nucleoside analog 3'-CTNU (3, Scheme 1) represents a particularly promising sub-class of nitrosoureas. In addition to being more effective than BCNU against L1210 cells (7), decomposition produces the potent anticancer nucleoside analog 3'-ATdR (1) as well as the alkylating (5) and carbamoylating (4) moieties (Scheme 2). The incorporation of radioactive labels in the parent drug at sites borne ultimately by the carbamoylating and alkylating moieties (3a and 3b respectively) will identify the cellular targets of these reactive species and allow quantitation of the extent of the interaction. The molecular basis of the antineoplastic activity of 3'-ATdR (1) has been investigated by Chen et al (8). The sub-cellular distribution of each of these biologically and chemically reactive species will serve to elucidate the target site of 3'-CTNU, and will aid in the understanding of the molecular basis for their action and the rational design of new nitrosoureas.

RESULTS AND DISCUSSION

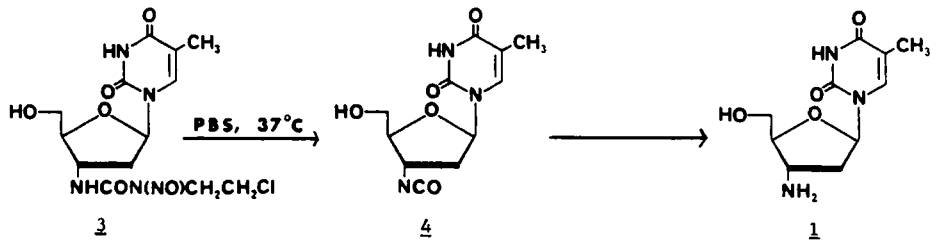
The synthesis of [^{3}H]- and [^{14}C]-radiolabelled 3'-CTNU was approached by first optimizing and adapting the synthesis of the unlabelled drug to the microscale. The procedure of Lin et al. (7) involves conversion of 3'-ATdR (1) to 3'-UTdR (2) by reaction with chloroethyl isocyanate, followed by aqueous nitrosation with $NaNO_2$ to produce 3'-CTNU (3) in a 43% yield. The extensive work-up following nitrosation was not adaptable to the <20 mg scale desired for the radiosynthesis; therefore the urea was nitrosated

Scheme 1



1, R = ^{1}H 1a, R = ^{3}H 2, R = ^{1}H 2a, R = ^{3}H 3, R = ^{1}H 3a, R = ^{3}H

Scheme 2



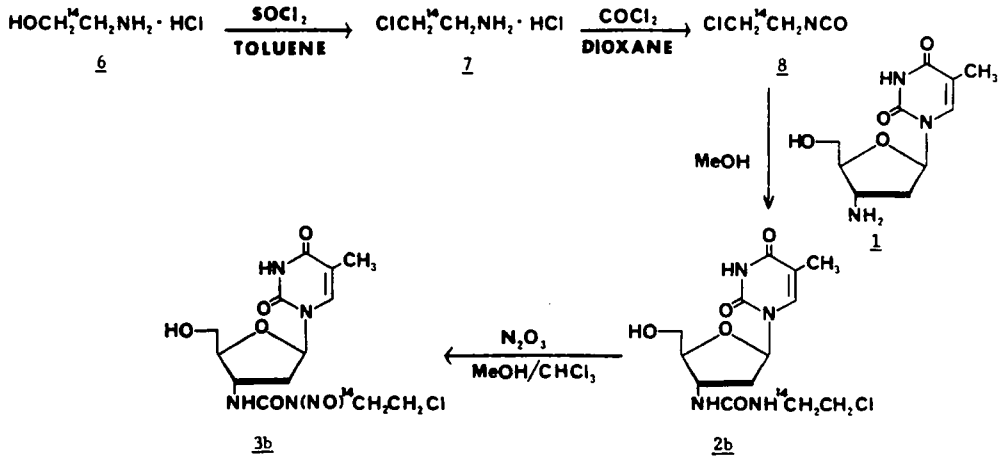
3 4 1

+

$ClCH_2CH_2NNOH$

5

Scheme 3



6 7 8 1 3b 2b

with N_2O_3 (9) in anhydrous 5% MeOH/$CHCl_3$ (Scheme 1). This sequence consistently afforded a >90% yield of 3'-CTNU.

