A Critical Appraisal of the Evolution of *N*-Nitrosoureas as Anticancer Drugs

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I. Introduction

The *N*-alkyl- and *N*-(2-haloethyl)-*N*-nitrosoureas represent one of the generally most useful classes of anticancer agents, with a wide range of activities against various leukemias and solid tumors.

Since the early l960s a huge volume of chemical, biological, and clinical research has been published. Thus, a number of reviews[1−6] were devoted to a collection of many aspects of the chemistry and biology of *N*-nitrosoureas. More restricted reviews appeared on special topics such as synthesis and structure−activity relationships,[7−16] antitumor activities,[17−20] metabolism and pharmacology,[21−27] toxicology,[28,29] mutagenicity and carcinogenicity[4,14,30−40] and clinical oncology.[41,42]

In the present review, the emphasis is on the synthetic and some mechanistic aspects of potential anticancer drugs of the *N*-nitrosourea type and the evaluation of their anticancer activities, although other topics closely related to the main theme, e.g. structure−activity relationships, fragmentation patterns of *N*-nitrosoureas, and their interactions with biological systems, such as DNA, are also included. An attempt was made to cover the material as comprehensibly as possible, but not necessarily ex-

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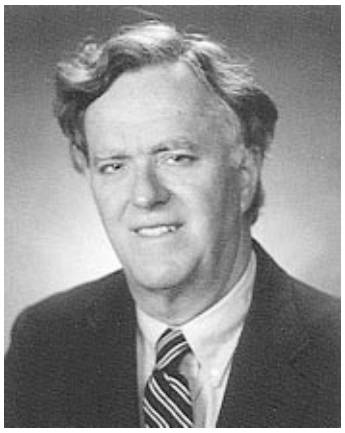


Thomas Gnewuch was born in Fond du Lac, WI. His academic record includes a B.S. degree from Georgetown University (1960), M.S. degree from Iowa State University (1963), and Ph.D. degree from Indiana University (1966). His doctoral thesis under Professor Ernest Wenkert was concerned with alkaloid total synthesis. Since that time he has held teaching or research positions at Marquette University, The Medical College of Wisconsin, The Upjohn Company, the University of Missouri—Columbia, the University of Wisconsin—Stout, and the University of Wisconsin—Milwaukee. His research work has been involved with the synthesis of adrenergic blocking agents, the synthesis of substrates for a lipase enzyme, chemical studies of blood platelet storage, chemical carcinogenesis studies of *N*-nitrosamines, the synthesis of spin-labeled compounds useful in both cancer treatment and NMR imaging, and the synthesis of *N*-nitrosourea anticancer agents.

George Sosnovsky has been professor of chemistry at the University of Wisconsin—Milwaukee since 1967. At present, he is Professor Emeritus and Adjunct Professor in the Department of Chemistry. He received his Ph.D. from the University of Innsbruck in 1948. After graduation, his experience included research at CSIRO and ICI, Australia, 1949–1956; Postdoctoral Research Associate, University of Chicago, 1956–1959; Senior Scientist, IIT Research Institute, Chicago, 1959–1963; Associate Professor, IIT, Chicago, 1963–1966; and Special Senior Research Fellow of Public Health Services at the University College, London, and the University of Tubingen, 1967–1968. He was the co-founding editor of *Synthesis, International Journal of Methods in Synthetic Organic Chemistry*, and served as editor from 1969 to 1985. He was regional director for the National Foundation for Cancer Research, 1980–1985. He has authored about 170 publications and a book *Free Radical Reactions in Preparative Organic Chemistry* (Macmillan, 1964). During those years his interests spanned over a wide range of topics, such as research and development of fungicides, herbicides, and insecticides; amidine derivatives as antiradiation agents; diazo dyes leading to a commercial copying process; free radical chemistry, in particular, metal ion-catalyzed redox reactions involving antioxidants and reactions with peroxides; discovery of the peroxyester reaction; photochemical synthesis; oxidative phosphorylation reactions; synthesis of spin-labeled phosphorus compounds of biological interest, including anticancer agents; transphosphorylation reactions using imidazole and related compounds as transfer agents; organosulfur and organoselenium compounds; and synthetic methodology. In more recent years, his research interest focused on medicinal chemistry, specially, on structure–activity relationships of anticancer drugs involving design and biological evaluations of new anticancer drugs. Related to this theme also were pursuits in the synthesis of contrast-enhancing agents for diagnostic NMR-imaging (MRI), radiation sensitizers, and syntheses of aminoxyl-labeled probes for studies of intracellular environments, such as hypoxic cells by EPR and MRI.

haustively, because of an overwhelming amount of literature. Nevertheless, it is believed that all important original contributions were critically appraised and included.

The review was arbitrarily organized following an approximate historical evolution of *N*-nitrosourea drugs. Thus, the first generation of these anticancer agents encompass compounds containing aliphatic, alicyclic, aromatic, and heteroaromatic groups. The second generation of compounds are represented by water-soluble hydroxyalkyl and hydroxycycloalkyl analogs. The third generation of compounds includes various carrier moieties, such as amino acids, peptides, carbohydrates, nucleosides, and steroids. Finally, the fourth generation of anticancer agents contains nitroxyl moieties and conjugates composed of amino acids, carbohydrates and steroids. It is hoped that this critical review will serve as a stimulant for new thoughts in the quest for rational designs of more active and less toxic anticancer drugs containing the *N*-nitrosourea moiety.

II. Scope and Limitations

The present review contains only those synthetic methods which are essential for a systematic procurement of the desired *N*-nitrosoureas for evaluation as anticancer agents. Minor variations in basic synthetic methods and the syntheses of most starting materials which can be found in the literature are not included. Throughout the review the selection of compounds for inclusion of their anticancer activities is restricted to chemically well-defined products. However, the physical constants and proof of structures are excluded. The anticancer activities are combined in tables in accordance with various classes of compounds. The inclusion of anticancer test results in tables is mainly restricted to data that have been obtained in vivo against the interperitoneally inoculated murine (mouse) lymphocytic P388 and lymphoid L1210 leukemias in accordance with the National Cancer Institute (NCI) protocol. This restriction allows, in many cases, an approximate comparison of anticancer activities within one class and among different classes of compounds, in spite of the fact that various investigators were found to deviate from the original NCI protocol. Also, the many vehicles, i.e. solvent combinations, which have been used for the administration of drugs to animals are excluded from the tables. In a few cases the results obtained with the rat leukemia L5222 are included. However, in vitro test results and many in vivo screenings against solid cancers could not be included, although these results are often mentioned in the text where appropriate.

Besides the already mentioned topics, the structure−activity relationships, including the dominant influence of lipophilicity on the anticancer activity of nitrosoureas, are critically analyzed. Also included in the review is an in-depth coverage of the mechanisms of chemical decomposition in vitro, and in vivo interactions of *N*-nitrosoureas with biomolecules

found within the cell nucleus. A final section covers selected aspects of the metabolism, pharmacology, toxicology, and carcinogenicity of *N*-nitrosoureas.

In order to be able to cope with the huge amount of references, a selective computer search, covering the period from 1966 to May 1995, and a manual search through February 1995 were used.

III. Selected Abbreviations and Definitions

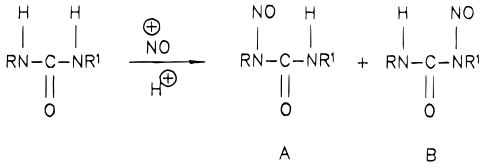
Various abbreviations and definitions are used routinely in the text, tables, and schemes.

AAT (AT)	alkyl acceptor transferase (alkyl transferase)
Ac	acetyl group, $CH_3C{=}O$
Ac_2O	acetic anhydride
AcOH	acetic acid
aminoxyl	nitroxyl compound with $=N\dot{-}O$ moiety
ANU	*N*-alkyl-*N*-nitrosourea
aq	aqueous
Ar	aryl, substituted phenyl moiety
BBB	blood−brain barrier
Bn	benzyl group
BCNU	*N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea (carmustine)
BOC	*tert*-butoxycarbonyl moiety
n-Bu	"normal" butyl $CH_3(CH_2)_3$
t-Bu	tertiary butyl $C(CH_3)_3$
Bz	benzoyl group C_6H_5CO, PhCO
Cbz	carbobenzoxy (benzoyloxycarbonyl) moiety, $PhCH_2OC{=}O$
CCNU	*N*-cyclohexyl-*N*′-(2-chloroethyl)-*N*′-nitrosourea (lomustine)
CENU	(2-chloroethyl)nitrosourea or *N*-(2-chloroethyl)-*N*-nitrosourea
CFU-C	granulocyte-committed stem cells
CFU-S	pluripotent stem cells
Ci	Curie, a unit of radioactivity
CIMS	chemical ionization mass spectrometry
conc	concentrated
CZT	chlorozotocin, *N*-(2-amino-2-deoxy-D-glucosyl)-*N*′-(2-chloroethyl)-*N*′-nitrosourea
d	day
DCC	*N*,*N*′-dicyclohexylcarbodiimide
dG	deoxyguanosine
DHT	dihydrotestosterone
DMBA	dimethylbenz(*a*)anthracene
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E	entgegen, opposite in German, geometric stereodescriptor, *trans*
ED	effective dose
ED_{50}	effective dose causing cures of 50% of the test animals
EPR	electron paramagnetic resonance (ESR, electron spin resonance)
Et_2O	ethyl ether, $CH_3CH_2OCH_2CH_3$
EtOH	ethyl alcohol
FMOC	(9-fluorenylmethoxy)carbonyl moiety
5FU	5-fluorouracil
G	guanine or guanosine
h	hour
HECNU	*N*-(2-hydroxyethyl)-*N*′-(2-chloroethyl)-*N*′-nitrosourea (elmustine)
HPLC	high pressure liquid chromatography
i	*iso* e.g. isopropyl
ic	intracerebral
ICL	interstrand cross-link
ILS	increase in life span, $((T - C)/C) \times 100$, where *T* is the median survival time of the treated animals and *C* is the median survival time of the untreated control animals
ILS_x	dose resulting in an *x*% increase in life span.
im	intramuscular
ip	intraperitoneal
iv	intravenous
LC	liquid chromatography
LD_{50}	lethal dose killing 50% of the test animals
log *P*	logarithm (common) of partition coefficient
MCNU	*N*-[methyl(6-amino-6-deoxy-D-glucosidyl)]-*N*′-(2-chloroethyl)-*N*′-nitrosourea (ranomustine)
MeCCNU	*N*-(*trans*-4-methylcyclohexyl)-*N*′-(2-chloroethyl)-*N*′-nitrosourea (semustine)
μCi	microcurie
mer^+	methylation repair sufficient human cell type
mer^-	methylation repair deficient human cell type
mCi	millicurie
Me	methyl group, CH_3
Me_2CO	acetone
MeOH	methyl alcohol
min	minute
mmol	millimole
mol	mole
MNU	*N*-methyl-*N*-nitrosourea
MRI	magnetic resonance imaging
MS	mass spectrometry
NBS	*N*-bromosuccinimide
NMR	nuclear magnetic resonance
nor	prefix indicating a parent compound
OD	optimal dose, the dose resulting in maximal anticancer activity
P	partition coefficient
Ph	phenyl, C_6H_5
po	peroral or by mouth
Pr	"normal" propyl moiety $CH_3CH_2CH_2$
py	pyridine, C_5H_5N
QSAR	quantitative structure−activity relationship
R	various univalent moieties, e.g. H, alkyl, etc.
RBA	relative binding affinity
RC=O	acyl group when R = aliphatic group
RNA	ribonucleic acid
RNCO	isocyanate derivatives
rt	room temperature
s	second
SAR	structure−activity relationship
sc	subcutaneous
SSB	single-strand break
SSS	single-strand scission
SZT	*N*-(2-amino-2-deoxy-D-glucosyl)-*N*′-methyl-*N*′-nitrosourea (streptozotocin)
sec-	secondary, e.g. *sec*-butyl
t-	*tert*, tertiary, e.g. *tert*-butyl, $(CH_3)_3C$ moiety
$t_{1/2}$	half-life, a measure of chemical stability
TD_{50}	total dose of carcinogen in mg/kg body weight required to reduce by 1/2 the probability of an animal remaining tumor-free throughout a standard life time
therapeutic index	therapeutic ratio = maximum effective dose (MED) or optimal dose (OD) divided by the minimum effective dose
THF	tetrahydrofuran
TLC	thin-layer chromatography
Tr	trityl or triphenylmethyl group, Ph_3C
U	uracil or uridine
Z	zusammen, together in German, opposite of *E*, *cis* in simple cases

IV. General Synthetic Methods

The general methods of synthesis of nitrosoureas, various factors affecting the position of the nitroso group in unsymmetrical ureas, and several preparations of isotopically labeled nitrosoureas are briefly discussed in this section.

Scheme 1



The basic reaction of an urea with a nitrosating agent can result in a mixture of two isomers, as shown in Scheme 1. The nitrosating agent, a source of the nitrosonium ion NO^+, can be either nitrous acid (HNO_2), nitrosyl chloride (NOCl), dinitrogen trioxide ($O{=}N{-}O{-}N{=}O$, N_2O_3), dinitrogen tetraoxide ($O{=}N{-}O{-}NO_2$, N_2O_4), or nitrosonium tetrafluoroborate ($NO^+BF_4^-$). The nitrous acid has been generated in situ[6,43] by the reaction of sodium nitrite with acids, such as hydrochloric, acetic, and formic acids. The resulting active nitrosating agents under these conditions are nitrosyl chloride (NOCl), acetyl nitrite ($CH_3COO^-NO^+$), and formyl nitrite ($HCOO^-NO^+$), respectively. The optimum pH for these reactions is about 3, and often a slight excess of nitrosating agent is employed. Generally, good yields of the *N*-nitrosoureas have been obtained[44] from symmetrical ureas (R = R′). Nitrosyl chloride,[45] which is no longer available commercially, and nitrosonium tetrafluoroborate are efficient nitrosating agents but are expensive reagents when compared to nitrous acid. The gaseous agents N_2O_3[46] and N_2O_4[47,48] also have been successfully employed as nitrosating agents. In some instances, the N_2O_3 has been used with aqueous hydrochloric acid for specific nitrosation.[46] Normally, the N_2O_4 has been dissolved[47,48] in a 2-fold excess of a solvent such as carbon tetrachloride at 0 °C. The resulting solution is added at −35 °C to a mixture of an appropriate urea in either tetrahydrofuran (THF) or ethyl acetate containing an excess of either sodium acetate or pyridine to neutralize the liberated nitric acid. The initial deep blue color of the reaction changes to a blue-green, and then to a deep yellow as the reaction mixture is allowed to warm slowly to 0 °C.

The *N*-nitrosoureas are isolated either by precipitation from water or by extraction with organic solvents, followed by washing with dilute sodium carbonate and water to remove all traces of acid. After drying, the solvent is removed in vacuum. The isolated compounds should be recrystallized from organic solvents at low temperatures to prevent decomposition. In some instances, the final products cannot be crystallized and must be purified by column chromatography either on silica gel or neutral aluminas. However, occasionally the chromatographic procedures cause decomposition of products. After purification the thoroughly dried compounds should be stored in a freezer at −20 °C or lower temperature to prevent decomposition. The purity of *N*-nitrosoureas should be checked periodically, particularly before biological tests, by thin-layer chromatography (TLC) using either ultraviolet (UV) light or a combination of UV light and the Griess reagent[49] as convenient indicators of the nitroso moiety.

A problem can arise in the nitrosation of unsymmetrical ureas caused by the formation of a mixture of mononitrosated compounds (A and B in Scheme 1). Purification of large quantities of such mixtures, for example, by TLC, can be laborious. The position of the nitroso group in unsymmetrical ureas can be determined by several methods. Thus, the addition of an amine, such as cyclohexylamine, to mixtures of the isomers **1** and **2** results in the formation of ureas **3** and **4** from whose structures the precursor of the *N*-nitrosoureas can be determined (Scheme 2).[44]

Spectroscopic methods are very useful for the determination of the *N*-nitroso group in nitrosoureas.[44] Infrared (IR) spectroscopy can be used, since nitrosation of a ureido function causes a shift of the carbonyl absorption to a higher wavelength. The extent of this shift can provide an estimate of the completeness of the nitrosation. In some instances, doublet absorptions (both C=O and NH) are indicative of a nitrosourea.[44] Nuclear magnetic resonance (NMR) spectroscopy is generally the most useful method for establishing the isomeric purity of *N*-nitrosoureas.[44] For the isomer **1** a symmetrical A_2B_2 spectrum would be observed, whereas for the isomer **2** an A_2B_2X spectrum resulting from coupling between the NH and the adjacent methylene groups would be obtained.[44] Singlet absorptions for the methyl group are characteristic for all *N*-methyl-*N*-nitrosoureas of type $CH_3N(NO){-}COR$.

Various factors affect the position of nitrosation of unsymmetrical ureas. Thus, the effect of water on the isomer ratio was observed[44] in the nitrosation of 1-(2-bromoethyl)-3-phenylurea. The reaction with sodium nitrite in 85% formic acid yielded an approximate l:l mixture of isomers **5** and **6** (Chart 1), whereas in 98% formic acid only **5** was obtained.[44] The nitrosation of 1-cyclohexyl-3-(2-chloroethyl)urea (**4**) in formic acid with an equal volume of aqueous

Scheme 2

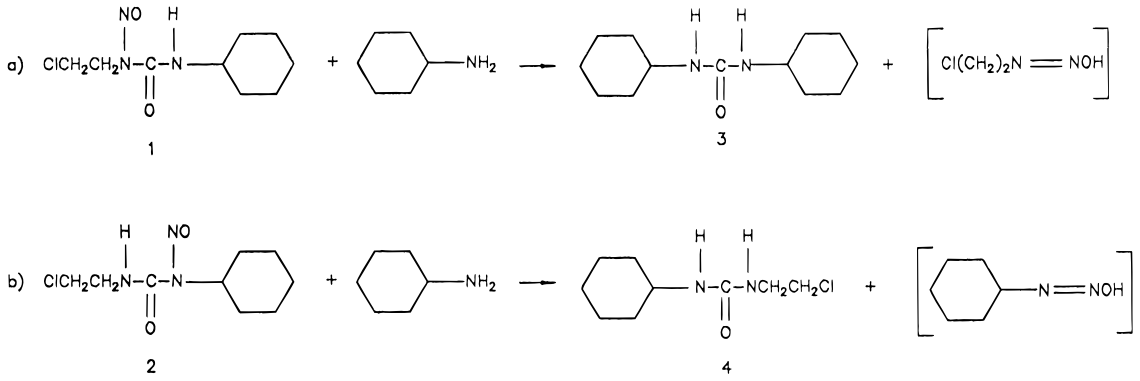


Chart 1. Structures for Section IV

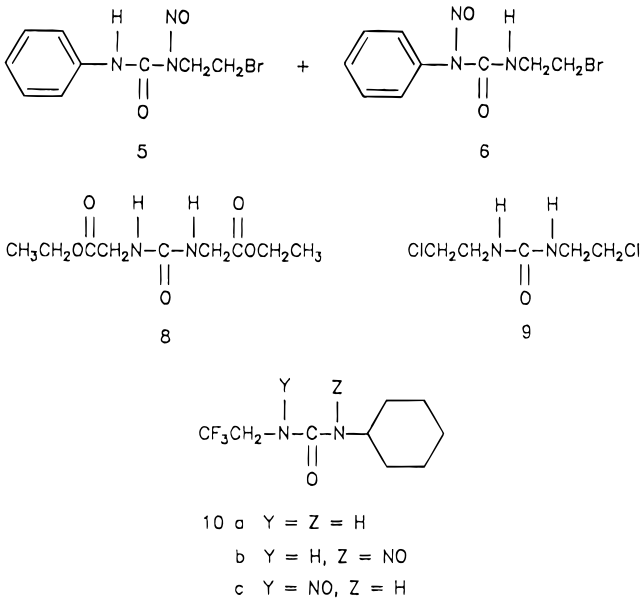
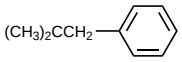
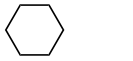
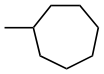


Table 1. Effect of Structure on the Position of the Nitroso Group. Nitrosation of Unsymmetrical Ureas

$$RNHCONHR^1 \rightarrow \underset{\mathbf{a}}{RN(NO)CONHR^1} + \underset{\mathbf{b}}{RNHCON(NO)R^1}$$

compound no.	R	R^1	%**a**	%**b**
7a	alkyl, aryl	H	100	0
7b	$ClCH_2CH_2$	CH_2CH_2F	50	50
7c	CH_3	CH_2CH_2Cl	100	0
7d	$ClCH_2CH_2$		100	0
7e	$ClCH_2CH_2$		100	0
7f	$ClCH_2CH_2$		100	0
7g	$ClCH_2CH_2$		95	5
7h	$ClCH_2CH_2$	$CH_3CH(CH_2)_4CH_3$	85−90	10−15
7i	$ClCH_2CH_2$		75−90	10−25
7j	$ClCH_2CH_2$		80	20

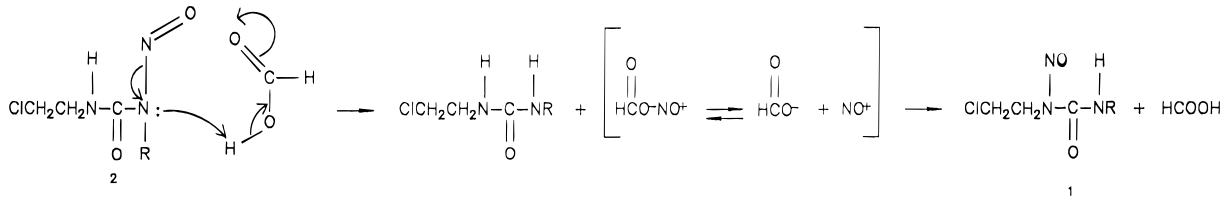
sodium nitrite gave a mixture of about 65% of **1** and 35% of **2**.[44] This isomeric mixture was converted to the desired **1** in high yield by dissolving the mixture in cold formic acid, followed by a precipitation of **1** with water. Under such conditions, the thermodynamically favored isomer **1** is isolated (Scheme 3).[44] The transfer of the nitroso group in formic acid probably proceeds in situ via the formation of formyl nitrite.

Several examples of the effect of structure on the position of nitrosation are shown for compounds **7a**−**j** in Table 1. The nucleophilicity of the urea nitrogen was found[6,44] to be an important factor in determining the site of nitrosation. Thus, for compounds **7a** and **7c** (Table 1) the site of the nitrosation is favored either by an adjacent alkyl or aryl group as compared to the effect of either a hydrogen atom or the electron-withdrawing 2-chloroethyl group. It was found[36] that diethyl *N*,*N*′-carbonyldiglycinate (**8**) was not nitrosated under conditions which favor the nitrosation of the bis-2-chloroethyl analog **9**. The nitrosation of the trifluoromethyl analog **10a** produced a slight excess of **10b** over **10c**[44] (Chart 1).

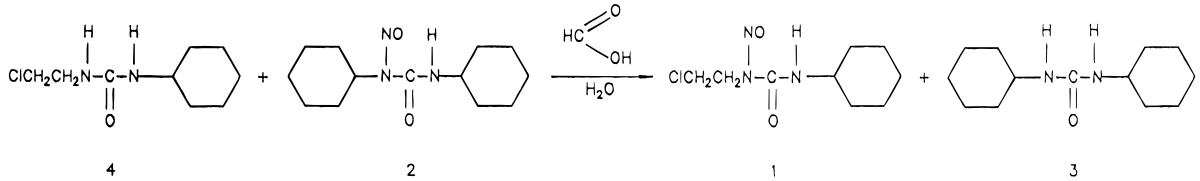
Steric control of nitrosation can decisively determine[6,45] the position of the nitrosations, as in the examples of compounds **7d**, **e**, **f**, and **h** (Table 1). There is some balancing between steric and nucleophilicity effects for compounds **7g**−**j**. Steric effects were also observed[6] in the intermolecular transfer of nitroso groups. Thus, the exclusive formation of the isomer **1** (Scheme 4) was attributed[6] to the steric hindrance of the cyclohexyl group.

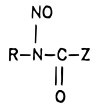
The uncertainty of the classical nitrosation reactions of unsymmetrical ureas prompted the search for alternative synthetic methods.[45,50−59] Thus, various active esters **11a**−**j** and acid azide **11k** of the *N*-nitrosocarbamoyl moiety **11** (Figure 1) were developed for a selective and unambiguous introduction of the nitroso group to give the final products (Scheme 5). The byproducts, *p*-nitrophenol and *p*-cyanophenol, from the use of reagents **11b** and **11d**, requires column chromatography for their removal which can cause a lowering of the yield of products. The azido reagent **11k** has potential explosive properties and can become hazardous when used in large quantities. Reagent **11h** is an attractive alternative because the liberated *N*-hydroxysuccinimide is water soluble, and the desired products can often be isolated by extraction with organic solvents. Another ap-

Scheme 3



Scheme 4





11, R = alkyl or 2-haloethyl (XCH_2CH_2, X = Cl, F)

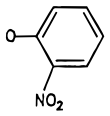
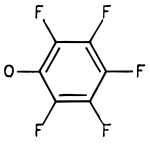
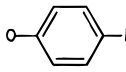
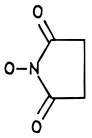
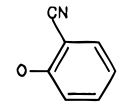
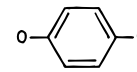
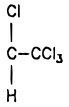
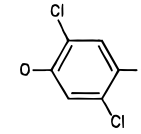
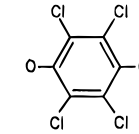
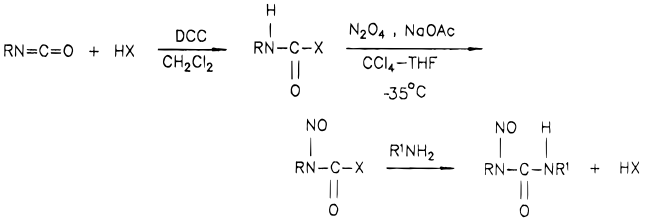
	Z	Ref.		Z	Ref.
a)		45,50	g)		45
b)		45,50-53	h)		45
c)		45,50	i)	various aromatic and heteroaromatic carbamates	54
d)		45,50	j)		55, 56
e)		45	k)	N_3	57-59
f)		45			

Figure 1. Transfer agents for the transformation of amines into *N*-Nitrosoureas.

Scheme 5



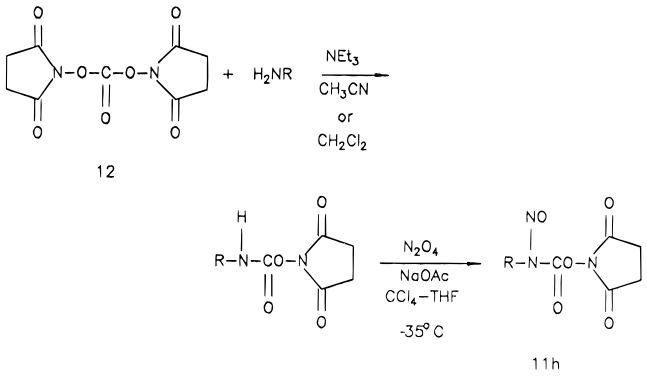
X = OZ, N_3, (11a=k)

proach[60] to the transfer agent **11h** arises from the reaction of disuccinimidyl carbonate **12** with amines followed by nitrosation (Scheme 6).

A combination of nitric acid and copper dust in acetic acid provided[61] good yields of *N*-nitrosoureas, as exemplified by the reaction of Scheme 7. The reaction of copper with nitric acid produces the nitrous acid. This reagent is particularly useful for the synthesis of ^{13}N-containing compounds.

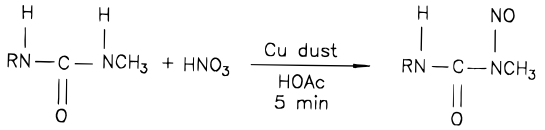
A number of radioisotopes, such as ^{13}N, ^{15}N, ^{11}C, ^{13}C, ^{14}C, and ^{18}F have been incorporated into the *N*-nitrosourea structure by various synthetic methods.[62−67] Such compounds, **13**−**23**, are useful for the study of the chemical decomposition and the metabolism of nitrosoureas. The incorporation of these isotopes is shown in Schemes 8−13. Examples

Scheme 6



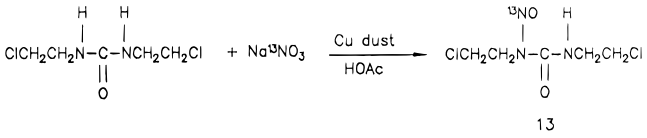
R = various groups

Scheme 7

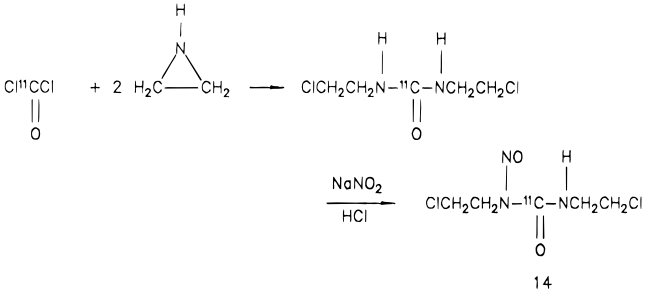


R = 2-Deoxy-2-D-glucopyranosyl

Scheme 8



Scheme 9

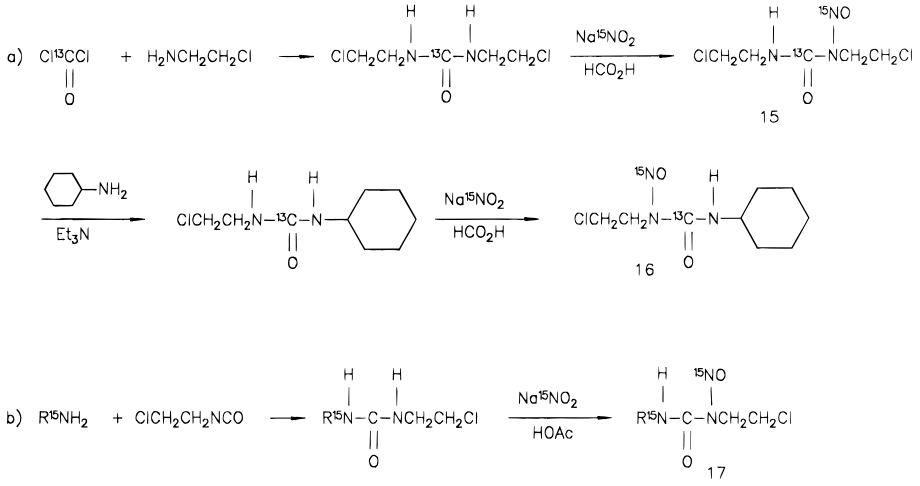


of isotopically labeled *N*-nitrosoureas can be found in sections IX and X of this review.

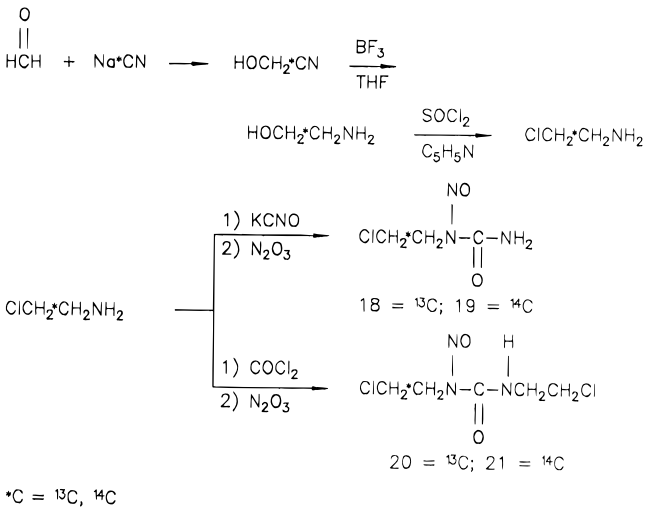
V. Cancer Lines and Screening Methodologies

During approximately 30 years of research on nitrosoureas as anticancer agents, a variety of cancer cell lines have been employed for both in vitro and in vivo testing. After much experience, the murine leukemias P388 and L1210 were chosen as the most sensitive and reliable cell lines for anticancer screening.[68,69] Standard protocols for the administration of these cell lines have been published by the National Cancer Institute.[70,71] The cancer cells can be administered either via the peritoneum, i.e. intraperitoneal (ip), the cerebellum, i.e. intracerebral (ic), under the skin, i.e. subcutaneous (sc), the vein, i.e. intravenous (iv), the muscle, i.e. intramuscular (im), or the mouth, i.e. (po). The ic administration of cancer cells is used for the screening of the effectiveness of drugs in crossing the blood−brain barrier, and the iv administration allows the testing of the ability of drugs to penetrate all areas of the body. Drugs and cancer cells can be given by different routes in the same

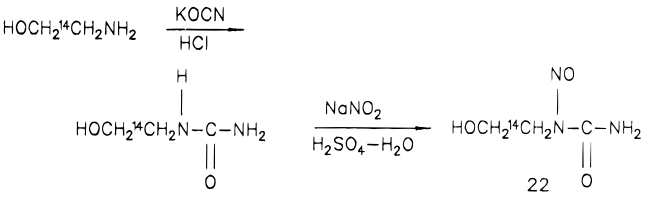
Scheme 10



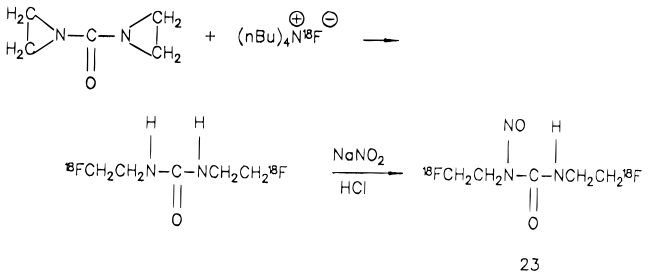
Scheme 11



Scheme 12



Scheme 13



experiment so that many cancer cell−drug combinations are possible. The standard protocol[70] contains detailed instructions concerning the number of cells injected per mouse, i.e. 10^6 cells for P388 and 10^5 cells for L1210 by the ip route, and the schedule of administration, i.e. day 0 for cells and one dose each on days 1−9 for the drugs. Unfortunately, some research groups have adopted variations of the standard NCI protocols and these deviations are indicated, where possible, in the tables. These variations in the standard protocol, i.e. different doses, dose schedules, numbers of cancer cells, affect the values of %ILS that are reported in the literature and cause difficulties in comparing the effectiveness of various anticancer agents.

The biological activity of the *N*-nitrosoureas has been expressed in different ways, but most commonly by T/C and %ILS values according to the NCI protocol. The percent increase in life span or %ILS is calculated from the formula $\%ILS = [(T - C)/C] \times 100$, where T is the median survival time of the treated animals and C is the median survival time of the control, i.e. of the untreated animals. The standard protocol defines a T/C value of 125% as the minimum value necessary for a statistically significant antitumor activity. The number of treated animals which survive a certain number of days are listed in the tables as either ratios or percentages, e.g. 8/10 or 80%. In many studies with leukemias a 60 day survival of the animals is considered to be a cure.

Drugs can be screened for anticancer activity by measuring their ability to reduce the population of cancer cells in an animal after a certain time period by counting the cells in a volume of withdrawn fluid. This activity is often expressed as a log kill number because the log expression is independent of the volume of the fluid.[44] Thus, a reduction of a cell count from 10^5 to 10^2 would represent a 3-log kill. The maximum log kill numbers are 6 for ip-implanted L1210 and 5 for ic-implanted L1210.

A series of structurally related drugs can be ranked according to their margin of safety. This is known as the therapeutic index or therapeutic ratio. The therapeutic index is defined by the relationship of maximum effective dose/minimum effective dose, where the maximum effective dose or optimum dose OD is the daily dose that will produce the maximum increase of life span and the minimum effective dose is the daily dose that will yield an increase of life span to a predetermined limit, such as 30% ILS or ILS_{30}. The larger the ratio, the greater is the margin of safety. In some cases, a lethal dose (LD) term is used in the denominator. Thus, a ratio of ED_{50}/LD_{10} is defined as the daily dose that will cause a 50% survival of treated animals after a certain number of days divided by the dose that will kill 10% of the

untreated normal animals. Both values are determined from the log−dose probit−survival plots.[44] In this instance, the smaller the ratio, the greater is the margin of safety.

The anticancer drugs also can be ranked for in vitro activity by a cytotoxicity index[44] that is defined as the concentration necessary to inhibit the growth of either KB or HEp-2 cells to 50% of the control growth which is determined from semilog plots of concentration against the ratio of growth of treated cells to the growth of control cells. In general, there was no correlation[44] between the cytotoxicity of drugs in vitro and their in vivo activity or toxicity and, therefore, the cytotoxicity indices are not included in the tables.

The biological data in the tables of this review are restricted to the in vivo results using the two aforementioned leukemia cell lines, and occasionally the rat leukemia L5222 and certain solid tumor lines. The in vivo results are better indicators of the activity of drugs against human cancers than the many in vitro screening experiments described in the literature. However, certain in vitro screening results are mentioned in the text where appropriate. In addition to the leukemia cell lines, a number of solid tumors have been employed by various investigators in order to determine the full range of anticancer activities. These types include carcinomas, i.e. epithelial tissue, Lewis lung, Walker mammary, 26 colon, melanomas, i.e. skin, B16, Harding−Passey, lymphomas, i.e. lymphoid tissue, TLX, C57BL6, sarcomas, i.e. nonepithelial connective tissue, fibrosarcoma, glioma, i.e. nervous tissue, myelomas, i.e. bone marrow, and many more. The degree of reduction in the weight and volume of the solid tumors is taken as the measure of the drug's anticancer activity. Solid tumor screening results of various investigators are even more difficult to compare than the NCI standardized P388 and L1210 leukemias, and, hence, are listed only occasionally in the tables and are mentioned in the text where appropriate.

Some of the important adverse side effects of the nitrosoureas are myelosuppression, i.e. decrease of the bone marrow, leukopenia, i.e. decreased white blood cell count, and thrombocytopenia, i.e. decreased blood platelet count. The level of the blood components decrease to a minimum level, the so-called nadir, followed by a return to normal levels within a certain number of days. In addition, various *N*-nitrosoureas were found to exhibit a variety of toxicities toward other organs, such as the kidney, i.e. nephrotoxicity, the liver, i.e. hepatotoxicity, intestine and stomach, i.e. gastroenteritis, the nerves, i.e. neurotoxicity, and many other toxicities. Some of these areas are mentioned in section XI.C but details have been excluded from this review.

VI. Assays of Chemical and Biological Parameters, Lipophilicity, and Structure–Activity Relationships

A. Assays of Chemical and Biological Parameters

The half-life was determined[72] by measuring the change in absorbance at 230 nm after incubation in phosphate buffer at pH 7.4 and 37 °C. The alkylating activity of nitrosoureas was determined[73] by incubation with 4-(*p*-nitrobenzyl)pyridine in acetate buffer at pH 6 and 37 °C for 2 h. At the end of the incubation period the solution was made alkaline and extracted with ethyl acetate, and the absorbance of the ethyl acetate solution was determined at 540 nm. The carbamoylating activity of nitrosoureas have been determined[73] by the incubation of a solution of 0.42 μmol of [^{14}C]lysine and 0.42 μmol of the *N*-nitrosourea in 0.45 mL of 0.1 phosphate buffer. The reaction mixture was separated by paper chromatography, and the radioactivity was assayed by a scintillation spectrometer. Radioactivity present on the paper at positions other than that of the [^{14}C]-lysine was considered to be from the lysine carbamoylation products.[73] Another carbamoylation measurement assay, based on the decrease of glutathione GSH on incubation with CENUs, was also developed.[74] The decrease in free thiol groups during the incubation is followed by the use of thiol regent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).[74] A rapid method for the determination of carbamoylating activity of *N*-nitrosoureas involves a reaction with 5′-amino-5′-deoxythymidine at pH 7.4 and 37 °C and analysis of the reaction mixture by combined HPLC−UV method.[75] The decrease of the absorbance at 254 nm as a function of time is used to calculate the carbamoylating activity. This method has several advantages over the radiolabeling method using [^{14}C]-lysine,[73] including greater speed, 30 min as compared to 24 h, the absence of expensive radioisotopes and a greater quantity of carbamoylation for a given *N*-nitrosourea.

Observations at the turn of the century on the relationships between narcotic activity of organic compounds and their oil/water partition coefficients (*P*) led to the use of such measurements as a means of defining lipophilicity of biologically active compounds.[76,77] Later, it was shown[78] that the rate of penetration of plant cell membranes by a variety of organic compounds could be correlated with their partition coefficients. Furthermore, it was shown[79,80] that the interactions of drugs with biomolecules in plasma and in cell membranes depend, not only on the lipophilicity of the molecule, but also on steric and electronic effects. Over the years, olive oil was replaced by *n*-octanol as a reference compound for the lipid, i.e. fatty phases of biological molecules.[81,82] The octanol−water solvent system has been considered by many investigators as a model for complex biological membranes.[81] However, the use of this system fails to accurately predict either the permeability of molecules across the blood−brain barrier or through the skin.[82] Hence, various hydrocarbon solvents are sometimes substituted for octanol to obtain a better understanding of the permeability of membranes.[79,82]

Lipophilicity is considered to be the ratio between the solubility of a substance in oil or lipid and its solubility in water. It is usually expressed as the logarithm of the partition coefficient *P*, where *P* is equal to the ratio: drug in the lipid phase/drug in the aqueous phase. The determination of the partition coefficient *P* by the "shake flask" method with presaturated solvents is generally used, and extensive experimental details for this method have been published.[83] For compounds that are partially ion-

ized at physiological pH, the use of distribution coefficients *D*, i.e. apparent partition coefficients at a given pH, was proposed.[84] The determination of partition coefficients for very lipophilic compounds with a log $P > 5$ was accomplished either by a "slow-stirring" method,[85,86] by the use of high-pressure liquid chromatography (HPLC),[87] or by centrifugal partition chromatography[88] procedures. In addition, the potentiometric (pH-metric) technique was used[82] to calculate log *P* values from the difference between the pK_a and poK_a, i.e. the apparent pK_a in the presence of octanol. A good discussion of the lipophilic and hydrophilic character of organic compounds is available.[89] A quantitative analysis of lipophilic CENU drugs in plasma involved[90] an ether extraction, conversion of the drugs to their *O*-methylcarbamate derivatives, and analysis of the latter by the GC−MS method.

B. Lipophilicity and Quantitative Structure−Activity Relationships

The binding of small molecules to biological macromolecules closely parallels the hydrophobicity factor as measured by the partition coefficient *P*.[80] Both linear (eq 1) and nonlinear (eq 2) relationships between biological response and partition coefficients have been promulgated.[80]

$$\log(1/C) = a \log P + b \quad (1)$$

$$\log(1/C) = k_1(\log P)^2 + k_2 \log P + k_3 \quad (2)$$

C is the concentration of a drug producing a biological response in a fixed-time period. A logarithmic plot of partition coefficients against the corresponding anticancer activities resulted in a parabolic curve with the most active drugs located at the apex of the curve.[91−93] A theoretically plausible bilinear model was also proposed.[94]

The basic equations can also be modified to include electronic and steric effects of substituents in a chemical structure. Thus, for a given set of drugs the equilibrium constant k_i for a physical or chemical process can be formulated, as shown in eq 3:[80]

$$\log k_i = k_1 \log P + k_2 \sigma + k_3 E_s + k_4 \quad (3)$$

where the first, second, and third terms in eq 3 represent the hydrophobic, electronic, and steric effects, respectively. The log *P* term can be calculated from the relative hydrophobicity factor π of substituents. Calculations of substituent factors from regression equations for lipophilic organic compounds have been published.[79] The electronic and steric effects in eq 3 are defined as highly specific effects not contained in the hydrophobic effect.[80] Since the hydrophobic term often has an overwhelming effect on the rate constant k_i,[80] it is not unreasonable to emphasize the lipophilicity of drugs in quantitative structure−activity relationship (QSAR) studies. However, in many cases, a simpler SAR correlation can be successfully used for forecasting drug activity (section VIII).

QSARs of antitumor agents have been correlated with log *P* values.[91,92,95] Thus, 14 nitrosoureas, which varied in log *P* values from 4.51 to −2.21, were evaluated[92] at a series of dose levels for their ability to delay the growth of the Lewis lung carcinoma, a solid tumor, in mice. A plausible correlation of activity with partition coefficients was obtained.[92] On the basis of this correlation the ideal log *P* range should be between −0.20 and +1.34 for nitrosoureas that inhibit this cancer line.[92] Further extensions[95] of this work to the screening of 90 nitrosoureas against the L1210 leukemia in mice revealed that nitrosoureas with log *P* values of −1.5 to −2.5 would have better therapeutic indices than those in use at that time. An optimum log *P* value of −0.6 was found[91] for a series of nitrosoureas against ic-inoculated L1210. However, ic-implanted L1210 leukemia is not a solid tumor per se and was considered[96] to be a poor model for the ability of drugs to penetrate the blood−brain barrier. Thus, the optimal log *P* for six nitrosoureas against ic-inoculated rats with NMU (*N*-methylnitrosourea)-induced sarcoma, a brain tumor, was +0.37.[96] The anticancer activity of 17 nitrosoureas against ip-inoculated L1210 was directly correlated[97] to the alkylating activity which increased with decreasing hydrophobicity. A "principle of minimal hydrophobicity" was proposed[98] by which it was implied that drugs should be made as hydrophilic as possible without loss of cytotoxicity.

Molecular orbital (MO) calculations by the MINDO/3 method on a variety of cytotoxic nitrosoureas revealed that the hydrophobic parameter, expressed as log *P* values, can be related[99] to bulk molecular parameters, to the total electrophilic superdelocalizability (SE) and to the heat of formation (H_f). The relative ease of transport of these nitrosourea compounds, as estimated by log *P*, appears to be a function of the molecular size and shape, as determined by SE and H_f.[99]

VII. Synthesis and Anticancer Activities of Specific Chemical Classes

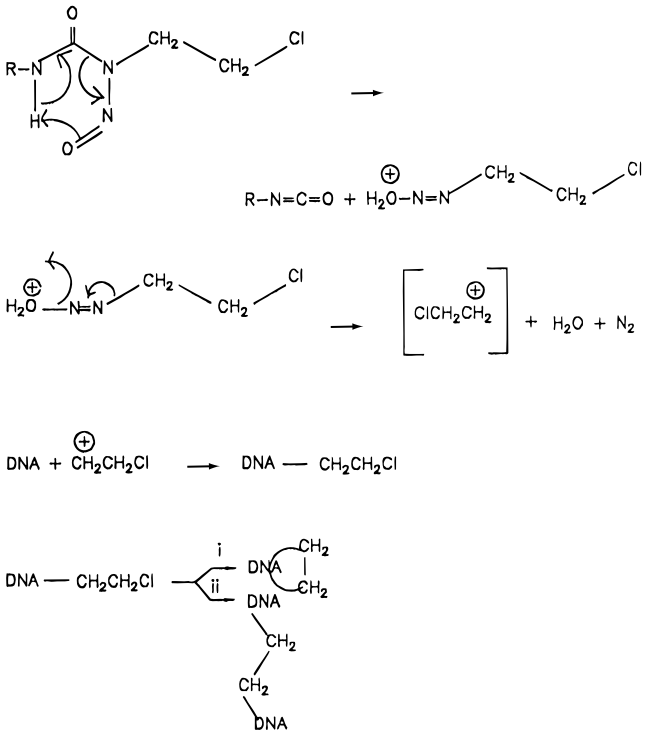
A. Aliphatic, Alicyclic, Aromatic, and Heterocyclic Analogs

The basic biochemical transformations of *N*-nitrosoureas are the intra- and intercellular alkylations, and carbamoylation reactions of various biological macromolecules, whereby the most important interactions involve the DNA. These interactions can significantly affect the healthy and cancerous DNAs during the induction, propagation, and suppression of cancerous cell growth. In the case where the N1 nitrogen of the *N*-nitrosourea is bonded to a 2-chloroethyl moiety, a decomposition can occur to initially give the isocyanate and diazohydroxide fragments (Scheme 14). The carbamoylation reaction of the isocyanate can involve various proteins and/or the DNA and RNA. A subsequent decomposition of the diazohydroxide molecule produces several electrophilic species via the 2-chloroethyl carbocation moiety. The intracellular reactions of this species with the purine and pyrimidine heterobases of the DNA primarily result in the O6 and N7 alkylation "adducts" which can further undergo reactions to form the intrastrand and interstrand cross-linked DNA.

1. Historical Background

During a random screening program of anticancer agents conducted at the Chemotherapy National

Scheme 14



Service Center (CCNSC), it was noted[100] that the compound 1-methyl-3-nitro-1-nitrosoguanidine (**24**, Chart 2), first synthesized in 1947,[101] was weakly active against the ip-inoculated murine L1210 leukemia with a %ILS = 150 at a daily dose of 20 mg/kg.[102,103] The activity against the ip-inoculated L1210 was reduced significantly when either the sc or po routes of administration were chosen.[100,102] However, compound **24** in clinical trials[104] caused lung edema and pleural effusion, venous thrombosis at the site of injection, pulmonary vascular damage, and no evidence of remissions.

The structurally related compound, 1-methyl-1-nitrosourea (MNU, **25**), first synthesized in 1888,[105] was tested and shown[102] to be more effective than **24** in increasing the life span of mice with ip-inoculated leukemia, and almost equally effective against ic-inoculated L1210 leukemia. MNU (**25**) was found[102] to be active against ip-inoculated L1210 whether administered by the ip, sc, or po routes with %ILS values of 187, 174, and 151, respectively. In addition, the activity of MNU (**25**) against the ic-inoculated leukemia with a %ILS of 152 indicated[102] that the drug was capable of crossing the blood−brain barrier. However, compound **25** was not used in clinical trials in the United States because of its instability and the subsequent introduction of more active analogs.[103] Nevertheless, in Russia MNU received much attention with reports[106] of response in patients with undifferentiated carcinoma of the lung and Hodgkin's disease.

A large number of analogs of **24** were synthesized and tested against the ip-inoculated L1210, and the most active compounds were found[107] to be the 1-(2-chloroethyl) and 1-(2-bromoethyl) analogs. Later, compound **26**, the 1-(2-chloroethyl) analog of MNU, was shown[108] to be even more effective against the ip-inoculated L1210 than either of the two aforementioned nitrosoguanidines.

This early work set the stage for an explosive growth in the synthesis and anticancer evaluation of aliphatic, alicyclic, aromatic, and heterocyclic analogs of *N*-nitrosoureas, the so-called first generation compounds.

2. Aliphatic Analogs

a. Mono Nitrosoureas. Among a group of monosubstituted *N*-nitrosoureas **25**−**31** (Table 2), the substitution with either the methyl or 2-chloroethyl group, i.e. compounds **25** and **26**, resulted[43,104] in

Chart 2. Structures for Section VII.A.2

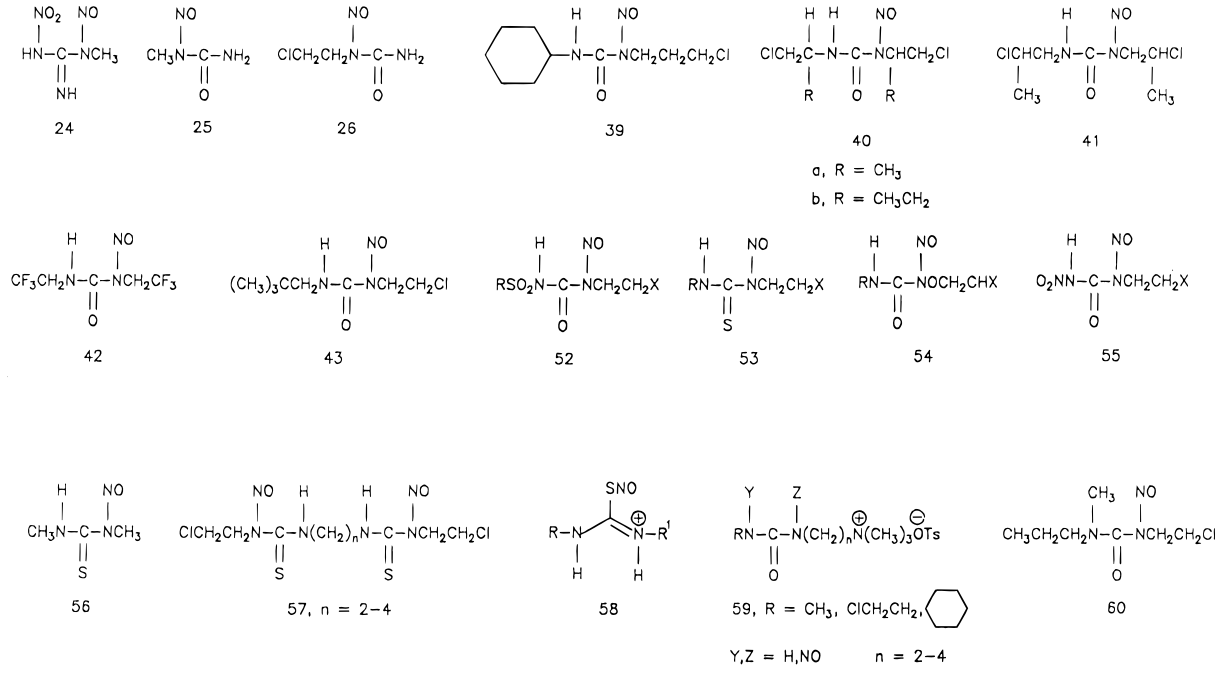


Table 2. Anticancer Activity and Chemical Properties of Aliphatic Nitrosoureas against Ip-Implanted L1210 Lymphoid Leukemia

	$R^1-NH-CO-N(NO)-R^2$				ip		ic						
compound no.	R^1	R^2	OD,[a] mg/kg	ILS_{max},[b] %	log kill[c]	surv,[d] %	log kill[c]	surv,[d] %	ther ratio[e]	$t_{1/2}$,[f] min	alkyl act[g]	log P[h]	ref(s)
25	H	CH_3	12	109					<2	27.6	0.15	−0.30	43,104
26	H	$ClCH_2CH_2$	0.9	131					2.5	34.1	0.03	−0.05	43,104
27	H	CH_3CH_2	50	34									43,104
28	H	$CH_3CH_2CH_2$	100	30						24.0	0.015	0.30	43,104
29	H	$CH_2{=}CHCH_2$	100	32									43,104
30	H	$CH_3(CH_2)_3$	200	29						26.7	<0.01	1.00	43,104
31	H	$(CH_3)_3CCH_2$								23.0	<0.01	1.00	43,104
32	CH_3	CH_3	75	61					<2				43,104
7c	$ClCH_2CH_2$	CH_3	150	46					<2				43,104
33	$ClCH_2CH_2$	$ClCH_2CH_2$	25−30	184	6	0−100	5	0−100		8.13	0.033	1.50	43
34	FCH_2CH_2	FCH_2CH_2	6−7		6	50−60	5	60					44
35	FCH_2CH_2	$ClCH_2CH_2$	32−40	500									44
7b	$F(Cl)CH_2CH_2$ (1:1)	$Cl(F)CH_2CH_2$	20		6	90−100	5	50−90					44
36	$BrCH_2CH_2$	$BrCH_2CH_2$	200		5	20	0	0					44
37	$Br(Cl)CH_2CH_2$ (1:1)	$Cl(Br)CH_2CH_2$	50−75		6	100	0	0					44
38	ICH_2CH_2	ICH_2CH_2	inactive										44
41	$ClCH(CH_3)CH_2$	$ClCH(CH_3)CH_2$	1000		5	20	4	0					44
43	$(CH_3)_3CCH_2$	$ClCH_2CH_2$	75	>275									111
44	$CH_3CH_2OCOCH_2$	$ClCH_2CH_2$	>100	26									44
45	$NCCH_2CH_2$	$NCCH_2CH_2$	75	53					<2				43
46	$NCCH_2CH_2$	$ClCH_2CH_2$	18	40	67								112,113
47	CH_3COCH_2	$ClCH_2CH_2$	10	186	16								113
48	H_2N	$ClCH_2CH_2$	58		100								113
49	$(CH_3)_2N$	$ClCH_2CH_2$	18	129	50								113
50	CH_3NCHO	$ClCH_2CH_2$	105	226	33								113,249
51	CH_3NCOCH_3	$ClCH_2CH_2$	189	229	50								113

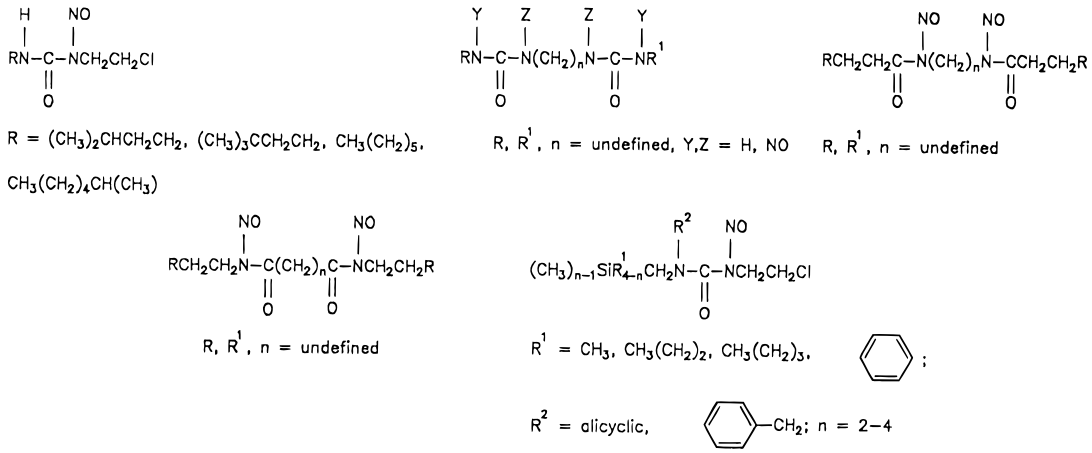
[a] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. Drugs administered on day 1 after tumor implantation. [b] Increase in life span = $[(T - C)/C] \times 100$. [c] Defined in section V. [d] Percentage of treated animals surviving on day 45 after drug administration. [e] Therapeutic ratio = OD/MED; MED, minimum effective dose = daily dose eliciting a 40% increase in life span (ILS_{40}) of the treated animals. [f] Half-life. [g] Alkylating activity, measured by the reaction with 4-(*p*-nitrobenzyl)pyridine, pH 6. [h] Partition coefficient: P = [compound in 1-octanol]/[compound in water].

higher anticancer activity than either a substitution with longer carbon chains or an unsaturated group. Substitution at both nitrogens of the urea with the 2-chloroethyl moiety (Table 2) resulted in the bis(*N*-2-chloroethyl)-*N*-nitrosourea (BCNU, carmustine, **33**) which was found[43] to be the most active agent of a large series of such analogs and more active than the N1 methyl analogs **7c** and **32**. Compounds **26** and **33** were clearly superior to **25** against both the ip- and ic-implanted L1210 leukemia, whether administered by the ip, sc, or po routes.[104] However, hazardous-delayed toxicity from chronic administration of **33** to dogs and monkeys was observed.[104] The ratio of ic-L1210 to ip-L1210 for **26** and **33** was in the range of 0.5−0.7, whereas for a series of anticancer agents such as 5-fluorouracil (5FU) and 6-mercaptopurine (6-MP), the ratio range was only 0.02−0.27.[104] This result indicates that compounds such as **26** and **33** have a balance of hydrophilic and hydrophobic properties at pH 7, as evidenced[104] by their appreciable solubility of 4.5−13 g/L in both physiological saline and 25−150 g/L in the lipid-soluble solvent butyl acetate. In contrast, the drugs 5FU and 6-MP are ionized in water at pH 7 and have low solubilities in both water and butyl acetate. This strong relationship of the water−lipid solubility, degree of ionization, and activity against ic-inoculated L1210 leukemia was in agreement[109] with a contemporaneous theory of drug action. The anticancer activity of BCNU (**33**) against a variety of cancer tumors has been comprehensively reviewed.[17,18]

Among various 1,3-bis(2-haloethyl)-1-nitrosoureas **33**−**38**, the bis-fluoroethyl (BFNU, **34**) and the fluoro−chloro analogs **7b** and **35** were active[44] against both ip- and ic-implanted L1210 leukemia, whereas the bis-bromoethyl compound (BBNU, **36**) and the bromo−chloro compound **37** were only active against ip-implanted L1210 and the bis-iodoethyl compound (BINU, **38**) was inactive against both ip- and ic-implanted L1210 (Table 2). The order of reactivity of the 2-haloethyl compound F, Cl > Br > I is reversed in the C−X bond strengths, i.e. I < Br < Cl < F. Thus, the iodo and bromo analogs **38** and **36** should be more susceptible to nucleophilic attack and, hence, could undergo a decomposition in the plasma before reaching the interior of the cells. The fluoro analogs **34** and **35** are less likely to undergo DNA interstrand cross-linking (section X.B) because the fluorine moiety is a poorer leaving group and, thus, less likely to generate an alkylating species than other halogen derivatives.[10] Consequently, the *N*-(2-fluoroethyl)-*N*-nitrosoureas may exert their anticancer activity by a different mechanism.[10]

The fluoro- and chloroethyl analogs have comparable alkylating and carbamoylating activities but the fluorine-containing compounds are more hydrophilic, i.e. they have lower log P values,[10] and have been reported[110] to be less toxic to bone marrow. However, the median day of animal death is in the range of 2−5 days for the (2-fluoroethyl)nitrosoureas compared with 6−27 days for the (2-chloroethyl)-nitrosoureas.[10] This toxicity result seems to indicate[10] a biochemical formation of fluoroacetate by the alcohol dehydrogenase-catalyzed oxidation of the liberated fluoroethanol. A reversal of this fluoroacetate toxicity is possible through the administration of sodium acetate in ethanol within 10 min of the administration of the drug.[10]

Chart 3. Structures for Section VII.A.2



The placement of the chlorine in the 3-position relative to the urea nitrogen yielded compound **39** which had no anticancer activity.[44] Branching at the 1-position relative to the urea nitrogen produced compound **40** which had no activity against the ip-inoculated L1210.[44] The addition of an alkyl group on the carbon bearing the halogen (**41**) decreased the activity against ip- and ic-inoculated L1210 while replacement of the halogen with a trifluoromethyl group (**42**) abolished the activity.[44] The substitution of a neopentyl group at the N3-position produced compound **43** which was moderately active against the L1210 in vivo.[111] However, the introduction of a 2-heptyl group on the N3-position yielded compound **7h** of low activity.[6] This result supported the contention[6] that either a five- or six-membered ring or a 2-haloethyl moiety attached to the unnitrosated nitrogen is essential for high anticancer activity. The introduction at the N3-position of ester (**44**), nitrile (**45**,**46**), ketone (**47**), amine (**48**), and substituted amines (**49**−**51**) moieties have been reported (Table 2).[43,44,112,113] Some of these compounds e.g. **48**−**51**, are actually semicarbazide and carbazide derivatives.

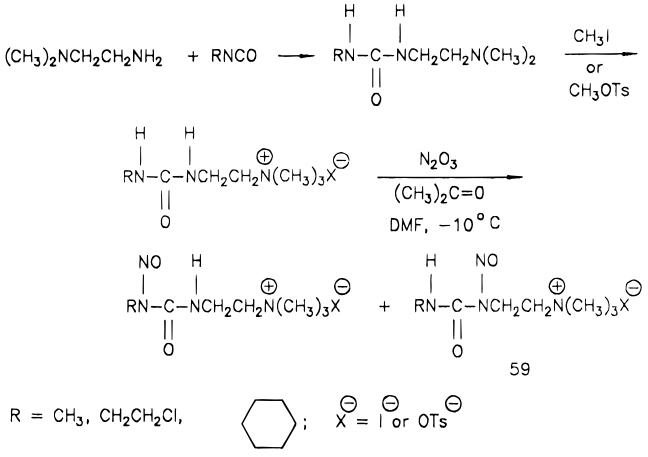
Several other highly substituted and longer chain analogs have been reported[111,114] and are shown in Chart 3. A set of silicon-containing CENU derivatives (Chart 3) were reported[115] to have activity against Ehrlich ascite carcinoma in mice. Alcohol analogs are discussed in section VII.B and carboxyl-containing analogs are discussed in the amino acid section VII.D.

Early attempts[116] to prepare sulfonyl ureas **52**, thioureas **53**, alkoxyureas **54**, and nitroureas **55** were unsuccessful, except for 1,3-dimethyl-1-nitroso-2-thiourea (**56**,[43] Chart 2) which was inactive against the ip-inoculated L1210.[43] Later, several *N*-nitrosothioureas **57** were synthesized;[117−119] however, their antitumor activity was not reported. The nitrosothioureas were synthesized from the respective thioureas, using either sodium nitrite and dilute aqueous hydrochloric acid (0.05−0.1 N),[117,118] or in the solvents dichloromethane or chloroform.[119] Dilute acidic conditions were chosen to favor formation of unprotonated nitrous acid and dinitrogen trioxide. These harder acid species were expected to favor electrophilic attack at the harder nitrogen of the thioureas rather than at the competing sulfur atom. If the nitrosation of the thioureas were carried out at higher acid concentrations (5−6 N) the transient thionitrosyl intermediate **58** was transformed[118] to the corresponding urea by either an elimination of HSNO to give the carbodiimide followed by an addition of water, or by direct attack of water on the amide carbon and displacement of the SNO group. If excess sodium nitrite was employed then the urea would be nitrosated to yield the corresponding *N*-nitrosourea. Detailed NMR studies of ^{13}C-^{15}N-labeled *N*-nitrosothioureas provided[117] explanations for their conformations and decomposition modes in aqueous solution.

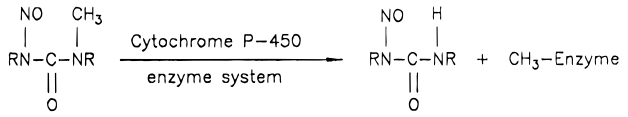
A set of monoquaternary ammonium analogs of general structure **59** were designed[120−122] to contain the choline group as a neuromediator carrier group. It was believed[123,124] that the choline-like fragment could have a selective effect because of the interaction between the increased negative surface charge of the cancer cells and the presence of a positively charged quaternary nitrogen atom. The compounds **59** also were expected[120] to have a high affinity for tumors of connective tissues, since quaternary ammonium salts were observed[125] to be strongly adsorbed by these tissues. The synthesis of the quaternary compounds **59** is outlined in Scheme 15, but no biological activity data was obtainable for these compounds. Studies of the chemical decomposition of these monoquaternary compounds at various pH values in water were reported.[126,127]

Trisubstituted alkylnitrosoureas, with N1 bonded to either an alkyl or a 2-haloethyl group, and N3 bonded to two alkyl groups, one of which is methyl,

Scheme 15



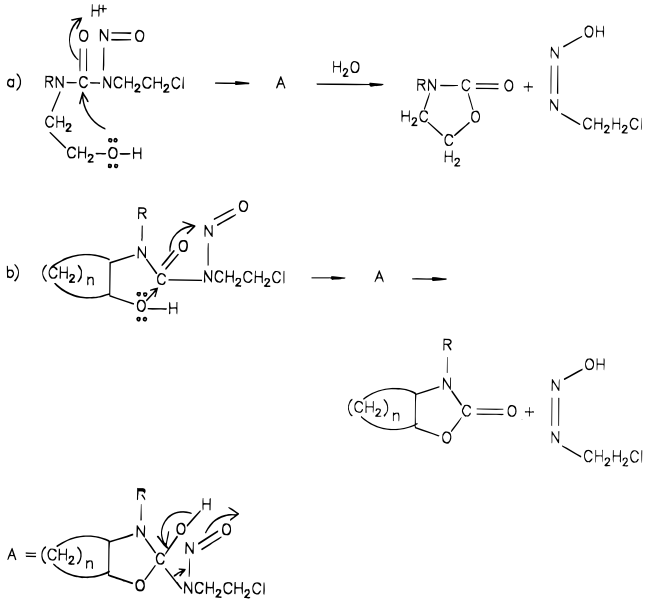
Scheme 16



can be considered as latent forms of biologically active *N*-nitrosoureas.[10] Such stable compounds are metabolized to the carcinogenic N3-monosubstituted nitrosourea presumably by a demethylation reaction[128,129] (Scheme 16). The compound *N*-(2-chloroethyl)-*N′*-methyl-*N*-nitroso-*N′*-propylurea (**60**) was stable in aqueous solution[130−132] and was active against L1210 leukemia only in vivo, lending further support[10] to the concept of an activation of the latent drug by an enzymatic demethylation. Furthermore, compound **60** had the highest therapeutic index of a series of such compounds in which the methyl group was replaced by 2-chloroethyl and cyclohexyl moieties.[130−132] A related series of N3,N3-disubstituted nitrosoureas **61**−**73** were prepared and tested[133] for anticancer activities against the L1210 leukemia (Table 3) and Ehrlich ascites carcinoma. Since these compounds lack a hydrogen on the N3-position they cannot undergo decomposition to an isocyanate and a diazohydroxide. Instead, compounds **65**−**71**, with a hydroxy group on the β-carbon, undergo a decomposition to an oxazolidinone (Scheme 17). This class of nitrosoureas possessed excellent anticancer activities and higher therapeutic ratios (Table 3) than other nitrosoureas.[133]

b. Bis-nitrosoureas. Much attention has been devoted to the synthesis and biological testing of bis-nitrosoureas. The first reported[104] bis-*N*-methylnitrosoamides **74**−**76** (Table 4) were somewhat more active than the simple analog **32**. The series of bis-*N*-methyl-*N*-nitrosoureas **77**−**82** were found[43,104] to have an overall modest increase in activity but no clear correlation was established[43] between the activity of the compounds and the distance between their *N*-nitroso moieties. The compounds with the urethane−urea structures **83** and **84** were much less active than **32** as anticancer agents.[43] Similarly, the biological evaluation[59,134,135] of the bis-*N*-(2-chloroethyl)-*N*-nitrosoureas **85**−**89** (Table 4) against the rat leukemia L5222 revealed little change from that of

Scheme 17



the parent compound BCNU (**33**), and no relationship could be found[59,134] between the polymethylene chain length linking the CENU moieties and their anticancer activities. These compounds decompose to a polymethylene bisisocyanate and a diazohydroxide (Scheme l8a). Thus, compounds **85**−**89** can be considered[59,134] as transport forms for polymethylene isocyanates and could act as bifunctional carbamoylating agents in vivo. The fact that there was so little difference between BCNU and these compounds indicated[59,134] that carbamoylation is not an important process for the anticancer activity.

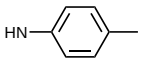
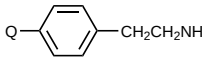
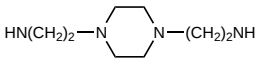
A series of isomeric polymethylenebis(1-nitrosoureas) **90**−**99** (Table 5) were synthesized and tested[136] for anticancer activity against rat ascites hepatoma AH13 and mouse leukemia L1210. These compounds would decompose to the polymethylenebis(diazohydroxide) and hydrogen isocyanate (Scheme 18b). The ethylene and tetramethylene analogs **90** and **92** were the most active compounds against the L1210 leukemia, but no activity was found[136] beyond the C9 compound. Studies were reported[137,138] on the hexamethylene analog **94**. A number of these com-

Table 3. Anticancer Activities of N3,N3-Disubstituted Nitrosoureas against the Ip-Implanted L1210 Lymphoid Leukemia[133]

	$R^1R^2N-CO-N(NO)-CH_2CH_2Cl$				
compound no.	R^1	R^2	OD,[a] mg/kg	ILS_{max},[b] %	ther ratio[c]
61	CH_3	CH_3	12.5	>249	5.2
62	CH_3	$CH_3(CH_2)_3$	100	421	5.3
63	CH_2=$CHCH_2$	CH_2=$CHCH_2$	50	103	6.6
64	$CH_2CH_2OCH_2CH_2$		50	623	4.8
65	CH_3	$HOCH_2CH_2$	25	140	6.6
66	CH_3	$HOCH_2(CHOH)CH_2$	12.5	>433	27.8
67	CH_3	$HOCH_2(CHOH)_4CH_2$	50	84	7.7
68	$CH_3CH_2CH_2$	$HOCH_2(CHOH)CH_2$	12.5	>231	7.6
69	$CH_3(CH_2)_3$	$HOCH_2(CHOH)CH_2$	25	>278	22.7
70	$CH_3(CH_2)_3$	$HOCH_2(CHOH)_4CH_2$	25	143	16.7
71	$HOCH_2CH_2$	$HOCH_2CH_2$	12.5	>688	22.7
72	$AcOCH_2CH_2$	$AcOCH_2CH_2$	3.12	>431	5.6
73	CH_3	$AcOCH_2C(CHOAc)_4CH_2$	6.25	62	2.2
137b (CCNU)			25.0	>757	5.1

[a] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. Drugs administered on days 1−5 after tumor implantation. [b] Increase in life span = $[(T - C)/C] \times 100$. [c] Therapeutic ratio = OD/ILS_{30}; ILS_{30} = daily dose eliciting a 30% increase in life span of the treated animals.

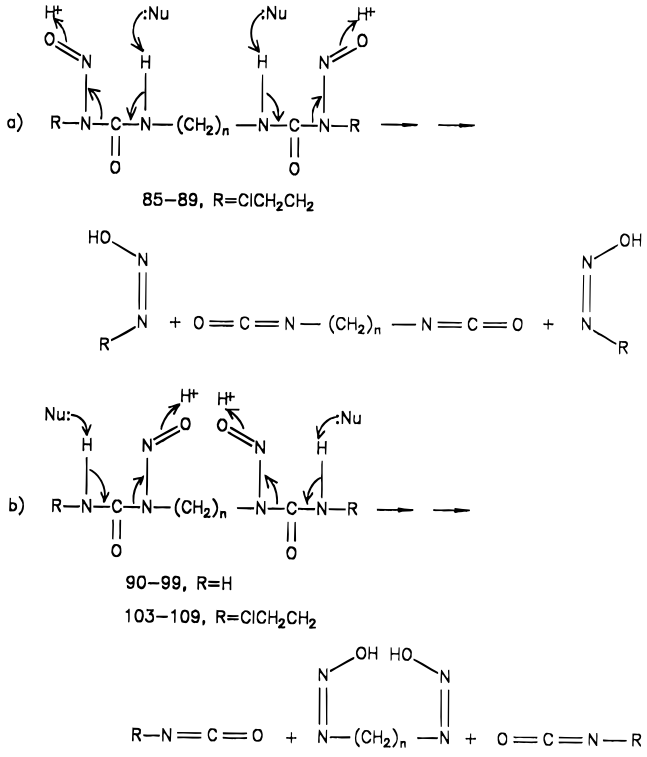
Table 4. Anticancer Activity of Bis-N1-nitrosoureas and Bis-N1-nitrosourea Urethanes against the Ip-Implanted L1210 Leukemia[a] and the L5222 Leukemia[b]

	R−N(NO)−CO−X−CO−N(NO)−R					
compound no.	R	X	OD,[c] mg/kg	ILS_{max},[h] %	survivors,[i] %	ther ratio[j]	ref(s)
74	CH_3	$(CH_2)_3$	100[a,d]	100			104
75	CH_3	$(CH_2)_4$	200[a,d]	63			104
76	CH_3	$(CH_2)_5$	150[a,d]	90			104
77	CH_3	HN-NH	20[a,e]	52		<2	43,104
78	CH_3	$HN(CH_2)_2NH$	<25[a,e]	<35			43
79	CH_3	$HN(CH_2)_3NH$	100[a,e]	100		2	43,104
80	CH_3	$HN(CH_2)_4NH$	200[a,e]	63		2	43,104
81	CH_3	$HN(CH_2)_5NH$	150[a,e]	90		<2	43
82	CH_3		30[a,e]	62		<2	104
83	CH_3	OCH_2CH_2NH	9[a,e]	24			43,104
84	CH_3		25[a,e]	24			43
85	$Cl(CH_2)_2$	$NH(CH_2)_2NH$	29[b,f]	560	60		59,134
86	$Cl(CH_2)_2$	$NH(CH_2)_3NH$	23[b,f]	560	60		59,134
87	$Cl(CH_2)_2$	$NH(CH_2)_4NH$	23[b,f]	560	75		59,134
88	$Cl(CH_2)_2$	$NH(CH_2)_5NH$	23[b,f]	165	30		59,134
89	$Cl(CH_2)_2$	$NH(CH_2)_6NH$	25[b,f]	535	50		59,134
110	$Cl(CH_2)_2$	$HN(CH_2)_2N(CH_3)(CH_2)_2N(CH_3)(CH_2)_2NH$	25[a,g]	181			149
111	$Cl(CH_2)_2$		10[a,g]	125			149

$[HN(CH_2)_2N^+(CH_3)_2(CH_2)_nN^+(CH_3)_2(CH_2)_2NH]\cdot 2Br^- = X$					
compound no.	R	*n*	OD,[c] mg/kg	ILS_{max},[b] %	ref
112	CH_3	4	300[a,g]	46	149
113	$Cl(CH_2)_2$	4	15[a,g]	108	
114	CH_3	6	25[a,g]	0	

[a] Murine lymphoid leukemia. [b] Rat leukemia. [c] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. [d] Drugs administered on days 1−15 after tumor implantation. [e] Drugs administered either on days 1 to death or on day 30 after tumor implantation. [f] Drug administered on day 6 after tumor implantation. [g] Drug administered ip on days 1−5 after tumor implantation. [h] Increase in life span = $[(T - C)/C] \times 100$. [i] Percentage of treated animals surviving on day 60 after tumor implantation. [j] Therapeutic ratio = OD/MED; MED, minimum effective dose = the daily dose resulting in a 40% increase in life span (ILS_{40}) of the treated animals.

Scheme 18



pounds were very active against the AH13 tumor line in vivo.[136,139] The comparison of the activities of the ethylene(bisnitrosourea) (EBNU) (**90**) and BCNU (**33**) revealed[136] that BCNU was more effective after a single ip injection and at lower doses, whereas with EBNU no apparent dose schedule dependency was observed. However, EBNU was found[136] to be more effective against the rat AH13 line than BCNU. A study of the chemical decomposition of EBNU under physiological conditions, including the effects of pH, amino acids, and thiol compounds, was reported.[140] In addition, the interaction of radioactively labeled EBNU with nucleic acids, proteins, and synthetic biopolymers was published.[141,142] The biological mechanisms of EBNU (**90**) are discussed in section X. Various aspects of the chemical properties of EBNU (**90**) could be correlated[143] with its high anticancer activity. Thus, among several polymethylene bisnitrosoureas **90, 91, 94**, and **97**, and mononitrosoureas **25, 26, 28, 30**, and **31**, EBNU (**90**) possessed the shortest half-life of 13.3 min, the greatest alkylating activity, with the exception of MNU (**25**), and the lowest lipophilicity, i.e. the lowest log *P* (*P* = partition coefficient) (Table 5).[143] The inverse relationship between the half-life and the alkylating activity agreed with the results of earlier work[97] on *N*-(2-haloethyl)-*N*-nitrosoureas and mono-*N*-nitrosoureas. The correlation of increased anticancer activity with decreasing hydrophobicity caused by shorter carbon chains, agreed with studies[95] on the relationship of the lipophilicity of *N*-(2-haloethyl)-*N*-nitrosoureas and their activity against ip-implanted L1210 leukemia. However, these correlations were imperfect

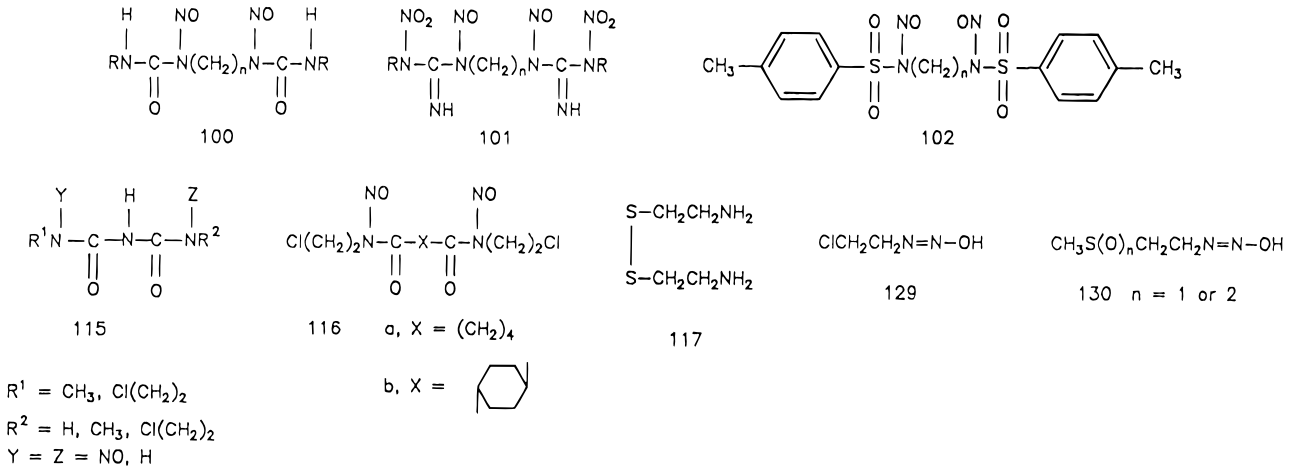
Table 5. Anticancer Activity and Other Data of Bis-N3-nitrosoureas against the Ip-Inoculated L1210 Lymphoid Leukemia

$R^1-NH-CO-N(NO)(CH_2)_nN(NO)-CO-NH-R^2$

compound no.	R^1	R^2	n	OD,[a] mg/kg	ILS_{max},[d] %	survivors,[e] %	$t_{1/2}$ min	alkyl act[h]	LD_{50},[k] nmol/kg	log *P*	ref(s)
90	H	H	2	100[b]	>262	67	13.3[f]	0.12[i]		−1.0[l]	136,144
91	H	H	3	20[b]	52	0	14.5[f]	0.02[i]			136,144
92	H	H	4	50[b]	>209	33					136
93	H	H	5	50[b]	63	0					136
94	H	H	6	100[b]	86	0	29.2[f]	0.02[i]			136,144
95	H	H	7	100[b]	71	0					136
96	H	H	8	400[b]	65	0					136
97	H	H	9	200[b]	34	0		0.02[i]			136,144
98	H	H	10	25[b]	12	0					136
99	H	H	12	200[b]	11	0					136
103	$Cl(CH_2)_2$	$Cl(CH_2)_2$	2	25[c]	0		14.4[g]	24.4[j]	1.24	1.36[m]	145−147
104	$Cl(CH_2)_2$	$Cl(CH_2)_2$	3	9[c]	16		14.8[g]	15.1[j]	0	1.74[m]	145−147
105	$Cl(CH_2)_2$	$Cl(CH_2)_2$	4	100[c]	0		15.4[g]	28.6[j]	>2.80	2.12[m]	145−147
106	$Cl(CH_2)_2$	$Cl(CH_2)_2$	5				16.7[g]	13.5[j]	0	2.47[m]	145−147
107	$Cl(CH_2)_2$	$Cl(CH_2)_2$	6	20[c]	63		17.2[g]	10.0[j]	1.08	2.61[m]	144−147
108	$Cl(CH_2)_2$	$Cl(CH_2)_2$	7	80[c]	93		15.1[g]	19.4[j]	2.00	2.79[m]	145−147
109	$Cl(CH_2)_2$	$Cl(CH_2)_2$	8	100[c]	47		25.1[g]	2.0[j]	3.15	2.97[m]	145−147

[a] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. [b] Drugs administered on days 2 and 6 after tumor implantation. [c] Drugs administered on days 1−10 after tumor implantation. [d] Increase in life span = $[(T - C)/C] \times 100$. [e] Percentage of treated animals surviving on day 60 after tumor implantation. [f] Half-life measured at pH 7.0, 37 °C, in 0.05 M cacodylate buffer containing 3.3% DMSO. [g] Half-life measured at pH 7.2, 37 °C, phosphate buffer. [h] Alkylating activity, measured by the reaction with 4-(*p*-nitrobenzyl)pyridine, pH 6. [i] Modification of literature procedures.[136,144] [j] Modification of literature procedures.[145−147] [k] Lethal dose, single ip-administered dose resulting in 50% death of treated animals. [l] Partition coefficient: *P* = [compound in 1-octanol]/[compound in water (97.5) + DMSO (2.5)]. [m] Partition coefficient: *P* = [compound in octanol]/[compound in water].

Chart 4. Structures for Section VII.A.2

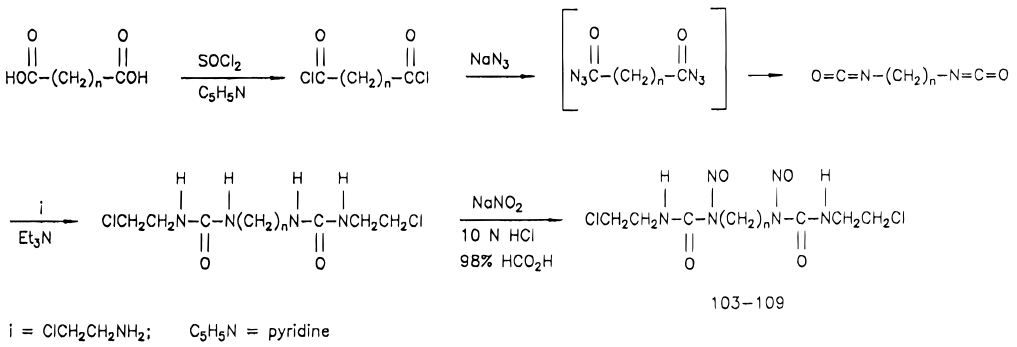


since the half-lives of the mononitrosoureas **25**, **26**, **28**, **30**, and **31** were very similar, i.e. 23.0−34.1min, but their alkylating activities decreased with increasing carbon chain length (Table 2).[143] Furthermore, the bis-*N*-(2-chloroethyl)-*N*-nitrosourea BCNU (**33**) possesses a long half-life, high alkylating activity, and high lipophilicity, i.e. high positive log *P* value (Table 2). Such a compound also has a carbamoylating activity derived from the liberated 2-chloroethyl isocyanate, an activity not available to the bis-nitrosoureas **90**−**99**.

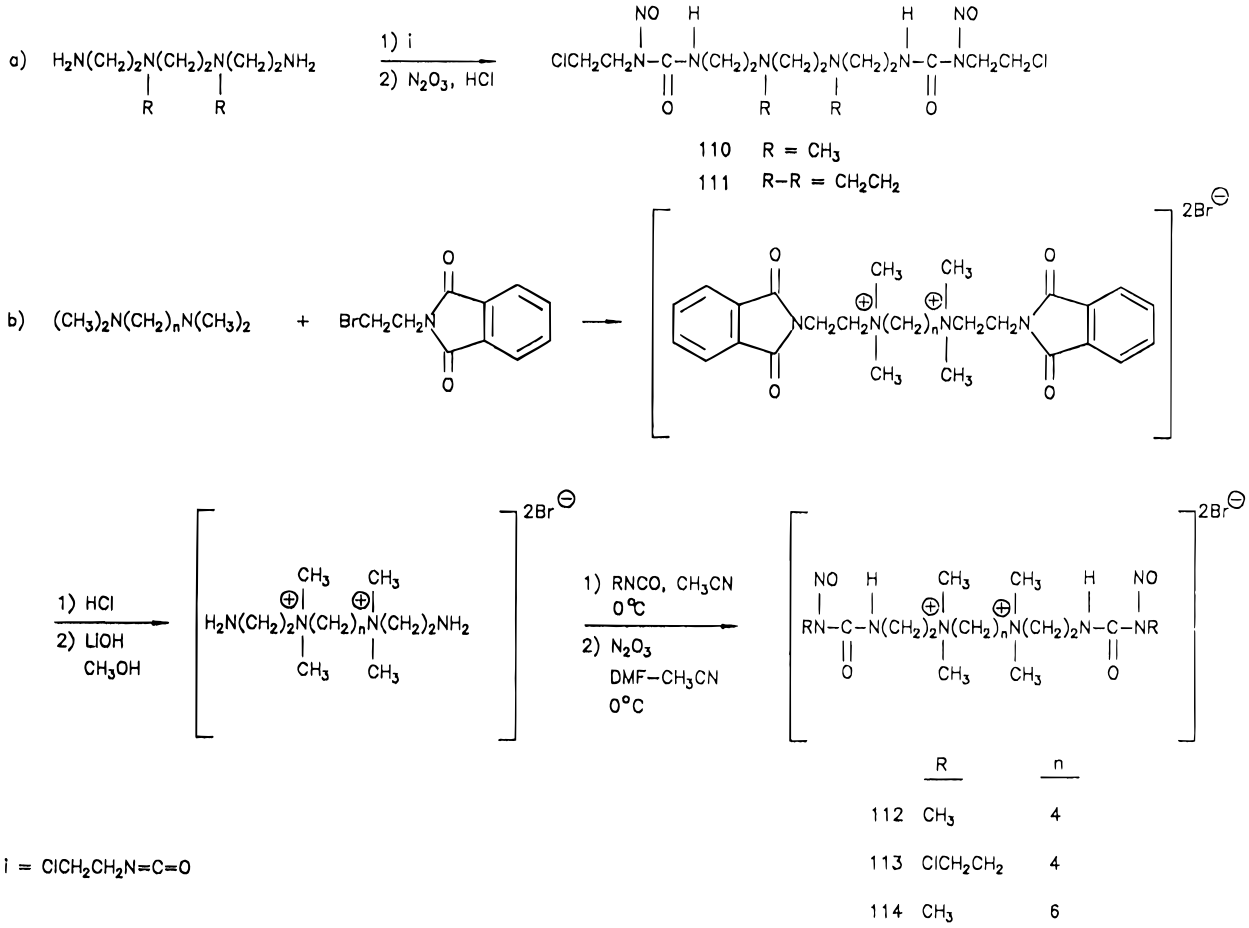
The activity of polymethylenebis(1-nitrosoureas) (**100**, Chart 4) was compared[136] with a series of polymethylenebis(1-nitroso-3-nitroguanidines) (**101**) and polymethylenebis(1-nitroso-*p*-toluenesulfonamides) (**102**). The bis-nitrosoureas were active against both the murine L1210 and the rat AH13 cancer lines, whereas the bisnitrosoguanidines were effective only against the AH13 and the bisnitrosotoluenesulfonamides were inactive against both cancer lines.[136] The following order of decreasing antitumor activities, nitrosoureas > nitrosonitroguanidines > nitrososulfonamides, was in agreement with the order of decreasing instabilities of the functional groups.[136]

A related series of polymethylenebis[*N*-(2-chloroethyl)-*N*-nitrosoureas] **103**−**109** (Table 5) was synthesized[144,145] as shown in Scheme 19. The diacids were converted to their corresponding bisisocyanates via the acid chlorides and acyl azides. Reaction of the isocyanates with 2-chloroethylamine followed by nitrosation with a mixture of hydrochloric acid−formic acid yielded the desired bis-nitrosoureas **103**−**109**. These compounds would be expected[146] to decompose to a polymethylene bisdiazohydroxide and 2-chloroethyl isocyanate (Scheme 18b). The highest activity among these polymethylene compounds was found for the C_6 to C_8 analogs **107**−**109** (Table 5). There were no direct correlations between the anticancer activity and alkylating activity, i.e. compounds **103** and **105** as compared to **107**, nor the expected inverse correlation between half-life and alkylating

Scheme 19



Scheme 20



activity (Table 5).[146] In this series the more lipophilic analogs were also the more cytotoxic. A comparison of the anticancer activities of the two sets of isomeric polymethylene bisCENUs **85**−**89** and **103**−**109** is presented in section VIII. Some undefined polymethylene bisCENUs were reported.[147]

The bistertiary **110** and **111**, and bisquaternary **112**−**114** ammonium analogs (Table 4) were synthesized,[148,149] because it was found[125] that the quaternary ammonium cations are capable of selectively accumulating in connective tissue as a result of ionic bonding with sulfate and phosphate anions present in the chondroitin sulfate and cellular DNA, resulting in bridged structures. The synthesis of these compounds is outlined in Scheme 20a,b. These bisquaternary congeners were more active[149] than the monoquaternary compounds **59**, and the bistertiary amine CENU (**110**) was the most active against L1210 leukemia in vivo (Table 4). Lengthening the distance between the nitrogen atoms caused an increase in the toxicity of these compounds.[149] These compounds also were evaluated[149] against the solid tumor lines of mammary adenocarcinoma, Lewis lung carcinoma, and sarcoma.

Several bisnitroureas, *N*-nitroso derivatives of biuret **115**, biurea **77** and carboxamides **116a**,**b** (Chart 4) possessed weak to moderate activity against the L1210 leukemia in vivo.[150] Their activities were generally inferior to those of the corresponding *N*-nitrosoureas and, thus, were not deemed worthy of further developmental work.[150]

c. Sulfur- and Phosphorus-Containing Nitrosoureas. An interesting set of bis-*N*-nitrosoureas **118**−**121** (Table 6) were derived from the disulfide diamine cystamine **117**. Such compounds should be sufficiently lipophilic to cross the blood−brain barrier because the disulfide bond could be metabolized in vivo by reductive cleavage of the S−S bond and

Table 6. Anticancer Activity of CENU Cysteamine Analogs against the Ip-Implanted L1210 Lymphoid Leukemia

	$R^1-CH_2CH_2-S-S-CH_2CH_2-R^2$						
compound no.	R^1	R^2	OD,[a] mg/kg	ILS_{max},[d] %	survivors,[e] %	LD_{50},[f] mg/kg	ref(s)
118	$CH_3N(NO)CONH$	$CH_3N(NO)CONH$	40[b]	40			43
119	$Cl(CH_2)_2N(NO)CONH$	$Cl(CH_2)_2N(NO)CONH$	10[c]			28	151,157
			12.5[c]		100		
			18[b]	900	90		151,156,157
120	$Cl(CH_2)_2NHCON(NO)$	$Cl(CH_2)_2NHCON(NO)$	20[c]		0	375	151,157
121	$Cl(CH_2)_2NHCON(NO)$	$Cl(CH_2)_2N(NO)CONH$	20[c]		100	95	151,157
CNCC = **120** + **121** + trace **119**			30[c]		70	75	151,157
122	$Cl(CH_2)_2N(NO)CONH$	N_3CONH	18[b]	900	100		156

[a] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. [b] Drug administered on day 1 to day of death after tumor implantation. [c] Drug administered on days 1, 5, and 9 after tumor implantation. [d] Increase in life span = $[(T - C)/C] \times 100$. [e] Percentage of treated animals surviving on day 60 after tumor implantation. [f] Lethal dose = single ip-administered dose resulting in a 50% death of treated animals.

