not be separated from *ara*-C in system A but was readily distinguishable in system B, which produced R_f values of 0.72 (*ara*-U) and 0.83 (*ara*-C). Unchanged starting material was observed in the incubation of **1b** even after 24 h, along with an approximately equal amount of a second compound which had the same R_f as *ara*-U. However, there was never more than a trace of *ara*-CMP (control experiments indicated that the TLC assay would be sensitive down to ~5% conversion of **1b** to *ara*-CMP). When *ara*-CMP itself was used in place of **1b**, the product comigrating with authentic *ara*-U was again observed, but no unchanged *ara*-CMP could be seen. It thus appeared that formation of *ara*-CMP from **1b** proceeds much more slowly than subsequent cleavage of the *ara*-CMP to *ara*-C and ultimately *ara*-U. As a result, the amount of free *ara*-CMP present at any one time when **1b** is exposed to the enzymes in mouse serum must be extremely small.

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Activated *N*-Nitrosocarbamates for Regioselective Synthesis of *N*-Nitrosoureas

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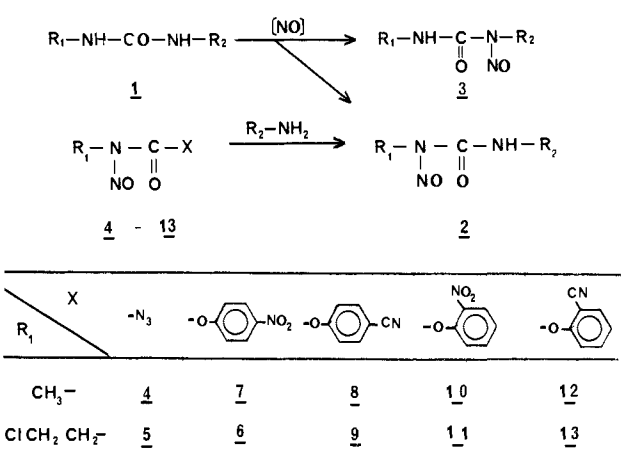
A practical and convenient method for synthesizing antitumor compounds, *N*-alkyl-*N*-nitrosoureas, regioselectively nitrosated on the nitrogen atom bearing the alkyl group is proposed. *N*-Alkyl-*N*-nitrosocarbamates are interesting intermediates in these syntheses and yield, by reaction with amino compounds, the regioselectively nitrosated *N*-alkyl-*N*-nitrosoureas. As an interesting example, *N*,*N*′-bis[(2-chloroethyl)nitrosocarbamoyl]cystamine, a new attractive oncostatic derivative, has been prepared. The cytotoxic activity of these various compounds were tested on L1210 leukemia.

Among significant compounds, nitrosoureas are an extremely active class of antitumor agents that are effective against solid tumors, as well as leukemias. In particular, 2-haloethyl derivatives and some of their metabolites show great promise as effective antitumor agents.[1-3] For the treatment of a number of experimental and clinical tumors, several *N*-(2-chloroethyl)-*N*-nitrosoureas have successfully been applied as chemotherapeutic agents.[2] Not only do these drugs show the ability to inhibit the growth and spread of many forms of solid tumors in men and animals,[2,4,5] but some of them, such as *N*,*N*′-bis(2-chloroethyl)-*N*′-nitrosourea (BCNU) and *N*-(2-chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea (CCNU), also have been found to rapidly enter the cerebrospinal fluid and control meningeal tumor implants.[6] All of these compounds are undergoing intense clinical trials, and some of them have recently been made commercially available.