[^{14}C]3'-CTNU (3b) was synthesized in four steps from [2-^{14}C]ethanolamine hydrochloride (Scheme 3). The conversion of (6) to (7), and subsequently to (8) was performed essentially by the procedure used by Chang and Narayan (10) to synthesize [1-^{13}C]2-chloroethyl isocyanate. The conversion of (6) to (7) is quantitative, as monitored by TLC and autoradiography. Formation of the isocyanate from (7) is the lowest yield step in the reaction sequence, proceeding in approximately 45% yield. Physical loss of the isocyanate while flushing with nitrogen to remove excess phosgene is the probable cause. Unfortunately, if the phosgene is not completely removed before reaction of the isocyanate with the nucleoside, the yield of the ureido analog is reduced by reaction of the nucleoside with phosgene. The subsequent steps, formation of [^{14}C]3'-UTdR (2b) and [^{14}C]3'-CTNU (3b), proceed in high yield (>90%) as in the case of the tritiated and unlabelled analogs (Scheme 1). The final two steps produce only a few trace by-products which are readily removed by HPLC purification.

Three HPLC systems were developed for the purification and identification of (1), (2), and (3) and to maximize recovery of the labelled compounds. Both normal and reversed phase systems were used for identification of the radiolabelled products. As shown in Table 1, all three compounds are well-resolved in the reversed phase systems. The normal phase system was developed for purification of the urea and nitrosourea, since nitrosoureas are considerably more stable in organic solvents (11). In order to assay the chemical and radiochemical purity of the products, a purified sample was injected with 10% of the effluent diverted to

Table I. Retention times of 3'-Analogs of Thymidine

compd	Retention Time in Minutes		
	A[a]	B[b]	C[c]
3'-ATdR	13.5	3.6	Retained
3'-UTdR	28.0	18.0	25.8
3'-CTNU	47.5	29.0	15.0

[a]System A: Magnum ODS-2 column, flowrate 4.5 ml/min, linear gradient (0-25%, 1%/min), 1°-0.01M KH_2PO_4 (pH 5.5), 2°-80% MeOH/20% 0.01M KH_2PO_4. [b]System B: Analytical ODS-2 column, flowrate 1.5 ml/min, linear gradient (0-100%, 3.33%/min), 1°-10% MeOH/90% 0.01M KH_2PO_4 (pH 3.0), 2°-40% MeOH/60% 0.01M KH_2PO_4. [c]System C: Magnum Partisil 10 column, flowrate 3.0 ml/min, convex gradient (0-60%, 2%/min), 1°-1% MeOH/$CHCl_3$, 2°-9% MeOH/$CHCl_3$.

an LKB fraction collector. The fractions were collected in scintillation vials and the radioactivity measured with a Beckman LS 7500 liquid scintillation counter. A plot of radioactivity vs. fraction number indicated a single radioactive peak superimposable on the chromatographic UV peak (Figure 1).

Two of the problems commonly encountered in small-scale radiosyntheses with multistep reaction schemes are contamination of the laboratory environment and the relatively large losses in yield due to transfers, evaporations, and other manipulations of the sample. To eliminate these problems, a reaction vial was constructed by fusion of a 14/20 ground glass joint to a 10mm x 80mm pyrex test tube. The tube dimensions were compatible with