Scheme 21

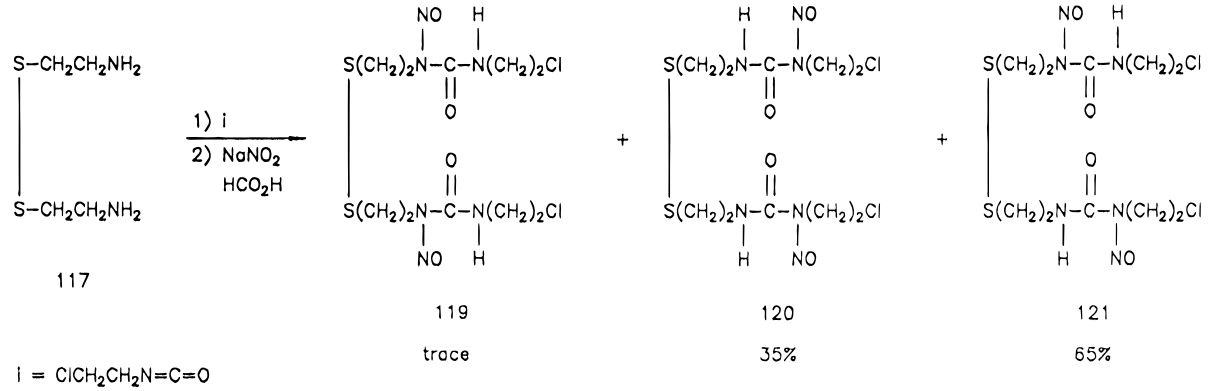


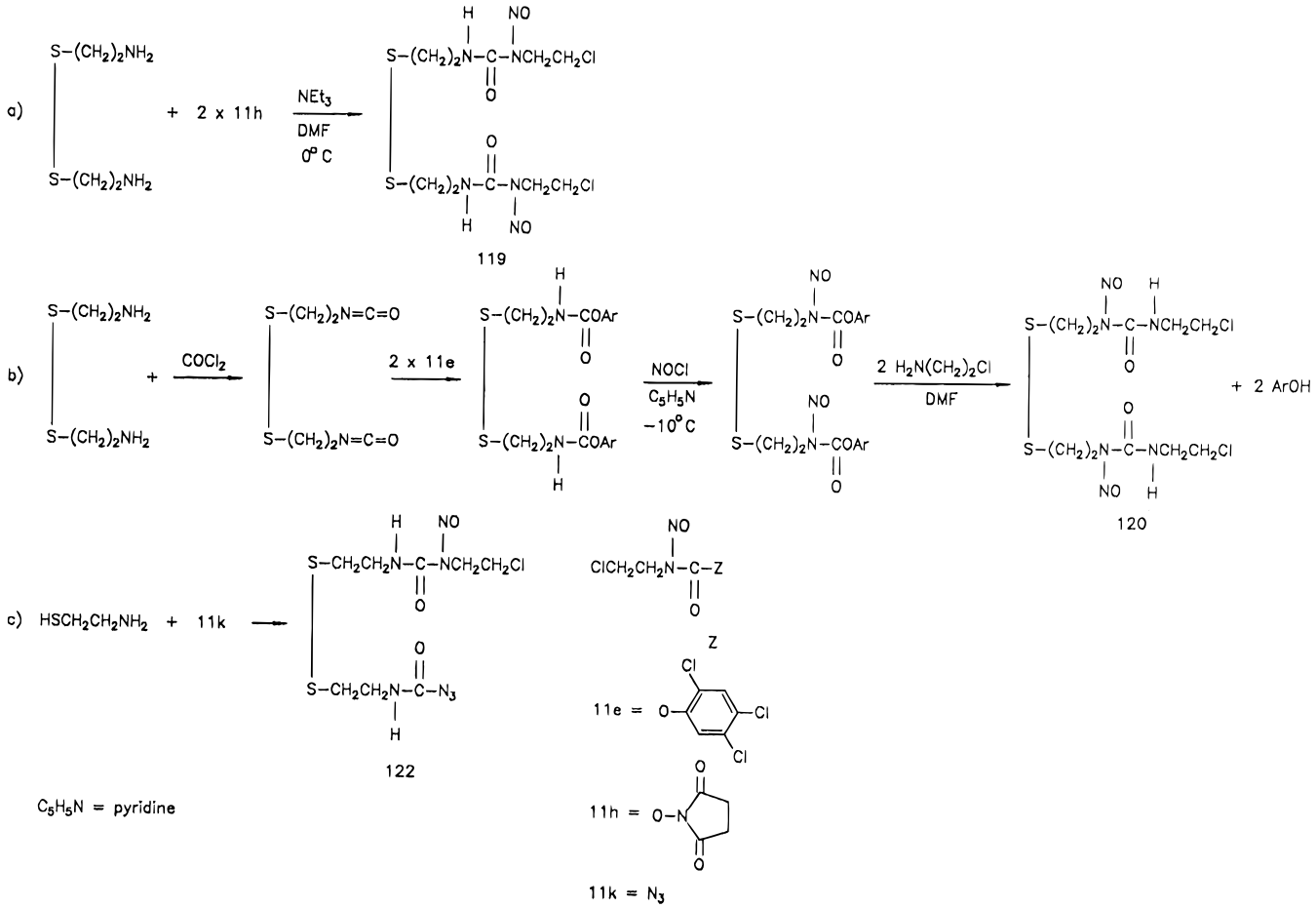
Table 7. Anticancer Activity of CENU Cysteamine Metabolites and CENU Taurine Analogs against the Ip- and Iv-Implanted L1210 Lymphoid Leukemia

	$R-CH_2CH_2N(Y)-CO-N(Z)-CH_2CH_2Cl$							
compound no.	R	Y	Z	OD,[a] mg/kg	ILS_{max},[f] %	survivors,[h] %	LD_{50},[i] mg/kg	ref(s)
123	CH_3SO	NO	H	40−250[b,d]	ns[g]		70	167,169
124	CH_3SO_2	NO	H	40−250[b,d]	ns[g]		160	167,169
125	CH_3SO	H	NO	40[b,d]		100	25[d]	167,169
126	CH_3SO_2	H	NO	40[b,d]		100	50[d]	167,169
CNCC				50[b,d]		100	75[e]	167,169
123 + **125** (70:30)				125[c,d]		100		167,169
				125[b,d]		100		167,169
124 + **126** (70:30)				115[c,d]		100		167,169
				115[b,d]		100		167,169
131	$(CH_3)_2NSO_2$	H	NO	31[c,e]	>500	90		170
132	CH_3NHSO_2	H	NO	31[c,e]	>500	100		170
133	H_2NSO_2	H	NO	16[c,e]	>500	78		170

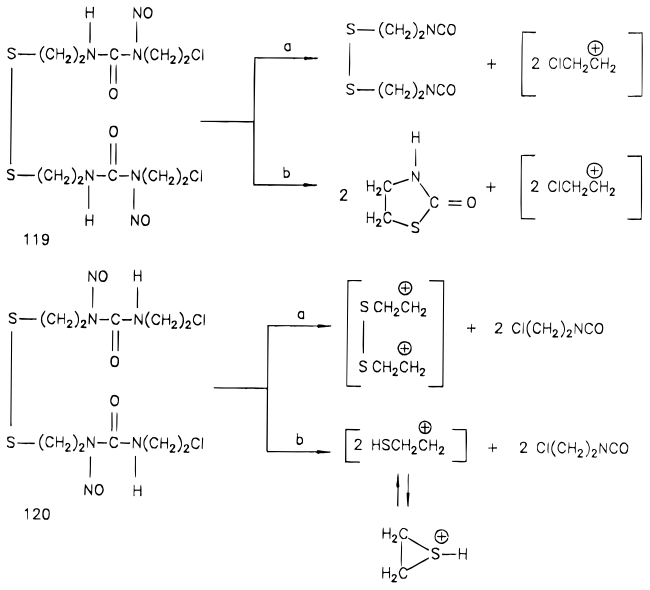
[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Tumor cells administered by the iv route. [c] Tumor cells administered by the ip route. [d] Drug administered on days 1, 5, and 9 after tumor implantation. [e] Drug administered on day 1 after tumor implantation. [f] Increase in life span = $[(T - C)/C] \times 100$. [g] ns = not significant, % ILS <25. [h] Percentage of treated animals surviving on day 90 after tumor implantation. [i] Lethal dose = single ip-administered dose resulting in 50% death of untreated animals.

subsequent oxidation of the thiol group.[151] The reaction of **117** with 2-chloroethyl isocyanate yielded the bisurea which was nitrosated with sodium nitrite in formic acid to give a mixture of bis-nitrosoureas **119** (trace), **120** (35%), and **121** (65%) (Scheme 21).[151−153] This mixture of chloroethylnitrosoureas of cystamine was called CNCC. Another synthetic method, utilizing *S*-tritylcysteamine as the starting material, produced[154] the same mixture **119**−**121**. The isomers **119** and **120** were synthesized[155,156] in pure form by the reactions shown in Scheme 22. The use of the transfer agents **11h** and **11e** proved to be important in the synthesis of the pure isomers. A byproduct of the use of the acyl azide transfer agent **11k** was the mono-nitrosourea−acyl azide **122**.[156] Significant activity was obtained[151,157−159] with the mixture CNCC against the P388 and L1210 murine cancers as judged by the percentage of survivors (Table 6), as well as against the five sc-transplanted solid tumors, i.e. the Lewis lung carcinoma, colon 26 carcinoma, TM2 mammary adenocarcinoma, M555 ovarian carcinoma, and B16 melanoma. However, no activity was evidenced against fibrosarcoma and glioma cancers.[159] A comparative study of the CNCC mixture with the individual isomers **119**−**121** revealed[160] that the activity of the mixture was attributable primarily to the N1−N3′ isomer **121**. CNCC was slightly less active than **119** and **121**

Scheme 22



Scheme 23

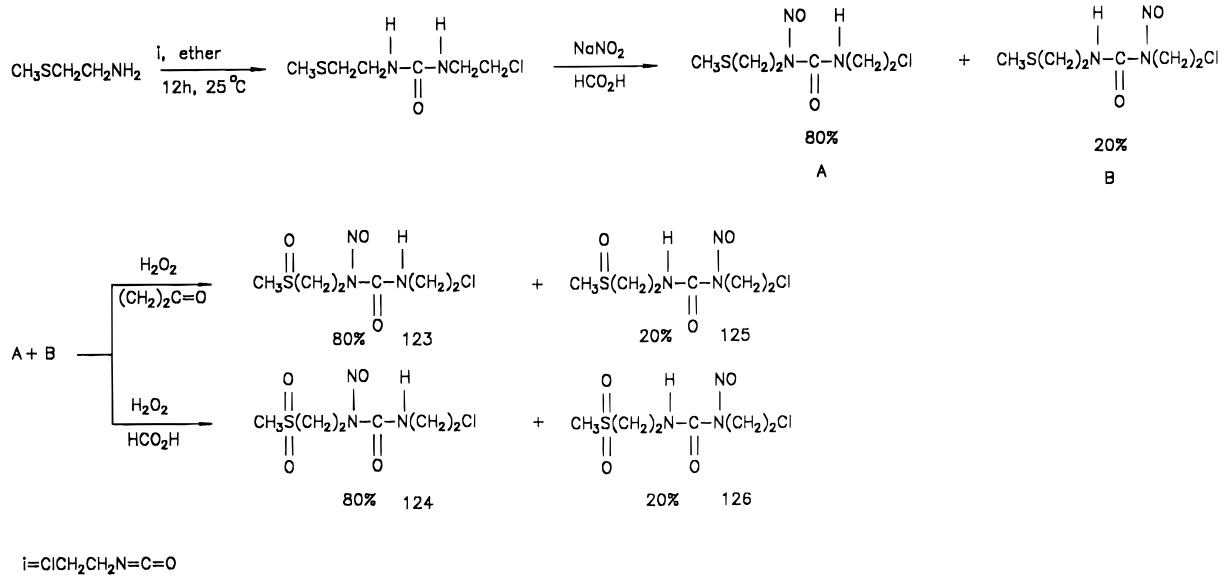


against L1210 leukemia. The 3,3′ isomer **120** was less toxic and less active than either the 1,1′ isomer **119** or the 1,3′ isomer **121** (Table 6). The greater activity and toxicity of the 1,1′ isomer **119** than that of the 3,3′ isomer **120** can be explained[157,160] by the Scheme 23. Thus, the decomposition of **119** results in the formation of the 2-chloroethyl carbocation which causes an alkylation and cross-linking of the DNA, whereas the decomposition of **120** forms a thioethyl carbocation which cannot participate in the cross-linking of the DNA. The aqueous decomposition of CNCC is discussed[161] in section IX.

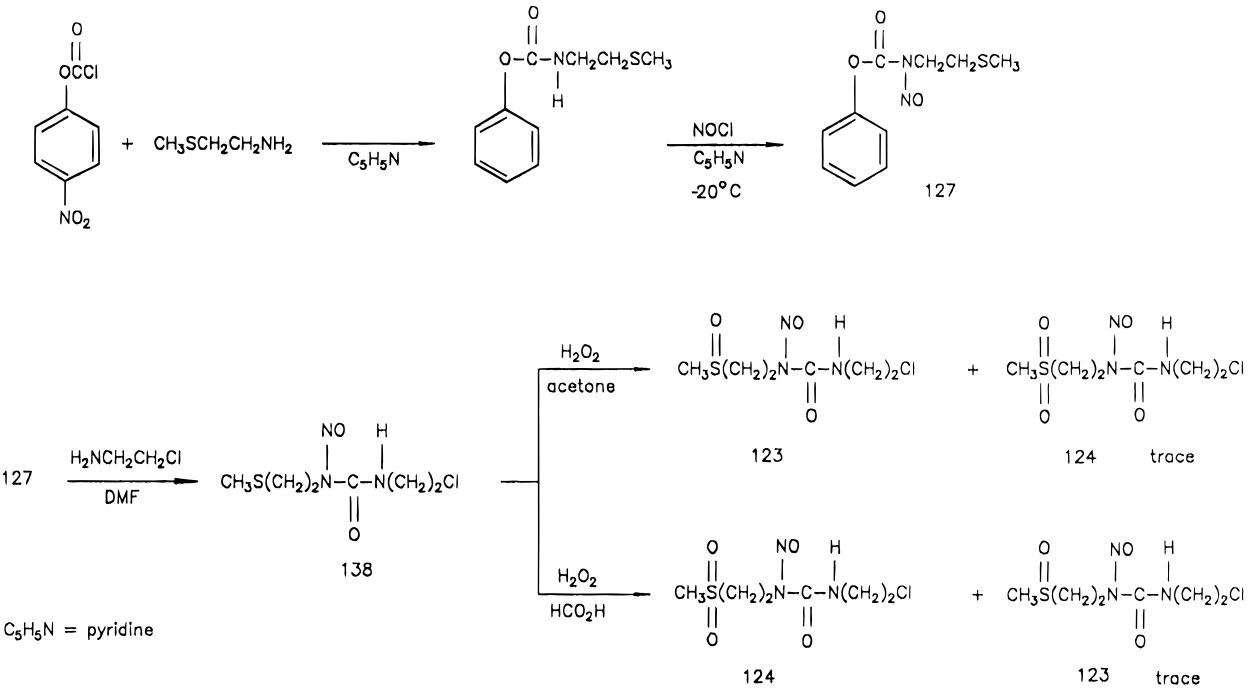
Metabolic studies of CNCC in vivo revealed[162−166] the formation of four metabolites **123**−**126** (Table 7) resulting from the cleavage of the disulfide bond followed by methylation and oxidation of the sulfur atom. The metabolites **123**−**126** have been synthesized[167,168] as mixtures of **123** and **124**, and **125** and **126** (Scheme 24), and as pure **123** and **124** (Scheme 25), and **125** and **126** (Scheme 26). The mixtures were separated by low-pressure liquid chromatography. An unambiguous positioning of the nitroso group was established by the use of the intermediate transfer agents **127** and **128** (Schemes 25 and 26). Oxidation of the intermediate sulfides with hydrogen peroxide in acetone produced the sulfoxides, while the use of hydrogen peroxide in formic acid yielded the sulfones.[167,168]

The four metabolites of CNCC were tested[167,169] for activity against both the ip-and iv-implanted L1210 (Table 7). In order to compare the anticancer activity of the CNCC mixture with those of its metabolites, a 70:30 mixture i.e. the expected cleavage ratio of the disulfide bond of CNCC, of either **123** and **125** or **124** and **126** were administered to mice by the ip route. In both cases 50−100% survivors at day 90 were observed.[167] After the intravenous administration of both mixtures high activity was also observed (Table 7). The four metabolites were also tested separately against iv-inoculated L1210.[167] Compounds **123** and **124** were inactive while compounds **125** and **126** exhibited complete 90 day cures at doses of 40 and

Scheme 24



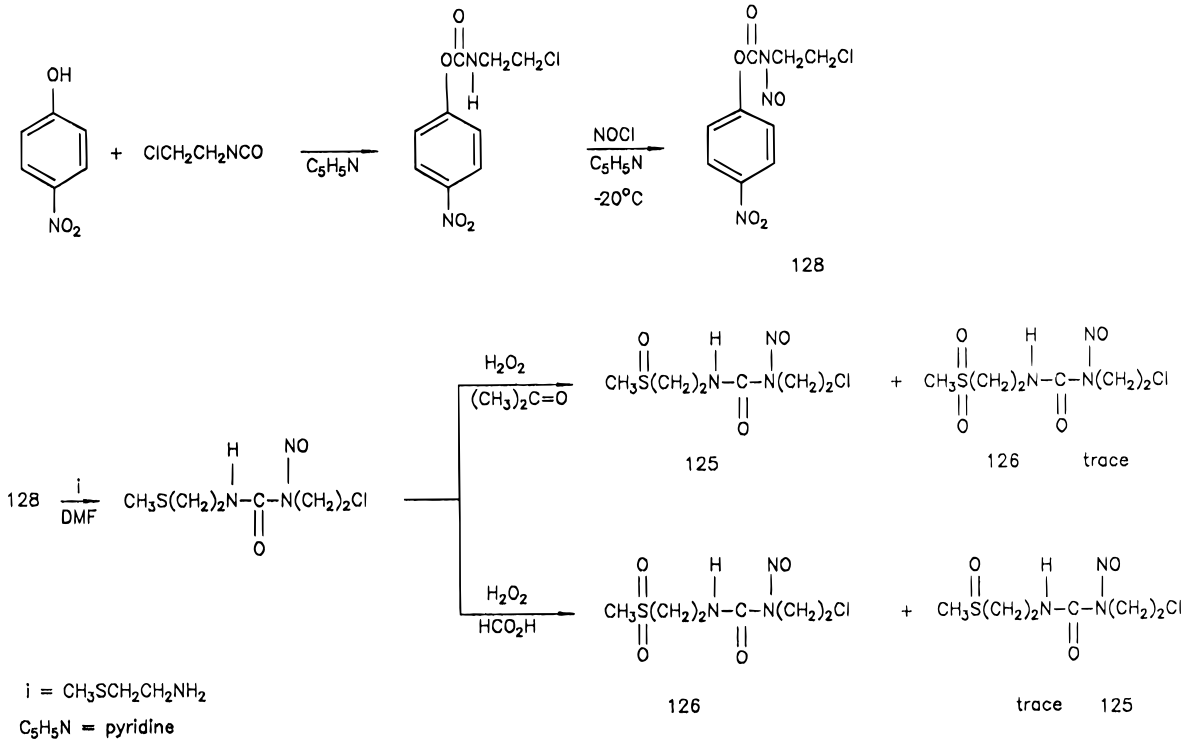
Scheme 25



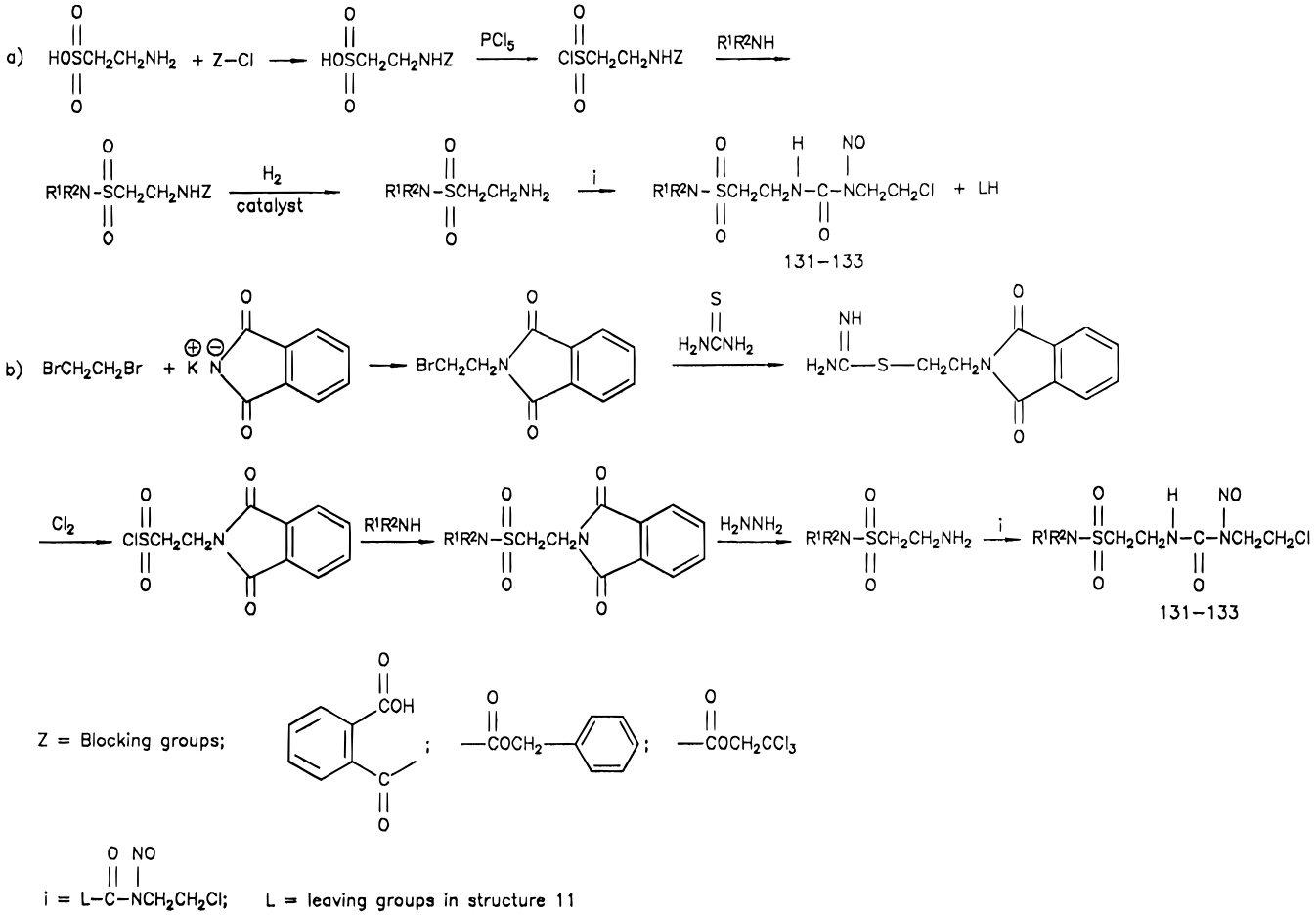
60 μmol/kg (Table 7). It was assumed[167,169] that the biochemical decomposition of **125** and **126** would lead to a very reactive bioalkylating species **129** which could cross-link the DNA, whereas **123** and **124** would decompose to a much less reactive monoalkylating species **130** (Chart 4). The two cytotoxic metabolites, the sulfoxide, perimustine (**125**), and the sulfone, cystemustine (**126**), were compared[169] with CNCC against 12 cancer models in vivo. Both compounds were found[169] to be at least as active as CNCC against eight of the leukemia lymphomas and solid cancers, but, more importantly, the two metabolites caused a larger percentage of long-term survivors on day 90 than the parent mixture CNCC. This result was particularly clear for cystemustine **126** as compared to CNCC against the B16 melanoma, 26 glioma, and ic-implanted L1210 leukemia.[169] A review was published[170] on cystemustine (**126**) containing the aspects of synthesis, pharmacology, pharmacokinetics, metabolism, and clinical studies. Fluorine analogs of **125** and **126** were reported[171] to be more active against L1210, but toxic effects resulting from the release of fluoroethanol limited their use in humans.

Tauromustine (TCNU, **131**), a CENU analog of the amino acid taurine, as well as its two probable metabolites **132** and **133** (Table 7) were synthesized by two methods,[172] as outlined in Scheme 27. The justification for choosing taurine as a carrier for nitrosoureas was based on the hypothesis[173] that taurine interacts with membrane phospholipids, thereby modifying their function. Thus, it was hypothesized that once inside the cell taurine could leak out slowly and, hence, was expected[172] to be a good carrier for anticancer moieties. The anticancer activity of compounds **131**−**133** against L1210 leukemia (Table 7), Walker mammary carcinoma, Lewis lung carcinoma, Harding−Passey melanoma, and colon carcinoma C26 was equal to or better than that of BCNU (**33**), and several other *N*-nitrosoureas. A

Scheme 26



Scheme 27



major difference between TCNU and other nitrosoureas was the high level of the unmetabolized drug found[172] in the plasma of dogs and cancer patients after oral administration. The presence of taurine in the structure was shown[174] not to influence either the cytotoxicity or the alkylating and carbamoylating properties of TCNU. TCNU was highly active against three murine adenocarcinomas of the colon, i.e. MAC 13, MAC 15A, and MAC 26.[175] The high activity was maintained in the ip, ic, and iv

Table 8. Anticancer Activity of Phosphorus-Containing CENU Analogs against Ip-Implanted L1210 Leukemia[a] and P388 Leukemia[b]

R = −NH−CO−N(NO)−CH_2CH_2Cl					
compound no.	parent	OD,[c] mg/kg	ILS_{max},[g] %	survivors,[h] %	ref
134	$(CH_3CH_2O)_2P(=O)CH_2CH_2R$	50[b,d]		88	179
		12.5[b,e]	195	33	
		30[a,d]		100	
		40[a,d,f]	100	17	
135	$(CH_3)_2P(=O)CH_2R$	22[b]	92		184
		25[a]	160		
136	$CH_3P(=O)(CH_2R)_2$	28[b]	149		184
		80[a]	435		

[a] Murine lymphoid leukemia. [b] Murine lymphocytic leukemia. [c] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. [d] Drug administered on day 1 after tumor implantation. [e] Drug administered on days 1−9 after tumor implantation. [f] Tumor implanted by ic route. [g] Increase in life span = $[(T - C)/C] \times 100$. [h] Percentage of treated animals surviving on day 60 after tumor implantation.

Table 9. Anticancer Activity of Alicyclic Nitrosoureas and Bisnitrosoureas against Ip- and Ic-Implanted L1210 Lymphoid Leukemia

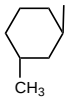
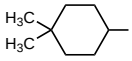
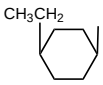
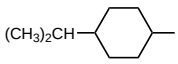
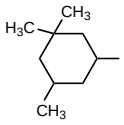
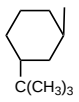
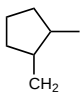
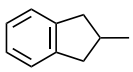
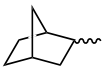
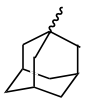
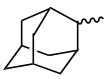
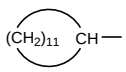
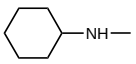
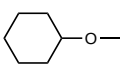
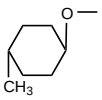
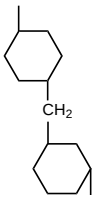
RNH−CO−N(NO)−CH_2CH_2X					ip			ic				
compound no.	R	X	OD,[a] mg/kg	ILS_{max},[d] %	log kill[e]	survivors, %	ther ratio[i]	log kill[e]	survivors, %	ther ratio[i]	log *P*[l]	ref(s)
137a		F	30−45[b]		6	70−90[f]		5	60−100[f]			44
137b		Cl	40−50[b]		6	80−100[f]	0.77	5	20−100[f]	0.53	2.8	44
137c		Br	300[b]		5	30[f]		0	0[f]			44
138a		F					0.57			0.76	2.7	116
138b		Cl	30−45[b]		6	90−100[f]	0.43	4	20−50[f]	>1	3.3	44,116
139		Cl	30−45[b]		6	90−100[f]						116
140		Cl	62[b]		6	70−100[f]	0.48	5	50−80[f]		3.3	116
141		Cl					0.38				3.3	116
142		F					0.94	nt[j]			3.83	116
143a		F					>1.00	nt[j]				116
143b		Cl					0.31			1.00		116
144		F					0.76	nt[j]				44
145		Cl	750[b]		6	100		nt[j]				44, 116
146		Cl	375−500[b]		6	20−80		4	20			44, 116
147		Cl					0.9	nt[j]				116
148		Cl					>1	nt[j]				116
149		Cl	64−93*b*		5	30[g]		6	70[g]		2.19	44
		Cl	16[c]	100		0[h]						119
	thiourea	Cl	16[c]	370		100[h]						119
150		Cl	125−150[b]		6	100[g]		5	90[g]			44

Table 9. (Continued)

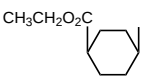
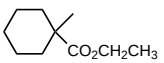
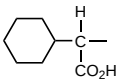
RNH−CO−N(NO)−CH_2CH_2X					ip			ic				
compound no.	R	X	OD,[a] mg/kg	ILS_{max},[d] %	log kill[e]	survivors, %	ther ratio[i]	log kill[e]	survivors, %	ther ratio[i]	log P[l]	ref(s)
151		Cl	62−125[b]		5	30[g]		2	0[g]			44
152a		F	46−59[b]		6	90−100[g]		5	80−90[g]			44
152b		Cl	58−62[b]		6	100[g]		5	80−90[g]			
153		Cl	300[b]		3	0[g]		nt[j]				44
154a		F	187[b]		5	20[g]		2	0			44
154b		Cl	62[b]		6	70[g]		<2	0			116
155a		F					>1	nt[j]				
155b		Cl					0.31			>1.0		116
156a		F					ns[k]		ns[k]			116
156b		Cl					0.79		ns[k]			
157		Cl	750−840[b]		5	30[g]	ns[k]	<2	0			44
158		OH	28[c]	0							1.46	146
159		Cl									4.31[m]	146
160		Cl	9[c]	19							4.81[m]	146
161		Cl	150[b]		6	80[g]		4	0[g]			44
162a		F	80−250[b]		6	100[g]						44,185
162b		Cl	255[b]		6	100[g]	0.26	5	60[g]		2.7	
163		Cl					0.48				2.7	185
164		Cl					0.38				3.1	185

[a] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. [b] Drug administered on day 1 or 2 after tumor implantation. [c] Drug administered on days 1−8 after tumor implantation. [d] Increase in life span = $[(T - C)/C] \times 100$. [e] Defined in section V. [f] Percentage of treated animals surviving on day 45 after tumor implantation. [g] Percentage of treated animals surviving on day 60 after tumor implantation. [h] Percent of treated animals surviving on day 30 after tumor implantation. [i] Therapeutic ratio = Ed_{50}/LD_{10}; ED_{50} = dose resulting in a 50% 45 day survival of treated mice as determined from log−dose probit−survival plots; LD_{10} = dose resulting in 10% death of untreated mice as determined from log−dose probit−survival plots. [j] Not tested. [k] Not significant. [l] Partition coefficient: P = [compound in 1-octanol]/[compound in water]. [m] Calculated values.

routes of administration. It was suggested[175] that TCNU could be useful in the treatment of human large bowel cancer; however, bone marrow toxicity[176] could be a limiting factor in a clinical application of TCNU. Discussion of the pharmacokinetics,[172] toxicology,[172] and metabolism[174,177] of TCNU as well as a brief review of TCNU[178] were reported.

A high anticancer activity for a novel phosphonate CENU analog (**134**, fotemustine, S 10036) was observed[179] against the ip-implanted P388 and L1210 leukemias (Table 8). The parent compound, β-aminoethylphosphonate, can be considered as an isotere of the amino acid β-alanine. Compound **134** was found[179] to be inactive against murine cancers which were resistant to BCNU. Various aspects of the metabolism and pharmacokinetics[180−182] of **134** were reported. Fotemustine was shown[180] to be a weak inhibitor of glutathione reductase activity from rat

Table 10. Anticancer Activity of Alicyclic Nitrosourea Analogs Containing Halogen, Acetoxy, Carboxylic Acid, and Ester Moieties against the Ip- and Ic-Implanted L1210 Lymphoid Leukemia

RNH−CO−N(NO)−CH_2CH_2X				ip			ic				
compound no.	R	X	OD,[a] mg/kg	log kill[b]	survivors,[c] %	ther ratio[d]	log kill[b]	survivors,[c] %	ther ratio[d]	log *P*[e]	ref(s)
165		Cl	46−61	6	80−100	0.56	5	30−70		2.7	44,185
166		Cl	50−72	6	30−90	0.63	4	50		2.7	44,185
167		Cl				0.69				2.7	185
168a		F				0.50				2.0	185
168b		Cl				0.40			0.55	2.6	116,185
169a		F				0.79				2.5	185
169b		Cl				0.50				2.1	185
170		Cl				0.45				2.6	185
171		Cl				0.62				-1.1	185
172		Cl				0.56		inactive		3.1	116,185
173		Cl				0.52 0.39			>1	3.1	185 116
174		Cl	250	6	70−80		4	20			44
175		Cl				0.63				0.0	185

[a] Optimal dose = daily ip-administered dose resulting in a maximum log kill and largest percentage of survivals. [b] Defined in section V. [c] Percentage of treated mice surviving on day 45 after tumor implantation. [d] Therapeutic ratio = ED_{50}/LD_{10}; ED_{50} = dose resulting in a 50% 45 day survival of treated mice as determined from log−dose probit−survival plots; LD_{10} = dose resulting in 10% death of untreated mice as determined from log−dose probit−survival plots. [e] Partition coefficient: *P* = [compound in 1-octanol]/[compound in water].

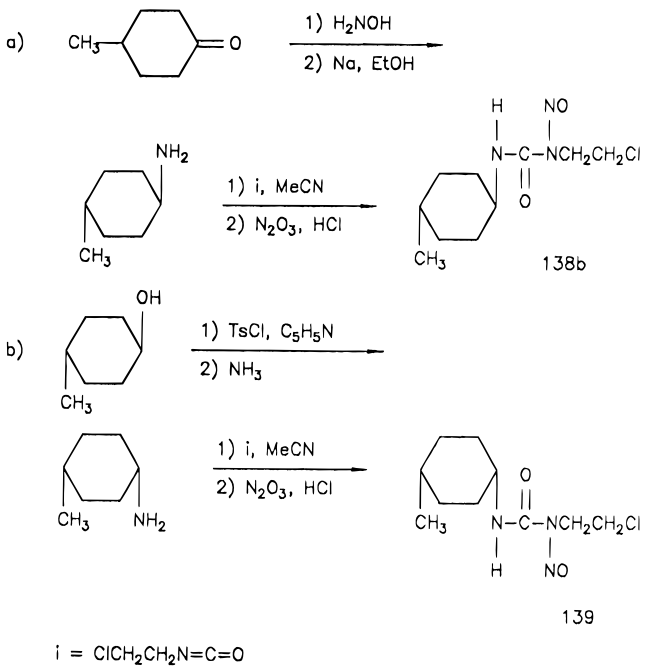
liver, lung, and kidney cytosols. Fotemustine-sensitive melanoma cells contained more thioredoxin reductase than glutathione reductase, whereas fotemustine-resistant cells contained more glutathione reductase than thioredoxin reductase.[182] The induction of alternative electron donors for ribonucleotides, such as thioredoxin reductase and glutathione reductase, was believed[182] to determine the response of the melanomas to the drug. An early clinical phase II study of **134** against disseminated malignant melanoma was reported.[183]

The CENU analogs of tertiary phosphine oxides **135** and **136** were synthesized[184] by the reaction of the corresponding monoamine and diamine with the acyl azide transfer agent **11k**. These compounds exhibited[184] moderate to high in vivo activity against the P388 and L1210 leukemias (Table 8). The bis-nitrosourea **136** was much more active and less toxic than the mono-nitrosourea **135**.

3. Alicyclic Analogs

Early in the history of nitrosourea research it was discovered[44] that the *N*-cyclohexyl-*N′*-(2-haloethyl)-*N′*-nitrosoureas (FCNU, **137a**) and CCNU (lomustine, **137b**) had excellent activities against both the ip- and ic-implanted L1210 leukemia, as was demonstrated by the number of survivors on day 45 (Table 9). As a result of this work a large number of alkyl-substituted cyclohexyl analogs **137**−**164** (Table 9) and halide, acetoxy, carboxy, carbomethoxy, and carbethoxy analogs **165**−**175** (Table 10) were synthesized and screened[44,116] against the L1210 cell line by means of the log kill and therapeutic ratio ED_{50}/LD_{10} criteria. A number of analogs which included substituted cyclohexyl **138**−**141**, **145**, cyclopentyl **149**, methylcyclopentyl **150**, and 2-norbornyl **152**, but did not include 2-indanyl **151**, bornyl **153**, 1-adamantyl **156**, or cyclododecyl **157**, were found[44,116] to be highly active against both the ip- and ic-inoculated L1210, with their therapeutic ratios ranging from 0.28 to 0.77 (Table 9). The *trans*-1,4-bis-nitrosourea analogs of cyclohexane **162a**,**b** also were very active (Table 9).[44] From this series of compounds the *trans*-(4-methylcyclohexyl)-CENU analog, MeCCNU (**138b**), was particularly effective. The pure *trans*-(4-methylcyclohexyl)-CENU (**138b**) and pure *cis*-(4-methyl-

Scheme 28

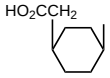
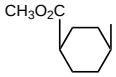


cyclohexyl)-CENU (**139**) were synthesized as shown in Scheme 28a,b.[116,185] An analysis of the infrared spectra of ureas and nitrosoureas prepared from *cis* and *trans* 2 chloroethyl cyclohexylamine led to the conclusion[44] that axial chlorine atoms are *cis* and equatorial chlorine atoms are *trans* and, thus, the ureido group is equatorial in every instance. By similar reasoning the *trans* isomer **138b** is diequatorial and the *cis* isomer structure **139** has the axial−equatorial conformation.[44]

In order to ascertain the structure−activity relationships of the 4-methylcyclohexyl analogs a series of *cis* and *trans* pairs **176** and **177**, **178** and **179**, **180** and **181**, and **182** and **183** were synthesized and tested[185] for activity against the L1210, as determined by the therapeutic index ED_{50}/LD_{10} (Table 11). These congeners of **138b**, with three exceptions, were highly active, affecting cure rates of > 50% at the LD_{10} dose and even lower doses (Table 11).[185] The therapeutic indices ranged from 0.28 to 0.69. Generally, the 4-substituted cyclohexyl analogs were the most active against the L1210 and among these compounds, the *trans* diequatorial isomers **177**, **179**, **181**, and **183** were either as active or more active than the *cis* axial−equatorial isomers **176**, **178**, **180**, and **182** in terms of more favorable therapeutic indices ED_{50}/LD_{10}.[185,186] A screening involving the iv-administered L1210 revealed that the *trans*-MeCCNU (**138b**) was twice as active at the LD_{10} dose and 2.5 times more toxic than the *cis* analog **139**.[185] No obvious correlations were found[185] between the therapeutic index and the partition coefficient among the hydrophobic compounds, for example, 0.77 and 2.8 for CCNU (**137b**), 0.43 and 3.3 for MeCCNU (**138b**) and 0.49 and 1.5 for BCNU (**33**), and among the hydrophilic compounds containing the carboxyl group, 0.53 and −0.6 for **180** and 0.30 and −0.6 for **181**.

The 2-fluoroethylcyclohexyl compounds generally exhibited[44] equal activity with CCNU against both the ip- and ic-implanted L1210 leukemia. In addition, the 2-fluoroethyl congener FCNU (**137a**) of CCNU (**137b**) was less myelosuppressive than CCNU,

Table 11. Anticancer Activity[185] of Stereochemical Pairs of Alicyclic Nitrosourea Analogs against the Ip- and Ic-Implanted L1210 Lymphoid Leukemia

RNH−CO−N(NO)−CH_2CH_2X				
compound no.	R	X	ther ratio[a]	log P[b]
176		Cl	0.53	2.4
177a		F	toxic	−1.7
177b		Cl	0.62	−1.1
178		Cl	0.52	2.4
179a		F	toxic	−1.7
179b		Cl	0.57	−1.1
180		Cl	0.53	−0.6
181		Cl	0.30	−0.6
182		Cl	0.28	2.6
183		Cl	0.54	2.6

[a] Therapeutic ratio = ED_{50}/LD_{10}; ED_{50} = dose resulting in a 50% 45 day survival of treated mice as determined from log-dose probit-survival plots; LD_{10} = dose resulting in 10% death of untreated mice as determined from log-dose probit-survival plots. [b] Partition coefficient: P = [compound in 1-octanol]/[compound in water].

using the spleen colony assay for hematopoietic stem cells.[110] However, **137a** was neurotoxic in the test animals but this toxicity could be decreased by an addition of either sodium acetate or sodium citrate.[110] Compound **137a** was found[187] to have high activity against the ip- and ic-implanted solid tumor B16 melanoma. Interestingly, the substitution of the cyclohexyl group in FCNU with either the 2,6-dioxo-3-piperidyl, 2,6-*cis*-dihydroxycyclohexyl, 2-*cis*-hydroxycyclohexyl, 2-D-glucopyranosyl, or 2-hydroxyethyl moieties did not markedly influence the therapeutic range, whereas similar substitutions enhanced the potency in the CCNU series.[187]

The Lewis lung carcinoma was adopted[186] as a secondary screen to differentiate between nitrosoureas with equal activities against the L1210 leukemia. Two forms of the disease were utilized,[186] i.e. the early form, treated 1−2 days post-implant before the metastases occur and the advanced form, treated 7 days post-implant when growth characteristics of the primary cancer have changed and metastases have occurred. With a few exceptions the most active compounds against the early form were also active against the advanced form.[186] The most active drugs were the *trans*-4-substituted cyclohexyl analogs of CCNU, including the *trans*-methyl **138b**, *trans*-*N*-(2-chloroethyl)-*N*-nitrosourea **162b**, *trans*-carboxy **179b**, and *trans*-carbethoxy **173**.[186] The *cis* analogs of these isomers, and several fluorine-containing analogs **177a** and **179a**, were all inactive against the advanced form of Lewis lung carcinoma.[186]

Scheme 29

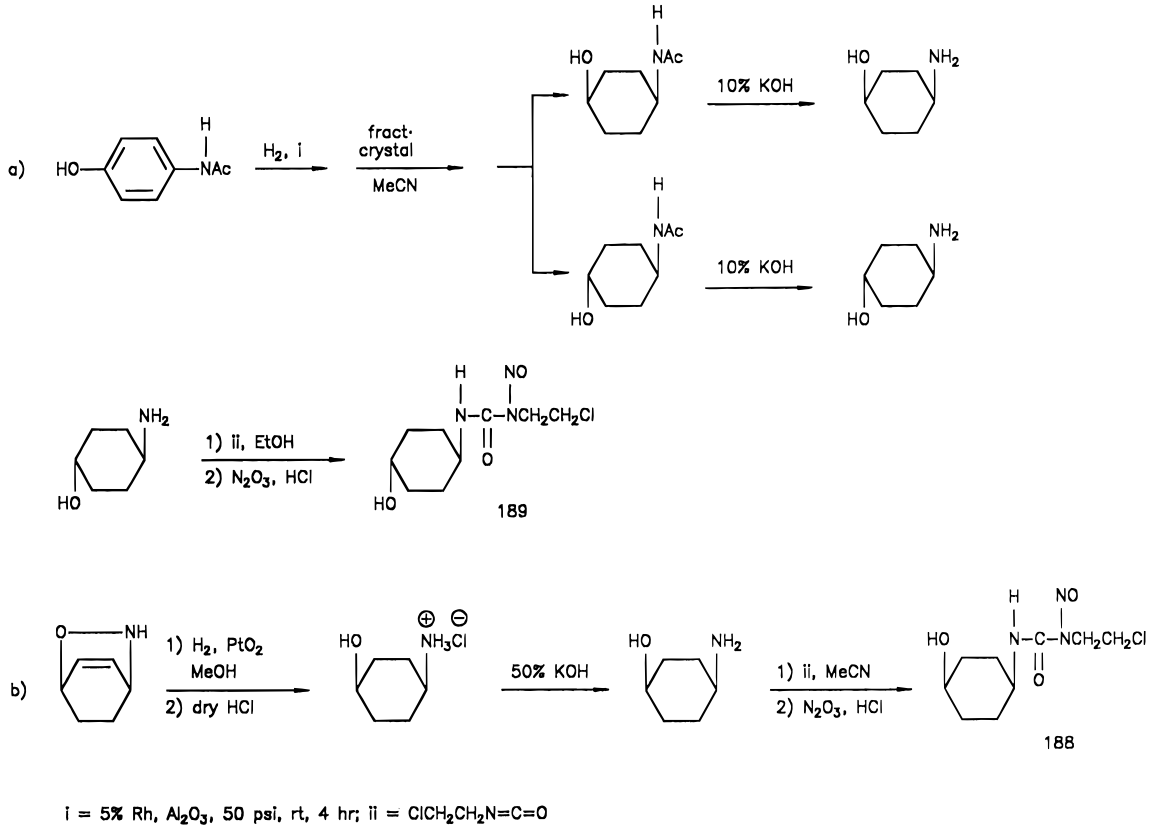


Table 12. Anticancer Activity of Monohydroxycyclohexyl Metabolites of CCNU (137b) against the Ip- and Ic-Implanted L1210 Lymphoid Leukemia

RNH−CO−N(NO)−CH_2CH_2Cl					ther ratio[h]			
compound no.	R	OD,[a] mg/kg	ILS_{max},[f] %	survivors,[g] %	ip	ic	log P[i]	ref(s)
184		25[b,d]	267	50	0.44	0.42	1.6	198−200
185		10[b,d]	410	90	0.29	0.52	1.3	198−200
186					0.37	0.54	1.25	198
187					0.42	0.53	1.28	198
188		40[c,d] 40[c,e]	58	90 100	0.47 0.65	0.41	1.1	185,197,198
189		20[c,d] 30[c,e]		100 100	0.33 0.40	0.43	1.0	185,197,198
137b		38[c,d] 57[c,e]	l44 111	90 70				197

[a] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. [b] Drugs were administered on day 2 after tumor implantation. [c] Drugs were administered on day 1 after tumor implantation. [d] Drugs were administered by the ip route. [e] Drugs were administered by the ic route. [f] Increase in life span = $[(T - C)/C] \times 100$. [g] Percentage of treated mice surviving on day 45 after tumor implantation. [h] Therapeutic ratio = ED_{50}/LD_{10}; ED_{50} = dose resulting in a 50% 45 day survival of treated mice as determined from log-dose probit-survival plots. LD_{10} = dose resulting in 10% death of treated mice as determined from log-dose probit-survival plots. [i] Partition coefficient: P = [compound in 1-octanol]/[compound in water].

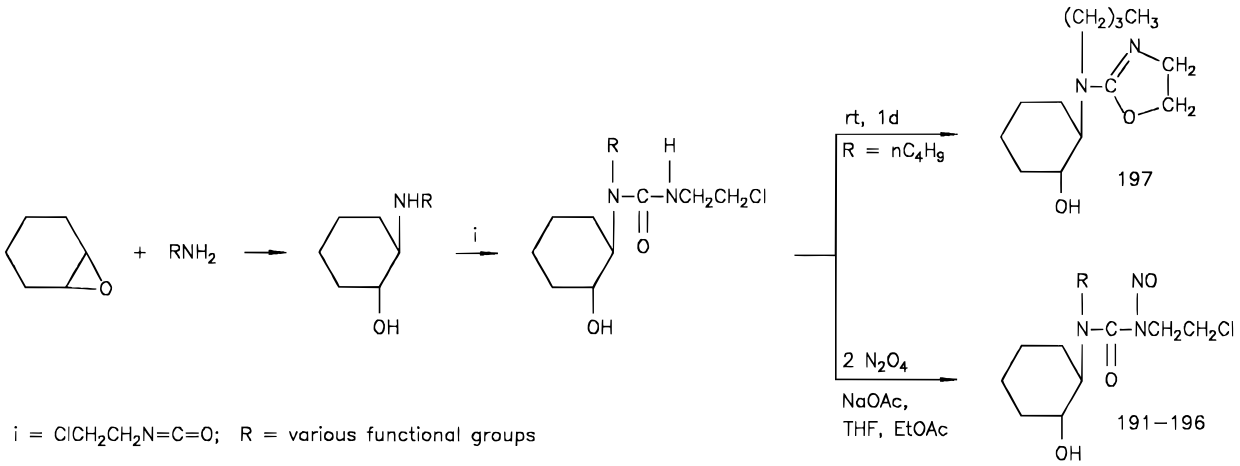
While it was possible, using the early and advanced Lewis lung carcinoma model, to differentiate nitrosoureas with the same activities against the murine L1210 leukemia, it was not possible to determine whether such differences also applied to human cancers.[186] In general, the most active compounds against the Lewis lung carcinoma also were very active against other rodent solid cancers, such as Walker adenocarcinoma 256, B16 melanotic melanoma, C3H mammary adenocarcinoma, and colon No. 36 adenocarcinoma. These similarities of responses were assumed[186] to indicate that there are

Table 13. Anticancer Activity of N3,N3-Disubstituted Alicyclic CENU Analogs against Lewis Lung Carcinoma[a,b] and Ip-Implanted L1210 Leukemia[c]

	$R^1R^2N-CO-N(NO)-CH_2CH_2Cl$						
compound no.	R^1	R^2	OD,[d] mg/kg	ILS_{max},[h] %	survivors,[i] %	ther ratio[j]	ref
190		CH_3	600[a,e] 400[a,e] 400[b,f]		70,90 40 40		201
							
191		CH_3	25[c,g]	757		12.5	202
192		$CH_3(CH_2)_3$	50[c,g]	757		10.0	202
193		$CH_3OCH_2CH_2$	25[c,g]	757		12.5	202
194		$HOCH_2CH_2$	12.5[c,g]	757		25.0	202
195		$CH_3CH(OH)CH_2$	12.5[c,g]	757		12.5	202
196		$HOCH_2CH(OH)CH_2$	12.5[c,g]	114		31.3	202

[a] Early form of Lewis lung carcinoma. [b] Late form of Lewis lung carcinoma. [c] Murine lymphoid leukemia. [d] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. [e] Drug administered on day 2 after tumor implantation. [f] Drug administered on days 7 and 14 after tumor implantation. [g] Drug administered on days 1−5 after tumor implantation. [h] Increase in life span = $[(T - C)/C] \times 100$. [i] Percentage of mice surviving on day 80 after tumor implantation. [j] Therapeutic ratio = OD/ILS_{30}; ILS_{30} = daily dose resulting in a 30% increase in life span of the treated animals.

Scheme 30



common structural characteristics which are essential for cytotoxicity against solid cancers. Reviews were published[13,17,18,188] on the antitumor activity of BCNU (**33**), CCNU (**137b**), and MeCCNU (**138b**) against leukemias and many solid cancers as well as combination therapies[189,190] involving these agents.

The incubation of CCNU (**137b**) in the presence of rat liver microsomes, NADPH, and oxygen resulted[191−195] in the monohydroxylation of the cyclohexane ring. Five of the six possible monohydroxy isomers (**184**−**189**)—*trans*-2-**185**, *cis*-3-**186**, *trans*-3-**187**, *cis*-4-**188**, and *trans*-4-**189** (Table 12) were obtained. However, the human plasma contained only a 1:1 mixture of the *cis*-4- and *trans*-4-hydroxy metabolites after administration of CCNU.[196] An important finding about the anticancer activity of CCNU was that the rate of metabolic hydroxylation exceeds the rate of chemical decomposition. On the basis of this result it was concluded[6,191] that the hydroxylated metabolites are the immediate precursors of the biologically active species. A more thorough discussion of the metabolism of both CCNU (**137b**) and MeCCNU (**128b**) is found in section XI.A.

All six of the monohydroxy-CCNU analogs **184**−**189** were synthesized.[194,197,198] Representative syntheses of the major metabolites, *cis*-4- and *trans*-4-hydroxy-CCNU, **188** and **189**, are illustrated in Scheme 29a,b. The catalytic reduction of (4-hydroxyphenyl)-*N*-acetamide produced a mixture of the *cis*- and *trans*-*N*-(4-hydroxycyclohexyl)acetamide. The stereoisomers were separated by fractional crystallization from acetonitrile, and then the pure *cis*-4- and *trans*-4-hydroxyl analogs were hydrolyzed to the free amines with aqueous potassium hydroxide solution.[197] The reaction of pure *trans*-4-aminocyclohexanol with 2-chloroethyl isocyanate, followed by nitrosation of the intermediate urea with a combination of 1 N hydrochloric acid and dinitrogen trioxide at 0 °C, produced the desired *trans*-4-hydroxy-CCNU (**189**) (Scheme 29a). The preferred route[197] (Scheme 29b) to the *cis*-4-hydroxy-CCNU (**188**) began with the catalytic reduction of 2-oxa-3-azabicyclohexyl[2.2.2]-oct-5-ene hydrochloride to give *cis*-4-aminocyclohexanol hydrochloride. Conversion of the amine to the urea, followed by nitrosation yielded **188**.[197]

A screening of the *cis*-4- and *trans*-4-hydroxy-CCNU **188** and **189** as well as CCNU (**137b**) against ip- and ic-implanted L1210 in mice revealed[197] that both monohydroxylated metabolites were more active and more toxic, i.e. had lower LD_{10} values, than the parent CCNU against both L1210 systems (Table 12). These results supported[197] the concept that the anticancer activity of CCNU is caused mainly by its hydroxylated metabolites. The *trans*-4-hydroxy ana-

Scheme 31

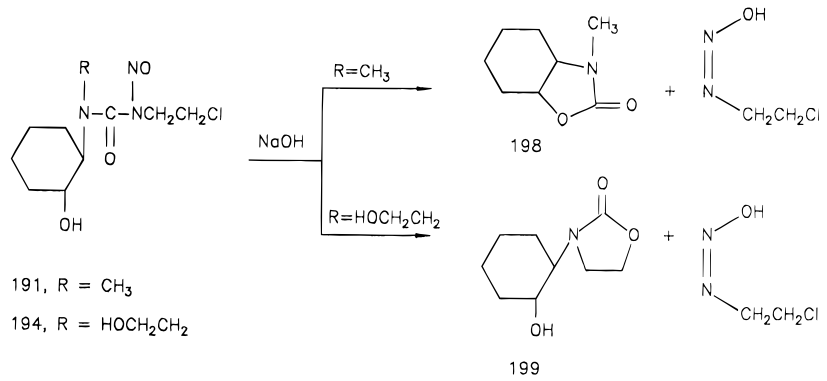
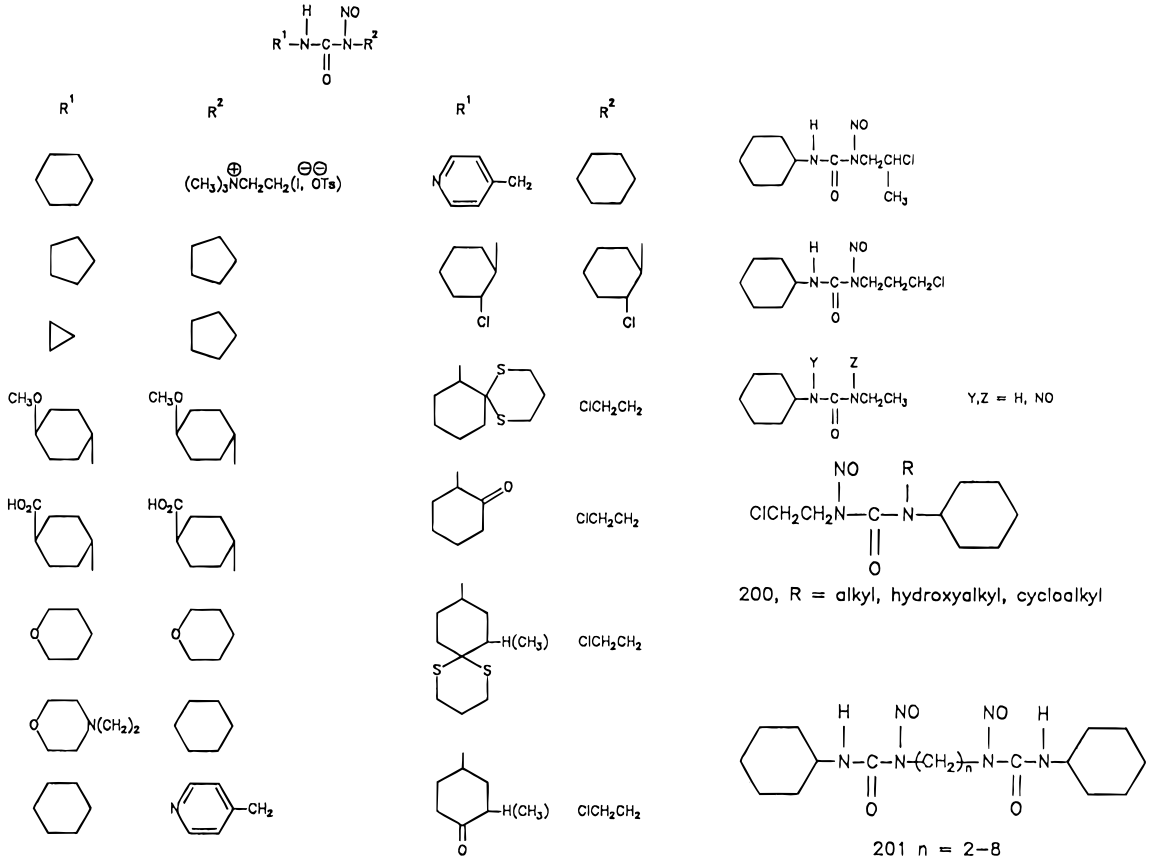


Chart 5. Structures for Section VII.A.3

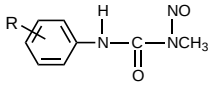
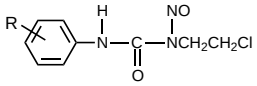


log **189** displayed[197] the most favorable therapeutic indices ED_{50}/LD_{10} of all the monohydroxylated CCNU metabolites against the L1210 leukemia.

Various chemical and biological properties[198−200] of the six monohydroxy cyclohexyl analogs of CCNU (**184**−**189**) and certain polyhydroxy and carbohydrate analogs are discussed in section VIII. The N2-fluoroethyl analog of *cis*-2-hydroxy-CCNU **184b** was shown[187] to have either equal or higher activity than the chlorine analog **184a** against the solid murine cancers B16 melanoma and Lewis lung carcinoma. This compound was synthesized by the reaction of the water-insoluble amine with the fluorinated transfer agent **11e**.

A series of N3,N3-disubstituted CCNU analogs **190**−**196** (Table 13) are further examples of the latent *N*-nitrosoureas.[10] The compound **190**, an analog of the *cis*-4-carboxyl-CCNU (*cis*-CCCNU, **178**), has a half-life of 4.2 h at pH 7.4 and a LD_{10} value of >400 mg/kg, compared to the values of 46 min and 26 mg/kg for **178**.[201] This disubstituted analog **190** had a significantly higher activity against both the early and late forms of the Lewis lung carcinoma as compared to **178** (Table 13). Thus, compound **190**, at a single dose of 600 mg/kg at day 2 postimplant, effected 90% cures of the early form and, at a dose of 400 mg/kg on days 7 and 14 postimplant, resulted in a 40% cure of the late form.[201] By comparison, administration of the N3-monosubstituted analog **178** resulted in 70% cures at doses of 13 and 20 mg/kg against the early form and 20% cures against the late form. The N3,N3-disubstituted *trans*-2-hydroxy-CCNU compounds **191**−**196** had outstanding anticancer activities and therapeutic indices (Table 13) as compared to CCNU (**137b**).[202] The overall syntheses of these compounds from epoxycyclohexane is shown in Scheme 30. The intermediate urea can decompose at room temperature to the aminooxazolidine **197**. A hydroxide ion-catalyzed decomposition

Table 14. Anticancer Activity of Aromatic Nitrosoureas against the Ip-Implanted Murine L1210 Lymphoid Leukemia

				ip		ic			
compound no.	R	OD,[a] mg/kg	ILS_{max},[d] %	log kill[e]	surv,[f] %	log kill[e]	surv,[f] %	ED_{50},[h] μg/mL	ref(s)
									
202	H	inactive						24	104
203	*p*-OCH_3	50[b]	22					32,36	43,104,208
204	*p*-F	50[b]	29					4.3,9.6	43,104,208
205	*p*-Cl	50[b]	28					26	208
206	*p*-CO_2H	25[b]	110					16	104
207	*p*-CO_2Et	50[b]	67						104
									
208	H	36[b]	89	3	0	<1	0	30.5	43,44,208
209	*m*-OCH_3	40[c]		5	20	nt[g]		4.5	44,208
210	*p*-OCH_3	40[c]		5	0	<1	0	16.5	44,208
211	*p*-F	25[c]		5	20	<1	0	4.2	44,208
212	*o*-Cl	50[c]		3	0	0	0	9.1	44,208
213	*m*-Cl	100[c]		5	0	0	0	4.7	44,208
214	*p*-Cl	75[c]		5	10	<1	0	3.2	44,208
215	*m*-NO_2	26[c]		4	0	<1	0	3.4	44,208
216	*p*-CF_3	75[c]		3	0	<1	0		44,208
217	*p*-$COCH_3$	75[c]		5	20	2	0		44
218	*p*-CN	150[c]		5	20	0	0		44
219	*p*-$CON(CH_3)_2$	16[c]		6	70	<2	0		44
220	*m*-CO_2H	12−18[c]		5	20	0	0		44
221	*p*-CO_2H	30[c]		5	30	<1	0		44
222	*p*-CO_2Et	9[c]		5	30	<1	0		44
223	*p*-CH_2CO_2H	26[c]		5	30	2	0		44
224	*p*-SCH_2CO_2H	18[c]		5	20	0	0		44
225	*p*-SO_2F	125[c]		3	0	nt[g]			44
226	2,6-di-CH_3	31[c]		6	70	<1	0		44

[a] Optimal dose = daily ip-administered dose providing the maximum increase in life span. [b] Drug administered on days 1−15 after tumor implantation. [c] Drug administered on day 1 after tumor implantation. [d] Increase in life span = $[(T - C)/C] \times 100$. [e] Defined in section V. [f] Percentage of mice surviving on day 45 after tumor implantation. [g] Not tested. [h] Effective dose = daily dose inhibiting the growth of L1210 cells to 50% of the untreated control.

of the compounds containing one hydroxyl group, e.g. **191**, yielded the perhydrobenzoxazol-2-one **198**, whereas decomposition of the compounds containing two hydroxyl groups, e.g. **194**, produced the oxazolidinone **199** (Scheme 31).[202]

A related series of N3,N3-disubstituted CCNU analogs of general structure **200** (Chart 5) were synthesized and their chemical stabilities and lipophilicities determined.[203] The long half-lives of these compounds, compared to CCNU (**137b**), are to be expected on the basis of the earlier work.[201,202] Speculations[203] were made concerning the decomposition products and the relative anticancer activity of compound **200** with no reference to the published research.[201,202] Oversights of this kind underline the need for the present review.

A series of polymethylene biscyclohexyl bis-nitrosourea compounds **201** (Chart 5) containing 2−8 methylene groups were synthesized[144] and shown[146] to be inactive against the ip-inoculated L1210, in contrast to the polymethylenebis[*N*-(2-chloroethyl)-*N*-nitroso]urea series **103**−**109** (Table 5). In the former case, the intermediate cyclohexyl isocyanate cannot participate in the second cross-linking step between protein and DNA.

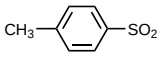
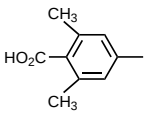
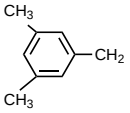
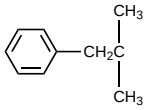
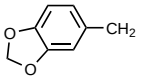
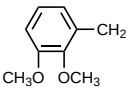
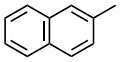
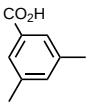
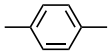
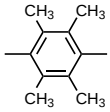
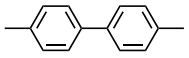
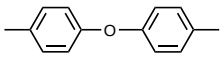
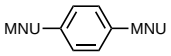
Miscellaneous alicyclic CENU analogs which were either inactive as anticancer agents or for which no published activity data could be obtained[204−207] are included in Chart 5.

4. Aromatic Analogs

A large series of *ortho*-, *meta*-, and *para*-substituted phenyl analogs of *N*-methyl-*N*-nitrosourea **202**−**207** and *N*-(2-chloroethyl)-*N*-nitrosourea **208**−**226** were synthesized and tested in vitro[208,209] for inhibitory activity against the L1210 leukemia (Table 14). In general, it was found[208,209] that the presence of electron-withdrawing groups in drugs caused an inhibition, i.e. $ED_{50} = 2.1-10.7$ μg/mL, whereas the presence of electron-donating groups resulted in no significant inhibition, i.e. $ED_{50} = 11-44$ μg/mL. By comparison, the ED_{50} value for MeCCNU (**138b**)) was 1.7 μg/mL.[208] However, the in vivo testing of various *ortho*-, *meta*-, *para*-substituted aryl nitrosourea analogs **202**−**226** (Table 14), **227**−**233** (Table 15), and aryl bis-nitrosoureas **234**−**240** (Table 15) revealed[44,104,112,116,146,210] that only a few compounds such as **206**, **207**, **208**, **219**, **226**, and **231** possessed activities against the ip-inoculated L1210, albeit all were found to be inactive against the ic-inoculated L1210. The conclusion drawn[44] from these results was that an aromatic ring prevents the passage of these drugs across the blood−brain barrier. The high activity[112,210] of the (methylenedioxy)phenyl analog **231** is somewhat surprising, considering the low activity[146] of the related dimethoxy analog **232**.

Several nitrosourea analogs of CCNU, **241**−**244**, which are more hydrophobic than BCNU and contain a tertiary amine group, were synthesized[211,212] from

Table 15. Anticancer Activity of Various Substituted Aryl CENU and Bis-CENU Analogs against Ip-Implanted L1210 Lymphoid Leukemia

RNH−CO−N(NO)−CH_2CH_2Cl				ip			ic					
compound no.	R	OD,[a] mg/kg	ILS_{max},[e] %	log kill[f]	surv, %	ther ratio[i]	log kill[f]	surv, %	ther ratio[i]	LD_{50}[l]	log *P*[m]	ref(s)
227						0.22			0.53			116
228						ns[j]			nt[k]			116
229						0.52			0.33			116
230						0.75			ns[j]			116
231		44[b]	650		40[g]							112,210
232		200[c]	60				<1	0		4.98	2.29	146
233		300[d]		5	0[h]							44
234		10[d]		3	0[h]		nt[k]					44
235		26−40[d]		5	30[h]		2	0				44
236		600[d]		5	50[h]		<2	0				44
237		500[d]		5	0−20[h]		<2	0				44
238		1500[d]		5	20[h]		nt[k]					44
239		1000[d]		5	30[h]		2	0				44
240	 MNU = $CH_3N(NO)C(O)NH$	30[d]	62									104

[a] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. [b] Drug administered on day 10 after tumor implantation. [c] Drug administered on days 1−10 after tumor implantation. [d] Drug administered on days 1−15 after tumor implantation. [e] Increase in life span = $[(T - C)/C] \times 100$. [f] Defined in section V. [g] Percentage of mice surviving on day 60 after tumor implantation. [h] Percentage of mice surviving on day 45 after tumor implantation. [i] Therapeutic ratio = ED_{50}/LD_{10}; ED_{50} = effective dose resulting in 50% 45 day survivors in a group of treated animals as determined from log-dose probit-survival plots; LD_{10} = dose required to kill 10% of a test group of non-treated mice as determined from log-dose probit-survival plots. [j] No survivors. [k] Not tested. [l] Lethal dose = ip-administered dose required to kill 50% of a test group of untreated mice. [m] Partition coefficient: *P* = [compound in 1-octanol]/[compound in water].

substituted diphenylacetonitrile, as shown in Scheme 32. The congeners **241**, **242**, and **244** had moderate and compound **243** high activity against the ip-inoculated P388 and L1210 leukemias over a wide dose range (Table 16). The 4-phenyl-4′-fluorophenyl analog **243** also exhibited high activity against solid cancers, including the B16 melanoma, colon adenocarcinoma 26, and Lewis lung carcinoma, but either low or no activity against the Harding−Passey melanoma and the ependymoblastoma brain tumor.[211,212] The in vitro toxicities of compounds **241**−**243**, expressed as the LD_{50} (Table 16), were appreciably lower than that of CCNU (**137b**), i.e. LD_{50} (po) = 77 ± 10 mg/kg.[211]

Colchicine and many of its derivatives are powerful mitotic poisons, antiinflammatory agents, and inhibitors of tumor growth.[213] The cytotoxic effect of colchicine is mediated through the formation of colchicine−tubulin complexes which prevent the microtubule polymerization.[214] Thiocolchicine has about

Scheme 32

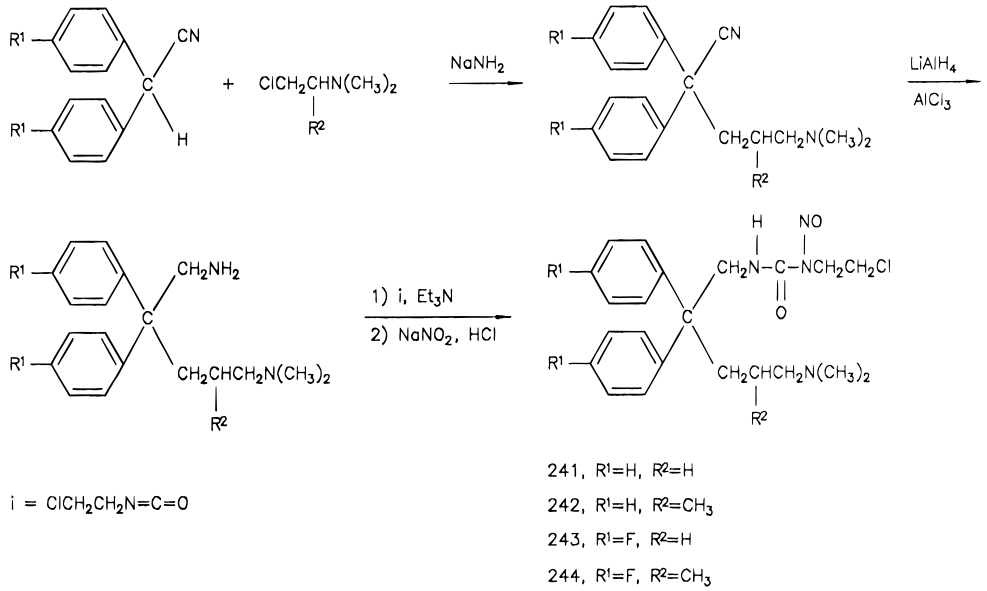
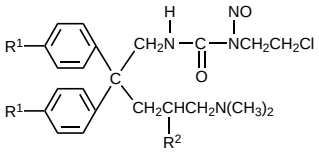
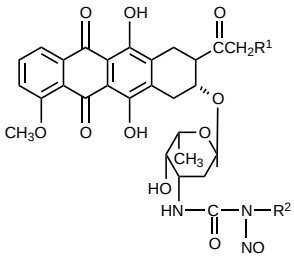


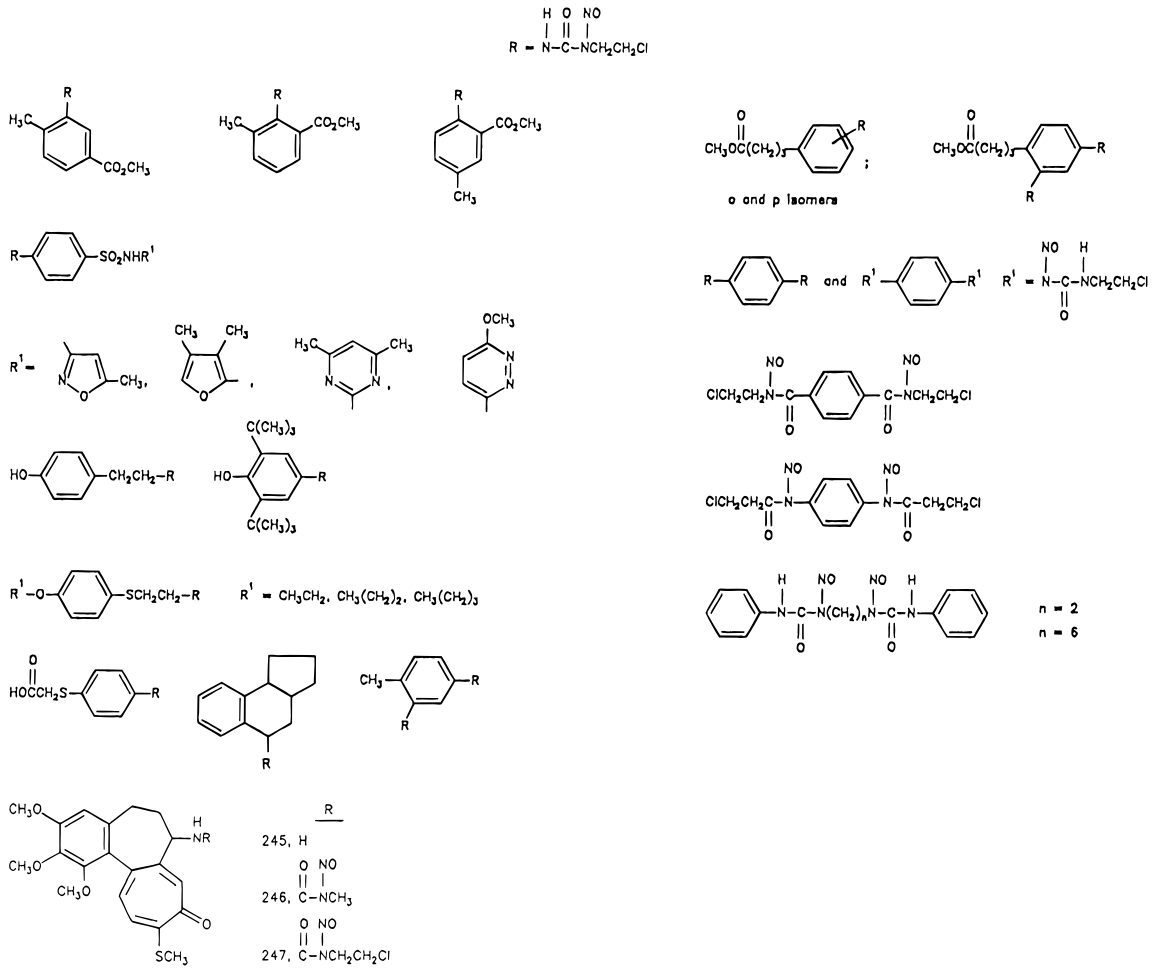
Table 16. Anticancer Activity of Various Aryl CENU Analogs against the Ip-Implanted P388[a] and Adriamycin-Resistant P388[b] Leukemia and L1210 Leukemia[c]

compound no.	R^1	R^2	OD,[d] mg/kg	ILS_{max},[i] %	survivors,[j] %	LD_{50},[k] mg/kg	ID_{90}[l] μM	ref(s)
								
241	H	H	25[a,e]	91−185		156 ± 30		211,212
242	H	CH_3	100[a,e]	44−107		182 ± 25		
			100[c,e]	137				
243	F	H	25[a,e]	74−169		128 ± 25		
			100[c,f]	497				
			25[c,e]	159				
244	F	CH_3	100[c,f]	50−166				
								
248	H	$ClCH_2CH_2$	17.5[a,f]	>540	100		0.26	217,218
			40.5[a,g]	>500	100			
			40.5[a,h]	>460	86			
			27−40[b,g]	>500	91			
			15−20[b,f]	>500	100			
249	OH	$ClCH_2CH_2$	16[a,f]	>500	57		0.58	
250 4′-epi	OH	$ClCH_2CH_2$		75−100	0		0.53	
DNR (daunorubicin)			2.0[a,f]	150−200			0.90	
			12−18[b,g]	0	0			
			1−4[b,f]	0	0			
ADR (adriamycin)			3.0[a,f]				0.31	
4′-epiADR (epirubicin)							0.43	

[a,b] Murine lymphocytic leukemia. [c] Murine lymphoid leukemia. [d] Optimal dose = daily dose resulting in the maximum increase in life span. [e] Drug administered on days 1−9 after tumor implantation. [f] Drug administered on days 1−4 after tumor implantation. [g] Drug administered on day 1 after tumor implantation. [h] Drug administered on day 3 after tumor implantation. [i] Increase in life span = $[(T - C)/C] \times 100$. [j] Percentage of mice surviving on day 60 after tumor implantation. [k] Lethal dose = ip-administered dose required to kill 50% of a test group of untreated mice. [l] Inhibitory dose = dose required to kill 90% of the L1210 leukemia cells after 1 h of drug exposure.

the same activity against L1210 as colchicine.[215] *N*-Nitrosourea analogs **246** and **247** (Chart 6) of 7-aminothiocolchicine **245** were tested in vitro for their ability to inhibit the growth of L1210 cells, sarcoma 180 cells, and herpes simplex virus type 1. The nitrosoureas **246** and **247** and their corresponding ureas were found[216] to inhibit the two cancer lines, L1210 and sarcoma 180 with $ED_{50} = (1.2-3.3)$

Chart 6. Structures for Section VII.A.4



$\times 10^{-8}$ M, to about the same extent as the starting amine **245**, with ED_{50} of 1.9×10^{-8} M, and were considerably more reactive in vitro than BCNU (**33**), with $ED_{50} = 400 \times 10^{-8}$ M.[216] The compounds inhibited the HSV-1 virus by 72−86% as compared with the 99% inhibition by the standard 5-iodo-2-deoxyuridine. These reported[216] in vitro tests against the L1210 and sarcoma 180 tumor lines are not necessarily good predictors of in vivo cytotoxicities.

The CENU analogs of the antibiotics daunorubicin, adriamycin, and epirubicin, **248**, **249**, and **250**, respectively (Table 16), contain the structural aspects of intercalation and alkylation.[217,218] Compound **248** was the most active of the CENU analogs and the parent antibiotics as measured by the inhibition of L1210 growth in vitro. Compounds **248** and **249** exhibited excellent in vivo activity against the murine P388 leukemia using various drug administration schedules (Table 16). Interestingly, the CENU analog of epirubicin **250** was found[217,218] to be inactive against the P388 leukemia. Against a special adriamycin-resistant P388 strain the daunorubicin analog **248** was[217,218] highly active, whereas daunorubicin, itself, was inactive (Table 16). Compound **248** was not myelosuppressive in cancer-bearing animals and caused only a small lowering of the white-blood cell count in noncancerous animals, with their full recovery by day 10.[217,218] The importance of the N2-chloroethyl group is illustrated[219] by the lack of in vivo anticancer activity of the *N*-methyl-*N*-nitrosoureas of daunorubicin, carminomycin, and 14-(adipoyloxy)-daunorubicin against the P388 leukemia. Furthermore, the *N*-methyl-*N*-nitrosoureas exhibited greatly reduced cytotoxicity compared with the parent anthracycline antibiotics.[219]

Structures of aryl *N*-nitrosoureas, which either have no in vivo activity against the leukemia cell lines or for which no anticancer data were obtainable are shown in Chart 6. The list includes *N*-nitrosourea congeners of benzoic esters,[144] phenyl-*n*-butyrate esters,[220] sulfa drugs,[221] antioxidant,[222] 4-phenyl sulfides,[223,224] benzo[3,4-*a*]bicyclo[4.3.0]nonanylurea,[146] bis-*N*-nitrosocarboxamides[137] and bis-*N*-nitrosoureas.[144,147,206] The precursor ureas to the CENU analogs of phenyl-*n*-butyrate esters were shown[220,225] to have weak in vitro and in vivo anticancer activity, whereas the corresponding *N*-nitrosoureas were inactive.

5. Heterocyclic Analogs

This section is divided into two main parts, i.e. heteroalicyclic compounds and heteroaromatic (heteroaryl) compounds. Within each part the rings are grouped by numbers and size.

a. Heteroalicyclic Analogs. Several *para*-substituted derivatives of phensuximide (**251**, Chart 7) were known to be good anticonvulsant agents,[226] and a relationship was established[227] between anticonvulsant activity and the ability to penetrate the central nervous system (CNS). On the basis of these results, several *N*-nitrosourea analogs **252**-**256** of phensuximide were synthesized and tested[228] as potential CNS anticancer agents. The succinimidyl

Chart 7. Structures for Section VII.A.5.a

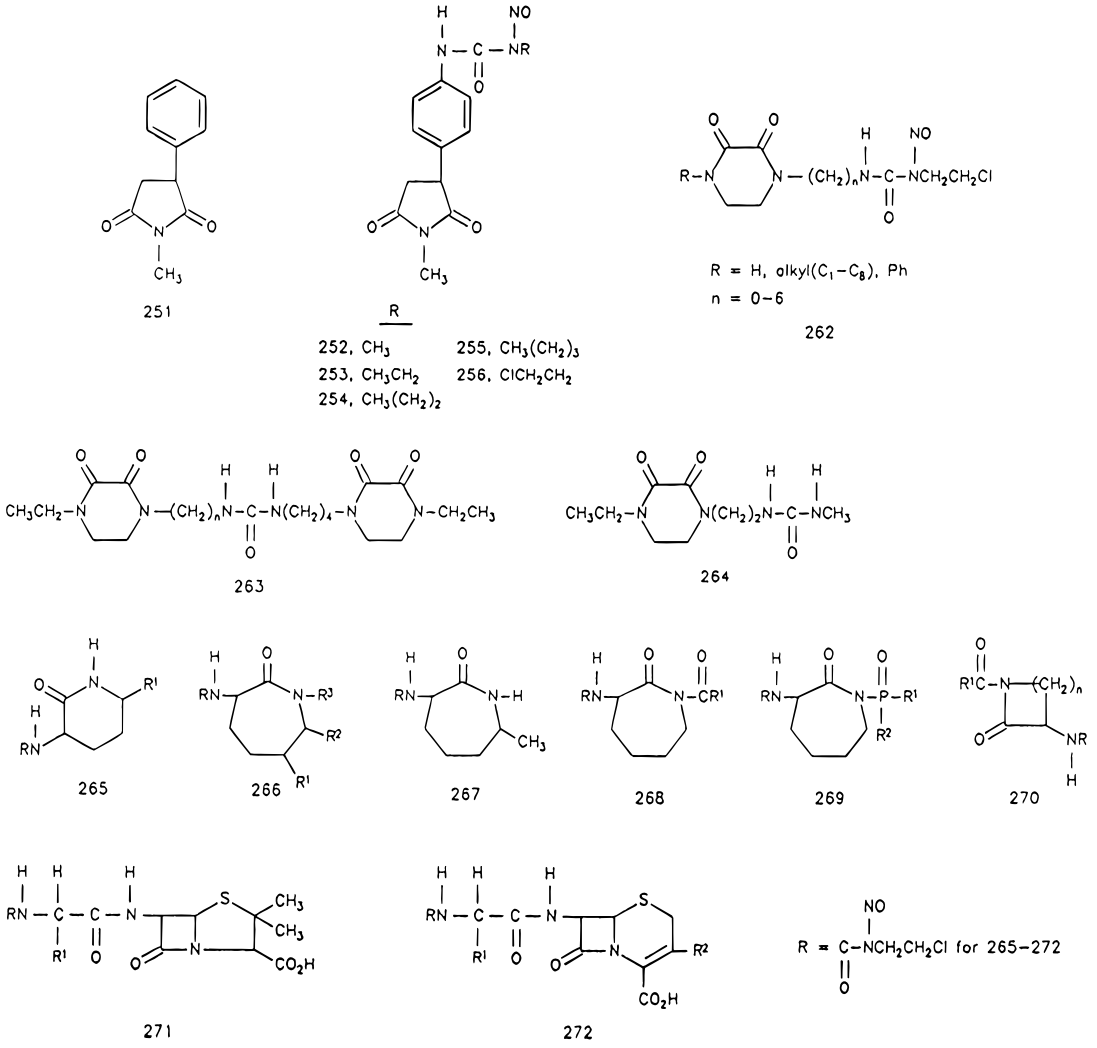


Table 17. Anticancer Activity of Heteroalicyclic CENU Analogs against the Ip-, Ic-, and Iv-Implanted L1210 Leukemia[a] and the Ip-Implanted L5222 Leukemia[b]

RNH−CO−N(NO)−CH_2CH_2X

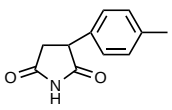
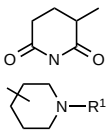
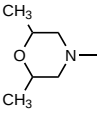
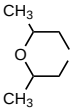
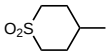
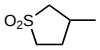
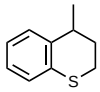
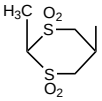
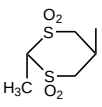
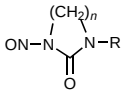
compound no.	R	X	OD,[c] mg/kg	ILS_{max},[j] %	log kill[k]	survivors, %	ther ratio[o] ip	ther ratio[o] ic	log P[r]	ref(s)
256		Cl	25[a,d,g]	96		33[l]				228
257		Cl	58.3[a,f,g]	200	>6	50[m]			0.37	96,113
	position	R^1								
273	3	$PhCH_2$	100[a,d,g]	240		50[l]				246
			100[a,e,g]	92						
274	3	CH_3CH_2	128[a,d,g]	135		16[l]				
275	4	$PhCH_2$	100[a,d,g]	279		83[l]				
			100[a,e,g]	51						
276	4	$CH_3(CH_2)_3$	64[a,d,g]	97						
278		Cl	22.1[b,d,h]	800		100[n]				112,247,248
279		Cl	27.9−44.2[b,d,h]	800		83−67[n]				112,247,248
64		Cl	88[b,d,h]	770		33[n]				249

Table 17. (Continued)

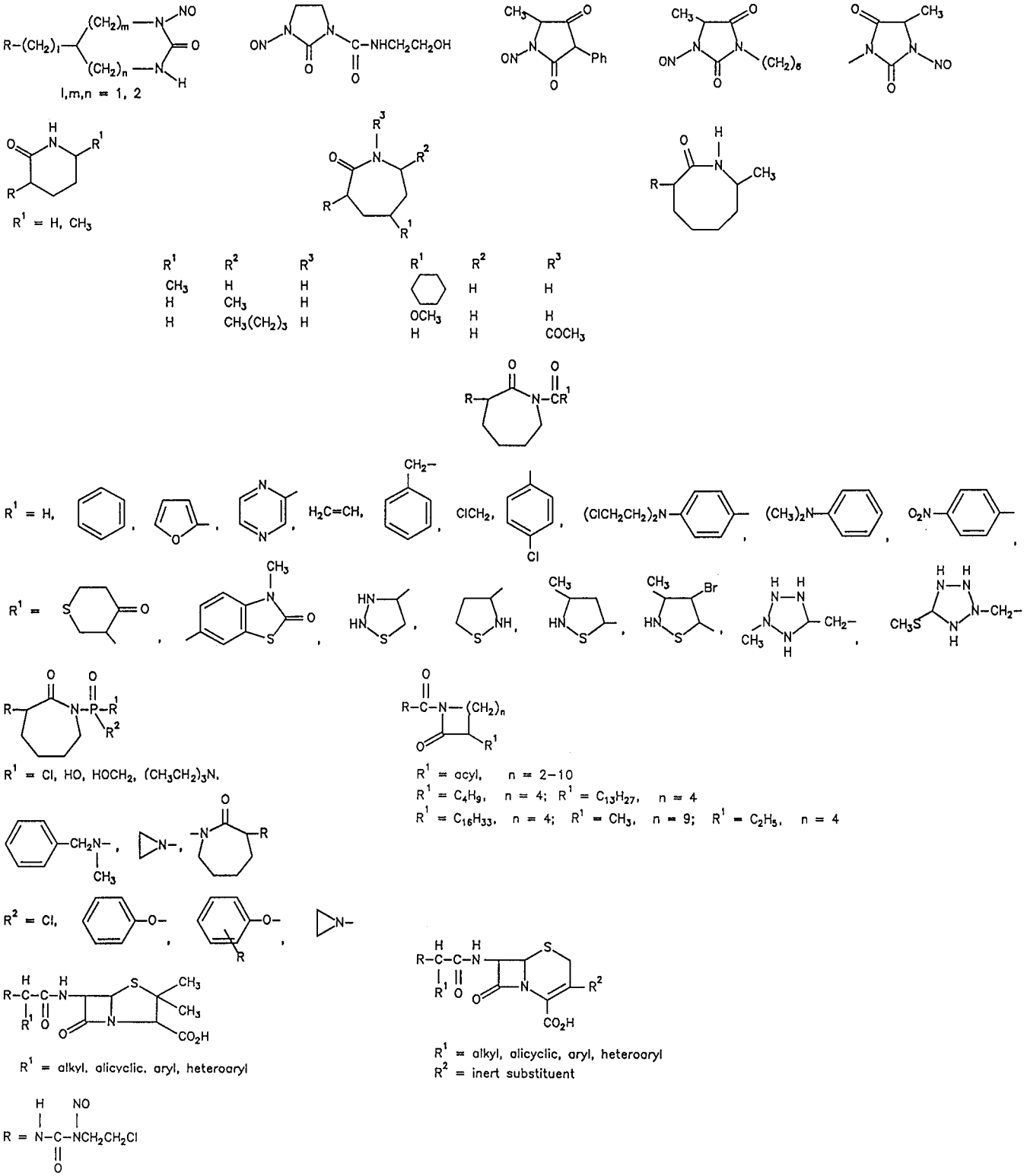
RNH−CO−N(NO)−CH_2CH_2X										
compound no.	R	X	OD,[c] mg/kg	ILS_{max},[j] %	log kill[k]	survivors, %	ther ratio[o] ip	ther ratio[o] ic	log P[r]	ref(s)
280		Cl	27.9[b,d,h]	740		16[n]				112,247,248
281		Cl	70[b,d,h]	195						249
282a		F					0.37	0.66		116
282b		Cl					0.46	0.86		
286a		F					0.31	0.58	−0.41	116
286b		Cl					0.48	0.75	0.19	
287		Cl					0.38	ns[p]		116
283a		F					0.80	nt[q]		116
283b		Cl					0.46	0.76		
284a		F					0.30	>1	1.54	116
284b		Cl					0.29	1	2.1	
288a		F					0.58	inactive	0.37	116
288b		Cl					0.20	ns[p]		
285		Cl					0.53		2.6	185
289		Cl					0.50		−0.4	185
290		Cl					0.37		−0.4	185
										
	R	*n*								
258	H	2	18[a,d,i]	71						43
260	NO	2	6[a,d,i]	24						
259	H	3	50[a,d,i]	81						43
261	NO	3	inactive							

[a] Murine lymphoid leukemia. [b] Rat leukemia. [c] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. [d] Tumor cells implanted by the ip route. [e] Tumor cells implanted by the ic route. [f] Tumor cells implanted by the iv route. [g] Drug administered on day 1 after tumor implantation. [h] Drug administered on day 10 after tumor implantation. [i] Drug administered on days 1−15 after tumor implantation. [j] Increase in life span = $[(T - C)/C] \times 100$. [k] Defined in section V. [l] Percentage of mice surviving on day 30 after tumor implantation. [m] Percentage of mice surviving on day 63 after tumor implantation. [n] Percentage of mice surviving on day 90 after tumor implantation. [o] Therapeutic ratio = ED_{50}/LD_{10}; ED_{50} = dose required to produce 50% 45 day survivors in a group of treated mice as determined from log-dose probit survival plots; LD_{10} = dose required to kill 10% of a test group of untreated mice as determined from log-dose probit-survival plots. [p] No survivors. [q] Not tested. [r] Partition coefficient: P = [compound in 1-octanol]/[compound in water].

portion was considered[228] to be a lipophilic carrier of the nitrosourea moiety. The 2-chloroethyl derivative **256** was the only analog with a significant activity of 96% ILS against ip-L1210 in mice (Table 17).[228] The compound was found to have a moderate activity against the CNS cancer ependymoblastoma with a 140% ILS, but the clinical drugs BCNU (**33**) and MeCCNU (**138b**) were much more effective against both cancer lines. By contrast, the glutarimide analog 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea (PCNU, **257**) had a high activity against both the ip- and ic-inoculated L1210 in mice (Table 17).[96,113] This lipophilic drug had lower carbamoylating and higher alkylating activities[96] than other nitrosoureas but similar cytotoxic and DNA repair-inhibiting capabilities.[229] A review[230] was published on the anticancer activity, toxicology, pharmacokinetics, and phase I clinical studies of PCNU (**257**). PCNU (**257**) was considered[231] to be less active against solid cancers than BCNU (**33**) because of lower plasma levels in rats and humans.

The *N*-nitrosated ureides **258** and **259** were reported[43] to have mild activity against leukemia L1210 and sarcoma 180 in mice, while the bisnitroso analogs

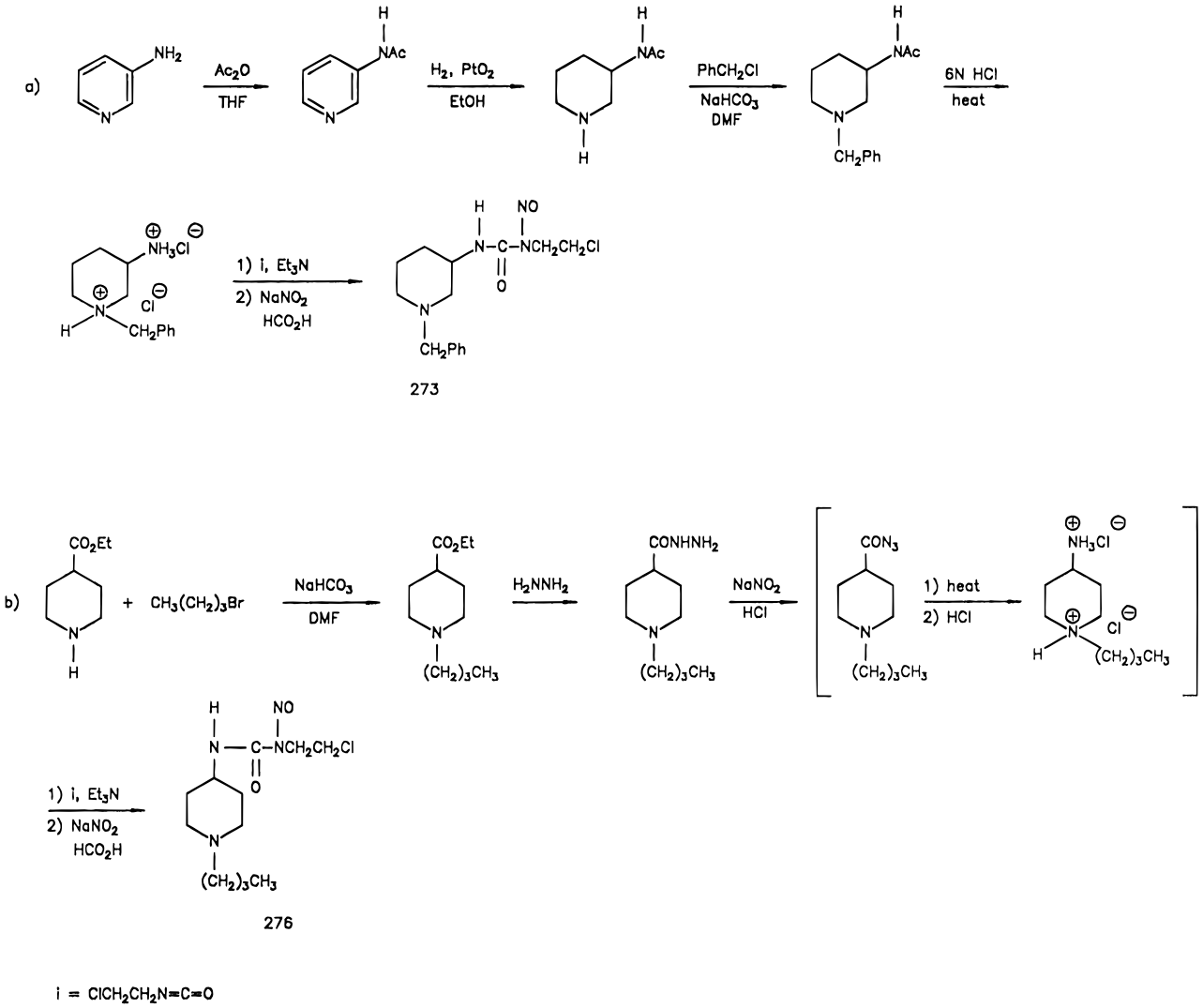
Chart 8. Structures for Section VII.A.5.a



260 and **261** exhibited either a low or no activity (Table 17). Additional *N*-nitrosoureides and nitrosohydantoins were reported[232−234] to have some anticancer activity (Chart 8).