N-Nitrosoureas are usually and readily obtained by the conventional route of preparing the urea structure first then subsequently nitrosating it with a variety of nitrosating agents, e.g., sodium nitrite in acidic medium, nitrous anhydride, nitrosyl chloride, dinitrogen tetroxide, or nitrosium tetrafluoroborate. However, it is known that when nitrosoureas are synthesized by one of the above-mentioned procedures, difficulties in achieving selective nitrosation of the urea **1** at the required position are encountered and both isomers **2** and **3** are sometimes produced.[7] Selective nitrosation at the nitrogen bearing the methyl or the 2-chloroethyl group is critical in syntheses of unsymmetrical N,N′-disubstituted 2-chloroethyl-*N*-nitrosoureas.[7] Nitrosation by those conventional methods have been shown to favor formation of *N*-(2-chloroethyl)-*N*-nitroso compound **2** in those cases where the geometry of the substituent at position N′ provides steric control and directs the nitroso group into the required position. Selective nitrosation fails, however, when there is no such steric control. Moreover, *N*-nitrosoureas of type **3** have been shown in many cases to present no antitumor activity.[8] Furthemore, separation of the unwanted byproduct **3** from the desired compound **2** requires complex

Scheme I



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procedures, resulting in a lowering of the yield of **2**.

Alternative processes, to circumvent these disadvantages, have been proposed recently. Thus, a synthetic pathway was proposed for the regioselective synthesis of *N*-alkyl-*N*-nitrosoureas of type **2** by Hardegger et al.[9] and by Meier et al.[10] This procedure involves the use of *N*-nitroso-*N*-alkylcarbamoyl azides **4** and **5** as intermediates, which in a one-step reaction with amines yield the corresponding *N*-alkyl-*N*-nitrosoureas of type **2** (Scheme I) with the nitroso group attached at the required position. This approach offers a useful alternative and has been used for the unambiguous regioselective synthesis of steroidal *N*-nitrosoureas.[14] However, the instability of **4** and **5** and their possible explosive properties[9,10] were a serious limitation in their further applications. On the other hand, this synthetic pathway was very attractive. Nakao et al.[12] synthesized and used *p*-nitrophenyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate **6** for the preparation of pure isomeric *N*-alkyl-*N*-nitrosoureas of type **2**. Very recently, K. Goro proposed and used *p*- and *o*-nitrophenyl *N*-alkyl-*N*-nitrosocarbamates, as well as *p*- and *o*-cyanophenyl *N*-alkyl-*N*-nitrosocarbamates **6–13**, as intermediates for the regioselective synthesis of compounds of type **2**.[13,14]

Results and Discussion

We have been engaged in similar endeavors at the same time. We proposed and obtained quite easily *p*-nitrophenyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**6**) and *p*-nitrophenyl *N*-methyl-*N*-nitrosocarbamate (**7**), and we proved their usefulness for the synthesis of some compounds of type **2** [streptozotocin[16] (**14**), chlorozotocin[17] (**15**), and CCNU[17] (**16**)].

The desired *N*-alkyl-*N*-nitrosoureas of type **2** were produced selectively, quite readily, and in high yield as compared with previous syntheses. Compounds **6** and **7** showed a pronounced reactivity toward amines and displayed fair stability upon storing. They proved to be interesting derivatives for the regioselective synthesis of *N*-alkyl-*N*-nitrosoureas of type **2**. However, in regards to the synthesis of compounds of type **2** from "active" *N*-alkyl-*N*-nitrosocarbamates, some problems related to their purification have been reported.[13] It was found that when *N*-nitrosocarbamates **6–9** with a *p*-nitro or a *p*-cyano group substituted on the phenyl nucleus were used, the desired nitrosourea **2** could be formed in the reaction; column chromatography was required for the separation of the *p*-nitrophenol or *p*-cyanophenol, which was formed as a byproduct.[13] Such chromatography lowered the overall yield of compounds of type **2**. It is believed that the above-mentioned difficulty of the separation of *p*-nitrophenol or *p*-cyanophenol from compounds of type **2** is due to a strong affinity between such substituted phenols and