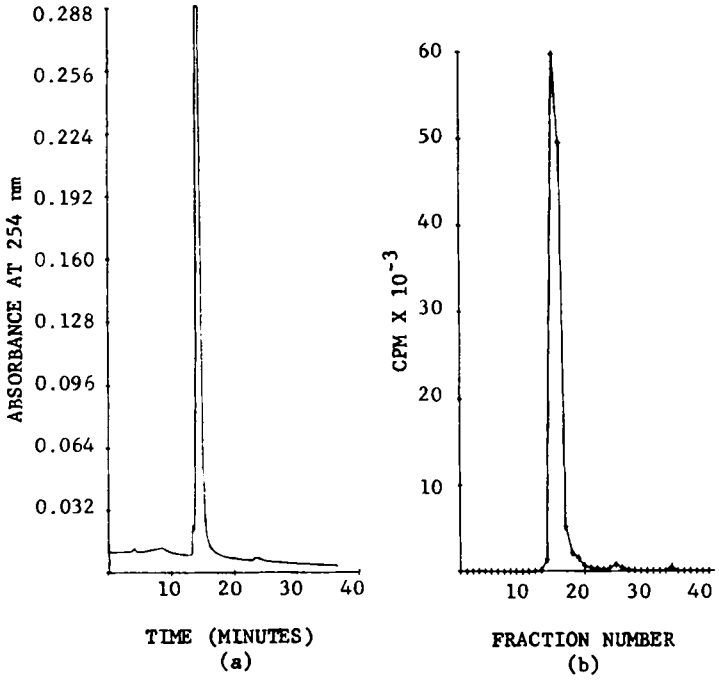


Figure 1. a) UV Chromatogram of [^{3}H]3'CTNU on system C and b) Corresponding radiochromatogram

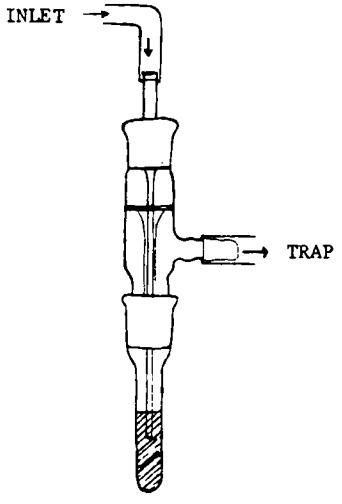


Figure 2. Microscale Reaction Apparatus

the rotor of a Savant Speed Vac Concentrator, allowing all solvent and gas removal steps to be performed in this apparatus. Introduction of phosgene or nitrogen trioxide gas was effected safely and efficiently by attaching a vacuum adapter to the vial and introducing the gas via a disposable glass pipette which pierced the rubber septum cap. A trap was on-line between the gas source and the reaction vessel, and an aqueous KOH trap was connected to the apparatus outlet (Figure 2). The entire reaction sequence can be performed in a single apparatus, eliminating most physical losses of material and protecting the laboratory environment.

EXPERIMENTAL

Melting points were determined with a Thomas-Hoover Unimelt apparatus and are not corrected. The UV spectra were recorded on a Beckman Model-25 spectrophotometer. ^1H NMR spectra were recorded at 500 MHz on a Bruker WM-500 spectrometer in Me_2SO-d_6 solution (δ relative to Me_4Si, 0.00 ppm). Mass spectra were obtained on a VG micromass 16F magnetic sector instrument in the CI mode. Isobutane was used as the reagent gas at a source pressure of approximately 0.5 Torr. Source temperature ranged from 250-270°C. Samples dissolved in MeOH were deposited on a direct exposure probe tip machined from a 1/4-inch Vespel rod (12). Gradient elution HPLC analyses were performed on a modified Dupont 830 liquid chromatography in which the Haskel pump was replaced with an Altex 100 pump. Radioactivity was measured with a Beckman LS7500 liquid scintillation counter. Thin layer chromatography was performed on Polygram Sil G/UV_{254} plates (Brinkmann Instruments, Inc., Westbury, NY) and autoradiograms produced on Kodak X-Omat AR film (Rochester, NY).

HPLC grade chloroform was obtained from Mallinckrodt (St. Louis, MO) and HPLC grade methanol was purchased from J.T. Baker

(Phillipsbury, NJ). Water was double-distilled prior to use. Mobile phases were filtered through Rainin nylon-66 filters (organic mobile phases) or Millipore HA filters (aqueous mobile phases). 3'-Amino-3'-deoxythymidine was generously provided by Dr. T-S. Lin and tritiated by New England Nuclear (Boston, MA). [2-^{14}C]Ethan-1-ol-2-amine hydrochloride was obtained from Amersham Corporation (Arlington Heights, IL). All other reagents were obtained from Aldrich Chemical Co., (Milwaukee, WI) and were gold label grade.