A number of *N*-nitrosoureas of the lactam type with differing ring sizes have been synthesized and tested for anticancer activity (Chart 7). Thus, the 2,3-dioxopiperazinyl analogs **262** were tested[235] for in vitro cytotoxicity against HeLa cells and for in vivo anticancer activity against L1210. The cytotoxicity of these analogs was reported[236] to be related to the partition coefficient *P*, while the anticancer activity was related to the substituent R and the value of *n* in **262**. An increase in the number of methylene groups caused a reduction in the alkylating activity, an increase in the stability in aqueous solution at pH 7−8, and a shift of the NH proton to a higher field in the NMR.[235] A symmetrical urea degradation product **263** and an unsymmetrical urea analog **264** exhibited weak activity against a solid form of Ehrlich carcinoma.[237] A wide variety of CENU lactam analogs of general structures **265**−**270** have been synthesized,[238−243] but few anticancer activity studies have been reported. Several penicillin CENU

Scheme 33



analogs **271** and cephem CENU analogs **272** were synthesized[244,245] by the reaction of the amines with the CENU transfer agents **11b**. These compounds had anticancer activities against the P388 leukemia as well as the expected activity against staphylococcus bacteria. Additional structural details of compounds **265**−**272** are given in Chart 8.

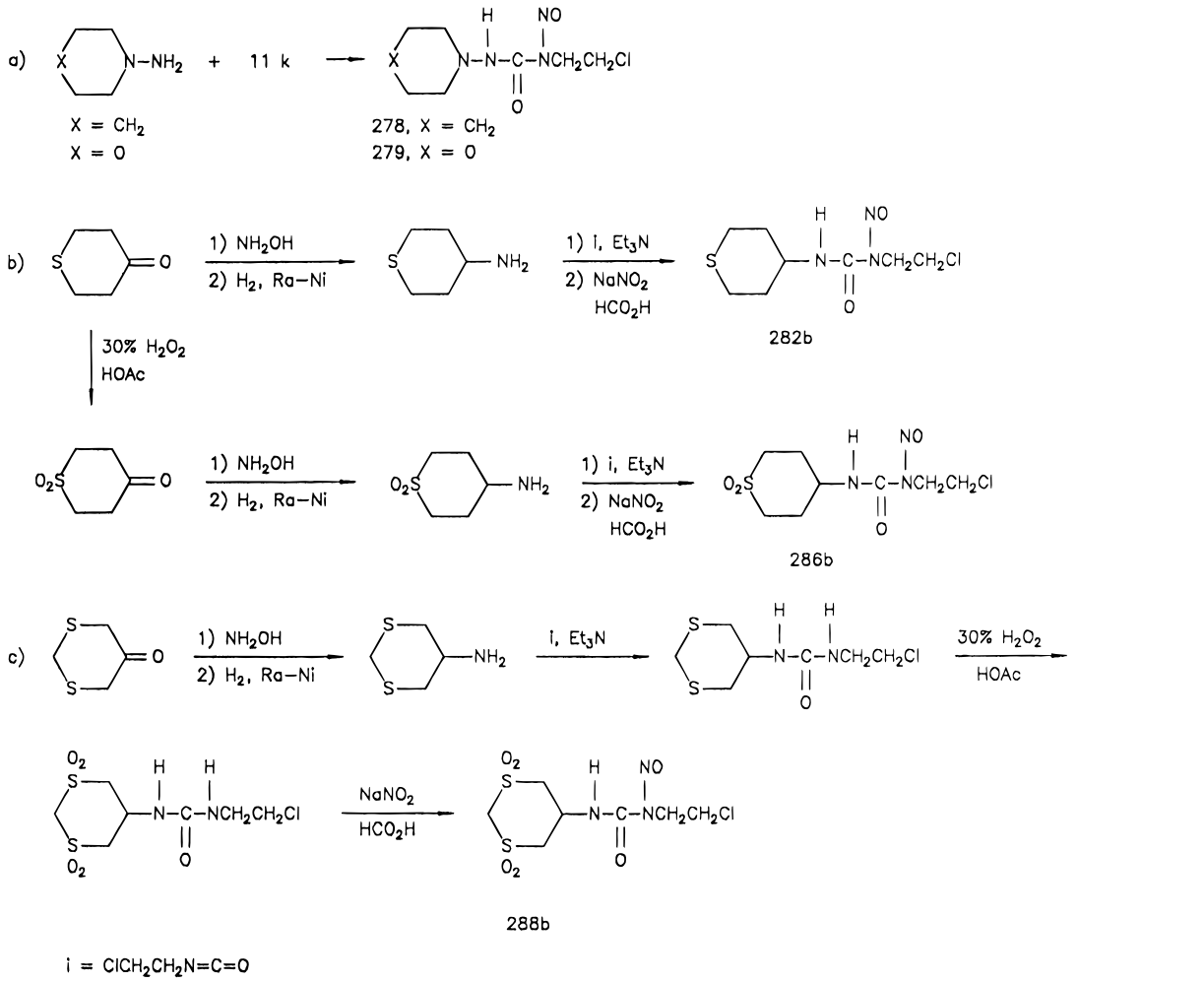
Several *N*-nitrosourea derivatives of *N*-alkylated piperidines **273**−**276** (Table 17) were of interest[246] because (1) they are nitrogen analogues of CCNU (**137b**), (2) they can be isolated as stable, water-soluble crystalline salts, (3) the *N*-substituent can be altered to vary the lipophilicities of the compounds, and (4) they are less leukopenic than BCNU (**33**) at equitoxic doses. Representative syntheses[246] of **273** and **276** are shown in Scheme 33a,b. The more lipophilic compounds **273** and **275** were shown[246] to be active against the ip-inoculated L1210 and P388 leukemias (Table 17). The less lipophilic compounds **274** and **276** were[246] somewhat less active against the L1210 (Table 17), and a corresponding 3-pyridyl analog **277** (Table 18) was both toxic and inactive. Compound **275** was also found[246] to have activity against Lewis lung carcinoma comparable to the clinical drug BCNU (**33**).

Substitution of the cyclohexyl ring of CCNU (**137b**) with either piperidine, morpholine, or 2,6-dimethylmorpholine rings yielded the corresponding CENU semicarbazides **278, 279**, and **280**, respectively (Table 17).[112,247,248] The water-insoluble piperidine analog **278** was very active against the rat leukemia L5222 and Yoshida sarcoma in the rat similarly to the water-soluble morpholino congeners **279** and **280**.[112,247−249] The unsubstituted morpholine CENU **279** was more active than **280** against two neurogenic cancers but both were less active than the clinical drug cyclophosphamide.[249] The compound **279** could not be used in clinical trials because of its high toxicity and carcinogenicity.[112] Similarly, the unsubstituted morpholine *N*-nitrosourea **64** was appreciably more cytostatic in vivo than the 2,6-dimethylmorpholine *N*-nitrosourea **281**.[249]

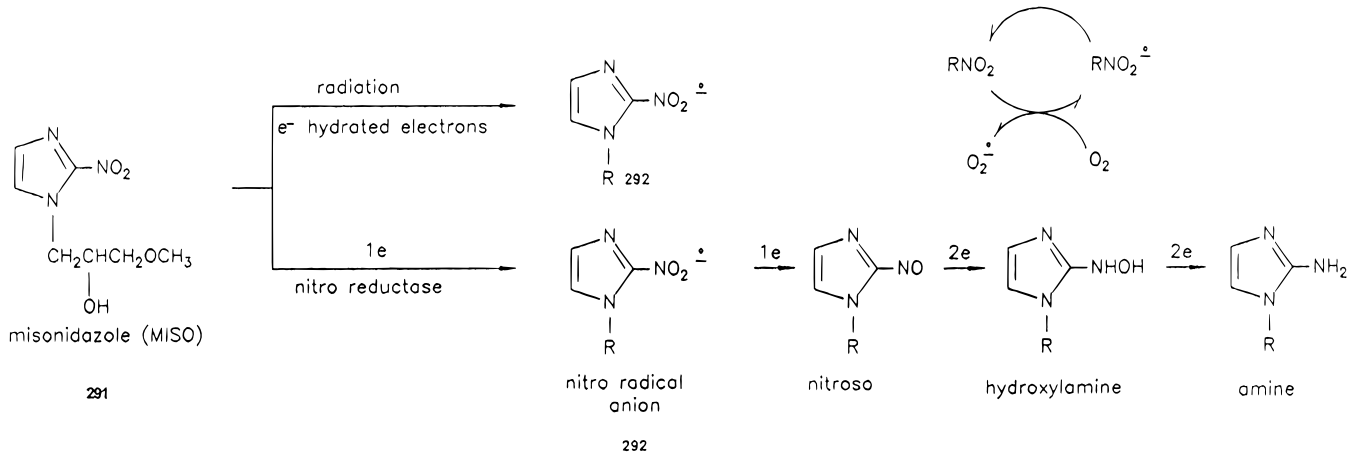
The cyclic sulfides **282**−**285** and sulfoxides **286**−**290** were synthesized[116] as analogs of the CCNU. Among this group the tetrahydro-2*H*-thiopyran-4-yl urea **282**, the corresponding *S*,*S*-dioxide **286** and the thiochroman-4-yl analog **283** were found[116] to have high activities against both the ip- and ic-inoculated L1210 (Table 17). The syntheses of the heterocyclic CENU analogs **278**, **279**, **282b**, **286b** and **288b** are outlined in Scheme 34.

b. Heteroaryl Analogs. Various nitroheterocyclic compounds, such as the 2-nitroimidazole misonidazole (MISO, **291**, Chart 9) and nitrofurans, were shown[250] to possess preferential toxicity to hypoxic cancer cells and increase their sensitivity to radia-

Scheme 34



Scheme 35

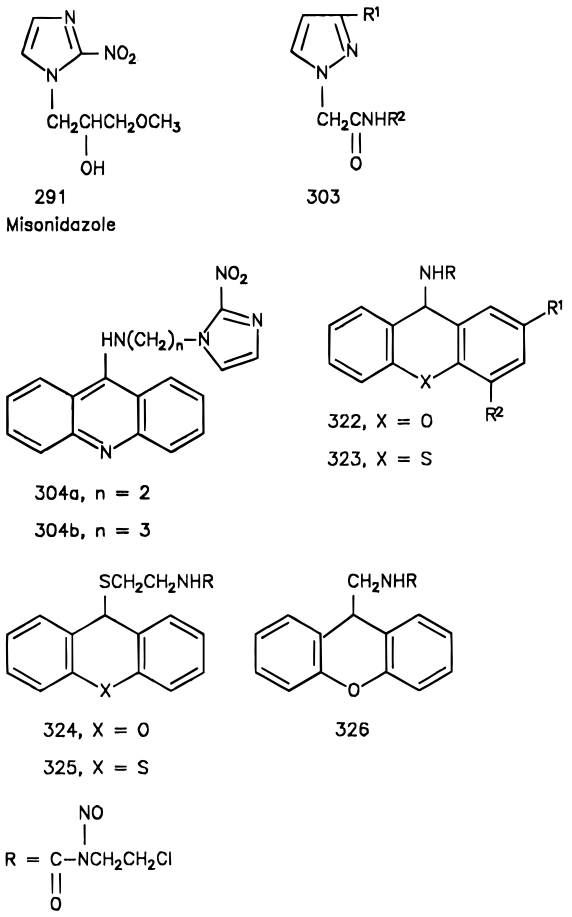


tion. Hypoxic cancer cells are much more resistant to irradiation than healthy aerobic cells,[251] as evidenced by the fact that the radiation dose−response curves for mammalian cells, irradiated under hypoxic conditions, are much less steep than those for the same cells irradiated in air.[252−254] Hypoxic cells often have either prolonged cell cycle times or are blocked in their progression through the G_1 phase and, thus, are very resistant to anticancer drugs which act mostly on the S phase of aerobic and rapidly proliferating cells. In addition, the drugs may not reach the sites of hypoxic cells located in regions of severe vascular insufficiency.[251]

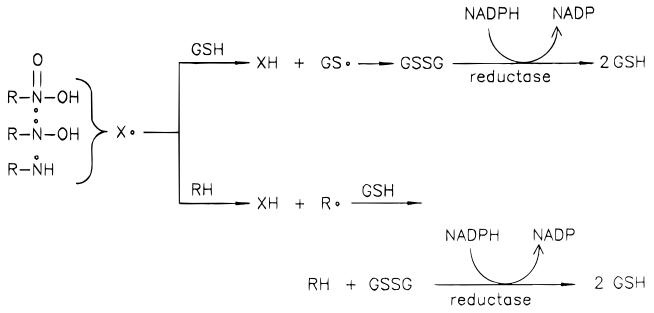
The cytotoxicity of a series of nitroaromatic and nitroheterocyclic compounds increased in the order of increasing electron affinity, i.e. the one-electron reduction potentials.[255,256] It is believed[256] that this property is the reason why the 2-nitroimidazoles, such as MISO (**291**) are more cytotoxic than either the 4- or 5-nitro congeners.

Hypoxic cancer cells can be sensitized in vitro and in vivo to anticancer agents, a process called chemopotentiation and/or chemosensitization, by a pretreatment with certain nitroheterocycles.[251] Superimposed on this so-called preincubation effect by chemosensitizers is the baseline toxicity of various

Chart 9. Structures for Section VII.A.5.b



Scheme 36



anticancer drugs toward aerobic, i.e. oxygenated, and anaerobic, i.e. hypoxic cancer cells.[257] However, chloroethyl nitrosoureas, such as BCNU and CCNU, exhibited[257] little selectively for cells based on the level of cellular oxygenation.

The cytotoxicity effect under hypoxic conditions can be explained[251,252,258,259] by a bioreduction mechanism shown in Schemes 35 and 36. The nitroheterocycle is converted to a nitro radical anion (**292**) either by the radiation or by a nitroreductase enzyme. The radical anion, in turn, can be further reduced by successive electron transfers to the nitroso, hydroxylamine, and amine groups. One or more of these intermediates could be responsible for the cytotoxicity of the nitroheterocycles under hypoxic conditions. These reactions are favored by hypoxic conditions, since oxygen would convert the nitro radical anion to the starting nitro compound by a one-electron transfer (Scheme 35).

The major portion of the cytotoxicity is the result of hydrogen abstraction by the radicals (Scheme 36).[258,259] The reaction of the reactive intermediate X• with cellular macromolecules and glutathione (GSH) results in an inactive covalently bound glutathione heterocyclic adduct. This binding would jeopardize the cellular protective mechanism, since the glutathione (GSH) diminishes the amount of target cell damage by either a reduction of the radical X• to XH, or by donating a hydrogen atom to the target cell radical R• (Scheme 36).[258,259] The resultant oxidized glutathione GSSG is converted to GSH by the NADPH-reductase enzymes.

Chemopotentiation can be explained in the following way. Alkylation of the DNA by the nitro radical anion would induce a sublethal cellular damage by the so-called preincubation effect. Subsequent treatment of the hypoxic cells with an anticancer agent, such as a CENU drug, would cause an additional DNA damage through alkylations as well as through additional DNA interstrand cross-links.[258] Furthermore, a nitrosourea with good carbamoylating activity, such as CCNU, can block the re-formation of the cell protective agent GSH by carbamoylating and inhibiting the enzyme GSSG reductase, which catalyzes the reduction of GSSG to GSH (Scheme 36).[258] The carbamoylation reaction also could block the repair of DNA adducts (section X.C). Therefore, the large potentiation of CCNU cytoactivity, which is observed when cells are pretreated with sensitizer for longer periods of time, suggests that GSH depletion is attributable to the function of the sensitizer, while the GSH regeneration is blocked by the subsequent exposure to CCNU.[258,259]

To obtain more information about this biological process, the chemopotentiation of CCNU on Mer^+ and Mer^- cell lines (section X.C) under hypoxic condition was studied.[260] The presence of MISO (**291**) produced an enhancement of the toxicity of CCNU by a factor of 1.4−1.6 on Mer^- cells but not on the Mer^+ cells. Thus, the increase in cross-linking can be attributed to deficient DNA repair systems which result in a greater amount of monoalkylated DNA available for the second, slower step in the cross-linking process.[260] Unfortunately, greater than 75% of human cancers are of the Mer^+ type, and thus, may not be susceptible to chemopotentiation by radiation sensitizers such as MISO (**291**).[260] The fact that the degree of chemopotentiation of nitrosoureas, such as BCNU, is greater than the hypoxic cancer cell fraction was explained[258] by a diffusion of a MISO metabolite from the hypoxic regions into aerobic regions of the cancer cells. The MISO chemopotentiation also occurred to some extent in cells at oxygen tensions of 2−3% which is intermediate between hypoxic and fully aerobic conditions.[252] Reviews have been published[254,258,259,261] on the chemopotentiation of cytotoxic drugs by nitroimidazoles. Other factors studied for their effect on chemopotentiation were the lipophilicity of the radiosensitizer,[262−264] inhibition of ring hydroxylation of CCNU by mouse mixed-function oxidase[265] and cell cycle distribution.[266]

A logical extension of this work was the synthesis[267] of the double-functioning nitroimidazole NI-CENU drugs **293**−**295** (series B) and **296**−**298** (series A) (Table 18) which contain both the radiosensitizing nitroimidazole and the alkylating CENU moieties. The synthesis of these compounds is detailed in

Table 18. Anticancer Activity of Heteroaryl CENU Analogs against Ip-Implanted L1210 Leukemia,[a] and L5222 Leukemia[b] and P388 Leukemia[c]

RNH−CO−N(NO)−CH_2CH_2Cl

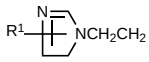
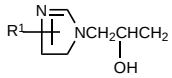
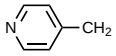
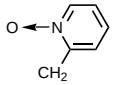
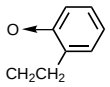
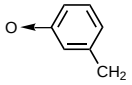
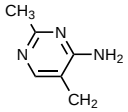
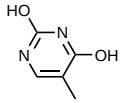
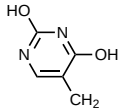
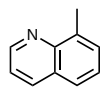
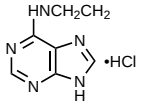
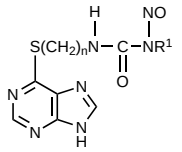
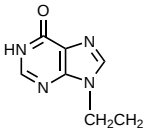
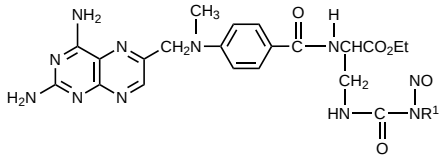
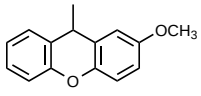
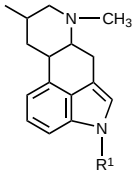
compound no.	R		OD,[d] mg/kg	ILS_{max},[l] %	log kill[m]	survivors, %	LD_{50},[s] mg/kg	ref(s)
								
B	position	R^1						
293	2	NO_2	55[a,e]			74[n]	150	267
294	4	NO_2	25[a,e]			83[n]	80	
295	5	NO_2	75[a,e]			75[n]	>100	
								
A								
296	2	NO_2	40[a,e]			100[n]	120	267
297	4	NO_2	203[a,e]			100[n]	80	
298	5	NO_2	30[a,e]			100[n]	60	
277			11.1[b,f]	70				112,246,247
			12.5[b,f]	26				249
	HCl salt		7.0[b,f]	70				249
305			27.9[b,f]	75		38[o]		112,249
	HCl salt		27.9[b,f]	700		75[o]		
306			17.6[b,f]	635		38[o]		112,249
	HCl salt					20[p]		247
307			17.6[b,f]	70				112,247
	HCl salt			85		25[o]		
308			60[a,f]	>273		100[q]		275
			25[a,g]	>265		100[q]		
			18[a,e]	>288		100[q]		
	tosylate salt		20[a,f]	>313		100[q]		
			50[a,g]	>286		100[q]		
			20[a,e]	>226		100[q]		
309			30[a,f]	>313		100[q]		275
			40[a,g]	>244		100[q]		
			30[a,e]	>226		100[q]		
310			18[a,f]	>288		100[q]		275
			20[a,g]	>331		100[q]		
			9[a,e]	>278		100[q]		249
311			27.9[b,f]	800		75[o]		280−282
312			40[a,h]	129	6	70[r]		43
313			65[a,h]	33				43
314			12[f]			5[r]		44
			25[i]	24				43
315			>400[a,h]	>26				43

Table 18. (Continued)

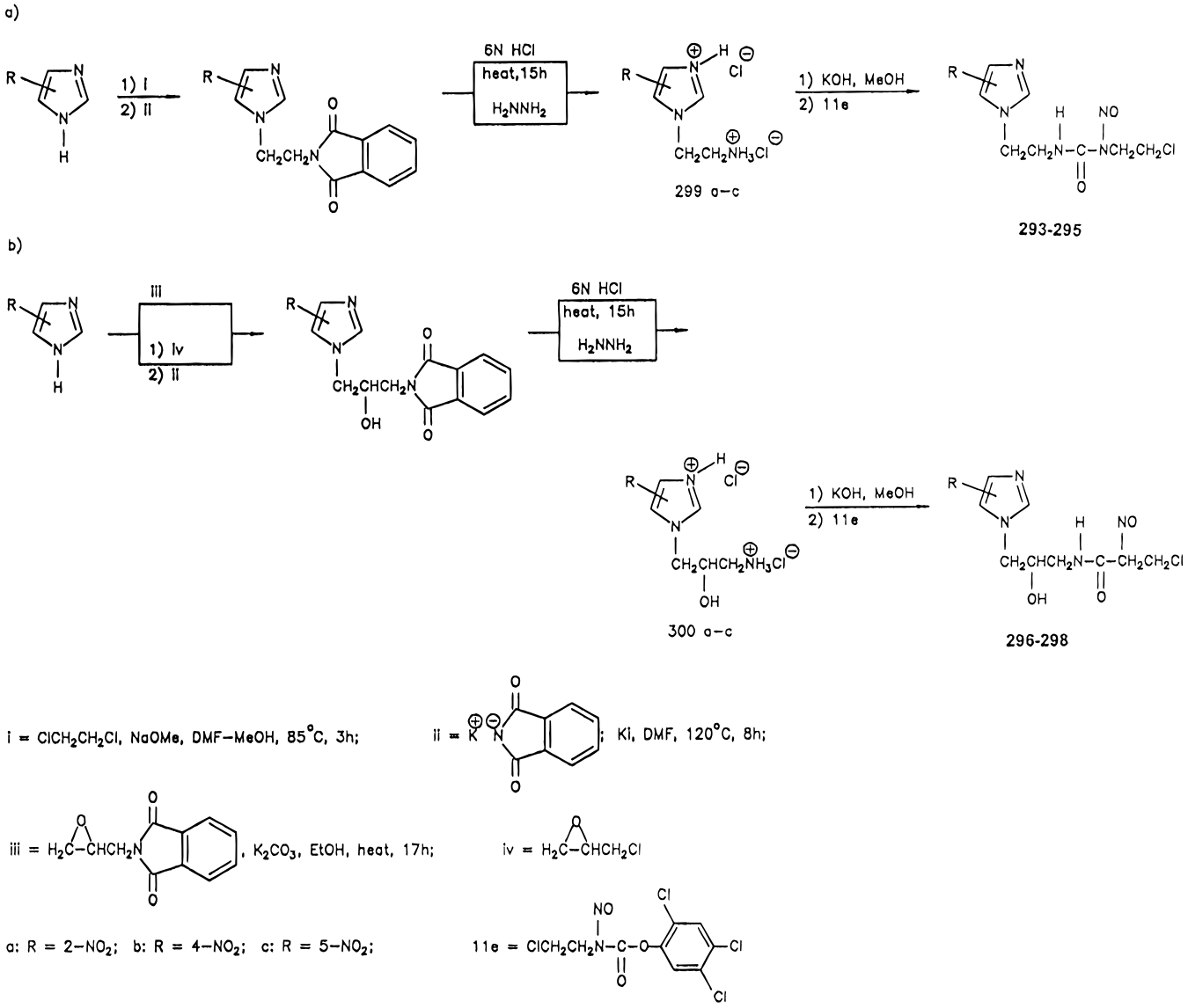
compound no.	R	OD,[d] mg/kg	ILS_{max},[l] %	log kill[m]	survivors, %	LD_{50},[s] mg/kg	ref(s)
	$RNH-CO-N(NO)-CH_2CH_2Cl$						
							
	n R^1						
316	2 CH_3	160[a,i]	113				104
317	2 $ClCH_2CH_2$	160[a,h]	113				43
318	4 $ClCH_2CH_2$	200[a,h]	151				291
319		100[a,h]	28				43
							
	R^1						
320	CH_3	200[a,j]	58				285
321	$ClCH_2CH_2$	400[c,j]	113				285
322		100−150[c,e]	63				286
							
	R^1						
327	H	200[a,k]	290		50[o]		287
328	NO	400[a,k]	189		66[o]		
	NO	100[c,k]	101		16[o]		

[a] Murine lymphoid leukemia. [b] Rat leukemia. [c] Murine lymphocytic leukemia. [d] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. [e] Drug administered on days 1, 5, and 9 after tumor implantation. [f] Drug administered on day 1 after tumor implantation. [g] Drug administered on days 2 and 6 after tumor implantation. [h] Drug administered on days 1 to death after tumor implantation. [i] Drug administered on days 1−15 after tumor implantation. [j] Drug administered on days 1−5 after tumor implantation. [k] Drug administered on days 1−9 after tumor implantation. [l] Increase in life span = $[T - C)/C] \times 100$. [m] Defined in section V. [n] Percentage of animals surviving on day 100 after tumor implantation. [o] Percentage of animals surviving on day 60 after tumor implantation. [p] Percentage of animals surviving on day 120 after tumor implantation. [q] Percentage of animals surviving on day 30 after tumor implantation. [r] Percentage of animals surviving on day 45 after tumor implantation. [s] Lethal dose = ip-administered dose required to kill 50% of a test group of untreated mice.

Scheme 37. Thus, the substituted imidazoles were converted to their corresponding phthalimide derivatives which were then transformed either by acid or hydrazine hydrate to the corresponding amines **299a**−**c** and **300a**−**c**. Reaction of the amines with the nitrosourea transfer agent **11e** produced the desired NI-CENU compounds **293**−**298**.[267] A high in vivo anticancer activity was observed[267] with both series A and B using the murine L1210 leukemia (Table 18). Low activities were found[267] for both series against the B16 melanoma. Compounds of the A series **296**−**298** could decompose to a cyclic urethane (**301**) while compounds of the B series **293**−**295** could degrade to an isocyanate **302** (Scheme 38).[267,268] A detailed study was reported[268] concerning the decomposition products of compounds of the A and B series. A much lower concentration of 2-NI, micromoles instead of millimoles, was required for chemosensitization when the NI and CENU groups were administered[269] as a single double-functional compound. Increased cross-linking occurred under hypoxic conditions, possibly, because of a synergistic interaction between the two functions.[269]

Preferential hypoxic toxicity, by a factor of 2.4, was found only in the 2-nitro analogs **293** and **296**. This result was attributable[269] to their superior electron affinities and to an increase of the CENU toxicity, i.e. chemosensitization, and not to any toxicity of nitroimidazole (NI) metabolites. This conclusion was supported[269] by the facts that the NI concentrations were very low, and there was a lack of cytoactivity of the purified 2-NI oxazolidinone decomposition product **301**, R = 2-NO_2 (Scheme 38). Furthermore, the Mer^+ HeLa 57 cell line was much more resistant to these NI-CENU drugs than the Mer^- HeLa-MR counterparts.[270] Thus, in the Mer^+ series the drug enhancement factor was only 1.3 for the 2-NI analogs **293** and **296**.[270] However, the B series compounds

Scheme 37



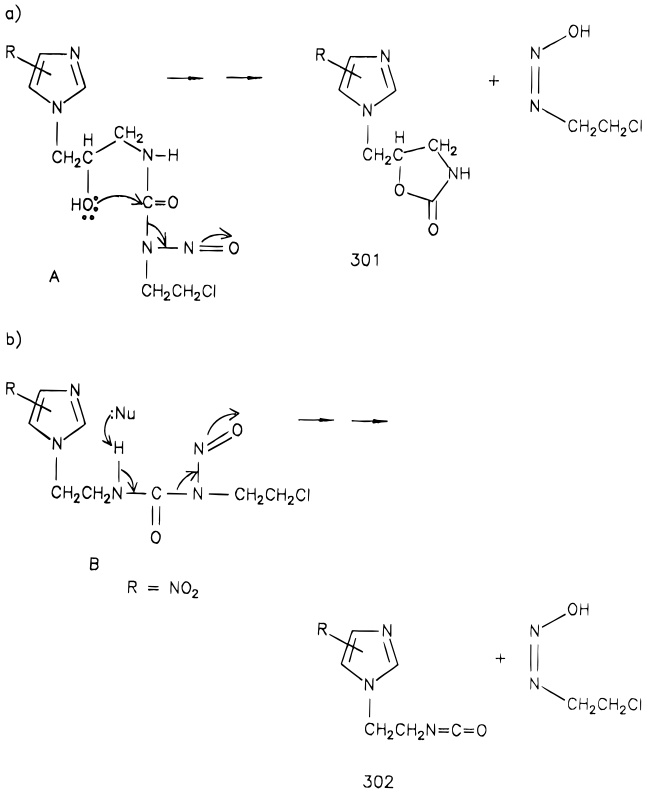
were somewhat more active against the Mer^+ cell lines, probably, because of the formation of an isocyanate and its subsequent carbamoylating activity which is not possible for the A series compounds (Scheme 38).

The chemopotentiation of nitrosoureas by other nitroheterocycles was also investigated.[271,272] Thus, the toxicity of CCNU (**137b**) against EMT-69 mouse mammary cancer cells in vitro was enhanced[271] by a series of acetohydroxamic acid derivatives of 3-nitropyrazole **303** (Chart 9). Under appropriate conditions these compounds should undergo a Lossen rearrangement to give isocyanates (Scheme 39).[271] While the anticancer activity of these analogs was not found to be proportional to the rate of the Lossen rearrangement, a strong correlation was established[271] between hypoxic toxicity and chemosensitizing potency. The 2-nitroimidazole acridines **304a**,**b** (Chart 9) were synthesized and shown[272] to be potentiators of the toxicity of CCNU (**137b**) under hypoxic conditions. A preincubation of V.79 cancer cells with these compounds for 4 h followed by CCNU administration was much more effective than a coincubation of the two agents. In addition, the potentiation of CCNU by **304a**,**b** could also be related to their ability to intercalate with DNA.[273] This property could explain the chemopotentiation after short preincubation periods.[273]

A series of pyridine analogs **277** and **305**–**307** were synthesized and tested[112,247,249] for anticancer activity. No clear structure–activity relationships were found in this series of compounds (Table 18). The hydrochloride salt, but not the free base, of the 2-picolyl analog **305** was active, the 3-picolyl analog **306** was very active, and the 3-pyridyl analog **277** and the 4-picolyl compound **307** were only slightly active.[112,249] All of these compounds were inactive[249] against the transplanted neurogenic cancers, glioma 1432 and malignant neurinoma 29792.

Interestingly, the corresponding *N*-oxides of the 2-pyridylalkyl and 3-pyridylalkyl analogs **308**–**310**, which were prepared[274] by oxidation of the corresponding nitrosoureas with *m*-chloroperbenzoic acid, were found[275] to have moderate activities in vivo against both the L1210 leukemia and AH13 hepatoma cell lines in the ip–ip (cell–drug) administered system and against the L1210 in the ip–iv and ip–po systems (Table 18). The corresponding N1 methyl analogs were inactive against both cell lines.[275] The anticancer activity of these chemically stable, water-soluble *N*-oxides was either equal or higher than that of the water-soluble 1-[(4-amino-2-methyl-5-pyrim-

Scheme 38



idinyl)methyl]-3-(2-chloroethyl)-3-nitrosourea hydrochloride salt (ACNU, **311**). All of the tested compounds produced a significant leucopenia.[275]

The aminouracil analog **312** and the thymine analog **313** exhibited[43] only weak in vivo anticancer activity against the ip-inoculated L1210 (Table 18). In contrast, ACNU (**311**) possessed high activity with cures over a broad dose range against the rat L5222[249] and mouse L1210[276,277] leukemias (Table 18). However, compound **311** was not as active over as broad a range as either CCNU (**137b**) or BCNU (**33**).[249] The administration of therapeutic doses of ACNU (**311**), with relatively low alkylating and carbamoylating activities,[277] resulted in an acute bone marrow toxicity in mice and a delayed and cumulative myelosuppression in man.[277,278] ACNU−HCl was shown[279] to be cytotoxic to several experimental brain tumors. ACNU is a weak base (pK_a 5.95) and highly soluble in water at pH 3.3 but weakly soluble at pH 6.8. It is sufficiently lipophilic, log P = 0.92, to cross the blood−brain barrier. Various pharmacokinetic[276] and metabolic[280−282] studies of ACNU have been published. The preclinical and early clinical results with ACNU have been reviewed.[283,284]

The 8-quinolylnitrosourea **314** and various purin-6-yl- and purin-9-ylnitrosoureas **315**−**319** were either inactive or weakly active against the L1210 leukemia (Table 18).[43]

The ester−*N*-nitrosourea analogs **320** and **321** of the folic acid antimetabolite methotrexate (MTX) were synthesized[285] by the reaction of the corresponding amine with the *N*-nitrosourea transfer agent **11e**. Relative to MTX the *N*-nitrosourea analogs were found[285] to have weak L1210 cell membrane influx rates, and poor inhibition of both L1210 dihydrofolate reductase (DHFR) and cell growth in vitro. Hence, their anticancer activity against the L1210 leukemia (Table 18) was not attributable to an antifolate action.[285] The carboxylic acid form of **321** was considered to be more desirable because it should bind tightly to the L1210 DHFR enzyme, while the *N*-nitrosoureido group would be transformed to an isocyanato group.[285] The latter moiety could irreversibly inhibit the enzyme through carbamoylation reactions. However, several attempts to prepare the carboxylic acid form by nitrosation of the corresponding ureas were unsuccessful.[285]

A series of tricyclic xanthen-9-yl- and thioxanthen-9-ylnitrosoureas **322**−**326** (Chart 9) were synthesized.[286] The compound **322** (R^1 = OCH_3, R^2 = H) was only weakly active against the P388 leukemia in vivo (Table 18). A number of the xanthen-9-yl-CENUs **322** containing R^1 = H, CH_3, OCH_3, and R^2 = H, CH_3, exhibited[286] high in vitro activity against a bronchial−epidermoid carcinoma, whereas the thioxanthen-9-yl analogs **323** and compounds **324**−**326** were inactive.

Mono- and bis-nitrosourea analogs of 8-amino-6-methylergoline, **327** and **328** (Table 18), respectively, were synthesized[287] as examples of compounds which contain both prolactin and anticancer activities. Ergolines are believed[288] to inhibit prolactin release from the anterior pituitary gland by interacting with the prolactin-inhibiting factor (PIF) receptor. It was hoped[287] that such compounds would target cancers located in the pituitary gland. The two compounds **327** and **328** exhibited[287] moderate in vivo antileukemic activity against the L1210 line (Table 18) but only weak antiprolactin activity, i.e. 28% and 17% inhibition, respectively.

A large number of additional heterocyclic *N*-nitrosourea derivatives related to the previously dis-

Scheme 39

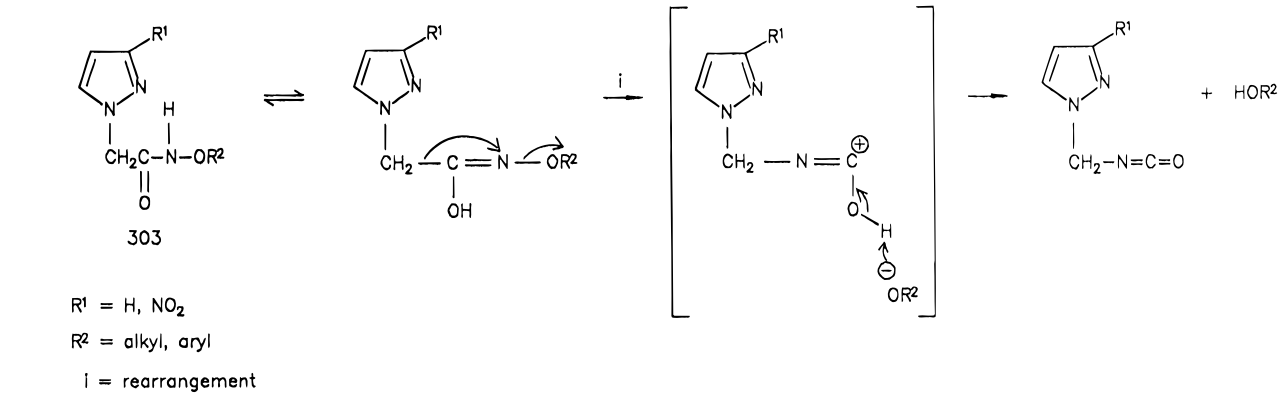


Chart 10. Structure for Section VII.A.5.b

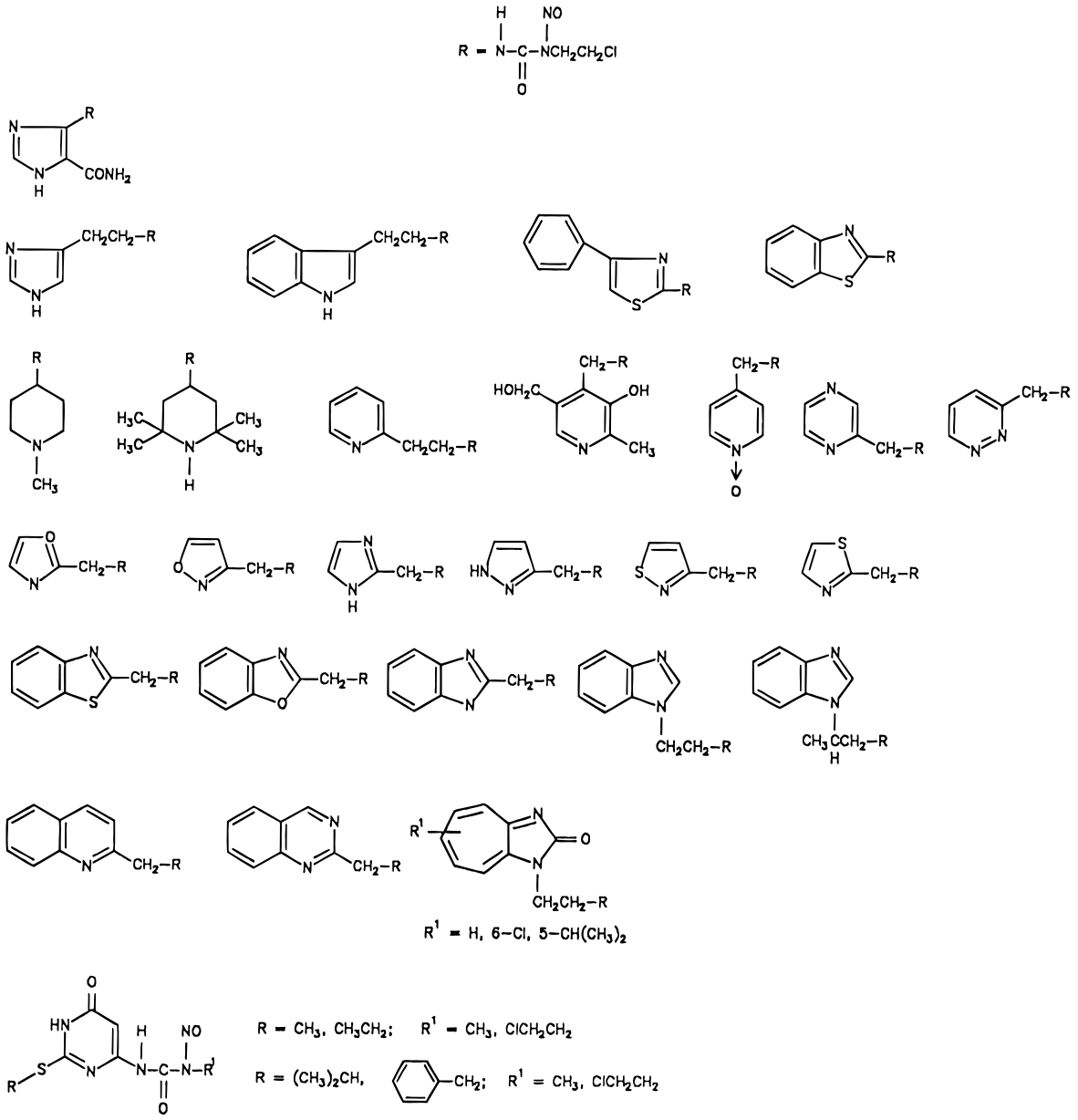
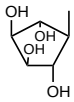
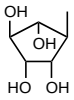
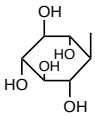
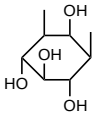
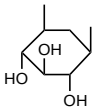
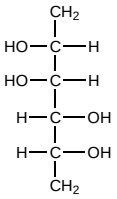
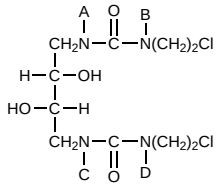


Table 19. Anticancer Activity of Hydroxyalkyl and Polyhydroxy-Alkyl and Alicyclic *N*-Nitrosoureas against Ip-, Ic-, and Iv-Implanted L1210 Leukemia[a] and Ip-Implanted L5222 Leukemia[b]

compound no.				OD,[c] mg/kg	ILS_{max},[n] %	survivors, %	ther ratio[s] ip	ther ratio[s] ic	LD_{50},[t] mg/kg	log P[u]	ref(s)
R–O–$(CH_2)_n$–NH–CO–N(NO)–CH_2CH_2X											
	R	X	*n*								
329	H	Cl	2	16[b,d,g]	560	90[o]				0.3	59,134
				8[b,d,h]	240	10[p]					134
				10[b,e,h]	718	100[p]					134
				17.6–22.1[b,d,i]	800	75[p]					112
				18[b,f,i]	329	68[q]					113
330	H	Cl	3	16[b,d,g]	170	10[o]				0.7	134
331	H	Cl	4	16[b,d,g]	160	5[o]				0.7	134
332	H	F	2	7[b,d,i]	255	0[p]					112
				32.4[b,f,j]	500	83[q]					113
333	CH_3SO_2	Cl	2	27.9[b,d,i]	395	33[p]					112
				22.1[b,d,i]	420	25[p]					112,134
				18[b,f,j]	100[q]						113
RNH–CO–N(NO)–CH_2CH_2Cl											
	R										
346	$(HOCH_2)_2C(CH_3)$			10[a,d,k]	305						306
350	$HOCH_2CH(OH)–CH_2$			17.6[a,d,j]	150	25[p]					112,134
				10[a,d,k]	320						306
				18[b,f,j]	343	83[q]					113

Table 19. (Continued)

compound no.		OD,[c] mg/kg	ILS_{max},[n] %	survivors, %	ther ratio[s] ip	ther ratio[s] ic	LD_{50},[t] mg/kg	log P[u]	ref(s)
351	$HOCH_2CH(OH)-CH(CH_3)$	10[a,d,k]	330						306
358		16[a,d,l]	371	100[r]				−1.63	308,310
359		8[a,d,l]	236	16[r]					308,310
360		16[a,d,l]	371	66[p]					308,310
361		4[a,d,m]	482		0.24	0.72			312 198
362		20[a,d,m]	530					−2.13	309,312
363		64[a,d,j]	150	83[r]					308,311
364		15[a,d,j]	180	63[p]			40		315
365		16[a,d,j]	371	16[r]					308,311
366		32[a,d,j]	371	50[r]					308,311
372		10[a,d,j]	140	80[p]			110		315



	A	B	C	D						
369	NO	H	NO	H	30[a,d,j]	230	88[p]		80	315
370	H	NO	H	NO					40	315
371	NO	H	H	NO					40	315

[a] Murine lymphoid leukemia. [b] Rat leukemia. [c] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. [d] Tumor cells implanted by the ip route. [e] Tumor cells implanted by the ic route. [f] Tumor cells implanted by the iv route. [g] Drug administered on day 6 after tumor implantation. [h] Drug administered on day 8 after tumor implantation. [i] Drug administered on day 10 after tumor implantation. [j] Drug administered on day l after tumor implantation. [k] Drug administered on days 1−5 after tumor implantation. [l] Drug administered on days 1−8 after tumor implantation. [m] Drug administered on days 1−3 after tumor implantation. [n] Increase in life span = $[(T - C)/C] \times 100$. [o] Percentage of treated animals surviving on day 60 after tumor implantation. [p] Percentage of treated animals surviving on day 90 after tumor implantation. [q] Percentage of treated animals surviving on day 63 after tumor implantation. [r] Percentage of treated animals surviving on day 30 after tumor implantation. [s] Therapeutic ratio = ED_{50}/LD_{10}; ED_{50} dose required to produce 50% 45 day survivors in a group of treated mice as determined from log-dose probit-survival plots; LD_{10} = dose required to kill 10% of a test group of untreated mice as determined from log-dose probit-survival plots. [t] Lethal dose = ip-administered dose required to kill 50% of a test group of untreated mice. [u] Partition coefficient: P = [compound in 1-octanol]/[compound in water].

cussed compounds have been reported in the literature[52,119,289−295] and some of them are collected in Chart 10.

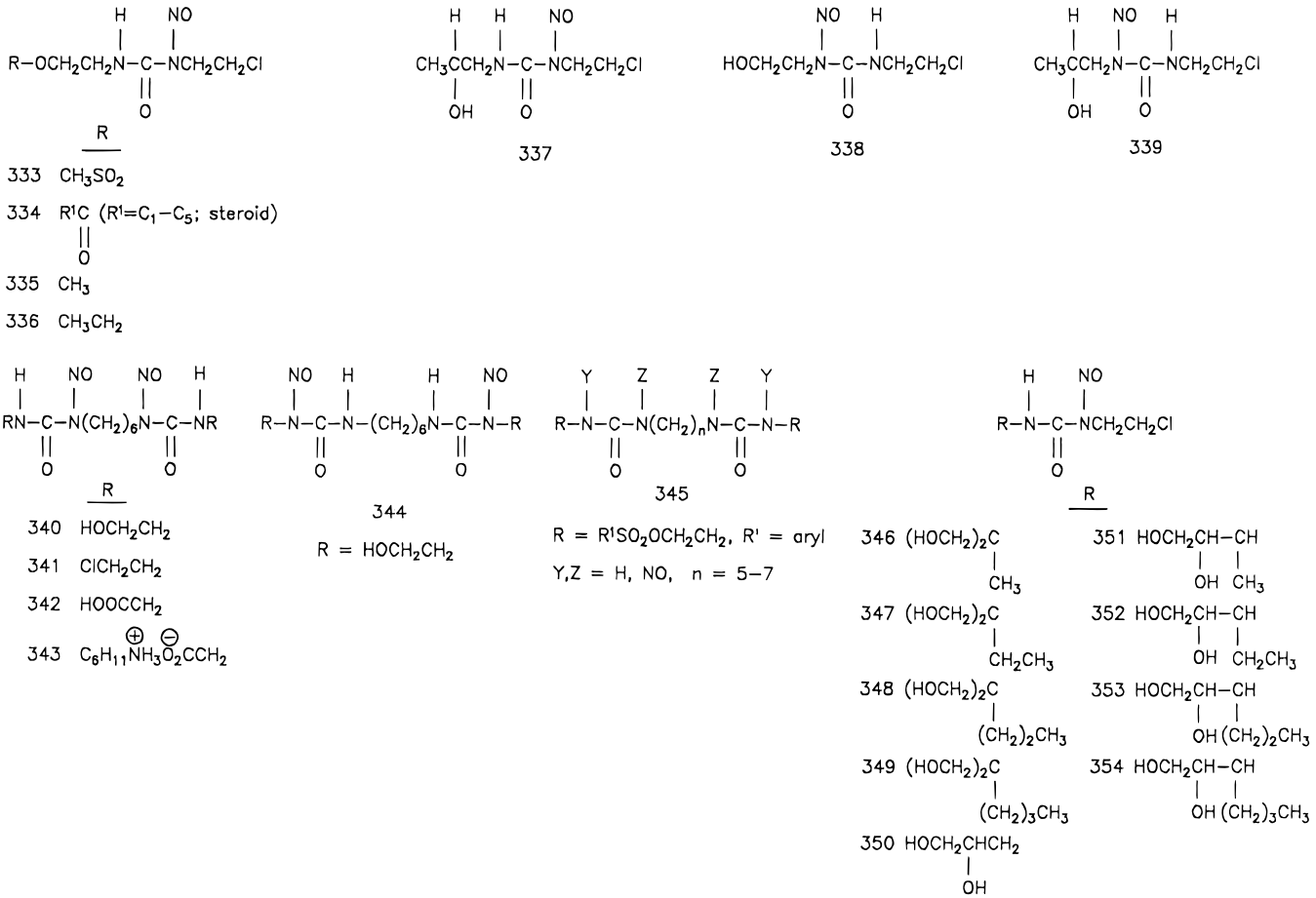
B. Hydroxyalkyl Analogs

The suggestion[91,95,98] that less lipophilic nitrosoureas should be more active and less toxic has led to the synthesis of more hydrophilic CENU compounds, containing one or more hydroxyl moieties. In 1976 a report appeared[59] that covered the synthesis of several polyethylene-CENUs **85**−**89** (Table 4) and hydroxyalkyl-CENUs **329**−**331** (Table 19) compounds. These compounds were prepared[59] by the reaction of the appropriate amino alcohols with the CENU azide transfer agent **11k**.

The three water-soluble analogs **329**−**331** possessed dramatically different anticancer activities.[59,134] Thus, the anticancer evaluation of 3-(2-hydroxyethyl)-1-(2-chloroethyl)-1-nitrosourea (HECNU, **329**) resulted in 90% cures against the ip-inoculated rat L5222 leukemia (Table 19) and an 85% cancer weight reduction against sc-implanted Walker carcinoma.[59] However, the corresponding figures for **330** and **331** were 10.5% and 5.0%, respectively. This result is a remarkable example of a structure−activity relationship involving a small molecular change. In comparison, the corresponding figures for the clinical drug BCNU (**33**) against the two cancers were 70% and 83%, respectively.[59] Further studies revealed[134,135] that HECNU (**329**) was more active than BCNU (**33**) against both the ip- and ic-implanted L5222 leukemia. This property of killing cells in both intraperitoneal and intracerebral areas could be interpreted[134] to mean that HECNU can accumulate in cytotoxic concentrations in these compartments more rapidly than BCNU. Although HECNU is about 30 times (11%) more water soluble than BCNU (0.4%), it has a good balance of hydrophobic and hydrophilic properties necessary for a rapid cell membrane transport.[134] This factor is also reflected in the comparative log *P* values of the two compounds, i.e. 0.3 for HECNU and 1.5 for BCNU. However, this explanation alone is not sufficient, since the compounds **330** and **331** have similar log *P* values of 0.7. Yet, they are much less active against the ip-inoculated L5222 (Table 19). Interestingly, the 1,1′-tetramethylene-bisCENU analog **87** (Table 4), with a log *P* value of 0.9, exhibited high activity of 560% ILS against the ip-inoculated L5222 but lower activity of 123% ILS against the ic-inoculated L5222. The LD_{50} values of BCNU (**33**), HECNU (**329**), and the bis-nitrosoureas **85**−**89** were all about 25 mg/kg.[134] A comparison of the in vivo activity of HECNU (**329**) with those of several other active CENU analogs against the L5222 and L1210 leukemias, and a variety of solid tumors was reported[296] and is discussed in section VIII.

Replacement of the chlorine atom in **329** with fluorine produced compound **332** which was much more toxic and less active than HECNU **329** (Table 19).[187] Since carbon−fluorine bonds are stronger than carbon−chlorine bonds, it is less likely that an electrophilic species would be generated from **332** which would be involved in cross-linking reactions with DNA molecules.[187] Another explanation[10] is

Chart 11. Structures for Section VII.B



that fluoroethanol, a possible metabolite, could be transformed to fluoroacetate, a known metabolic poison.

A number of esters **333** and **334** and ethers **335** and **336** of HECNU have been found[297] to have high activity against the rat L5222 leukemia and rat glioma G616 (Chart 11). In particular, the methanesulfonate analog HECNU-MS (**333**) possessed excellent antileukemic activity (Table 19). HECNU-MS (**333**) was the weakest carcinogen of a series of open-chain compounds.[296] Compounds **329** and **333** were characterized by a specific neoplastic effect on nervous tissue[296] and by a reduction of spontaneous tumors.[5,296]

The *N*-nitroso analogs **329** and **337** were more active as anticancer agents than the isomeric compounds 3-nitroso-3-(2-hydroxyethyl)-1-(2-chloroethyl)-urea (**338**) and 3-nitroso-3-(2-hydroxylpropyl)-1-(2-chloroethyl)urea (**339**, Chart 11).[298] Compounds **338** and **339** exhibited[298] both a low toxicity and a high mutagenicity and carcinogenicity. The evidence that hydroxyethylation is not relevant for anticancer activity was provided[298,299] by the fact that the hydroxyethylating nitrosoureas **338** and **339** had either little or no antileukemic activity in rodents, in contrast to their chloroethylating and cross-linking counterparts **329** and **337**. Detailed discussions of the biological mechanisms of action of *N*-(hydroxyethyl)-*N*-nitrosoureas, such as HECNU (**329**), are to be found in section X. The chronic toxicity and carcinogenicity of HECNU and of other CENUs have been reviewed[5,296] and are discussed in section XI.D. A summary was presented[300] of the phase I and II clinical studies of HECNU (**329**).

Several bis-*N*-nitrosourea analogs of HECNU (**340**−**343**, Chart 11) were synthesized and tested[301] for anticancer activity. The bis-hydroxyethyl analog **340** was the most cytotoxic of the compounds, inhibiting Walker carcinosarcoma by 54% and sarcoma 45 by 90%. The bis-chloroethyl compound **341** also was quite active against these cancers, but the bis-acetic acid and acid salt analogs **342** and **343** were less toxic than **340** and **341** and had no anticancer activity.[301] Compound **340** and its asymmetric isomer **344** possessed comparable stabilities at pH 7 and 37 °C, with half-lives of 324−393 min, and both had anticancer activity.[302] The pharmacokinetics of these compounds has been reported.[303,304] Various methanesulfonate esters of bis-hydroxyalkyl-CENUs with general structure **345** were synthesized according to Scheme 40, but no anticancer activity was reported.[305]

The polyhydroxy-CENU compounds **346**−**354** (Chart 11) were synthesized[306] via reactions of various amino alcohols with 2-chloroethyl isocyanate to give the corresponding ureas, which were nitrosated with a mixture of sodium nitrite and an acid. Alternatively, the amino alcohols were reacted with the CENU transfer agents **11b** and **11k**.[306] Several of these water-soluble compounds **346**, **350**, and **351** possessed moderate antileukemic activity in vivo (Table 19). These compounds also were active when administered by the oral route and caused lower nephrotoxicity than the water-insoluble BCNU (**33**).[306]

A series of water-soluble polyhydroxycyclopentyl- and polyhydroxycyclohexyl *N*-nitrosoureas **355**−**357**

Scheme 40

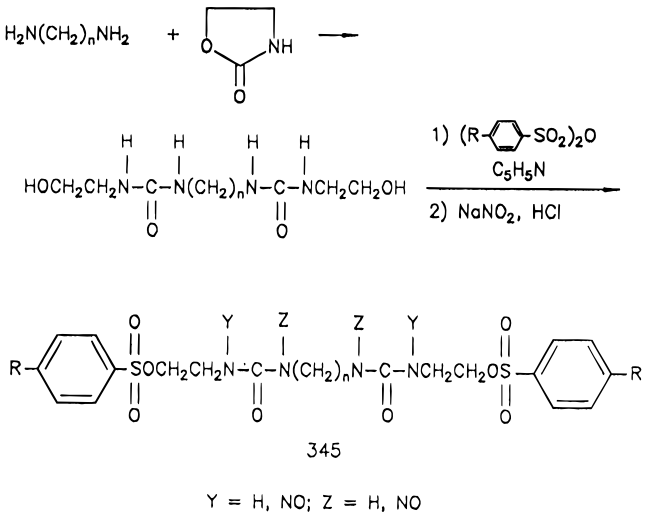
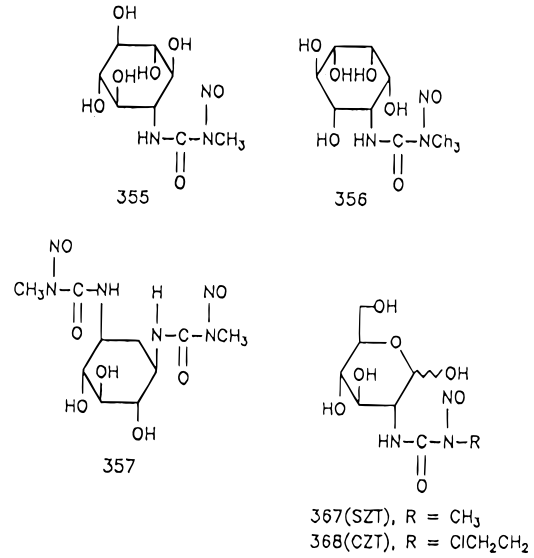


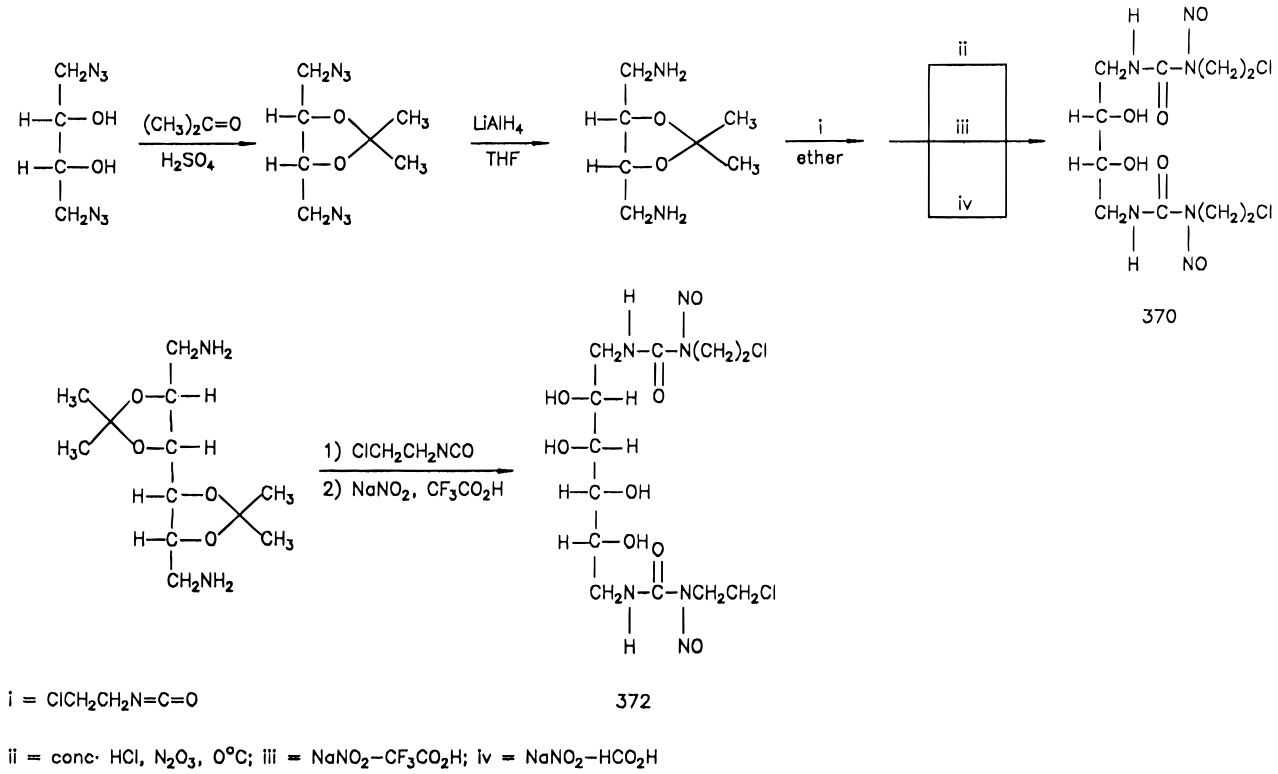
Chart 12. Structures for Section VII.B



(Chart 12) and **358**−**366** (Table 19) were synthesized[307] as potential substitutes for the monosaccharide derivatives streptozotocin (**367**) and chlorozotocin (**368**). The *N*-methyl-*N*-nitrosourea analogs of *scyllo*-inosamine **355**, *epi*-inosamine **356**, and *myo*-inosodiamine **357**, were synthesized[307] by the conversion of the starting amines with methyl isocyanate in acetonitrile, in the presence of a silver carbonate catalyst, to yield the corresponding *N*-methylureas. Nitrosation of the ureas with sodium nitrite in acetic acid yielded the nitrosoureas. These three compounds were reported[307] to be active against Ehrlich ascites tumor and HeLa carcinoma.

Similarly, *N*-(2-chloroethyl)-*N*-nitrosourea congeners of cyclopentanetetrols (**358**−**360**), cyclohexanediol (**361**, DONU), and cyclohexanepolyols (**362**−**366**) were synthesized and tested[308−314] for activity against the L1210 leukemia. The *trans*,*trans*-2,6-dihydroxy-CCNU (DONU **361**) was synthesized[198,309] by a sequence involving a condensation reaction of glutaraldehyde with nitromethane and reduction of the resulting (1α,2*β*,3α)-2-nitro-1,3-cyclohexanediol. Reaction of the resulting amine with 2-chloroethyl isocyanate in DMF, followed by nitrosation of the urea with dinitrogen trioxide in 0.5 M hydrochloric

Scheme 41



acid at 0 °C, yielded the desired product **361**. The anticancer activity[198] of DONU (Table 19) against ip-implanted L1210 leukemia was comparable to that of *trans*-2-hydroxy-CCNU (**185**) (Table 12), but its activity against the ic-implanted L1210 was somewhat lower.[198] The water-soluble DONU (**361**) was reported to have activity against human undifferentiated breast carcinoma,[313] poorly differentiated colon adenocarcinoma,[313] and amelanotic melanoma[314] in mice. Various chemical and biological properties of DONU were compared[198] with those of the monohydroxyl-CCNU metabolites **184**-**189** and other hydroxylated CENU compounds and are discussed in section VIII. The *N*-(2-fluoroethyl) analog of DONU (**361**) was synthesized by the reaction of the corresponding amine with the fluorinated *N*-nitrosourea transfer agent **11b**.[187] A screening against the solid murine cancers B16 melanoma and Lewis lung carcinoma revealed[187] that the fluoro analog had equal or higher activity than the clinical chloro analog **361**. Several of these polyhydroxy-CENU analogs **358**, **360**−**362**, **365**, and **366**, displayed as high activity as that of BCNU (**33**) against the L1210 leukemia (Table 19). The water-soluble polyhydroxy analogs were strongly myelosuppressive, as measured by the depression of peripheral blood neutrophil count on day three, the nadir of white blood cell suppression.[199] These results agreed with the my-

Scheme 42

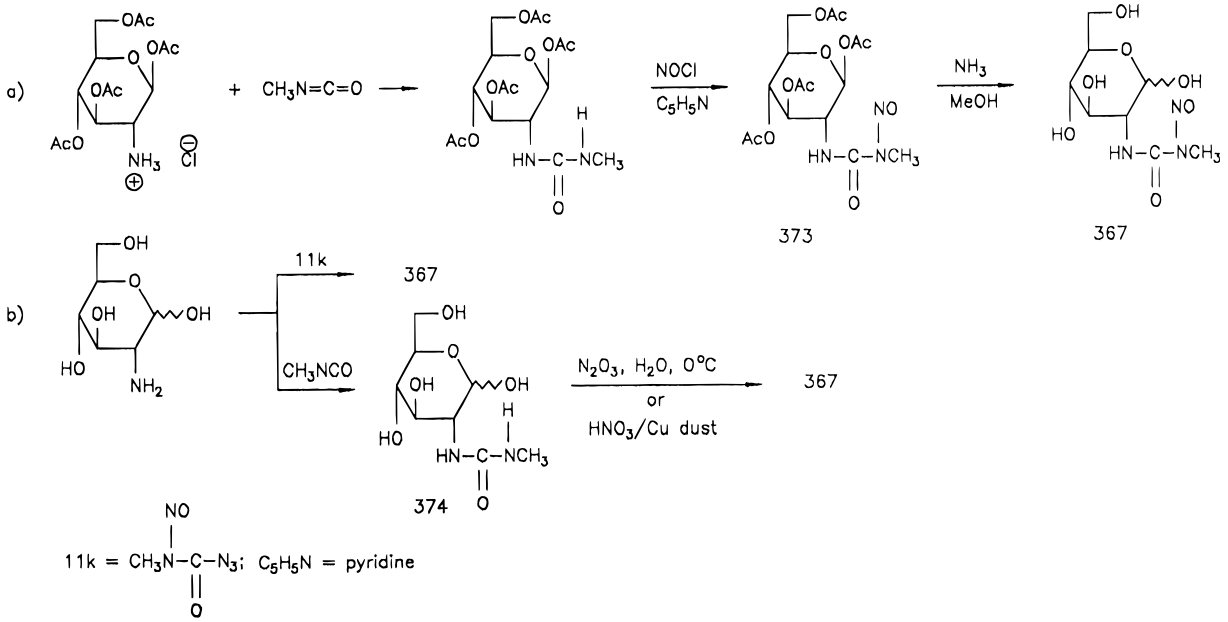
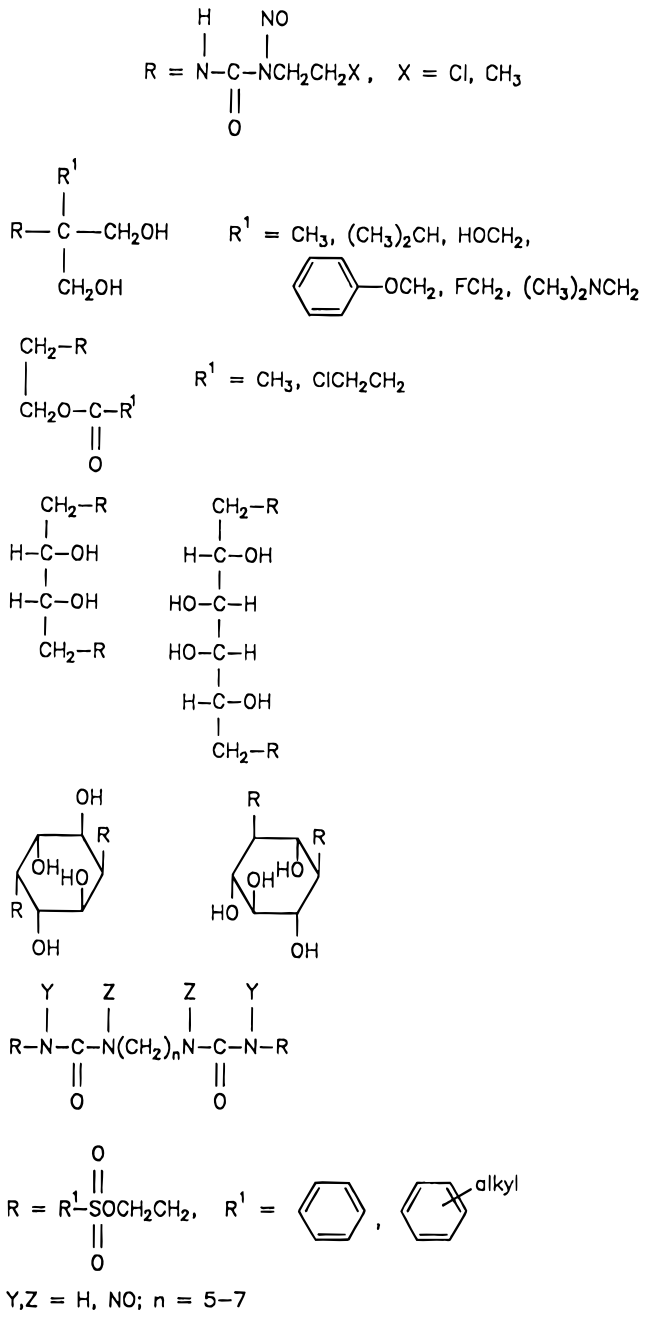


Chart 13. Structures for VII.B



elosuppression of other water-soluble hydroxyl-containing *N*-nitrosoureas which are not carbohydrates (section X.F).[199]

A number of bisCENU congeners of the sugar alcohols, such as D-threitol **369**–**371** and D-mannitol **372** were synthesized and tested for anticancer activity.[315–318] Representative syntheses of these analogs are outlined in Scheme 41. Compounds **369**–**371** were shown to have anticancer activity against the P388 and L1210 leukemias (Table 19) as well as several solid tumors. These compounds also were active when given by the oral route, had a low toxicity, and were more active than BCNU.[317] In addition, **371** was shown[318] to have a long-term total regression effect on the human adenomatous colorectal tumor xenografts.

It is difficult to draw meaningful conclusions[319] about structure–activity relationships among these polyhydroxy-containing CENU compounds **355**–**366** and **369**–**372**, except to indicate that a number of these compounds have similar anticancer activities like those of the monosaccharide CENU analogs (section VII.C). Hence, it can be concluded[319] that a carbohydrate structure is not an essential requirement for the anticancer activity among the water-soluble *N*-nitrosoureas. Several additional hydroxyalkyl-CENU analogs[305,312,315,320] for which either no activity was reported or biological results were unavailable are listed in Chart 13.

The *N*-nitrosourea analogs of carbohydrate alcohols D-arabinitol, D-glucitol, D-mannitol, and D-galacitol are discussed in the monosaccharides section VII.C. A series of N3,N3-disubstituted hydroxyalkyl-CENUs (**65**–**71**) are discussed in section VII.A.2.

C. Carbohydrate Analogs

1. Historical Background

A broad spectrum antibiotic was discovered[321–324] as a metabolite from a fermentation of *Streptomyces achromogenes* var. *streptozotocin*. After several years the active compound was isolated,[324] and its chemical structure was shown to be the *N*-methyl-*N*-nitrosourea derivative of 2-amino-2-deoxy-D-glucose.[324–326] This compound, known as streptozotocin (SZT, **367**), was first synthesized[325] (Scheme 42a) in a low yield by the reaction of tetra-*O*-acetyl-*β*-D-glucosamine hydrochloride salt with methyl isocyanate to give the urea, followed by reaction of the urea with nitrosyl chloride in pyridine to give streptozotocin tetraacetate **373**. The removal of the acetate groups was effected by the use of methanolic ammonia solution. Later, improved syntheses (Scheme 42b) of SZT were developed starting with D-glucosamine which was either converted to SZT **367** by the two-step sequence[57] of urea **374** formation followed by nitrosation with nitrogen trioxide, or by a one-step reaction[327] using the *N*-nitrosourea transfer agent **11k**. The intermediate urea **374** also was nitrosated in 81% yield by the nitrite ion obtained in situ[61] from the reduction of nitric acid with copper dust (Scheme 42b).

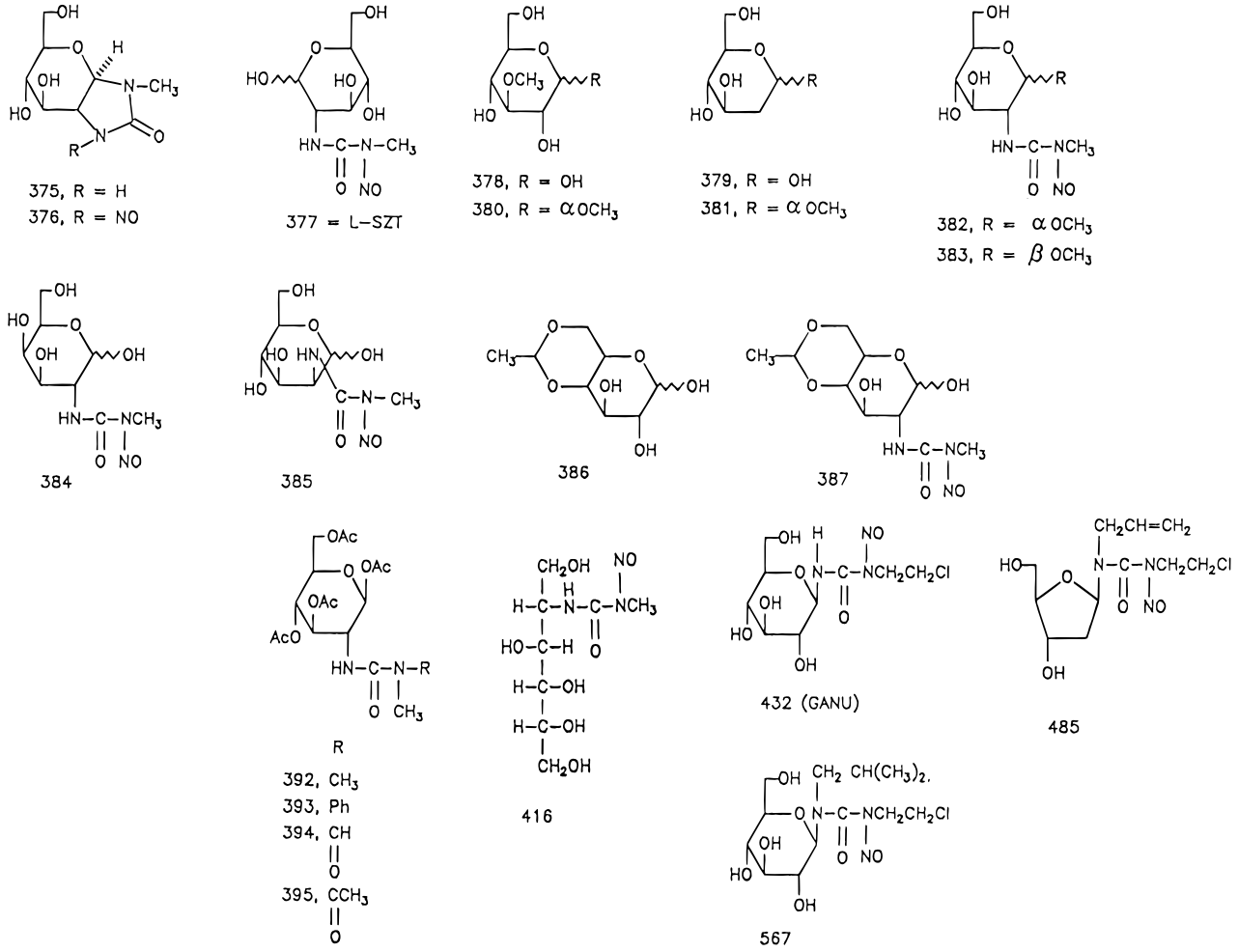
The azide reagent **11k** can become hazardous when used on a large scale and the intermediate urea can cyclize to the cyclic urethane **375** (Chart 14), whose *N*-nitrosated derivative **376** was devoid of activity against leukemia L1210 cells in vivo. Streptozotocin is best obtained by lyophilization of neutral aqueous solutions at 0 °C. A review was published[328] of the early chemistry of streptozotocin (**367**) as well as its interesting transformations at various pH levels.

The L-streptozotocin (**377**) was prepared[329] from L-glucosamine via the *N*-methylurea derivative and had a somewhat greater activity against L1210 than the D isomer.

Streptozotocin has antileukemic (Table 20),[330–332] mutagenic,[333–336] and diabetogenic[337–341] activities. Biochemical[335,342,343] and pharmacological[344–347] studies of SZT have been reported. SZT has been used clinically[348,349] to treat malignant islet cell carcinoma of the pancreas and carcinoid carcinoma, but the clinical use has been limited[349] because of the damage to the pancreas and kidneys. A review was published[350] which covers the chemical and biological aspects of streptozotocin.

The diabetogenic activity of streptozotocin in mice and rats was correlated[339] with the depression of the

Chart 14. Structures for Section VII.C



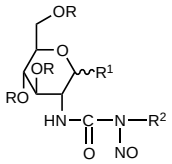
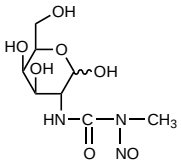
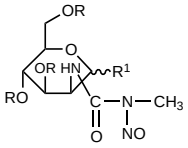
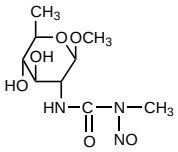
biosynthesis of nicotinamide adenine dinucleotide (NAD) and it could be prevented by the administration of nicotinamide.[13,339,351] Nicotinamide-pretreated animals were protected against morphological disruption of the endoplasmic reticulum, mitochondria, and either partial or complete degranulation.[339] In addition, the pancreatic islet cells of mice and rats were protected as determined[340] by electron microscopic examinations of the β-cells and blood sugar data. Furthermore, the antitumor activity of SZT was not caused by the depression of NAD levels, since the addition of nicotinamide failed to lower the cytotoxicity of SZT against the L1210 cells.[339]

The importance of the free glucose moiety of SZT (**367**) for its biological activities was emphasized in several studies.[352−356] Thus, 3-*O*-methylglucose (**378**) and 2-deoxyglucose (**379**, Chart 14), both nonmetabolized glucose analogs that are transported into cells in the same way as glucose, protected[352] β-cells of the pancreas from SZT cytotoxicity when injected with or just before SZT. However, the α-methylglucosides of 3-*O*-methylglucose (**380**) and 2-deoxyglucose (**381**) and the α- and β-methylglycosides of SZT, **382** and **383** respectively, failed to protect against the SZT pancreatic toxicity and caused no diabetes in rats.[353] Hence, an unsubstituted C1-hydroxyl of the glucose moiety in SZT (**367**) is essential for allowing it to accumulate in the β-cells of the pancreas.[354] The D-glucose structure is important for the binding of the drug to the outer membrane and for the transport into the cells since the membrane binding affinities are very low for galactose, the C4 epimer of glucose, and for mannose, the C2 epimer, and the compounds 4-*epi*-SZT (**384**) and 2-*epi*-SZT (**385**) have no diabetogenic properties.[355]

The compound 4,6-*O*-ethylidene glucose (EG, **386**, Chart 14) was known[353] to bind to the outer surface of the D-glucose transporter in the membranes of human erythrocytes and other mammalian cells. When EG and SZT were coinjected into rats, the diabetogenicity of SZT was blocked[356] as judged by their normal blood glucose levels, normal response in a glucose-tolerance test, and normal insulin levels in response to feeding. This blockage of the cellular uptake of SZT by EG without the influx of EG into the cells was supported[354] by [^{14}C]EG and [^{14}C]glucose distribution studies in rats. A conclusion was drawn[354] that a specific glucose transport system exists on the membrane surface of pancreatic β-cells which recognizes the glucose moiety of SZT. This recognition can be blocked by competitive inhibition with EG. Further support for this conclusion came from the synthesis and testing[354] of 4,6-*O*-ethylidene streptozotocin (**387**) which was shown to be nondiabetogenic and noncytotoxic to the β-cells of the pancreas.

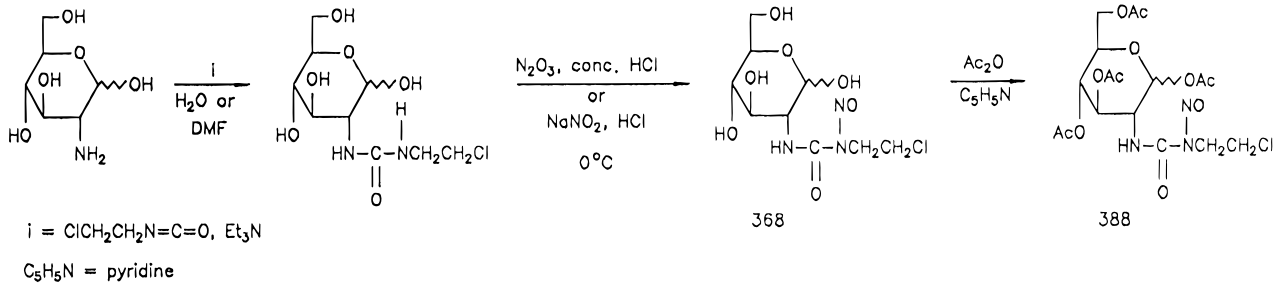
The pioneering work[43,44,116] on the synthesis of aliphatic and cycloaliphatic nitrosoureas demonstrated that the substitution of a β-chloroethyl group for the alkyl group of alkylnitrosoureas greatly increased the cytotoxic activity against the P388 and L1210 cell lines. Chlorozotocin (CZT, **368**), the 2-chloroethyl analog of streptozotocin, was synthe-

Table 20. Anticancer Activity of C2-Substituted *N*-Nitrosourea Monosaccharide Analogs against the Ip-Implanted Murine L1210 Lymphoid Leukemia

compound no.	R	R^1	R^2	OD,[a] mg/kg	ILS_{max},[h] %	log kill[i]	survivors, %	ID_{50},[l] μg/mL	LD_{50},[m] mg/kg	ref(s)
										
367	H	OH	CH_3	120[b]	85			84	360	46,362
373	Ac	β-OAc	CH_3	100[b]	69			10		332
389	H	OH	CH_3CH_2	100[b]	0				190	332
390	Ac	β-OAc	CH_3CH_2	100[b]	7			29		332
391	Ac	β-OAc	$CH_3(CH_2)_3$	100[b]	7			35		332
368	H	OH	$ClCH_2CH_2$	15[c]	701	≥6	60[j]		45−47	46,362
				20[d]	401		30[j]			
				2.5[f]	63		0			
				12.5[e]	418		67[k]			374
				6.3[g]	622		83[k]			374
388	Ac	OAc	$ClCH_2CH_2$	15[c]	137	≥5	40[j]			46,362
				20[e]	100		40[k]			361
				7.5[f]	36		10[k]			
382	H	α-OCH_3	CH_3	50[g]	35			47	1930	368
383	H	β-OCH_3	CH_3	50[g]	40			80	1700	368
396	H	β-OCH_3	CH_3CH_2	50[g]	14				1700	368
397	H	β-OCH_3	$CH_3(CH_2)_2$	100[g]	7					368
398	H	β-OCH_3	$CH_3(CH_2)_3$	50[g]	12					368
399	H	α-OCH_2CH_3	CH_3	125[g]	64				2050	368
400	H	α-OCH_2CH_3	CH_3CH_2	100[g]	14					368
401	H	β-OCH_2CH_3	CH_3CH_2	100[g]	10					368
402	H	α-$O(CH_2)_2CH_3$	CH_3	50[g]	29				1467	368
403	H	β-$O(CH_2)_2CH_3$	CH_3	50[g]	30				2150	368
404	H	α-$O(CH_2)_3CH_3$	CH_3	50[g]	30				2000	368
405	H	β-$O(CH_2)_3CH_3$	CH_3	50[g]	33				1580	368
406	H	α-$O(CH_2)_3CH_3$	$CH_3(CH_2)_3$						660	368
417	H	α-OCH_3	$ClCH_2CH_2$	10[e]	717		100[k]		48	374
				3.2[g]	394		50[k]			
				6.3[g](po)	380		50[k]			
407		dicaproate	$ClCH_2CH_2$	1[f]	94				141	373
408		monopalmitate	$ClCH_2CH_2$	1[f]	72				162	373
409		dipalmitate	$ClCH_2CH_2$	1[f]	106				800	373
410		monomyristate	$ClCH_2CH_2$	1[f]	74				61	373
411		dimyristate	$ClCH_2CH_2$	1[f]	104				800	373
										
412								75		355,369
										
413	H	OH						62		355,369
414	Ac	α-OAc		50[g]	46			5×10^{-5} M		355,369
										
415				200[g]	78					367

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered on days 1−7 after tumor implantation. [c] Drug administered on day 2 after tumor implantation. [d] Drug administered on day 6 after tumor implantation. [e] Drug administered on day 1 after tumor implantation. [f] Drug administered on days 1−9 after tumor implantation. [g] Drug administered on days 1−5 after tumor implantation. [h] Increase in life span = $[(T - C)/C] \times 100$. [i] Defined in section V. [j] Percentage of mice surviving on day 90 after tumor implantation. [k] Percentage of mice surviving on day 60 after tumor implantation. [l] Inhibitory dose = single dose resulting in the killing of 50% of the L1210 leukemia cells. [m] Lethal dose = ip-administered dose resulting in the death of 50% of the untreated mice.

Scheme 43



sized (Scheme 43) by the reactions of D-glucosamine with 2-chloroethyl isocyanate in either water or DMF, to give the (2-chloroethyl)urea which was nitrosated selectively either with nitrogen trioxide in concentrated hydrochloric acid at 0 °C[46] or with nitrous acid generated from sodium nitrite and concentrated hydrochloric acid in a 2:1 water−ethanol mixture.[357] The use of concentrated hydrochloric acid resulted in the synthesis of the pure desired N1-nitroso isomer whereby the generation of nitrosyl chloride occurs in situ under these conditions.[46] GCNU (**388**), the tetraacetate of CZT, was obtained[116,357] by the acetylation of CZT (**368**) with acetic anhydride in pyridine (Scheme 43). The corresponding 2-fluoroethyl analog of streptozotocin (**367**) was synthesized and tested in vivo against the B16 melanoma.[187]

The L-CZT was synthesized[358] in several steps from L-arabinose. This L isomer had about the same activity against the leukemia L1210 in vivo, and equivalent therapeutic index, ED_{50}/LD_{10}, as the D isomer. Administration of either D- or L-CZT produced comparable inhibitions of bone marrow DNA, as measured by the [^{3}H]thymidine incorporation, and comparable bone marrow-sparing activity, as measured by circulating white blood cell counts at a maximum effective dose $<LD_{10}$.[359] Thus, the absolute configuration of the glucose moiety in CZT (**368**) and SZT (**367**) is not a factor in their active transport into cells.[359]

Nitrosoureas and nitrosamine compounds containing the *N*-alkyl-*N*-nitrosourea moiety depress pyridine nucleotide concentration in liver and this depression is related to the diabetogenic properties of SZT (**367**).[339,360] However, nitrosoureas containing a chloroethyl group attached to either N1 and/or N3, as in BCNU (**33**), CCNU (**137b**), and CZT (**368**) have no effect on NAD concentrations.[360] An administration of GCNU (**388**) caused no depression of the white blood cell count of bone marrow 24 h after administration, whereas BCNU (**33**) and CCNU (**137b**), containing the chloroethyl group but no glucose moiety, caused a severe depression of the white blood cell count.[361] Comparative studies[361,362] of the carbohydrate CENU analogs CZT (**368**) and GCNU (**388**) with the noncarbohydrate CENU analogs BCNU (**33**) and CCNU (**137b**) revealed that 24 h after administration the carbohydrate analogs inhibited the DNA synthesis, as measured by the [^{3}H]thymidine uptake, in the L1210 cells and GI mucosa but not in the bone marrow, whereas the noncarbohydrate analogs inhibited DNA synthesis in all three tissue types. The bone marrow-sparing activity of GCNU (**388**) was not complete because there was a decrease in the bone marrow uptake of [^{3}H]thymidine after 48 h at a dose of 20 mg/kg.[362] The nonmyelosuppressive activity of carbohydrate CENU analogs is discussed further in sections X.F and X.I.

2. Structural Variations of the C2-Nitrosoureas

Studies of streptozotocin (**367**) structure revealed[58,332,355,363] important structure−activity relationships. Streptozotocin β-tetraacetate (**373**) exhibited[332] only a slightly lower anticancer activity than SZT against the L1210 cells (Table 20). The replacement of the N3-methyl with longer carbon chains resulted in analogs **389**-**391** which were inactive against the L1210 cells in vivo[332] (Table 20). Substitution of the nitroso group either in SZT or in its acetylated analog **373** with various groups gave compounds **392**−**395** (Chart 14) which were about as active as **372** against L1210 in vitro but were inactive in vivo against P388 leukemia in mice.[364] The importance of the *N*-NO group for cytotoxicity was confirmed[332] by the fact that the urea **374** was inactive in vivo.

Important early studies[355,365−368] were concerned with the synthesis (Scheme 44) and biochemical testing of the α- and β-methyl glycosides **382** and **383** of SZT. The α-methyl glucoside **382** was 1.7 times more active than the β-methyl anomer **383** against the L1210 leukemia cell line.[355] A thorough study[367,368] of various alkyl glycosides **382**, **383**, and **396**−**406** of SZT was reported (Table 20). Substitutions of the methyl group of the nitrosourea side chain with longer chain alkyl groups, as in compounds **396**−**398**, **405**, and **406**, caused large decreases in the in vivo anticancer activities. The variation of the alkyl group

Scheme 44

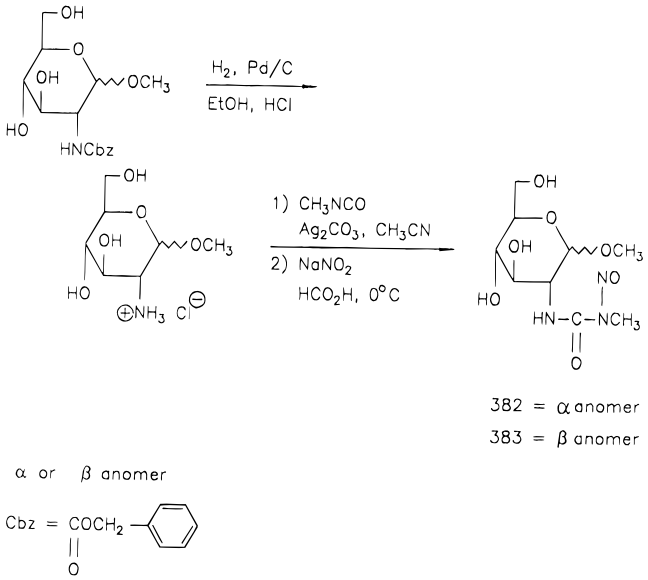
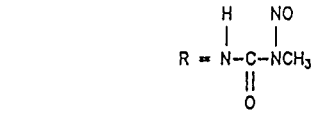
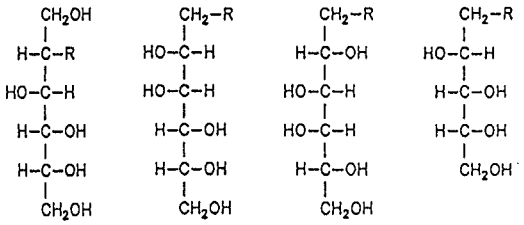
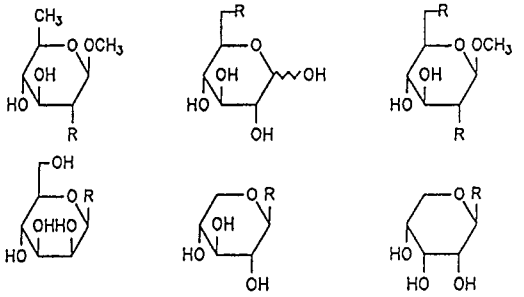
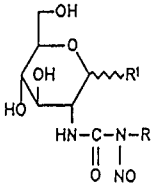


Chart 15. Structures for Section VII.C

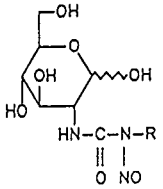




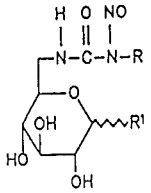




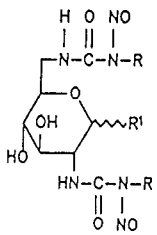
R	R¹
$ClCH_2CH_2$	β OCH_3
$ClCH_2CH_2$	α, β OCH_2CH_3
$ClCH_2CH_2$	α, β $O(CH_2)_2CH_3$
$ClCH_2CH_2$	α, β $O(CH_2)_3CH_3$
CH_3	α, β OCH_3
CH_3CH_2	β OCH_3
$CH_3(CH_2)_2$	α OCH_3
CH_3CH_2	α, β OCH_2CH_3
CH_3	α $O(CH_2)_2CH_3$



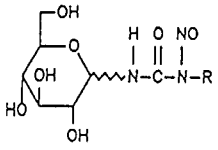
R
CH_3
CH_3CH_2
$CH_3(CH_2)_2$



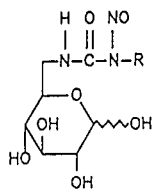
R	R¹
$ClCH_2CH_2$	α OCH_3
$ClCH_2CH_2$	α, β OCH_2CH_3
$ClCH_2CH_2$	α, β $O(CH_2)_2CH_3$
$ClCH_2CH_2$	α, β $O(CH_2)_3CH_3$
CH_3	α, β OCH_3
CH_3	α, β OCH_2CH_3
CH_3CH_2	α, β OCH_2CH_3



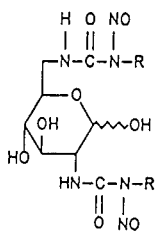
R	R¹
$ClCH_2CH_2$	β OCH_3
$ClCH_2CH_2$	α, β OCH_2CH_3
$ClCH_2CH_2$	α, β $O(CH_2)_2CH_3$
$ClCH_2CH_2$	α, β $O(CH_2)_3CH_3$
CH_3	α, β OCH_3
CH_3	α OCH_2CH_3
CH_3	α $O(CH_2)_2CH_3$
CH_3	β $O(CH_2)_3CH_3$
CH_3CH_2	α OCH_3



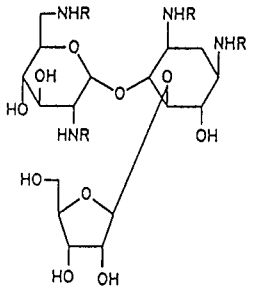
R	
CH_3	β
CH_3CH_2	β
$CH_3(CH_2)_2$	β
$CH_3(CH_2)_3$	β
CH_3	α
CH_3CH_2	α



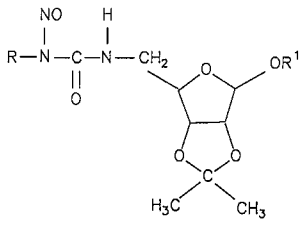
R
$ClCH_2CH_2$
CH_3
CH_3CH_2
$CH_3(CH_2)_2$



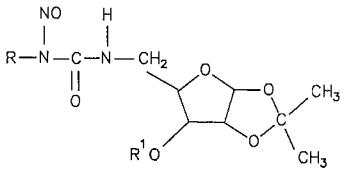
R
$ClCH_2CH_2$
CH_3
CH_3CH_2
$CH_3(CH_2)_2$
$CH_3(CH_2)_3$



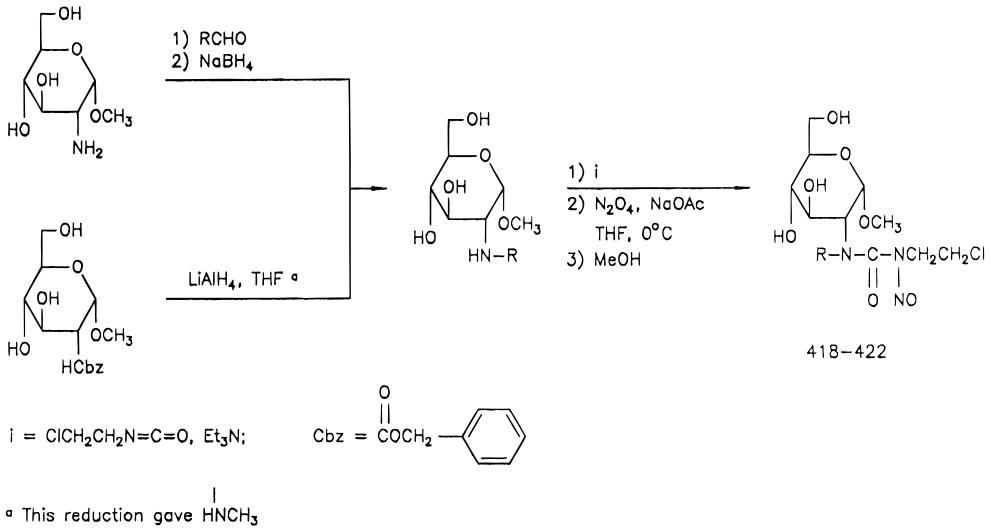
ribostamycin (R = $-C(=O)-NHCH_3$)



R = CH_3, CH_3CH_2, $CH_3(CH_2)_2$, $ClCH_2CH_2$
R^1 = H, CH_3, CH_3CH_2, $CH_3(CH_2)_2$



Scheme 45

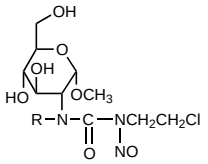


size at the glycosidic position, as in compounds **399**−**405**, had little effect on the anticancer activity while the difference in anticancer activity between the α and β anomers, i.e. **382** and **383**, was minimal. Overall, these various glycosides had lower activities than the parent compound SZT against the L1210 leukemia in mice (Table 20).[368] However, all the alkylglycosides shown in Table 20 had no diabetogenic properties and significantly lower acute toxicities than SZT as judged[368] by their LD_{50} values in mice. The therapeutic index of the β-methyl glycoside **383** was three times that of the SZT against the L1210 cells. Additional glycosides of ANU analogs are listed in Chart 15.