Scheme II

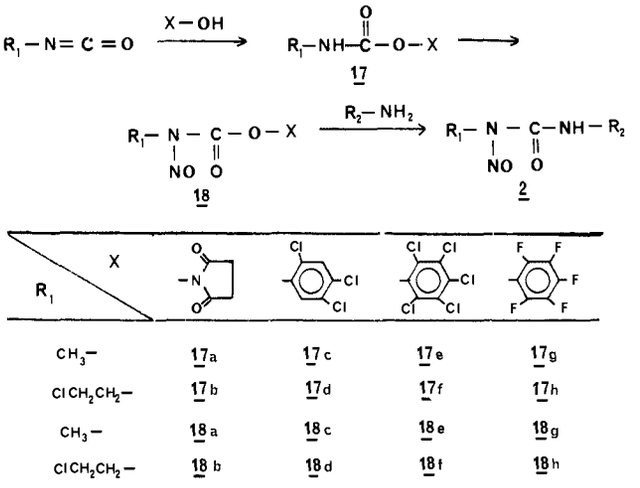
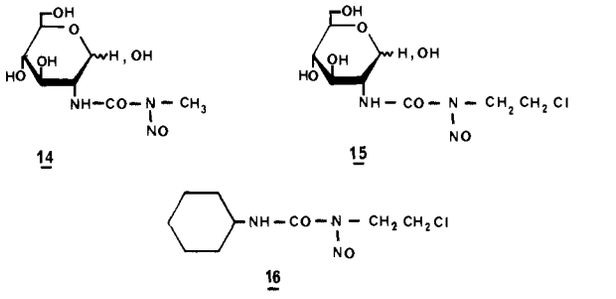


Chart I



compounds of type **2**, such affinity being a characteristic feature of **2**.[13]

In recent years, some other active esters of acyl-substituted amino acids (such as 2,4,5-trichlorophenyl,[18] pentachlorophenyl,[19] pentafluorophenyl,[20] and *N*-hydroxysuccinimide[21]) have been of great value in the synthesis of peptide derivatives. Consequently, we have undertaken an extensive investigation of the corresponding "active" *N*-alkyl-*N*-nitrosocarbamates **18**: *N*-(2-chloroethyl)-*N*-nitrosocarbamic acid *N'*-hydroxysuccinimide ester (**18b**), *N*-methyl-*N*-nitrosocarbamic acid *N'*-hydroxysuccinimide ester (**18a**), 2,4,5-trichlorophenyl *N*-methyl-*N*-nitrosocarbamate (**18c**), 2,4,5-trichlorophenyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**18d**), pentachlorophenyl *N*-methyl-*N*-nitrosocarbamate (**18e**), pentachlorophenyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**18f**), pentafluorophenyl *N*-methyl-*N*-nitrosocarbamate (**18g**), and pentafluorophenyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**18h**). These derivatives are interesting because of their high reactivity toward nucleophiles, particularly amines, and, in addition, they are usually readily crystallized. Moreover, *N*-hydroxysuccinimide derivatives **18a** and **18b** readily give removable byproducts, allowing an easy purification of compounds of type **2**. In fact, the *N*-hydroxysuccinimide

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byproduct of the reaction of **18a** or **18b** with an amine is water soluble. Furthermore, it is known that pentafluorophenyl esters are very reactive compounds; for example, derivatives such as **18g** and **18h** are capable of reacting with such weak nucleophiles as aniline. Their syntheses were performed according to Scheme II.

Methyl or 2-chloroethyl isocyanate was allowed to react with the appropriate phenol (2,4,5-trichlorophenol, pentachlorophenol, or pentafluorophenol) or with *N*-hydroxysuccinimide to produce active *N*-alkylcarbamates of type **17**. Convenient solvents in these processes are, for example, pyridine or ethyl acetate containing a tertiary amine. *N*-Alkylcarbamates **17g** and **17h** were not isolated as pure compounds and were used as crude material for the following nitrosation. Nitrosation of compounds of type **17** were performed using nitrosyl chloride in pyridine. Active *N*-alkyl-*N*-nitrosocarbamates **18**, were isolated as crystalline, stable compounds in very good yields. These derivatives meet the important requirements of crystallinity and reactivity. As examples, streptozotocin, chlorozotocin, and CCNU (Chart I) were conveniently prepared by the reaction of the corresponding *N*-alkyl-*N*-nitrosocarbamate of type **18** with glucosamine or cyclohexylamine in organic solvents (dichloromethane or dimethylformamide) at 0 °C. Usually, a 40-min reaction time was used, although a shorter period may suffice.