3'-[3-(2-Chloroethylureido)]-3'-deoxythymidine (2):

2-Chloroethyl isocyanate (6.33 mg, 0.06 mmol) was added to a solution of 3'-amino-3'-deoxythymidine (1, 12.06 mg, 0.05 mmol) in 2.0 ml of anhydrous MeOH at 0°C. The reaction mixture was stirred at room temperature for 3 h followed by HPLC purification (multiple injections) using system C (t_R = 25.8 min) to yield 16.43 mg (0.0475 mmol, 95%) of an analytically pure sample: UV λ max (EtOH) 266 nm (ϵ 7947); UV λ min (EtOH) 233 nm (ϵ 1719); mass spectrum, m/z (rel intensity) 311 (30.5), 222 (11.5), 185 (87), 127 (100); NMR 11.265 (s, 1H, NH-3), 7.738 (S, 1H, H-6), 6.557 (d, 1H, J = 7.35, NH-3'), 6.121 (t, 1H, J = 6.55, H-1'), 6.107 (t, 1H, J = 5.866, $CH_2\underline{NH}$), 5.036 (t, 1H, J = 5.29, OH-5'), 4.151 (m, 1H, H-3'), 3.695 (m, 1H, H-4'), 3.566 (m, 4H, CH_2N and H-5'), 3.291 (m, 2H, CH_2Cl), 2.118 (m, 2H, H-2'), 1.769 (s, 3H, CH_3-5).

3'-[3-(2-Chloroethyl)-3-nitrosoureido]-3'-deoxythymidine (3):

A solution of 2 (17.3 mg, 0.05 mmol) dissolved in 2.0 ml of anhydrous 5% MeOH/$CHCl_3$ was placed in a specially constructed 3 ml reaction vial. The vial was surmounted by a vacuum adapter, and the solution cooled to 0°C in an ice bath. Nitrogen trioxide gas was introduced through a rubber septum cap pierced by a glass

pipette which intruded below the surface of the solution. The outlet was connected to a KOH trap. The gas was allowed to bubble through the solution for 1 h, at which time the residual gas and solvent were removed by vacuum centrifugation in a Savant Speed Vac Concentrator. The resulting oil was purified by HPLC using system C (t_R = 15.0 min) to yield 17.62 mg (0.047 mmol, 94%) of an analytically pure sample. The retention times in system B (t_r = 29.0 min) and system C (t_R = 15.0 min) were identical to those of an authentic sample of 3'-CTNU; UV λ max (ETOH) 266 nm (ε 12400); UV λ min (ETOH) 235 nm [lit[7]. UV λ max (ETOH) 266 nm (ε 12400); UV λ min (ETOH) 235 nm]; NMR spectra were identical to those of an independently synthesized sample of 3'-CTNU.

[6-^{3}H]3'-Amino-3'-deoxythymidine (1a):

3'-Amino-3'-deoxythymidine (1) was tritiated at New England Nuclear by catalytic exchange with tritium gas. The resulting crude product was purified by adsorption onto a 18cm x 2cm column of Dowex AG 50W-X8 (H^+) resin followed by elution with a gradient of H_2O to 1M NH_4OH. Following removal of the NH_4OH by vacuum evaporation, the partially purified product was further chromatographed using system A (t_R = 13.5 min). This afforded a chemically and radiochemically pure product with retention times identical to those of an authentic sample of unlabelled 3'-ATdR in system A (t_R = 13.5 min) and system B (t_R = 3.6 min). Specific activity: 1467 mCi/mmole; UV λ max (0.1N HCl) 266 nm (ε 9190); UV λ min (0.1N HCl) 234 nm (ε 2250) [lit[7]. UV λ max (0.1N HCl) 266 nm (ε 9190); UV λ min (0.1N HCl) 234 nm (ε 2250)]; NMR spectra were identical to those of authentic sample of unlabelled 3'-ATdR.