The synthesis and biochemical testing of the *N*-methyl-*N*-nitrosourea analogs of D-galactosamine (**412**) and D-mannosamine (**413** and **414**),[355,369] and of the β-methyl-6-deoxyglycoside **415**[312,367] were the focus of important earlier studies. The corresponding sugar alcohol, D-glucosaminol analog **416** (Chart 14), was synthesized[370] but no biological studies were reported. The 4-*epi*-galactosamine and 2-*epi*-mannosamine analogs **412** and **413** respectively, and the tetraacetate analog **373** were slightly more active in vitro than SZT (**367**)[355] but were less active in vivo[332] than SZT against the L1210. All of these analogs of SZT, the tetraacetate **373**, the α- and β-methyl glycosides **382** and **383**, and the 4-*epi* and 2-*epi* isomers **412** and **413**, were nondiabetogenic at the dose of 65 mg/kg which in the case of SZT caused a 4-fold increase in blood glucose concentration.[355]

Much attention has been devoted to the study of the CENU monosaccharide analogs because chlorozotocin (**368**) has a significantly higher in vivo activity against the L1210 cells, eliciting a 701% ILS, than the tetraacetate GCNU (**388**) with a 137% ILS, or SZT (**367**) with a 85% ILS (Table 20).[46,362] Both CZT and GCNU were nondiabetogenic and nonmyelosuppressive.[361,362,371,372] A series of long-chain esters **407**−**411** of CZT containing the C_{10}−C_{18} alcohol moieties, were synthesized[373] by the reaction of CZT with varying ratios of the corresponding acid chlorides in pyridine. Thus, there were produced the mono-, di-, and tricaprates, mono-, di-, and trilaureates, mono-, di-, and trimyristates, mono-, di-, and tristearates, and mono-, di-, tri-, and tetrapalmitates.

Table 21. Anticancer Activity of C2-Substituted N3,N3-Disubstituted Monosaccharide CENU Analogs against Ip-Implanted Murine L1210 Lymphoid Leukemia[377]



compound no.	R	OD,[a,b] mg/kg	ILS_{max},[c,d] %	ther ratio[d,e]
418	CH_3	50	700	26
419	$CH_3(CH_2)_2$	50	650	31
420	$CH_3(CH_2)_3$	50	745	33
421	$(CH_3)_2CHCH_2$	50	689	36
422	$CH_3OCH_2CH_2$	25	650	56
368 CZT		12.5	240	9

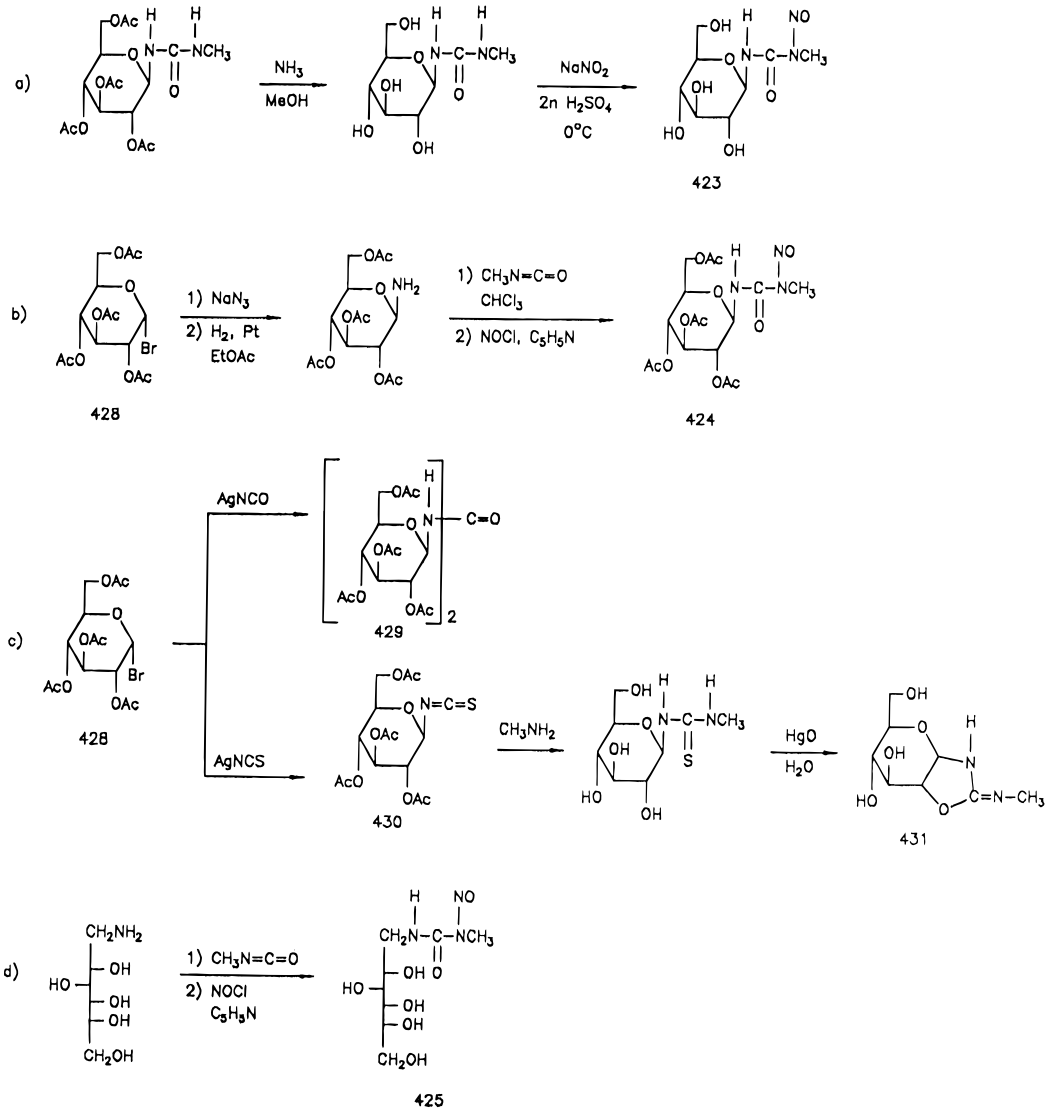
[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered on days 1−5 after tumor implantation. [c] Increase in life span = $[(T - C)/C] \times 100$. [d] Values have been rounded. [e] Therapeutic ratio = OD/ILS_{30}; ILS_{30} = daily dose resulting in a 30% increase in life span of the treated mice.

Several of these esters of CZT were either as active or more active than CZT in the P388 in vivo test system (Table 20).[373] The toxicity of these esters was considerably lower than that of CZT, as judged by their LD_{50} values (Table 20).

A number of CZT (**368**) analogs were reported in patents.[374,375] The overall synthetic scheme involved reaction of various carbohydrate amines with *N*-(2-chloroethyl)-*N*-nitrosourea transfer agents **11a** and **11c**. The alkyl glycosides of CZT with one to four carbon atoms were synthesized[312,370,374] and their structures are shown in Chart 15. The in vivo anticancer activity of the α-methyl glycoside **417** (Table 20) was much higher than that of the *N*-alkyl-*N*-nitrosourea monosaccharides **396**−**406**.[374,376]

A series of N3,N3-disubstituted CENUs located on the C2-position of the D-glucose ring, **418**−**422**, were synthesized[377] according to the sequence of Scheme 45. The secondary amine intermediates were obtained either by reductive amination of the methyl α-D-glucosaminide or, in the case of the methyl substituent, by a hydride reduction of the carboben-

Scheme 46



zoxyamine derivative. The crude amines were converted to the corresponding ureas with 2-chloroethyl isocyanate. After purification, the ureas were nitrosated by means of dinitrogen tetraoxide according to a standard procedure (Scheme 45).[377] In this case, 4 equiv of the nitrosating agent were used and the resulting nitrite ester-containing intermediates were hydrolyzed with acidic methanol to the final products. As with earlier examples in this series (Tables 3 and 13) compounds **418**−**422** are chemically activated by the intramolecular cyclization of a β-hydroxy group to yield a cyclic urethane. These N3,N3-disubstituted congeners were found[377] to have significantly higher in vivo antileukemic activity and higher therapeutic indices (Table 21) than the parent drug CZT (**368**). Survivors on day 60 were found[377] for all of the compounds **418**−**422** at their optimal doses. These analogs also have lower acute toxicities than CZT, as evidenced by their higher optimal doses against the L1210 cell line (Table 21).[377]

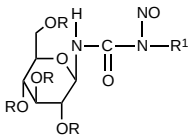
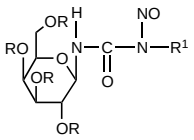
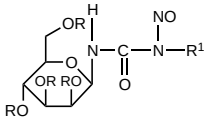
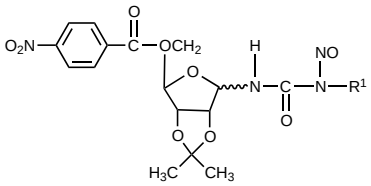
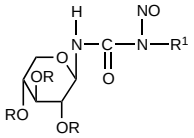
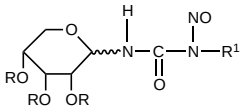
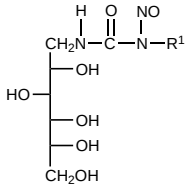
3. Structural Variations of the C1-Nitrosoureas

A large number of C1-substituted glycosylamine ANU analogs (**423**−**427**) have been reported.[355,370] The syntheses of a number of these analogs **423**-**425** are outlined in Scheme 46. In an attempt to prepare the 1-substituted urea, the acetobromoglucose **428** was reacted with silver isocyanate to give the symmetrical urea **429** rather than the 1-isocyanate derivative.[355] The reaction of **428** with silver thiocyanate yielded the corresponding 1-isothiocyanate **430** which was converted to the corresponding methyl thiourea. However, the reaction of this urea with mercuric oxide in water produced the *trans*-1,2-bicycloisourea **431** rather than the expected methylurea.[355] These SZT analogs **423**-**427**, generally, had lower antileukemic activities than SZT (Table 22).

A variety of CENUs substituted at the C1-position of monosaccharides were synthesized by the reaction of the corresponding glycosylamine with 2-chloroethyl isocyanate, followed by nitrosation with nitrous acid, in analogy to the sequence of Scheme 46.[309,312,378−382]

The parent C1-CENU compound GANU (**432**, Chart 14) was synthesized[312] and tested[312,383,384] against the L1210 cell line by the ip, iv, and po routes (Table 22). Administration of a LD_{10} dose, 10 mg/kg, resulted in no depression of the normal bone marrow DNA synthesis; however, it caused a 96% inhibition of L1210 DNA synthesis.[384] A comparison of GANU with CZT (**368**) revealed[384,385] that at a dose of 15 mg/kg GANU was less active, with 117% ILS, than CZT, with 306% ILS, against the L1210 in vivo.

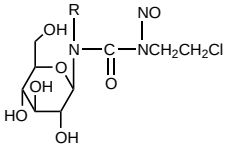
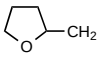
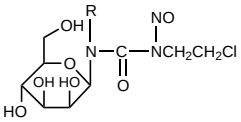
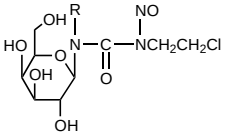
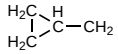
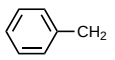
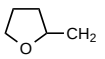
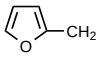
Table 22. Anticancer Activity of C1-Substituted Monosaccharide *N*-Nitrosourea Analogs against the Ip-Implanted Murine L1210 Lymphoid Leukemia

compound no.	R	R^1	OD,[a] mg/kg	ILS_{max},[j] %	survivors, %	ID_{50},[n] μg/mL	ref(s)
							
423	H	CH_3	100[b,e]	56		55	332
			200[b,f]	107			312
424	Ac	CH_3	100[b,c]	70	15[l]	18	332
432	H	$ClCH_2CH_2$	8[b,f]	931			312
			8[b,f]	512	100[m]		383
			20[b,g]	297	33[m]		383
			20[b,h]	504	80[m]		383
			20[c,h]	305	20[m]		383
			15[c,h]	507	83		374
							
426	H	CH_3	100[b,e]	56		76	332
427	Ac	CH_3	100[b,e]	43		28	332
433	H	$ClCH_2CH_2$	4[b,f]	405	100[m]		312
							
434	H	$ClCH_2CH_2$	8[b,f]	103	0[m]		312
							
437		$ClCH_2CH_2$	15−30[b,i]	∞[k]			388,389
							
435	H	$ClCH_2CH_2$	8[b,f]	445	100[m]		312
438	Ac	$ClCH_2CH_2$	10−15[b,i]	∞[k]			388,389
							
439	Ac	$ClCH_2CH_2$	10−15[b,i]	∞[k]			388,389
							
425		CH_3	100[b,e]	33		51	332
			200[b,f]	40			312
436		$ClCH_2CH_2$	16[b,f]	402			312

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered by the ip route. [c] Drug administered by the iv route. [d] Drug administered by the po route. [e] Drug administered on days 1−7 after tumor implantation. [f] Drug administered on days 1−3 after tumor implantation. [g] Drug administered on day 1 after tumor implantation. [h] Drug administered on day 2 after tumor implantation. [i] Drug administered on days 1, 5, and 9 after tumor implantation. [j] Increase in life span = $[(T - C)/C] \times 100$. [k] ≥50% cures. [l] Percentage of mice surviving on day 45 after tumor implantation. [m] Percentage of mice surviving on day 60 after tumor implantation. [n] Inhibitory dose = dose resulting in the killing of 50% of the L1210 leukemia cells.

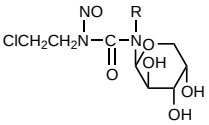
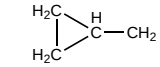
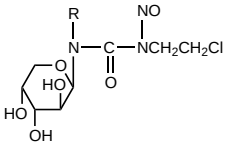
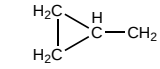
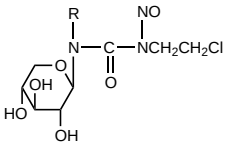
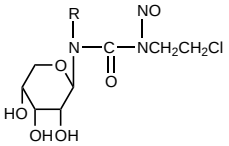
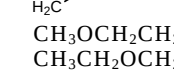
The two compounds produced similar DNA alkylation levels but GANU had seven times the carbamoylating activity of CZT.[384] This result was used to explain[384] why GANU (**432**) had a 2-fold greater lethal toxicity, on a molar basis, than CZT. Some activity by GANU was also shown by the oral route resulting in a 115%

Table 23. Anticancer Activity of C1-Substituted N3,N3-Disubstituted Aldohexose CENU Analogs against Ip-Implanted Murine L1210 Lymphoid Leukemia[394]

compound no.	R	OD,[a,b] mg/kg	ILS_{max},[c,d] %	ther ratio[e,d]
				
440	CH_3	50	689	11
441	CH_3CH_2	50	721	18
442	$CH_3(CH_2)_2$	25	711	20
443	$CH_3(CH_2)_3$	25	689	17
444	$(CH_3)_2CHCH_2$	25	669	36
445	$CH_3O(CH_2)_3$	50	721	29
446		50	757	29
				
447	$CH_3OCH_2CH_2$	200	529	36
				
448	$(CH_3)_2CH$	50	669	24
449	$CH_3(CH_2)_3$	100	590	48
450	$(CH_3)_2CHCH_2$	50	711	50
451	$(CH_3)_3CCH_2$	100	711	25
452	$CH_2{=}CHCH_2$	50	669	36
453	$HC{\equiv}CCH_2$	50	669	25
454	$CH_3OCH_2CH_2$	50	700	15
455	$CH_3O(CH_2)_3$	100	700	38
456	$CH_3OCH_2CH(CH_3)$	50	722	36
457	$CH_3OC(=O)CH_2CH_2$	25	757	30
458		50	700	28
459		100	623	33
460		100	700	24
461		100	689	59
462		50	650	15
432 (GANU)		6.25	199	8
137b (CCNU)		25	757	5
311 (ACNU)		25	757	9

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered on days 1−5 after tumor implantation. [c] Increase in life span = $[(T - C)/C] \times 100$. [d] Values have been rounded. [e] Therapeutic ratio = OD/ILS_{30}; ILS_{30} = daily dose resulting in 30% increase in life span of the treated mice.

Table 24. Anticancer Activity of C1-Substituted N3,N3-Disubstituted Aldopentose CENU Analogs against Ip-Implanted Murine L1210 Lymphoid Leukemia[395]

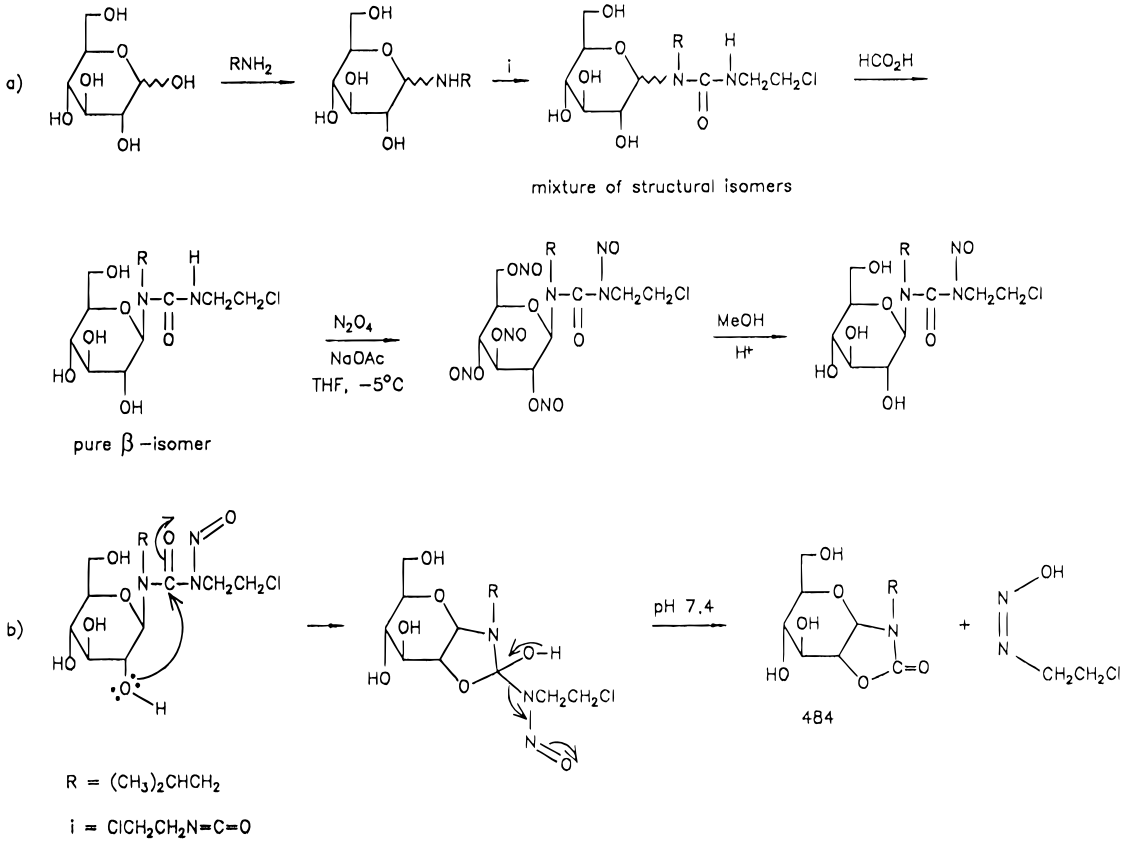
compound no.	R	OD,[a,b] mg/kg	ILS_{max},[c,d] %	ther ratio[e,d]
				
463	$CH_3(CH_2)_2$	50	711	33
464	$(CH_3)_2CH$	25	758	32
465	$(CH_3)_2CHCH_2$	50	711	28
466		50	723	45
467	$CH_2{=}CHCH_2$	50	700	38
468	$HO(CH_2)_3$	50	641	24
469	$CH_3CH_2CH(CH_2OH)$	50	722	64
470	$CH_3OCH_2CH_2$	50	733	65
471	$CH_3O(CH_2)_3$	50	650	52
				
472	$CH_3O(CH_2)_3$	50	641	57
473	$CH_3OCH(CH_3)CH_2$	25	733	50
474	$CH_3OCH_2CH(CH_3)$	25	722	58
475		25	679	24
				
476	$CH_3(CH_2)_2$	50	623	25
477	$(CH_3)_2CH$	25	757	38
478	$(CH_3)_2CHCH_2$	25	733	26
479	$CH_3OCH_2CH_2$	25	733	38
				
480	$CH_3(CH_2)_3$	100	614	13
481		25	757	21
482	$CH_3OCH_2CH_2$	50	757	57
483	$CH_3CH_2OCH_2CH_2$	50	733	3

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered on days 1−5 after tumor implantation. [c] Increase in life span = $[(T - C)/C] \times 100$. [d] Values have been rounded. [e] Therapeutic ratio = OD/ILS_{30}; ILS_{30} = daily dose resulting in 30% increase in life span of the treated mice.

ILS, which was greater than that for CZT. On the basis of this result, it was concluded[383] that attachment of the CENU group to the C2-position of the glucose moiety decreases the absorption of drugs from the gastrointestinal (GI) tract. GANU was found[383] to have additional activities against ascites sarcoma 180, ascites hepatomas AH-130, and Walker carcinosarcoma 256. The D-galactose (**433**) and D-xylose (**435**) analogs had much greater activity than the D-mannose derivative (**434**) (Table 22). Phase 1 clinical studies of GANU were reviewed.[283,284] The 1-CENU-glucosaminol derivative **436** was shown[312] to have a high activity against the L1210 leukemia cell line as compared with the 1-MNU analog **425** (Table 22).

Several CENU analogs of 1-deoxyaldopentose, namely the ribofuranosyl-CENU (RFCNU, **437**), ribopyranosyl-CENU (RPCNU, **438**), and xylopyranosyl-CENU (XPCNU, **439**) were synthesized[386,387] and shown[387−389] to have some activity in vivo against the L1210 (Table 22). In these studies the prolongation of survival (PS) values are given as ∞ when more than 50% of the animals are cured. These com-

Scheme 47



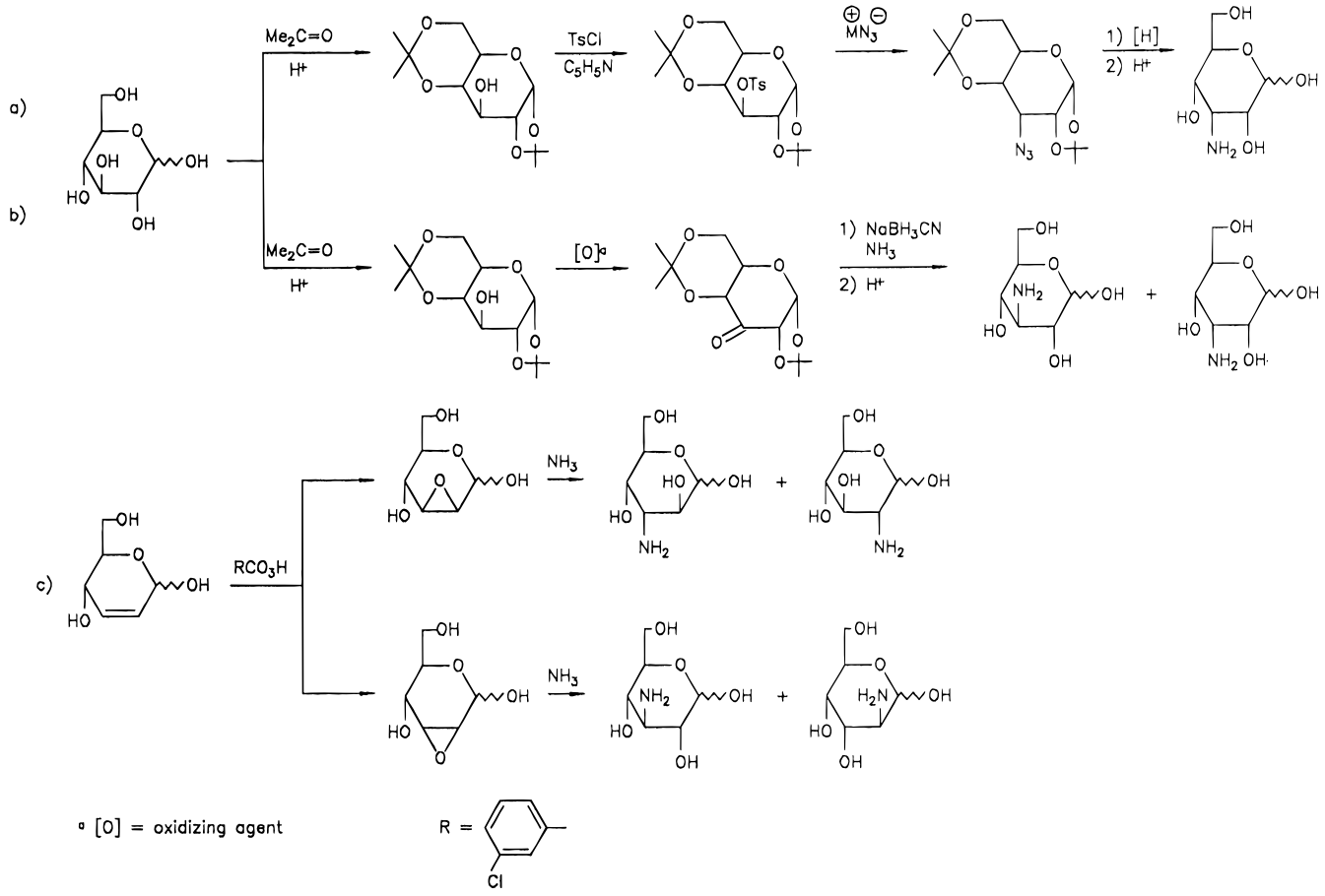
pounds were less toxic and had greater therapeutic indices than CCNU (**137b**) or MeCCNU (**138b**). Of all of the compounds tested, only RFCNU (**437**) was not immunosuppressive in the hemolytic plaque-forming cell (PFC) test, either before or after the addition of the antigen in the form of fresh sheep red blood cells administered ip to mice.[389] The other compounds **438** and **439** were immunosuppressive whether given before or after the antigen. The RFCNU was shown[390] to have a low cumulative hematoxicity. Immunological[391,392] and metabolic[393] studies of RFCNU and RPCNU were reported.

A large series of N3,N3-disubstituted-CENU analogs containing the glucopyranosyl, mannopyranosyl, and galactopyranosyl moieties **440**−**462**[394] (Table 23) and the arabinopyranosyl, xylopyranosyl, and ribopyranosyl moieties **463**−**483**[395] (Table 24) were synthesized as illustrated in Scheme 47a and also reported in several patents.[396−399] Reaction of the carbohydrates with primary amines yielded the corresponding glycosylamines which, in turn, were converted to the ureas with 2-chloroethyl isocyanate. The products were mixtures of structural isomers which were smoothly transformed to the pure C1-substituted ureas by an acid-catalyzed isomerization. These intermediates were nitrosated with an excess of dinitrogen tetraoxide which nitrosated all the hydroxyl groups as well as the N1-nitrogen. The resulting *O*-nitrite esters were hydrolyzed with acid in methanol to generate the desired *N*-nitrosourea analogs. These CENU congeners were unstable yellow caramels and oils which were characterized by spectroscopic methods.[394,395] Earlier research[133,202] on N3,N3-disubstituted nitrosoureas with a hydroxyl group in the β-position had shown that their chemical decomposition produced a mixture of a cyclic urethane and the chloroethyldiazohydroxide (Scheme 17b). The mechanism of this decomposition was verified[394] by isolation of the cyclic urethane **484** at pH 7.4 (Scheme 47b). Since in these cases no isocyanate product can be formed, the toxicities of this class of compounds should decrease.

A large number of variations of the R group were employed in both series (Tables 23 and 24). Included in the tables are only those compounds which produced cures of all mice on 60 days. Some general structure−activity relationships were apparent.[394,395] Thus, in the D-glucose series, the compounds with straight-chain and branched alkyl groups up to C_4 were highly active, followed by a sharp decrease in the activity for compounds with C_5−C_{18} groups. Several compounds with side-chain alcohol moieties had low activity, but there was good activity for compounds with ether and alicyclic ether functions. Compounds containing unsaturated and alicyclic groups also showed high activities. The acetylated derivatives had low anticancer activity. Variations of the carbohydrate structure revealed[394,395] that the D-glucose and D-galactose series were more active than the D-mannose series (Table 23) and that the L- and D-arabinose analogs were more active than the D-xylose and D-ribose analogs (Table 24). The 2-deoxyribose compound **485** (Chart 14) was inactive.[395] This result was in support of the importance of the C2-hydroxyl group for intramolecular cyclization to the cyclic urethane, according to Scheme 17b.

The therapeutic indices of these compounds were much higher than those for the N3-monosubstituted nitrosoureas, such as CCNU (**137b**), ACNU (**311**), and GANU (**432**) (Tables 23 and 24). These N3,N3-disubstituted analogs also had lower acute toxicities as seen by the higher optimum doses which were

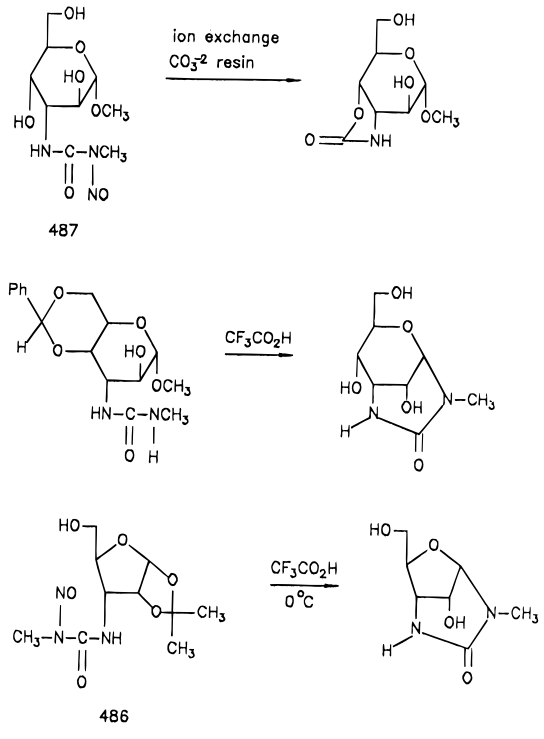
Scheme 48



required for optimal activity against the leukemia L1210.[394,395]

Additional ANU and CENU analogs of C1-substituted monosaccharides are included in Chart 15.[312,370,374]

Scheme 49



4. Structural Variations of the C3-Nitrosoureas

The location of the *N*-nitrosourea moiety at the 3-position of the monosaccharide ring resulted in a number of biologically active compounds. The general synthesis of these 3-amino-3-deoxyglucose analogs requires the prior blocking of the C-1, C-2, C-4, and C-6 hydroxyl groups. The unsubstituted C3-hydroxyl then can be converted to the amino group by a sequence shown in Scheme 48a where the hydroxyl is converted to a good leaving group, such as tosylate. This intermediate is subjected to a S_N2 reaction with azide ion, and the resulting azide reduced to the amine with an overall inversion of configuration. Alternatively, the C3-hydroxyl can be oxidized to the ketone which then can be reductively aminated to the amine function with ammonia, thus yielding either one of the stereoisomers, depending on the conditions and any neighboring group effects (Scheme 48b). The 2,3-anhydro derivatives also can be ring opened by the reaction with an amine to

Scheme 50

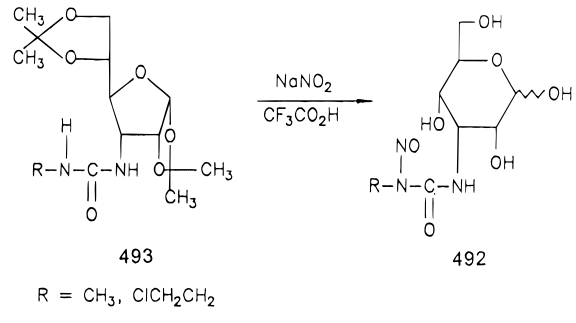


Table 25. Anticancer Activity of C3-Substituted Monosaccharide *N*-Nitrosourea Analogs against Ip-Implanted Murine L1210 Lymphoid Leukemia

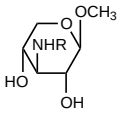
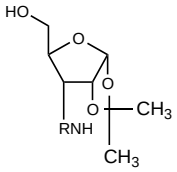
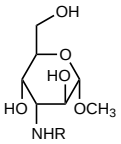
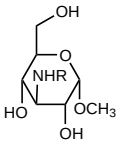
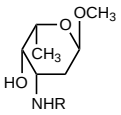
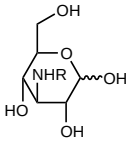
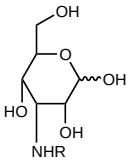
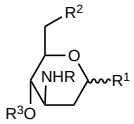
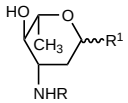
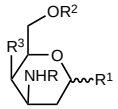
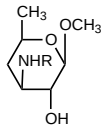
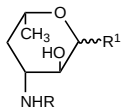
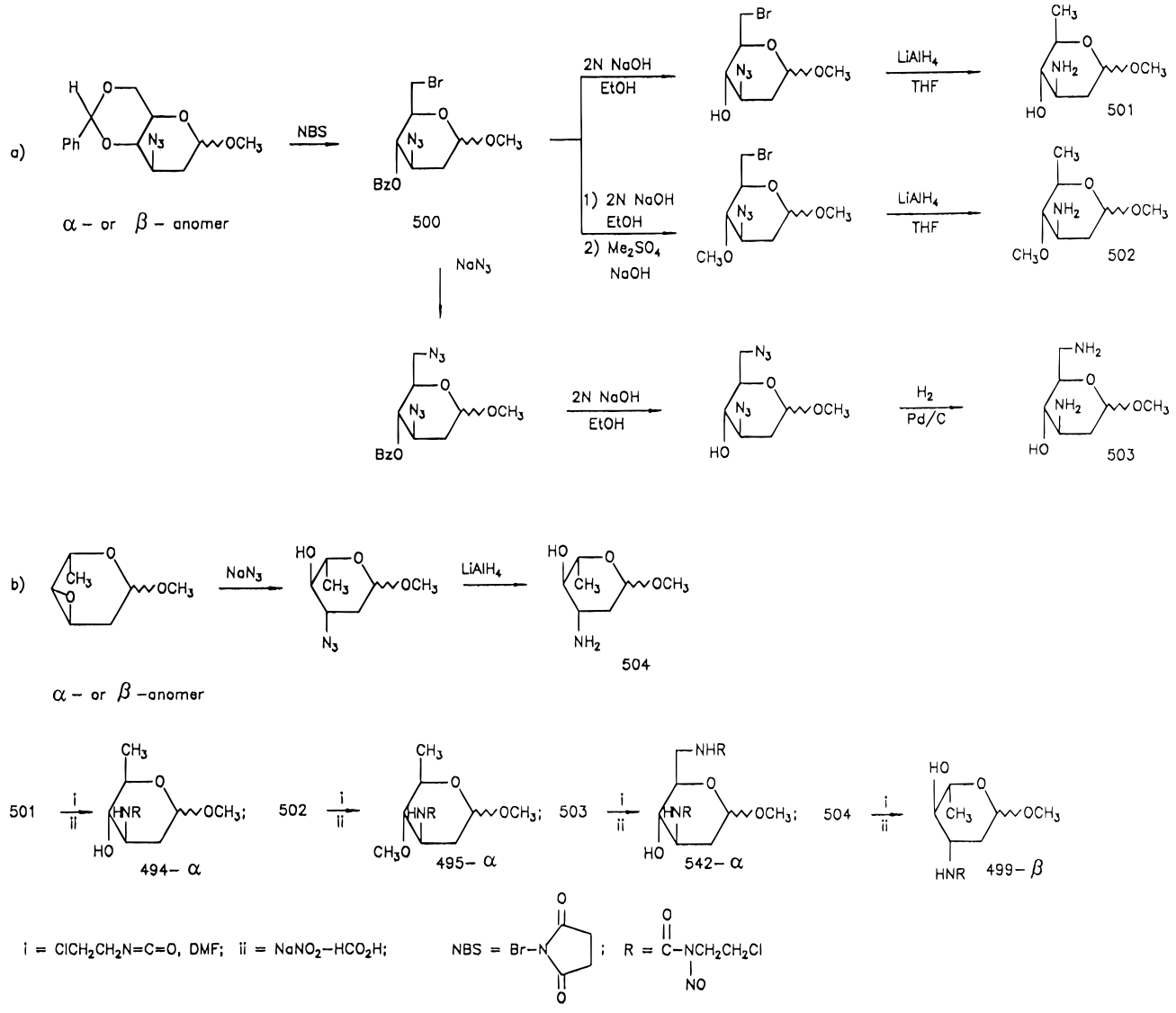
compound no.		OD,[a] mg/kg	ILS_{max},[i] %	survivors, %	LD_{50},[l] mg/kg	log P[m]	ref(s)
		R = −CO−N(NO)CH_3					
486		200[b,e]	191			−1.08	400
		100[b,f]	124	30[j]			
487		400[b,e]	7			−0.40	400
		200[b,f]	(29,98)				
488		100[b,e]	42,58,18			−0.82	400
		100[b,f]	118,3,31				
489		400[b,e]	114			−1.57	400
		200[b,f]	77,97				
490		400[b,e]	32,6			0.61	400
		R = −CO−N(NO)CH_2CH_2Cl					
491[o]		40[b,g]	500	100[j]			403
		8.9[b,f]	171	43[j]			
		60[c,g]	143	14[j]			
		320[d,g]	250	43[j]			
492		40[b,g]		100[j]			406
		114[b,h]		100[j]			
		40[c,g]	214	86[k]			
		200[d,g]	250	86[k]			
							
	R^1 R^2 R^3						
494	α-OCH_3 H H	5[b,e]	50	0[j]			408
495	α-OCH_3 H CH_3	20[b,e]		100[j]			
496	α-OCH_3 OH H	5[b,e]		60[j]			
497	β-OCH_3 H H	10[b,e]	98	0[j]	43		
498	β-OCH_3 OH H	5[b,e]	526	60[j]			
							
499	β-OCH_3	5[b,e]	419	40[j]			408
							
505	β-OCH_3 H Cl	5[b,e]	30				413
506	β-OCH_3 C(=O)Ph H	5[b,e]	30	0[j]	40		
507	β-OCH_3 H H	5[b,e]	500	50[j]	40		
508	α-OCH_3 H Cl	5[b,e]	88	0[j]	40		
509	α-OCH_3 H H	5[b,e]	>500	60[j]	40		

Table 25. (Continued)

compound no.		OD,[a] mg/kg	ILS_{max},[i] %	survivors, %	LD_{50},[l] mg/kg	log P[m]	ref(s)
		R = −CO−N(NO)CH_2CH_2Cl (Continued)					
510		10[b,e]	>500	80[j]			413
							
511	α-OCH_3	10[b,e]	500	60[j]			413
512	β-OCH_3	10[b,e]	175	30[j]			413

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drugs administered by the ip route. [c] Drugs administered by the iv route. [d] Drugs administered by the po route. [e] Drug administered on days 1, 5, and 9 after tumor implantation. [f] Drug administered on days 1−9 after tumor implantation. [g] Drug administered on day 1 after tumor implantation. [h] Drug administered on days 1−5 after tumor implantation. [i] Increase in life span = $[(T - C)/C] \times 100$. [j] Percentage of mice surviving on day 60 after tumor implantation. [k] Percentage of mice surviving on day 86 after tumor implantation. [l] Lethal dose = single ip-administered dose resulting in the killing of 50% of the untreated mice. [m] Partition coefficient: P = [compound in 1-octanol]/[compound in water].

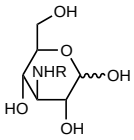
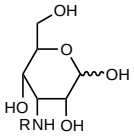
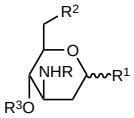
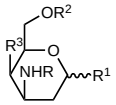
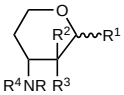
Scheme 51



produce a mixture of the 2- and 3-substituted amine products (Scheme 48c).

Various *N*-methyl-*N*-nitrosoureas of 3-amino-3-deoxypentoses and hexoses, **486**−**490**, were synthesized and tested[400] for anticancer activity against L1210 in vivo (Table 25). In all of these compounds the C1-anomeric position was protected either as a methoxy or an isopropylidene group. The nitrosations of the corresponding ureas were achieved with aqueous dinitrogen trioxide, and the isolation proce-

Table 26. Anticancer Activity of C3-Substituted Monosaccharide CENU Analogs against the Sc-Implanted Murine B16 Melanoma

compound no.	R^1	R^2	R^3	R^4	OD,[a] mg/kg	ILS_{max},[h] %	survivors,[i] %	tumor volume, mm^3	ref(s)
				R = −CON(NO)CH_2CH_2Cl					
									
491					40[b,e]	72	0		403
					67[b,f]	65	0		
					69[c,f]	112	0		
									
492					30[c,e]	158	71		406
					200[d,e]	58	43		
									
494	α-OCH_3	H	H		20[b,g]	160	5		408,409
495	α-OCH_3	H	CH_3		20[b,g]	70	0		
496	α-OCH_3	OH	H		20[b,g]	139	15		
									
507	β-OCH_3	H	H		10[b,g]	158	80		413
508	α-OCH_3	H	Cl		10[b,g]	102	30		
509	α-OCH_3	H	H		10[b,g]	157	70		
									
514	β-OCH_3	OH	H	H	10[b,g]	74		308	414
515	α-OCH_3	OH	H	H	10[b,g]	45		2071	
516	β-OCH_3	H	OH	H	10[b,g]			3701	
517	β-OCH_3	OH	H	CH_3	10[b,g]			3109	
518	β-OCH_3	OH	H	$(CH_2)_3OCH_3$	10[b,g]			4771	
control								6425	
137b (CCNU)					10[b,g]	48		2247	
436 (RFCNU)					15[b,g]	17		2181	

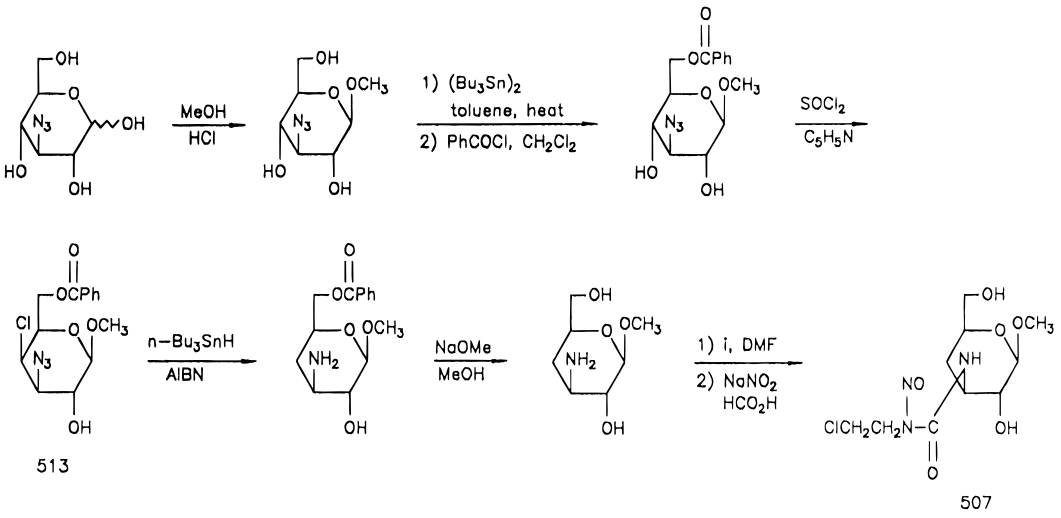
[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered by the ip route. [c] Drug administered by the iv route. [d] Drug administered by the po route. [e] Drug administered on day 1 after tumor implantation. [f] Drug administered on days 1−9 after tumor implantation. [g] Drug administered on days 1, 5, and 9 after tumor implantation. [h] Increase in life span = $[(T - C)/C] \times 100$. [i] Percentage of mice surviving on day 60 after tumor implantation.

dure involved lyophilization at low temperatures to prevent undesirable cyclization reactions,[400] such as those shown in Scheme 49. All the 3-substituted analogs had anticancer activities in vivo that were either equal to or greater than that of SZT (**367**). On the basis of the variety of carbohydrate structures which were employed, it was concluded[400] that the carbohydrate moiety functions as a relatively nonspecific hydrophilic carrier for the *N*-methyl-*N*-nitroso urea group.

The corresponding C3-CENU analogs are shown in Table 25. The 3-amino-D-3-deoxyglucose analog CNUG (**491**) was synthesized[401,402] and shown[403] to be active against various murine tumors (Table 25). A synthesis of the 3-amino-3-deoxy-D-allose analog CNUA (**492**) involved the use of blocking groups in the final nitrosation step (Scheme 50).[404,405] In this case, the acid used for the nitrosation reaction also caused the hydrolysis of the blocking groups of compound **493** in one step. CNUA (**492**) was found[406] to be active against L1210 leukemia (Table 25), Lewis lung carcinoma, and B16 melanoma. The immunosuppression and myelotoxicity of **492** was equal to that of CZT (**368**) but lower than that of ACNU (**311**).[406,407]

The synthesis[408,409] of a series of 2-deoxy- and 2,6-dideoxy-3-CENU carbohydrate analogs **494**−**499** resulted in a number of active compounds (Table 25). A general synthesis adopted[408] for the preparation of the 2-deoxy-3-amino and 2-deoxy-3,6-diamino analogs is illustrated in Scheme 51. The starting material, containing the 3-azido-4,6-benzylidene moieties, was ring opened with *N*-bromosuccinimide to produce the 3-azido-4-(benzyloxy)-6-bromo intermediate **500**. This crucial compound could either be converted to the 2,6-dideoxy-3-amino compounds **501** and **502** by a hydride reduction or to the 2-deoxy-3,6-diamino compound **503** by an azide displacement of the C6-

Scheme 52



i = $ClCH_2CH_2{=}C{=}O$

AIBN = azobisisobutyronitrile, $(CH_3)_2C(CN)-N{=}N-C(CN)(CH_3)_2$, a free radical initiator; C_5H_5N = pyridine

bromide, followed by a catalytic reduction of the 3,6-diazide. The isomeric 2,6-dideoxy-3-amino congener **504** (Scheme 51b) was synthesized by a ring opening of a 3,4-anhydro intermediate followed by a hydride reduction of the 3-azide. The resulting amines then were converted to the corresponding CENU derivatives (**494**−**499**) by a standard sequence of urea formation, followed by nitrosation.[408]

These compounds were found[408] to be intermediate in lipophilicity between the water-soluble and lipid-soluble CENUs. Their preparation was undertaken[408] with the triple aim of attaining a better therapeutic index, a lack of cross-resistance with a broader spectrum of activity, and an increased stability near the physiological pH. The lack of adjacent *cis*-hydroxyl groups was expected[408] to increase the stability by preventing intramolecular cyclizations (Scheme 17).

Compounds **494**−**499** were tested[408] against the L1210 leukemia (Table 25), the 3LL Lewis lung carcinoma and the B16 melanoma (Table 26). These compounds possessed high activities against the L1210 in vivo, with several of them resulting in cures after 60 days.[408] A number of these analogs were much more active than BCNU. In addition, the acute toxicities of 40−50 mg/kg for these compounds were lower than that of 25 mg/kg for BCNU. Compounds **494** and **496** exhibited improved activities against the ip-implanted B16 melanoma as compared with the more hydroxyl-containing **491** (Table 26). There appeared to be no consistent SAR between the cytoactivity and the number of hydroxyl groups in the compounds, although the analog **498** containing two hydroxyl groups was the most active against the L1210 leukemia in vivo[408] (Table 25).

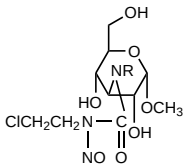
There was no significant difference in activity against the L1210 between the α and β anomers of the same molecular structure. Thus, for the α and β anomers **494** and **497** the %ILS values were 50 and 98, respectively. The L isomer (**499**) was more active and more toxic than the D isomer (**497**).

Compound **498** (ecomustine, CY233) had high activity[408] against the L1210 leukemia (Table 25), the Lewis lung carcinoma, the B16 melanoma[408,410] and the sc-implanted colon adenocarcinoma,[409] a cancer line resistant to both BCNU (**33**) and CZT (**368**).[410] Ecomustine was found[411] to be highly active via the intravenous route against the human xenografted tumor, the amelanotic melanoma Me X274, and partially active against the colon adenocarcinoma CXF 243. Compound **498** had limited hematological toxicity and it failed to cross the blood−brain barrier or enter the bone marrow, as judged from autoradiographic results with a ^{14}C-labeled analog.[408] The syntheses of ^{14}C- or ^{13}C-labeled **498** which can be used for biochemical mechanism studies was reported.[412] In this work, the radioactive label was placed either on the carbonyl group of the urea, the second carbon of the chloroethyl group, or the glycosidic methyl group.

Several 4-deoxy-3-CENU analogs **505**−**512** (Table 25) have been synthesized[413] according to a representative sequence shown in Scheme 52. The C1- and C6-positions were blocked as methoxy and benzoyl ester groups, respectively, and the C4-hydroxyl converted to a chloride of opposite configuration. The reduction of the intermediate 3-azido-4-chloro derivative **513** by means of a combination of tributylstannate and the free radical initiator azobis(isobutyronitrile) (AIBN) resulted in the replacement of the 4-chloro group by hydrogen and the reduction of the azide to the 3-amine moiety. An alkaline hydrolysis of the C6-ester was followed by a conversion of the 3-amine to the corresponding CENU derivative **507** by a standard two-step sequence.[413] The other compounds can be obtained[413] either from some of the intermediates in this sequence or by starting with the α-methyl glycoside.

Compounds **507** and **509**−**511** possessed[413] high activities against the L1210 leukemia in vivo (Table 25) and compounds **507** and **509** were more active than **496** against the B16 melanoma (Table 26). A number of C3-CENU analogs were active[409] against the ip-administered C38 colon carcinoma and against the iv-administered B16 melanoma.

Table 27. Anticancer Activity of C3-Substituted N3,N3-Disubstituted Monosaccharide CENU Analogs against Ip-Implanted Murine L1210 Lymphoid Leukemia[415]



compound no.	R	OD,[a,b] mg/kg	ILS_{max},[c,d] %	ther ratio[e,d]
519	CH_3	12.5	614	48
520	$CH_3(CH_2)_3$	25	745	38
521	$(CH_3)_2CHCH_2$	12.5	598	19
522	$CH_3OCH_2CH_2$	25	344	38
523	$CH_2{=}CHCH_2$	12.5	598	32

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered on days 1−5 after tumor implantation. [c] Increase in life span = $[(T - C)/C] \times 100$. [d] Values have been rounded. [e] Therapeutic ratio = OD/ILS_{30}; ILS_{30} = daily dose resulting in 30% increase in life span of the treated mice.

A series of similar 4-deoxy-3-CENU pentose analogs **514**−**518** (Table 26) were synthesized and tested[414] for activity against the B16 melanoma. Compound **514** had the highest %ILS and greatest reduction of the tumor volume of any member of this series as well as CCNU (**137b**) and RFCNU (**437**). In this case, the β anomer **514** was more active than the α anomer **515**. The percentage of mice developing the melanoma after 39 days was 42% for **514** and 65% for CCNU, but at 90 days the values were approximately 65% for both compounds.[414]

Several analogs containing the N3,N3-disubstituted-*N*-nitrosoureas moiety attached to the C3-position of the D-glucose ring **519**−**523** (Table 27) were synthesized[415,416] according to the sequence outlined in Scheme 53a. The reaction of 2,3-anhydro-α-D-allopyranoside **524** with various primary amines in sealed tubes, followed by the reaction with 2-chloroethyl isocyanate produced a mixture of urea products **525** and **526**. After chromatographic separation, the major product, methyl glucoside **525**, was nitrosated with dinitrogen tetraoxide according to a standard procedure[415] for these N3,N3-disubstituted congeners. The *N*-nitrosourea products were unstable, yellow powders and their chemical identities were determined[415] by spectroscopic methods. The structures of the intermediate isomeric ureas **525** and **526** were established by an independent synthetic route, as outlined in Scheme 53b. Thus, the reaction of sodium azide with the starting 2,3-epoxide **524** yielded a mixture of 2- and 3-azido intermediates which were chromatographically separated and then catalytically reduced to the 2-amino and 3-amino isomers. Reductive amination of the amines with *n*-butyraldehyde and sodium borohydride produced the secondary amines which, in turn, were converted to their corresponding 2-chloroethylureas by a reaction with

Scheme 53

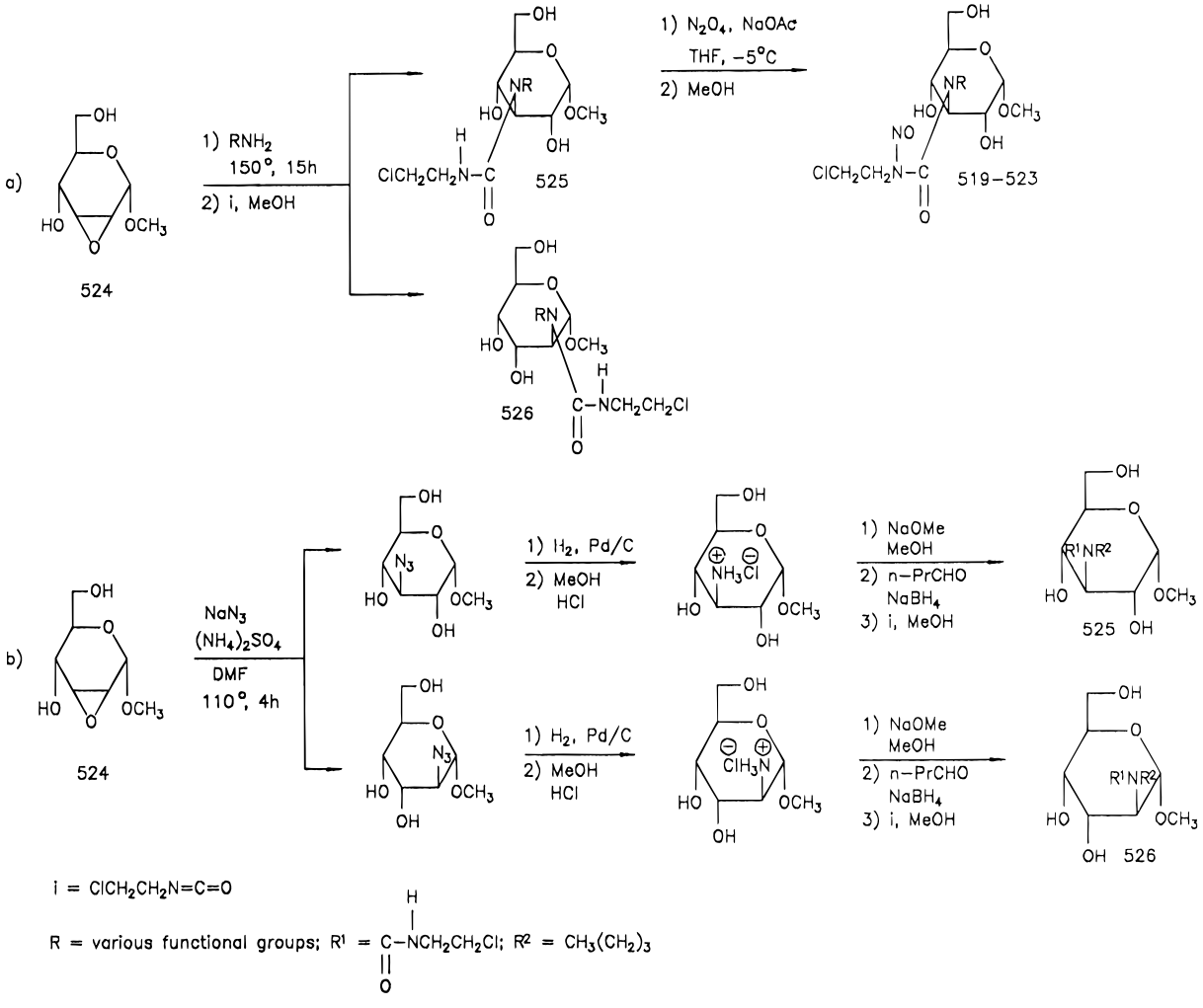
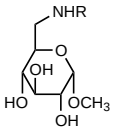
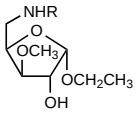
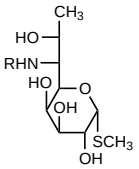
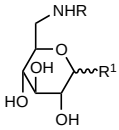
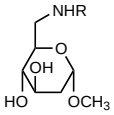


Table 28. Anticancer Activity of C6-Substituted Monosaccharide *N*-Nitrosourea Analogs against Ip-Implanted Murine L1210 Lymphoid Leukemia

compound no.		OD,[a] mg/kg	ILS_{max},[i] %	survivors,[j] %	LD_{50}, mg/kg	log P[m]	ref(s)
		R = −CON(NO)CH_3					
527		400[b,d] 300[b,e]	50,60 73			−1.45	400
528		250[b,f] 250[b,g] 250[c,g]	57 87 43		920[k] 1450[l]		372,421
529		400[b,e]	39				329
		R = −CON(NO)CH_2CH_2Cl					
530	 R^1 = α,β-OH	15[b,f] 50[b,h] 50[c,f]	650 606 >442	100 80 50			374,429
531	R^1 = α-OCH_3	18[b,h] 12.5[b,f] 12.5[c,f]	632 722 700	100 100 100		−0.70	374,429
532		20[b,d]	163	20			408

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered by the ip route. [c] Drug administered by the po route. [d] Drug administered on days 1, 5, and 9 after tumor implantation. [e] Drug administered on days 1−9 after tumor implantation. [f] Drug administered on days 1−5 after tumor implantation. [g] Drug administered on days 1−4 after tumor implantation. [h] Drug administered on day 1 after tumor implantation. [i] Increase in life span = $[(T - C)/C] \times 100$. [j] Percentage of mice surviving on day 60 after tumor implantation. [k] Lethal dose = single ip-administered dose resulting in the death of 50% of the untreated mice. [l] Lethal dose = single po-administered dose resulting in the death of 50% of the untreated mice. [m] Partition coefficient: P = [compound in 1-octanol]/[compound in water].

2-chloroethyl isocyanate. The identity of the C3-substituted analogs was determined by a comparison of optical rotation and spectroscopic data.[415] These N3,N3-disubstituted CENU congeners were shown[415] to have high anticancer activities and therapeutic indices (Table 27). There were small structure−activity differences among the C2- and C3-substituted glucose analogs as can be seen from a comparison of data in Tables 21 and 27. In general, the C3-substituted analogs were more toxic than the C1- and C2-substituted analogs, as evidenced by lower optimal dose levels.

5. Structural Variations of the C5-Nitrosoureas

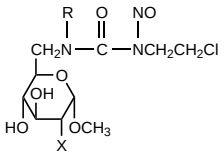
A series of nitrosourea analogs of 5-deoxy-D-ribofuranose were reported[417] and are shown in Chart 15.

6. Structural Variations of the C6-Nitrosoureas

Several C6-*N*-alkyl-*N*-nitrosoureas **527**−**529** are shown in Table 28.[369,370,400,418] A comparison[369] of the 6-substituted nitrosourea analog **528** (EDOMEN, GCP-6809)[419,420] with the 2-substituted analog **414** (MAZ) (Table 20) revealed that MAZ was 10 times more toxic to β-cells than to pancreatic fibroblasts, whereas EDOMEN was equally toxic to both β-cells and pancreatic fibroblasts at 10^{-3} M concentrations and, thus, was not considered to be diabetogenic. Since SZT (**367**) and MAZ (**414**) are diabetogenic and EDOMEN (**528**) is nondiabetogenic, it was proposed[369] that β-cells selectively transport the pyranose forms of D-glucose and not the glucofuranoside form. EDOMEN (**528**) had a half-life of 45 h at pH 6 and 37 °C which was five times longer than that observed for SZT.[421] The alkylating activity of **528** was the same as that of SZT, 18% and 19% as compared to 100% for BCNU, while its carbamoylating activity of 46% was similar to the value of 32% for CCNU (**137b**).[421] Thus, EDOMEN has a combination of both lipid and water (2.5%) solubility, low alkylating but high carbamoylating activity, and a different anticancer profile from the clinical nitrosoureas, such as BCNU, CCNU, SZT, or CZT.[421]

The inhibition by MAZ and EDOMEN of L1210 cell growth in vitro was found[369] at levels below that of SZT (**367**) and only a weak cytotoxicity was observed

Table 29. Anticancer Activity of C6-Substituted N3,N3-Disubstituted Monosaccharide CENU Analogs against Ip-Implanted Murine L1210 Lymphoid Leukemia[434]



compound no.	R	X	OD,[a,b] mg/kg	ILS_{max},[c,d] %	ther radio[e,d]
533	CH_3	OH	400	752	27
534	$CH_3(CH_2)_3$	OH	400	143	6
535	$(CH_3)_2CHCH_2$	OH	400	136	12
536	$CH_3OCH_2CH_2$	OH	200	371	20
537	CH_3	NHAc	200	757	17
538	$CH_3(CH_2)_3$	NHAc	200	180	7
539	$(CH_3)_2CHCH_2$	NHAc	400	110	10
540	$CH_3OCH_2CH_2$	NHAc	200	226	13

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered on days 1−5 after tumor implantation. [c] Increase in life span = $[(T - C)/C] \times 100$. [d] Values have been rounded. [e] Therapeutic ratio = OD/ILS_{30}; ILS_{30} = daily dose resulting in 30% increase in life span of the treated mice.

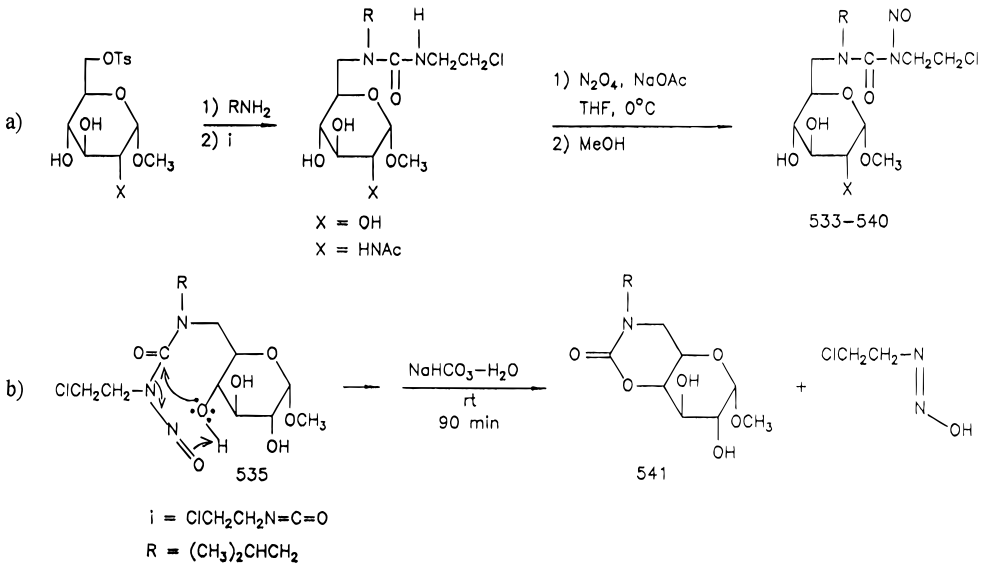
in vivo against the L1210 leukemia in mice (Table 28). While the activity of EDOMEN against the murine leukemia L1210 was low and certain mammary cancer lines and Lewis lung carcinoma were relatively resistant, the drug was quite effective against solid transplantable melanomas, such as Harding−Passey and B16, which were resistant to BCNU (**33**) and CCNU (**137b**).[421] In addition EDOMEN (**528**) was able to reduce the development of metastatic lung nodules. EDOMEN was nonmyelosuppressive at therapeutic doses of 125 and 250 mg/kg and failed to depress serum glucose levels at a toxic dose of 285 mg/kg.[369] Compound **528** was active against several human tumor xenograft lines growing sc in nude mice, especially those of the large bowel and epidermoid lung cancer.[422] Combination therapy of EDOMEN and cyclophosphamide resulted in a large reduction in tumor weight of im-transplanted Lewis lung carcinoma and almost total elimination of lung metastases.[421]

The corresponding C6-substituted CENU analogs[374,423] are represented by compounds **530**−**532** in Table 28. In particular, the CENU α-methyl glucoside **531**, (MCNU, ranomustine, Cymerin) exhibited very high activity against both the ip- and po-implanted L1210 leukemia (Table 28).[374] The unsubstituted C1-hydroxyl analog of MCNU, compound **530**, was shown[374] to have high activity against the ip-implanted L1210 in vivo, while **532,** the 2-deoxy analog of MCNU, was significantly less active than MCNU (**531**) (Table 28).[374] In the case of MCNU there is no β-hydroxyl group on the ring to participate in an intramolecular cyclization with the elimination of the CENU group,[374] as was shown in Scheme 17b.

The biological properties of MCNU were compared[374] with those of several other carbohydrate CENU analogs such as CZT (**368**), **530**, 2-CENU α-methyl glycoside (**417**), and the 1-CENU analog GANU (**432**). Compared with the other monosaccharide CENU analogs, MCNU (**531**) was the most active against the L1210 leukemias by both ip and po routes of administration, had a relatively low acute toxicity by the oral route and had the least diabetogenic activity.[374] Thus, the drug MCNU was a promising candidate for further testing and was the subject of several pharmacological studies[424−428] and anticancer activity studies.[429−432] MCNU was effective against various murine tumors, such as L1210 and P388, sarcoma 180, Ehrlich ascites carcinoma, adenocarcinoma 755, Nakahara−Fukuoka sarcoma, Lewis lung carcinoma, B16 melanoma, and BC47 bladder carcinoma as determined either by ip or sc administrations.[430] It also was active against intracerebrally implanted glioma of mice by the ip route.[431] MCNU (**531**) binds to the acid-insoluble fraction by alkylation rather than carbamoylation of biomolecules in the cell and, thus, induces the DNA strand breakage. An overview of MCNU with regard to antitumor activity, mechanism of action, pharmacokinetics, clinical evaluation, adverse reactions, drug interactions, dosage, and administrations was published.[433]

A series of analogs **533**−**540** (Table 29), containing the N3,N3-disubstituted CENU moiety at the C6-position of either methyl α-D-glucoside or methyl

Scheme 54



N-acetyl-α-D-glucosaminide, were synthesized[434] as outlined in Scheme 54a. The reaction of the C6-tosylate with the appropriate amines produced the crude secondary amines which were converted to the ureas by further reaction with 2-chloroethyl isocyanate. Nitrosation of the purified ureas was effected by means of dinitrogen tetraoxide, using a standard procedure[434] for these N3,N3-disubstituted congeners.

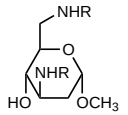
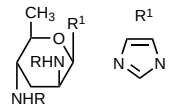
The antileukemic activities and therapeutic indices of these C6 analogs were greatly reduced (Table 29) as compared[434] with those of the C1- (Tables 23 and 24), C2- (Table 21), and C3- (Table 27) substituted compounds. In the C6 series the chemical activation by formation of a cyclic urethane requires the participation of the β-hydroxy group at C4. Such a six-membered cyclic urethane **541** was obtained[434] from the decomposition of **535** in an alkaline medium at room temperature (Scheme 54b). In this case, the completion of the cyclization required 90 min, whereas for the C1 and C3 analogs the cyclization reaction occurred in 10 and 6 min, respectively.[434] These large time differences in the activation reactions could explain the large differences in antileukemic activities. These results are believed[434] to support the importance of the β-hydroxy group for the intramolecular activation of the N3,N3-disubstituted CENU analogs.

Additional ANU and CENU analogs of C6-substituted monosaccharides are included in Chart 15.[374]

7. Structural Variations at Two Monosaccharide Positions. Bis-N-nitrosourea Analogs

Various 2,6-bis-ANU[370,418] and 2,6-bis-CENU[374] monosaccharide analogs have been synthesized and their structures are shown in Chart 15. The 2-deoxy-3,6-bis-CENU analog **542**[408] and the 3,6-dideoxy-2,4-bis-CENU glucose analog **543**[435] were reported to have high activity against the L1210 cell line (Table 30), and the use of **542** resulted in 75% 60 day survivors against the Lewis lung carcinoma and 10% survivors against the B16 melanoma.[408]

Table 30. Anticancer Activity of Bis-Substituted Monosaccharide CENU Analogs against Ip-Implanted Murine L1210 Lymphoid Leukemia

compound no.		OD,[a,b] mg/kg	ILS_{max},[c] %	survivors,[d] %	ref(s)
	R = −CON(NO)CH_2CH_2Cl				
542		20[b]		66	408
543		12.5	650		435

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered on days 1, 5, and 9 after tumor implantation. [c] Increase in life span = $[(T - C)/C] \times 100$. [d] Percentage of mice surviving on day 60 after tumor implantation.

8. Disaccharide N-Nitrosourea Analogs

Sucrose analogs **544** and **545** of SZT were synthesized and tested[436] in vivo against the L1210 leukemia (Table 31). The rationale for the synthesis of these compounds was that sucrose penetrates cell membranes of brain tumors but not those of normal brain tissue.[437] Sucrose appears to be a selective agent for absorption by the brain tumor cells, because it is too polar to pass through cell membranes by passive diffusion.[437] The large molecular radius of 4−5 Å of sucrose prevents its passage through cell pores, and it is not actively transported into cells.[436] The synthesis of these sucrose−SZT analogs is out-

Scheme 55

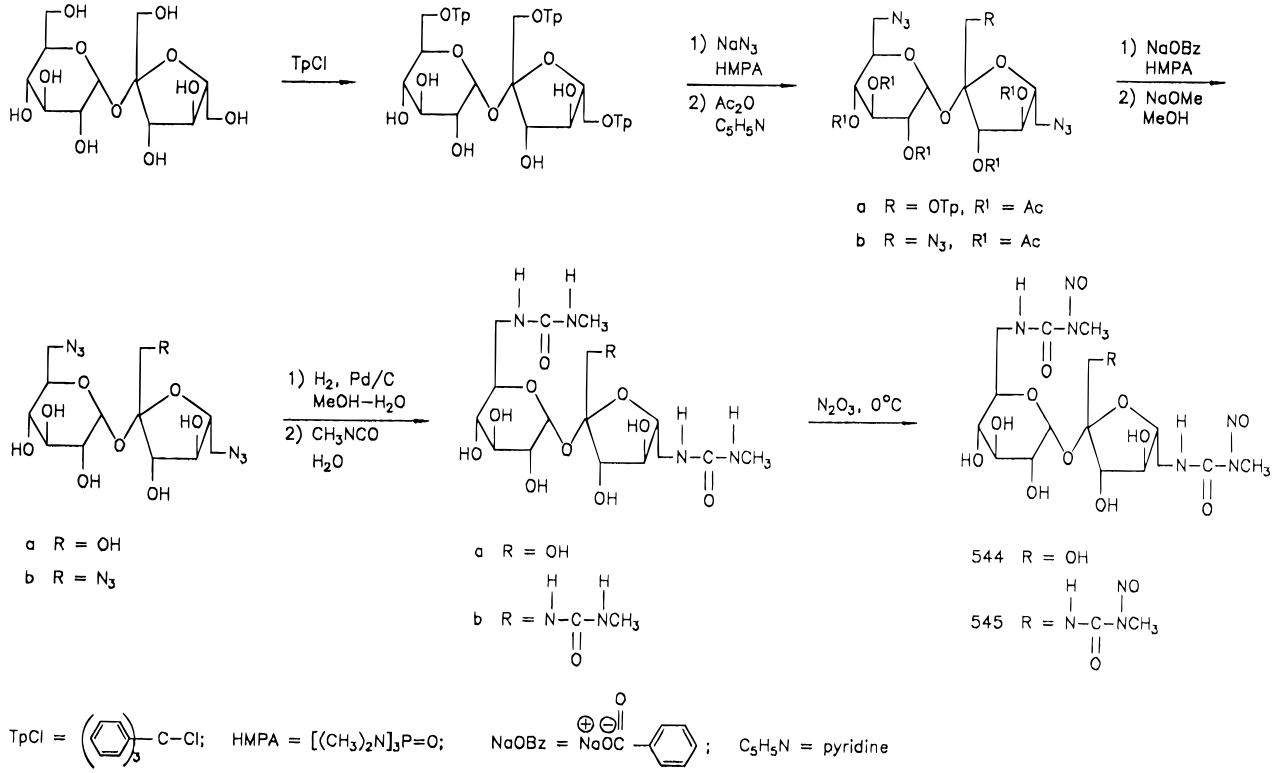


Table 31. Anticancer Activity of Disaccharide *N*-Nitrosourea Analogs against Ip-Implanted Murine L1210 Lymphoid Leukemia

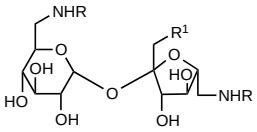
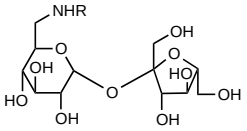
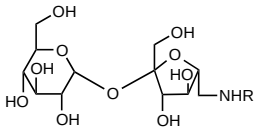
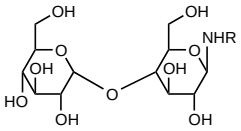
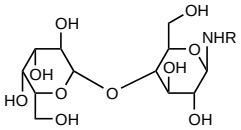
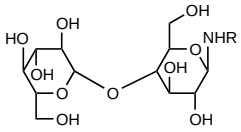
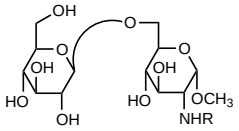
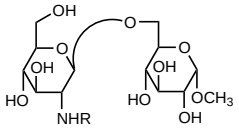
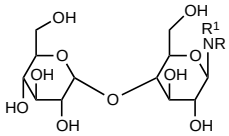
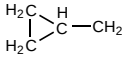
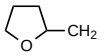
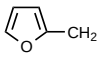
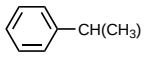
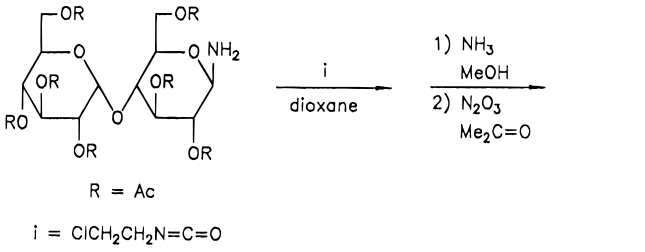
compound no.	R^1	dose[a] mg/kg	ILS_{max},[f,g] %	survivors,[h] %	ther ratio[i,g]	ref(s)
		$R = -CO-N(NO)-CH_3$				
						
544	OH	200[b]	37			436
545	NHR	200[b]	37			
		$R = -CO-N(NO)-CH_2CH_2Cl$				
						
546		100[c]	650	100		440
						
547		100[c]	650	70		440
						
548		20[d]	403	66		443
						
549		10[d]	384	66		443
						
550		8[d]	483	60		443
						
551		40[d]	226	20		445
						
552		32[d]	467	60		445
						
553	CH_3	100[e]	711		11	446
554	$(CH_3)_2CH$	100[e]	700		18	
555	$(CH_3)_2CHCH_2$	100[e]	733		83	
556	$CH_3CH_2CH(CH_3)$	100[e]	757		67	
557	$CH_2{=}CHCH_2$	100[e]	689		50	
558	$CH_3CH{=}CHCH_2$	100[e]	757		59	
559	$CH_3C(=CH_2)CH_2$	200[e]	757		62	
560	$CH_3CH_2OCH_2CH_2$	100[e]	669		37	
561	$CH_3OCH_2CH(CH_3)$	200[e]	700		67	
562		100[e]	689		29	

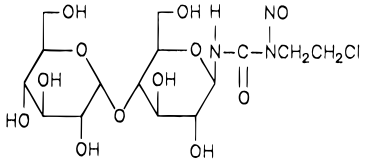
Table 31. (Continued)

compound no.	R^1	dose[a] mg/kg	ILS_{max},[f,g] %	survivors,[h] %	ther ratio[i,g]	ref(s)
		R = −CO−N(NO)−CH_2CH_2Cl				
563		200[e]	689		33	
564		200[e]	733		50	
565		200[e]	757		73	

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered on days 1−9 after tumor implantation. [c] Drug administered on day 1 after tumor implantation. [d] Drug administered on days 1−3 after tumor implantation. [e] Drug administered on days 1−5 after tumor implantation. [f] Increase in life span = $[(T - C)/C] \times 100$. [g] Values are rounded. [h] Percentage of mice surviving on day 60 after tumor implantation. [i] Therapeutic ratio = OD/ILS_{30}; ILS_{30} = daily dose resulting in a 30% increase in life span of the treated mice.

Scheme 56





548

lined in Scheme 55. Selective tripsylation of the primary 6,6′-dihydroxyls and the 1-hydroxyl groups of sucrose yielded the tristripsylated intermediate, which was acetylated and then reacted with the azide ion to yield either the di- or triazido intermediates. Removal of the acetate groups produced the unblocked azido intermediates. Reduction of the azido functions yielded the corresponding amines which were converted to their *N*-methylureas with methyl isocyanate. Nitrosation with dinitrogen trioxide produced the desired bis- and tris-*N*-methyl-*N*-nitrosourea analogs **544** and **545**.[436] The attempted synthesis of the bis-CENU analogs of sucrose was unsuccessful because the acidic conditions of the final nitrosation step caused hydrolysis of the sucrose glycosidic bond.[436]

The anticancer activities of compounds **544** and **545** against the L1210 leukemia were disappointing, with %ILS values of only 37 (Table 31).[436] Similarly, compounds **544** and **545** had only low activities against ependymoblastomas brain tumor, with %ILS values of 48 and 33, respectively, compared with a 44% ILS value for SZT (**367**). These low activities were attributed[436] to possible cleavage of the molecules to the corresponding D-glucose and D-fructose moieties by glycosidases. The corresponding peracetates of **544** and **545** proved to be inactive.[436] It would be interesting[319] to study the bioactivity of the corresponding thioglycoside which should be more stable to acidic and enzymatic hydrolytic reactions.

The two mono-CENU analogs of sucrose **546** and **547** were synthesized[438−440] by a reaction sequence similar to that for the analogs **544** and **545**. These CENU derivatives exhibited a high anticancer activity against the L1210 leukemia[440] (Table 31) and also

Scheme 57

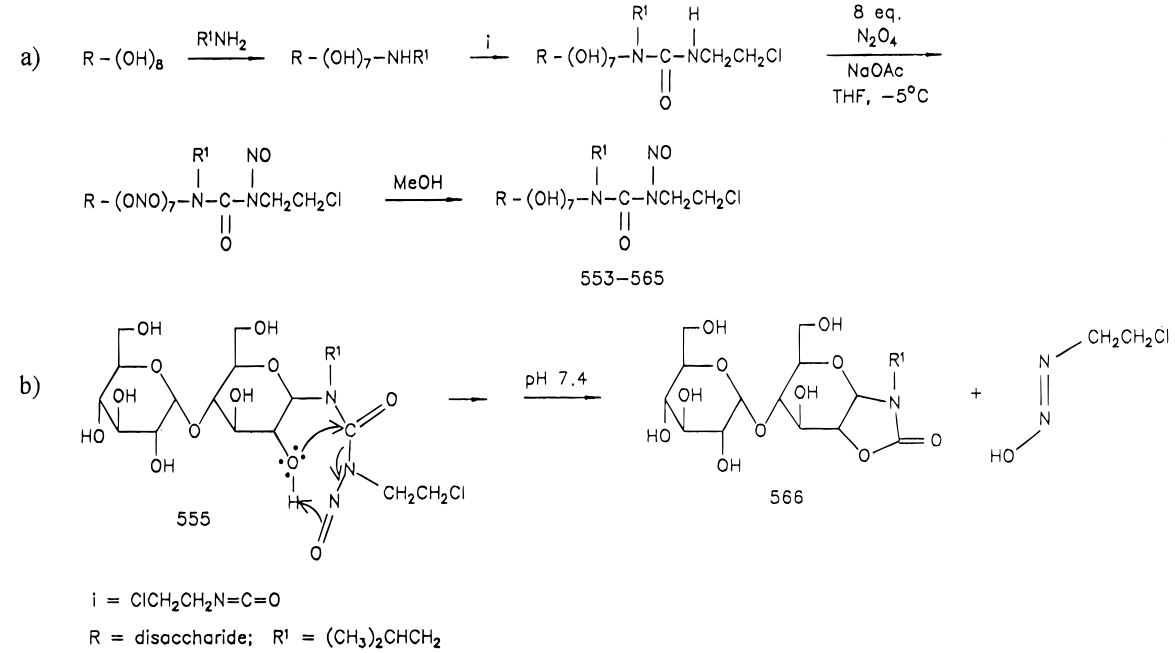
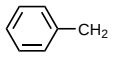
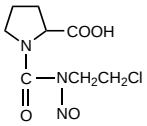
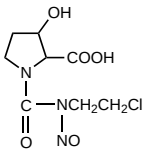


Table 32. Anticancer Activity of CENU Amino Acid Analogs against Ip-Implanted L5222 Leukemia,[a] L1210 Leukemia,[b] and P388 Leukemia[c]

$ClCH_2CH_2N(NO)$-CO-NH-$(CHR)_n$-COOH								
compound no.	R	*n*	OD,[d] mg/kg	ILS_{max},[i] %	survivors,[j] %	LD_{50},[k] mg/kg	log *P*[l]	ref(s)
570	H	1	44[a,e]	135			−1.25	467
			40[b,f]	270	33			465
571	H	2	15[b,g]	10		50		289
572	CH_3	1	20[b,f]	176	0		−1.11	465
573	$(CH_3)_2CH$	1	44[a,e]	155				467
			50[c,h]	197	16	72		466
	$(CH_3)_2CH$ + cyclodextrin	1	200[c,f]	130	16	320		466
574	$CH_3CH_2CH(CH_3)$	1	40[c,g]			70		289
575	$HOCH_2$	1	28[a,e]	130				467
			40[b,g]	111		160		289
576	$HOCH(CH_3)$	1	70[a,e]	155				467
			60[b,g]	111		110		467
577	$CH_3SCH_2CH_2$	1	70[a,e]	120				467
			20[b,f]	141	16		−0.85	465
578		1	30[b,f]	132	0		−0.28	465
579	H_2NCOCH_2	1	44[a,e]	150				467
580	$H_2NCOCH_2CH_2$	1	70[a,e]	155				467
581			80[b,g]	67		80		289
582			500[b,g]	8		>500		289

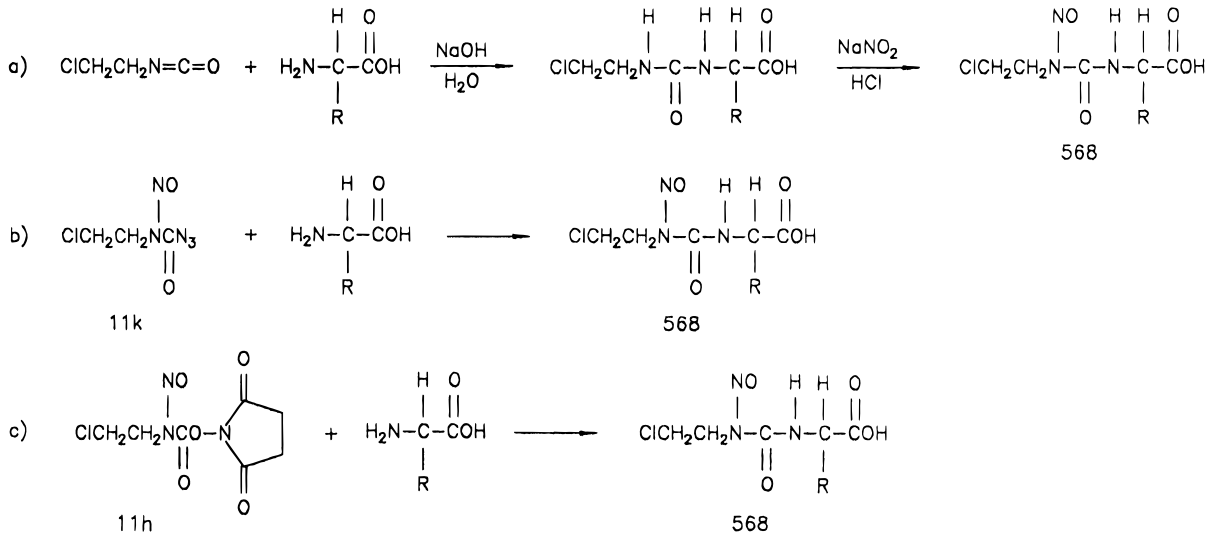
[a] Rat leukemia. [b] Murine lymphoid leukemia. [c] Murine lymphocytic leukemia. [d] Optimal dose = daily ip-administered dose resulting in the maximum increase of life span. [e] Drug administered 24 h before the median day of death. [f] Drug administered on days 1−9 after tumor implantation. [g] Drug administered on day 6 after tumor implantation. [h] Drug administered on days 1−5 after tumor implantation. [i] Increase in life span = $[(T - C)/C] \times 100$. [j] Percentage of animals surviving on day 60 after tumor implantation. [k] Lethal dose = single ip-administered dose resulting in the death of 50% of the untreated animals. [l] Partition coefficient: *P* = [compound in 1-octanol]/[compound in water].

against several solid murine tumors.[441] In this respect compounds **546** and **547** were superior to both CZT (**368**) and ACNU (**311**).

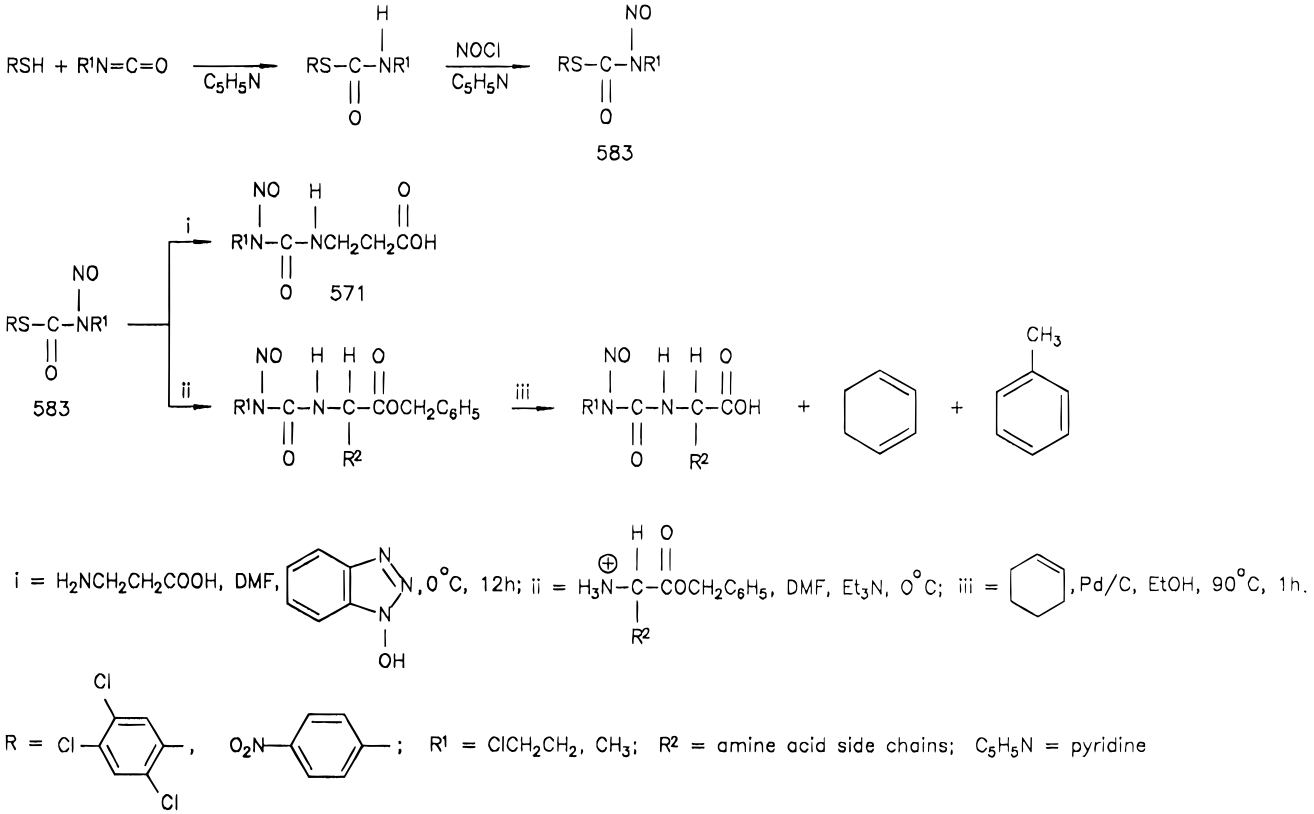
The disaccharide CENU analogs **548**−**550** were synthesized[442,443] according to the overall synthetic sequence illustrated in Scheme 56 for the D-maltose series. High activity in vivo against the L1210 leukemia was reported[443] for these water-soluble compounds (Table 31). The D-maltosyl compound **548** appeared to be less toxic than the D-lactosyl **549** and D-cellobiosyl **550** analogs.[443] The gentiobioside derivatives **551** and **552** also exhibited good antileukemic activity (Table 31).[444,445]

A large series of N3,N3-disubstituted 1-CENU disaccharide derivatives **553**−**565** were synthesized[396,397,446] according to Scheme 57a and tested[446] for anticancer activity (Table 31). The formation of a cyclic urethane **566** following the decomposition reaction was proven[446] (Scheme 57b). Only those analogs which resulted in 60 day cures are included in the Table 31. It is evident that many of these compounds have outstanding anticancer activities and therapeutic indices, as compared to CCNU (**137b**), ACNU (**311**), and GANU (**432**). The β-D-maltosyl analogs **553**−**565** were found[446] to be much more active than the β-D-lactosyl and β-D-meliobiosyl analogs, i.e. the isobutyllactosyl analog had only a 33% ILS and 1.3 value for the therapeutic index, as compared with the corresponding values of 733 and 83 for the isobutylmaltosyl analog **555** (TA-077) (Table 31). However, no large differences could be found for the N3-monosubstituted β-D-maltosyl- and β-D-lactosyl-CENU analogs **548** and **549**, respectively (Table 31). The latter compounds were used in smaller optimum doses than the disubstituted compound **555** and, hence, were more toxic. Compound **555** had a much higher therapeutic index and exhibited a higher cytotoxicity against the L1210 when administered by multiple injections, e.g. 1−5 ip/day, than by a single 1 ip/day injection, whereas other clinical nitrosoureas were found[396,447] to result in higher activities and therapeutic ratios on a single injection regimen. Compound **555** had the highest therapeutic ratio against a variety of cancer lines, including L1210 leukemia, Ehrlich carcinoma, sarcoma 180, Lewis lung carcinoma, Yoshida sarcoma, rat ascites hepatomas, and Walker 256 carcinoma, when compared with GANU, ACNU, and CCNU.[447] High therapeutic ratios were also found for solid cancers, regardless of the cancer and the route of administration, such as ip, iv, and po.[447] The therapeutic ratios in both ip and iv treatments with **555** were nearly equal and were half of those in the po treatment.[447] Compound **555**, like MCNU (**531**), was found[447] to be active against both the early and advanced forms of Lewis lung carcinoma, a cancer that is resistant to many anticancer agents. Furthermore, five intravenous injections of **555** over a five consecutive day period, rather than a single dose, provided[448,449] the best anticancer activity against the

Scheme 58



Scheme 59

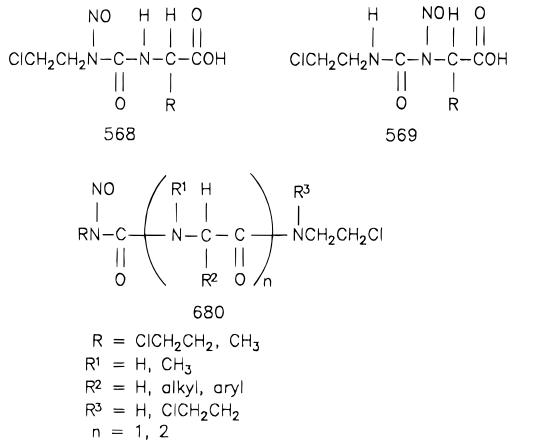


advanced Lewis lung carcinoma and a human mammary carcinoma MX-1 as compared to five other CENU derivatives, MCNU (**531**), GANU (**432**), ACNU (**311**), CZT (**368**), and MECCNU (**138b**).The toxicity of **555** did not increase with the consecutive five day treatment, indicating its suitability for chronic administration in cancer treatment.[450] Compound **555** also was active against the amelanotic melanoma in nude mice.[314,451]

Interestingly, **555** was inactive against L1210, L5175, and HeLa cells in vitro.[452,453] This compound is hydrolyzed by a maltase enzyme to the active metabolite TA-G (**567**, Chart 14).[453] The uptake of **567** by cancer cell lines is much greater than that for **555** and this result could account for the greater cytotoxicity of **567** to the cancer cells.[453] The alkylation of calf thymus DNA with **555** was as effective as that with **567**;[454] hence, the chemical reactivity is not an important differential. The lower cellular uptake of **555** could explain the difference between its in vitro and in vivo activities since the in vitro test system lacks the maltase enzyme which is essential for the conversion of **555** to the more easily membrane transportable metabolite **567**.[453,454]

Various pharmacokinetic studies[455–457] demonstrated the importance of the maltase enzyme for the biological activity of **555**. Thus, the tissue level of **567** in the kidney, a maltase-rich organ, was always the highest among the organs examined in both guinea pigs and VX-2 tumor-bearing rabbits.[455] Compound **555** was hydrolyzed to **567** by homogenates of guinea pig organs, rabbit VX-2 carcinoma, and rat Yoshida sarcoma, as well as by suspensions of various tumor lines.[456] A phase 1 study[457] of **555** in humans

Chart 16. Structures for Section VII.D



revealed that the time-course patterns and pharmacokinetic parameters of **555** and **567** were similar to those in the guinea pig which, like humans, lacks the plasma maltase activity. The in vitro sensitivity among various tumor lines to **555** or **567** could neither be explained[456] by differences in cellular maltase activity nor by the cell permeability to the drug. Some phase 1 clinical studies of **555** were reviewed.[283,284]

Additional carbohydrate *N*-nitrosourea analogs which either had no anticancer activity or for which no testing data could be obtained are collected in Chart 15.