Furthermore, as we described earlier, CNCC (**20**; Scheme III) is a mixture of isomeric derivatives obtained by nitrosation, using conventional methods, of the corresponding urea[22] and presents very interesting oncostatic properties (see Results and Conclusions).

It was, therefore, interesting to reach regioselectively the expected active compound, *N*,*N*′-bis[(2-chloroethyl)-nitrosocarbamoyl]cystamine (**19**, ICIG 1725), nitrosated at the nitrogen atoms bearing the 2-chloroethyl group and to determinate the cytotoxic capacity of this isomer as compared to the isomeric mixture **20** (ICIG 1325). The synthesis of **19** was performed according to Scheme III. Cystamine was allowed to react with one of the active *N*-nitrosocarbamates of type **18** (**b**, **d**, **f**, or **h**) in dimethylformamide as solvent. Compound **19** was secured in an excellent yield and was pure as confirmed by ^{1}H NMR spectroscopy. On the other hand, this regioselective synthesis of compound **19** allowed us, with the help of HPLC, to determine the isomeric composition of **20** (CNCC, ICIG 1325).

Results and Conclusions

The results from these studies provide a sound methodology for the synthesis of biologically important *N*-alkyl-*N*-nitrosoureas. Compounds **18a–h** proved to be a great value for the synthesis of chlorozotocin, streptozotocin, CCNU, isomeric pure CNCC **19**, and their applications in the synthesis of other isomeric, pure *N*-alkyl-*N*-nitrosoureas are promising, particularly for compounds which could not be nitrosated by conventional methods. Further work in this direction is projected.

Examination of the "in vivo" data presented in Table I demonstrated that the tested active *N*-nitrosocarbamates did not show significant oncostatic activities. For compounds **6** and **18a**, values of *I* (% T/C) were found not significant even at doses as high as half of the LD_{50} (LD_{50}, respectively, of 17 and 115 mg/kg). For compound **18b**, at 18 mg/kg little activity has been noted (*I* = 137), whereas **19** and **20** showed very high activity against tumor growth. At 10 mg/kg, **19** afforded a high percent T/C value (*I* = ∞), the same value being obtained with **20** at 30 mg/kg. Moreover, isomer **19** was more toxic (LD_{50} = 28 mg/kg) than CNCC (**20**; LD_{50} = 75 mg/kg). However, as expected, isomer **19** showed a maximum effective dose (MED) range not as large as the corresponding range observed with CNCC (**20**). Results collected with **19** and **20** on L1210 leukemia proved the significance of these compounds on antitumor activity. There is much interest to continue studies on these two derivatives, "in vivo", on other tumor systems. Works in these areas are in progress.

Experimental Section

All capillary melting points were determined on a Buchi apparatus and are reported uncorrected. Thin-layer chromatography (TLC) experiments were carried out on Merck silica gel GF_{254} plates. Column chromatography was conduced with Merck silica gel, 60–230 mesh, ASTM. Elemental analyses were performed by "Le Service Central de Microanalyse du CNRS" de Montpellier. IR spectra were determined on a Beckmann Acculab 4 spectrophotometer in KBr pellets, and ^{1}H NMR spectra were determined for 10% solutions in $CDCl_3$ on a Varian A60 spectrophotometer using Me_4Si as internal standard (chemical shifts are reported in δ units).

General Procedure for the Synthesis of Active *N*-Alkylcarbamates 17. All compounds of formula **17** were prepared according to the procedure given below, either with methyl isocyanate or with 2-chloroethyl isocyanate and the corresponding phenol or *N*-hydroxysuccinimide. As an example, the synthesis of *N*-(2-chloroethyl)carbamic acid *N*′-hydroxysuccinimide ester (**17b**) is described.