[6-^{3}H]3'-[3-(2-Chloroethylureido)]-3'-deoxythymidine (2a):

A solution of 1a (10.0 mg, 0.041 mmole, specific activity adjusted to 53.8 mCi/mmole by addition of unlabelled 3'-ATdR) in 1.5 ml anhydrous MeOH was placed into a 3 ml reaction vial. The vial was cooled to 0°C by means of an ice bath. 2-Chloroethyl isocyanate (6.1 mg, 0.057 mmol) was added to the stirred solution, with an additional 1.5 mg added at 1h and 2h. After 3h, purification (multiple injections) by HPLC system C afforded 13.2 mg (0.038 mmole, 93%) of chemically and radiochemically pure 2a, with retention times identical to those of an authentic sample of unlabelled 3'-UTdR in system B (t_R = 18.0 min) and system C (t_R = 25.8 min). Specific activity: 52.5 mCi/mmol; UV and NMR spectra were identical to those of 2.

[6-^{3}H]3'-[3-(2-Chloroethyl)-3-nitrosoureido]-3'-deoxythymidine (3a):

A solution of 2a (10.0 mg. 0.029 mmol) dissolved in 2.0 ml of anhydrous 5% MeOH/$CHCl_3$ was placed in a 3 ml reaction vessel (as described for 3). Nitrogen trioxide gas was bubbled through the solution for 1h, at which time the solvent and residual gas were removed by vacuum centrifugation. Purification using HPLC system C afforded 9.86 mg (0.026 mmol, 91%) of chemically and radiochemically pure [^{3}H]3'-CTNU. Retention times were identical to those of 3 in system B (t_R = 29.0 min) and system C (t_R = 15.0 min). Specific activity: 54.6 mCi/mmol; UV and NMR spectra were identical to those of 3.

[1-^{14}C]2-Chloroethylamine Hydrochloride (7):

A solution of [2-^{14}C]ethan-1-ol-2-amine hydrochloride (6, 15.0 mg, 0.154 mmol, specific activity: 124.4 uCi/mmol) in 1.0 ml dry toluene was placed in a 3 ml reaction vial. Distilled

thionyl chloride (18 ul, 0.234 mmol) was added, the vial capped with a $CaSO_4$ drying tube, and the reaction mixture heated to 65°C. At 2h an additional 12 ul of thionyl chloride was added, and another 6 ul at 4h. After 6h, 100 ul of dry MeOH was added and the solvents removed by vacuum centrifugation at 45°C. This afforded 17.6 mg (0.152 mmol, 99%) of a white powder, m.p. 148-150°C (lit[6]. m.p. 148.5-150°C).

The autoradiogram indicated a homogenous product with R_f 0.38 [lit[9]. R_f 0.37, n-BuOH/AcOH/H_2O, 3:1:1), identical with an authentic sample. Specific activity: 118.4 uCi/mmol.

[1-^{14}C]2-Chloroethyl isocyanate (8):

A slurry of [1-^{14}C]2-chloroethylamine hydrochloride (7, 10.0 mg, 0.086 mmol) in 1.5 ml of dry dioxane was placed in a 3 ml reaction vial. The vial was surmounted by a vacuum adapter, and the solution heated to 66-68°C. Phosgene was introduced through a rubber septum cap pierced by a glass pipette which intruded below the surface of the solution. The outlet was connected to a KOH trap. When a clear solution resulted, the phosgene line was removed from the inlet and replaced by a nitrogen line. Heating was continued for 30 min with a slow flow of nitrogen. The solution of [1-^{14}C]2-chloroethyl isocyanate was used for the next step without further purification.

3'-[3-([1-^{14}C]2-Chloroethylureido)]-3'-deoxythymidine (2b):

A solution of 3'-amino-3'-deoxythymidine (1, 20.7 mg, 0.086 mmol) in 1.5 ml of anhydrous MeOH was placed in a 3 ml reaction vial and cooled to 0°C. The dioxane solution of 8 was added all at once. After stirring for 3h at room temperature, purification (multiple injections) by system C afforded 10.33 mg (0.029 mmol, 34.7%, based on 6) of chemically and radiochemically pure 2b, with retention times identical to those of an authentic sample of

unlabelled 3'-UTdR in system B (t_R = 18.0 min) and system C (t_R = 25.8 min). Specific activity: 118 uCi/mmol; UV spectra identical to those of 2.