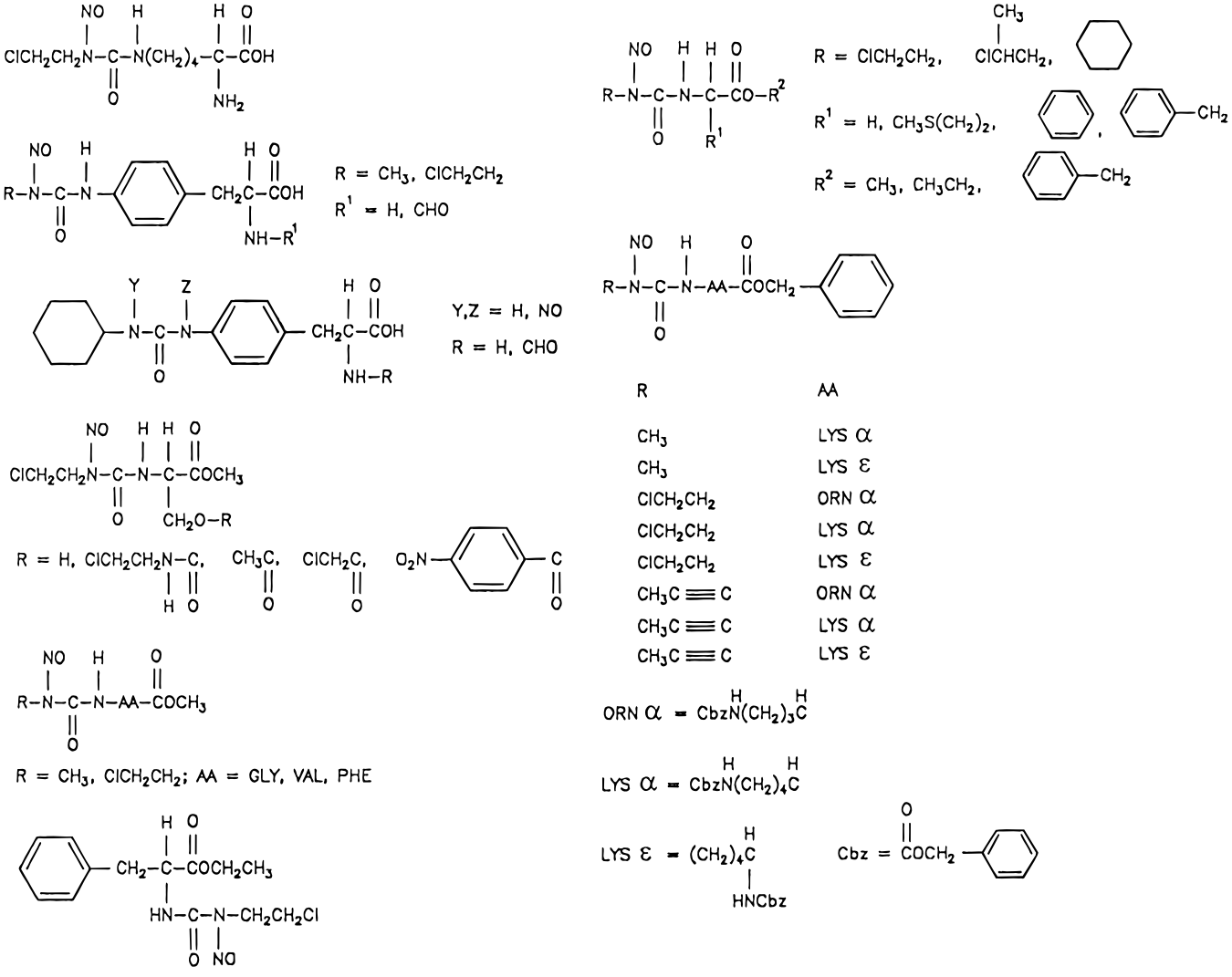
D. Amino Acid and Peptide Analogs

1. Amino Acid Analogs

The structure−activity studies of *N*-2-(chloroethyl)-*N*-nitrosourea (CENU) analogs of hydroxyalkyl compounds, such as HECNU **329**, and of carbohydrates, such as chlorozotocin (CZT, **368**), indicated that such water-soluble analogs possessed greatly reduced bone marrow toxicity and improved therapeutic indices. A balancing of hydrophobic and hydrophilic properties appeared[91,97,98] to be an important consideration in the design of these anticancer drugs.

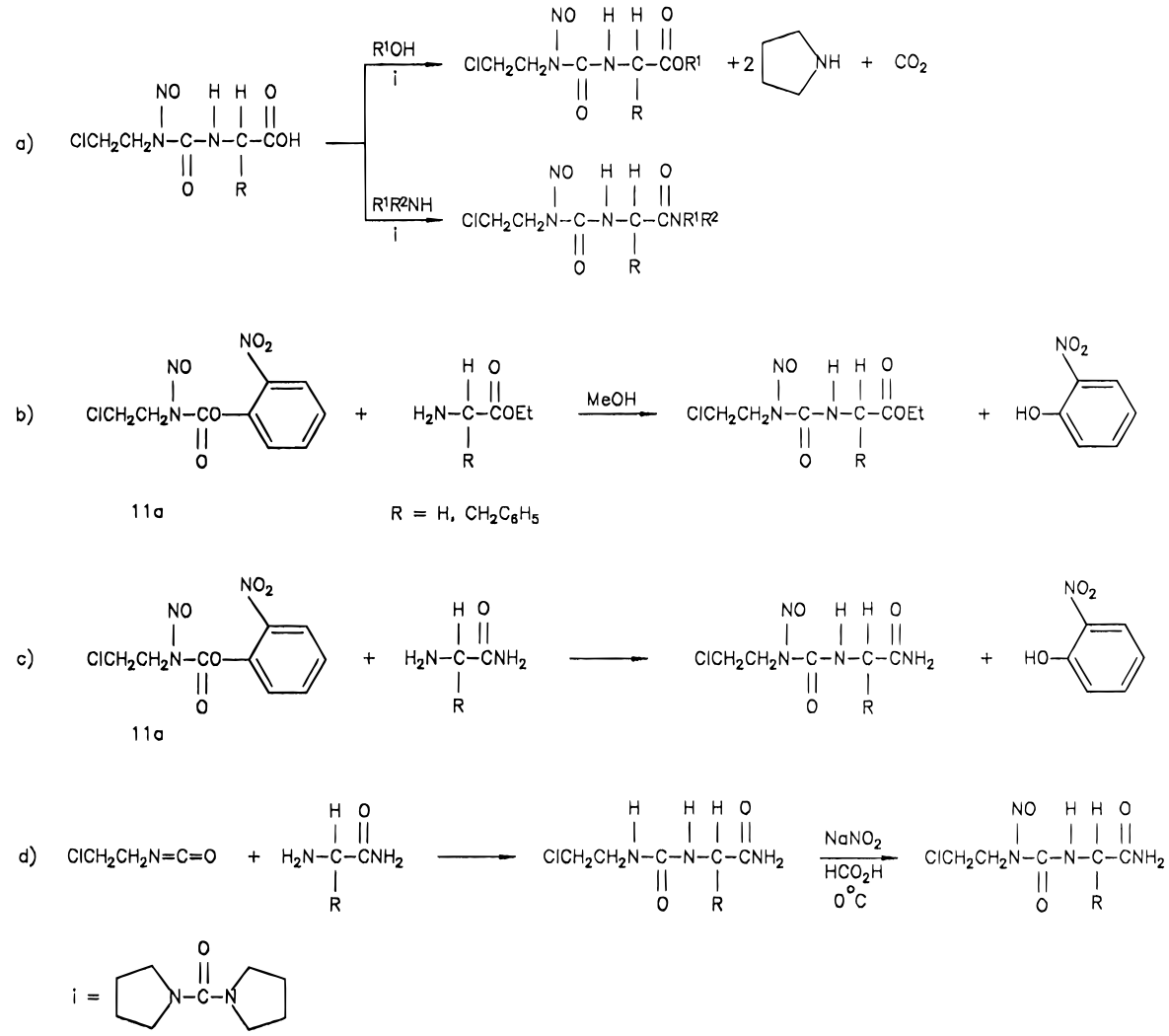
The attachment of L-amino acids to the CENU moiety could add desirable hydrophilic properties. In addition, the L-amino acids are actively transported into mammalian tissues and thus could serve as carriers for the CENU group.[458] It was shown[459−462] that the polyoma virus-transformed 3T3 cells accumulated α-aminobutyric acid, cycloleucine, and L-glutamine about twice as rapidly as 3T3 cells. Thus, the preferred permeability of some amino acids through the membranes of transformed cells could serve to make L-amino acids as excellent carriers for cytotoxic groups, such as the CENU moiety.

Chart 17. Structures for Section VII.D



R	AA
CH_3	LYS α
CH_3	LYS ε
$ClCH_2CH_2$	ORN α
$ClCH_2CH_2$	LYS α
$ClCH_2CH_2$	LYS ε
$CH_3C\equiv C$	ORN α
$CH_3C\equiv C$	LYS α
$CH_3C\equiv C$	LYS ε

Scheme 60



A large number of CENU amino analogs (Table 32) were synthesized[463−466] according to either a two-step or one-step reaction sequence, as outlined in Scheme 58a−c. The preparation of analytically pure amino acid derivatives **568** by either reaction sequence proved to be difficult.[463,464] Thus, from a series of 12 amino acids there were obtained[463] satisfactory microanalyses for only the five analogs of glycine, L-alanine, L-methionine, L-glutamine, and L-tryptophan. The remainder of the series were converted[463] to crystalline anilides or toluidides in order to obtain products of sufficient purity. The isomeric N3-nitroso analogs **569** were not detected[463] in this series (Chart 16).

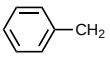
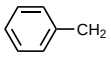
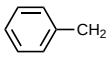
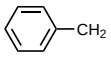
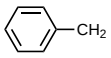
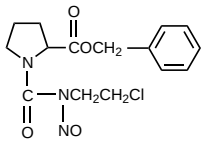
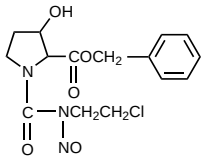
The in vivo screening of the CENU amino acid analogues **570**−**582** against either the rat leukemia L5222 or the mouse leukemias L1210 and P388 revealed[289,465−467] that these compounds have only moderate antileukemic activity (Table 32).

Several CENU amino acids **571**, **572**, **574**−**576**, **581**, **582**, and CENU-biogenic amines (Chart 17) were synthesized[289] by the reaction sequence outlined in Scheme 59. Thus, the thiophenols were reacted with the appropriate isocyanate to yield the thiourea intermediates which were converted to the *N*-nitrosourea **583** with nitrosyl chloride in pyridine. The intermediate **583** was used in two different reaction sequences. In the first reaction sequence, the amino acid β-alanine was condensed with **583** in the presence of 1-hydroxybenzotriazole, with a probable in situ formation of a reactive carbamate intermediate, to give only a 25% yield of **571**. In the second reaction sequence, **583** was condensed with amino acid benzylic esters to produce the corresponding CENU amino acid benzylic esters. The benzyl groups were then removed by a mild *trans*-hydrogenation procedure using cyclohexene, without affecting the *N*-nitroso moiety.[289]

Various CENU amino acid esters and amides were synthesized[458,464,468,469] according to the reactions of Scheme 60a−d. The esters were obtained[464,468] as yellow oils either by an esterification of the corresponding CENU amino acid (Scheme 60a) or by a reaction of the amino acid esters with a CENU transfer agent **11a** (Scheme 60b). Similarly, the amides were synthesized either by the amidation of the CENU amino acid (Scheme 60a),[464] the direct reaction of the amino acid amide with the CENU transfer agent **11a** (Scheme 60c),[458] or by the *N*-nitrosation of the corresponding 2-chloroethylurea derivatives (Scheme 60d).[458] The secondary amides were crystalline solids and the tertiary amides were oils.

Evaluation of the amino acid esters **584**−**591** for anticancer activity demonstrated[470] that these analogues had similar activities to those of the corresponding acids (Table 33). A notable exception was the CENU isoleucine ethyl ester (**590**) which had a

Table 33. Anticancer Activity of CENU−Amino Acid Esters against the Ip-Implanted L5222 Leukemia[a] and the L1210 Leukemia[b]

$ClCH_2CH_2N(NO)-CO-NH-CHR-COOR^1$							
compound no.	R	R^1	OD,[c] mg/kg	ILS_{max},[e] %	survivors,[f] %	LD_{50},[g] mg/kg	ref(s)
584	H	CH_3CH_2	28[a,d]	169			470
585	CH_3	CH_3	18[a,d]	33			470
586	CH_3	CH_3CH_2	28[a,d]	229			470
587	CH_3	$CH_3(CH_2)_3$	18[a,d]	200			470
588	CH_3	$(CH_3)_2CHCH_2$	18[a,d]	200			470
589	CH_3	$CH_3CH_2CH(CH_3)$	18[a,d]	163			470
590	$CH_3CH_2CH(CH_3)$	CH_3CH_2	44[a,d]	644	50		470
			70[a,d]	750	33		470
591		CH_3CH_2	44[a,d]	429			470
592	CH_3		10[b,d]	16		40	289
593	$CH_3CH_2CH(CH_3)$		40[b,d]	100		100	289
594	$HOCH_2$		75[b,d]	58		110	289
595	$HOCH(CH_3)$		70[b,d]	111		80	289
596			80[b,d]			160	289
597			200[b,d]	56		250	289

[a] Rat leukemia. [b] Murine lymphoid leukemia. [c] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [d] Drug administered on day 6 after tumor implantation. [e] Increase in life span = $[(T - C)/C] \times 100$. [f] Percentage of animals surviving on day 90 after tumor implantation. [g] Lethal dose = single ip-administered dose resulting in the death of 50% of the untreated animals.

644% ILS against the L5222 rat leukemia. The benzylic ester derivatives **592**−**597** had lower activities[289] against the L1210 murine leukemia than those of the other ester analogs (Table 33).

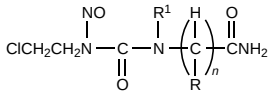
In contrast, the CENU amino acid primary amides **598**−**611**[458,469] (Table 34) and the secondary and tertiary amides **612**−**637**[470,471] (Table 35) exhibited high anticancer activity in vivo against the rat L5222 and murine L1210 leukemias. Among the primary amides (Table 34) the CENU sarcosinamide **610** was particularly attractive because of a combination of high anticancer activity of 711% ILS, very high chemical stability with a half-life of 330 min, and low toxicity with a LD_{50} of 392 mg/kg.[458] The L-proline analog **611** also had high chemical stability and low toxicity but also a greatly reduced anticancer activity, so there was no obvious correlation between the % ILS and the chemical half-life values. The CENU L-serinamide (**606**) and CENU L-threoninamide (**607**) had excellent therapeutic indices equal to 40.[458]

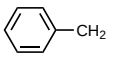
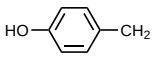
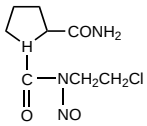
The CENU-containing L-sarcosinamide (**610**) and the L-prolinamide (**611**) derivatives would not be expected to decompose to an isocyanate because of the disubstituted N3-nitrogen.[458] Rather, they would decompose to a carbamic acid and 2-chloroethyldiazohydroxide,[458] as shown in Scheme 61a. Studies of the decomposition of the CENU L-prolinamide (**611**) and CENU L-valinamide (**602**) at pH 7 and 37 °C revealed[469] that an intramolecular carbamoylation reaction occurs with formation of the corresponding hydantoins (Scheme 61b). This same mode of decomposition, leading to oxazolidinones, was shown for similar N3,N3-disubstituted aliphatics and alicyclics (Tables 3 and 13) and carbohydrates (Tables 21, 23, 24, 27, 29, and 31) which possessed high anticancer activities. The in vitro myelotoxicities of CENU L-asparaginamide (**604**) and CENU L-sarcosinamide (**610**) were significantly lower in the CFU-C assay than that of BCNU (**33**) at equal concentrations.[472]

Most of the secondary and tertiary amino acid amides **612**−**637** possessed high antileukemic activities in vivo against the L5222 leukemia, with a few exceptions (Table 35). The morpholine analog **621** had higher activity than either the piperidine (**620**) or the piperazine (**622**) derivatives. In two cases, i.e. **622** and **626,** the substitution of a 2-hydroxyethyl moiety on the amide nitrogen resulted in a reduction of anticancer activity.[470]

A series of CENU amino acid amides containing a 1,3,4-thiadiazole ring system (**638**−**650**) (Table 36) had moderate activity against the P388 leukemia and a reduced toxicity, as indicated by their LD_{50} values.[466]

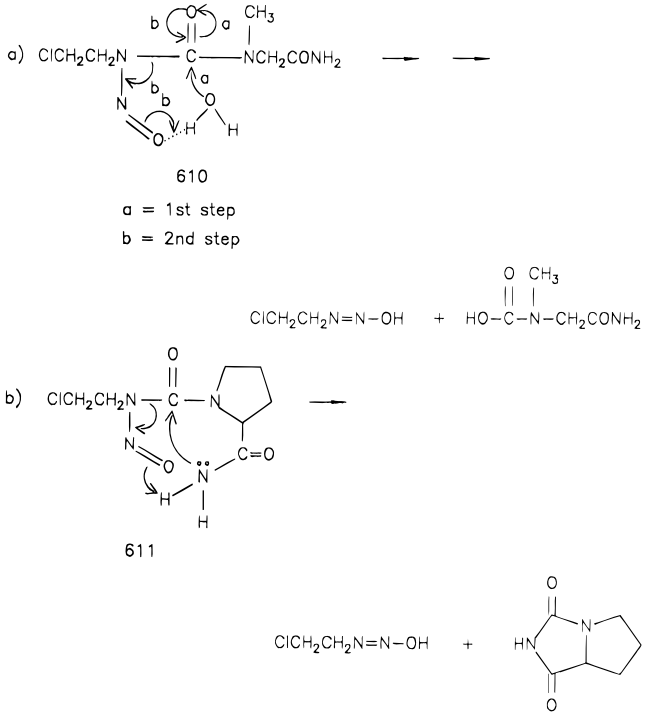
Table 34. Anticancer Activity of CENU−Amino Acid Primary Amides against Ip-Implanted Murine L1210 Lymphoid Leukemia



compound no.	R	R^1	*n*	OD,[a] mg/kg	ILS_{max},[e,f] %	survivors,[h] %	ther ratio[i,f]	$t_{1/2}$[j,f] min	LD_{50},[k] mg/kg	ref(s)
598	H	H	1	16[b]	679	100	4	44	21	458
599	H	H	2	16[b]	733	100	15	61		458
600	H	H	3	16[b]	733	100	7	72		458
601	CH_3	H	1	8[b]	711	100	14	37		458
602	$(CH_3)_2CH$	H	1	8[b]	757	100	16	44		458
				20[c]	212					469
603	$(CH_3)_2CHCH_2$	H	1	24[b]	757	100	11	48		458
				15[d]	111				40	486
604	$H_2NCOCH_2CH_2$	H	1	8[b]	494	40	16	32		458
605	$CH_3SCH_2CH_2$	H	1	16[b]	278	0	6	38		458
				20[d]	136				30	486
606	$HOCH_2$	H	1	8[b]	757	100	40	20		458
607	$HOCH(CH_3)$	H	1	8[b]	757	100	40	23		458
608		H	1	16[b]	446	20	4	50		458
				40[d]	∞[g]				55	486
609		H	1	16[b]	342	40	10			458
610	H	CH_3	1	225[b]	711	100	15	330	392	458
611				64[b]	250	20	6	295	220	458
				100[c]	420					469

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered on days 1−3 after tumor implantation. [c] Drug administered on day 1 after tumor implantation. [d] Drug administered on days 1, 5, and 9 after tumor implantation. [e] Increase in life span = $[(T - C)/C] \times 100$. [f] Values are rounded. [g] >50% cures at one or more doses. [h] Percentage of mice surviving on day 60 after tumor implantation. [i] Therapeutic ratio = MED/ED_{30}; MED = maximal effective dose; ED_{30} = the daily dose resulting in a 30% ILS among the treated mice. [j] Half-life, measured in phosphate buffer, pH 7.4, after seven days at room temperature. [k] Lethal dose = single ip-administered dose resulting in the death of 50% of the untreated mice.

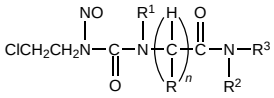
Scheme 61

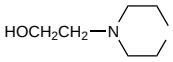
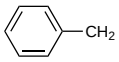
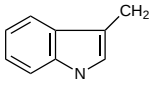
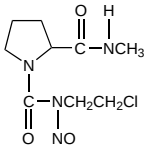
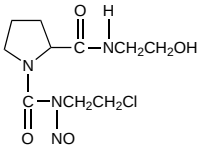
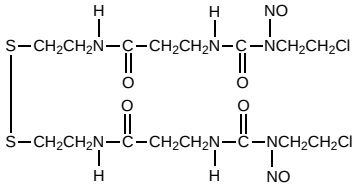


A hypothesis was advanced[467] that the ester analogs (Table 33) are hydrolyzed to the free acids in vivo more readily than the amide analogs (Tables 34 and 35), and the ionized free acids cannot easily penetrate the cell membrane. Hence, the CENU amino acid amides are more available to the cell interior because of a greater membrane transport. However, the CENU L-Ala derivative **572** exhibited bioavailability, volume of distribution, and terminal half-life comparable to similar clinical CENUs after iv administration to mammary carcinoma-bearing rats.[473]

Plots of the toxicity of amino acid derivatives, expressed as log $1/LD_{50}$, against the hydrophobicity of the side chain of the amino acids revealed[289] two regression lines. In the first case, the hydrophilic hydroxylated amino acids Tyr, Thr, and Ser were found to have an increasing toxicity with increasing hydrophobicity. In the second case, the hydrophobic amino acids Ala, Cys, Pro, Val, and Leu were shown to have a decreasing toxicity trend with increasing hydrophobicity. The amino acid tryptophan was outside of both linear regression lines. While such correlations are interesting it is a fact that the CENU amino acids are only moderately cytoactive against the L1210 leukemia and solid tumors.[289,465−467] More useful correlations involve the relationship between % ILS values and the lipophilicity parameter, as measured by the partition coefficient *P*, of the CENU amino acids.[465]

Table 35. Anticancer Activity of CENU−Amino Acid Secondary and Tertiary Amides against Ip-Implanted Rat L5222 Leukemia



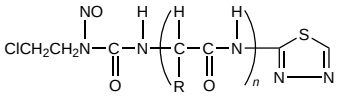
compound no.	R	R^1	R^2	R^3	n	OD,[a] mg/kg	ILS_{max},[e] %	survivors,[g] %	ther ratio[i]	LD_{50},[k] mg/kg	ref(s)
612	H	H	H	CH_3	1	11[c]	800	66			470
613	H	CH_3	H	CH_3	1	469[b,c]		++[h]	1.6		471
614	H	H	H	CH_3	2	118[b,c]		+++[h]	1.6		471
615	CH_3	H	H	CH_3	1	11[c]	800	100			470
616	CH_3	H	CH_3	CH_3	1	18[c]	800	83			470
617	CH_3	H	H	CH_3CH_2	1	18[c]	638	50			470
618	CH_3	H	CH_3CH_2	CH_3CH_2	1	44[c]	800	100			470
619	CH_3	H	H	$CH_3(CH_2)_7$	1	56[c]	650	33			470
620	CH_3	H			1	18[c]	229	33			470
621	CH_3	H			1	28[c]	800	83			470
622	CH_3	H			1	28[c]	286	17			470
623	CH_3	H	H	$HOCH_2CH_2$	1	18[d]	619	50			470
624	$(CH_3)_2CHCH_2$	H	H	CH_3	1	15[d]	∞[f]		2.20[j]	33	486
625	$CH_3CH_2CH(CH_3)$	H	H	CH_3	1	28[c]	800	83			470
626	$CH_3CH_2CH(CH_3)$	H	H	$HOCH_2CH_2$	1	28[c]	243	33			470
627	$CH_3SCH_2CH_2$	H	H	CH_3	1	28[c]	800	83			470
628	DL-$CH_3SCH_2CH_2$	H	H	CH_3	1	28[c]	800	83			470
629	$CH_3SCH_2CH_2$	H	H	$HOCH_2CH_2$	1	28[c]	779	50			470
630	$HOCH_2$	H	H	CH_3	1	70[b,c]		+[h]	1.6		471
631	$CH_3NHCOCH_2CH_2$	H	H	CH_3	1	91[b,c]		++[h]	2.5		471
632		H	H	CH_3	1	22[d]	∞[f]		1.7[j]	38	486
						112[b,c]		+++[h]			471
633		H	H	CH_3	1	250[b,c]		++[h]	2.0		471
634	$Cl(CH_2)_2N(NO)CONH(CH_2)_4$	H	H	CH_3	1	103[b,c]		+++[h]	2.5		471
635						423[b,c]		+++[h]	2.5		471
636						601[b,c]		+++[h]	1.6		471
637						50[b,c]		++[h]	1.6		471

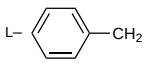
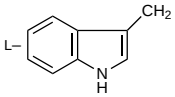
[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Dose = μmol/kg. [c] Drug administered on day 6 after tumor implantation. [d] Drug administered on days 1, 5, and 9 after tumor implantation. [e] Increase in life span = $[(T - C)/C] \times 100$. [f] >50% cures at one or more doses. [g] Percentage of animals surviving on day 90 after tumor implantation. [h] + = ≥100% ILS, ++ = ≥50% cures at one dosage or <50% cures at more than one dosage, +++ = ≥50% cures at more than one dosage. [i] Therapeutic ratio = toxic dose/OD; toxic dose = lowest dose resulting in the reduced survival of the test animals. [j] Therapeutic ratio = LD_{50}/OD. [k] Lethal dose = single ip-administered dose resulting in the death of 50% of the untreated animals.

2. Peptide Analogs

A natural extension of research in this area was the utilization of peptides as carriers of the CENU group. A series of CENU peptides were reported in a patent.[474] The anticancer activity[471] of some of these peptide derivatives **651**−**662** against the L1210 leukemia are shown in Table 37. The amides **657**−**662** were more active and had higher therapeutic

Table 36. Anticancer Activity of CENU−Amino Acid Amides against Ip-Implanted Murine P388 Lymphocytic Leukemia[466]



compound no.	R	*n*	OD,[a,b] mg/kg	ILS_{max},[c] %	survivors,[d] %	LD_{50},[e] mg/kg
638		0	25	73		78
639	L-$(CH_3)_2CH$	1	25	191	83	49
640	L-$(CH_3)_2CHCH_2$	1	50	203	83	82
641	DL-$(CH_3)_2CHCH_2$	1	25	203	50	85
642	L-$CH_3CH_2CH(CH_3)$	1	50	197	50	78
643	L-$CH_3SCH_2CH_2$	1	50	194	83	67
644	D- 	1	50	197	83	69
645	L- 	1	25	197	83	74
646	cycloleucyl	1	25	76		53
647	DL-$CH_3CH(OH)CH_2$	1	12.5	53		65
648	L- 	1	25	194	83	73
649	D-$(CH_3)_2CH$	1	25	117	16	68
650	D-$(CH_3)_2CHCH_2$	1	50	117		61

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered on days 1−5 after tumor implantation. [c] Increase in life span = $[(T - C)/C] \times 100$. [d] Percentage of surviving treated mice, no day of survival given. [e] Lethal dose = single ip-administered dose resulting in the death of 50% of the untreated animals.

Table 37. Anticancer Activity of CENU Oligopeptides against Ip-Implanted Murine L1210 Lymphoid Leukemia[471]

$ClCH_2CH_2N(NO)-CO-NH-X-CO-R$

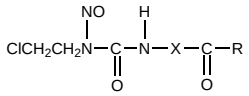
compound no.	X	R	OD,[a,b] μmol/kg	curative activity[c]	ther ratio[d]
651	Gly-Gly	OH	200	+	2
652	Ala-Ala	OH	400	++	2.5
653	Gly-Leu	OH	400	++−+++	4
654	Ala-Ala-Ala	OH	400	++−+++	4
655	Gly-Gly	OCH_3	150	+++	4
656	Gly-Gly		300	+++	3
657	Gly-Gly	NH_2	150	+++	6
658	Ala-Ala	NH_2	150	+++	6
659	Gly-Gly-Gly	NH_2	300	+++	3
660	Gly-Gly-Tyr	NH_2	300	+++	4
661	Gly-Gly	$NHCH_3$	100	+++	8
662	Ala-Ala	$NHCH_3$	50	+++	6

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drug administered on day 3 after tumor implantation. [c] + = ≥100% ILS; ++ = ≥50% cures at one dosage or <50% cures at more than one dosage; ++−+++ = ≥50% cures at one dosage and <50% cures at other dosages; +++ = ≥50% cures at more than one dosage. Percentage determined on day 90 after tumor implantation. [d] Therapeutic ratio = OD/ILS_{40}; ILS_{40} = daily dose resulting in a 40% increase in life span among the treated animals.

indices than the carboxylic acids **651**−**654**, while the dipeptide secondary amide analogs **661** and **662** exhibited the highest therapeutic ratios but also were the most toxic, as judged by their lower optimal doses. A CENU-Gly-Gly dipeptide **657** had a more favorable therapeutic index than the corresponding tripeptide **659**.

A large number of CENU analogs of carboxylic acids and amides of amino acids **570**, **572**, **598**, **601**, **612**, **615**, dipeptides **651**, **652**, **657**, **658**, **661**, **662**, and tripeptides **654**, **659**, **663**−**666** were screened for in vivo activity against three transplantable mouse adenosarcomas of the colon (MAC)[475−477] and MNU-induced mammary carcinoma[478] (Table 38). The amide derivatives **612**, **615**, and **661** had higher activity against the sc-administered solid tumors MAC 13 and MAC 26 than the acid analogs **572**, **651**, and **652** (Table 38). The free acid dipeptide analog CENU-Ala-Ala (**652**) was highly active against the ascitic MAC 15A tumor line but was either weakly active or inactive against the sc-administered MAC 13 and MAC 26 tumor lines.[475,476] It was proposed[476,477] that since the dipeptide **652** had a minimal systemic absorption it may have a role in the treatment of either local peritoneal disease or in treatment of various solid tumors by a localized administration of the drug to the tumor. However, against the MNU-induced mammary carcinoma a different pattern of activity was found.[478] In this case, the administration of the acid glycine analogs **570**, **651**, and **663** produced small but significant increases in the % ILS values, whereas the administration of the corresponding primary and secondary amides caused no such increases in % ILS (Table 38). All of the alanine-based analogs, except the dipeptide **652**, caused either a decrease or a small increase in the life span of the mice implanted with the mammary carcinoma.[478] Thus, no clear structure−activity

Table 38. Anticancer Activity of CENU−Amino Acids and Oligopeptides against Three Mouse Adenosarcomas of the Colon (MAC) and a Methylnitrosourea (MNU)-Induced Rat Mammary Carcinoma



			MAC 13[a]			MAC 15[b]		MAC 26[a]		MNU mammary carcinoma[c]				
compound no.	X	R	dose[d] mg/kg	TWI,[e,f] %	survivors,[e] %	dose,[d] mg/kg	ILS,[g] %	dose,[h] mg/kg	TVI,[i] %	dose,[j] mg/kg	tumor vol,[k,f] cm^3	mortality,[n] %	ILS,[g] %	ref(s)
570	Gly	OH								10	13	0	+23	478
572	Ala	OH	37	72	100	37	33	50	47	45	21	10	−5	475,476,478
						50	127							
598	Gly	NH_2								20	13	0	4	478
601	Ala	NH_2								20	9	0	−3	478
612	Gly	$NHCH_3$	20	92	100	20	100	50	55	30	2	0	-15	476,478
			37	100		50	300						19	475
615	Ala	$NHCH_3$	25	97	100	25	213	25	58	30	7	0	−7	475,476,478
651	Gly-Gly	OH	45	73		30	91	30	0	45	20	0	+46	475,476,478
652	Ala-Ala	OH	100	81	100	50	300	50	6	45	19	10	+15	475,476,478
			50	62		50	275							
657	Gly-Gly	NH_2								20	15	10	−3	478
658	Ala-Ala	NH_2								20	18	10	−1	478
661	Gly-Gly	$NHCH_3$	20	92		20	100	20	78	30	10	20	−9	475,476,478
662	Ala-Ala	$NHCH_3$								20	26	0	−19	478
663	Gly-Gly-Gly	OH								30	24	0	+38	478
654	Ala-Ala-Ala	OH								45	17	0	−1	478
659	Gly-Gly-Gly	NH_2								30	22	0	+7	478
664	Ala-Ala-Ala	NH_2								30	27	20	−7	478
665	Gly-Gly-Gly	$NHCH_3$								30	18	0	+14	478
666	Ala-Ala-Ala	$NHCH_3$								30	11	0	+5	478
control[l,m]											32[l]	10[l]		478
											19[m]	15[m]		478

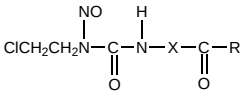
[a] MAC 13 tumors transplanted into female mice and MAC 26 tumors into male mice by sc implantation of tumor fragments. [b] MAC 15A ascite tumors transplanted into male mice by ip inoculation of 10^5−10^6 tumor cells in 0.2 mL of 0.9% saline. [c] Mammary carcinomas induced by iv administration of MNU (**25**) according to published procedures. [d] Drugs administered by a single ip injection on day 2 after tumor implantation. [e] Tumor weight inhibition and percentage of survivors determined on day 14 after tumor implantation. [f] Values are rounded. [g] Increase in life span = $[(T - C)/C] \times 100$. [h] Drugs administered by a single ip injection on day 17 after tumor implantation. [i] Tumor volumes assayed twice weekly by two-dimensional caliper measurements. Percentage of tumor volume inhibition obtained from the growth curves once parallel growth had been achieved. [j] Drugs administered by single ip injections to female SD mice on days 1, 8, 22, and 29 following randomization after the tumor volumes were at least 0.8 cm^3. [k] Tumor volumes are median of each group (95% confidence limits) at the end of week 6. [l,m] Untreated control values for the glycine series (*l*) and alanine series (*m*). [n] Percentage of mice dead at the end of therapy on week 6.

relationship existed among this collection of CENU acid and amide derivatives against the solid tumors.[319]

The bone marrow toxicity, i.e. myelotoxicity, of the acids **572**, **651**, and **652** and the *N*-methyl amides **612**, **615**, and **661** was determined[476] by means of a spleen colony-forming unit assay. A marked myelosupression was observed for all the three methylamides when administered by the ip route while a reduced bone marrow toxicity was found for the acid analogs. In fact, the dipeptide **651** was nonmyelosuppressive at the maximum tolerated dose of 50 mg/kg administered by the ip route,[476] but the marrow-sparing effect of **651** was lost when the drug was administered by the oral route.

A series of [(2-chloroethyl)nitroso]carbamoyl (CENU) derivatives of the polypeptide hormones, α-melanotropin and gastrin, were investigated.[479−481] The polypeptide hormones have specific receptors on the cell surfaces and, hence, could be expected[479] to direct the CENU moiety to specific tissues. Melanotropin exhibits strong effects on the adenylate cyclase and the tyrosinase activity in mouse melanoma cells.[479] Several fragments of the melanotropin[482] and gastrin[483] peptides can recognize the hormone receptor and also possess biological activity, albeit less than that of the parent hormones. More specifically, the CENU-containing small peptides **668**−**670**, a pentapeptide **671** representing the 9−13 amino acid fragments of α-melanotropin, and a tetrapeptide **672** representing the C-terminus of gastrin (Table 39) were synthesized[479] and tested[480] for in vivo activity against the L1210 leukemia and several solid tumors. The syntheses of the various CENU peptides is outlined in Scheme 62. Among these compounds the dipeptide Pro-Val **668**, the tripeptide Lys-Pro-Val **669** and the complete pentapeptide Trp-Gly-Lys-Pro-Val **671** analogs were the most effective against the L1210 leukemia with 60 day cures of 80%, 60%, and 60%, respectively (Table 39). The amino acid amides L-Val-NH_2 **602**, Lys-NH_2 **667** and the tetrapeptide Gly-Lys-Pro-Val-NH_2 **670** were only weakly active as anticancer agents.[480] However, the CENU-L-Pro-NH_2 analog **611**, with the longest half-life, exhibited a 420% ILS at a dose of 100 mg/kg.[480] The tetrapeptide **672** of the gastrin polypeptide was only moderately active. Further screening[480] of the CENU-tripeptide **669** against several solid tumors revealed that it had no activity against sarcoma 180 and Lewis lung carcinoma but a relatively high growth inhibition of the B16 melanoma. This tripeptide also inhibited several human tumor xenografts, i.e. melanoma (77%), three colon carcinomas (13%, 52%, and 42%) and a pancreatic carcinoma (38%).[480] The combination of the clinical drug BCNU (**33**) and **669** resulted in a synergistic effect on the activity against the murine L1210 leukemia.[480]

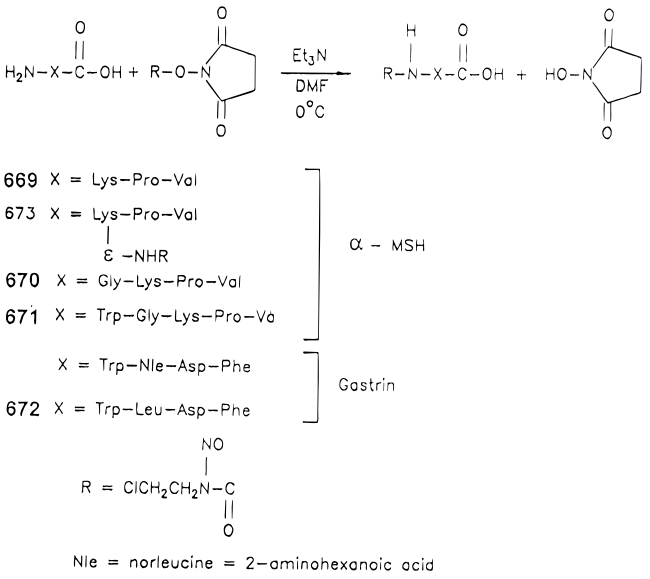
Table 39. Anticancer Activity of CENU Peptide Analogs of α-Melanotropin and Gastrin and Proline-Containing Peptides against Ip-Implanted Murine L1210 Lymphoid Leukemia



compound no.	X	R	OD,[a] mg/kg	ILS,[e] %	survivors,[f] %	$t_{1/2}$[g,h] min	LD_{50},[i] mg/kg	ref(s)
602	Val	NH_2	20[c]	212		15		480
611	Pro	NH_2	100[c]	420		255	140	480,481
667	Lys	$^+NH_3Cl^-$	20[c]	28		20		480
668	Pro-Val	NH_2	100[c]	600	80	188	150	480,481
669	Lys-Pro-Val	$^+NH_3Cl^-$	130[c]		60		175	480,481
670	Gly-Lys-Pro-Val	$^+NH_3Cl^-$	100[c]	100		26		480
671	Trp-Gly-Lys-Pro-Val	$^+NH_3Cl^-$	100[c]		60	20		480
672	Trp-Leu-Asp-Phe	NH_2	150[c]	230				480
673	α,ε-Lys-Pro-Val	NH_2	100[c]	380	0		250	481
674	Pro-Lys-Pro-Val	NH_2	100[c]		100			481
675	Pro-Lys(ε-HNBOC)-Pro-Val	NH_2	200[c]	30			300	481
676	Lys(ε-HNFor[b])-Pro-Val	NH_2	200[c]	190			260	481
677	Pro-Lys(ε-HNFor[b])-Pro-Val	NH_2	200[c]	80			250	481
678	Pro-Leu	NH_2	80[d]	100			260	484
679	Pro-Leu-Gly	NH_2	150[d]	111			260	484

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] For = formaldehyde moiety, HC=O. [c] Drug administered on day 1 after tumor implantation. [d] Drug administered on days 1, 5, and 9 after tumor implantation. [e] Increase in life span = $[(T - C)/C] \times 100$. [f] Percentage of mice surviving on day 60 after tumor implantation. [g] Half-life, determined by measuring the decrease of extinction (ϵ) at λ_{max} in phosphate buffer, pH 7.4, 37 °C. [h] Values are rounded. [i] Lethal dose = single ip-administered dose resulting in the death of 50% of the untreated mice.

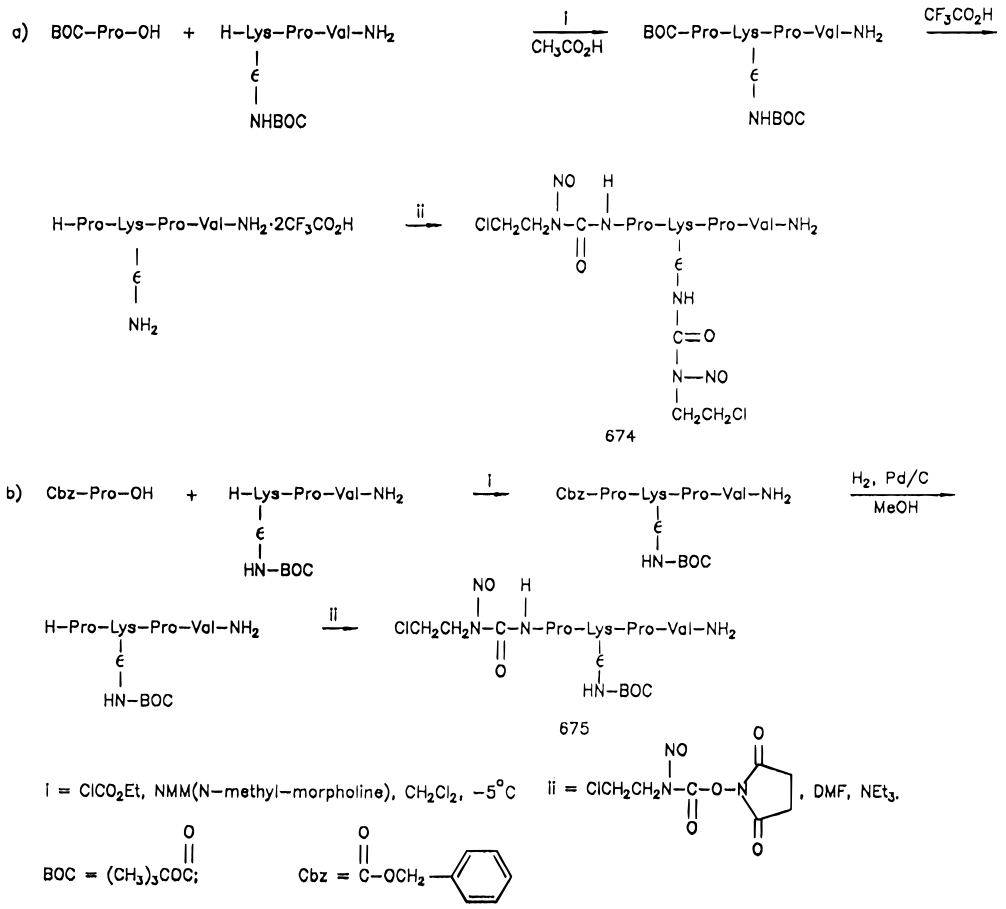
Scheme 62



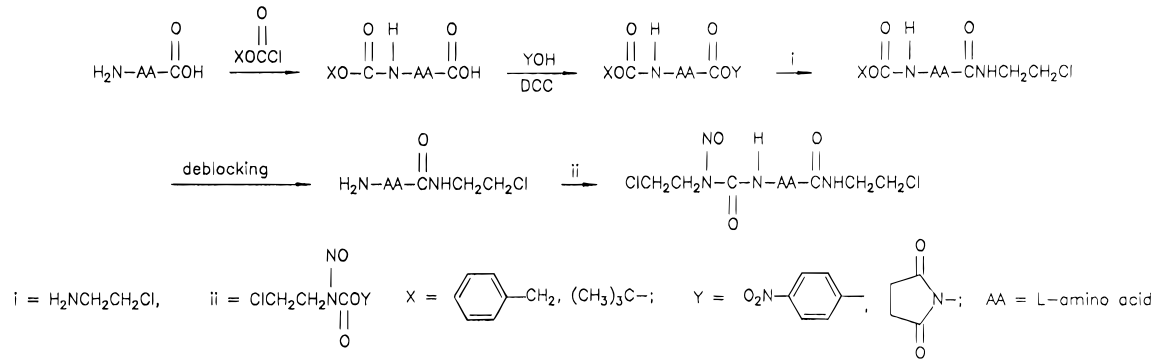
Since the *N*-terminal proline peptide CENU-Pro-Val-NH_2 **668** exhibited significant anticancer activity, a series of related proline-containing peptides **673**−**677** were synthesized and screened[481] for anticancer activity (Table 39). Examples of the syntheses are shown in Scheme 63a,b. It was not possible to prepare the mono-CENU-lysine-containing peptide Pro-Lys-Val-NH_2, and, therefore, only the bis-CENU-substituted peptides α,ε-Lys-Pro-Val-NH_2 **673** and Pro-Lys-Pro-Val-NH_2 **674** could be compared. The *N*-terminal proline containing-dipeptide Pro-Val-NH_2 **668** and the tetrapeptide **674** had 80% and 100% 60 day cures, respectively, while the *N*-terminal lysine tripeptide **673** had no 60 day cures (Table 39).[481] The tetrapeptide **675**, containing a BOC-blocked lysine residue, was marginally active and the peptides **676** and **677**, containing a *N*-formyl protected lysine residue, were only moderately active (Table 39).[481] The related CENU-containing Pro-Leu **678** and Pro-Leu-Gly **679** peptides were moderately cytotoxic, with no 60 day cures.[484]

Some "pseudo-peptides' (**680**, Chart 16), containing either an alkylnitrosourea or (2-chloroethyl)nitrosourea at the *N*-terminus and 2-chloroethylamine or bis(2-chloroethylamine) moieties at the C-terminus have been synthesized and screened for anticancer activity.[484−488] The general synthetic scheme for these compounds is outlined in Scheme 64. Thus, the *N*-terminus was blocked with either a carbobenzoxy (Cbz) or *tert*-butyloxycarbonyl (BOC) group, and the C-terminal carboxyl converted to an active ester, i.e. *p*-nitrophenyl or succinimidyl moiety. Reaction of the active ester with 2-chloroethylamine produced the resulting 2-chloroethylamide. The removal of the Cbz group by hydrogenolysis and the BOC group by acid hydrolysis yielded the free *N*-terminal amino group which was reacted with the CENU transfer agents **11b** and **11h**. Most of the L-amino acid derivatives **681**−**696** (Table 40) were reported[485,486] to have greater than 50% cure rates for at least one dose. However, the number of days for a cure was not listed.[485] A reevaluation of the L-Ala **685**, L-Phe **690**, and L-Asp **694** analogs demonstrated[487,488] only a weak antileukemic activity for these compounds. The sarcosine analog **684** had the lowest toxicity, as judged from its LD_{50} value of 400 mg/kg. The

Scheme 63



Scheme 64



therapeutic indices, LD_{50}/OD, for these pseudopeptides were low, except for the GABA (**683**), L-Asp (**694**) and L-Lys (**695**) analogs (Table 40).[485,486]

A further extension of this work involved the synthesis[484,487,488] of bis-substituted dipeptides **697**–**701** (Scheme 65). An intermediate Cbz-protected amino acid was converted to the active ester and reacted with 2-chloroethylamine to yield the 2-chloroethylamide. After removal of the Cbz group, the free amine was reacted with a second Cbz-protected amino acid active ester. The subsequent removal of the Cbz group by either acid or by hydrogenolysis, followed by the reaction of the free amine group with the CENU transfer agent **11h**, produced the desired bis-CENU-substituted dipeptides. The in vivo anticancer activities of the di- and tripeptides **697**–**701** against the L1210 leukemia were low to moderate (Table 40).[484,488] An initial screening against the P388 leukemia in CDF1 male mice had indicated[488] a high activity for the bis-substituted amino acids **685**, **694**, and **690** and low activity for the dipeptides **699**–**701**. The corresponding peptides prepared from the D-amino acids were inactive.[488] A general correlation was noted[488] between higher cytotoxicity and lower hydrophobicity, i.e. smaller positive log *P* values, of these amino acids and dipeptides and the clinical drugs CCNU (**137b**) and MeCCNU (**138b**) (Table 40).

A number of *N*-nitrosourea analogs of amino acid derivatives for which no anticancer activity could be obtained[489–496] are included in Chart 17. The series of *N*-methyl-, *N*-(2-chloroethyl)- and *N*-propargylnitrosoureas of amino acid benzyl esters (Chart 17) were synthesized and tested for anti-HIV activity.[497]

Scheme 65

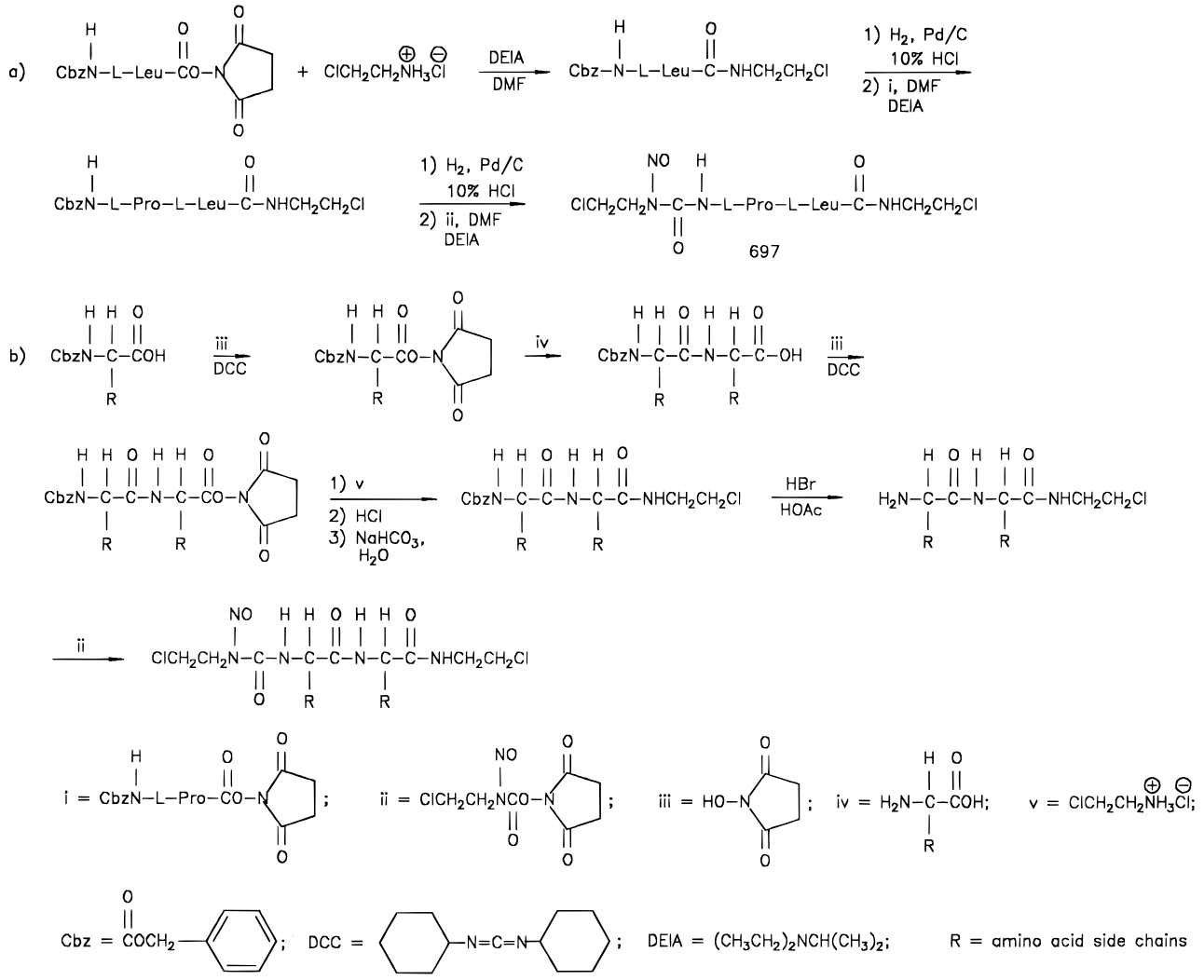
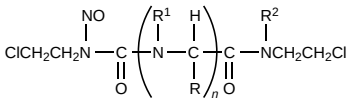


Table 40. Anticancer Activity of Amino Acids and Pseudopeptides Containing the N-Terminal CENU Group and the C-Terminal 2-Chloroethylamine or Bis(2-chlorethyl)amine Groups against Ip-Implanted Murine L1210 Lymphoid Leukemia



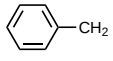
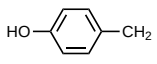
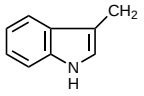
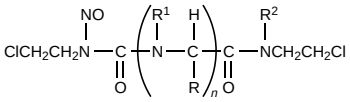
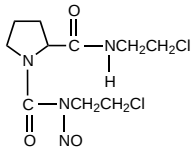
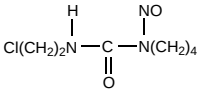
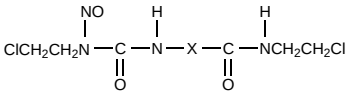
compound no.	R	R^1	R^2	*n*	OD,[a] mg/kg	ILS_{max},[d] %	ther ratio[f]	LD_{50},[g] mg/kg	log *P*[h]	ref(s)
681	H	H	H	1	12[b]	∞[e]	2.0	25		485,486
682	H	H	H	2	35[b]	∞	2.9	100		485,486
683	H	H	H	3	35[b]	∞	3.6	125		485,486
684	H	CH_3	H	1	200[b]	∞	2.0	400		485,486
685	CH_3	H	H	1	20[b]	∞	1.75	35		485,486
					40[c]	56			3.22	488
686	$CH_3CH_2CH(CH_3)$	H	H	1	50[b]	∞	2.6	130		485,486
687	$CH_3SCH_2CH_2$	H	H	1	20[b]	∞	1.6	32		485,486
688	$CH_3CH(OH)$	H	H	1	15[b]	∞	1.6	35		485,486
689	H_2NCOCH_2	H	H	1	20[b]	∞	1.75	35		485,486
690		H	H	1	50[b]	∞	1.6	80		485,486
					40[c]	48			3.25	488
691		H	H	1	25[b]	111		40		485,486
692		H	H	1	27[b]	∞	2.4	65		485,486

Table 40. (Continued)



compound no.	R	R^1	R^2	*n*	OD,[a] mg/kg	ILS_{max},[d] %	ther ratio[f]	LD_{50},[g] mg/kg	log *P*[h]	ref(s)
693					35[b]	94		80		485,486
694	$HOOCCH_2$	H	H	1	37[b]	∞	3.5	130		485,486
					40[c]	64			3.15	488
695		H	H	1	30[b]	∞	4.5	135		485,486
696	H	H	$ClCH_2CH_2$	1	40	∞	2.7	110		486
	 X									
697	Pro-Leu				250[b]	120		320		484
698	Pro-Leu-Gly				240[b]	90		410		484
699	Phegly-Val				40[c]	46			3.32	488
700	Phe-Val				40[c]	31			3.46	488
701	Val-Phe				40[c]	26			3.47	488

[a] Optimum dose = daily ip-administered dose resulting in the maximum increase in lifespan. [b] Drug administered on days 1, 5, and 9 after tumor implantation. [c] Drug administered on days 1−9 after tumor implantation. [d] Increase in life span = $[(T - C)/C] \times 100$. [e] >50% cures at one or more doses. [f] Therapeutic ratio = LD_{50}/OD. [g] Lethal dose = single ip-administered dose resulting in the death of 50% of the untreated mice. [h] Partition coefficient: *P* = [compound in 1-octanol]/[compound in water].

E. Steroid Analogs

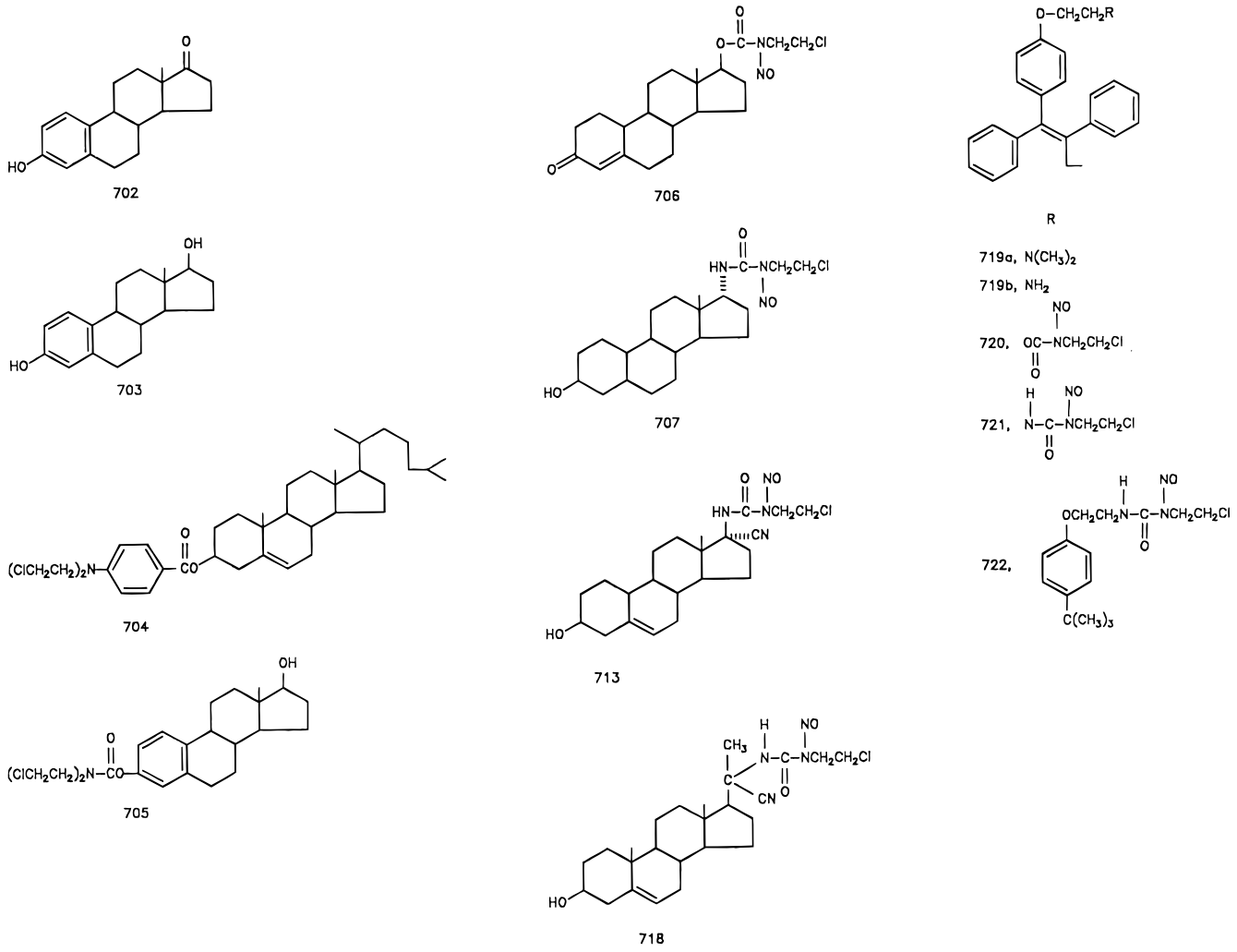
The discovery[498] of estrogen receptors (ER) in human breast cancer has led to significant progress in the management of the disease. The ER specifically concentrates estrogens into the target tissues. Hence, it was assumed[498] that a drug with a high binding affinity for ER could be concentrated in the cancerous tissue in the same manner as the estrogens. The various factors affecting drug interactions with estrogen receptors have been reviewed.[498−500] An analysis of the structure−activity relationship of various estrogens and antiestrogens led to the concept[498] of tight hydrophobic interactions between the estrogens and the receptor for high binding affinity. Specifically, the ER contains a narrow and deep cleft, i.e. a "hydrophobic pocket", with two small hydrogen-bonding regions within the pocket for interactions with the two polar groups of the estrogens, such as estrone (**702**) and estradiol (**703**) (Chart 18). In addition, it was proposed[498] that an antiestrogen with an aminoalkyl side chain has an additional binding capability in an antiestrogenic region.

For several decades, attention has been devoted to attempts of increasing the organ and/or tissue specificity of anticancer agents by their linkage to various steroidal hormones, androgens, and estrogens, which were expected to interact with specific receptors on the cell surface.[498−500] Nitrogen mustard analogs, such as phenestrin (**704**)[501−503] and estramustine (**705**),[502−504] were studied in detail.

The alkylating moiety of cytotoxic steroidal alkylating agents, such as **704** and **705**, must be readily either hydrolyzed by water without enzyme catalysis or mediated by enzymes in vivo.[501] No activity was found for those compounds whose steroid and alkylating moieties were linked by a stable bond which could not be cleaved.[501] In order to obtain a high binding to the ER, the estrogenic steroid hormone with an estral-1,3,5(10)-triene skeleton must have either unblocked 3 and 17β hydroxyl groups[505−509] or at least an unblocked oxygenated function in the form of either a carbonyl or a hydroxyl group[502,504] which could form hydrogen bonds with the ER protein of the binding site. For several nitrogen mustard analogs[501−504] these hydroxyl groups are linked to the alkylating moiety. This arrangement greatly reduces their binding affinity to the ER, and thus lowers their anticancer activities.[498,510,511]

A series of androgen-linked nitrosocarbamates which are related to the estrogen analog estramustine (**705**) were synthesized.[512] From this series the *N*-(2-chloroethyl)-*N*-nitrosocarbamate of 19-nortestosterone (**706**) was studied in detail.[513,514] Compound **706** exhibited[513] excellent in vitro activity against the L1210 leukemia but it had only a low in vivo activity against the L1210, Ehrlich ascites, and Walker 256 carcinoma. It was established[514] that **706** was rapidly hydrolyzed to 19-nortestosterone and polar oxidized metabolites in the liver of the mouse and dog as well as in several tumor tissues. This rapid metabolism could prevent[514] the alkylating moiety from reaching the site of the androgen receptor, and thus its subsequent absorption by the cells. Compound **706** possessed alkylating but no carbamoylating properties.[514] The administration of **706** caused a dose-dependent reduction of the growth of dimethylbenz(*a*)anthracene (DMBA)-induced mammary tumors in rats,[514] and a greater reduction of tumor

Chart 18. Structures for Section VII.E



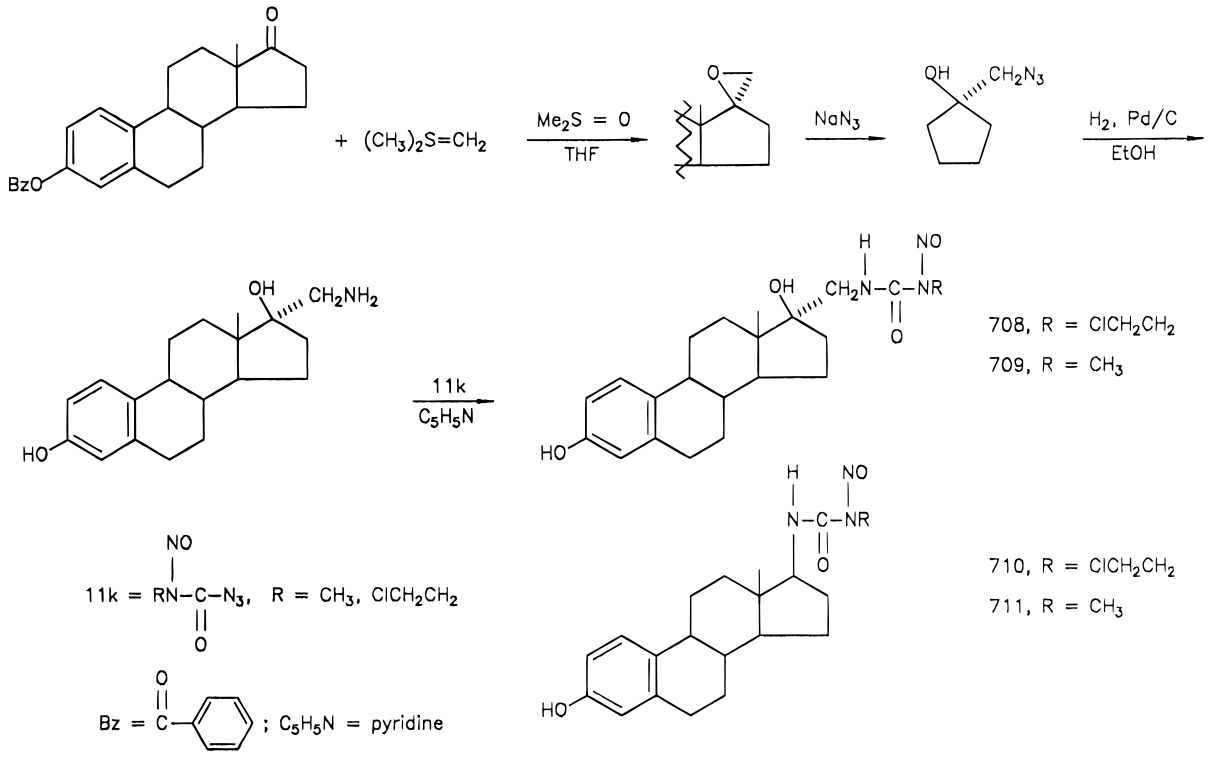
growth and tumor DNA synthesis than either administration of 19-nortestosterone or CCNU (**137b**). The specificity of binding to the androgen receptor was shown[514] by experiments in which pretreatment of tumor tissue with **706** significantly reduced the binding of [^{3}H]dihydrotestosterone in the tumor tissue, but not in muscle, uterus, and blood. Furthermore, treatment with **706** did not affect the uptake of [^{3}H]progesterone in tumor tissue, uterus, blood, and muscle.[514] Compound **706** reduced the uptake of [^{3}H]estradiol in the uterus but not in the tumor tissue, blood, and muscle, and this observation indicated an additional estrogenic activity for **706**.[514]

The first reported[502] steroid-linked *N*-nitrosourea **707** caused, at a daily dose of 40 mg/kg, an 80−100% inhibition of a rat mammary tumor. The first estrogen-linked *N*-nitrosoureas **708**−**711** were synthesized[515] by the reaction of the corresponding amines with the azide transfer agent **11k** (Scheme 66). The CENU analogs **708** and **710**, at a daily dose of 40 mg/kg, were found[515] to inhibit the growth of DMBA-induced rat transplantable mammary cancer by factors of 85% and 100%, respectively whereas the corresponding *N*-methyl-*N*-nitrosoureas **709** and **711** at the same daily doses inhibited the cancer growth by only 23% and 15%, respectively.

An extension of this work[516] led to the synthesis of related 17β and 20 CENU derivatives of dehydroepiandrosterone **712** and **713**, estrone **714** and **715**, and pregnenolone **716**−**718** (Scheme 67a, Chart 18). The reduction of the oximes produced[522] the corresponding 17β-amines and 20-amines which were reacted with the CENU transfer agent **11b** to yield the analogs **712**, **714**, **716**, and **717**. However, no reaction could be achieved[516] between the α-aminonitriles and the transfer agent **11b**. Instead, the α-cyano-CENU derivatives **713**, **715**, and **718** were prepared by the reaction sequence for compound **715** outlined in Scheme 67b. Thus, the 3-hydroxyl group of the starting material was blocked as a tetrahydropyranyl ether, and this derivative converted to the α-aminonitrile by the Strecker amino acid synthesis. The amine function then was converted to the corresponding *N*-(2-chloroethyl)-*N*-nitrosocarbamoyl moiety by the two-step sequence involving formation of the urea with 2-chloroethyl isocyanate and *N*-nitrosation with nitrosyl chloride in pyridine. A mild acid hydrolysis of the THP ether yielded **715** (Scheme 67b).[516]

The relative binding affinities of compounds **712**−**718** for the estrogen receptor of calf uterus were determined[516] by competitive binding experiments with radiolabeled [^{3}H]estradiol. The estrone analogs **714** and **715** were bound specifically to the estrogen receptor as evidenced by their apparent affinity constants K_a of 1.4×10^9 and 0.58×10^9 LM^{-1}, respectively, compared to a value of 0.34×10^{12} LM^{-1} for estradiol.[516] The other compounds, **712**, **713**, and

Scheme 66

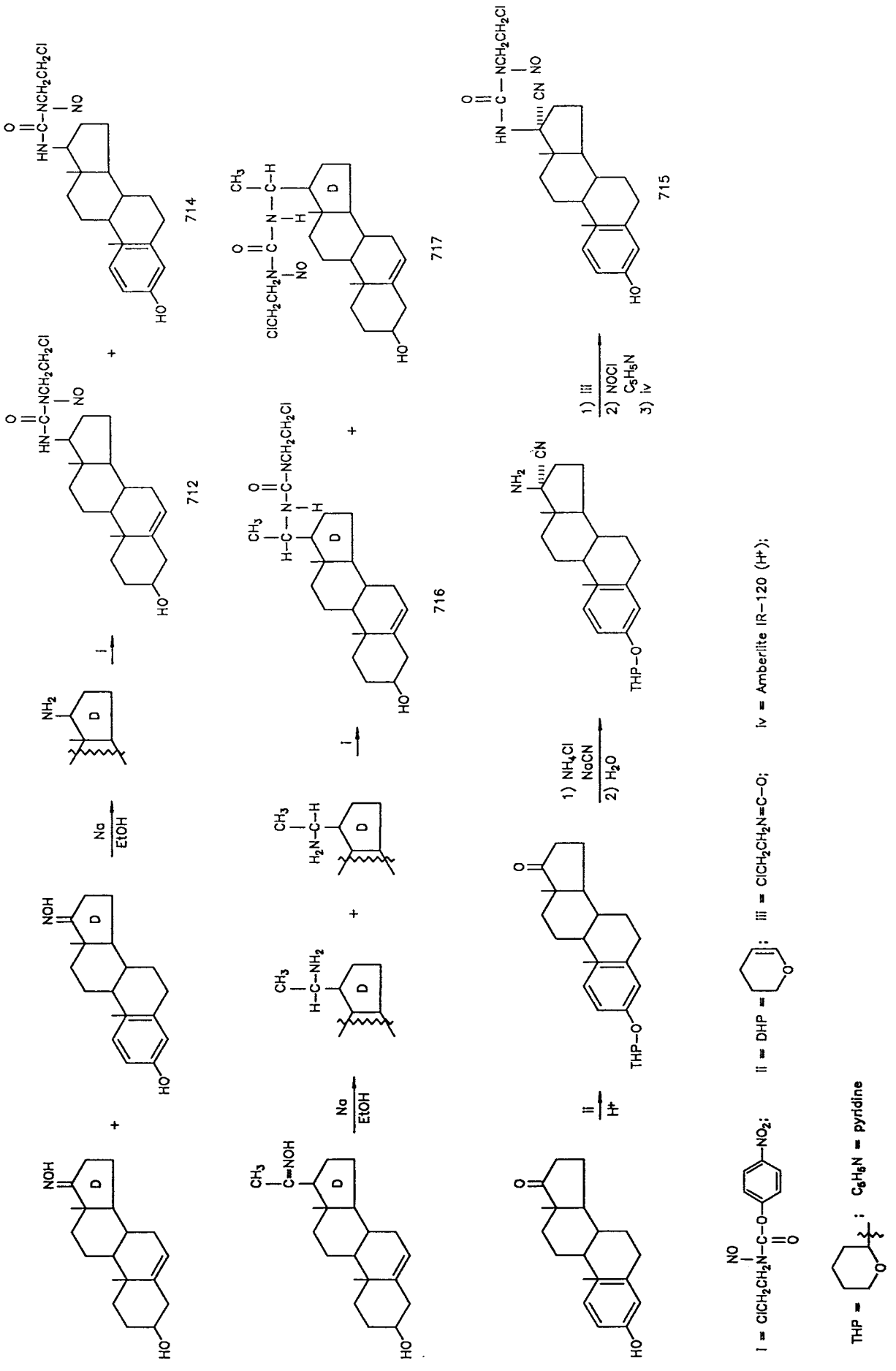


716−**718**, had little affinity for the ER, as evidenced by their K_a values which were lower than 4×10^6 LM^{-1}. While the 17β-position is blocked in compounds **714** and **715** the hydroxyl group is free for binding to the estrogen receptor, and hence this feature could account[516] for their relatively high binding affinities. Since the specific binding of [^{3}H]-estradiol in the presence of **714** and **715** increased after a period of time, the conclusion was reached[516] that the CENU moiety had no modifying effect on the receptor by an alkylation reaction.

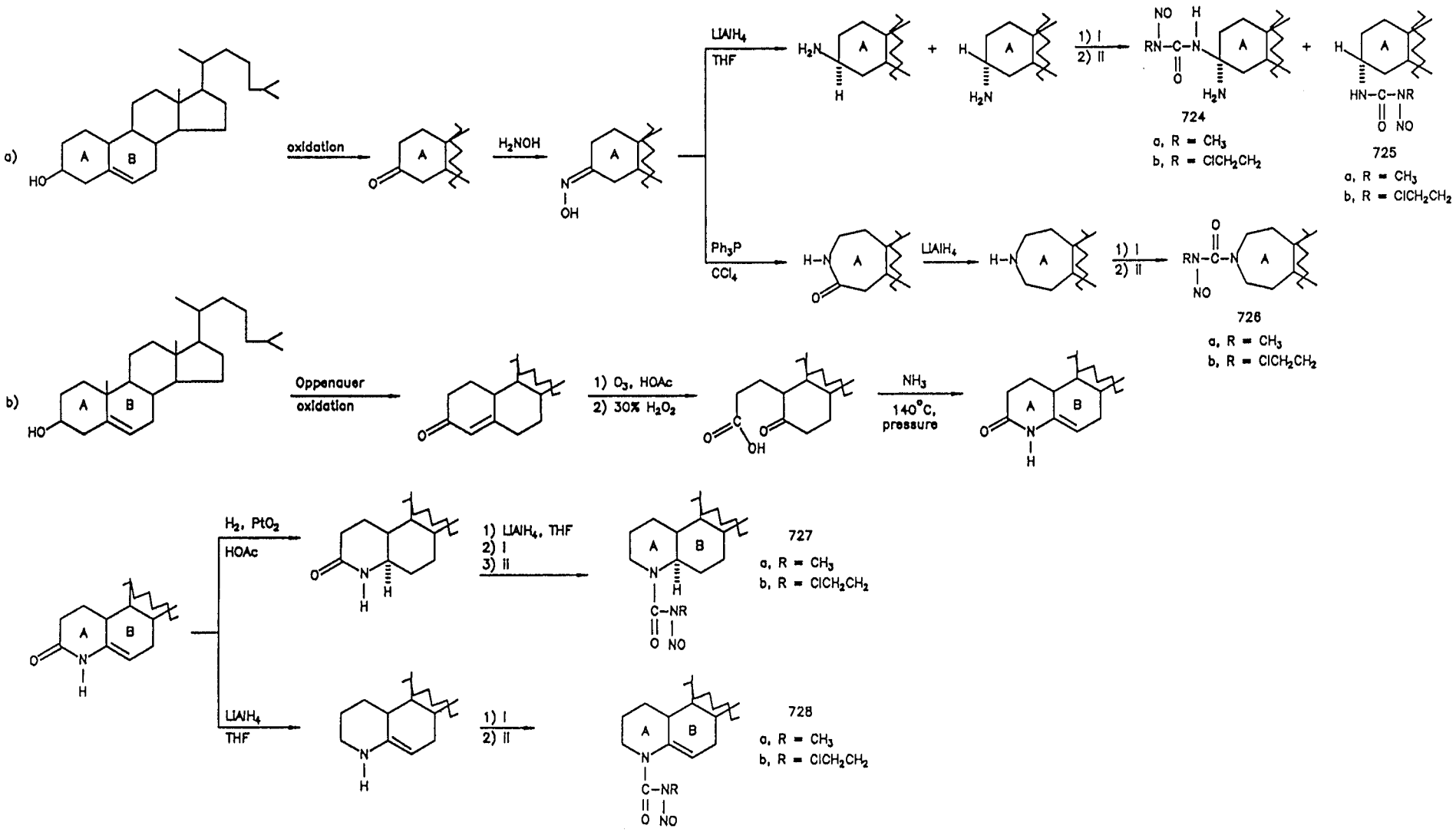
The antiestrogen tamoxifen (**719a**) was converted[517] to the nitrosocarbamate **720** and the nitrosourea **721** (Chart 18) which then were screened for cytotoxic activity in cultures of human breast cancer cells. In addition, the binding affinities to the estrogen receptor of MCF-7 cells were 0.18% for **720**, 0.35% for **721**, 1.8% for **719a**, and 100% for estradiol.[517] The activity of the nitrosocarbamate **720** was not related to a receptor binding since the addition of estradiol failed to block the cytotoxicity against either the estrogen receptor-positive MCF-7 cells or the estrogen receptor-negative MDA-MB 231 cells. In contrast, estradiol selectively blocked the cytotoxicity of the nitrosourea **721** against the receptor-positive cells but not against the receptor-negative cells.[517] In addition, the compound **721** was more active against the receptor-positive cell line. A control *N*-nitrosourea **722**, which could not bind to the estrogen receptor, was active against the receptor-negative, but not the receptor-positive, breast cancer cells. This effect excluded an incidental cytotoxicity of the *N*-nitrosourea moiety of **721** against the receptor-positive cells. Because the activity of **721** was apparent only after a prolonged treatment, and because the *N*-nitrosourea moiety would decompose during that time period, it was proposed[517] that the cytotoxicity of **721** was attributable to a hydrolysis product, bisdemethyltamoxifen **719b**, and not to the antiestrogen−*N*-nitrosourea conjugate structure. The importance of adequate control experiments was emphasized[517] for studies involving receptor-binding and structure−activity relationships.

A number of CENU analogs **723**−**728** (Chart 19) of cholesterol were synthesized[224,518−520] as potential anticancer agents. The precursor amines for compounds **724**−**728** were prepared according to the reactions of Scheme 68. Thus, a hydride reduction of the 3-oxime group yielded the 3-β and 3-α amines which were used for the synthesis of **724** and **725**.[518] Alternatively, the 3-oxime group was converted, by a Beckmann rearrangement and a hydride reduction, to the 3-aza-A-homo-5a-cholestane precursor, which was converted to **726** (Scheme 68a).[519] The Oppenauer oxidation of cholesterol followed by an ozonization and a reaction with ammonia under elevated temperatures and pressure, resulted in the ene-lactam A ring system (Scheme 68b).[520] The reduction of this ene−lactam by either lithium aluminum hydride or hydrogen in the presence of platinum oxide produced the two piperidine-containing ring systems which were used for the syntheses of **727** and **728**.[520] The conversion of the amines to the final CENU products **723**−**728** was accomplished via the preparation of the ureas, followed by a selective *N*-nitrosation using sodium nitrite in concentrated formic acid. These compounds were screened[224,518−520] in vitro for activity against the L1210 leukemia. Compounds **723**−**725** had no significant antileukemic activity[224,518] while for compound **726** an ID_{50} value of 1.6 μg/mL was found[519] which compared favorably with the 1.7 μg/mL value for the clinical drug MeCCNU (**138b**). Compounds **727** and **728** were reported[520] to have only low activity against the sarcoma 180 cell line.

Scheme 67

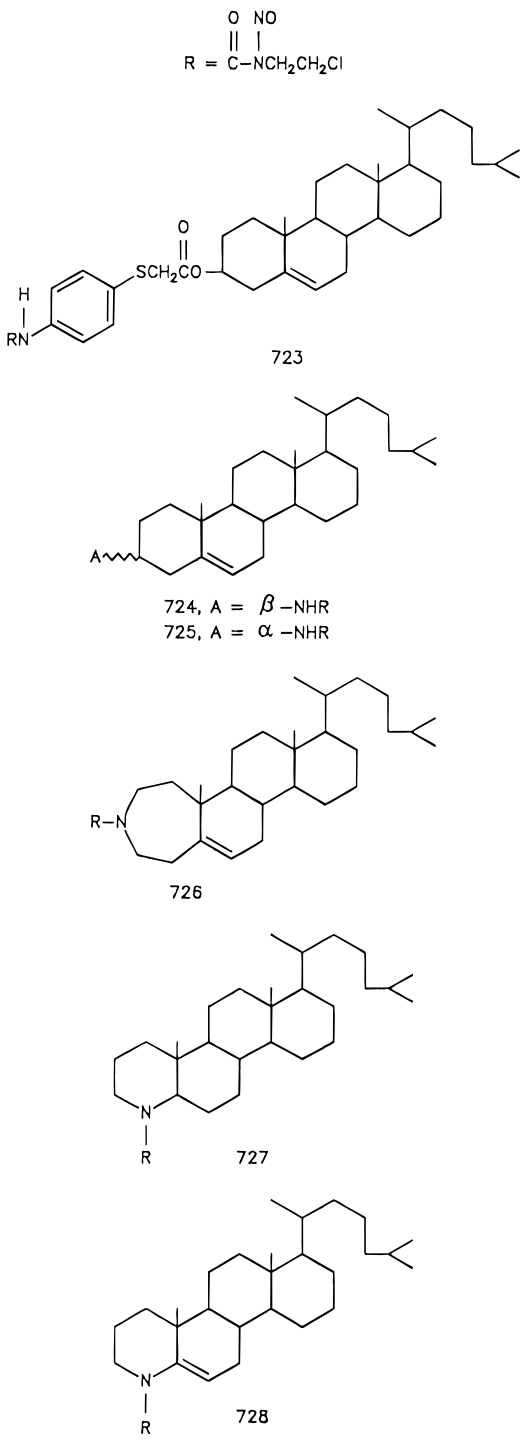

a)
b)
Na
EtOH
1) NH4Cl
NaCN
2) H2O
1) III
2) NOCl
C5H5N
3) IV
712
714
715
716
717
I = ClCH2CH2N(NO)C(=O)O-C6H3(NO2)
II = DHP
III = ClCH2CH2N=C=O;
IV = Amberlite IR-120 (H+);
THP = O-C5H9;
C5H5N = pyridine

Scheme 68



I = $RN{=}C{=}O$, R = CH_3, $ClCH_2CH_2$; II = $NaNO_2$, HCO_2H, 0°C

Chart 19. Structures for Section VII.E



F. Nucleoside Analogs

Various themes have been enunciated in the design of nucleoside nitrosourea analogs. The nucleoside moiety can be considered as a specific carrier for the nitrosourea moiety with altered pharmacological properties such as increased water solubility, lower myelotoxicity, and increased cell membrane transport. In addition, these analogs can be considered as irreversible inhibitors of nucleotide-metabolizing enzymes. They could also be a form of a prodrug of the nucleoside which would allow the slow release of the active drug in the cancer cells. All of these concepts will be discussed in this section.

Early research on the attachment of the *N*-nitrosourea moiety to the pyrimidine and purine bases resulted in the synthesis of compounds **311**−**313** and **315**−**319** (Table 18) which exhibited, in general, moderate anticancer activities. However, a substituted CENU pyrimidine derivative ACNU (**311**) (Table 18) had excellent anticancer activity and was subjected to clinical trials.

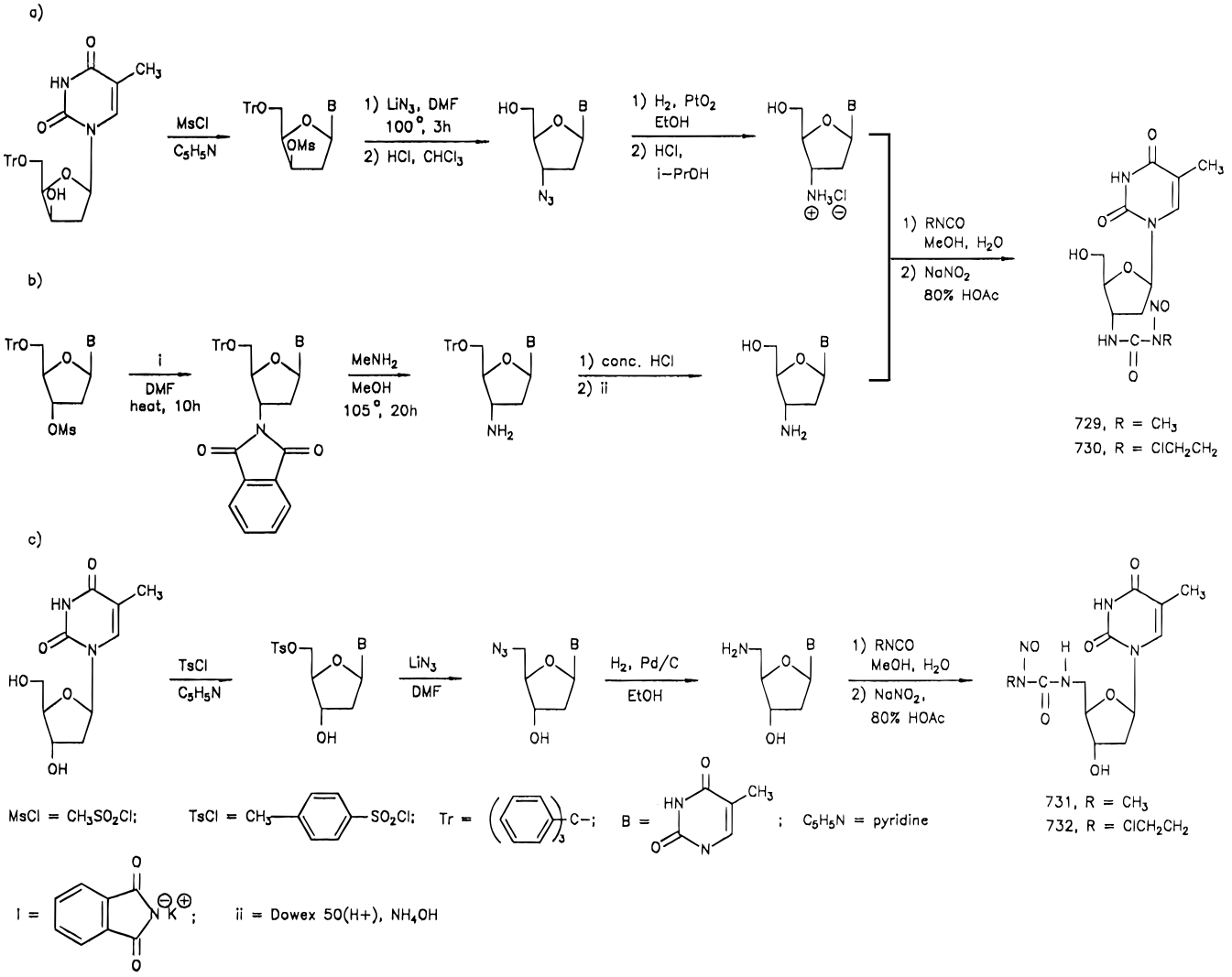
Most of the reported work on the nucleoside compounds has focused on the attachment of the *N*-nitrosourea group to the 3′- and 5′-positions of the carbohydrate portion of the molecules. The first reported[521] nucleoside *N*-nitrosoureas, 3′- and 5′-2-deoxythymidine analogs **729**−**732**, were synthesized from the corresponding 3′-amino-3′-deoxy-[522,523] and 5′-amino-5′-deoxythymidines,[524] as outlined in Scheme 69a−c.

The C3′ *N*-methyl- and *N*-chloroethyl-*N*-nitrosourea containing compounds **729** and **730** had approximately an equal growth inhibitory effect on the H.Ep-2 cells while the C5′ *N*-chloroethyl analog **732**, but not the *N*-methyl analog **731**, was shown[521] to have good in vitro activity. No clear correlation was found[521] between the cytotoxicity of compounds **729**−**732** and their carbamoylating and alkylating activities relative to BCNU (**33**). Thus, the C3′ compounds **729** and **730** had widely differing alkylating activities but nearly equal growth inhibitory properties.[521] The C5′ analogs **731** and **732** had low alkylating activities but only **732** was cytotoxic. Radiolabeled analogs of **732** containing ^{14}C in the CENU group, the alkylating moiety, and ^{3}H in the pyrimidine base, the carbamoylating moiety, were reported.[525]

Several interesting papers[526−529] were concerned with the biological activity of the precursor compounds to the CENU thymidine analogs **730** and **732**. Thus, 3′-amino-3′-deoxythymidine **733** (Chart 20), a hydrolysis product of 3′-CTNU **730**, was shown[526,527] to be a potent inhibitor of L1210 cell replication. Compound **733** was formed from **730** by an incubation for 5 h at 37 °C.[528] In contrast, 5′-amino-5′-deoxythymidine (**734**), the hydrolysis product of 5′-CTNU (**732**), failed to inhibit the L1210 cell replication.[526] The cytotoxicity of **733** could be explained[527] by a selective inhibition of DNA metabolism of the L1210 leukemia cells.

An important discovery[527,528] was the fact that pyrimidine-2′-deoxyribonucleosides, such as 2′-deoxythymidine **735**, but not pyrimidine ribonucleosides and purine nucleosides, could specifically prevent and reverse the inhibition of L1210 leukemia and B16 melanoma cell growth caused by 3′-CTNU **730** or its hydrolysis product **733** (Chart 20). The reversal of the cytotoxicity of **730** against L1210 could be achieved by the addition of **735** to the cell cultures as long as 24 h after the beginning of the incubation.[528] This time factor supports the contentions[528] that the pyrimidine-2′-deoxyribonucleosides fail to interfere with the cellular transport of the 3′-nucleoside nitrosourea and that alkylation is not the primary mechanism of action of **730**. All of these results indicate that the observed cytoactivity of **730** is attributable to its hydrolysis product **733**.[528] While the in vitro studies[526−528] seemed to support a cytotoxic role for **733**, the results of in vivo studies[529] revealed that **733** had little toxicity and anticancer activity when given to L1210-bearing mice at a dose of 320 mg/kg three times on day one. Administration

Scheme 69

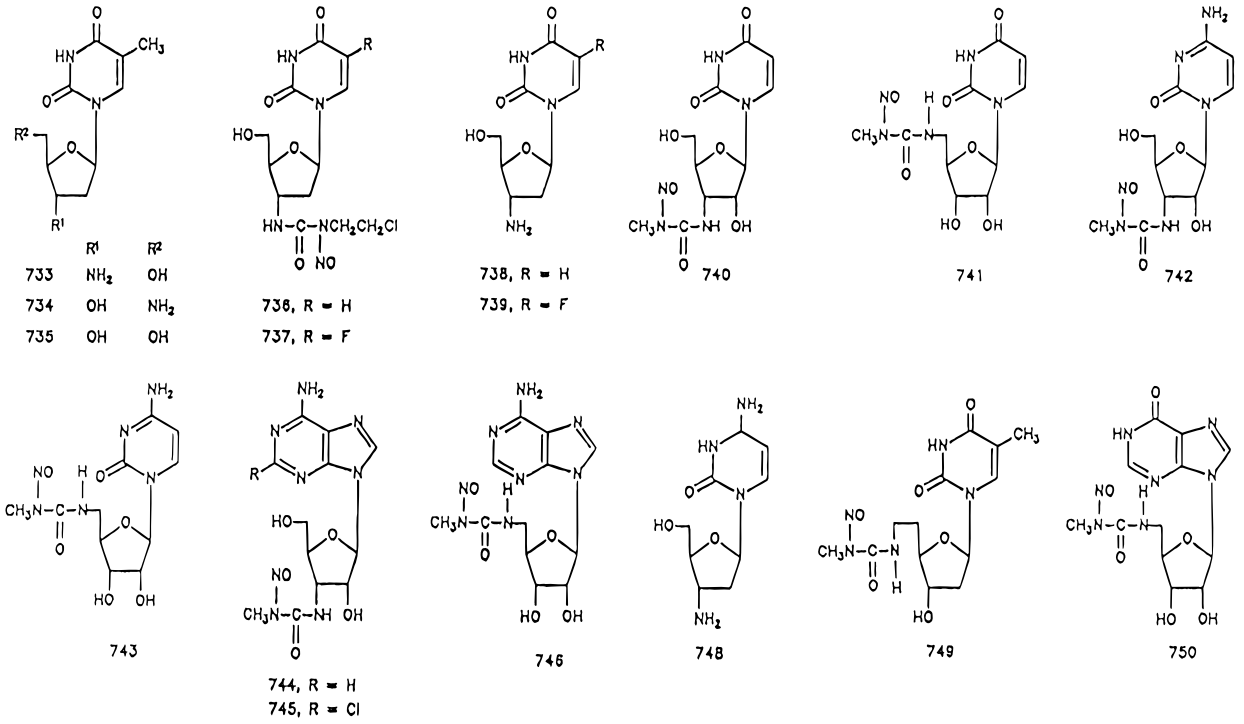


of the intact *N*-nitrosourea analog 3′-CTNU **730** showed a definite toxicity and anticancer activity at a single dose of 40 mg/kg (Table 41).[529] The simultaneous addition of the thymidine **735** and **730** resulted in a reduction of the toxicity of **730** against non-tumor-bearing mice but not a reduction in the anticancer activity of **730** against the L1210 and P388 leukemias in mice.[529] This selective effect was shown[529] not to be the result of any reduction in the bone marrow toxicity of **730**. Furthermore, the presence of thymidine **735** failed to block the initial weight loss and did not exhibit any anticancer activity against the leukemia-bearing mice.[529]

The C3′ CENU analogs of 2′-deoxyuridine (**736**) and 2′-deoxy-5-fluorouridine (**737**) (Chart 20) were synthesized[530] from the corresponding amines[531] by formation of the ureas followed by selective *N*-nitrosation with formyl nitrite. The analogs **736** and **737** had high activity[530] against the L1210 leukemia in vivo and exhibited higher alkylating and lower carbamoylating activity relative to BCNU (Table 41). There was no correlation between the cytotoxicity, as measured by the ED_{50} values, and the half-lives, alkylating and carbamoylating activities of **730**, **736**, and **737**.[530] The ED_{50} values for the corresponding 3′-amino-3′-deoxy analogs **738** and **739** were much larger than those of **736** and **737**. This result indicated that the anticancer activities of the latter were not attributable to a prior chemical decomposition to 3′-amino-3′-deoxynucleosides.[530]

A number of *N*-methyl-*N*-nitrosoureido analogs of 3′-amino- and 5′-aminouridine, **740** and **741**, 3′-amino and 5′-aminocytidine, **742** and **743**, and 3′-amino- and 5′-aminoadenine, **744**−**746** were synthesized,[531,532] according to the reactions outlined in Schemes 70 and 71. The positioning of the nitrosourea group at either the 3′- or 5′-positions required a series of protection and deprotection steps. A rationale for the synthesis of the *N*-nitrosoureido nucleosides was their potential as active, site-directed irreversible enzyme inhibitors.[532,533] The *N*-methylnitrosoureido function was chosen[532] because it would be expected to have greater carbamoylating activity than the *N*-(chloroethyl)nitrosoureido moiety. It was thought[532] that the 3′-deoxy-3′-*N*-methylnitrosoureido nucleosides could either inhibit the nucleotide-metabolizing enzymes or be phosphorylated to the corresponding nucleotides which could act as enzyme inhibitors. The 5′-deoxy-5′-*N*-methylnitrosoureido nucleosides might inhibit the enzymes by carbamoylating a guanidinium moiety of an arginine at the active site, a group known to be essential for the binding of the 5′-phosphate moiety of nucleotides.[533] None of these analogs proved[532] to be cytotoxic toward H.Ep-2 cancer cells in culture and against the L1210 leukemia in vivo.

Chart 20. Structures for VII.F



The lack of anticancer activity for compounds **740**−**746** can be explained[319,532] by an intramolecular cyclization of the neighboring C2′-hydroxyl group to form a cyclic urethane **747** and a concomitant release of the *N*-methylnitrosoureido function (Scheme 72). Such intramolecular cyclization reactions have been encountered in the carbohydrate *N*-nitrosourea compounds (section VII.C). Furthermore, the 3′-amino-2′,3′-dideoxycytidine **748**, which lacks the 2′-hydroxyl, was shown[534] to have a moderate anticancer activity of 183% ILS against the L1210 leukemia in mice. The 5′-*N*-methyl-*N*-nitrosoureido analogs of thymine **749** and inosine **750** were found[535] to be ineffective as active site inhibitors of the metabolizing enzymes and as anticancer agents.

Further attempts were made[533,536] to exploit the carbamoylating potential of the *N*-nitrosourea moiety at the C5′-position as irreversible inhibitors of the active site of nucleotide-metabolizing enzymes. Molecular modeling revealed[533] that for effective binding with the active site, the reactive group, i.e. *N*-nitrosourea, should be located at one to three carbons removed from the 5′ carbon of a monophosphate and one, three, or five carbons from a diphosphate group. These different spacings were achieved in the analogs **751**−**761** (Chart 21 and Table 41).