***N*-(2-Chloroethyl)carbamic Acid *N*′-Hydroxysuccinimide Ester (17b).** To a cooled (0 °C) solution of pyridine (100 mL) or ethyl acetate containing triethylamine (or diisopropylethylamine) (0.15 mol) and *N*-hydroxysuccinimide (0.1 mol, 11.5 g) was added dropwise 2-chloroethyl isocyanate (0.12 mol, 12.7 g) during a period of 30 min under magnetic stirring. Stirring was continued for 24 h at room temperature. The solution was concentrated in vacuo, and the crystalline residue was recrystallized from a mixture of ethyl acetate and ether: yield 18.1 g (82%); mp 106–109 °C; IR 3300 (NH) cm^{-1}; ^{1}H NMR δ 8.55 (1 H, t, NH), 3.53 (4 H, m, $ClCH_2CH_2$), 2.75 (4 H, s, succinimide). Anal. ($C_7H_9ClN_2O_4$) C, H, N; M_r 220.5

***N*-Methylcarbamic acid *N*′-hydroxysuccinimide ester (17a):** yield 80%; mp 148–152 °C; IR 3340 (NH) cm^{-1}; ^{1}H NMR δ 8.10 (1 H, d, NH), 2.74 (4 H, s, succinimide). Anal. ($C_6H_8N_2O_4$) C, H, N; M_r 172.

2,4,5-Trichlorophenyl *N*-methylcarbamate (17c): yield 85%; mp 157–158 °C; IR 3340 (NH) cm^{-1}; ^{1}H NMR δ 7.96 (1 H, d, NH), 7.90 (2 H, s, aromatic), 2.70 (3 H, d, CH_3). Anal. ($C_8H_6Cl_3NO_2$) C, H, N; M_r 254.5.

2,4,5-Trichlorophenyl *N*-(2-chloroethyl)carbamate (17d): yield 81%; mp 105–108 °C; IR 3340 (NH) cm^{-1}; ^{1}H NMR δ 8.36 (1 H, t, NH), 7.86 and 7.68 (2 H, s, aromatics), 3.70 (2 H, t, $ClCH_2$), 3.48 (2 H, m, CH_2NH). Anal. ($C_9H_7Cl_4NO_2$) C, H, N; M_r 303.

Pentachlorophenyl *N*-methylcarbamate (17e): yield 85%; mp 185–186 °C; IR 3240 (NH) cm^{-1}. Anal. ($C_8H_4Cl_5NO_2$) C, H, N; M_r 323.5.

Pentachlorophenyl *N*-(2-chloroethyl)carbamate (17f): yield 82%; mp 170–174 °C; IR 3300 (NH) cm^{-1}; ^{1}H NMR δ 8.66 (1 H, t, NH), 3.72 (2 H, t, $ClCH_2$), 3.44 (2 H, m, CH_2NH). Anal. ($C_9H_5Cl_6NO_2$) C, H, N; M_r 372.

General Procedure for the Synthesis of Active *N*-Alkyl-*N*-nitrosocarbamates 18. All compounds of formula **18** were prepared according to the procedure given below for the synthesis of **18b**.

***N*-(2-Chloroethyl)-*N*-nitrosocarbamic Acid *N*′-Hydroxysuccinimide Ester (18b).** To a cooled solution (−20 °C) of pyridine (3 mL) containing **17b** (4.4 g, 0.02 mol) was added nitrosyl chloride (3 mL). The reaction was monitored by thin-layer chromatography. After the mixture stirred for an additional 30 min at −20 °C, no more starting material could be detected. The reaction mixture was poured into ice–water (200 mL), and the resulting precipitate was filtered off, washed with water, and dried