3'-[3-([1-^{14}C]2-Chloroethyl)-3-nitrosoureidol-3'-deoxythymidine (3b):

A solution of 2b (10.0 mg, 0.029 mmol) dissolved in 2.0 ml of anhydrous 5% MeOH/$CHCl_3$ was placed in a 3 ml reaction vial (as described for 3). Nitrogen trioxide gas was bubbled through the solution for 1h followed by removal of the solvent and residual gas by vacuum centrifugation. Purification (multiple injections) by HPLC system C afforded 9.75 mg (0.026 mmol, 90%) of chemically and radiochemically pure [^{14}C]3'-CTNU. Retention times were identical to those of 3 in system B (t_R = 29.0 min) and system C (t_R = 15.0 min). Specific activity: 118 uCi/mmol; UV spectra were identical to those of 3.

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REFERENCES

1) Carter, S.K., Schabel, F.M., Jr., Broder, L.E., and Johnston, T.P. - 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) and Other Nitrosoureas in Cancer Treatment. A Review. Advan. Cancer Res., 16:273-332 (1972).

2) Prusoff, W.H., Lin, T.-S., Chen, M.S., Fischer, P.H., Mancini, W.R., Brubaker, W.F., Jr., Lee, J.J., Woods, K. - Development of Nitrosourea Nucleosides as Anticancer Agents in "Development of Target-Oriented Anticancer Drugs, ed. Y.-C. Cheng et al. Raven Press, New York 57-73, (1983).

3) Montgomery, J.A., James, R., McCaleb, G.S., and Johston, T.P. - The Modes of Decomposition of 1,3-Bis(2-chloroethyl)-1-nitrosourea and Related Compounds. J. Med. Chem., 10:668-674 (1967).

4) Cheng, C. - J., Fujimura, S., Grunberger, D., and Weinstein, I.B.-Interaction of 1-(2-chloroethyl)-3-cyclohexyl-1-Nitrosourea (NSC 79037) with Nucleic Acids and Proteins in Vivo and in Vitro. Cancer Res., 32:22-27 (1972).

5) Erickson, L.C., Bradley, M.O., Ducore, J.M., Ewig, R.A.G., and Kohn, K.W. - DNA Crosslinking and Cytotoxicity in Normal and Transformed Human Cells Treated with Antitumor Nitrosoureas. Proc. Natl. Acad. Sci., USA Vol. 77, No. 1, 467-471 (1980).

6) Schmall, B., Cheng, C.-J., Fujimura, S., Grunberger, D., and Weinstein, I.B. - Modification of Proteins by 1-(2-Chloroethyl)-3-cyclohexyl-1-Nitrosourea. Proc. Am. Assoc. Cancer Res., 13:65 (1972).

7) Lin, T.-S., Fischer, P.H., Shiau, G.T., and Prusoff, W.H. - Antineoplastic Agents. 1. Synthesis and Antineoplastic Activities of Chloroethyl- and Methylnitrosourea Analogs of Thymidine. J. Med. Chem., 21:130-133 (1978).

8) Chen, M.S., Woods, K.L. and Prusoff, W.H. - Molecular Basis of the Antineoplastic Activity of 3'-Amino-3'-deoxythymidine. Molec. Pharmac., 25: 441-445 (1984).

9) Yanko, W.H., and Sharp, D.E. - U.S. Pat., 4,028,410 (1977).

10) Chang, C.-j. and Narayan, R. - J.of Label. Compound. and Radiopharmac., Vol. XIX, No. 1, 129-137 (1982).

11) Loo, T.L., Dion, R.L., Dixon, R.L., and Rall, D.P. - J. of Pharm. Sci., Vol. 55, No.5, 492-497 (1966).

12) Cotter, R.J. - Anal. Chem., 51:317-318 (1979).