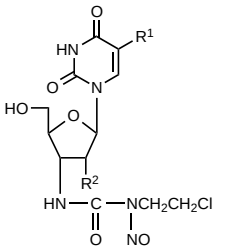
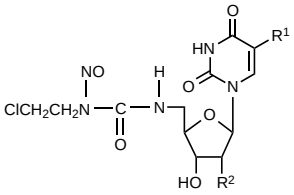
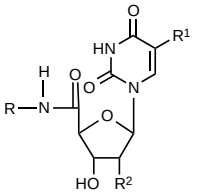
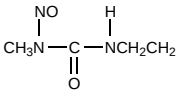
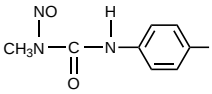
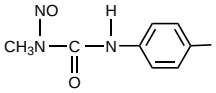
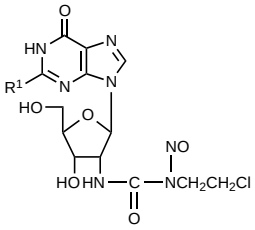
The compounds **753**−**761** were prepared[533,536] from the corresponding 5′-ribofuranuronic acids,[537,538] as outlined in Scheme 73. Spacer arms were used to place the *N*-nitrosoureido group at different distances from the C5′-position.[536] The reaction of the 5′-acid with diphenylphosphoryl azide and triethylamine formed a mixed anhydride which, in turn, reacted smoothly with either aminoethylureas or with anilinoureas to yield the desired urea intermediates.[533,536] *N*-Nitrosation was effected either by the use of sodium nitrite in concentrated formic acid or by means of the CENU transfer agent **11e** for **751** and its ethyl analog for **752**.[536] Nitrosation of the N3-methylureas produced the N3-nitroso products **753**, **754**, **757**, **758**, and **760**, whereas the same nitrosation of the N3-cyclohexyl ureas yielded the N1-nitroso products **755**, **756**, and **761**. The possibility of nitrosation of the C5′-amide nitrogen was eliminated by means of its MS(EI) fragmentation pattern.[533]

Because of the different *N*-nitroso positions, the reaction of different compounds would give different decompositions of isocyanates and diazohydroxides, as shown in Scheme 74. The normal C5′-CENU analogs **732** and **751** were the most cytoactive of the C5′-series, with % ILS values of 135 and 191, respectively (Table 41).[521,536] The inactive **752** would yield the same isocyanate but a different diazohydroxide than **751** (Scheme 74a,b). This fact supports the role of alkylation, and not carbamoylation, in the anticancer activity of *N*-nitrosourea drugs. Compounds **753**−**756**, **760**, and **761** have long half-lives which resulted in very low alkylating activity (Scheme 74c,d), and they exhibited little or no activity against the L1210 leukemia.[533] Compound **760** failed to inhibit hypoxanthine phosphoribosyl-transferase even after incubation with the enzyme for 1 h before addition of the substrate.[533]

The compounds **757**−**759** had different spacer arms containing phenyl rings. Decompositions of **757** and **758** would yield methanediazonium hydroxide, while the decomposition of **759** would produce the diazohydroxide connected to the nucleoside group (Scheme 74e,f). The former compounds were somewhat more cytotoxic (Table 41).[533] All three of these compounds had shorter half-lives than the other C5′ *N*-nitrosoureas.

A series of purine analogs based on the 2′-amino-2′-deoxyguanosine skeleton **762** were reported[539] (Scheme 75). Intermediate guanine ureas could be converted to the *N*-nitrosoureas of either xanthine **763** and **764** or guanine **765** and **766**, depending on the reaction conditions. The [(dimethylamino)methylene]amino derivatives **767** and **768** were much more water soluble than **763**−**766**, and they were

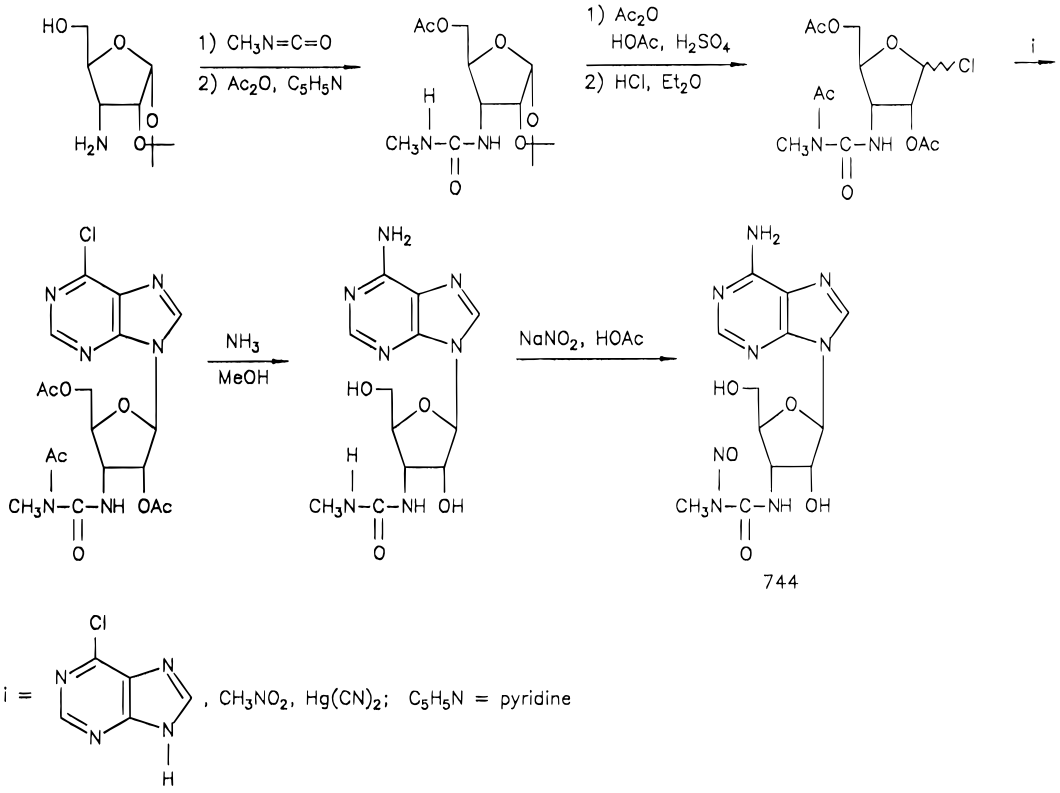
Table 41. Anticancer Activity of Nucleoside *N*-Nitrosourea Analogs against Ip-Implanted L1210 Leukemia[a] and P388 Leukemia[b]

compound no.	R^1	R^2	OD,[c] mg/kg	ILS_{max},[g] %	survivors, %	ED_{50},[k] μM	alkyl act[l]	carbamoyl act	$t_{1/2}$,[p] min	ref(s)
										
736	H	H	40[a,d]	269	91[h]	12.5	1.65	0.85[m]	27.3	530
737	F	H	20[a,d]	123	33[h]	2.5	1.78	0.77[m]	36.1	530
730	CH_3	H	40[a,d]	140	50[i]		2.00	0.85[m]		521,529,530
			40[b,d]	197	17[h]	1.5	2.1	0.90[m]		
										
751	H	H	25[b,e]	191		14			240	521,536
732	CH_3	H	25[b,e]	135		10	0.47	1.6[n]	360	
 R										
										
753	H	OH	200[b,f]	28			0	1.91[o]	1020	533
										
757	H	OH	100[b,f]	56	33[j]				120	536
										
758	CH_3	H	200[b,f]	79	46[j]				24	536
										
764	OH		45[a,d]	59						539
766	NH_2		40[a,d]	46						539

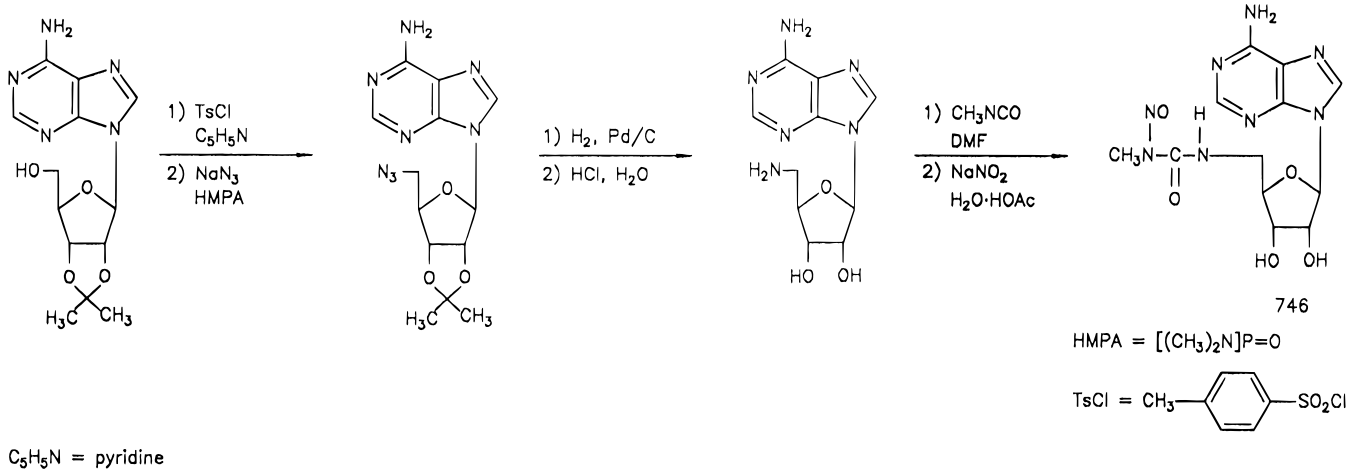
[a] Murine lymphoid leukemia. [b] Murine lymphocytic leukemia. [c] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. [d] Drug administered on day 1 after tumor implantation. [e] Drug administered on days 1−5 after tumor implantation. [f] Drug administered on days 1−9 after tumor implantation. [g] Increase in life span = $[(T - C)/C] \times 100$. [h] Percentage of treated animals surviving on day 60 after tumor implantation. [i] Percentage of treated animals surviving on day 120 after tumor implantation. [j] Percentage of treated animals surviving on day 90 after tumor implantation. [k] Dose which results in the killing of 50% of the HEp-2 cells. [l] Alkylating activity, relative to that of BCNU (**33**) = 1.0; measured as the absorbance at 540 nm after reaction with 4-(*p*-nitrobenzyl)pyridine, pH 6.4, at 37 °C for 2 h. [m−o] Carbamoylating activity, measured as (m) the extent of carbamoylation of 5′-amino-5′-deoxythymidine in phosphate buffer, pH 7.4, at 37 °C for 6 h; (*n*) the extent of carbamoylation of [^{14}C]lysine relative to BCNU (**33**) = 1.0: (*o*) actual ^{14}C radioactivity. [p] Half-life, determined by measuring the decrease of extinction (ϵ) at λ_{max} in phosphate buffer, pH 7.4, at 37 °C.

expected[539] to slowly degrade to the 2-amino derivative under in vivo conditions. All of these analogs were either inactive or slightly active in the in vivo screening against the L1210 leukemia (Table 41).[539] Compared with the clinical drug MeCCNU (**138b**), the CENU analog **766** had a greatly reduced bone

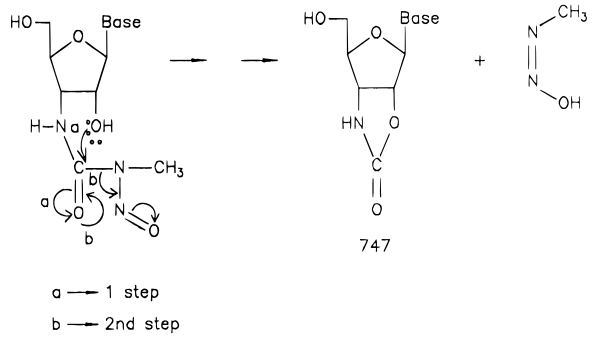
Scheme 70



Scheme 71



Scheme 72



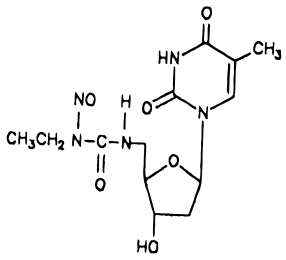
marrow toxicity.[539] The low activity of these purine analogs could be explained[319] by an intramolecular cyclization involving the neighboring C3′-hydroxyl to form a cyclic urethane, in analogy to the mechanism of Scheme 17b. Furthermore, the 2′-amino-2′-deoxyguanosine (**762**) was reported[540] to be an antimetabolite which exhibited anticancer activity.

Additional nucleoside derivatives **769** and **770** were synthesized.[329,541] Compound **769** was essentially inactive at a dose of 400 mg/kg per day against the L1210 leukemia in mice.[329]

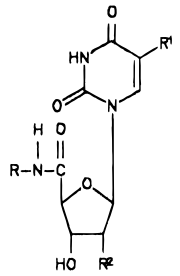
A combination of two anticancer agents, such as 5-fluorouracil (5FU, **771**) and a (chloroethyl)nitrosoureido (CENU) moiety, by an attachment to a common carbohydrate structure offers another approach to chemotherapy.[542,543] In principle, it should be possible to tailor the chemical structure for a gradual release of the antimetabolite 5FU.[542] This combination of a prodrug form of 5FU and CENU is more effective than a single agent therapy, both experimentally[544,545] and clinically.[188]

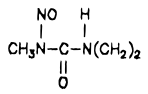
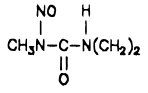
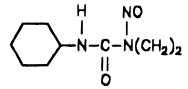
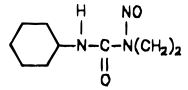
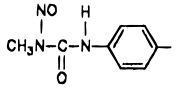
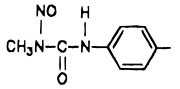
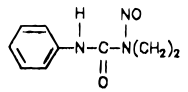
The general structure envisioned for such a chemical combination **772** involves a seconucleoside containing both the nucleoside and the CENU group.[542] The biochemical activation mechanisms of this type

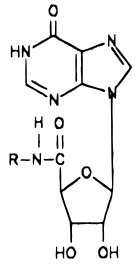
Chart 21. Structures for Section VII.F

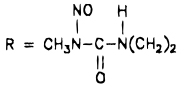


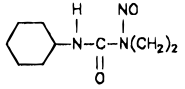
752

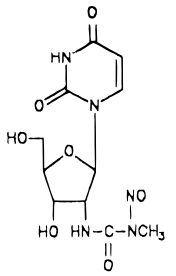


	R	R¹	R²
753		H	OH
754		CH_3	H
755		H	OH
756		CH_3	H
757		H	OH
758		CH_3	H
759		CH_3	H

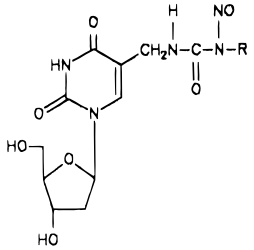


760 R = 

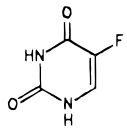
761 R = 



769

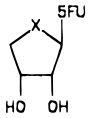


770, R = CH_3, $ClCH_2CH_2$

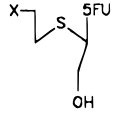


771 (5FU)

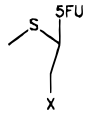
772 Y = CENU
B = 5FU
X = O or S



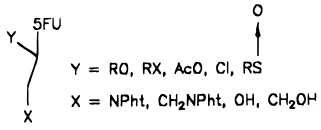
773, X = S
789, X = SO



774
X = OH, NH_2



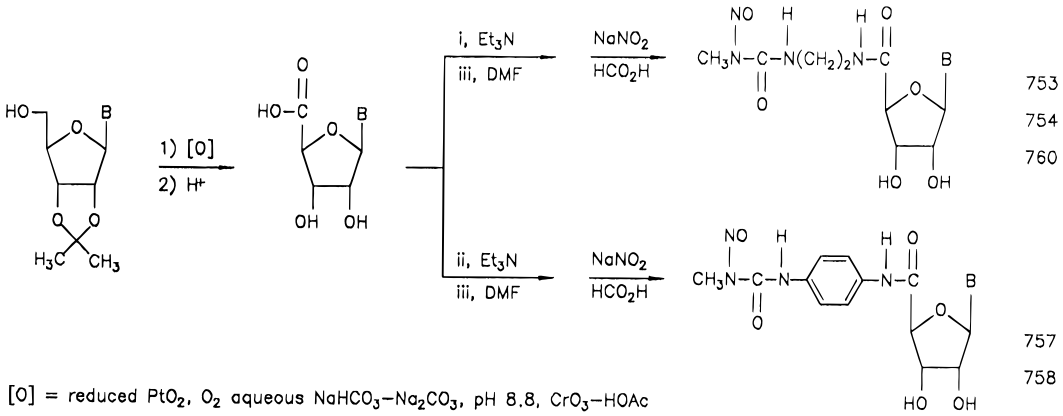
775, X + NH_2 or OH
5FU is N'–substituted



788

Y = RO, RX, AcO, Cl, RS
X = NPht, CH_2NPht, OH, CH_2OH

Scheme 73



[O] = reduced PtO_2, O_2 aqueous $NaHCO_3$–Na_2CO_3, pH 8.8, CrO_3–HOAc

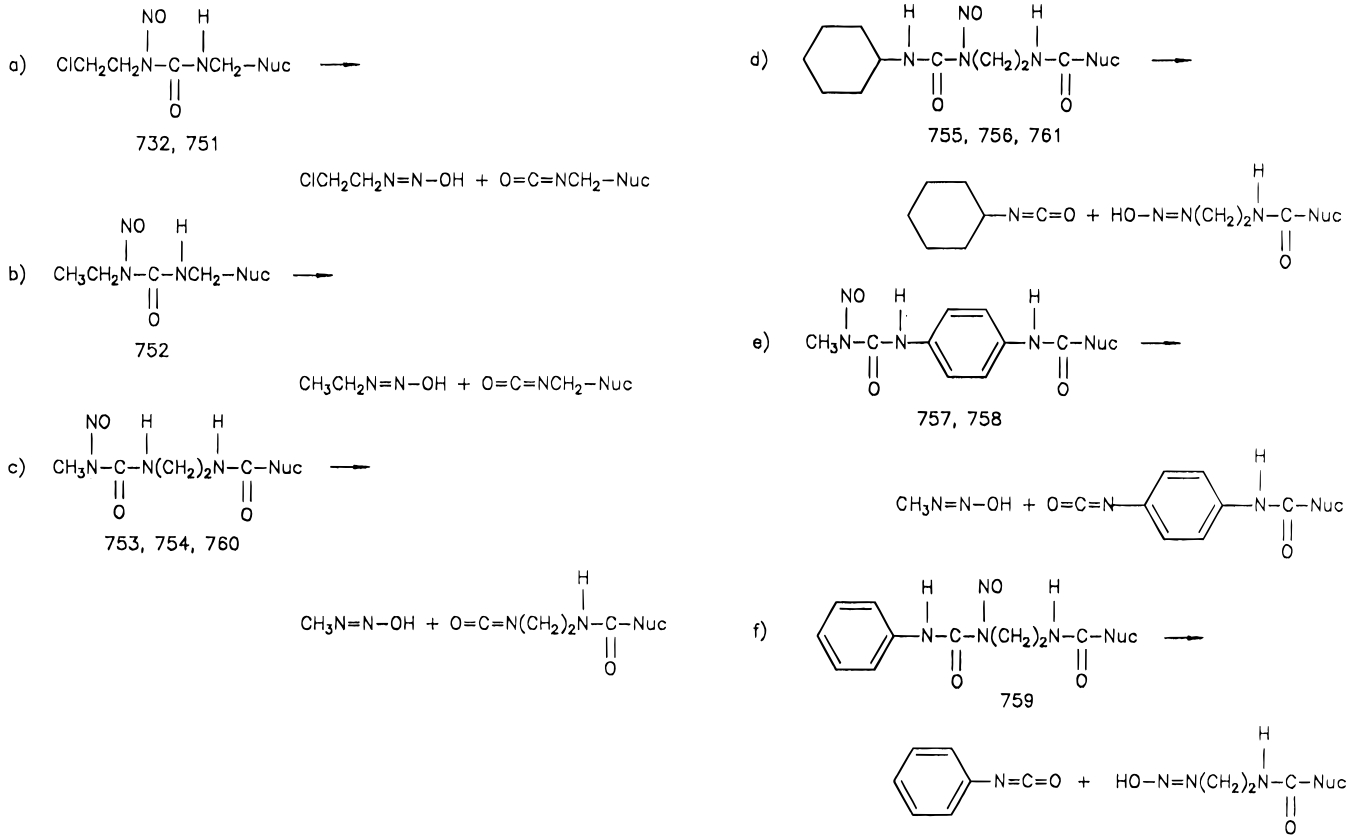
B = uracil–1–yl (753, 757); B = thymin–1–yl (754, 758); B = hypoxanthin–9–yl (760)

i = $CH_3N(H)C(=O)NHCH_2CH_2NH_2$, from $CH_3N=C=O + H_2NCH_2CH_2NH_2$

ii = $CH_3N(H)C(=O)N(H)$–C$_6$H$_4$–NH_2, from $CH_3N=C=O + H_2N$–C$_6$H$_4$–NH_2

iii = $Ph_2P(=O)N_3$

Scheme 74

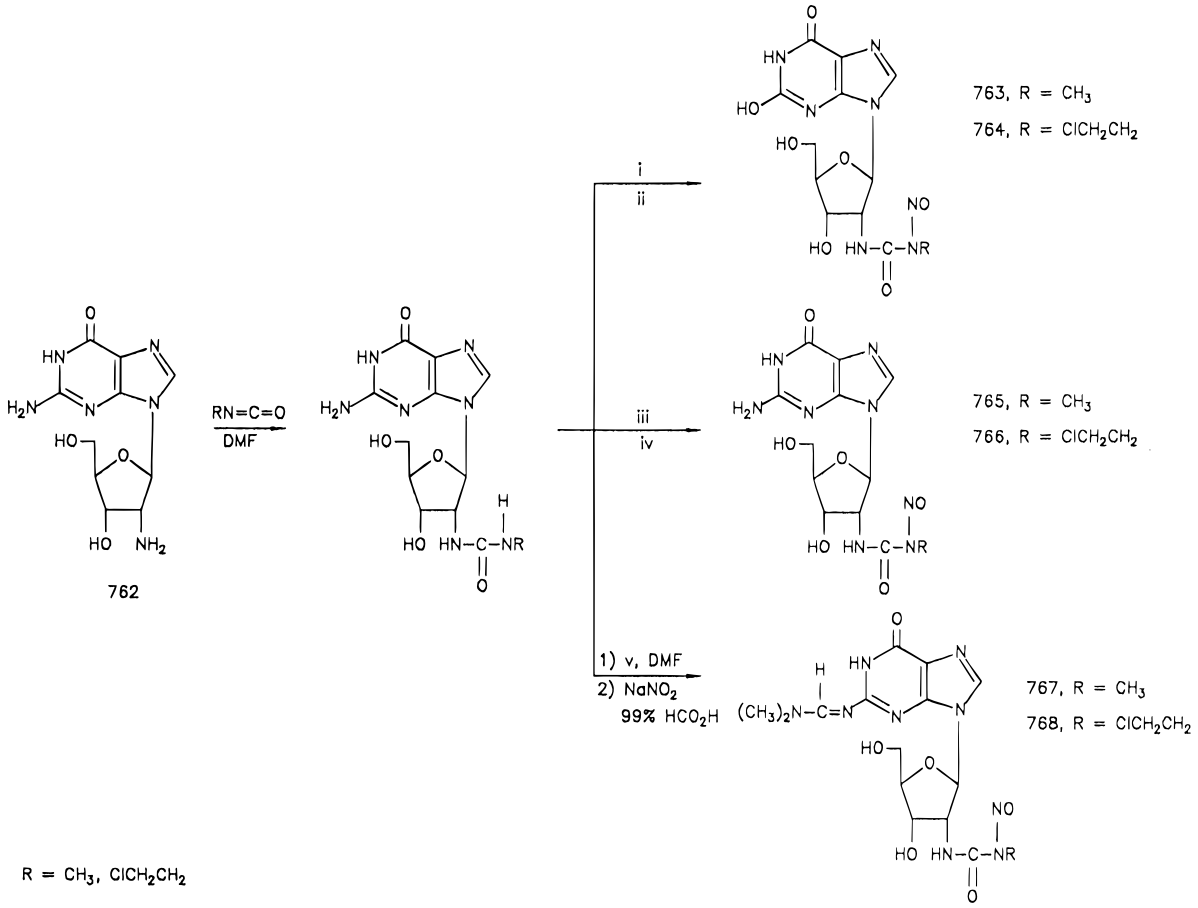


Nuc = Nucleosides

of compounds is shown in Scheme 76. The products are free 5FU, an alcohol or thiol, an aldehyde connected to the CENU moiety (Scheme 76a) or formaldehyde, and an alcohol or thiol connected to the CENU moiety (Scheme 76b). Designations of α and α′ were used to identify the two attachment points on the side chain. The following discussion will first cover the α-attachment.

Several 5FU nucleosides with sulfur in a ring[546] and in a side chain,[547] **773**–**775** (Chart 21), were

Scheme 75

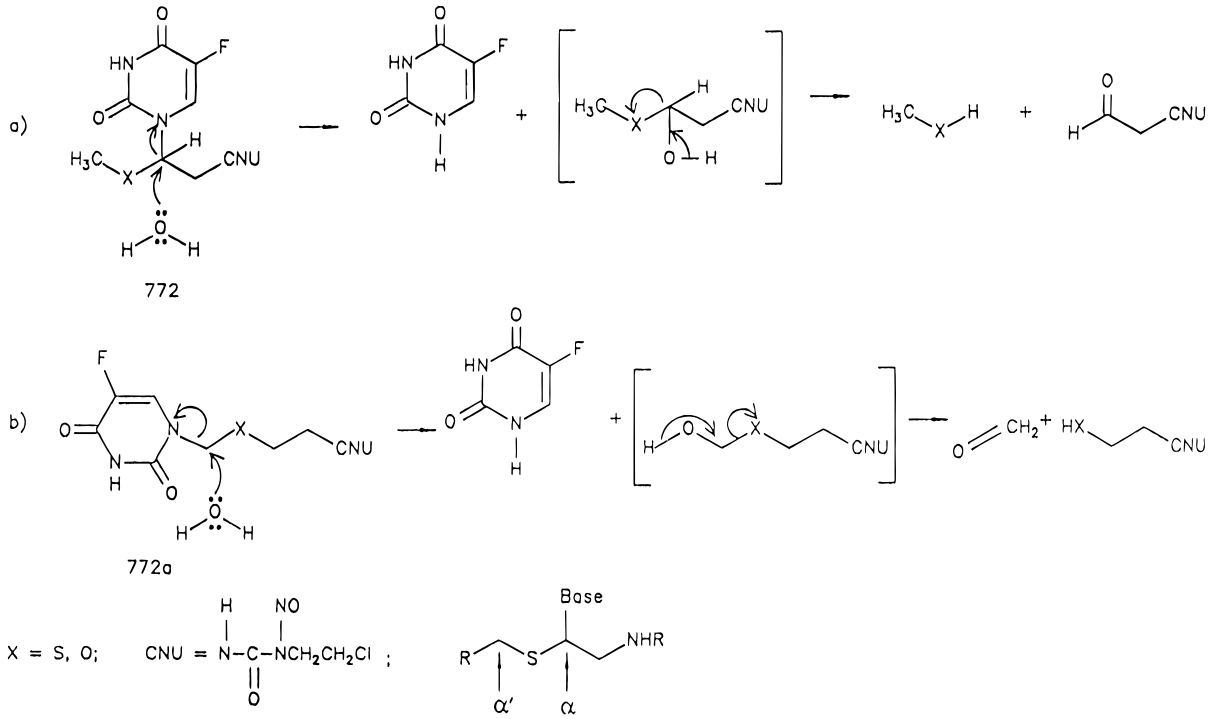


R = CH_3, $ClCH_2CH_2$

i = $NaNO_2$, HCl (4:3), 763; ii = $NaNO_2$, NHCl (3:4), 764; iii = 1eq. $NaNO_2$, HCO_2H, 766;

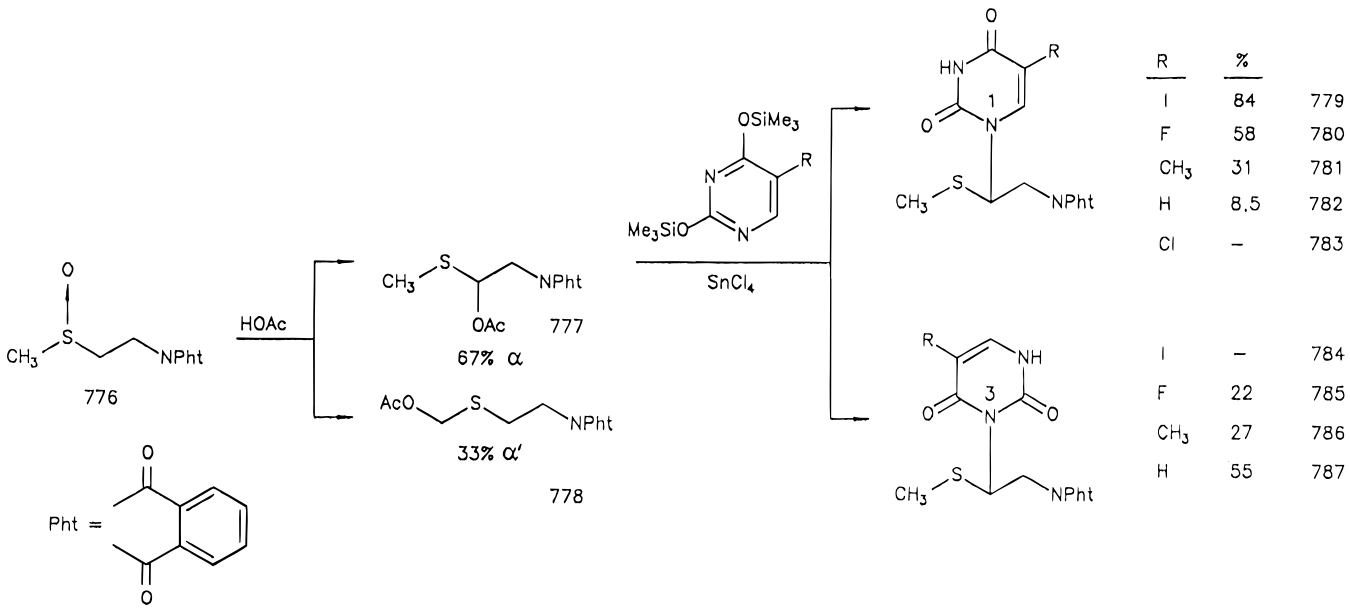
iv = 2.4eq. $NaNO_2$, HCO_2H, 765; v = $(CH_3)_2NC(OCH_3)_2$

Scheme 76



synthesized by a general method based on the Pummerer rearrangement of sulfoxides, as illustrated in Scheme 77. The reaction of **776** with acetic acid at elevated temperatures produced a migration of the oxygen moiety to the α- and α′-positions, resulting in the acetoxy sulfides **777** and **778** in a 2:1 ratio.[547]

Scheme 77



The amine function was blocked as a phthalimide in these reactions. The subsequent reaction of bis(trimethylsilyl)pyrimidines with **777** was studied in detail.[547,548] Nucleophilic attack by different pyrimidines on the α-position of **777** resulted in various proportions of the N1 **779**−**783** and N3 **784**−**787** substituted pyrimidines (Scheme 77).[548]

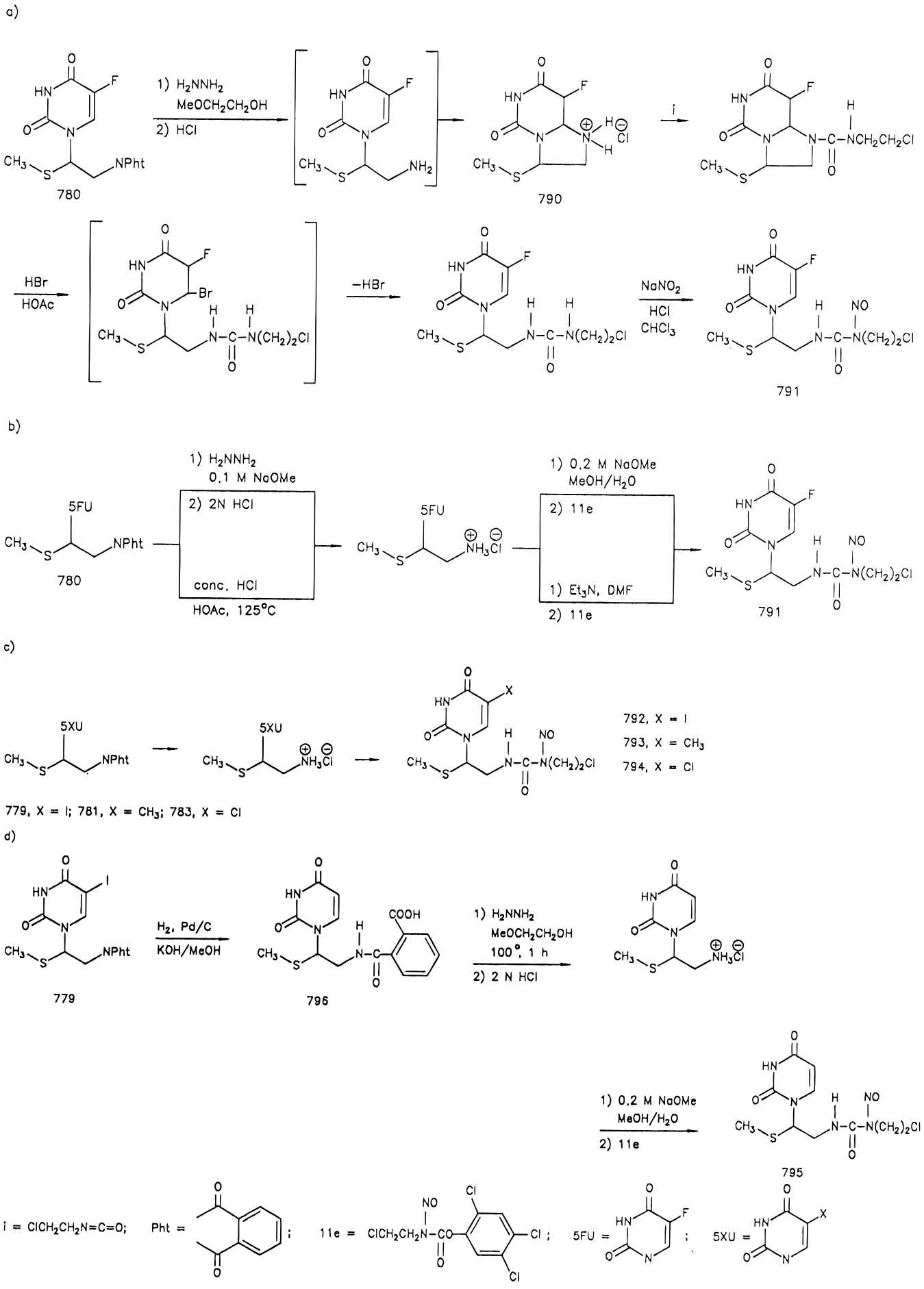
Some model 5FU−seconucleosides of general structure **788** (Chart 21) were synthesized,[549,550] and their rates of acid hydrolysis were determined.[549] Under these acid conditions there were large variations in the release of 5FU, as determined by ultraviolet spectroscopy, ranging from 10 min to 15 h or longer. The cyclic sulfide and sulfoxide **773** and **789** had very low hydrolysis rates.[549] This work provided a basis for the chemical tailoring of the drug structure **772**, containing two antineoplastic moieties.

An early transformation of the α-5FU1-S seconucleoside **781** to its CENU derivative **791** was carried out[547] as illustrated in Scheme 78a. Thus, dephthaloylation of **781** with hydrazine hydrate in 2-methoxyethanol at 100 °C for about 1 h produced[547,548] a bicyclic amine **790**. This intermediate was converted to the urea with 2-chloroethyl isocyanate, followed by a ring opening of the bicyclic amine with hydrogen bromide in acetic acid. A selective *N*-nitrosation with nitrosyl chloride produced[547] compound **791**, the first 5FU−CENU combination drug of this class. Later, milder conditions were used[548] for the removal of the phthaloyl group from **781**. Thus, as shown in Scheme 78b, the reaction of **781** with either a mixture of hydrochloric acid and acetic acid or with hydrazine hydrate and sodium methoxide followed by hydrochloric acid allowed the isolation of the desired amine salt.[548] The conversion of the amine to its CENU derivative **791** was carried out efficiently by neutralization with triethylamine in DMF followed by a reaction with the CENU transfer agent **11e**.[551] It was shown[548,552] that the transformation of the 5-iodo- (**779**), 5-methyl- (**781**), and 5-chloro- (**783**) uracil seconucleosides to their corresponding CENU analogs **792**−**794** (Scheme 78c) did not involve a bicyclic amine intermediate as was the case for the 5FU derivative **791**.

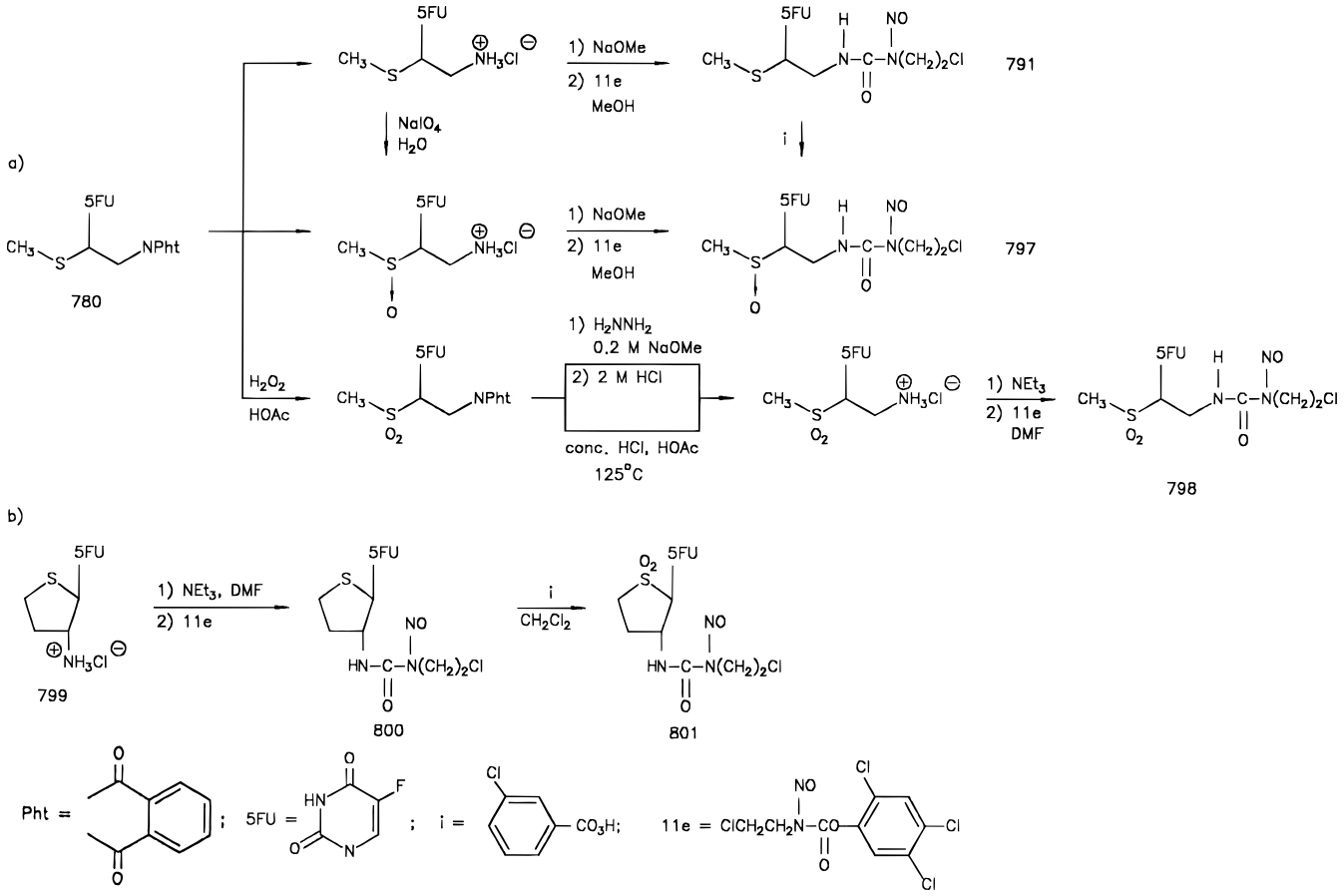
The preparation of the *N*-substituted uracil seconucleoside α-U1-S analog **795** proved to be a special problem because of the low yield of the precursor compound **782** (Scheme 77). A solution to this problem, as shown in Scheme 78d, involved a deiodination of the corresponding 5-iodouracil derivative **779** by a catalytic hydrogenation.[553] An opening of the phthalimido group was caused by the basic reaction conditions. The resulting amide **796** was converted[553] to the N1-uracil CENU analog **795** by the same reaction sequence as was used for the 5FU analog **791** (Scheme 78b).

Many permutations of the structure **791** are possible. Thus, the sulfur can be either oxidized or replaced with oxygen, the distance between the reactive groups can be either increased or decreased, and the N3-substituted pyrimidine can be substituted for the N1-substituted pyrimidine. Hence, the sulfoxide CENU analog **797** was synthesized[548,551,554] by two routes from **780** (Scheme 79a). A sequence of hydrolysis of the phthalimide to the amine salt, periodate oxidation of the amine salt to the sulfoxide and subsequent reaction with the CENU transfer agent **11e** was the preferred route.[551,554] Oxidation of **780** with hydrogen peroxide in acetic acid yielded[554] the sulfone which was transformed to the CENU analog **798** by an analogous reaction sequence (Scheme 79a). Similarly, the cyclic sulfide amine salt **799**[555] was converted[554] to the corresponding CENU analog **800** which was oxidized to the homologous sulfone **801** (Scheme 79b). Another molecular change[548,554] was the replacement of the sulfur atom with oxygen in the α′ side chain (Schemes 80 and 81). An important reaction was the first step in Scheme 80a, i.e. the reaction of the sulfide with sulfuryl chloride in dichloromethane to give the chloro intermediate **802**.[548,554] Subsequent reactions of **802** with alcohols produced the alkoxy intermediates, **803** and **804**, whereby the formation of the methyl analog involved methanol and a silver carbonate catalyst, and the formation of the ethylene glycol analog involved heating with the neat alcohol. In earlier work[548] the removal of the phthaloyl group from **803** and **804** by the reaction with hydrazine hydrate in

Scheme 78


a)
1) H_2NNH_2
$MeOCH_2CH_2OH$
2) HCl
780
790
i
HBr
HOAc
−HBr
$NaNO_2$
HCl
$CHCl_3$
791
b)
1) H_2NNH_2
0.1 M NaOMe
2) 2N HCl
conc. HCl
HOAc, 125°C
1) 0.2 M NaOMe
$MeOH/H_2O$
2) 11e
1) Et_3N, DMF
2) 11e
5FU
780
791
c)
5XU
792, X = I
793, X = CH_3
794, X = Cl
779, X = I; 781, X = CH_3; 783, X = Cl
d)
H_2, Pd/C
KOH/MeOH
1) H_2NNH_2
$MeOCH_2CH_2OH$
100°, 1 h
2) 2 N HCl
779
796
1) 0.2 M NaOMe
$MeOH/H_2O$
2) 11e
795
i = $ClCH_2CH_2N{=}C{=}O$;
Pht =
11e =
5FU =
5XU =

Scheme 79



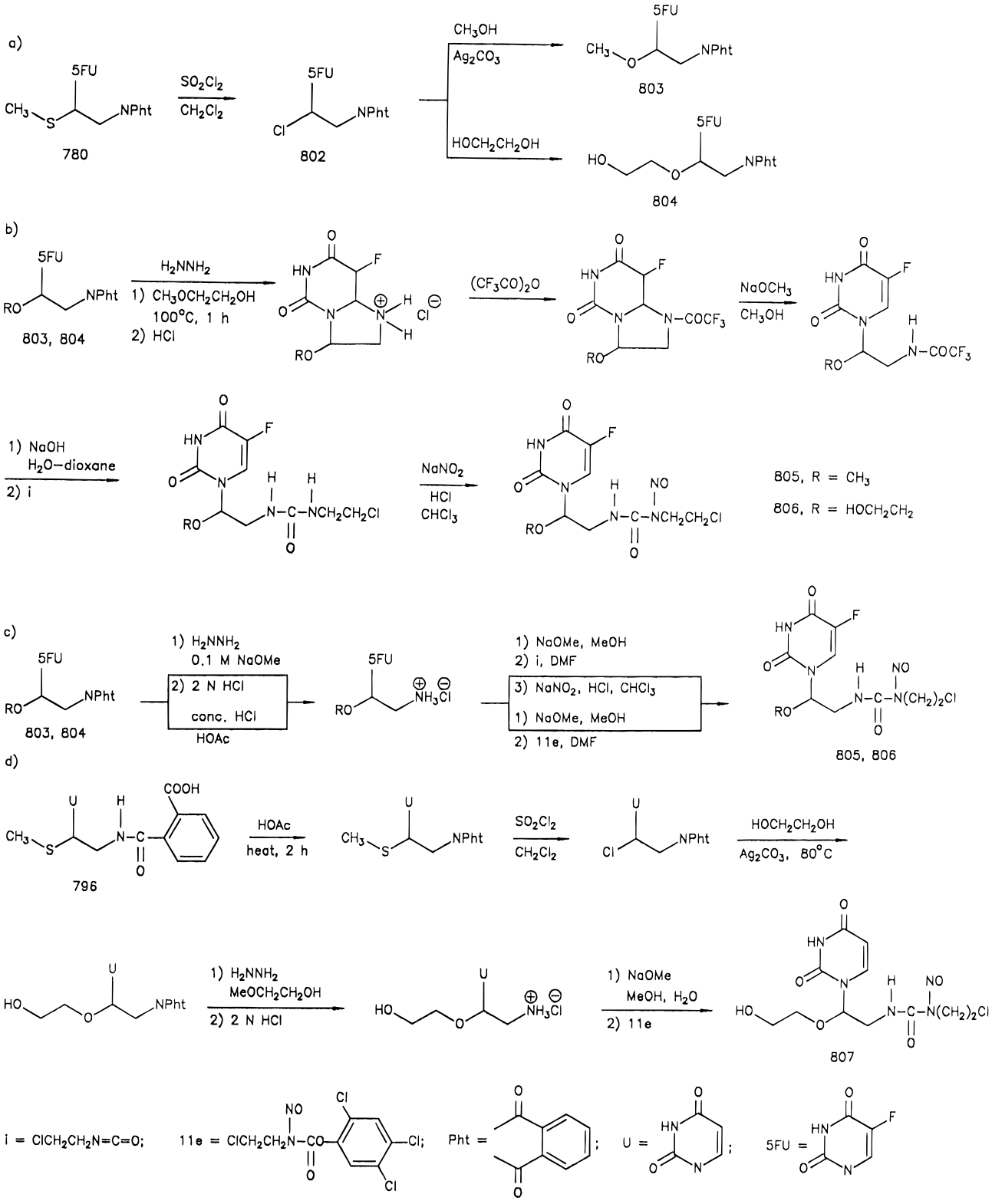
dimethoxyethanol produced a bicyclic amine (Scheme 80b). Conversion of this bicyclic ring system to a trifluoroacetamide derivative, followed by sodium methoxide treatment, resulted in the ring opening of the bicyclic amine. Alkaline hydrolysis of the amide, followed by urea formation and *N*-nitrosation yielded the desired alkoxy CENU analogs **805** and **806**. In later work[554] the intermediates **803** and **804** were hydrolyzed directly to the amine hydrochloride (Scheme 80c). The neutralization of the amine salts was followed by the reaction with the CENU transfer agent **11e** to yield the derivatives **805** and **806**. The (2-hydroxyethyl)oxy CENU N1-uracil analog **807** was prepared[553] from the amide **796** via a chloro intermediate (Scheme 80d).

Several variations for the synthesis of the alkoxy product are illustrated in Scheme 81. Compound **808**,[549] the homolog of **802**, was fused[551] either with ethanolamine hydrochloride or the (hydroxyethyl)-methylamine hydrochloride and the intermediates neutralized and reacted with **11e** to produce **809** and **810**. In these compounds the CENU group is attached at the α′-position of the original structure. Interestingly, the biochemical decomposition of **809** via the mechanism of Scheme 76a would produce the active drug HECNU (**329**) (discussed in section VII.B). The tolyl sulfide alcohols **811** and **812**[549] were the starting materials for a reaction sequence (Scheme 81b) which resulted[551] in the production of the α′-N1-O alcohol analogs **813** and **814** related to the amine **809**. Thus, protection of the hydroxyl groups as their *p*-nitrobenzoates, conversion to the reactive chloro intermediates, nucleophilic displacement of the chloro group with ethanolamine hydrochloride, alkaline hydrolysis of the benzoate ester, neutralization of the amine salts to the amines, and reactions with the CENU transfer agent **11e** yielded **813** and **814**.[551,554] A carboxylic acid CENU analog **815** was obtained[556] by the reaction of **808** with 2-mercaptoacetic acid followed by dephthaloylation, neutralization, and reaction of the amine with **11e** (Scheme 81c).

Another important structural variation involved the synthesis of N3-substituted bases. Various α-N3-S CENU derivatives **816**,[548,557] **817**,[548] and **818**[552] were synthesized by the general sequence illustrated in Scheme 82a,b. There was no problem with bicyclic amine formation in the N3-series. The iodinated intermediate for the synthesis of **818** was prepared[552] by the iodination with *N*-iodosuccinimide of the corresponding N3-uracil (U-N3) compound **784** (Scheme 82b), since the later compound was obtained by the direct nucleophilic substitution of the acetoxy-sulfide (Scheme 77). The α-5FU-3-S analog **816** exhibited[558] an anomalous ^{19}F NMR spectrum with two peaks. An equilibration of the *Z* and *E* isomers of the N-NO moiety at a rate slower than the NMR time scale was the explanation for this anomaly.[558]

Special procedures were required to obtain the α-N3-O CENU analogs **819**,[556] **820**,[552] and **821**.[552] Thus, for the 5-FU3-O analog **819**, the N1-position was blocked as a thioamide and the N3-position reacted with a methoxy chloride intermediate to produce, after hydrolysis, compound **822** (Scheme 82c).[556] By a similar process the U3 *p*-tolyl sulfide **823** was converted[552] to a methoxy phthalimide

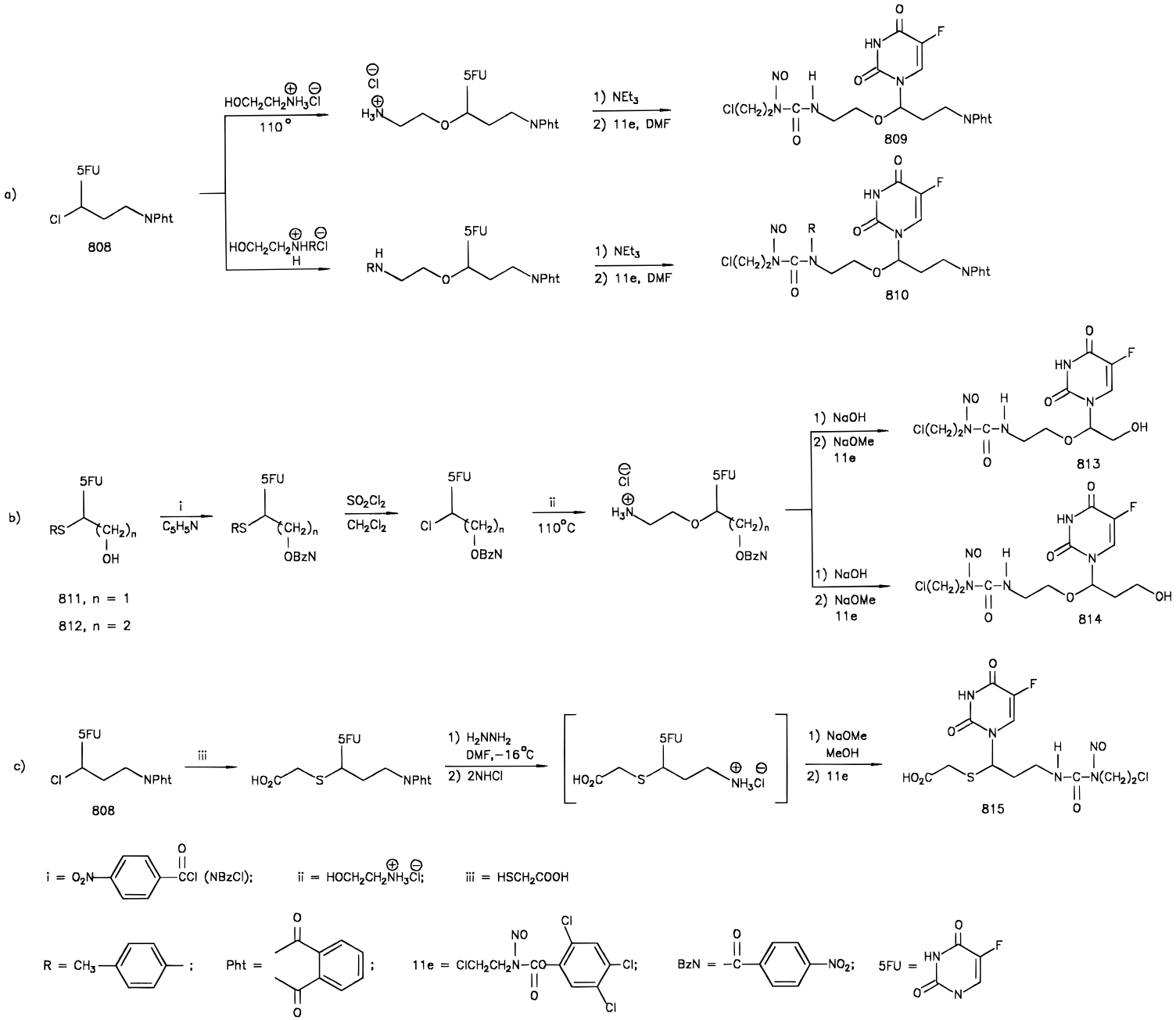
Scheme 80



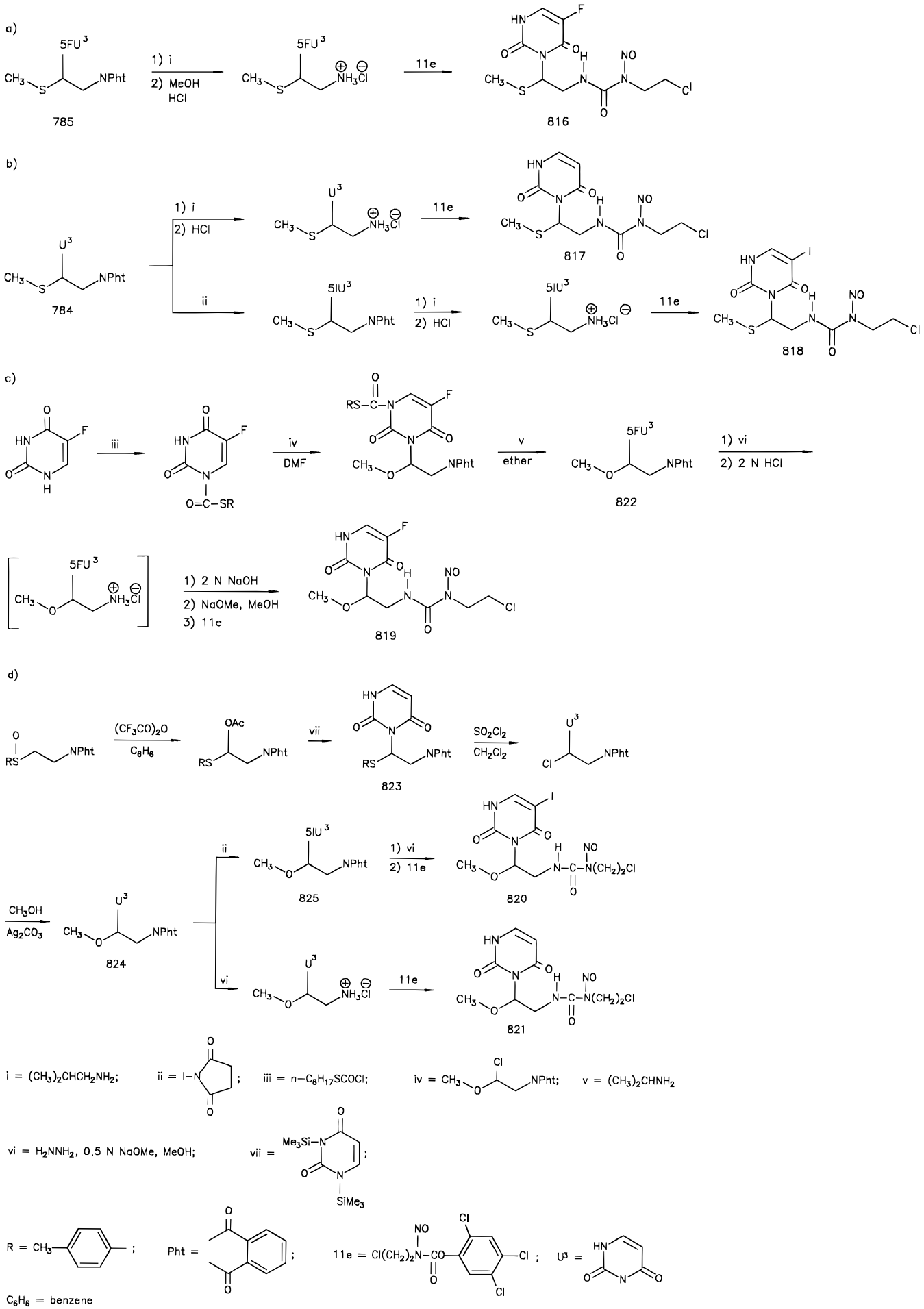
intermediate **824** (Scheme 82d). The 5-iodouracil (5IU) base **825** was obtained[552] by an iodination of **824**. These intermediates **822**, **824**, and **825** were then transformed into compounds **819**−**821** as illustrated in Scheme 82c,d.

A series of substitutions on the α′-position of the minor acetoxy sulfide **778** (Scheme 77) led to a variety of CENU−seconucleoside analogs (Scheme 83). The reaction of **778** with the bis(trimethylsilyl)-5-fluorouracil produced[557] the α′-5FU1-S analog **826**, the α′-5FU3-S analog **827** and a disulfide **828**. By reactions already discussed[557] the intermediate **826** was converted to the CENU-containing sulfide **829** and sulfoxide **830**. Similarly, the α′-5FU3-S **827** was transformed to the CENU-containing sulfide **831** and sulfone **832**. Oxidation of the disulfide **828** and further reactions yielded the bis-sulfone **833**.[557] The α′-U3 analog **834** was also synthesized from the α′-acetoxy sulfide **778** as shown in Scheme 83b. Further structural variations are represented by the α′-U5-S and α′-U6-S analogs **835** and **836** which were prepared[553] from 5-(hydroxymethyl)uracil and 6-(chlo-

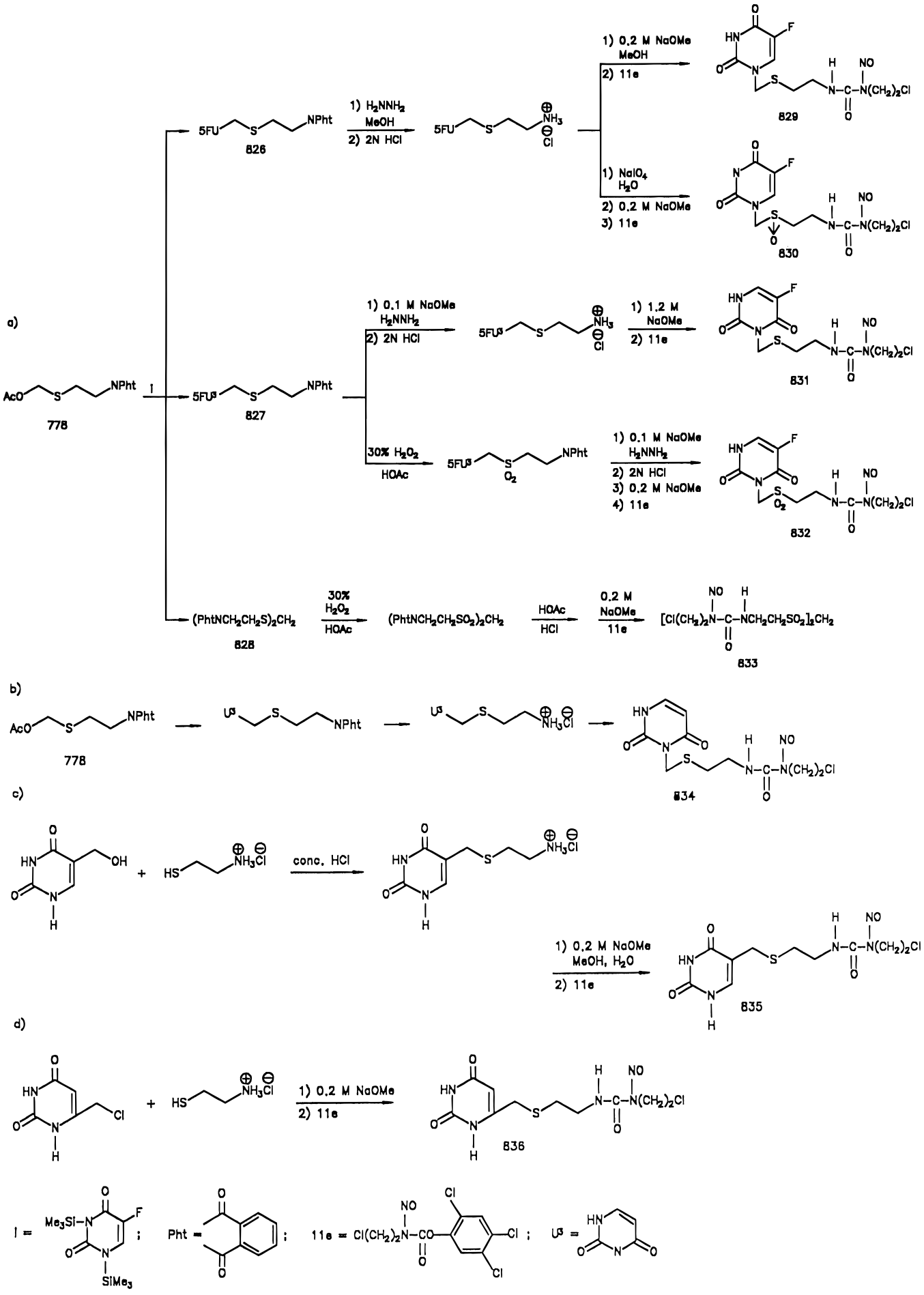
Scheme 81


a)
5FU
Cl
NPht
808
HOCH2CH2NH3Cl
110°
HOCH2CH2NHRCl
H
1) NEt3
2) 11e, DMF
809
810
b)
RS
(CH2)n
OH
OBzN
811, n = 1
812, n = 2
i
C5H5N
SO2Cl2
CH2Cl2
ii
110°C
1) NaOH
2) NaOMe
11e
813
814
c)
iii
1) H2NNH2
DMF, −16°C
2) 2NHCl
HO2C
S
NH3Cl
1) NaOMe
MeOH
2) 11e
815
i = O2N
CCl (NBzCl);
ii = HOCH2CH2NH3Cl;
iii = HSCH2COOH
R = CH3
Pht =
11e = ClCH2CH2N
CO
Cl;
BzN =
NO2;
5FU =

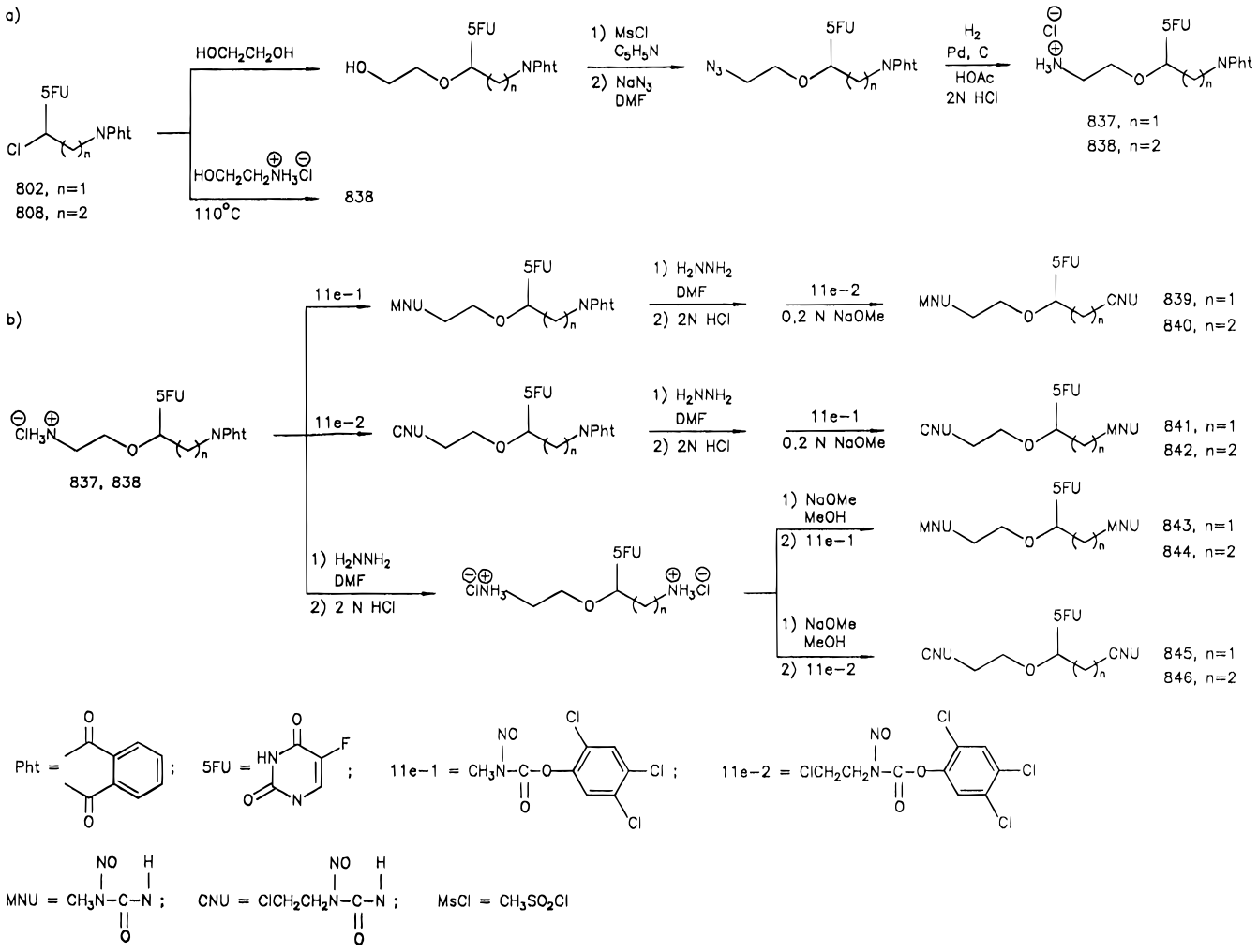
Scheme 82


a)
5FU3
1) i
2) MeOH
HCl
11e
785
816
b)
1) i
2) HCl
ii
11e
784
817
5IU3
818
c)
iii
iv
DMF
v
ether
1) vi
2) 2 N HCl
822
1) 2 N NaOH
2) NaOMe, MeOH
3) 11e
819
d)
(CF3CO)2O
C6H6
vii
SO2Cl2
CH2Cl2
823
CH3OH
Ag2CO3
824
825
820
821
i = (CH3)2CHCH2NH2;
ii =
iii = n-C8H17SCOCl;
iv =
v = (CH3)2CHNH2
vi = H2NNH2, 0.5 N NaOMe, MeOH;
vii =
R = CH3
Pht =
11e =
U3 =
C6H6 = benzene

Scheme 83


a)
b)
c)
d)
778
826
827
828
829
830
831
832
833
834
835
836
1) 0.2 M NaOMe MeOH 2) 11e
1) NaIO4 H2O 2) 0.2 M NaOMe 3) 11e
1) 0.1 M NaOMe H2NNH2 2) 2N HCl
1) 1.2 M NaOMe 2) 11e
30% H2O2 HOAc
1) 0.1 M NaOMe H2NNH2 2) 2N HCl 3) 0.2 M NaOMe 4) 11e
1) H2NNH2 MeOH 2) 2N HCl
0.2 M NaOMe 11e
conc. HCl
1) 0.2 M NaOMe MeOH, H2O 2) 11e
1) 0.2 M NaOMe 2) 11e
I = ; Pht = ; 11e = ; U =

Scheme 84



romethyl)uracil, respectively (Scheme 83c,d).

An interesting series of 5FU1-O bis-nitrosoureas were synthesized, as outlined in Scheme 84.[556] The two chloro 5FU−phthalimides **802** and **808** were transformed to the corresponding (2-aminoethyl)oxy compounds **837** and **838**, respectively. These intermediates were then employed to synthesize bis-nitroso isomers **839**−**842** by the sequential addition of the two *N*-nitroso transfer agents **11e-1** and **11e-2** (Scheme 84b).[556] Similarly, the symmetrical bis-nitroso analogs **843**−**846** were obtained from the intermediate bis-amine hydrochloride salts which were treated with the two transfer agents **11e-1** and **11e-2**.[556] These compounds contain the antimetabolite 5FU and two potential *N*-nitrosourea moieties MNU and CENU. The bis-nitrosourea analogs **839**−**842** are particularly interesting because the *N*-methyl-*N*-nitrosourea (MNU) moiety could inhibit the DNA repair enzyme, i.e. the *O*-alkylguanine alkyltransferase (GATase). This inhibition would allow the cross-linking of the DNA strands by the CENU moiety to occur[556] (section X.C). However, no anticancer activity studies were reported[556] for the series **839**−**846**.

Many of these CENU−seconucleoside compounds were screened for anticancer activity against several solid tumors, and the results are shown in Table 42. Among the compounds with the N1-substituted bases at the α-position, the parent compound **791** was shown[554] to have excellent activity against the solid colon 38 adenosarcoma (Table 42), a very low activity against the MAC 13, and moderate activity against MAC 15A. The drug 5FU also exhibited excellent activity against the colon 38 tumor line.[554] Substitutions at the C5-position with iodine (**792**), methyl (**793**), chlorine (**794**), and hydrogen (**795**) caused[552] variable activity changes against the MAC 13, and an increased activity against the MAC 15A cancer cell lines, i.e. **792** was found[552] to elicit a % ILS value of 325 while **792** and **794** were shown to have a greatly increased activity against the mammary carcinoma with five out of six survivors (Table 42).

Oxidation of the sulfide **791** to the corresponding sulfoxide **797** and sulfone **798** resulted[554] in almost complete loss of activity against the colon 38 tumor, and a smaller effect on the activity against the MAC 13 and MAC 15A tumors (Table 42). The sulfoxide **797** was much more water soluble than **791**, and it was hydrolyzed in acidic media to 5FU (**771**) to a much greater extent (Table 43); however, its biological activity against several of the solid tumors was reduced as compared to **791**.[554] This result is contrary to the hypothesis[542] that the ease of release of 5FU, which would occur by an enzymatic mediated hydrolysis in vivo, is an important factor in determining the overall anticancer activities of these CENU−seconucleosides. Furthermore, no significant difference in terms of anticancer activity against the Walker tumor model and the bone marrow toxicity existed[559] between the coadministration of BCNU and

Table 42. Anticancer Activity of CENU Analogs of Seconucleosides against the Mouse Adenosarcoma of the Colon (MAC 13, MAC 15A), Colon 38 Adenosarcoma, Mammary Carcinoma, and *N*-Methyl-*N*-nitrosourea (MNU) 1-Induced Lung Cancer

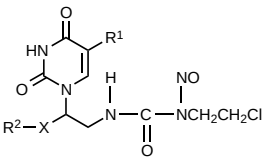
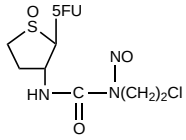
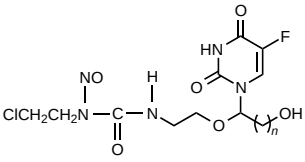
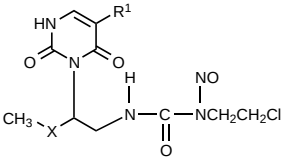
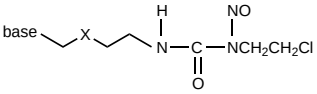
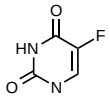
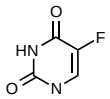
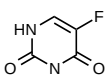
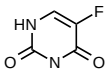
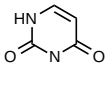
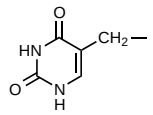
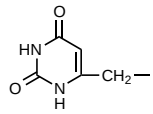
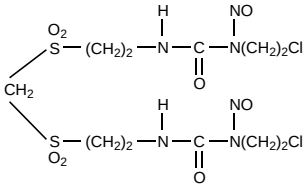
com-pound no.				MAC 13[a]		MAC 15A[b]		colon 38[c]		mammary carcinoma[d]			MNU-1 lung tumor[e]				
				OD,[f,g,h] mg/kg	TWI,[i] *T*/*C* %	OD,[g,h] mg/kg	ILS$_{max}$,[j] %	OD,[g,k] mg/kg	rel act[l]	OD,[g,m] mg/kg	ILS$_{max}$,[j] %	survivors,[n] %	OD,[g,o] mg/kg	TWI,[i] %	ILS$_{max}$,[j] %	*T*−*C*,[p] days	ref(s)
																	
	R^1	R^2	X														
791	F	CH_3	S	31	122	62	56	100	++++	60	89	12	60	88	−20	20	552
				50 (oral)	12												
792	I	CH_3	S	25	153	100	325			100		83					552
793	CH_3	CH_3	S	25	60	50	188			75	30	33					552
794	Cl	CH_3	S	25	20	50	263			100		83					552
795	H	CH_3	S	25	41	50	110			80	38	12					552
797	F	CH_3	SO	25	37	25	54	25	±	80	67	0					554
798	F	CH_3	SO_2	27	88	40	110	50	0								554
																	
801				50−200	toxic	50	10			100	21	0					554
805	F	CH_3	O	25	38	50	100	100	±	60	70	50	55	85	29	17	554
806	F	$HOCH_2CH_2$	O	50	10	50	55	50	+	80	58	12	60	62	5	9	554
807	H	$HOCH_2CH_2$	O	50	7	150	78	50	+	80	64	20					554
																	
	n																
813	1			50	129	50	8	50	+								554
814	2			50	90	100	36	50	±								554
																	
	R^1		X														
816	F		S	60	30	100	233	100	+	80	104	38	60	55	−19	12	552
				80	9								30	32	0	5	
817	H		S	50	24	100	144			100		75	60	61		12	552
818	I		S	25	94	100	222										552
819	F		O	50	11	100	>750	200	toxic	50		83	60	89	−3		552
820	H		O	25	46	50	>567										552
821	I		O	25	107	100	233										552
																	
	base		X														
829			S	100	25	150	111			75	58	25					557
830			SO	25	44	25	67										557
831			S	50	33	150	256	50	±	80	96	25	60	69	−3	12	557
832			SO_2	75	53	37	100										557
834			S	37	33	75	113			75	150	60					553

Table 42. (Continued)

compound no.		MAC 13[a] OD,[f,g,h] mg/kg	MAC 13[a] TWI,[i] *T/C* %	MAC 15A[b] OD,[g,h] mg/kg	MAC 15A[b] ILS_{max},[j] %	colon 38[c] OD,[g,k] mg/kg	colon 38[c] rel act[l]	mammary carcinoma[d] OD,[g,m] mg/kg	mammary carcinoma[d] ILS_{max},[j] %	mammary carcinoma[d] survivors,[n] %	MNU-1 lung tumor[e] OD,[g,o] mg/kg	MNU-1 lung tumor[e] TWI,[i] %	MNU-1 lung tumor[e] ILS_{max},[j] %	MNU-1 lung tumor[e] *T−C*,[p] days	ref(s)
835	 S	50	72	100	156	50	0	75	60						553
836	 S	37	109	150	200	50	0	100	130	29					553
															
833		50	20	50	210										557
771 (5FU)		120	65	120	25	70	++++	125	37	0	66	25	−4	4	
138b (MeCCNU)		20	8	20	54	64	+++								
131 (TCNU)		30	1	30	110	25	+++								
368 (CZT)		10	112	60	220	48	+								
847 (Ftorafur)								100	17	0	140	−2	−5	3	

[a] The MAC 13 tumors were implanted sc into female mice. [b] The MAC 15A tumors (1×10^6 cells) were implanted ip into male mice. [c] The colon 38 adenosarcoma tumors were implanted sc into BDFI hybrid mice. [d] The mammary carcinoma tumors (1×10^6 cells) were implanted in CBA/H mice. [e] The NMU-1 mouse lung tumor line was maintained by serial sc passage in BALB/C male mice. [f] All values are rounded. [g] Optimal dose = daily ip-administered dose resulting in a maximum increase in life span. [h] Drugs were administered on day 2 after tumor implantation. [i] Tumor weight inhibition. Tumor weight in treated over control mice (%*T*/*C*). Determined on day 21 after tumor implantation. [j] Increase in life span = $[(T - C)/C)] \times 100$. [k] Drugs were administered on days 2 and 9 after tumor implantation. [l] Percentage tumor growth inhibition 0 = ≤20%; ± = 20−50%; + = 50−80%; ++ = 80−90%; +++ = 90−100%, <50% of mice tumor free; ++++ = 90−100%, >50% of mice tumor free. [m] Drugs were administered on day 1 after tumor implantation. [n] Percentage of long-term survivors. [o] Drugs were administered once a week for three weeks starting on day 1 after tumor implantation. [p] Delay in tumor growth ($T - C$) calculated as the difference in number of days required for tumors to reach 2 g in treated (T) and control (C) mice.

Table 43. Solubility and Hydrolysis Rates of CENU−Seconucleosides

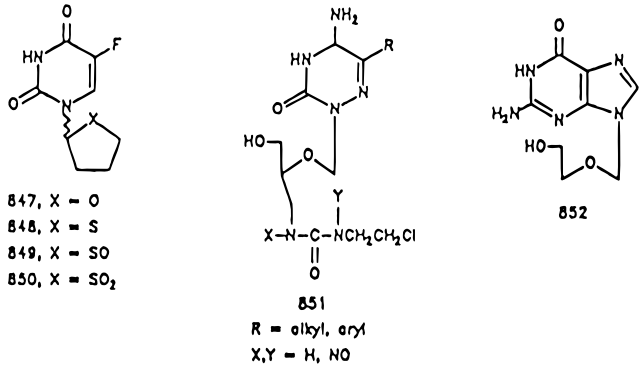
compound no.	solubility in water (mg/mL) at 20 °C	hydrolysis to 5FU at 100 °C in 2 M HCl,[a] %	ref
791	1	10	554
795	1		553
797	11	100	554
798	4	8/48	554
801	11	75	554
847 (Ftorafur)		100/0.2	554
805	1	85	554
806	13	45	554
813	8	75	554
814	25	100/4	554
819	4		552
829		0/48	557
831		0/48	557
833	0.4		557
834	3		553
835	0.3		553
836	0.7		553
131 (TCNU)	3		553

[a] A 24 hour period, except where indicated, e.g., 8/48 means 8% hydrolysis at 48 h.

5FU, and their chemical combination in the form of the drug **791**. Both methods increased the gastrointestinal toxicity to the same extent.

The cyclic sulfoxide **801** also had a greatly reduced activity as compared to the acyclic sulfide **791**.[554] These results contrast with those obtained with the sulfur analogs **848**−**850** of the 5FU prodrug Ftorafur (**847**)[560] (Chart 22). The substitution of sulfur in **847**

Chart 22. Structures for VII.F



would increase the electronegativity at the position next to the N1−C2′ bond and, possibly, increase the rate of release of 5FU from compounds **848**−**850**.[560] An in vivo screening with the P388 murine leukemia[560] revealed the following order of cytotoxicity: 5FU > **849** > **850** > **848** > **847**. An in vitro screen using the L1210 cell line resulted[560] in the following order of growth inhibition: 5FU > **850** > **849** > **847** > **848**. In both cases, the oxidized sulfur analogs **849** and **850** were more active than the compounds **848** and **847**. However, the more active compounds **849** and **850** were also the more water soluble of the four analogs and thus the solubility factor may be of greater importance.[560]

The substitution of oxygen for sulfur in **791** resulted in the compounds **805**−**807**, **813**, and **814** (Table 42). This substitution in **805**−**807** increased[554] the activity against the MAC 13 and the mammary

carcinoma and the MNU 1 induced lung tumor but it also abolished the activity against the colon 38 tumor. These oxygen isosteres exhibited[554] increased water solubility and increased acid catalyzed hydrolysis rates as compared to the sulfur analog **791** (Table 43). The repositioning of the CENU group to the α′-position of the molecule, and thereby generating compounds **813** and **814**, did not significantly change[554] the activity of these oxygen isosteres (Table 42). These compounds were of interest because they could be converted to the water soluble drug HECNU (**329**)[554] by the hydrolysis mechanism of Scheme 76. HECNU was considered a promising drug and was subjected to clinical trials (section VII.B).

Another structural variation was the attachment of the CENU side chain to the N3-position of the bases, as illustrated in compounds **816**−**821** (Table 42).[552] The most dramatic change occurred against the ascitic MAC 15A tumor line with compounds **819** and **820** eliciting activities of 750% ILS and 567% ILS, respectively.[552] The activity against the mammary carcinoma was increased to a lesser extent. In this case, the N3-O analog **819** had a higher water solubility than the N1-O analog **805** but the same degree of 5FU release under acidic conditions (Table 43).

A group of CENU−seconucleosides, compounds **829**−**832** and **834**−**836**, with heterobases attached at either the N1- or N3-positions to the α′-position of the side chain (Table 42), can undergo the enzymatic hydrolysis[561] resulting in the release of formaldehyde which is known to have cytotoxic properties (Scheme 76b). As can be seen from Table 42 these compounds[553,557] generally exhibited only moderate activities against any of the five cancer lines, with the exception of **831** against the MAC 15A ascitic tumor.

The bis-sulfone **833** is a bifunctional alkylating agent, similar to the polymethylene CENU drugs **85**−**89** (Table 4, section VII.A.2). Compound **833** elicited[557] higher activity against the ascitic tumor MAC 15A and moderate activity against the solid tumor MAC 13. A number of sulfone CENU analogs were shown to have good anticancer activities and some were subjected to clinical trials. Thus analog **288**, the bis-sulfone analog of CCNU, exhibited high activity against the L1210 leukemia (Table 17). The sulfoxide **125** and sulfone **126** metabolites of the bisCENU analog CNCC, as well as the CENU sulfoxamide **131** (TCNU) (Table 7) had high antileukemic activities.

A summary of the anticancer activity of several CENU−seconucleotides against the MAC 13 and MAC 15A colon tumors was published.[542] Generally, there was no structure−activity relationship between anticancer activity and either the water solubility or the acid-catalyzed release of 5-FU. In comparison to the parent sulfide analog **791**, the sulfoxide **797** had an increased activity against the MAC 13 tumor, and the sulfone **798** an increased activity against the MAC 15 tumor.[554] However, oxidation of the sulfur in **791** abolished the activity against the colon 38 adenosarcoma.[554] The oxygen analogs **805** and **806** exhibited greatly increased activity against the MAC 13 tumor in comparison with **791**.[552] The location of the CENU moiety is important since the 2′-position of **806** was much more advantageous than the 5′-position of **813**.[555]

The CENU−azapyrimidine seconucleosides of general structure **851** (Chart 22) were obtained[562] as mixtures of the N1- and N3-nitrosourea analogs. All of these compounds were reported[562] to be inactive in vitro against the growth of the HeLa and KB carcinoma cells. Several purine acyclonucleosides were synthesized and found[563] to have some in vitro activity against the P388 leukemia line.

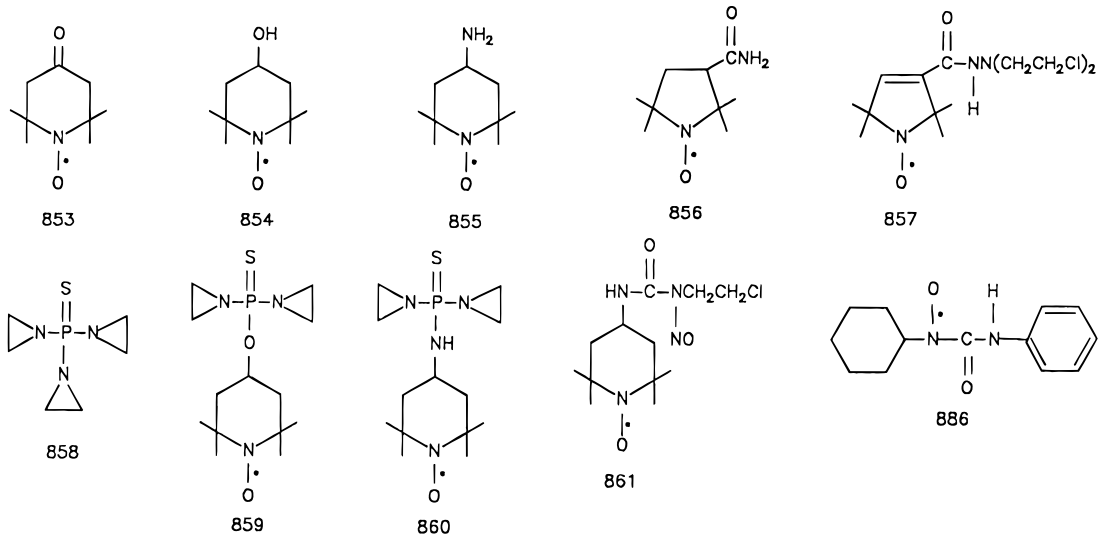
Throughout the series of papers on the CENU−seconucleotides the authors have drawn chemical structures to emphasize their formal relationships with the furanose ring of nucleosides. This formalism arose in the field of antiviral compounds, such as acyclovir (**852**, Chart 22) where it was found that the intact carbohydrate ring is not essential for the biological activity. However, from a purely chemical point of view, there is no reason to write these structures in such a manner and the seconucleosides in this review have been redrawn deliberately to deemphasize such a relationship.[319]

G. Nitroxyl (Aminoxyl) Analogs

Stable free radicals containing the nitroxyl (aminoxyl) group N·−O in sterically hindered cyclic amines, such as compounds (4-oxo-2,2,6,6-tetramethylpiperid-1-yl)oxyl **853** (Tempone), (4-hydroxy-2,2,6,6-tetramethylpiperid-1-yl)oxyl (**854**, Tempol), (4-amino-2,2,6,6-tetramethylpiperid-1-yl)oxyl (**855**, Tempamine), and (3-carboxamido-2,2,5,5-tetramethylpyrrolid-1-yl)-oxyl **856** (Chart 23), have been used to spin label the lipid and protein portions of biomembranes, such as RNA, DNA, and cellular proteins.[564−567] Electron paramagnetic resonance (EPR) spectroscopy has been extensively used to study the molecular environment of these covalently bound nitroxyl radicals. The attachment of nitroxyl compounds to anticancer agents presents the attractive possibility of monitoring by EPR spectroscopy the interaction of the drugs with various cell biopolymers, such as, DNA, RNA, proteins, lipids, and carbohydrates, as well as their metabolism and tissue distribution. It was shown that while the nitroxyl moiety imparts a beneficial influence on the anticancer properties of a drug,[48,568−570] the nitroxyl group by itself has no anticancer activity,[571,572] is relatively nontoxic,[572] is not carcinogenic or mutagenic,[573,574] exhibits no synergistic effect,[575] and has little effect on the cell growth and the cell cycle kinetics.[576] These facts can be explained[48,577] by assuming that the nitroxyl radical is a carrier group which facilitates the transport of the drug through the biological membranes to the cellular DNA. In support of this hypothesis it was found[578] that neutral or weakly acidic or basic nitroxyls rapidly permeate through cell membrane and that a plausible correlation of the anticancer activity of spin-labeled compounds with their lipophilic properties can be established.[579,580]

Previous research revealed[104,581] that BCNU (**33**), CCNU (**137b**), and MeCCNU (**138b**) rapidly enter the cerebrospinal fluid and, thus, can be used to control meningeal tumors. Further, the nitroxyl

Chart 23. Structures for Section VII.G



radicals are known[582] to cross the blood−brain barrier at the site of the diseased tissue. Thus, a combination of the (chloroethyl)nitrosourea and the nitroxyl moieties should lead to active anticancer drugs which could be followed pharmacokinetically by EPR[583] in animal models and diagnostically during therapy by NMR imaging (MRI).[582]

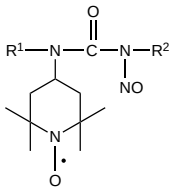
In early studies[584] spin-labeled derivatives of nitrogen mustards were used to label DNA. The hydrazine mustard spin label **857** was reacted[585] with DNA, and the bioenvironment of the nitroxyl in the DNA was analyzed by EPR spectroscopy. Such EPR analysis revealed[585] that **857** is a base-specific reagent, alkylating preferentially guanine, as well as indicating the difference between the spectra of immobilized double-stranded DNA and the spectra of nonimmobilized single-stranded DNA.

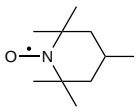
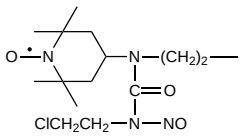
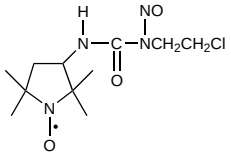
The anticancer drug thio-TEPA **858** was transformed[571,586−588] to spin-label (SL) analogs **859** and **860**. The oxygen analog SL-O-TT (**859**) was moderately active[572] against the P388 leukemia (% ILS = 142) and had a higher therapeutic index (5.15) than that of the parent compound **858** (2.75). The nitrogen analog SL-NH-TT (**860**) had about the same therapeutic index (2.73), similar cytotoxicity against P388, and a decreased toxicity when compared with **858**.[572] Thus the covalent bonding of a nitroxyl group to anticancer agents could result in an increase in the therapeutic index, i.e. a broader range of safe doses.[572] Analysis of EPR spectra of L1210 cells incubated with **859** has shown[572] a diminution of the high-field line of the triplet, attributable to a hindered rotation of the nitroxyl group which probably was covalently bound by an alkylation reaction. The nitroxyl moiety was rapidly reduced[572,589−591] by cells in culture to the corresponding hydroxylamine derivative which were less active against the P388 leukemia.[577] Such reduced compounds can be reoxidized for EPR studies to 90−95% of the original nitroxyl content by a 0.01−0.1 M solution of potassium ferricyanide.[572]

These earlier studies formed the basis for the extension of spin labeling to the CENU class of anticancer drugs.[48,568−570,592−595] In some cases, the spin-labeled CENU analogs were used in attempts to alkylate DNA.[592] Under the conditions of the DNA labeling, the nitroxyl group of **861** was lost, resulting in a DNA with no EPR signal.[592] This result was in sharp contrast to the nitrogen mustard spin-labeled compounds which were found[585] to alkylate the double-stranded DNA. Compound **861** was used also to spin label erythrocyte ghosts.[593,594] Studies, using EPR spectroscopy and preincubation experiments with *N*-ethylmaleimide (NEM), revealed[593,594] that the label was bound to two classes of mercapto (SH) groups of membrane proteins, i.e. with, strong (s) and weak (w) mobilities of the nitroxyl moiety. Both binding sites, s and w, were inaccessible to the bulky paramagnetic anion $Fe(CN)_6^{3-}$ from the aqueous medium, resulting in no line broadening of the s and w spectral components, but were accessible to ascorbic acid, a smaller reducing agent, resulting in a quenching of the EPR spectrum up to 95% during 13 min at 22 °C.[593,594] The reaction of **861** with amino groups could account for the small portion of binding not inhibited by NEM.

The replacement of the cyclohexyl moiety in CCNU (**137b**) with nitroxyl radicals led[48,568,569,595] to the development of the spin-labeled compounds **861**−**867** (Table 44). The synthesis of these compounds is outlined in Scheme 85. Thus, in the first sequence[595] the 4-aminopiperidine analog **868** was prepared from the corresponding ketone via the oxime, followed by the reaction of **868** with the appropriate isocyanates generated the ureas. Oxidation of the ureas to the nitroxyls was accomplished[48,569,570,595] by means of a 30% aqueous hydrogen peroxide solution in the presence of catalytic amounts of sodium tungstate (Na_2WO_4). The final nitrosation step, resulting in compound **861**, was accomplished by the use of either sodium nitrite and aqueous acetic acid[595] or a mixture of dinitrogen tetraoxide and sodium acetate in methylene chloride at −35 °C.[48,569,570] Nitrosation of the intermediate ureas before oxidation yielded a mixture of mono- (**869**−**871**) or dinitrosated (**872**) ureas.[595] The second synthetic sequence (Scheme 85b) involved[48,569,570] the reductive amination of **853** to the substituted amines which were converted to the ureas with 2-chloroethyl isocyanate. Nitrosation of the ureas with dinitrogen tetraoxide led to the spin-labeled CENU analogs **861**−**865**. Analogously, the *N*-nitrosourea analog **867** was prepared from the pyrrolidinyl-1-oxyl amine via the urea (Scheme 85c).

Table 44. Anticancer Activities of Spin-Labeled Nitrosoureas against Ip-Implanted L1210 Leukemia[a] and P388 Leukemia[b]



compound no.	R^1	R^2	OD,[c] mg/kg	ILS_{max},[d] %	survivors,[e] %	LD_{50},[f] mg/kg	ther ratio[g]	log P[h] EPR	log P[h] UV	ref(s)
861	H	$ClCH_2CH_2$	60[a]	713	100	123	40	1.60	1.58	48,569
			36−53[a]	19		99				595
				400						568
			20[b]	542	100					48,569
862	CH_3	$ClCH_2CH_2$	126[a]	117	0	353		1.72	1.70	48,569
			90[b]	329	17					
863		$ClCH_2CH_2$	120[a]	−3	0	337		2.30	2.21	48,569
			75[b]	31	0					
864		$ClCH_2CH_2$	120[a]	2	0			1.63	1.56	48,569
			60[b]	21	0					
865		$ClCH_2CH_2$	120[a]	84	0			2.35	2.32	48,569
			60[b]	53	0					
866	H	CH_3				20				595
867			60[a]	620	50		25	1.64	1.67	48,569
			25−40[a]	300						568
			35[b]	514	83					
137b (CCNU)			25[a]	646	67	56	5.7		2.55	48
138b (MeCCNU)									3.25	48

[a] Murine lymphoid leukemia. [b] Murine lymphocytic leukemia. [c] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. Drugs were administered on days 1−9 after tumor implantation. [d] Increase in life span = $[(T - C)/C] \times 100$. [e] Percentage of animals surviving on day 60 after tumor implantation. [f] Lethal dose = ip-administered dose resulting in the death of 50% of the untreated animals. [g] Therapeutic ratio = Optimal dose/ILS_{30}; ILS_{30} = daily dose eliciting a 30% ILS. [h] Partition coefficient: P = [compound in 1-octanol]/[compound in water].