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Scheme III

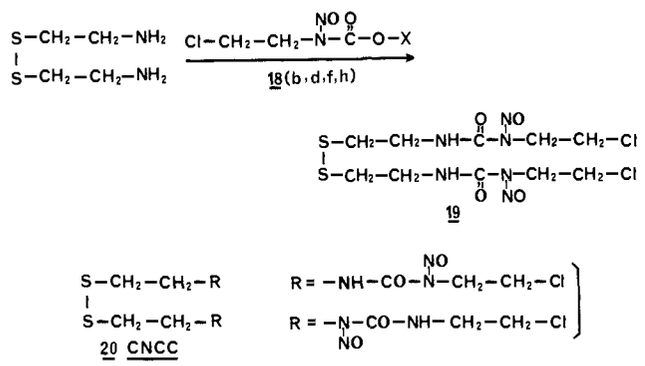

Table I. Activity on L1210 Leukemia in Mice

compd	dose, mg/kg ip	I^a	acute LD_{50}, mg/kg ip
6 (ICIG 1733)	8	NS	17
	3.2	NS	
18a (ICIG 1732)	50	NS	115
	20	NS	
	8	NS	
18b (ICIG 1734)	20	toxic	36
	8	137	
	3.2	125	
19 (ICIG 1725)	30	toxic	28
	20	∞	
	10	∞	
	5	212	
	2	111	
CNCC (ICIG 1325)	90	toxic	
	60	∞	75
	30	∞	
	20	228	
	10	151	
	5	133	
	2	NS	

[a] NS = not significant.

in the dessicator over phosphorus pentoxide. Compound **18b** was recrystallized as slightly yellow needles from a mixture of ether–light petroleum ether and was homogeneous on TLC, in different solvent systems: yield 4.8 g (96%); mp 104–105 °C. ^{1}H NMR δ 4.14 (2 H, t, $ClCH_2$), 3.68 (2 H, t, CH_2NNO), 2.90 (4 H, s, succinimide). Anal. ($C_7H_8CLN_3O_5$), C, H, N; M_r 249.5.

***N*-Methyl-*N*-nitrosocarbamic acid *N'*-hydroxysuccinimide ester (18a)** was recrystallized from a mixture of ether–light petroleum ether: yield 98%; mp 121–122 °C dec; ^{1}H NMR δ 3.18 (3 H, s, CH_3), 2.90 (4 H, s, succinimide). Anal. ($C_6H_7N_3O_5$) C, H, N; M_r 201.

2,4,5-Trichlorophenyl *N*-methyl-*N*-nitrosocarbamate (18c): reaction time of nitrosation 2 h; yield 94%; recrystallized from light petroleum ether; mp 79–80 °C dec; ^{1}H NMR δ 8.10 and 8.08 (2 H, s, aromatics), 3.22 (3 H, s, CH_3). Anal. ($C_8H_5Cl_3N_2O_3$) C, H, N; M_r 283.5.

2,4,5-Trichlorophenyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (18d): reaction time of nitrosation 1 h; recrystallized from light petroleum ether; yield 96%; mp 68 °C; ^{1}H NMR δ 8.08 (2 H, s, aromatic) 4.18 (2 H, t, $ClCH_2$), 3.71 (2 H, t, CH_2NNO). Anal. ($C_9H_6Cl_4N_2O_3$) C, H, N; M_r 332.

Pentachlorophenyl *N*-methyl-*N*-nitrosocarbamate (18e): reaction time of nitrosation 2 h; recrystallized from light petroleum ether; yield 94%; mp 118–119 °C dec; ^{1}H NMR δ 3.26 (3 H, s, CH_3). Anal. ($C_8H_3Cl_5N_2O_3$) C, H, N; M_r 352.5.

Pentachlorophenyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (18f): reaction time of nitrosation 2 h; recrystallized from light petroleum ether; mp 107–108 °C; ^{1}H NMR δ 4.24 (2 H, t, $ClCH_2$), 3.74 (2 H, t, CH_2NNO). Anal. ($C_9H_4Cl_6N_2O_3$) C, H, N; M_r 401.

Pentafluorophenyl *N*-methyl-*N*-nitrosocarbamate (18g): purified on a column of silica gel (dichloromethane–light petroleum ether as eluent); recrystallized from light petroleum ether; yield 55%, based on amounts of pentafluorophenol used for the synthesis of **17g**; mp 76–78 °C; IR 1775 (CO) cm^{-1}; ^{1}H NMR δ 3.20 (3 H, s, CH_3). Anal. ($C_8H_3F_5N_2O_3$) C, H, N; M_r 270.

Pentafluorophenyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (18h): purified and recrystallized as described for **18g**; yield 50% based on amounts of pentafluorophenol used for the synthesis of **17h**; mp 64–65 °C; IR 1780 (CO) cm^{-1}; ^{1}H NMR δ 4.2 (2 H, t, $ClCH_2$), 3.7 (2 H, t, CH_2NNO). Anal. ($C_9H_4ClF_5N_2O_3$) C, H, N; M_r 318.5.

Preparation of CCNU (16). 16 was synthesized from one of the *N*-nitrosocarbamates of type 18 (**b**, **d**, **f**, or **h**) according to the procedure described below. To a cooled (0 °C) solution of dimethylformamide (5 mL) containing either **18b**, **18d**, **18f**, or 18h (11 mmol) was added, under stirring, cyclohexylamine (0.99 g, 10 mmol). When no more cyclohexylamine could be detected by TLC (after about 2 h), the reaction mixture was poured into cold water (200 mL). The precipitate which formed was filtered, washed several times with water, and recrystallized from a mixture of acetone–water or ether–light petroleum ether. In each case, yields were around 80%. When **18d**, **18g**, or 18h was used in this reaction, purification of CCNU was achieved by filtration on a column of silica gel. The CCNU's obtained in all examples were identical and identified by comparison with the physical properties of an authentic sample of CCNU.

Preparation of Streptozotocin (14). To a cooled solution of pyridine (10 mL) or dimethylformamide (5 mL) containing **18a**, **18c**, **18g**, or **18e** (11 mmol) was added glucosamine (10 mmol, 1.79 g). The mixture was stirred at 0 °C during 12 h. Then, ethyl acetate (100 mL) was added, and the precipitate was collected by filtation and washed several times with ethyl acetate and ether to give 1.4 g of pure streptozotocin (**14**). From the mother liquors and washings was obtained an additional 0.8 g of **14** after concentration in vacuo ($t < 50$ °C) and recrystallization from ethanol: total yield 80%. Using **18g**, the reaction was faster (1 h). The homogeneity and identification of **14** were ascertained by comparison with the physical properties of an authentic sample of streptozotocin.

Preparation of Chlorozotocin (15). Chlorozotocin (**15**) was synthesized from **18b**, **18d**, **18f**, or 18h as previously described for the synthesis of **14**. In all cases, **15** was obtained with an overall yield of 80%.

Preparation of *N*,*N'*-Bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl]cystamine (19). To a cooled solution (0 °C) of dimethylformamide (10 mL) containing either **18b**, **18d**, **18f**, or **18h** (0.01 mol) was added dropwise cystamine hydrochloride (0.05 mol, 1.12 g) in dimethylformamide (20 mL) and triethylamine (or diisopropylethylamine). After the solution was stirred for about 40 min at 0 °C, no more starting material could be detected by TLC. The reaction mixture was poured into ice–water (200 mL), and the resulting precipitate was filtered off, washed with water, and dried in the dessicator over phosphorus pentoxide. The yellow compound was purified by column chromatography (ethyl acetate as eluent) and then recrystallized from a mixture of ether–light petroleum ether: yield 75%; mp 95 °C; IR 3360 and 3320 (NH), 2960 and 2930 (CH_2), 1695 (CO), 1480 (NO) cm^{-1}; ^{1}H NMR δ 8.80 (2 H, t, NH), 4.05 (4 H, t, CH_2NNO), 3.60 (4 H, t, CH_2NH), 2.95 (4 H, t, CH_2S). Anal. ($C_{10}H_{18}Cl_2N_6S_2O_4$) C, H, N; M_r 421.

Antitumor Evaluation. The oncostatic activities and acute LD_{50} values of active *N*-alkyl-*N*-nitrosocarbamates, as well as those of CNCC (**20**) (mixture of isomers) and **19** (pure isomer), were evaluated on L1210 leukemia. The method used for this evaluation will be described briefly: on day 0, adult B6D2F1 mice were inoculated ip with 10^5 leukemia cells. On day 1, the mice received various doses of compound to be tested in olive oil (2–150 mg/kg). On days 5 and 9, drug or solvent injections were repeated only in mice with no signs of toxicity. The mortality of mice was monitored daily and autopsies were performed to find out whether or not deaths were due to leukemia or to a toxic action of the drug. The acute LD_{50} of each compound was determined graphically. For each compound, the oncostatic effects of specified doses were expressed as an oncostatic index, *I*. $I = T/C \times 100$ (T = median survival time in the treated group of mice; C = median survival time in the control group). When $I > 125$ and the difference between treated and control groups was statistically significant

according to the Wilcoxon nonparametric W test, the agent was considered active at the given dose. The value ∞ means that more than 50% of treated animals in the group had been cured.