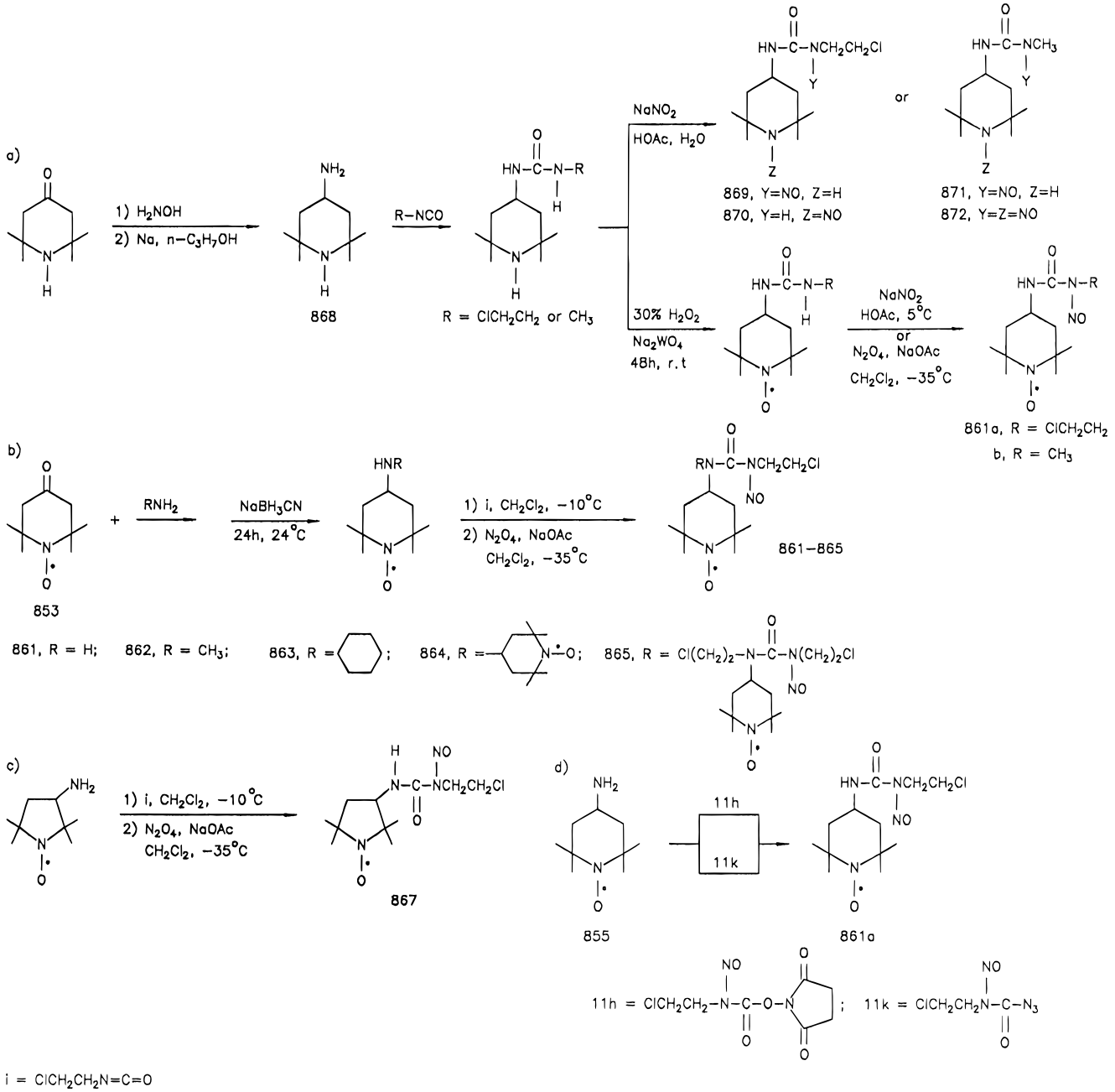
The position of the nitroso group in the final nitrosoureas was confirmed[48,568] by the reaction of the amine **855** with the CENU transfer agents **11h** and **11k**, as shown in Scheme 85d.

The six- and five-membered ring analogs **861** and **867** had equal or greater cytotoxicities[48,569] against the L1210 leukemia than the clinical drug CCNU (**137b**) (Table 44), were less toxic as judged by the LD_{50} data, and had a significantly greater safety factor, as evidenced by their higher therapeutic indices. The N3,N3-disubstituted analogs **862**−**865** were synthesized and shown[48,569] to be either inactive or only slightly active against the L1210 leukemia (Table 44). In contrast, the non-spin-labeled N3,N3-disubstituted analogs of CCNU **190**-**195** (Table 13) have excellent anticancer activity[201,202] against the L1210, and an enzymatic demethylation was proposed[132] for activation of the N3-methyl analogs to a cytotoxic form. In all these cases, the N3-nitrogen lacks a tautomeric proton and, thus, would not decompose biologically to an isocyanate and a diazohydroxide by the usual mechanism, as shown in Scheme 14. The highly active N3,N3-disubstituted analogs **191**−**195** contain a β-hydroxyl group which allows for a concerted cyclic decomposition to a cyclic urethane and a diazohydroxide (Scheme 31). The lack of β-hydroxy groups in compounds **862**−**865** could explain their low in vivo activities.[319] The bisnitroxyl−bisCENU analog **865** was synthesized because of the reported[59,134,135] high activity of certain bisCENU compounds **85**−**89** (Table 4) against the Walker carcinoma in rats, but the latter analogs were monosubstituted on the N3-position.

A comparison[48] of the partition coefficients, log P values (Table 44), among compounds **861** and **867**, CCNU (**137b**), and MeCCNU (**138b**) showed poor correlation with their cytotoxicity against L1210. However, against the P388 lymphocytic leukemia there was a definite correlation[48] of increased cytotoxicity with decreasing hydrophobicity, i.e. **861** > **867** > CCNU > MeCCNU.

In order to investigate further the balancing of hydrophilic and hydrophobic properties, a series of nitroxyl-labeled CENU−carbohydrate analogs **873**-

Scheme 85



876 were synthesized[596,597] as shown in Scheme 86. The *N*-nitrosourea transfer agents **877** and **878** were prepared by reactions of *N*-hydroxysuccinimide with corresponding isocyanates, followed by nitrosation of the resulting carbamates. Reactions of **877** and **878** with either D-glucosamine or its tetraacetate produced the spin-labeled carbohydrate analogs **873**–**876**. The unblocked spin-labeled carbohydrates derivatives **873** and **875** exhibited[596] excellent in vivo activity against the L1210 (Table 45), comparable with chlorozotocin (CZT, **368**) but somewhat lower activity than that of MCNU (**531**). The latter two compounds possess a 2-chloroethyl group which can cross-link the DNA strands (section X.B). However, compounds **873** and **875** were much more active[596] than streptozotocin (SZT, **367**) which contains a non-cross-linking methyl group instead of a nitroxyl moiety on the N1-position. On the basis of these facts, a conclusion[319] can be drawn that the greatly increased membrane transport of the nitroxyl derivatives **873** and **875** across the cell membranes allows for a greater amount of the initial DNA alkylation step which compensates for the lack of the cross-linking step available to CZT and MCNU. The greatly reduced activities of the tetraacetates **874** and **876** can be explained on the basis of their greater hydrophobicities which do not allow an efficient transmembrane transport.

An analysis of the partition coefficients, log *P* values in Table 45, reveals that hydrophobic nitrosoureas were increasingly cytotoxic with decreasing hydrophobicity i.e. **873**, **875** ≫ **874**, **876**, and the hydrophilic nitrosoureas were increasingly cytotoxic with decreasing hydrophilicity i.e. MCNU > CZT > SZT. These results support the hypothesis[48,91,95,577] that the most active anticancer drugs of this type would have a balance of hydrophilic and hydrophobic properties.

Scheme 86

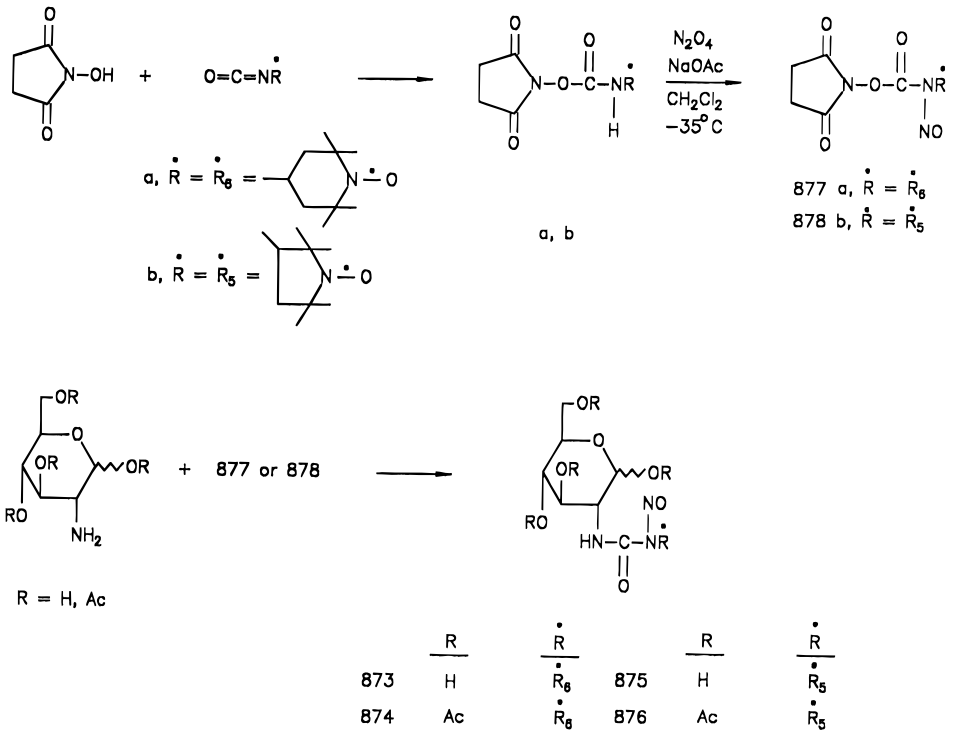
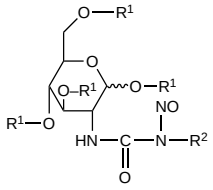
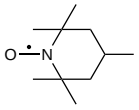
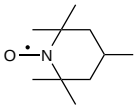
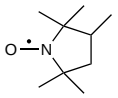
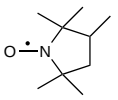


Table 45. Anticancer Activities of Spin-Labeled Carbohydrate Nitrosoureas against Ip-Implanted L1210 Leukemia[a] and P388 Leukemia[b,596]

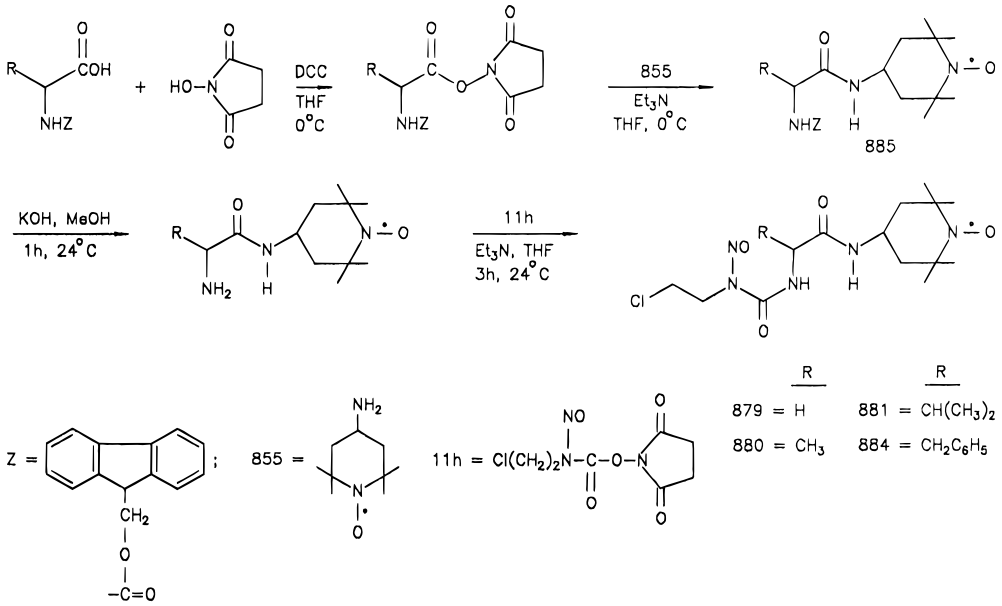


compound no.	R^1	R^2	OD,[c] mg/kg	ILS_{max},[d] %	survivors,[e] %	log P[f] EPR	log P[f] UV
873	H		50[a]	557	100	1.89	1.87
			20[b]	437	100		
874	Ac		20[a]	180	0	2.28	2.27
			20[b]	174	0		
875	H		50[a]	428	83	1.82	1.81
			20[b]	437	100		
876	Ac		20[a]	171	0	2.56	2.25
			20[b]	156	0		
367 (SZT)	H	CH_3	100[a]	55	0		−1.52
			8[b]	178	0		
368 (CZT)	H	$ClCH_2CH_2$	30[a]	517	100		−0.85
			8[b]	194	0		
531 (MCNU)			25[a]	713	100		−0.70
			20[b]	603	100		

[a] Murine lymphoid leukemia. [b] Murine lymphocytic leukemia. [c] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. Drugs were administered on days 1−9 after tumor implantation. [d] Increase in life span = [(*T* − *C*)/*C*] × 100. [e] Percentage of animals surviving on day 60 after tumor implantation. [f] Partition coefficient: *P* = [compound in 1-octanol]/[compound in water].

In the last decade a number of amino acid and peptide derivatives containing the CENU moiety have been synthesized and evaluated for in vivo anticancer activity (section VII.D). The rationale for this approach was that the L-amino acids are actively transported into mammalian tissue[458] and that some

Scheme 87



peptides accumulate in cancerous cells and, hence, may serve as carriers of the CENU moiety.[458,598] In particular, L-amino acid congeners of BCNU with general structure **680** (section VII.D), containing the (2-chloroethyl)amino group at the C-terminus and the CENU group at the N-terminus, have been shown[45,485,488] to have high in vivo activities against the P388 lymphocytic leukemia and low activities against the L1210 lymphoid leukemia.

On the basis of previous work,[48,596] the substitution of a less hydrophobic nitroxyl for the 2-chloroethyl group of **680** should lead to more active and less toxic anticancer agents. Accordingly, a series of nitroxyl−CENU−amino acid analogs **879**−**884** were synthesized[599−601] as outlined in Scheme 87. The Fmoc-protected L-amino acids were converted to their active succinimide esters[602] whose subsequent reaction with the spin-labeled amine **855** produced the nitroxyl amides **885**. Removal of the Fmoc-blocking group by alkaline hydrolysis yielded the free amines which were reacted with the CENU transfer agent **11h** to yield the spin-labeled amino acid analogs **879**, **880**, **881**, and **884**.[599] Alternatively, the compounds **879**, **880**, and **882**−**884** were independently synthesized by reactions of the corresponding CENU amino acids with **855**.[600,601]

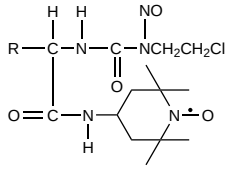
The chosen amino acids had a wide range of hydrophobicities,[603,604] ranging from the low hydrophobic glycine to the highly hydrophobic L-phenylalanine. The cytotoxicities of compounds **879**−**884** were moderate to high against the P388 and high against the L1210 murine leukemia and of the same order of magnitude as CCNU (**137b**) (Table 46).[599] The reported activity of the L-phenylalanyl analog **884** differed widely in two reports.[599,600] There was an imperfect correlation of increasing cytotoxicity with decreasing hydrophobicity, i.e. **880**, **881** > **884** (Table 46).[599] However, the glycine analog **879**, which had the lowest hydrophobicity, was somewhat less cytotoxic than the analogs **880**, **881**, and **884**. By comparison, the non-spin-labeled glycyl analog (**681**) and the L-phenylalanyl analog (**690**) had much lower activities against the L1210 leukemia in vivo, with 56% and 48% ILS values at day 30, respectively.[488]

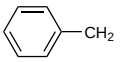
It appears that the presence of a spin label in these nitrosourea drugs increases their activities against the L1210 murine leukemia. Generally, the more active nitroxyl−CENU−amino acid analogs **879**−**884** had higher alkylating and lower carbamoylating activities,[600] in agreement with the predominant role of DNA alkylation for cytotoxicity.[97] Compounds **879**, **880**, **882**, and **883** exhibited[605] a high inhibition of sc-implanted melanoma B16 growth, i.e. tumor weight inhibition (TWI) = 62−100%. In this regard, the spin-labeled Tempone (**853**) was shown[606] to be selectively taken up by hamster and mouse melanotic melanomas, but not by the liver, kidney and lung. Possible explanations offered[606] for these results included enzyme defects in the melanotic melanoma cells, different metabolism in the normal and cancerous tissues, and/or retention of **853** by melanin.

Other aspects of nitroxyl-labeled nitrosoureas have been investigated. The tolerance of mice and rats to the injection of normally lethal doses of various anticancer agents was increased[607] by injection of nitroxyl radicals **854** and **856**. Thus, the injection of **854** or **856** with thio-TEPA, 6-mercaptopurine, cyclophosphamide, sarcolysine, daunorubicin, vincristine, nitrosomethylurea, and Ftorafur resulted[607] in a decreased toxicity and greatly increased survivals of the animals. Nitroxyl radicals normalize[607] the level of the P450 cytochrome oxidase enzyme that is decreased by the anticancer agents. This enzyme has the important function of detoxifying exogenous compounds. The elevation of the tolerance to the toxicity of cytostatic agents is probably associated with the activation of anabolic processes.[607] On this basis, the nitroxyl radical can be considered[607] a biological response modifier.

The biodistribution of **861** in the liver, spleen, blood, brain, and kidneys of C57BL/6 mice after the ip administration of a 40 mg/kg dose was studied[568] by EPR spectroscopy. A sharp increase in the concentration of **861** in the spleen occurred within 30 min followed by a return to the initial value by

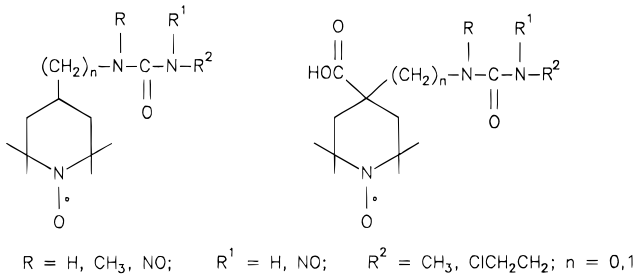
Table 46. Anticancer Activities of Spin-Labeled Amino Acid Nitrosoureas against Ip-Implanted L1210 Leukemia[a] and P388 Leukemia[b]



compound no.	R	dose,[c] mg/kg	ILS_{max},[e] %	survivors,[f] %	log *P*[g]	alk act,[h] %	carbamoyl act,[i] %	$t_{1/2}$,[j] min	ref(s)
879	H	12[a,c]	496	67	1.23				599
		12[b,c]	247	17					599
		67[a,d]	525	83		0.831	37.8	29	600,601
880	CH_3	12[a,c]	663	100	1.28				599
		12[b,c]	447	100					599
		120[a,d]	614	100		0.620	24.5	28	600
881	$(CH_3)_2CH$	12[a,c]	663	100	2.09				599
		12[b,c]	456	83					599
882	$CH_3SCH_2CH_2$	33[a,d]	472	50		0.850	32.4	25	600,601
883	$(CH_3)_2CHCH_2$	67[a,d]	289	50		0.670	40.0	33	600,601
884		15[a,c]	581	83	2.30				599
		12[b,c]	170	0					599
		67[a,d]	153	0		0.118	46.3	40	600
137b		25[a]	646	83					

[a] Murine lymphoid leukemia. [b] Murine lymphocytic leukemia. [c] Drugs were administered ip on days 1−9 after tumor implantation. [d] Drugs were administered ip on day 1 after tumor implantation. [e] Increase in life span = $[(T - C)/C] \times 100$. [f] Percentage of animals surviving on day 60 after tumor implantation. [g] Partition coefficient: P = [compound in 1-octanol]/[compound in water], as measured by a UV method.[83] [h] Alkylating activity determined as the absorbance at 560 nm according to a literature procedure.[97] [i] Carbamoylating activity determined by an EPR procedure.[613,614] [j] Half-life determined by a literature procedure.[97]

Chart 24. Structures for Section VII.G



R = H, CH_3, NO; R^1 = H, NO; R^2 = CH_3, $ClCH_2CH_2$; n = 0,1

60 min. A smaller increase of the **861** concentration occurred in the brain tissue while there was no increase in the kidney tissue and a decrease in blood and liver.[568]

Ultraviolet irradiation of BCNU (**33**) and CCNU (**137b**) in benzene[608,609] caused the formation of free radical intermediates, and a stable nitroxyl radical **886** (Chart 23) from CCNU was discovered[609] by EPR spectroscopy. The formation of the stable nitroxyl **853** from the corresponding piperidine under UV radiation increased considerably in the presence of CCNU.[609] Therefore, a suggestion[609] was made that the CENU drugs, such as CCNU (**137b**), can form stable nitroxyl radicals in situ which can act as photosensitizers and cause the production of more free radicals. As a result, various toxic oxygen species could be formed in vivo and these could contribute to the toxic side effects of CCNU.[609]

The tissue distribution of poly-(butyl cyanoacrylate) nanoparticles, carrying the spin-labeled nitrosourea **861**, in normal and cancerous melanoma B16 tissue in mice was reported.[610] The incorporation of this polymeric material caused an altered tissue distribution of **861**, with a greater accumulation in tumor tissue and a reduced concentration in the liver, brain, and blood.[610]

Some nitroxyl derivatives related to compounds **861** and **867** were reported in patents (Chart 24).[611,612]

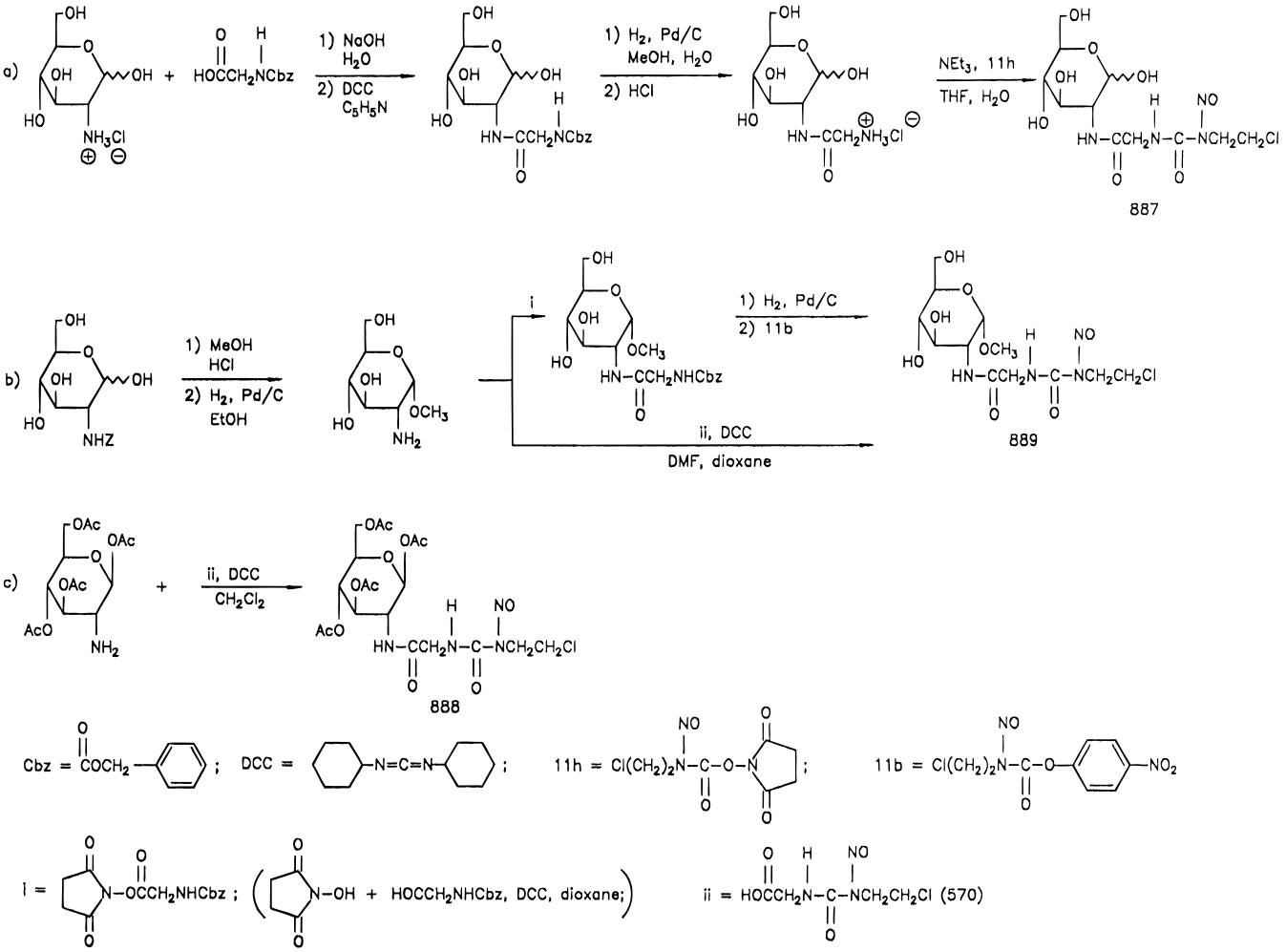
H. Carbohydrate–Amino Acid Conjugates

The conjugation of carbohydrates and CENU-containing amino acids was undertaken to adjust the relative polarity of the molecule and to use the carbohydrate as a hydrophilic carrier for the CENU moiety. The side-chain CENU−amino acid amides have excellent antileukemic activities (section VII.D, Table 33).

A series of unblocked carbohydrate−amino acid conjugates, their tetraacetates, and methylglycosides (**887**−**902**) were synthesized[465,615−617] and screened[465,616,617] against the ip-implanted L1210 leukemia (Table 47). Representative synthetic sequences are illustrated in Scheme 88a−c. The N-protected amino acids were condensed either with the D-glucosamine or its methyl α-glycoside followed by attachment of the CENU moiety by means of the CENU transfer agents **11h** or **11b** (Scheme 88a,b). Alternatively, the entire CENU−amino acid side chain moiety was condensed with the carbohydrate in one step, as shown in Scheme 88b,c. The unblocked D-glucosamine analogs **887** and **892** were not characterized fully in the patents[615,617] and their unequivocal syntheses were reported later.[465]

In general, the anticancer activities of these conjugates against the L1210 leukemia was high, with the exception of the tetraacetates **888** and **893**.[465] The difference in the reported[465,616] activities for compounds **887**, **889**, and **894** could be attributed to the different protocols which were employed. Some structure−activity relationships can be deduced[465,616] for the series **887**−**897**. Compounds with straight-chain substituents had higher anticancer activity than those with branched chains, e.g. **889** compared to **894**. The L-amino acid substituent induced a

Scheme 88



higher activity than the corresponding D isomer moiety, e.g. **895** compared to **894**. Longer side-chain substituents result in analogs with longer half-lives, e.g. **891** and **902** compared to **889** and **900**. In this series the conjugates **890** and **891** with longer half-lives and **895** with a higher therapeutic index exhibited high anticancer activity.[616] This relationship is opposite to that normally found in the CENU class of drugs in which a shorter half-life is correlated with higher alkylating activity and higher in vivo cytotoxicity.[97] The high activity of compounds **898** and **899** indicates that the attachment of the CENU−amino acid to the 2-position of the carbohydrate is not essential, and the high activity of compounds **900**−**902** related to the drug DONU **361**, demonstrates that other water-soluble ring structures can be substituted for the carbohydrate structure.[616,617]

A comparison of the activity of the unlinked CENU−glycineamide (**598**) with the conjugates **887**−**902** (Table 47) reveals that the conjugates possess little advantage in terms of cytotoxicity, therapeutic index, and half-life.

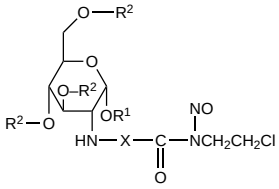
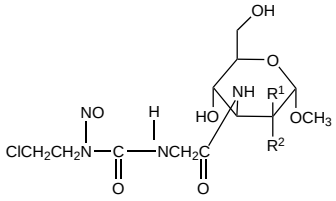
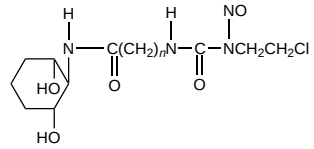
Another type of carbohydrate−amino acid conjugates is the Amadori compounds of general structure **903** (Scheme 89), which arise from the reaction of D-glucose and amino acids, both in vitro and in vivo. These products have been found[618,619] in stored and heat-processed foodstuffs containing ingredients of reducing sugars and amino acids (the Maillard Browning reaction). Such browning products are found[620] also in the biological environment.

Whereas the chemistry of biological systems involving Maillard transformations is extremely complex and results in a wide variety of products, the reactions involving amino acids can be delineated with well-defined monomeric products.[619] The initial step in the Maillard reaction between carbohydrates and amino acids probably results in the formation of Schiff base intermediates that cyclize to *N*-glucosyl amino acids in the case of D-glucose (Scheme 89). The intermediates then can undergo the Amadori rearrangement in acidic media to give *N*-(1-deoxy-D-fructos-1-yl)-L-amino acids (**903**). The analogous Heyns rearrangement of fructose derivatives results in the formation of *N*-(1-deoxy-D-glucos-1-yl)-L-amino acids. These compounds readily react with nitrites found in such sources as cured meats, tobacco, saliva, and stomach to give the *N*-nitroso derivatives (Scheme 89).[621,622]

It is well documented[623] that the biological action of *N*-nitrosamines varies considerably with structure, ranging from nonmutagenic and/or noncarcinogenic to highly mutagenic and/or carcinogenic. A question arises whether the ubiquitous Maillard−Amadori−Heyns products are involved in either cancer promotion or inhibition, or both.

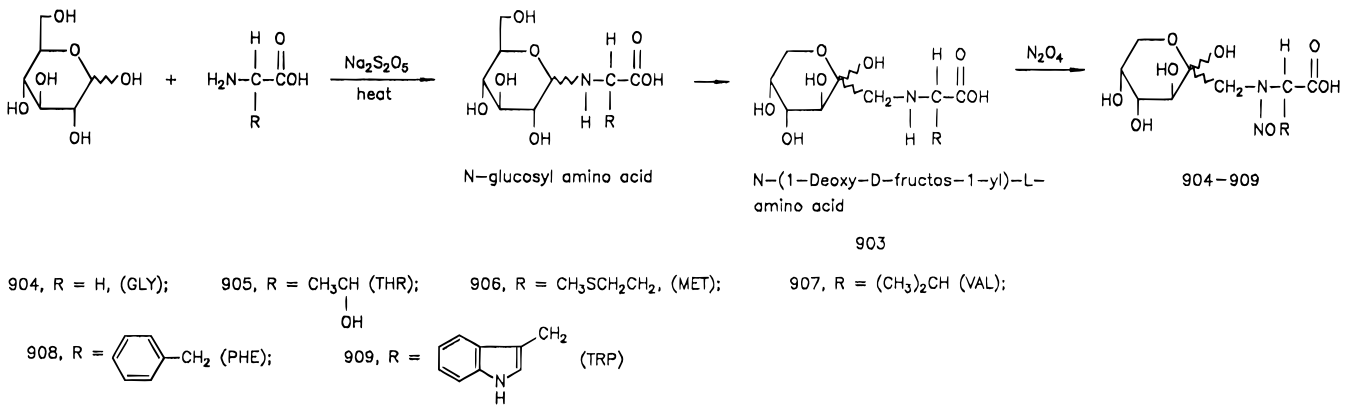
The *N*-nitrosated Amadori compounds **904**−**909** were synthesized[624,625] by the condensation reaction of D-glucose and the L-amino acids Gly, Thr, Met, Val, Phe, Trp, according to the reaction sequence of Scheme 89. This grouping indicated three nonmutagenic compounds (**904**, **906**, and **908**) one border-

Table 47. Anticancer Activity of Carbohydrate- and Dihydroxycyclohexyl-Amino Acid CENU Conjugates against Ip-Implanted L1210 Leukemia[a] and P388 Leukemia[b]

compound no.	X	R^1	R^2	OD,[c] mg/kg	ILS_{max},[f,g] %	survivors,[h] %	ther ratio[i]	$t_{1/2}$[j] min	log *P*[k]	ref(s)
										
887	$COCH_2NH$	α,β-OH	H	32[a,d]	689	0				616,617
				5[b,e]	93	0			−1.10	465
888	$COCH_2NH$	β-OAc	Ac	20[a,e]	110	0			0.61	465
889	$COCH_2NH$	α-OCH_3	H	32[a,d]	457	60	34	39	−1.04	616,617
				20[a,e]	137	0			−1.04	465
890	$CO(CH_2)_2NH$	α-OCH_3	H	48[a,d]	733	100	19	59		616,617
891	$CO(CH_2)_3NH$	α-OCH_3	H	64[a,d]	733	100	18	79		616,617
892	$COCH(CH_3)NH$ (S)	α,β-OH	H	10[a,e]	477	83			−1.02	465
893	$COCH(CH_3)NH$ (S)	β-OAc	Ac	10[a,e]	106	0			0.71	465
894	$COCH(CH_3)NH$ (S)	α-OCH_3	H	32[a,d]	379	33	19	35		616,617
				10[a,e]	152	0			−0.76	465
895	$COCH(CH_3)NH$ (R)	α-OCH_3	H	32[a,d]	568	80	63	44		616,617
896	$COCH(CH_2OH)NH$ (S)	α-OCH_3	H	24[a,d]	157	0	10	20		616,617
897	$COCH_2NHCOCH_2NH$	α-OCH_3	H	64[a,d]	340	40	16	49		616,617
										
898		H	OH	32[a,d]	614	80	17	43		616,617
899		OH	H	32[a,d]	643	83	52	45		616,617
										
		n								
900		1		24[a,d]	757	100	35	47		616,617
901		2		32[a,d]	689	100	37	64		616,617
902		3		64[a,d]	757	100	32	75		616,617
361 (DONU)				8[a,d]	669	100	20	31		616,617
598 (acetamido-CNU)				8[a,d]	679	100	35	44	−0.30	616,617

[a] Murine lymphoid leukemia. [b] Murine lymphocytic leukemia. [c] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [d] Drug administered on days 1−3 after tumor implantation. [e] Drug administered on days 1−9 after tumor implantation. [f] Increase in life span = $[(T - C)/C] \times 100$. [g] Values are rounded. [h] Percentage of treated mice surviving on day 60 after tumor implantation. [i] Therapeutic ratio = optimal dose/ILS_{30}, ILS_{30} = daily dose eliciting a 30% ILS. [j] Half-life, determined by measuring the decrease of absorbance at 230 nm in phosphate buffer, pH 7.4, at 37 °C. [k] Partition coefficient: *P* = [compound in 1-octanol]/[compound in water].

Scheme 89



line compound (**907**) and two mutagenic compounds (**905** and **909**).[622] The results[625] of the biological testing of compounds **904**−**909** were surprising. All compounds had very low toxicity, as exemplified by the acute toxicity of 2000 mg/kg and the LD_{50} dose of 1777 mg/kg for the L-Thr analog **905**. Further-

more, all of the *N*-nitrosated products had a low in vitro toxicity in a primary tumor screen and no cytotoxicity in vivo against the P388 and L1210 leukemia cell lines in mice.[625] It is apparent that the bis-oxidized nitrosamines **904**−**909** are not metabolically activated either by α- or β-oxidation or by retroaldol cleavage reactions.[626,627] Thus, the nitrosated Amadori compounds **904**−**909** can be considered[625] as detoxified water-soluble products that are either excreted without degradation or denitrosated by cytochrome P450 in the liver.

I. Steroid–Amino Acid Conjugates

Steroids containing the CENU group bind specifically to estrogen and androgen receptors, albeit with a much lower relative binding affinity than that of the parent steroid hormones (section VII.E). In comparison to CENU−steroid and CENU−amino acid derivatives only a small number of studies were undertaken in the area of CENU−steroid−amino acid conjugates. The first reported steroid−CENU analogs **708** and **710** were shown[515] to have high growth inhibition of a rat DMBA-induced mammary tumor. The attachment of the CENU moiety to amino acid amides yielded compounds with very high activities against the murine leukemias (Tables 34 and 35). Thus, a conjugate of a steroid and a CENU-containing amino acid would allow the possibility of targeting specific receptors by the steroid, followed by a hydrolysis liberating the cytotoxic CENU amino acid which could attack the cancer cells.

Conjugates containing CENU-amino acids joined to corticosteroids **910**−**913**, androgens **914** and **915**, and estrogens **916**−**922**, (Table 48) were synthesized[628,629] as illustrated in Scheme 90. Thus, estradiol 3-esters **916**, **917**, and **922** were prepared by reactions of estradiol with the active esters of CENU−L-Ala (**572**) and CENU−L-Ala-L-Ala (**652**) (Scheme 90a), while the synthesis of the estradiol 17-ester **918** required a blocking of the phenolic hydroxyl of estrone, as shown in Scheme 90b. The estradiol 6-ester **921** was synthesized from estradiol diacetate according to the sequence outlined in Scheme 90c.

The activities of compounds **910**−**917** against the rat L5222 leukemia were generally higher than that of the unlinked L-Ala analog **572** (Table 48).[630] The L5222 leukemia was used because it has higher levels of corticosteroid receptors than the L1210 leukemia.[630] Among the corticosteroids **910**−**913** the cortisone 21-ester **911** was the most active with a 756% ILS and 33% 90 day survivors. Both androgens, dihydrotestosterone 17-ester **914** and *cis*-androsterone 3-ester **915** had high activity of 800% ILS against the L5222 leukemia. In the estrogen series **916**−**922** the compound with the highest anticancer activity and greatest toxicity was the estradiol 3,17-diester **919** while the estradiol 3-ester **917** was the least toxic of the entire steroid group and also possessed a relatively high antileukemic activity.[630] In contrast, the stilbestrol 4,4-diester **920** exhibited low anticancer activity and relatively high toxicity (Table 48). The therapeutic ratios of the steroid−amino acid conjugates were not improved over that of the unlinked CENU−L-Ala (**572**) (Table 48).[630] Further short-term (1 and 2 weeks)[631] and long-term (4, 7, and 10 weeks)[628] studies involved the testing of CENU−L-Ala-estradiol analogs **917**−**919**, **921**, and **922** against a hormone-dependent MNU-induced rat mammary carcinoma (Table 48). This cancer contains measurable concentrations of estradiol and progesterone receptors and is considered[631,632] to be a better model of human cancers than transplantable tumors. The estradiol 17-ester **918** was the most active analog, causing the highest reduction in tumor volume of 90%, highest % ILS value of +18, and a low 30% mortality at week 7 postimplant.[631] The CENU−amino acid **572** and CENU−dipeptide **652** analogs, in comparison to the estradiol 3-ester conjugates **917** and **922**, were much more cytotoxic, as judged by the % ILS values of −5% and +39%, and much less chronically toxic, as judged by the lower seven-week mortality values of 20% and 10% (Table 48).

The high activity of the estradiol 17-ester **918** is understandable because it has a free 3-phenolic hydroxyl group which is required for bonding to the estrogen receptor.[505−509] Since the estradiol 3-ester **917** and the 3,17-diester **919** both have a blocked 3-hydroxyl, their large in vivo activity, as measured by tumor volume inhibition, is probably the result of a prior hydrolysis to estradiol which has intrinsic anticancer activity.[628] Compared to the other estradiol esters, the estradiol 6-ester **921** contains free hydroxyl groups at positions 3 and 17 but, nevertheless had a very low activity in terms of a 6% tumor volume inhibition, a −32% ILS value, and a high 60% mortality (Table 48). The estradiol 3-esters **917** and **922** exhibited the lowest toxicity but their administration resulted in a higher mortality compared to the 17-ester **918**.[628]

The importance of the estrogen receptors for the biological activity of these conjugates was supported[628] by the use of ovariectomized rats. When rats were ovariectomized before the manifestation of mammary carcinomas in order to induce non-hormone-dependent tumors, then the activity of the estradiol 17-ester **918** against the tumor decreased to a level comparable to that of the unlinked CENU−L-Ala **572** in both the nonovariectomized and ovariectomized rats.[628,631,632] Removal of the ovaries causes a large decrease in estradiol receptor content and, therefore, the activity of the CENU−L-Ala-estradiol ester conjugates, such as **917**−**919**, are strongly dependent on the estrogen receptor. Ovariectomy by itself resulted in a 53% ILS and the resultant therapeutic efficacy, as measured by the tumor volume inhibition, was 84% at week 4 but only 31% at week 7. However, the estradiol 17-ester **918** maintained high tumor inhibitions of 82% and 90% at weeks 4 and 7, respectively (Table 48).[628,631,632]

A comparison of the conjugate estradiol 17-ester **918** and the unlinked combinations of CENU−L-Ala + estradiol **923** and CENU−L-Ala-L-Ala + estradiol **924** was instructive (Table 48).[628,631,632] At equimolar doses of 75 μmol/kg for compound **918** and 75 μmol/kg for each of the unlinked components, the conjugate **918** had superior tumor volume inhibition at weeks 4 and 7, i.e. 82% and 90% respectively, compared to 58% and 76% for **923** and 68% and 84% for **924**. Furthermore, the administration of the conjugate **918** resulted in a lower mortality of 30% at weeks 7 and 10 compared to 70% and 90% for **923** and 30% and

Table 48. Anticancer Activity of CENU−Steroid−Amino Acid Conjugates against Ip-Implanted L5222 Rat Leukemia and MNU-Induced Rat Mammary Carcinoma

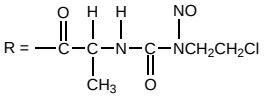
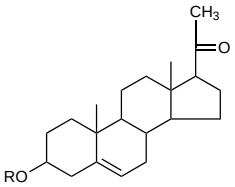
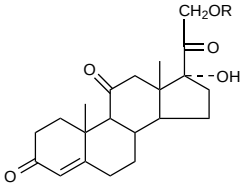
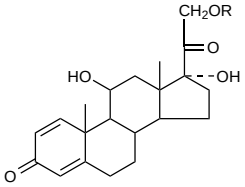
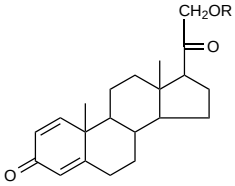
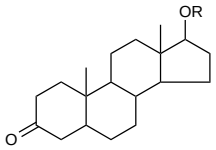
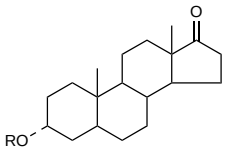
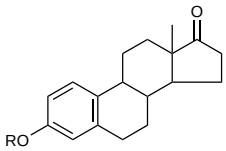
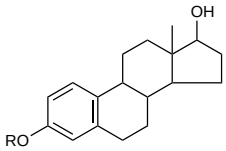
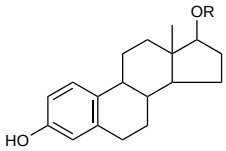
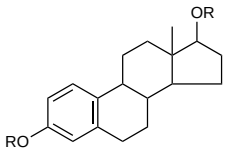
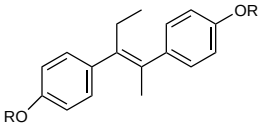
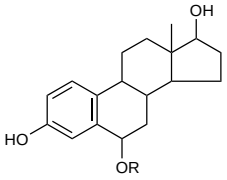
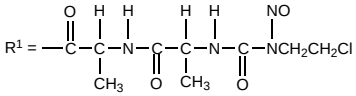
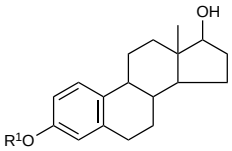
compound no.		L5222 OD,[a,b] mg/kg	L5222 ILS_{max},[c] %	L5222 survivors,[d] %	L5222 ther ratio[e]	MNU mammary carcinoma dose,[f] mg/kg	MNU mammary carcinoma TVI,[g] $T/C \times 100$	MNU mammary carcinoma mortality,[h] %	MNU mammary carcinoma ILS[c] %	ref(s)
	R = 									
910		442	181							630
911		176	756	33	2.0					630
912		111	238	33	1.6					630
913		88	238	33	2.5					630
914		111	800	83	2.0					630
915		111	800	100	1.6					630
916		176	425	17	1.6					630
917		279	513	33		75 147	 23	50	−28	628,630
918		176	369			75 75	 10	30	+18	628,633
919		70	769	50	2.5	75 105	 29	20	−29	628,630

Table 48. (Continued)

compound no.		L5222 OD,[a,b] mg/kg	L5222 ILS_{max},[c] %	L5222 survivors,[d] %	L5222 ther ratio[e]	MNU mammary carcinoma dose,[f] mg/kg	MNU mammary carcinoma TVI,[g] $T/C \times 100$	MNU mammary carcinoma mortality,[h] %	MNU mammary carcinoma ILS[c] %	ref(s)
920		70	225	17	1.6					630
921						38		60	−32	628
						75	94			
572	ROH	28	210	14	2	45		20	−5	628
						67	39			
923	ROH + estradiol					54 (each)		70	+30	628
						75 (each)	24			
922						75		40	−24	628
						147	13			
652	R^1OH					45		10	+39	628
						67	58			
924	R^1OH + estradiol					75 (each)		30	−13	628
						75 (each)	16			
ovariectomy							69	10	+53	628

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Drugs were administered on day 6 after tumor implantation. [c] Increase in life span = $[(T - C)/C] \times 100$. [d] Percentage of animals surviving on day 90 after tumor implantation. [e] Therapeutic ratio = toxic dose/optimal dose. [f] Drugs were administered on days 1, 8, 22, and 29 after randomization. Randomization of rats occurred when their total tumor volume equalled more than 0.8 cm^3. [g] Tumor volume inhibition. Total tumor volumes of treated groups versus control groups ($T/C \times 100$). $T/C \times 100$ values for weeks 1 and 4 and % mortality values for weeks 4 and 10 were provided in ref 628. [h] Results obtained at week 7.

70% for **924**. A similar activity was found[633] for **918** in comparison with the unlinked CENU−L-Ala (**572**) against the MXT mammary carcinoma, a hormone-dependent murine tumor.

As is common in steroid research the relative binding affinities (RBA) of estradiol analogs **917**−**919**, **921**, and **922** for the estrogen receptor from calf uterus cytosol were determined by means of competitive binding experiments with [^{3}H]estradiol (Table 49).[628] The estradiol 3-esters **917** and **922** had relatively high receptor binding affinities. This result can be explained by an enzymatic hydrolysis of the esters liberating estradiol which would have an artificially high apparent binding. The low RBA for the estradiol 6-ester **921** would explain its low cytotoxicity against the hormone-dependent rat mammary carcinoma.[632,633] However, the estradiol 17-ester **918** had a low binding affinity but the highest anticancer activity.[628] Therefore, the high activity of the other estradiol analogs cannot be fully explained by a specific binding to the cytosolic estrogen receptor.[628] Similarly, a series of CENU−amino acids linked to the androgens testosterone, dihydrotestosterone, and 19-nortestosterone **925** and **926** (Chart 25), were shown[632] to have no binding to estrogen receptors, a relatively high binding, 3−7.5% RBA, to the androgen receptors, and a relatively low binding to the progesterone receptors.

The CENU−Gly and CENU−Ala esters of 19-nortestosterone **925** and **926** were screened[634] for activity against the androgen-dependent Noble Nb-R sc prostate carcinoma of the rat. An administration of 50 mg/kg of the drugs on days 1, 7, and 21, beginning three weeks after tumor inoculation, resulted in a very high tumor inhibition, with tumors no longer detectable after termination of therapy.[634] An equimolar 24 mg/kg dose of CENU−Ala **572** resulted in the death of the animals during week 2 of therapy. Hence, the conjugation of the amino acid with the androgen drastically reduced the toxicity of the drug.[634]

The high activities of the estrogen, CENU−L-Ala-estradiol 17-ester **918**, and the androgen CENU−L-Ala-dihydrotestosterone (DHT) 17-ester **914**, against the MNU-induced rat mammary carcinoma could be correlated[628,635] with their effects on the cytosolic receptor contents. A single 75 μmol/kg dose of the estradiol 17-ester **918** resulted in[628] a rapid disappearance of estradiol receptor content and a strong induction of progesterone receptors, with a maximum value at 16 h after the ip injection. While the progesterone receptor content returned[628] to normal after 48 h, the estradiol receptor content remained depressed, and began to return to normal levels only 192 h after treatment. Similarly, the administration of the DHT 17-ester (**914**) depleted[635] the androgen

Scheme 90

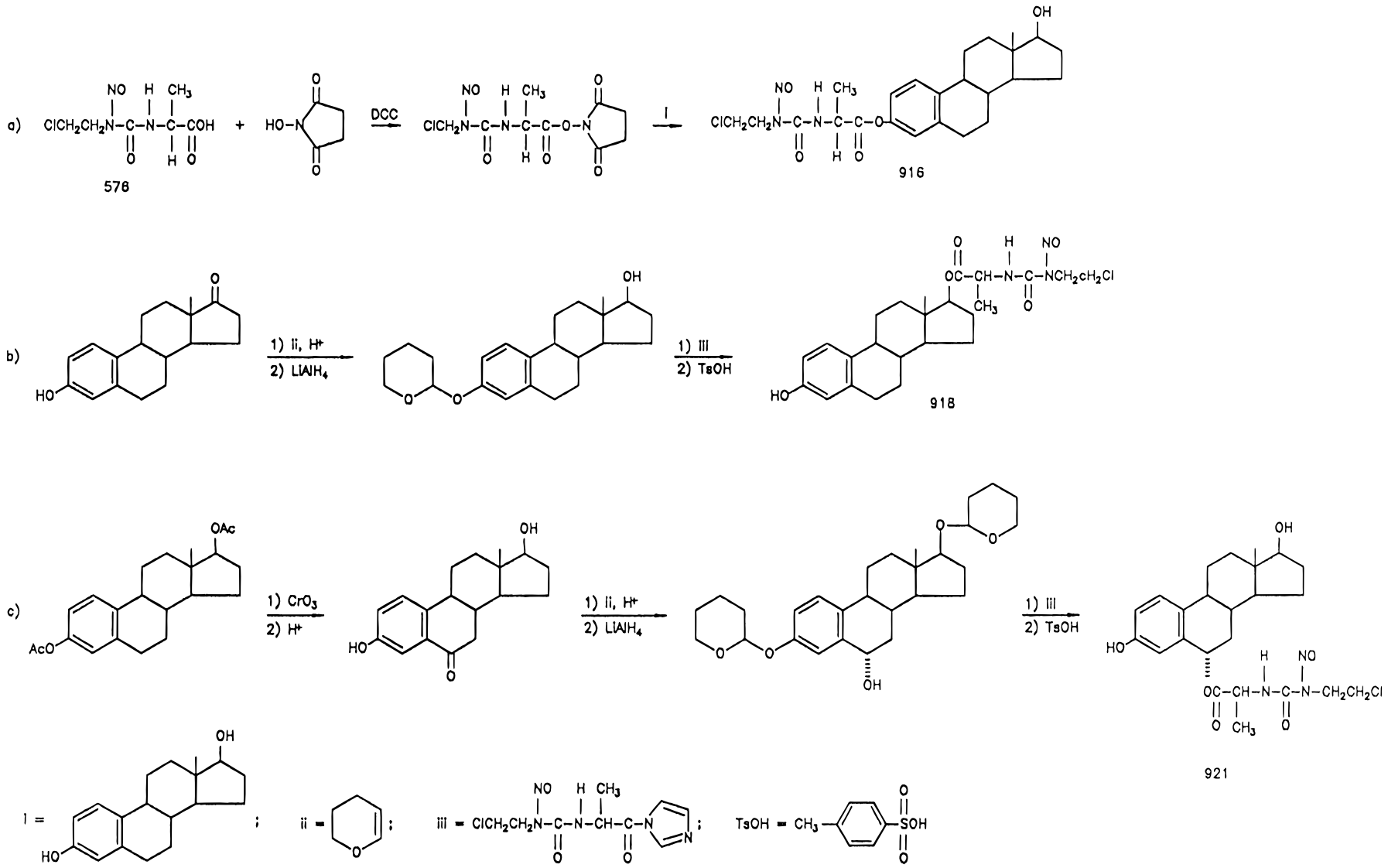

a)
DCC
I
576
916
b)
1) Ii, H+
2) LiAlH4
1) III
2) TsOH
918
c)
1) CrO3
2) H+
1) Ii, H+
2) LiAlH4
1) III
2) TsOH
921
I =
ii =
iii =
TsOH =

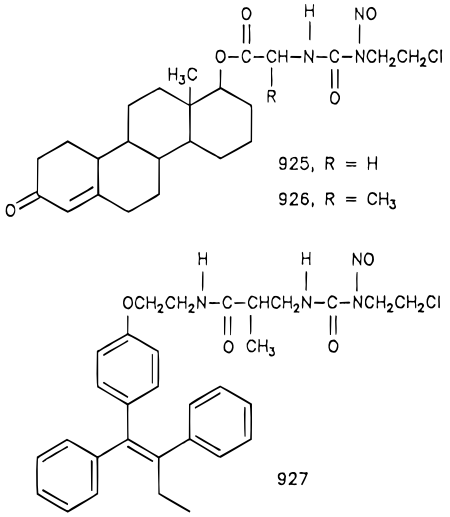
Table 49. Relative Binding Affinities of CENU−L-Alanine−Estradiol Esters to the Estrogen Receptor from Calf Uterus Cytosol[628,632,634]

compound no.		RBA,[a] %
917	RN(H)-L-Ala-estradiol 3-ester	4.70
922	RN(H)-Ala-L-Ala-estradiol 3-ester	10.20
918	RN(H)-L-Ala-estradiol 17-ester	0.80
919	RN(H)-L-Ala-estradiol 3,17-diester	<0.05
921	RN(H)-L-Ala-6α-OH-estradiol 6α-ester	0.28
	6-α-OH-estradiol	6.30

R = $ClCH_2CH_2N(NO)C(O)-$

[a] Relative binding affinity; RBA of estradiol = 100.

Chart 25. Structures for Section VII.I



receptors as well as the progesterone receptors. The androgen receptor content remained low for a week while the progesterone receptor content recovered within eight days.

Various aspects of the pharmacokinetics of the cytotoxic CENU−L-Ala-estradiol 17-ester **918** compared with those of CENU−L-Ala **572** were reported.[636−638] The amino acid−estradiol conjugate had a 3-fold longer terminal plasma half-life, three times larger volume of distribution, three times faster distribution following iv administration, and two times faster absorption after peroral administration.[636−638] The conjugate **918** was shown to have[636,638] a greater availability in all tissues of female rats studied, i.e. liver, lung, spleen, uterus, and mammary carcinoma. In the mammary carcinoma tissue conjugate **918** exhibited[638] a two times higher peak concentration, five times longer half-life, a 10 times increased peak accumulation, and a 20 times greater tissue availability compared with CENU−L-Ala (**572**). The greater lipophilicity of **918** could account for its greater tissue availability.[636] Similarly, the greatly increased plasma half-life could be attributed to a strong hydrophobic bonding with serum proteins which would delay the metabolism.[636] Furthermore, steroid−CENU−amino acid esters are inhibitors of serum esterases.[639] After administration of the conjugate **918** the metabolite CENU−Ala **572** was found[636] in the plasma, indicating a cleavage of the ester bond. The fact that the compound **572** had a longer plasma half-life when formed by hydrolysis of the conjugate **918** rather than by direct application implies[636] that there is a continuous release of the metabolite. Hence, the longer the plasma half-life, the longer the exposure of cancer cells to the drug concentrations which exert cytotoxic effects in vivo.[640] Thus, although the inherent activity of CENU−L-Ala **572** against the MNU-induced rat mammary carcinoma is modest (Table 48), its continued release from the conjugate by enzymatic hydrolysis permits an effective anticancer activity. It should be emphasized that both the prolonged plasma half-life and the presence of estradiol receptors are important factors for the observed anticancer activity.[636]

At the biochemical level, the DNA damage, in the form of single-strand breaks and cross-linking, caused by CENU−L-Ala-estradiol conjugate **918** was two times greater in the bone marrow but 10 times greater in the MNU-induced rat mammary carcinoma than that caused by the unlinked component CENU−L-Ala **572**.[14,641,642] However, the inhibition of bone marrow, as measured by the stem-cell assay using the CFU-S and CFU-C markers, was less for **918** than for **572**.[14,641,642] Therefore, another reason for the improved activity of the conjugate **918** against the hormone-dependent tumor was a decreased toxicity toward the bone marrow relative to that toward the cancer cells.

The administration of the steroid−CENU−L-Ala conjugates **914** and **918** and the didemethyltamoxifen CENU conjugate **927** (Chart 25) resulted[643] in a high level of DNA cross-links when applied to receptor-positive MCF-7 human breast cancer cells but a greatly reduced level of cross-links when applied to receptor-negative MDA-MB-231 cell line, indicating that the DNA cross-linking was directly correlated with the availability of the steroid receptors. This correlation was further substantiated by the finding[643] that the high DNA cross-linking of the estrogen receptor-positive cells by the conjugates **914** and **918** was greatly reduced by its simultaneous addition of the steroid hormones 4-dihydrotestosterone and estradiol, respectively.

VIII. Structure−Activity Relationships

This section is devoted to a critical analysis of the chemical and biochemical properties of various *N*-nitrosoureas. Some of the more important compounds which were discussed in sections VII.A−I will be highlighted. These discussions will serve as a bridge to the following sections on the chemical decomposition mechanisms and biochemical mechanisms of anticancer activity. The important parameters are the alkylating activity, carbamoylating activity, chemical half-life, partition coefficient and therapeutic index. Typical assays for these parameters are discussed in section VI.

At the outset it should be emphasized that there are inherent difficulties in the use of in vitro "test tube" experiments to measure in vivo parameters. Thus, the in vitro assay with lysine underestimates the carbamoylating reaction of *N*-nitrosoureas.[12] The ^{14}C distribution of ^{14}C-ring labeled CCNU in urinary metabolites included higher molecular-weight fractions which were conjugates of peptides derived from active site-directed inactivations of specific enzymes.[644] Lysine was not a major amino acid in such urinary metabolites.[644] The complexity of carbamoylation reactions is discussed in section X. The alkylation activities of CCNU (**137b**) and BCNU (**33**)

are significantly different as measured by the alkylation of 4-(4-nitrobenzyl)pyridine at pH 6 and 37 °C. However, the major product of the aqueous decomposition of BCNU and CCNU at pH 7.4 were equivalent amounts of 2-chloroethanol.[645,646] Consequently, the in vitro assay might not accurately represent the in vivo situation.

Arguments have been made[647] for a greater reliance on various solid tumors, especially drug resistant cell lines, for preclinical evaluation of new anticancer agents. The poor clinical experience with many compounds with promising activity obtained using the murine models, P388 and L1210 leukemias, gave rise to these ideas. The importance of the therapeutic index as a parameter for selecting anticancer agents for clinical trials was emphasized.[647]

An early study[97] concerning the relative roles of chemical half-life, alkylating activity and partition coefficient in determining the activities of *N*-(2-haloethyl)-*N*-nitrosoureas against ip-implanted L1210 leukemia was very instructive. Thus, in this study it was demonstrated[97] that an inverse relationship exists between the half-life and alkylating activity, i.e. the shorter the half-life and, thus, the greater the chemical instability, the greater the alkylating activity. The alkylating activity was the major factor in correlations with the anticancer activity, as expressed with ED_{50} and ED_{99} values,[97] with the carbamoylation supplementing this factor more than the distribution coefficient *P*.

Therapeutic indices are measures of the relative toxicities of the compounds to the cancer cells compared to the normal cells. It was found[97] using the regression equations that the therapeutic indices are increased by greater alkylating activity and more positive partition coefficients *P* and are decreased by greater carbamoylating activity. The carbamoylating activity contributes relatively more to the toxicity LD_{10} values of the tested compounds than the alkylating activity.[97] From one study, it was concluded[97] that nitrosourea drugs having optimal activity against ip-implanted L1210 leukemia would have the combination of low carbamoylating activity, high alkylating activity, and greater lipophilicity.

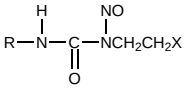
Quantitative structure−activity relationship (QSAR) studies of large series of *N*-nitrosoureas revealed[91,92,95] good correlations between the lipophilicity and anticancer activity. Thus, plots of log 1/*C*, where *C* is the molar concentration of the drug required to produce a delay in tumor growth of 4 days, against the log 1/*P* values of a group of 14 nitrosoureas tested against the Lewis lung carcinoma[91,92] was a parabolic curve, with the optimum log *P* range of −0.20 to + 1.34. A similar QSAR study[95] of the antileukemic activity of 90 nitrosoureas against the L1210 leukemia in mice was correlated with a QSAR of the LD_{10} for 96 nitrosoureas. It was found[95] that six times the concentration of the nitrosourea drug was needed to produce a LD_{10} dose as opposed to that producing a 3 log kill in leukemia cells, a measure of the therapeutic indices of these compounds. From this study it was concluded[95] that more hydrophilic compounds with a log *P* range of −1.50 to −2.50 would be expected to yield more active agents. Plots of either log 1/*C* or log LD_{10} against the log *P* produced similar but overlapping parabolic curves. On the basis of these results and partition coefficients, it was suggested[95] that there was no separation of activity and toxicity.

The structure−activity relationships of the first generation of nitrosoureas, consisting of the aliphatic, alicyclic, aromatic, and heterocyclic analogs, was excellently reviewed.[6] In these early studies it was shown that the *N*-(2-chloroethyl)- or *N*-(2-fluoroethyl)-*N*-nitrosoureido group was necessary for high anticancer activity against the L1210 leukemia (Table 2). A variety of groups could be attached to the N3-nitrogen resulting in corresponding differences in toxicity (LD_{10}), effective dose (ED_{50}), therapeutic index (LD_{10}/ED_{50}) and partition coefficient P. The N3-substituents associated with high activity against the L1210 leukemia include the 2-chloroethyl as in BCNU (**88**), cyclohexyl as in CCNU (**137b**), 4-substituted cyclohexyl as in MeCCNU (**138b**), cyclohexyl rings substituted with hydroxyl, carboxyl, carboxymethyl, and carbethoxy moieties and cyclohexyl rings substituted at the 3, the 4, and the 3,5 positions with S or SO_2 moieties (Tables 9, 10, and 17). Generally, aromatic substituents on the N3-nitrogen (Tables 14 and 15) caused a drastic reduction in activity, especially against intracerebrally implanted L1210. This result was interpreted[6] to indicate an inability of such compounds to cross the blood−brain barrier.

The polymethylene bis-CENU N3-NO series **85**−**89** and N1-NO series **103**−**109** were synthesized[59,134,135,146] because they could, in theory, result in bridging across the DNA double helix through dialkylation or dicarbamoylation reactions. The bis N3-NO series (Table 4) would be expected[59,134,135] to decompose to a poly(methylene diisocyanate) and 2-chloroethyldiazohydroxide, as depicted in Scheme 18a. The activity of these analogs, with $n = 2-6$, against the ip-implanted L5222 leukemia was approximately equal to that for BCNU (**33**)[59,134,135] while their activity against the solid cancer Walker 256 carcinoma was somewhat lower than that for BCNU[134] (Table 4). This anticancer activity of compounds **85**−**89** can be attributed to the common alkylating intermediate, 2-chloroethyldiazohydroxide, which can ultimately form interstrand cross-links with DNA. Since the anticancer activities of the compounds in this series are very similar, a conclusion can be reached[59,134,135] that the polymethylene bis(isocyanate) intermediates are not important for the observed activities. Interestingly, the tetramethylenebis(nitrosourea) **87** exhibited no activity against the ic-implanted L1210 (Table 4). This result was interpreted[134] to indicate that such compounds are not capable of wide tissue distribution and of crossing the blood−brain barrier. However, **89** had a lipophilicity of log $P = 0.9$ which is not sufficiently different from that of BCNU, log $P = 1.5$, to explain this large activity difference.[134]

The decomposition of the bis-N1-NO series **103**−**109** (Table 5) would yield the polymethylenebis(diazohydroxides) and the 2-chloroethyl isocyanate (Scheme 18b),[146] but these compounds had lower activity against the ip-L1210 leukemia than BCNU and appreciable activity against the Walker carcinoma (Table 5). In this case, it was suggested[146] that the observed activity resulted from the 2-chloroethyl isocyanate moiety which could form a cross-link

Table 50. Comparative Activity of N3-Substituted Aliphatic CENU Analogs against the Ip-Implanted Rat L5222 Leukemia



compound no.	R	X	dose,[a,b] mg/kg	ILS_{max},[g] %	survivors, %	log P[j]	rel alkyl act,[k] %	H_2O sol, %	ref(s)
329	$HOCH_2CH_2$	Cl	18−22[c]	800	67[h]		130	11.0	59,134,135
			16[d]	220	90[i]				
			10[e,f]		100[i]				134
332	$HOCH_2CH_2$	F	7[c]	255					59,134,135
330	$HO(CH_2)_3$	Cl	16[c]	170	10[h]	0.7			59,134,135
331	$HO(CH_2)_4$	Cl	16[c]	160	5[h]	0.7			59,134,135
333	$CH_3SO_2OCH_2CH_2$	Cl	28[c]	395	33[h]	−0.2	125	0.3	210,296
350	$HOCH_2CH(OH)CH_2$	Cl	18[c]	150	25[h]				210
571	$HOOCCH_2CH_2$	Cl	28[c]	170	10[h]				210
572	$HOOCCH(CH_3)$	Cl	28[c]	210	14[h]				210
46	$NCCH_2CH_2$	Cl	18[c]	325	17[h]				210
598	H_2NCOCH_2	Cl	11[c]	800	83[h]	−0.2	155	2.5	210
			18[c]	615	33[h]				
33	$ClCH_2CH_2$	Cl	18[c]	675	33[h]	1.53	100	0.4	210
			16[d]	180	70[i]				
			10[e,f]	200	37[i]				
137b (CCNU)			18−28[c]	800	100−83[h]	2.73			210
138b (MeCCNU)			18−44[c]	800	100−83[h]	3.30			210

[a] Optimal dose = daily ip-administered dose resulting in the maximum increase in life span. [b] Values are rounded. [c] Drug administered on day 10 after tumor implantation. [d] Drug administered on day 6 after tumor implantation. [e] Drug administered on day 8 after tumor implantation. [f] Drug administered by the ic route. [g] Increase in life span = $[(T - C)/C] \times 100$. [h] Percentage of treated animals surviving on day 90 after tumor implantation. [i] Percentage of treated animals surviving on day 60 after tumor implantation. [j] Partition coefficient: P = [compound in 1-octanol]/[compound in water]. [k] Alkylating activity relative to BCNU (**33**) = 100.

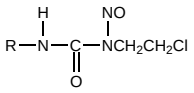
between nuclear proteins and DNA. The polymethylenebis(diazohydroxide) was discounted as the active alkylating agent because compounds in the corresponding cyclohexane series (structure shown in Chart 5), which would decompose to the same polymethylenebis(diazohydroxide) and cyclohexyl isocyanate, were inactive against the L1210 leukemia.[146]

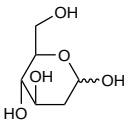
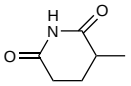
Several chemical and biological parameters were determined for compounds **103**−**109** (Table 5). Approximate linear correlations exist between the half-life, the lipophilicity, i.e. the log P parameter, and the number of methylene groups. The alkylating activity was directly related[146] to the cytotoxicity, expressed as % ILS. QSAR analyses, using linear and multilinear equations, demonstrated strong correlations between the toxicity and the alkylating activity, and the lipophilicity and alkylating activity. The bis-nitrosoureas **85**−**89**[648] and **103**−**109**[146] were both mutagenic in the Ames test.

An important advance occurred with the syntheses of various water soluble nitrosoureas, along with certain hydroxyalkyl, carbohydrate, heterocyclic, and amino acid analogs. The relative chemical properties and biological activities of these compounds have been compared in a number of studies with the more lipophilic drugs BCNU (**33**), CCNU (**137b**), and MeCCNU (**138b**). Several comparative studies have included the compound HECNU (**329**), a water-soluble hydroxy analog of BCNU (**33**). HECNU was shown[59,134,135] to be superior to BCNU against the ip-implanted rat L5222 leukemia, with 90% and 70% of 90 day cures, respectively, and against the ic-implanted L5222, with 100% and 37% of 90 day cures, respectively (Tables 19 and 50).[134] The corresponding 3-hydroxypropyl (**330**) and 4-hydroxybutyl (**331**) analogs of BCNU (**33**) were much less active (Tables 19 and 50) than HECNU (**329**),[59,134] even though all three compounds decompose to the same alkylating species, namely, the 2-chloroethyldiazohydroxide. These compounds have similar log P values of 0.3 and 0.7 respectively, so that their relative lipophilicities cannot be used to explain the difference in anticancer activity. HECNU (**329**) is about 30 times more water soluble than BCNU (**33**), i.e., 11% and 0.4% respectively, but it is still sufficiently lipophilic to be found in twice the concentration in the lipid phase than in the aqueous phase. This balance of hydrophilic and lipophilic properties may ensure its better distribution in the ic-implanted L5222 experiments.[134] HECNU (**329**) was found to be particularly active against intracerebrally (ic) implanted tumors, such as rat L5222 leukemia[134,296] (Table 50), the murine anaplastic mammary carcinoma,[649] murine ependymoblastoma,[649] and astrocytoma Ht60 in the rat.[650]

Other variations of the N3-substituent are instructive. Thus, the methanesulfonate (mesyl) ester of HECNU **333** is less water soluble than HECNU (**329**) and has a lower activity against the rat L5222 leukemia (Table 50),[5,210,296] but it had a high activity against the sc-implanted Walker carcinoma, and the highest activity of several nitrosoureas against the DMBA-induced mammary carcinoma.[296,649] The dihydroxypropyl analog **350** was appreciably less active than HECNU (**329**) (Table 50).[649] The presence of either a carboxyl group in the N3 side chain, e.g. as in the β-alanine **571** and L-alanine **572** analogs, or the cyanoethyl group in compound **46**, resulted in a large reduction in cytotoxicity (Table 50).[649] In contrast, the CENU-glycineamide (acetamido-CNU, **598**) exhibited high activity against the murine L1210[458] and rat L5222[210,249] at two doses. In order to explain the higher anticancer activity of **598** as compared to compounds **571** and **572**, it was hypoth-

Table 51. Comparative Activity of CENUs against Iv-Implanted Murine L1210 Leukemia[113]



compound no.	R	dose,[a,b] mg/kg	ILS,[c] %	survivors,[d] %
33	$ClCH_2CH_2$	32	243	50
137b		18	200	83
		32	250	74
138b		18		100
		32	200	67
368		32	100	17
257		58	200	50
329	$HOCH_2CH_2$	18	329	68
		32	100	39
332	$HOCH_2CH_2$[e]	18	143	67
		32	500	83
46	$NCCH_2CH_2$	18	407	67
47	CH_3COCH_2	10	186	17
350	$HOCH_2CH(OH)CH_2$	10	350	67
		18	343	83
87	$Cl(CH_2)_2N(NO)CONH(CH_2)_4$	58		100
333	$CH_3SO_2OCH_2CH_2$	10	207	67
		18		100
		32	214	50
49	CH_3NH	58	7	
		18	129	50
50	CH_3NCHO	105	235	33
51	CH_3NCOCH_3	189	229	50

[a] Drug was administered ip on day 1 after tumor implantation. [b] Values are rounded. [c] Increase in life span = $[(T - C)/C] \times 100$. [d] Percentage of treated animals surviving on day 63 after tumor implantation. [e] Fluorine analog of **329**.

esized[467] that the un-ionized amide **598** can more readily penetrate cell membranes than compounds **571** and **572** containing ionized carboxyl groups.

Eleven short-chain nitrosoureas were screened[113] against an iv-implanted L1210 leukemia (Table 51). The iv-implanted leukemia cells were shown[113] to be distributed to a much greater extent in the spleen, liver, lung, and brain than in the ip-implanted test system, and thus the intravenous system was considered[113] to be a better model for human cancers. As can be seen from Table 51 several of the nitrosoureas with short-chains, i.e. HECNU **329**, HEFNU **332**, $CNCH_2CH_2CNU$ **46**, dihydroxypropyl-CNU **350**, tetramethylene-bisCNU **87**, and HECNU-mesylate **333** were more active than BCNU in the iv systems. In comparison, the carbohydrate CZT (**368**) was only marginally active in this iv system. This result is also reflected in the disappointing clinical trials of CZT (**368**) using the iv regimen.[113] HECNU (**329**) was compared[210,296,649] with a variety of other nitrosoureas against ip- and ic-inoculated leukemias (Table 52) and a variety of solid tumors. Thus, when compared with BCNU (**33**), CCNU (**137b**) and MeCCNU (**138b**), HECNU (**329**) had equal or superior activity to the other analogs against ip- and ic-inoculated L5222, colon 26 and mammary 16 carcinomas, and Lewis lung carcinoma.[5] Both HECNU (**329**) and CZT (**368**) were shown[296] to have about the same activity against either nonimmunogenic or immunogenic lines of L1210 leukemias, whereas BCNU (**33**) and CCNU (**137b**) were significantly more active against the immunogenic line (Table 53). This difference could be important because many human cancers have low immunogenicity.[296]

The four analogs BCNU (**33**), MeCCNU (**138b**), CZT (**368**), and HECNU (**329**) were compared[296] according to their immunodepressive activity in mice. A prolonged immunodepression could contribute to infections during chemotherapy and to the carcinogenicity of these agents. HECNU (**329**) was found[296] to be more immunodepressive than BCNU (**33**), MeCCNU (**138b**), and CZT (**368**) on a milligram/kilogram basis but it exhibited about the same immunodepression when the therapeutic indices LD_{50}/ED_{50} and OD/ED_{50} were calculated (Table 53). HECNU (**329**) and acetamido-CNU (**598**) had equal or greater activity than either BCNU (**33**) or MeCCNU (**138b**) against ip-L5222 and sc-Walker carcinoma[649] as well as against mouse transplantable tumors, colon 26, mammary Ca16, and Lewis lung (Table 53).[5,296] The carbohydrate analog CZT (**368**) was generally less active. The administration of either HECNU (**329**) or acetamido-CNU (**598**) against the mouse anaplastic mammary carcinoma resulted[5,296] in a greatly reduced number of lung metastases.

A further set of aliphatic CENU agents, the disulfides CNCC, a mixture of the N1- and N3-NO isomers **119**−**121**, HECNU (**329**), and three carbohydrate analogs CZT (**368**), RPCNU (**437**), and RFCNU (**436**) were compared[151] for anticancer activity using the L1210 leukemia and seven solid tumors (Table 52). The CNCC and RFCNU (**436**) were the least toxic and HECNU (**329**) was the most toxic of this set of five compounds. The disulfide CNCC had the broadest median effective dose (MED) range, followed by RFCNU (**436**), RPCNU (**437**), HECNU (**329**), and CZT (**368**).[151] Both HECNU and CNCC displayed a broad spectrum of anticancer activity against ip-L1210, sc-Lewis lung carcinoma, sc-B16 melanocarcinoma, sc-26 glioma, sc-TM2 mammary adenocarcinoma, colon 26 carcinoma, and M555 ovarian carcinoma (Table 52). None of these agents were active against a fibrosarcoma cell line.[151] The CNCC mixture (**119**−**121**) can be considered a prodrug because the disulfide bond is reductively cleaved in vivo, and the resulting thiol groups are oxidized to sulfoxide and sulfone groups (section VII.A.2). The sulfoxide **125** and sulfone **126** metabolites (Table 7) were shown[169] to be at least as active as CNCC against eight of 12 cancer lines and resulted in a larger percentage of 90 day survivors. The relatively broad MED range of CNCC could be attributed,[319] possibly, to the mixture of CNCC and its active metabolites **125** and **126**.

Heterocyclic analogs of CCNU (Tables 17 and 18) were screened[112,210,248,249] against the rat L5222 leukemia. The most active compounds were the morpholino-CNU (**279**), pyrimidinomethyl-CNU (ACNU, **311**), 2-picolinyl-CNU (**305**), and its hydrochloride salt. A comparison[248] of the cyclic nitrosoureas CCNU (**137b**), MeCCNU (**138b**), morpholino-CNU (**279**), and piperidino-CNU (**278**) revealed that MeC-

Table 52. Comparative Activity of Some Selected Nitrosoureas against Solid Murine Tumors

			sc Walker 256 carcinoma[a]		sc colon 26 carcinoma[b,c]		mammary Ca carcinoma[b,c]		Lewis lung carcinoma[b,c]			ic L1210
no.	compound	ED_{50}, mg/kg	LD_{50}, mg/kg	LD_{50}/ED_{50}	dose[f] mg/kg	survivors,[g] %	dose[j] mg/kg	T/C,[k] %	dose,[l] mg/kg	TWI,[m] %	metasteses, T/C %	leukemia:[b] T/C[k,n] %
33	BCNU	11.9[d]	25.5	2.1	40	35	30	178	40	61	55	203
329	HECNU	8.6[d]	25.3	2.9	20	55	20	187	20	69	50	215
333	HECNU-MS	9.7[d]	23.2	2.7								
598	Acetamido CNU	7.4[d]	19.7	2.4								
368	CZT	not reached	27.2		40	25	40	179	30	54	70	191
138b	MeCCNU				40	60	40	181	30	75	35	>225
368	CZT	15[e]	35			∞[h]		ns[i]		ns[i]		ns[i]
438	RPCNU	15[e]	50			ns[i]		134		ns[i]		ns[i]
437	RFCNU	15[e]	90			ns[i]		ns[i]		ns[i]		167
329	HECNU	10[e]	25			∞[h]		165		∞[h]		∞[h]
119−121	CNCC	30[e]	75			∞[h]		185		∞[h]		∞[h]

[a] Reference 649. [b] Reference 151. [c] Reference 296. [d] Drug administered on day 4 only. [e] Drug administered ip on days 1, 5, and 9 after tumor implantation. Dose is median of maximum effective dose (MED) range.[b] [f] Drug administered ip on day 1; 2×10^5 cells injected im in $CD2F_1$ mice or 10^6 cells injected ic in BALB/C mice. [g] Percentage of animals surviving on day 90 after tumor implantation. [h] Test animals cured ≥50%.[b] [i] Not significant. [j] Drug administered ip on day 10 after tumor implantation; 10^5 cells injected im in C3H mice[c] or 10^6 cells injected ic in C3H mice.[b] [k] Median survival time of treated animals/median survival time of control animals. [l] Drug administered ip on day 1 after tumor implantation; 2×10^5 cells injected im in C57BL/6 mice[c] or 10^6 cells injected ic in C57BL/6 mice.[b] [m] Tumor weight inhibition on day 21 after tumor implantation. [n] 10^4 cells injected ic in BD62F1 mice.[b]

Table 53. Comparative Activity against Immunogenic and Nonimmunogenic L1210 Leukemias and Comparative Immunodepressive Activity of Selected Nitrosoureas[296]

		L1210 Cr[a] ip			L1210 Cr[a] ic		L1210 Ha[b] ip		humoral antibody inhibition			cellular reactivity inhibition		
no.	compound	OD,[c,d] mg/kg	T/C,[e] %	survivors,[f] %	T/C,[e] %	survivors,[f] %	T/C,[e] %	survivors,[f] %	ED_{50},[g] mg/kg	LD_{50}/ED_{50}[h]	OAD/ED_{50}[i]	ED_{50},[j] mg/kg	LD_{50}/ED_{50}[h]	OAD/ED_{50}[i]
33	BCNU	30	205	55	232	50	292	85	7.1	7	4.3	15	3.3	2.0
138b	MeCCNU	30	177	40	163	35	225	60	9.2	6	3.2	15	3.1	1.7
368	CZT	20	206	50	187	50	198	45	7.6	8	2.6	17.5	3.4	1.1
329	HECNU	20		100		100		100	4.0	10	5.0	10	4.0	2.0

[a] Nonimmunogenic cell line. [b] Immunogenic cell line. [c] Optimal dose = daily dose resulting in a maximum increase in life span. [d] Drugs were administered on day 1 after tumor implantation. [e] Median survival time of treated animals/median survival time of untreated controls. [f] Percentage of treated animals surviving on day 90 after tumor implantation. [g] Effective dose = dose resulting in a 50% reduction in the number of anti-SRBC (sheep red blood cells) AFC (antibody-forming cells) in spleen. [h] Therapeutic ratio = lethal dose/effective dose. [i] Therapeutic ratio = optimal activity dose in ip nonimmunogenic L1210 cell line/effective dose. [j] Effective dose = dose resulting in a reduction from 100% to 50% of the long time survivors.

CNU (**138b**) was superior in terms of the dosage interval at which the maximal survival of 90 days was achieved. The morpholino-CNU (**279**) required a somewhat higher minimum dose to achieve a 90 day cure while the piperidino-CNU (**278**) had a narrow dose−response curve, with a 90 day cure at only one dose. A glutarimide-CNU analog PCNU (**257**) (Table 17) was compared[96] to BCNU (**33**), CCNU (**137b**), and MeCCNU (**138b**) with regard to activity against a MNU-induced rat sarcoma and against iv-implanted L1210 leukemia (Table 51). It was found[230] that PCNU (**257**) had the shortest half-life, highest alkylating activity, lowest carbamoylating activity, lowest lipophilicity, and highest anticancer activity of the four drugs.[230] In this series the drug activity was linearly related to the log *P* within the range of 0.37 to 3.3.[230]

Another important concept is that of carrier groups for the CENU moiety. Thus, carbohydrates (section VII.C), amino acids and peptides (section VII.D), steroids (section VII.E), nucleosides (section VII.F), nitroxyls (section VII.G), and carbohydrate−amino acid (section VII.H) and steroid−amino acid (section VII.I) conjugates were explored as groups that could modify the water solubility and various biological and chemical properties.

It is instructive to compare[198] the anticancer activity and chemical and biological properties of water-soluble chlorozotocin (CZT, **368**) with those of the six monohydroxy metabolites of CCNU (**184**−**189**), DONU (**361**), the *trans*,*trans*-2,6-dihydroxylcyclohexyl analog of CCNU (**137b**), and the lipophilic drug CCNU (**137b**) (Table 54). The compounds with the highest alkylating activity, namely, CZT (**368**) and the *cis*-2- and *trans*-2-hydroxy-CCNU congeners **184** and **185**, also had the lowest carbamoylating activity and the highest toxicity.[198,200] All the low carbamoylating compounds can decompose to a cyclic urethane by an intramolecular reaction of a *β*-hydroxyl group, as shown in Scheme 17b. The *cis*-3-hydroxy-CCNU congener **186** also has a relatively low carbamoylating activity. This result indicates that the equatorial C3-hydroxyl group can react to form a six-membered cyclic urethane. The fact that some of the higher alkylating compounds in this series also have the greater toxicity supports the concept that alkylation, as well as carbamoylation of cell components, can be toxic to the organism.[200]

Some interesting correlations were found[198,200,651] to exist between the partition coefficient *P* and the therapeutic ratio ED_{50}/LD_{10}. Thus, all of the CCNU metabolites and CZT (**368**) were found to be less

Table 54. Comparative Chemical and Biological Properties and Anticancer Activities of Selected Water-Soluble *N*-(2-Chloroethyl)-*N*-nitrosoureas

no.	compound	dose,[a] mg/kg	ILS,[b] %	survivors,[c] %	alkylating activity,[d] %	carbamoylating activity,[e] %	$t_{1/2}$,[f] min	LD_{10},[g] μmol/kg	ther ratio[h] ip	ther ratio[h] ic	absolute neutrophil count,[i] %	log *P*[j]	ref(s)
368	CZT	15	306	60	100	2.0	48	64	0.62		110	−1.02	199,200,651
432	GANU		90		111	25.0	20	32			113	−1.02	200,651
362	DSI		158		108	0.6	64	35			47	−2.13	200
358	TE6		429		65	20.0	70	70			36	−1.63	200,651
184	*cis*-2-OH-CCNU	25	267	50	36	3.7	79	100	0.44	0.42	31	+1.6	198−200
185	*trans*-2-OH-CCNU	40	410	90	67	6.0	69	48	0.29	0.52	12	+1.3	198−200
186	*cis*-3-OH-CCNU				27	21	95	98	0.37	0.54		+1.25	198
187	*trans*-3-OH-CCNU				23	61	88	74	0.42	0.53		+1.28	198
188	*cis*-4-OH-CCNU		66	80	28	104	97	90	0.47	0.41		+1.11	197,198
189	*trans*-4-OH-CCNU		20	100	29	100	91	82	0.33	0.43		+1.00	197,198
361	*trans*,*trans*-2,6-diOH-CCNU			118	0.3	73	33		0.29	0.52			198
311	ACNU	30	492	100	61	3.0	75	97			15	+0.39	200
137b	CCNU	30	413	90	20	45	104	171	0.77	0.73	4	+2.83	197−200
33	BCNU		260		47	27	98	116			25	+1.53	651

[a] LD_{10} dose administered ip on day 2 after tumor implantation. [b] Increase in life span = $[(T - C)/C] \times 100$. [c] Percentage of mice surviving on day 45 after tumor implantation. [d] Percentage relative to that of chlorozotocin (**368**) = 100; measured as the absorbance at 540 nm after reaction with 4-(*p*-nitrobenzyl)pyridine, pH 6. [e] Percentage of [^{14}C]lysine carbamoylation; measured as the radioactivity of chromatographically separated components after carbamoylation of the [^{14}C]lysine, pH 7.4. [f] Half-life, measured in 0.1 M phosphate buffer, pH 7.4, at 37 °C. [g] Lethal dose = ip-administered dose resulting in the death of 10% of the untreated mice. [h] Therapeutic ratio = ED_{50}/LD_{10}; ED_{50} = single ip-administered dose on day 1, after ip- or ic-inoculated L1210 leukemia, resulting in 50% 45 day survivors. [i] Percentage of central peripheral blood neutrophil count on day 3, the nadir of white blood cell suppression. [j] Partition coefficient: *P* = [compound in 1-octanol]/[compound in water].

lipophilic than CCNU, and all had more favorable therapeutic ratios against the ip-implanted L1210. The compounds which are either hydrophilic such as CZT (**368**) and the 2,6-dihydroxy-CCNU (DONU, **361**) or highly hydrophobic, such as CCNU (**137b**), cannot cross the blood−brain barrier, as attested by either a lack of activity as is the case for CZT (**368**), or low activity as is the case for DONU (**361**) and CCNU (**137b**), against the ic-implanted L1210.[198] In a similar study the water-soluble carbohydrate-CENU analogs CZT (**368**) and GANU (**432**) were compared[200] with the water-soluble polyhydroxy alicyclic analogs DSI (**362**) and TE6 (**358**), the *cis*- and *trans*-2-hydroxy-CCNU (**184** and **185**), the *cis*- and *trans*-4-hydroxy-CCNU (**188** and **189**), the hydrochloride salt of the heterocyclic ACNU (**311**) and the water-insoluble CCNU (**137b**). Regression analysis of the different biological and chemical parameters, listed in Table 54, revealed[200] an inverse correlation between alkylating activity and half-life and an inverse relationship between alkylating activity and log *P* values, i.e. the more hydrophilic compounds with lower log *P* values had the higher alkylating activity.

No direct relationship was found between carbamoylating activity and several biochemical parameters. There are two groups of carbamoylators, i.e. strong, such as, CCNU (**137b**), TE6 (**358**), GANU (**432**), *cis*-4-OH-CCNU (**188**), and *trans*-4-OH-CCNU (**189**), and weak, such as, CZT (**368**), DSI (**362**), *cis*-2-OH-CCNU (**184**), *trans*-2-OH-CCNU (**185**), and ACNU (**311**).[200] All of the weak carbamoylators can, in principle, undergo an intramolecular cyclization with adjacent hydroxyl groups to form cyclic urethanes. GANU (**432**) and TE6 (**358**) have adjacent *trans*-hydroxyl groups which may account for their slower rate of intramolecular carbamoylation. ACNU (**311**) was shown[280] to form nonenzymatically an intramolecular carbamoylation product. The carbamoylating activity was not correlated[200,651] linearly with the toxicity, as evidenced by comparisons of the LD_{10} values of GANU (**432**) with DSI (**362**) and GANU (**432**) with that of CCNU (**137b**). The highest carbamoylator also was the least toxic. This result is contrary to the earlier work[97] where a strong correlation was found between the carbamoylation and toxicity. Furthermore, there was no linear correlation between carbamoylation and anticancer activity, as judged by the comparison of CZT (**368**) with GANU (**432**), GANU with TE6 (**358**), and *trans*-2-OH-CCNU (**185**) with CCNU (**137b**) (Table 54). Compounds TE6 (**358**) and ACNU (**311**) have similar alkylating activities and anticancer activities but very different carbamoylating activities. The same lack of correlations with carbamoylating activity was observed[652] for several *N*-methylnitrosoureas, including streptozotocin (**367**) and related monosaccharides. No direct correlation existed between the carbamoylation and bone marrow toxicity, as measured by the absolute neutrophil count on day 3 after drug administration.[200,651] Thus, only the two D-glucose analogs, the weak carbamoylating CZT (**368**) and the stronger carbamoylating GANU (**432**) were nonmyelosuppressive (Table 54). The other water-soluble compounds, i.e. DSI (**362**), TE6 (**358**), *cis*- and *trans*-2-OH-CCNU (**184** and **185**) and ACNU (**311**) were highly myelosuppressive, in some instances similar to the highly lipophilic CCNU. It is apparent[200] that the D-glucose structure of CZT and GANU is an essential element for the reduction of myelotoxicity (see also section X.F for a discussion of proposed biochemical mechanisms for myelotoxicity). The position of the CENU group on the glucose ring is not important since both the C2 isomer CZT (**368**) and the C1 isomer GANU (**432**) produced equal neutrophil counts after 3 days. ACNU (**311**) was shown[200] to have high antileukemic activity, similar to CZT, but strong myelosuppressive properties. The difference in bone marrow toxicity between CZT and ACNU, both weak carbamoylators, may be related[12,277] to different rates of intramolecular carbamoylation. A good correlation was found[653] between the ability of ACNU and CCNU to inactivate glutathione reductase and their myelosuppressive properties, whereas CZT and GANU are not able to

inactivate glutathione reductase and are not myelosuppressive. Thus, ACNU could carbamoylate certain proteins in vivo even though it appears to be a weak carbamoylator of L-lysine in the in vitro assay.[12] Furthermore, the nonmyelosuppressive GANU (**432**) and CZT (**368**) were shown[654] to alkylate subnucleosomal sites of bone marrow and cancer cells which are different from those alkylated by the strong myelosuppresive drugs, ACNU (**311**) and CCNU (**137b**) (section X.I).

A strong relationship existed between alkylating activity and the toxicity, as measured by the LD_{10} dose (Table 54), i.e. the strongest alkylators like GANU (**432**) are the most toxic.[200] The more water-soluble drugs, with lower positive log *P* values, were the most toxic, as can be seen by comparing CZT (**368**) and CCNU (**137b**). The inclusion of log *P* values improved the correlation coefficients of both toxicity and alkylating activity.[200]

The anticancer activity, as measured by %ILS values, was not correlated linearly[200] with any of the parameters (Table 54). This fact underscores the difficulty of using in vitro assays to predict in vivo activities. However, a parabolic relationship was shown[200] to exist between alkylating activity and anticancer activity.

The promising C6-CENU monosaccharide MCNU (**531**) (Table 28) was compared[430] with the water-soluble CZT (**368**), GANU (**432**), and ACNU (**311**) and the water-insoluble MeCCNU (**138b**) against the murine tumors L1210 and P388 leukemias, B16 melanoma, Lewis lung carcinoma, and BC-47 rat bladder carcinoma. The anticancer activity of MCNU (**531**) was superior to that of CZT (**368**) and GANU (**432**) and similar to that of ACNU (**311**) and MeCCNU (**138b**).[430] The weight loss caused by the administration of LD_{10} doses of the nitrosoureas was regained most rapidly by the BDF1 female mice given MCNU (**531**).

Amino acids were considered as carriers of the CENU group because of their hydrophilicity and the fact that certain L-amino acids were selectively transported into tumor cells.[458] However, the anticancer activity of the CENU-amino acid analogs (section VII.D) is probably not attributable to a specific membrane transport.[319] The important point about this class of drugs is that the amides (Tables 34−36) are much more active than the corresponding acids and esters. This same activity was extended to various oligopeptides (Tables 37−39). The glycineamide-CENU (acetamido-CNU, **598**) had excellent antileukemic activity,[458] and its cytotoxic activity compared favorably with representatives of other structural classes of nitrosoureas as discussed earlier in this section.

CENU−steroid analogs (section VII.E) were shown[498−500] to bind to the estrogen receptor, but their activities were disappointing. The presence of a free hydroxyl at the C3-position of the aromatic ring was required for binding to the estradiol receptor.[505−509] Among the steroid−amino acid conjugates (section VII.I) the L-alanylestradiol 17 ester (**918**) displayed[628] an activity against a hormone-dependent mammary carcinoma which was greater than that of the corresponding estradiol 3-ester (**917**), estradiol 3,17-ester (**919**), and the estradiol 6-α-ester (**921**) (Table 48). The anticancer activity of the estradiol conjugate **918** was appreciably higher than that of the unlinked CENU−L-Ala (**572**) and of a l:1 mixture of estradiol and **572**.[628,631,632] Furthermore, compound **572**, the active metabolite of estradiol 17-ester **918**, was obtained by an enzymatic hydrolysis.[636] A greatly prolonged plasma half-life of the metabolite **572** could account[636−638] for the effective anticancer activity of the estradiol−L-alanine conjugate **918**.