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Notes

Syntheses of α- and γ-Substituted Amides, Peptides, and Esters of Methotrexate and Their Evaluation as Inhibitors of Folate Metabolism[1]

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N-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-L-glutamic acid α-benzyl ester (**2**) and γ-benzyl ester (**6**) served as key intermediates in syntheses of precursors to amides and peptides of methotrexate (MTX) involving both the α- and γ-carboxyl groupings of the glutamate moiety. Coupling of **2** and **6** at the open carboxyl grouping with amino compounds was effected by the mixed anhydride method (using isobutyl chloroformate); carboxyl groupings of amino acids coupled with **2** and **6** were protected as benzyl esters. *N*-[4-[[(Benzyloxy)carbonyl]methylamino]benzoyl]-L-glutamic acid γ-methyl ester (**5**), a precursor to MTX γ-methyl ester, was prepared from L-glutamic acid γ-methyl ester and 4-[[(benzyloxy)carbonyl]methylamino]benzoyl chloride (**1**) in a manner similar to that used to prepare **2** and **6**. The precursor to MTX α-methyl ester was prepared from γ-benzyl ester **6** by treatment with MeI in DMF containing $(i\text{-Pr})_2$NEt. Benzyl and (benzyloxy)carbonyl protective groupings were removed by hydrogenolysis, and the deprotected side-chain precursors were converted to α- and γ-substituted amides, peptides, and esters of MTX by alkylation with 6-(bromomethyl)-2,4-pteridinediamine hydrobromide (**12**). Biochemical–pharmacological studies on the prepared compounds aided in establishing that the α-carboxyl grouping of the glutamate moiety contributes to the binding of MTX to dihydrofolate reductase while the γ-carboxyl does not. Other studies on the peptide MTX-γ-Glu (**13h**) are concerned with the contribution toward antifolate activity of this metabolite of MTX. The compounds prepared were also evaluated and compared with MTX with respect to cytotoxicity toward H.Ep.-2 cells and effect on L1210 murine leukemia.

Methotrexate (MTX) has been in clinical use for more than 30 years.[2,3] It remains a mainstay in the treatment of acute leukemia[4] and choriocarcinoma[5] and has also proved beneficial in treatment of osteogenic sarcoma[6,7] and carcinoma of the head and neck.[8] The usefulness of MTX in the treatment of human leukemia is limited by the ability of the malignant cells to develop resistance to the drug.[8,9] Some tumors are naturally resistant while others acquire resistance after a period of response. Two factors known to be connected with resistance are (1) increased intracellular levels of dihydrofolate reductase (DHFR), the enzyme whose inhibition is the main cause of the arrest of cell proliferation by MTX, and (2) loss of the active-transport system by which MTX enters cells.[10-13] Certain murine tumors deficient in this transport system are resistant to MTX but sensitive to other inhibitors of DHFR which enter cells by passive diffusion.[11,14] Acquired resistance in some leukemia cell lines has been shown to result from loss of the active-transport mechanism.[12,15-17] These observations have prompted searches for synthetic analogues of MTX which might have favorably altered transport characteristics and still have the capacity to bind to DHFR.

The tight binding of MTX to DHFR is readily demonstrated in vitro, and, until recently, the concept that MTX acted without metabolic activation was accepted.[18]

(1) Most of the synthetic work described in this paper was briefly summarized earlier [J. R. Piper and J. A. Montgomery, "Chemistry and Biology of Pteridines", Proceedings of the International Symposum on the Chemistry and Biology of Pteridines, 6th, La Jolla, CA, 1978. See *Dev. Biochem.*, 4, 261 (1979)].
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