Nucleosides with the CENU group attached to the 2′-, 3′-, and 5′-positions had disappointingly low anticancer activities (section VII.F) (Table 41). In most cases, there were no direct correlations[521,530] between the cytotoxicity, the half-life, and the alkylating and carbamoylating activities. The lack of activity could be the result of an intramolecular attack of a neighboring hydroxyl group on the urea carbonyl group to form a cyclic urethane and a concomitant loss of the *N*-methyl or *N*-(-2-chloroethyl)-*N*-nitrosourea function before the compounds could reach the cancer cells.[319]

Since it was shown[542] that the combination chemotherapy with 5-FU and CENUs is superior to that using a single agent therapy, a method for the combination of 5-FU and the CENU group by means of attachment to a seconucleoside was developed[542,544,547,548,551,554,556,557] so that two different cytostatic agents, acting by different biochemical mechanisms, could be enzymatically released inside the cancer cells (section VII.F). Some model studies[549] of the acid-catalyzed release of 5-FU from seconucleosides provided support for this structure−activity approach. The parent compound **791** had a high activity against the solid colon 38 adenosarcoma, similar to that of the clinical drug 5-FU.[542] Oxidation of the sulfur moiety in **791** gave the sulfoxide **797** and sulfone **798**. These compounds were found[542] to have an increased activity against the MAC 13 and MAC 15A cancer lines but a loss of activity against the colon 38 adenoscarcoma (Table 42). The substitution of the oxygen atom for the sulfur atom in compounds **805**−**807** increased the activity against the MAC 13, the mammary carcinoma, and an NMU-induced lung tumor but abolished the activity against the colon 38 tumor. The N3-substituted oxygen compounds **819** and **820** were shown[542] to have a very high activity against the ascitic MAC 15A cancer. In general, there was no correlation between the anticancer activity and either water solubility or acid-catalyzed release of the 5-FU moiety (Table 43).[542]

The nitroxyl group can be considered[48,577] a carrier group which can facilitate the transport of the anticancer drug through the cell membrane and across the blood−brain barrier[582] (section VII.G). Also the nitroxyl moiety can affect the relative hydrophobic/hydrophilic properties of molecules.[577] Thus, substitution of the cyclohexyl group of CCNU (**137b**) with nitroxyl moieties produced compounds **861** and **867** which were less hydrophobic than CCNU (Table 44). Compound **861** had a greater antileukemic activity, a lower toxicity, and a greatly improved therapeutic index when compared to those of CCNU.[48] Substitution of the *N*-methyl- and the *N*-(2-chloroethyl) moieties of the monosaccharide analogs SZT (**367**) and CZT (**368**) with nitroxyls yielded[596] the less

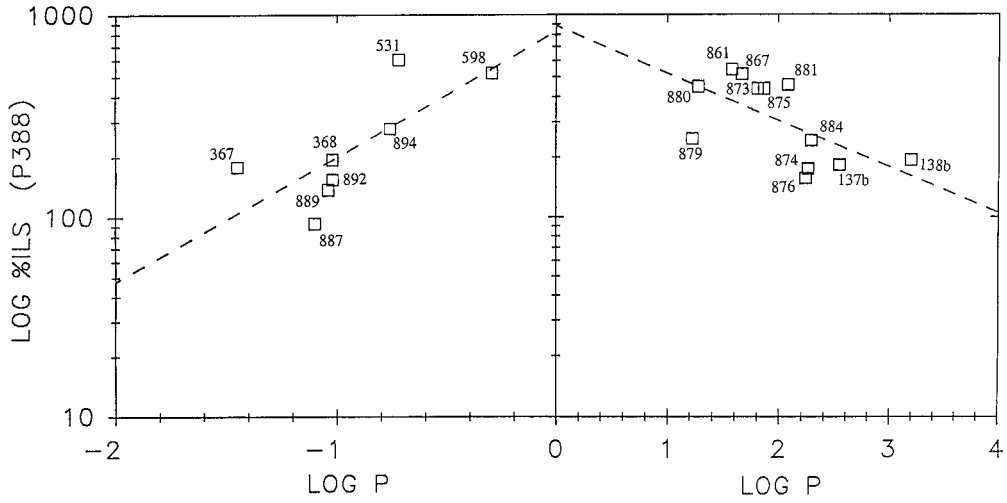


Figure 2. Anticancer activity−lipophilicity correlations of various nitrosoureas. The dotted lines are averaging linear regression lines which are used only to indicate a general trend.

hydrophilic analogs **873** and **875** (Table 45). The activity of **873** against both P388 and L1210 leukemia was superior to that of SZT (**367**) and CZT (**368**). A similar substitution of the CENU-amino acid amides with the (2,2,6,6-tetramethylpiperid-1-yl)oxyl moiety produced[599−601] active analogs **879**−**884** (Table 46). The CENU-glycine nitroxylamide **879** was less hydrophilic, log $P = 1.23$, but also less active than the CENU-glycineamide (**598**), log $P = -0.2$.[599] In the CENU-amino acid nitroxylamide series there was an imperfect correlation of increased cytostatic activity with decreased hydrophobicity when evaluated against the P388 and L1210 leukemias. An important potential use of the nitroxyl derivatives of *N*-nitrosoureas would be the study of their metabolism and tissue distribution by EPR spectroscopy and, in a clinical setting, by magnetic resonance imaging (MRI).[577]

The N3,N3-disubstituted nitrosoureas, also called latent nitrosoureas,[10] have important biological and chemical activities. The simple dialkyl compounds, some of which were shown to be carcinogenic[128,129] (section XI.D), require an in vivo enzymatic dealkylation to the corresponding N3-monosubstituted compounds (Scheme 16) in order to form the active drug. The morpholine analog **64**, a cyclic N3,N3-disubstituted compound, possessed excellent antileukemic activity (Table 3). Certain β-hydroxyethyl analogs, such as **66** and **71**, had both high antileukemic activity and therapeutic indices (Table 3).[133] In these cases, the β-hydroxyl group can undergo a reaction with the ureido carbonyl group to form an *N*-substituted oxazolidinone and no isocyanate (Scheme 17).[10] These compounds were active against the Ehrlich ascites murine carcinoma, whereas the nitrosoureas requiring an enzymatic dealkylation were only weakly active.[133] This same use of N3,N3-disubstituted nitrosoureas was applied to alicyclic compounds (Table 13), to monosaccharides substituted at the C1- (Tables 23 and 24), C2- (Table 21), C3- (Table 27), and C6- (Table 29) positions and to disaccharides (Table 31). In particular, the disaccharide analog **555** was subjected to clinical trials, since it had a very high antileukemic activity of 733% ILS and a high therapeutic index of 83.[446]

The same concept of latency is evident in the CENU-amino acid amides of sarcosine **610** and proline **611** (Table 34).[458] The CENU-sarcosinamide analog **610** had excellent antileukemic activity, the longest half-life, highest optimum dose, and lowest toxicity of the entire set of amino acid primary amides.[458] In this case an enzymatic demethylation may be required to generate the active drug.[458] Such a process is not possible for the proline analog **611** which had, consequently, a much lower in vivo anticancer activity (Table 34).

Because of the importance of lipophilicity in determining the activities of the *N*-nitrosourea class of anticancer drugs, plots of % ILS against log *P* for a wide variety of N3-substituted compounds have been published.[48,465,577,597,599] As can be seen in Figure 2 there are trends toward a maximum of anticancer activity at the minimal log *P* values for both hydrophilic and hydrophobic analogs. Thus, the less hydrophilic carbohydrate, amino acid, and carbohydrate conjugate *N*-nitrosourea analogs and the less hydrophobic alkyl, alicyclic, and heteroalicyclic analogs were found to be the more cytotoxic toward the P388 and L1210 leukemias. The introduction of a nitroxyl moiety was shown[48,465,577,597,599] to have a significant moderating effect on the hydrophobic/hydrophilic properties of these anticancer agents. Thus, the replacement of the cyclohexyl group in CCNU (**137b**) with nitroxyl moieties yielded compounds **861** and **867** which were both less hydrophobic and more cytotoxic in vivo toward the leukemia cell lines.[48] The carbohydrate analogs **873** and **875** containing the nitroxyl moiety were more cytotoxic and less hydrophilic than the drugs SZT (**367**) and CZT (**368**).[596,597] The replacement of the 2-chloroethyl group with nitroxyl moieties in the pseudopeptide **680** resulted in a series of nitroxyl-labeled CENU amino acid analogs **879**−**884** which were much more cytotoxic than the non-spin-labeled analogs.[599,600]

The cytotoxic activity at the cellular activity of five lipophilic CENUs, BCNU (**33**), CNU (**26**), PCNU (**257**), CCNU (**137b**), and MeCCNU (**138b**) against the P388 leukemia cells[655] and 9L rat brain tumor cells[656] in culture was correlated with a dose-function, $\Delta C = C_0 (1 - e^{-k_2 t})$, which is related to the amount of the active species, i.e. the (2-chloroethyl)diazonium ion, formed during exposure to the drugs. In the case of the lipophilic drugs, the passage into the cells is very rapid in vitro so that the concentration of drug

inside the cell equals that of the medium within a few seconds.[657] The activity is not influenced either by the structure of the N3-substituent, the lipophilicity, or the carbamoylating activity.[655,656] The drugs have the same ED_{50} dose of 6−7 μM active species per cell volume but a wide range of carbamoylating activity relative to CCNU (100%). Plots of the ED_{50} C_0 values against the log P values resulted in a hyperbolic curve, with a maximum anticancer activity at log $P = 0.5-0.6$.[655] The related plots of ED_{50} ΔC against the log P values gave a straight line for the hydrophobic compounds.[655] The apparent lower activity of the more lipophilic analogs, BCNU (**33**) and CCNU (**137b**), in the ED_{50} C_0 curves was an artifact of their slower activation rates.[655]

The hydrophilic drug CZT (**368**) required a 2-fold higher effective dose to produce the same toxic effect on the cancer cells in culture as the aforementioned lipophilic drugs **26**, **33**, **137b**, **138b**, and **257**.[655] This decreased activity was attributed[655] to the relatively slow uptake[658,659] of CZT (**368**) into cancer cells relative to the rate of decomposition of CZT to the active alkylating species. Therefore, CZT can undergo a decomposition in the medium to a greater extent than the lipophilic drugs before passage through the cell membrane. However, the decompositions of BCNU (**33**), CCNU (**137b**), and CZT (**368**) in aqueous media gave a 45% yield of 2-chloroethanol. This result indicated[655] that the low activity of CZT was not attributable to competing decomposition reactions.

An important implication of this work is that the structural differences among the lipophilic CENU drugs are not that important, since the plots of ED_{50} values against the log P values, using the dose−function ΔC, gave very similar curves for all of these drugs. Hence, attention was shifted to the concentration of the alkylating species inside the cell rather than on the structure of the parent drug which is not cytotoxic itself. The observation that the lipophilic CENU analogs have the same activity at the cellular level leads to the conclusion[655,656] that the observed differences in the in vivo activity are attributed primarily to differences in biodistribution. An example of this effect is the conjugate estradiol-17-CENU-L-Ala (**918**) (section VII.I). As discussed earlier, the conjugate had a 3-fold longer plasma half-life, three times larger volume of distribution, three times faster distribution following the iv-administration, and a two times faster absorption after peroral administration.[636−638] The CENU-L-Ala (**572**) is the active metabolite of the conjugate, and when generated from the conjugate it had a longer half-life than when it was applied directly.[636] This fact implies[636] that there is a continuous release of the metabolite. The presence of a nitroxyl moiety in the CENU structure can also change the biodistribution of the drug by changing its relative hydrophilic/hydrophobic properties.[577]

In recent reviews[10,16,660] the emphasis was on those *N*-nitrosoureas which have been the subject of clinical trials.

IX. Mechanisms of Decomposition

The chemistry of the (chloroethyl)nitrosoureas is complex and has been the object of intensive study for at least 25 years. The literature through 1980 was covered in an excellent review.[3]

The stability of (chloroethyl)nitrosoureas is pH dependent. Thus, for example, BCNU (**33**) is relatively stable at pH 4.5, with a half-life of greater than 500 min, whereas at pH 7.4 and 8 its half-life is 50 and 5 min, respectively.[3,661,662] In highly acidic solutions (pH < 2), the compounds decompose in a matter of seconds.[663] Consequently, on the basis of this pH dependence, it was found that alkylnitrosoureas undergo both acid (pH < 2)[663] and base (pH > 5)[664,665] catalyzed mechanisms of decomposition. In the pH 3−5 range, the rate of decomposition increases as the pH decreases which is a characteristic of general acid catalysis.[665] In general the decompositions at pH > 5 are attributable to a general base catalysis.[664] The rate of decomposition of BCNU (**33**) was shown to increase as the pH was increased from 4.4 to 8.0.[666−668]

Direct current (DC) and differential pulse polarographic analyses were used[669] to measure the rates of decomposition of a series of (2-haloethyl)nitrosoureas in aqueous solution. In the 1,3-bis(haloethyl)-1-nitrosourea series, i.e. compounds BCNU (**33**), BFNU (**34**), BBNU (**36**), and BINU (**38**), the nature of the halogen atom had only a small effect on the rate of decomposition. The substitution of the β-chloro atom in CCNU (**137b**) with the amino moiety or methoxy moiety resulted in a large decrease in the decomposition rate. *N*-Nitrosoureas containing a free NH_2 moiety at the N3-position, i.e. MNU (**25**), CNU (**26**), ENU (**27**), decomposed much faster than any of the N3-monosubstituted analogs.[669]

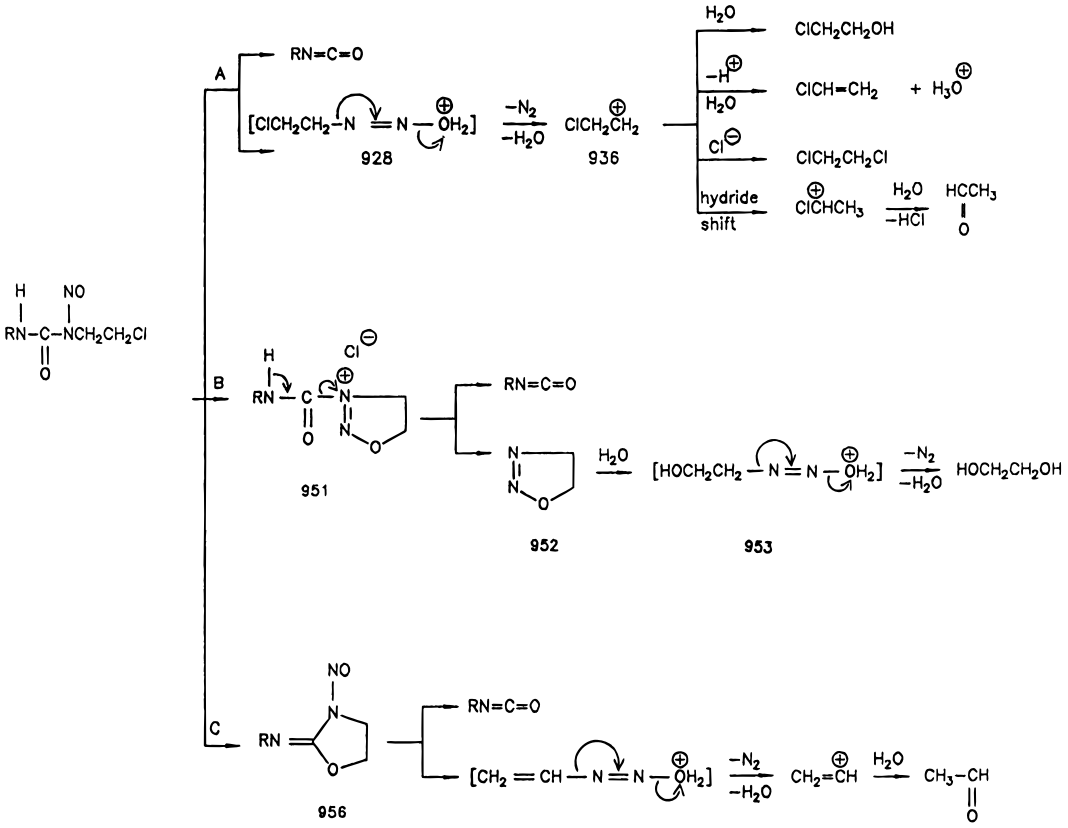
Much attention has been devoted[3,661,662] to the mechanism of decomposition of *N*-nitrosoureas at physiological pH = 7.4 and higher pH because of its pertinence to an understanding of the anticancer properties in vivo. Various mechanisms have been proposed to explain the activation of the nitrosoureas for reactions with the purine and pyrimidine bases of DNA. Three mechanistic pathways, A−C, for the chemical decomposition of *N*-nitrosoureas at pH > 5 are outlined in an idealized scheme (Scheme 91). Evidence for each pathway will be discussed in details in the following sections.

A. Pathway A

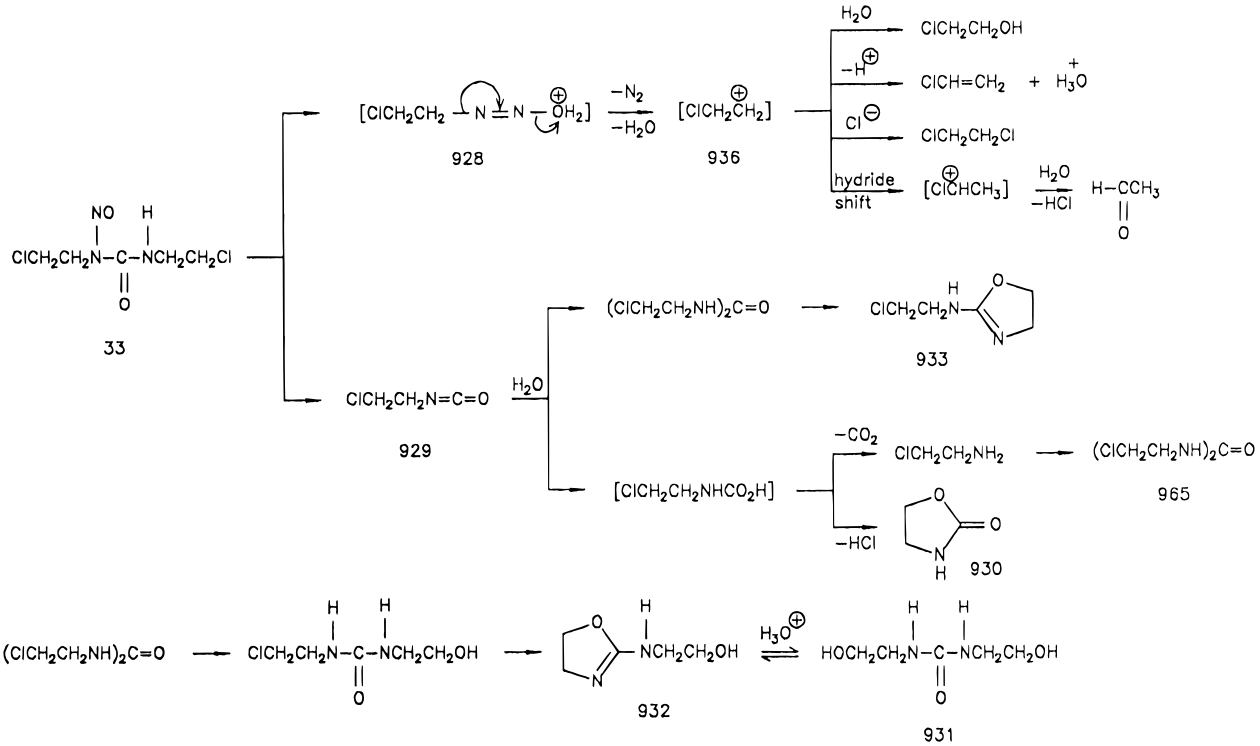
This pathway, characterized by an initial decomposition to a diazohydroxide and an organic isocyanate, is considered to be the chief route under physiological conditions (Scheme 14). The acidity of the hydrogen (pK_a 8−9) at the N3-nitrogen in nitrosoureas renders it liable for abstraction by a base.

The isocyanate intermediate is the carbamoylating species which can react with amino acids and proteins and/or RNA in vivo.[73] The diazohydroxide intermediate is the alkylating species which is considered to be chiefly responsible for the anticancer activity of CENU compounds by reactions with the DNA[72,97] (sections X.A and B). Because of its importance the nature of the alkylating species has been the subject of much research and discussion.[670] Evidence for a methyldiazonium ion in the decomposition of 1-methyl-1-nitroso-3-nitroguanidine (MNNG, **24**),[671] 1-methyl-1-nitrosourea (MNU, **25**),[672,673] and 1-ethyl-1-nitrosourea (ENU, **27**)[674] were reported. Furthermore, evidence against the forma-

Scheme 91



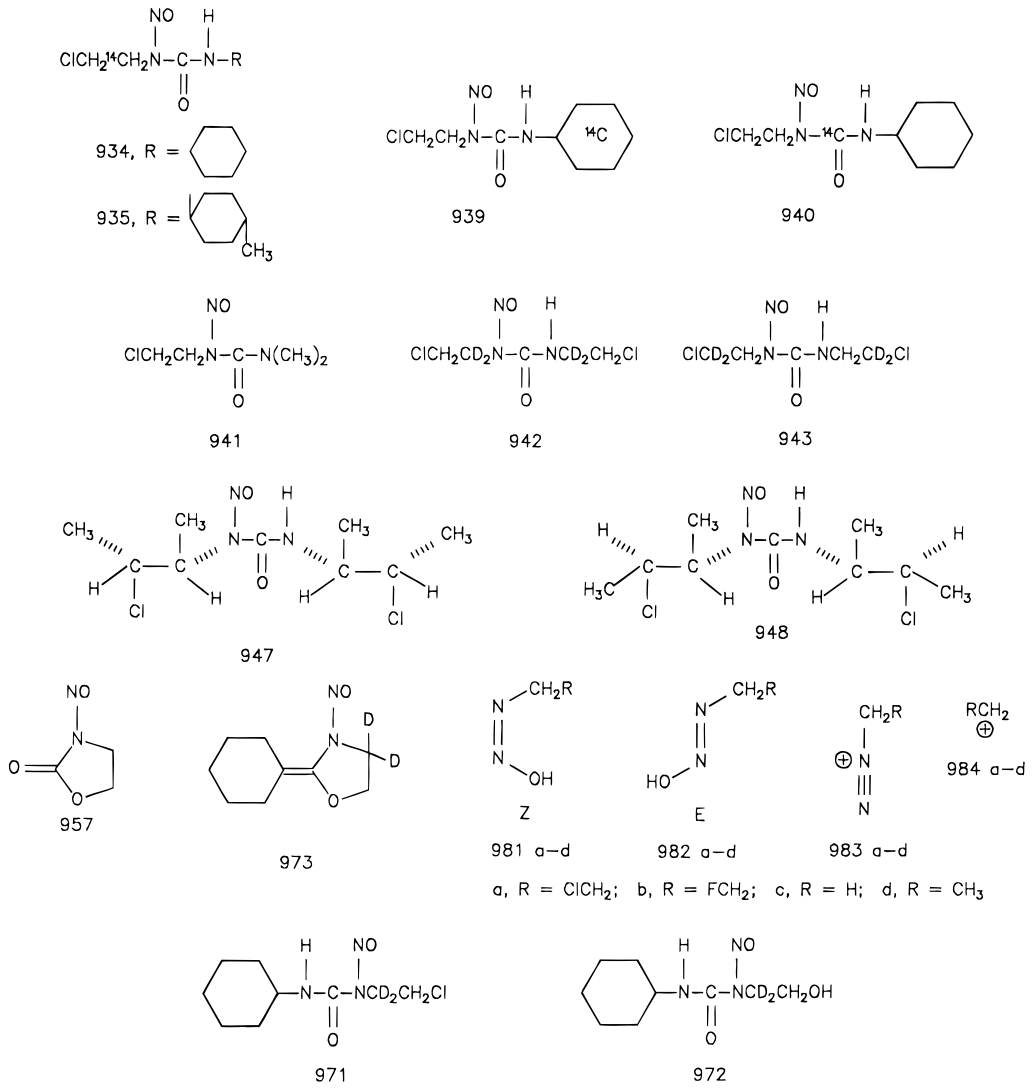
Scheme 92



tion of diazoalkanes under physiological conditions was reported[3] for *N*-alkyl-*N*-nitrosoureas, *N*-alkyl-*N*-nitrosoamides, *N*-alkyl-*N*-nitrosocarbamates, and *N*-nitrosourethanes.

The decomposition of bis(2-chloroethyl)-*N*-nitrosourea (BCNU, **33**) by the pathway A, as outlined in an idealized scheme (Scheme 92), leads to a surprisingly large number of products from both the diazohydroxide **928** and isocyanate **929** intermediates.[645,646,670,675] An early study[646] of ^{14}C-labeled BCNU (**21**) revealed that about 50% of the reactivity was present as nonvolatile material and this fraction was analyzed at a later date. The volatile products were analyzed by gas chromatography−mass spectrometry (GC−MS)[645,646,670,675] and the nonvolatile products by gas chromatography coupled with isobutane chemical ionization mass spectrometry (CI-MS).[645] A representative product mixture of BCNU

Chart 26. Structures for Section IX



decomposition at pH 7.4 and 37 °C has been analyzed[645] as follows: acetaldehyde (13−20%), 2-chloroethanol (25−40%), vinyl chloride (2−4%), 1,2-dichloroethane (1−2%), 2-oxazolidine (**930**, 26−33%), 2-chloroethylamine (11−14%), 1,3-bis(hydroxyethyl)-urea (**931**, 2%), 2-[(2-hydroxyethyl)amino]-2-oxazoline (**932**, 2%), 2-[(2-chloroethyl)amino]-2-oxazoline (**933**, 12%), and unreacted BCNU (**33**, 19%).

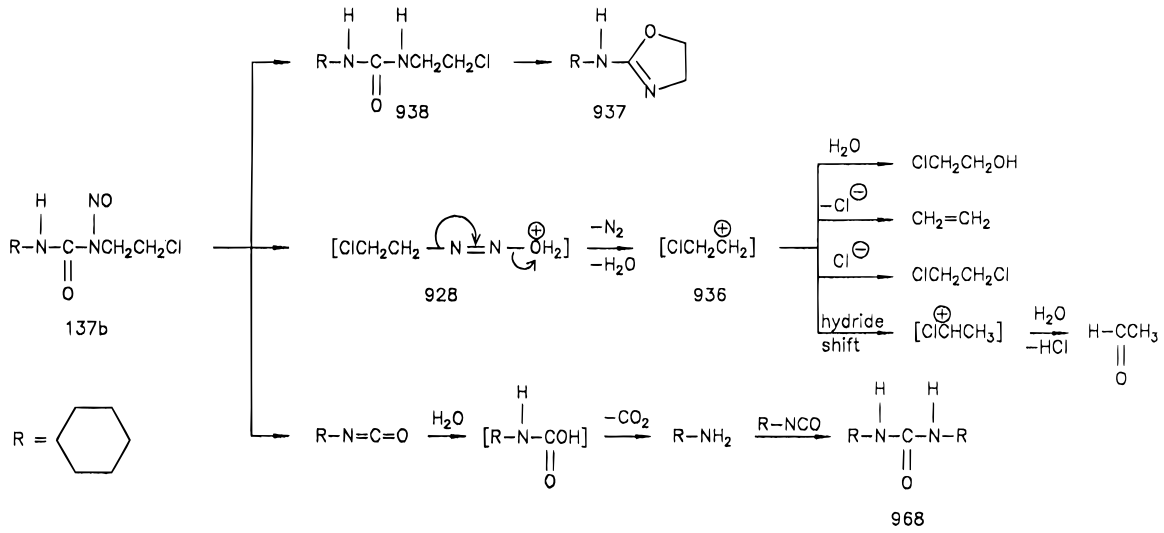
In an early study[670] was observed the formation of acetaldehyde, but not 2-chloroethanol, from the buffered decompositions of BCNU. This result led to the proposal[670,675] that the alkylating species was a vinyl cation derived from an ethylenediazohydroxide. In later studies[132] with [^{14}C]BCNU (**21**), [^{14}C]CCNU (**934**), and [^{14}C]MeCCNU (**935**, Chart 26) it was shown that a large quantity of 2-chloroethanol as well as acetaldehyde, were present among products of the decomposition in aqueous media, and a 2-chloroethyl carbocation (**936**, Scheme 92) was proposed[132,676] as the important alkylating species.

Nitrosation of 2-chloroethylamine hydrochloride by sodium nitrite in an aqueous solution, resulted[132] in a mixture of 2-chloroethanol, vinyl chloride, and ethylene in yields similar to those observed during the degradation of CCNU at pH 7.4. Presumably, a 2-chloroethyldiazonium ion (**928**) and/or 2-chloroethyl carbocation (**936**) are the reactive intermediates in both cases.[132]

The aqueous decomposition products of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU, **137b**) (Scheme 93) at pH 7.4 and 37 °C in aqueous media were determined[645,676] by GC−MS, high-pressure liquid chromatography (HPLC), and reverse isotope dilution analysis. The major products were found[645] to be as follows: 2-chloroethanol (18−25%), acetaldehyde (5−10%), followed by vinyl chloride (1−3%), ethylene (1−3%), cyclohexylamine (32%), 2-(cyclohexylamino)-2-oxazoline (**937**, 3−5%), 1-(2-chloroethyl)-3-cyclohexylurea (**938**, 3−5%), and cyclohexylisocyanate. When [^{14}C]chloroethyl CCNU (**934**) was incubated in phosphate buffer at 37 °C, 20−25% of the radioactivity was found in the 2-[^{14}C]chloroethanol which was extractable into hexane and ether.[132,676] Similarly, incubation of the (cyclohexyl-1-^{14}C)CCNU (**939**) and (carbonyl-^{14}C)CCNU (**940**) resulted in 65−70% of the ^{14}C in the nonextractable portion. Further analysis revealed[676] that 30−35% of the cyclohexyl moiety of CCNU had degraded to cyclohexylamine, presumably via cyclohexylisocyanate (Scheme 93). Similar product mixtures were found[677] with other alkylnitrosoureas.

The observation that the decomposition of 1-(2-chloroethyl)-3,3-dimethyl-1-nitrosourea (**941**, Chart 26) in aqueous solution yielded[132] only acetaldehyde, but not the other volatile products, supports the requirement of a hydrogen on N3 for the decomposi-

Scheme 93



tion to a diazohydroxide and an isocyanate (pathway A, Scheme 92). Compound **941** also is nontoxic to L1210 leukemia cells in vitro. The fact that the aqueous decomposition of the monosubstituted (2-chloroethyl)nitrosourea **26** failed to generate an organic isocyanate, but the compound **26** was, nevertheless, a very active anticancer agent in vitro and in vivo, led to the conclusion[132] that it is either a 2-haloethyl carbocation or a similar alkylating species, and not the organic isocyanate, which is responsible for the anticancer activity of **26**. Further support for a 2-haloethyl carbocation intermediate was provided by a study[678] of the decomposition in aqueous media at pH 7.4 of BCNU-α,α′-d_4 (**942**) and BCNU-β,β′-d_4 (**943**). The decomposition of **942** via a vinyl carbocation (path a, Scheme 94) was expected to yield a vinyl chloride with one D and an acetaldehyde with one D, while the actual results based on a chloroethyl carbocation (paths c and d) included a vinyl chloride with two D and an acetaldehyde with no D. A diazoalkane intermediate (path b) should lead to a chloroethanol and dichloroethane containing only one D while the experimental analysis resulted in 2-chloroethanol and 1,2-dichloroethane both with two D (path c). A similar analysis was obtained[678] by the use of compound **943** (paths e and f, Scheme 94). Since the isolated 2-chloroethanols **944** and **945** were obtained in a 90% unrearranged form from both compounds **942** and **943** it was proposed[678] that these products arose from an S_N2 attack of water on the 2-chloroethyldiazohydroxide and not by a S_N1 mechanism.[645] The 10% of rearranged 2-chloroethanol products **945** and **944** could arise from a cyclic chloronium ion **946** (Scheme 94).[678] The S_N2 and E2 character of reactions of primary alkanediazohydroxides was demonstrated[679] by the fact that the decomposition of 1,3-bis(*erythro*-3-chloro-2-butyl)-1-nitrosourea (**947**, Chart 26) yielded predominantly *threo*-3-chloro-2-butanol and *cis*-2-chloro-2-butene while the decomposition of 1,3-bis(*threo*-3-chloro-2-butyl)-1-nitrosourea (**948**) yielded mostly the *erythro* butanol and the *trans*-butene.

A further study[680] of the aqueous decomposition of BCNU-β-β′-d4 (**943**) revealed different product mixtures at pH 7.4 and at pH 5.0. Thus, at pH 7.4 the main products were acetaldehyde (19−21%), 2-chloroethanol (51−53%), and ethylene glycol (2%), whereas at pH 5 the mixture was acetaldehyde (58−59%), ethylene glycol (25%), and 2-chloroethanol (6%). In this case, the acetaldehyde was removed as quickly as it was formed and not allowed to exchange either the hydrogen or deuterium with water. The resulting acetaldehyde, CH_2DCDO, at both pH 7.4 and 5.0, contained two D, one on the aldehydic carbon and one on the methyl carbon (path f, Scheme 94). This result lends strong support for a 1,2 hydride shift in the 2-chloroethyl carbocation to yield the acetaldehyde (Schemes 92 and 93). Additional evidence for a 1,2 hydride shift was provided[132] by the isolation of some 1,1-dichloroethane, not shown in Schemes 92 and 93, which could arise from the interaction of the chloride ion with the rearranged secondary carbocations **949** and **950** in Scheme 94, paths d and f.

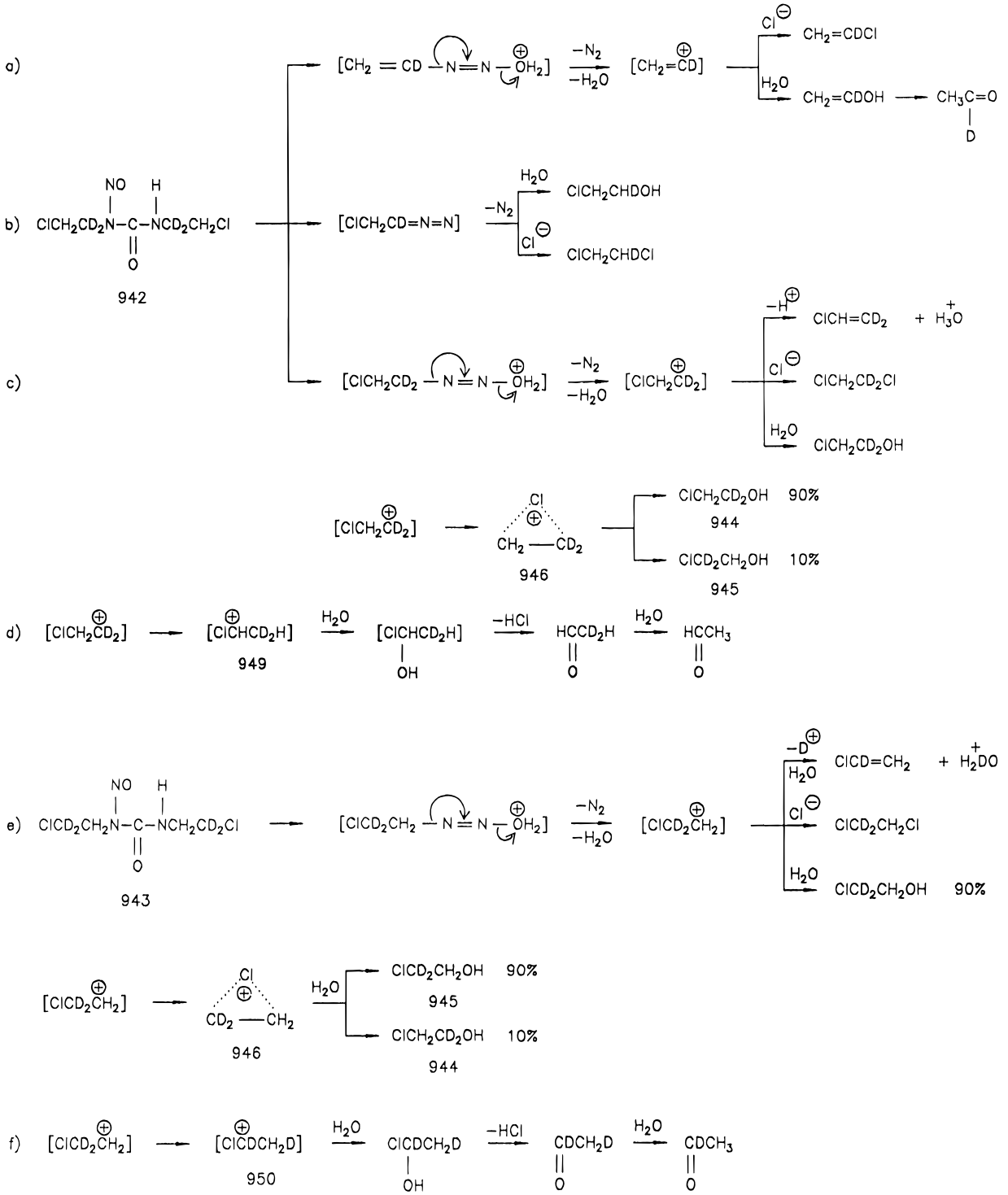
The decomposition products of 1,3-bis(2-chloropropyl)-1-nitrosourea (BCNU-β-CH_3, **41**) and 1,3-bis[1-(chloromethyl)ethyl]-1-nitrosourea (BCNU-α-CH_3, **40a**) were interpreted[670] to support a decomposition mechanism involving a diazohydroxide and a cyclic chloronium ion for **41** and the diazohydroxide and/or 2-chloro-1-methylethyl carbocation for **40a**, analogous to Scheme 91, pathway A. Also the presence of a 1,2,3-oxadiazoline intermediate could explain the observed decomposition products.

The product composition at pH 7.4 can be explained by a pathway A mechanism but the large amount of ethylene glycol formed at pH 5 led to the proposal[680] of a different decomposition mechanism, pathway B (Scheme 91).

B. Pathway B

In this minor decomposition mode[132,680] of (2-chloroethyl)nitrosoureas the nitroso group is involved in an intramolecular displacement reaction which yields a substituted 1,2,3-oxadiazolidine (**951**, Scheme 95). Further fragmentation would lead to the reactive intermediate 4,5-dihydro-1,2,3-oxadiazole (**952**) which could fragment in water to 2-hydroxyethyldiazohydroxide (**953**). The latter intermediate could be degraded via the 2-hydroxyethyl carbocation to ethylene oxide, ethylene glycol, and acetaldehyde, as shown in Scheme 95. An oxadiazole intermediate

Scheme 94



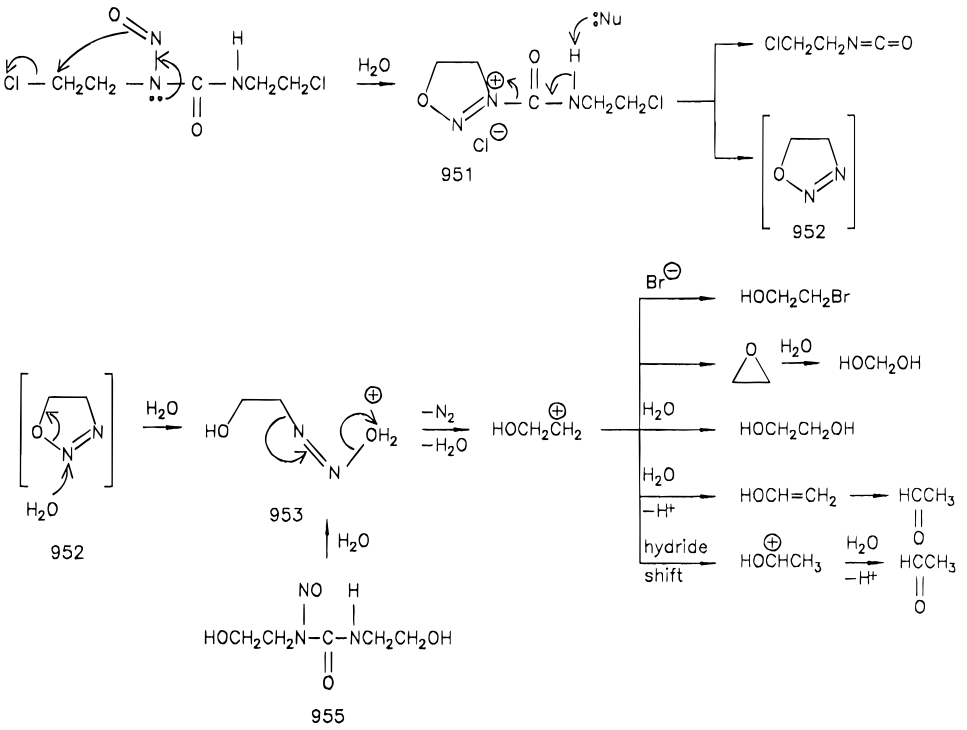
(**952**) was proposed earlier[665] in a mechanism for a decompositions of nitrosoureas such as BCNU (**33**), BFNU (**34**), CCNU (**137b**), MeCCNU (**138b**), SZT (**367**), and CZT (**368**).

The anchimeric assistance of the nitroso group for a displacement of a leaving group to form a 1,2,3-oxadiazoline is supported[681] by the facile reaction of the nitrosamine **954** (Scheme 96).

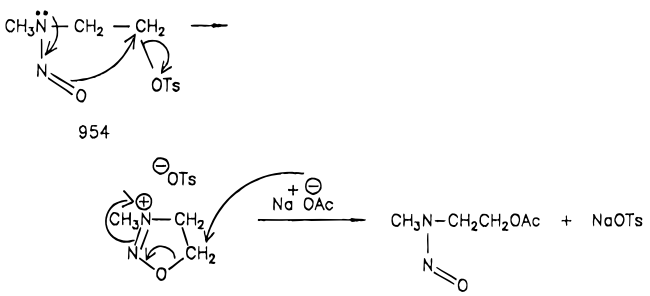
The decomposition of bis(hydroxyethyl)-*N*-nitrosourea (**955**) also yielded ethylene glycol and acetaldehyde as major products at pH 7.4 (Scheme 95). These results support[680] a 2-hydroxyethyldiazohydroxide intermediate **953**. An addition of bromide resulted[680] in the formation of 2-bromoethanol as a major product at pH 5, but not at pH 7.4, lending support to the oxadiazole **952** (Scheme 95).

This shift in decomposition mechanism is attributable to the fact that the rate-limiting reaction to form 2-chloroethyldiazohydroxide (pathway A) is base catalyzed and at lower pHs becomes slower than cyclization (pathway B). The oxadiazole **952** probably has no anticancer effect since compound **941**, which fragments to acetaldehyde and ethylene glycol at pH 7.4 according to Scheme 97, is not cytotoxic to L1210 leukemia cells.[680] Thus, this cyclization mechanism plays only a minor role in the decomposition of CENUs at physiological pH.

Scheme 95



Scheme 96



C. Pathway C

Another decomposition mechanism involves the intramolecular cyclization of the carbonyl oxygen and displacement of halogen to produce 2-(alkylimino)-3-nitrosooxazolidines **956** (Scheme 91). In earlier work[669] the decomposition rate for the *N*-nitrosooxazolidinone (**957**, Chart 26) was much faster than that of the CCNU analogs **137a** and **137b**, and it could not be determined from the electrochemical data whether 2-imino-*N*-nitrosooxazolidinone **956** was an intermediate in such decompositions. The synthesis[682] of the intermediates **958**−**960** related to CCNU, MeCCNU, and BCNU is shown in Scheme 98. A base-catalyzed nitrosation of the corresponding 2-(alkylamino)oxazolines **933**, **937**, and **961** was chosen[682] to avoid conditions that would destroy the products. The desired products **958**−**960** were separated from the other *N*-nitroso isomers **962**−**964** by fractional crystallization.

The reactions of **959** in dry hydrogen chloride and in aqueous phosphate buffer at pH 7.2 are detailed in Scheme 99.[682] The products were identified by their ^{13}C and CIMS spectra and also by the synthesis of authentic samples.[682]

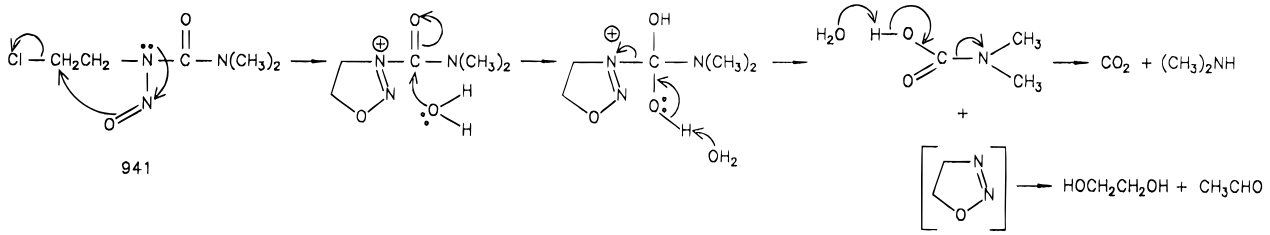
Interestingly, the reactions of **959** and **960** in anhydrous hydrogen chloride gave CCNU **137b** and MeCCNU **138b**, respectively.[682] This result indicates that **959** and **960** could be intermediates in the decomposition of **137b** and **138b**. However, the decomposition of **958** under the same acid conditions, produced only denitrosated products **933** and bis(2-chloroethyl)urea **965** (Scheme 92). This result indicates that the decomposition of BCNU **33** probably does not proceed through the cyclized intermediate **958**.[682]

Decomposition of **959** in aqueous solution at pH 7.2 resulted[682] in the formation of acetaldehyde and the nonvolatile products represented by the carbamate **966**, the two ureas **967** and **968**, 2-oxazolidinone **930**, and the denitrosated starting material **937** (Scheme 99a). The compounds **958** and **960** in phosphate buffer at pH 7.2 and 37 °C decomposed to the corresponding range of products as was obtained for **959**. The decomposition of compound 3-cyclohexyl-1-(2-hydroxyethyl)-1-nitrosourea (**969**) gave the same product mixture as **959** under the same conditions (Scheme 99b).[682]

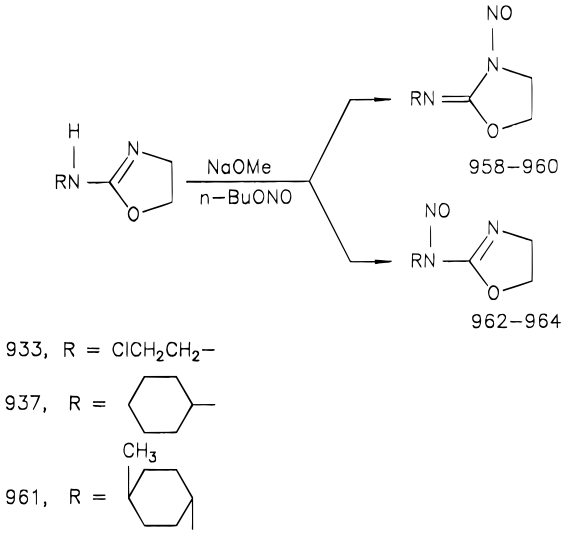
A mechanism that explains[682] many of the products formed from 2-(cyclohexylimino)nitrosoxazolidine (**959**) is shown in Scheme 100. A ring opening of the **959** with hydrogen chloride yields CCNU (**137b**) and, with water, the 2-hydroxyethyl analog **969**. Denitrosation of **959** would give **937**. Decomposition of **969** would generate the denitrosated product **967** and the 2-hydroxyethyldiazohydroxide **953** which can decompose to a variety of products (Scheme 100a). Alternatively, **959** could ring open either with hydrogen chloride or water to give the carbamate diazohydroxide **970** which could degrade to the isolated 2-hydroxyethyl *N*-cyclohexylcarbamate **966** (Scheme 100b).[682]

The intermediate 2-hydroxyethyldiazohydroxide (**953**) from both the 4,5-dihydro-1,2,3-oxadiazole **952** (Scheme 95) and 2-(alkylimino)-3-nitrosooxazolidine **959** (Scheme 100) could account for the formation of hydroxyethylated nucleosides which were isolated

Scheme 97



Scheme 98



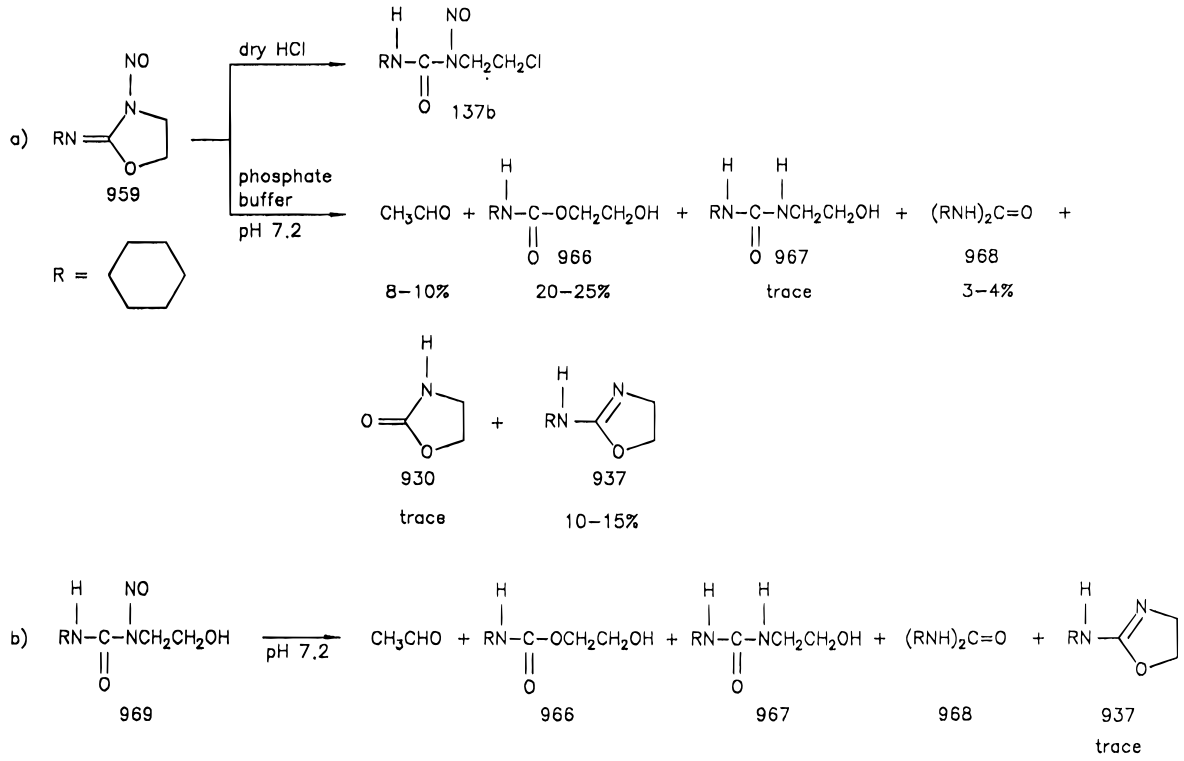
from the reaction of (2-haloethyl)nitrosoureas with polynucleotides (section X.A).

More details on the proposed decomposition of the 2-(cyclohexylimino)-3-nitrosooxazolidine **959** were provided[683] by a study of the decomposition reactions of the deuterated compounds **971**−**973** (Scheme 101a,b). Incubation of **971** in phosphate buffer or incubation of **972** and **973** with the addition of chloride ion to the buffer resulted in a set of common products, including deuterium-free acetaldehyde, a mixture of two deuterio-2-chloroethanols **944** and **945**, the deuterated 2-hydroxyethyl cyclohexylcarbamates **974** and **975**, vinyl chloride containing one or two deuterium **976** and **977**, and the denitrosated starting materials **934**, **978**, and **979**.

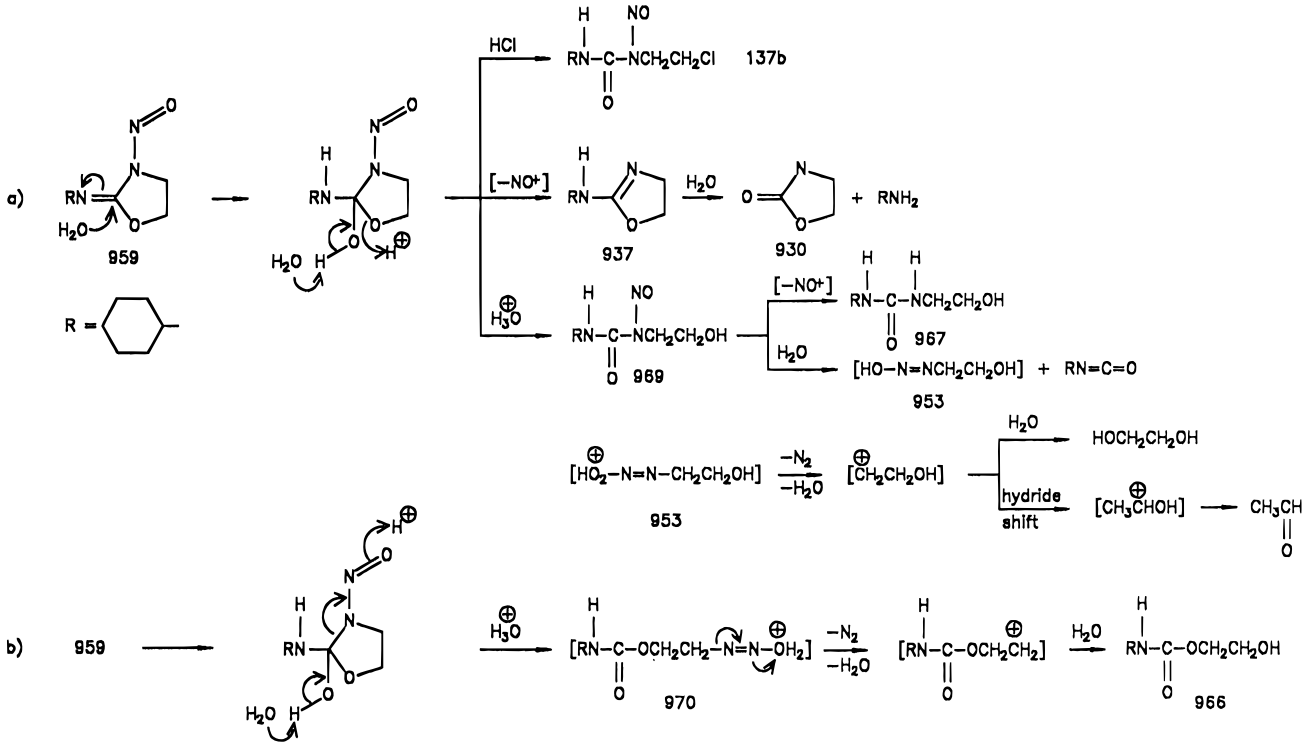
On the basis of the foregoing, two pathways for CCNU (**137b**) decompositions were proposed.[683] The first major pathway involves a direct decomposition of **971** as detailed in Scheme 101. The second minor pathway involves a reversible conversion of **971** to **973** followed by a ring opening of the latter to **972**, as shown in Scheme 101. The vinyl chloride could arise from **971** and **973** (Scheme 101a). The two carbamates **974** and **975** could form directly from **973** (Scheme 101a) or from intramolecular reactions of **972** (Scheme 102).

An interesting set of experiments were conducted[684] to determine the relative importance of the pathways A−C (Scheme 91) for the decomposition of BCNU (**33**). The compound BCNU-β-β'-d_4 (**943**) was hydrolyzed in 99% $H_2{}^{18}O$ at pH 7.1 and 25 °C and the acetaldehyde produced was immediately reduced to ethanol with liver alcohol dehydrogenase and NADH in order to avoid any $^{18}O-{}^{16}O$ exchange in the carbonyl group (Scheme 103). A GC−MS analysis of the D and ^{18}O labels revealed that the ethanols

Scheme 99



Scheme 100



$CH_2DCDHOH$ and CH_3CDHOH contained 79 ± 10% of ^{18}O and 21 ± 10% of ^{16}O. Since the incorporation of the external ^{18}O would occur by pathway A and/or *C* but not by B, then the relative proportions of the pathways are A + C/B = 79:21 (Scheme 103a).[684]

The decomposition of the hindered BCNU analog **40a** (Scheme 103b) under the same conditions as **943** produced propanol containing 11% ^{18}O and 89% ^{16}O. This result indicated that the B pathway was favored by a 89:11 ratio.[684] In this case, the intramolecular cyclization to a 1,2,3-oxadiazole intermediate is highly favored because of steric hindrance. As a check of these results the decomposition of the N=^{18}O labeled compound **980** in $H_2{}^{16}O$ was investigated (Scheme 103c). There were obtained the propanols $CH_3CDHCDHOH$ and CH_3CH_2CDHOH containing 88 ± 10% ^{18}O and 12 ± 10% ^{16}O, in support of the preponderance of pathway B by a 88:12 ratio.[684]

The observation of the $CH_2DCDHOH$ ethanol and the $CH_3CDHCDHOH$ propanol products indicated that the 1,2,3-oxadiazole intermediate may decompose partially by a concerted pathway requiring a 5 to 4 deuterium shift[684] (Scheme 104).

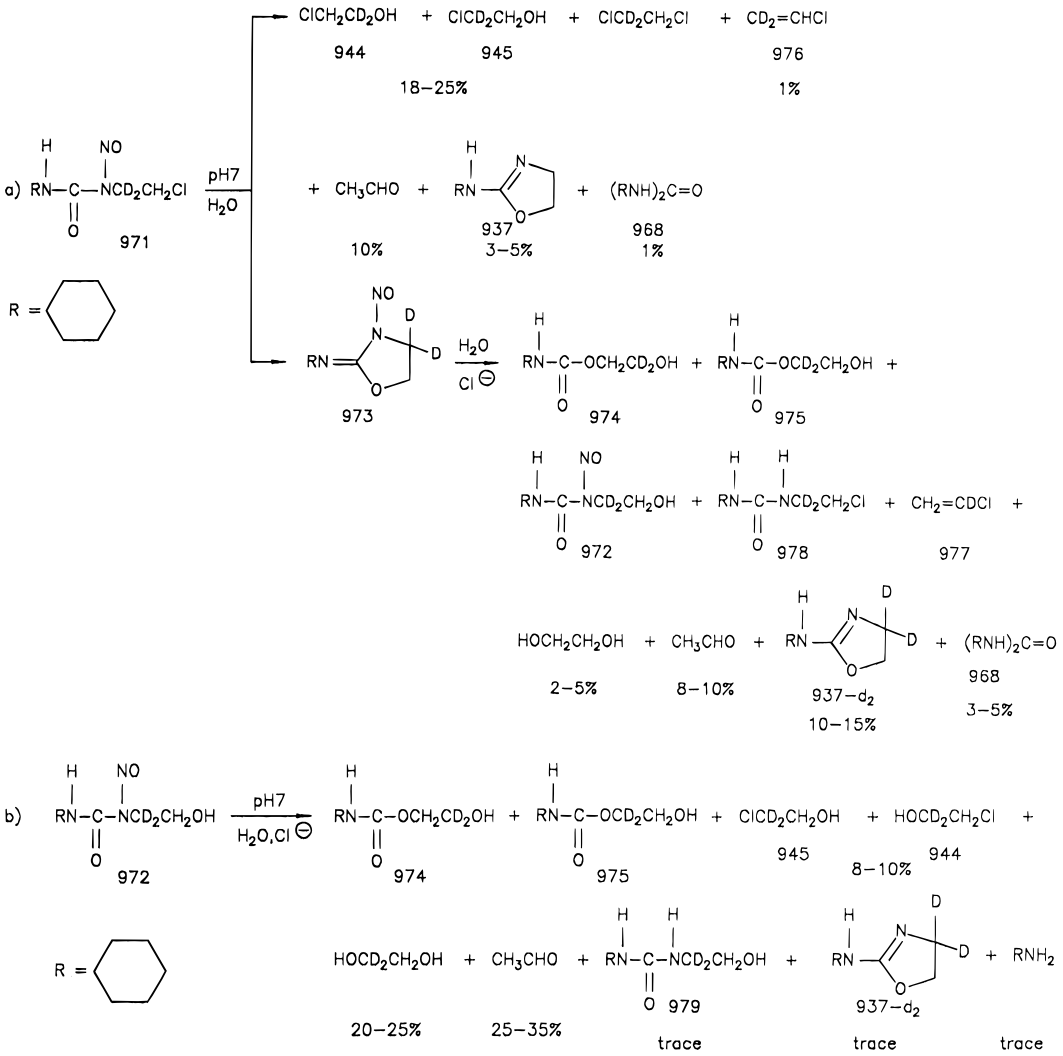
Thus, it appears that the structure of the (2-chloroethyl)nitrosoureas, especially the substituents on the N3-nitrogen, can influence the mode of decomposition under physiological conditions.[319]

The nature of the ultimate electrophilic species from the decomposition of nitrosoureas has been the subject of much study. The three species considered as likely candidates for the carcinogenic nitrosamines, the mutagenic nitrosoamides and the cytotoxic nitrosureas are the (*Z*)- and (*E*)-alkyldiazohydroxides **981** and **982**, the alkydiazonium ions **983**, and the corresponding carbocations **984** (Chart 26). Clearly, the diazohydroxide form is involved in the ^{18}O-labeling experiments[684] via the pathways A, B, or *C* (Scheme 103), whereas the alkyldiazonium ion **983** is not involved in the route B because the oxygen of the 1,2,3-oxadiazoline is derived from the original nitrosourea.[684] A carbocation **984** is ruled out by several lines of evidence. First, less than 10% of the decomposition products of the deuterated BCNU analogs **942** and **943** (Scheme 94) under physiological conditions correspond to a rearrangement involving a hydride shift in an intermediate carbocation[678] and second, because the alkylation by 1-*n*-propyl-1-nitrosourea (**28**) of calf thymus DNA in vitro produced 90−93% unrearranged guanosine N7-alkylated product **985a** and 72−77% unrearranged guanosine O6-alkylated product **986a** (Scheme 105a).[685] This result strongly supports an S_N2 process via an alkane diazohydroxide (Scheme 105b) and is against an S_N1 process via a free carbocation (Scheme 105c).[685]

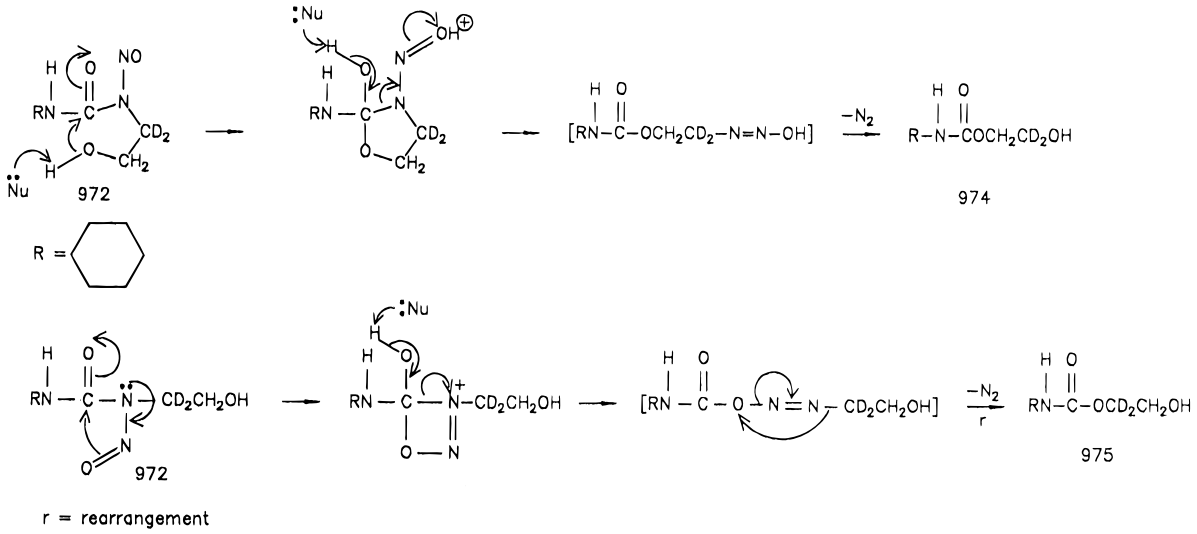
The study[64] by ^{15}N and ^{13}C NMR spectroscopy of the decomposition of specifically ^{15}N- and ^{13}C-labeled (2-chloroethyl)nitrosoureas **987**−**991** (Chart 27) in aqueous phosphate-buffered media provided important new evidence about the reaction mechanisms.[64,686] Thus, the ^{13}C NMR spectra of these compounds revealed the existence of four transient tetrahedral intermediates A−D. In all cases, the nitroso group is positioned *syn* to the chloroethyl side chain. Only conformers C and D are shown in Scheme 106. In these conformers the one pair of electrons on the oxygen and nitrogen heteroatoms are antiperiplanar to the bond being broken. This element of stereoelectronic control allows the formation of the (*Z*)-chloroethyldiazohydroxide **981a** from conformer *C* and the *E* isomer **982a** from conformer D. The conformer D is also stabilized by a hydrogen bond between the NH and the N=O groups which would favor formation of the (*E*)-diazohydroxide.[64]

A variety of products can be accounted for by the further decomposition of the intermediates **981a** and

Scheme 101



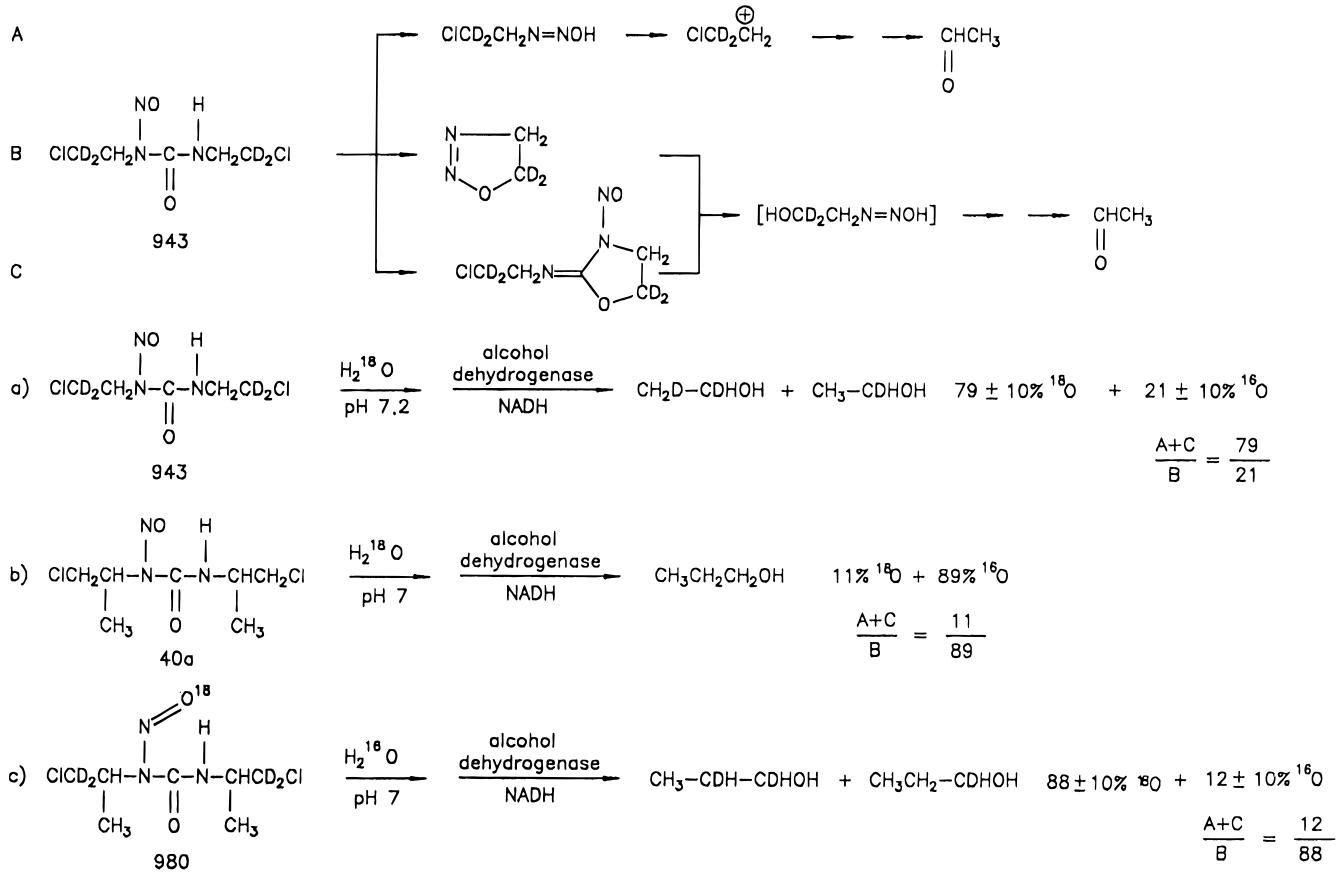
Scheme 102



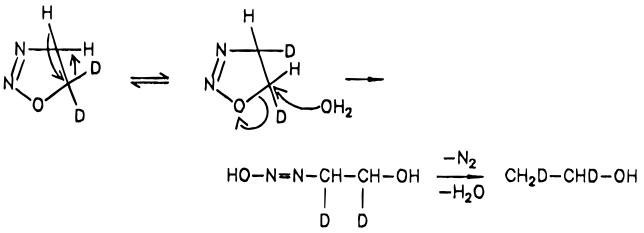
982a.[64] In the case of the *syn* (*Z*) intermediate **981a** the groups are correctly aligned to afford the 1,2,3-oxadiazole **952** by the intramolecular displacement of chloride (Scheme 106a). Compound **952** was previously suggested[665,680] as a reactive intermediate precursor for the formation of acetaldehyde and ethylene glycol.

A further sequence of steric changes can occur (Scheme 106b)[64] by a suitable bond rotation of the 2-chloroethyl side chain to be positioned for bond formation with the oxygen anion at the tetrahedral center leading to the oxazolidine-type intermediates **992** and **993**. In the case of **992**, the lone-pair orbitals of the two heteroatoms are correctly aligned

Scheme 103



Scheme 104



to promote the cleavage of the C2−N3 bond of the oxazolidine to give the alkylcarbamate diazohydroxide **970** which, in turn, can be converted by loss of molecular nitrogen to the carbamate **966** in Scheme 100. The antiperiplanar alignment of the lone-pair orbital of oxygen and the hydrogen bonding in **993** would lead to a cleavage of the 1,2-oxazolidine bond, and formation of compound **969** which had been shown[682] to decompose to acetaldehyde, ethylene glycol and **966**, among other products (Schemes 99b and 100). Intermediate **993** also may decompose to the 2-(alkylamino)oxazoline **937** (Scheme 106).[64] Evidence for the tetrahedral intermediates was provided[64] by ^{18}O exchange of the carbonyl group of BCNU (**33**). Such intermediates have been proposed for the fragmentation of alkylnitrosoureas in aqueous solution.[687,688]

A study[689] of the hydrolysis of BCNU (**33**) and CCNU (**137b**) in H_2O and D_2O at pH 7 and 37 °C revealed a large primary hydrogen isotope effect, i.e., $k_H/k_D = 6.04$, and in 1:1 H_2O/D_2O $k_{HD}/k_D = 4.05$. On the basis of this evidence the rate-limiting step in the mechanism A of Scheme 91 is the addition of

Scheme 105

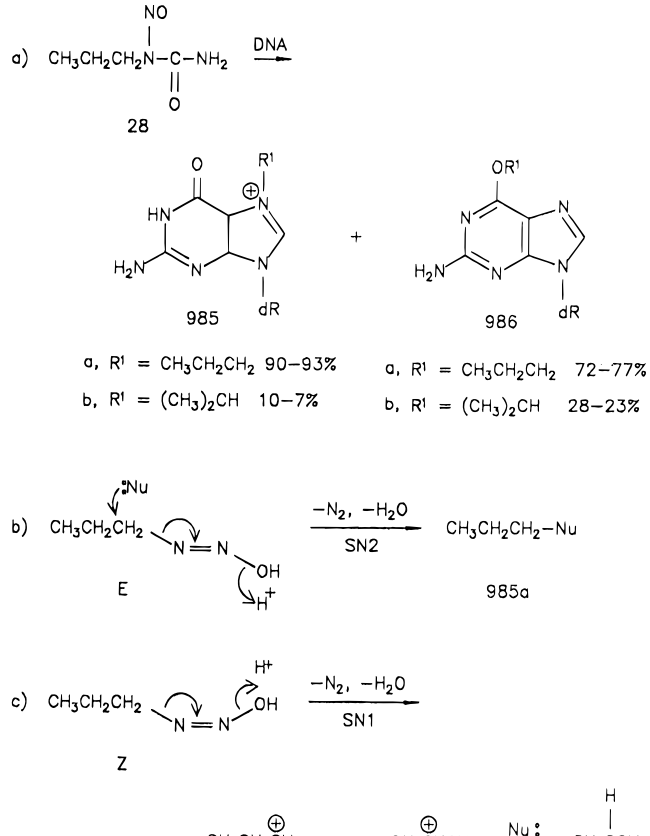
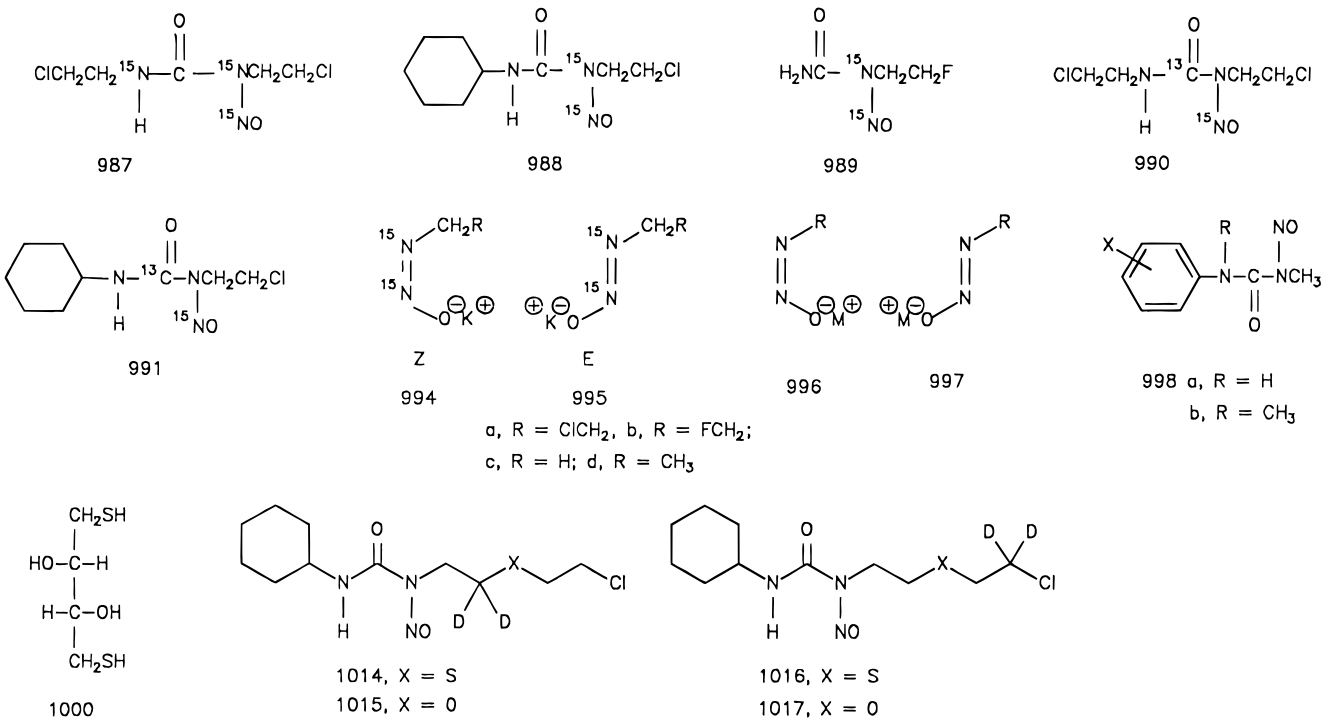
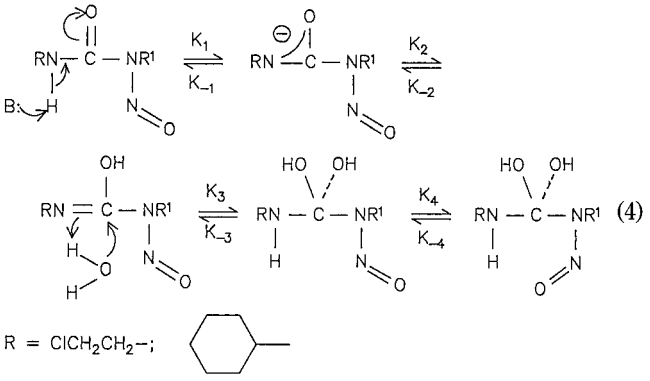


Chart 27. Structures for Section IX



water to the imino urea (k_3 in eq 4 and Scheme 106a) and not the direct addition of water to the carbonyl group.[689] For alkylnitrosoureas there is an inverse



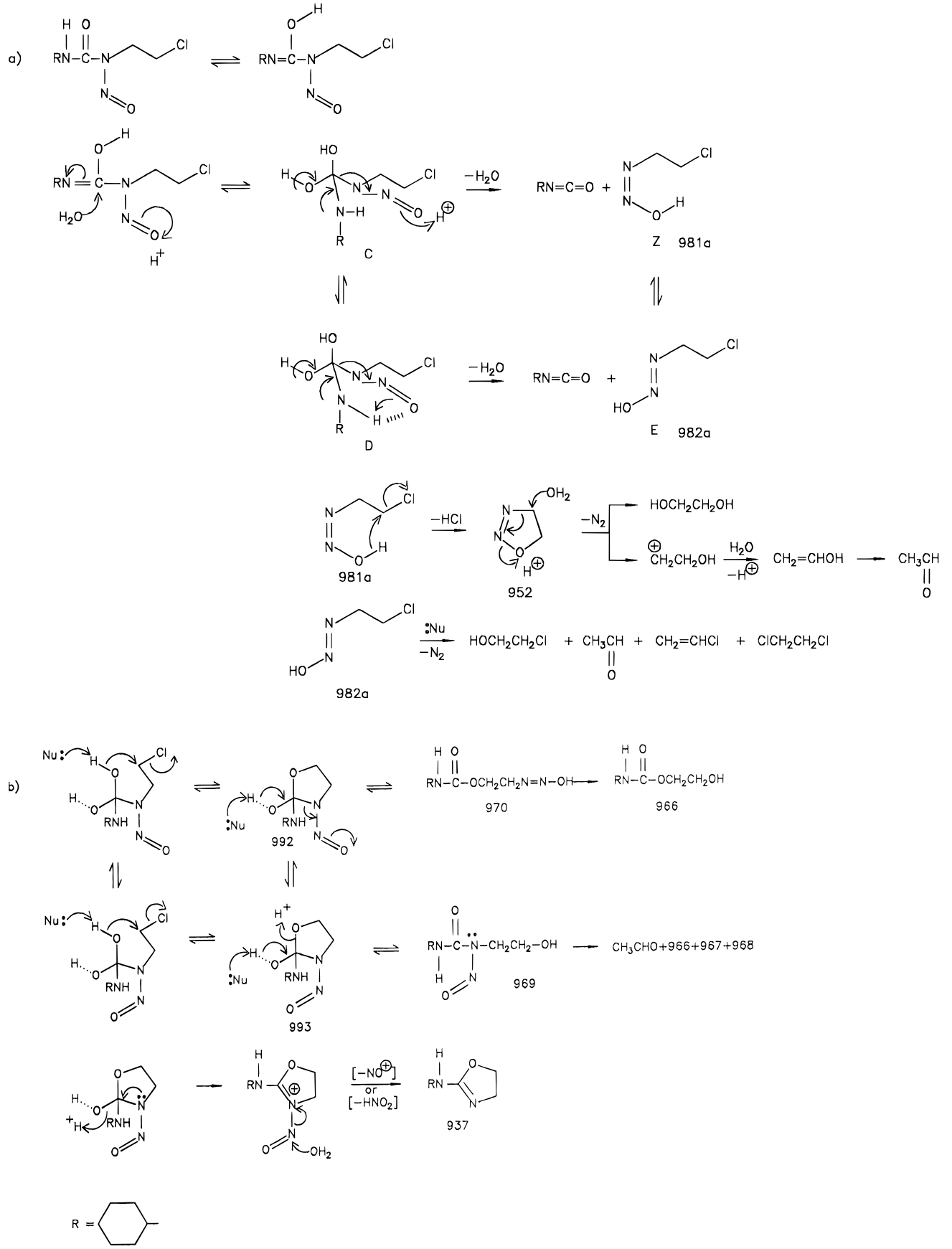
(4)

deuterium isotope effect[688] which could be attributed to a slower rate of collapse of the tetrahedral intermediate rather than to the rate of addition of water to the imino urea. The aforementioned mechanism was challenged.[690] Thus, studies of the decomposition in aqueous phosphate buffer at pH 7 of [^{13}CO]-*N*-methyl-nitrosourea by ^{13}C NMR and of the decomposition of [$^{15}NH_2$]*N*-methyl-N-nitrosourea by ^{15}N NMR spectroscopy revealed[690] that the initial product is a cyanate rather than a carbamate. The further reaction of the cyanate with phosphate yielded the carbamoyl phosphate. This result is consistent with an initial deprotonation of MNU (**25**) at its amido group followed by a fragmentation to cyanic acid and methanediazoate. However, it is not consistent with the mechanism[687,688] which involves the attack of water on the imidourea form of the nitrosourea to give a tetrahedral intermediate with subsequent formation of methanediazoate and carbamate.

Additional information about the *syn*-(*Z*) and *anti*-(*E*) diazohydroxides **981a**−**d** and **982a**−**d** in Scheme 106 was provided by the synthesis and study[691−693] of the ^{15}N-labeled (*Z*)- and (*E*)-alkanediazotates **994a**−**d** and **995a**−**d**, respectively (Chart 27). Their stability and solubility in organic solvents was increased by the formation of their crown ether complexes.[693] The ^{15}N and ^{13}C NMR spectra of these compounds were found[691−693] to indicate that the *E* and *Z* pairs do not interconvert at room temperature in aprotic solvents and, thus, react more or less independently. The interrelationships among the various structures are shown in Scheme 107. The *Z* isomer **996** was rapidly decomposed to the diazoalkane with stereoelectronic control from the antiperiplanar lone pair orbital on nitrogen, while the *E* isomer **997** was very stable.[691,692] Further, the facts that the *Z* isomer **981a** in Scheme 106 is more reactive and is selectively removed by a cyclization reaction to the 1,2,3-oxadiazoline, while the *E* isomer **982a** is more thermodynamically stable can explain why the *E* isomer accounts for greater than 80% of the reaction pathways of BCNU.[691−693] Furthermore, the (*E*)-2-chloroethane diazotate **997a** cross-links DNA, in contrast to the *Z* isomer **996a**.[693] The *E* isomer **997a** exhibited[693] an anticancer activity of 40% ILS against P388 leukemia at a dose of 200 mg/kg per day for five days, supporting its role as the ultimate electrophile obtained from CENU compounds.

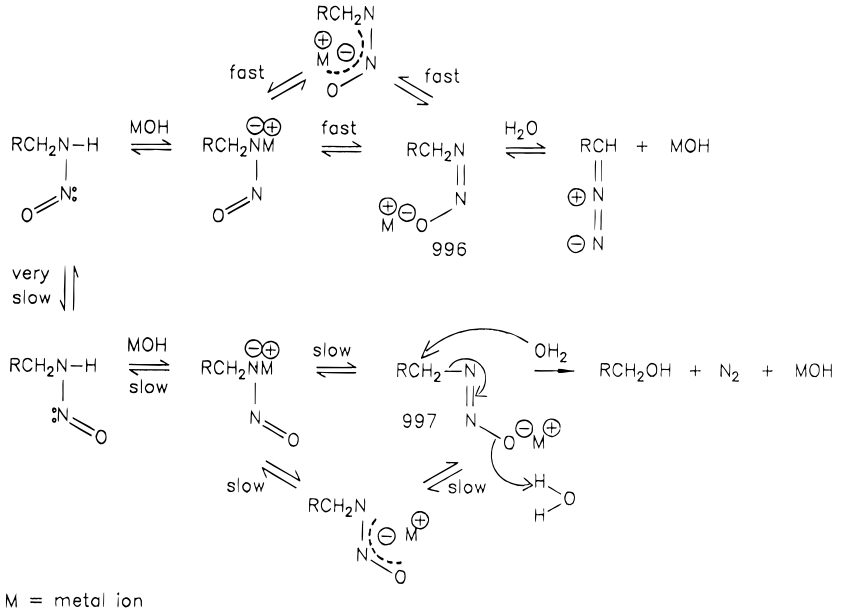
It was shown[694,695] that the (*E*)-alkanediazotates decompose in aqueous solution by a rate-limiting unassisted heterolytic bond fission of the diazoic acid, R−N=N−OH, to give the diazonium ion, $R-N_2^+$. There was no evidence of an S_N2 attack by nucleophiles on the *E*-diazoic acid form. The decomposition of the *Z*-diazoic acids by general acid catalysis involves a proton transfer to the N−O oxygen, followed by a bond heterolysis to yield the diazonium ion.[694] Furthermore, the *Z* isomers of a series of trifluoroethanediazoic acids were found to be more readily hydrolyzable than the *E* isomers by a factor

Scheme 106



of 2600.[694] It was also estimated[694] that the (*Z*)-2-haloethanediazotates, such as **996a**, would decompose in water in about 1 ms. Thus, in both in vitro and in vivo studies, much of the *Z* form would be hydrolyzed before it could alkylate the DNA in cells. Hence it was concluded[694] that the *E* isomer **997a** is not the ultimate electrophile from CENU drugs, since its demonstrated DNA cross-linking and antileukemic activity[693] is the result of its slower hydrolytic decomposition, relative to the *Z* isomer **996a**.

Scheme 107



The (*Z*)-alkanediazohydroxide **981** has a longer C−N bond and a softer carbon than the *E* isomer **982** (Chart 26) and should preferentially react with the softer guanine (G)-O6 center of DNA, according to the hard and soft acids and bases (HSAB) theory.[696] The greater reactivity and the longer C−N bond of the *Z* isomer **981** also would favor an S_N1 attack to give G-O6 alkylated products with rearrangement, as in the observed case of propylation (Scheme 105c). By contrast, the *E* isomers **982a**−**d** have shorter C−N bonds and harder carbons than the *Z* isomers **981a**−**d** and would react at the harder G-N7 center of DNA. The lower reactivity and shorter C−N bond of the *E* isomer would favor a S_N2 mechanism, leading to the observed unrearranged products (Scheme 105b).[691−693] The site of DNA alkylation appears to be crucial in terms of the biological response because guanine-O6 alkylation may correlate with carcinogenicity.[697]

Ab initio Hartree−Fock calculations were performed[693,698] on the diazohydroxide, diazonium ions, and diazotate salts of (2-haloethyl)nitrosoureas. It was found[692,693,698] that ethyl- and 2-fluoroethyldiazohydroxides **981d** and **981b** are more prone to a soft electrophilic attack on the DNA bases than methyl- and 2-chloroethyldiazohydroxides **981c** and **981a**, and the same trend was found for their diazonium ions. The geometry-optimized conformations of the *Z* and *E* isomers **981** and **982** of the ethyl-, 2-fluoroethyl-, and 2-chloroethyldiazohydroxides, the *Z* and *E* isomers **996** and **997** (Chart 27) of the corresponding diazotate salts and of the diazonium ions **983** were reported.[692,693,698,699]

Several additional quantum chemical studies of *N*-nitrosoureas have been reported.[700−702] INDO-MO calculations of *N*-methylnitrosourea (NMU, **25**), and related alkyl analogs and their radical anions and cations indicated that the anticancer activity of **25** is associated with its electron-acceptor and electron-donor properties. In an EPR study[702] of the electron-acceptor and electron-donor properties of methylnitrosourea it was shown that NMU in the presence of an electron donor formed an anion radical $H_2NCON(CH_3)N^{\bullet}-O^{-}$ which can decompose to nitric oxide NO. In cells the formation of NO may inhibit the electron transfer in the mitochondrial respiratory chain.

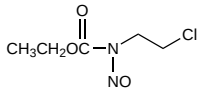
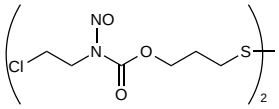
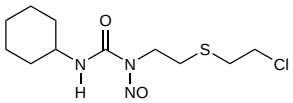
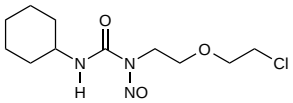
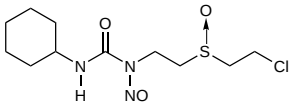
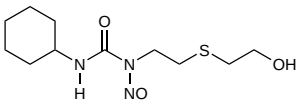
Studies of the pH dependence (pH 5.4 to 7.6) of the chemical decomposition of *N*-methyl-*N*′-aryl-nitrosoureas **998a**,**b** (Chart 27) were reported.[703] The N3,-N3-disubstituted analogs **998b** were stable at this pH range because of the absence of a hydrogen on the N3-nitrogen. The decomposition of the 1,1′-ethylenebis(1-nitrosourea) (EBNU, **90**) under acid, neutral, and alkaline conditions was reported.[140] The half-lives of EBNU are 0.9 min at pH = 9.0, 13.3 min at pH = 7.0 and 45 h at pH = 1.0.

D. Modifications of Nitrosourea Decompositions

Attempts were made to enhance the fragmentation of *N*-nitrosoureas via the alkanediazohydroxide intermediate (path A, Scheme 91), and to lessen the decomposition by paths B and C (Scheme 91).The reasoning behind this work was that only the alkane diazohydroxide of path A, but neither the 1,2,3-oxadiazoline of path B nor the 2-imino-*N*-nitrosooxazolidinone of path C, contains the 2-haloethyl side chain which is necessary for a cross-linking of the DNA, an important aspect of the anticancer activity of these agents (section X.B).

The in vitro interstrand cross-linking of DNA engendered by ethyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**999**)[108] resulted[704] in a 12% cross-linking by **999** itself and a 28% cross-linking in the presence of a thiol compound such as dithiothreitol (**1000**, Chart 27) (Table 55). According to the mechanism of Scheme 108a[704] the thiol compound could increase the nucleophilic attack at the carbonyl group of the carbamate, resulting in a greater fragmentation to the diazohydroxide and greater cross-linking. On the basis of these findings, the compound 3,3′-bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamyl]propyl disulfide (**1001**) was synthesized[704] which, in principle, could be reduced in vivo to a thiol derivative which could, in turn, react either intermolecularly or intramolecularly with the carbamate carbonyl. A mechanism proposed[704] for the intramolecular attack is shown in Scheme 108b. The existence of the intermediate

Table 55. In Vitro and in Vivo Activities of *N*-Nitrosourea−DNA Cross-Linking Models[704]

no.	compound	DNA ICL,[b] %	$t_{1/2}$,[c] min	DNA alkylation,[d] rel %	dose,[e] mg/kg	ILS,[f] %
999		12	81 ± 8		0.9	40
	+ 15 mM DTT[a]	28				
1001		8				
	+ 15 mM DTT[a]	25				
1008		90			32	94
1009		0		66	200	52
1010		0		84	100	59
1011		0		96	200	10

[a] Dithiothreitol. [b] Percentage of CLC (covalently linked complementary) sequence in λ DNA determined at 6 h by the ethidium fluorescence after heat denaturation (96 °C/3 min) and rapid cooling, compared with the control sample; CLC-DNA, a DNA molecule containing at least one cross-link per sample. [c] Half-life, determined by measuring the rate of decrease of the amplitude of the polarographic wave at pH 7.1 and 37 °C. [d] Alkylation of PM2-CCC (covalently closed circular) DNA (90% CCC) determined by the rate of decrease of ethidium fluorescence, compared to control, after heating (96 °C/4 min) followed by rapid cooling. [e] Dose administered ip once daily on days 1−9 after ip implantation of L1210 leukemia cells in mice. [f] Increase in life span = $[(T - C)/C] \times 100$.

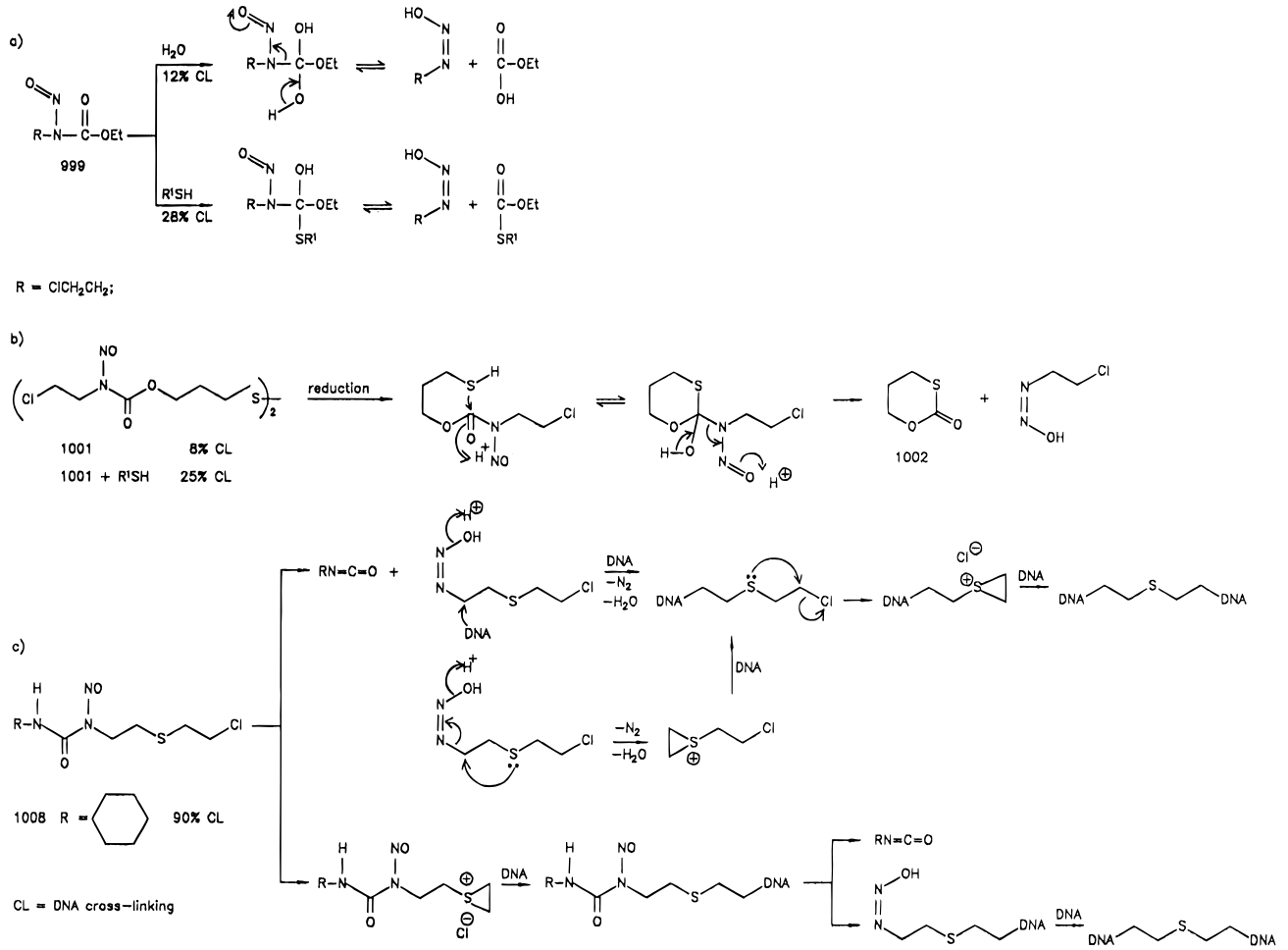
1,3-oxathian-2-one (**1002**) was supported by a GC−MS analysis of the decomposition mixture arising from **1001** at pH 7.2 and 37 °C. In this instance, the low cross-linking (8%) of the DNA by the compound **1001** was increased to 25% by the addition of the dithiothreitol (Table 55).

The aqueous decomposition of the bis-CENU disulfide analog CNCC (**119**−**121**), which can undergo bioreduction to the corresponding thiol compounds, was related[161] to the decomposition of the disulfide **1001**. CNCC is a mixture of 1,1′- (**119**), 3,3′- (**120**), and 1,3′- (**121**) bis-*N*-nitrosoureas (section VII.A.2). The decomposition of the 1,1′-bis-nitroso isomer **119** at pH 7.0 and 37 °C resulted in the formation of 2-chloroethyl isocyanate, bis(2-chloroethyl)urea **1003**, and bis(2-hydroxyethyl) disulfide **1004**, whereas in the presence of dithiothreitol (DTT, **1000**) it yielded 2-chloroethyl isocyanate, bis(2-chloroethyl)urea **1003**, bis(2-hydroxyethyl) disulfide **1004**, thiirane **1005**, and 2-mercaptoethanol (Scheme 109a).[161] No cross-linking of λ DNA was found in the presence of **119** either in the absence or the presence of DTT. In this case no reactive electrophilic intermediate was formed. The aqueous decomposition[161] of the 3,3′-bis-*N*-nitroso isomer **120** produced acetaldehyde, vinyl chloride, 2-chloroethanol, and bis(2-cyanatoethyl) disulfide **1006**, whereas in the presence of DTT the products obtained were acetaldehyde, vinyl chloride, 2-chloroethanol, and *N*,*N*′-bis(2-mercaptoethyl)urea **1007** (Scheme 109b). The DNA cross-linking caused by isomer **120** in the absence and presence of DTT was 20% and 38%, respectively.[161] The increase in DNA cross-linking for **120** is attributable to an initial intermolecular nucleophilic attack by DTT (**1000**) on the carbonyl group of **120** followed by a stereoelectronically controlled decomposition of the tetrahedral intermediate to the 2-chloroethyldiazohydroxide. No intramolecular thiol participation, analogous to that found for compound **1001**, occurred in this case.

The relative carbamoylating activity of the three isomers, as judged by the [^{14}C]lysine assay and by inhibition of glutathione reductase, was **119** ≈ **121** > **120**.[161] Apparently the 2-chloroethyl isocyanate is a more effective carbamoylating agent than the bis-(2-cyanatoethyl) disulfide **1006**. The order of relative alkylating activity for the isomers was **119** > CNCC mixture > **121** > **120**. The order of in vitro cytotoxicity against human DU 145 prostatic carcinoma cells was **121** > **120** > **119** during a 24-h period.[161] This order of reactivity reflects the formation of 2-chloroethanediazohydroxide from **121** and **120** which can cross-link the DNA and cause cell damage. It was proposed[161] that the reduced cytotoxicity of **119** to the cancer cells is attributable to an interaction between the GSH and the drug, thereby protecting other more critical nucleophilic targets within the nucleus.

Another approach to the enhancement of path A (Scheme 91) was to design a compound which would allow a selective anchimeric assistance for the production of the alkane diazohydroxide and ultimately an increase in the amount of DNA cross-linking.[704] A compound **1008**, which contains the elements of both a *N*-nitrosourea and a sulfur mustard, was synthesized and shown[704] to produce a 90% cross-linked DNA in 10 min at pH 7.2 and 37 °C, and also to have a moderate anticancer activity of 94% ILS

Scheme 108

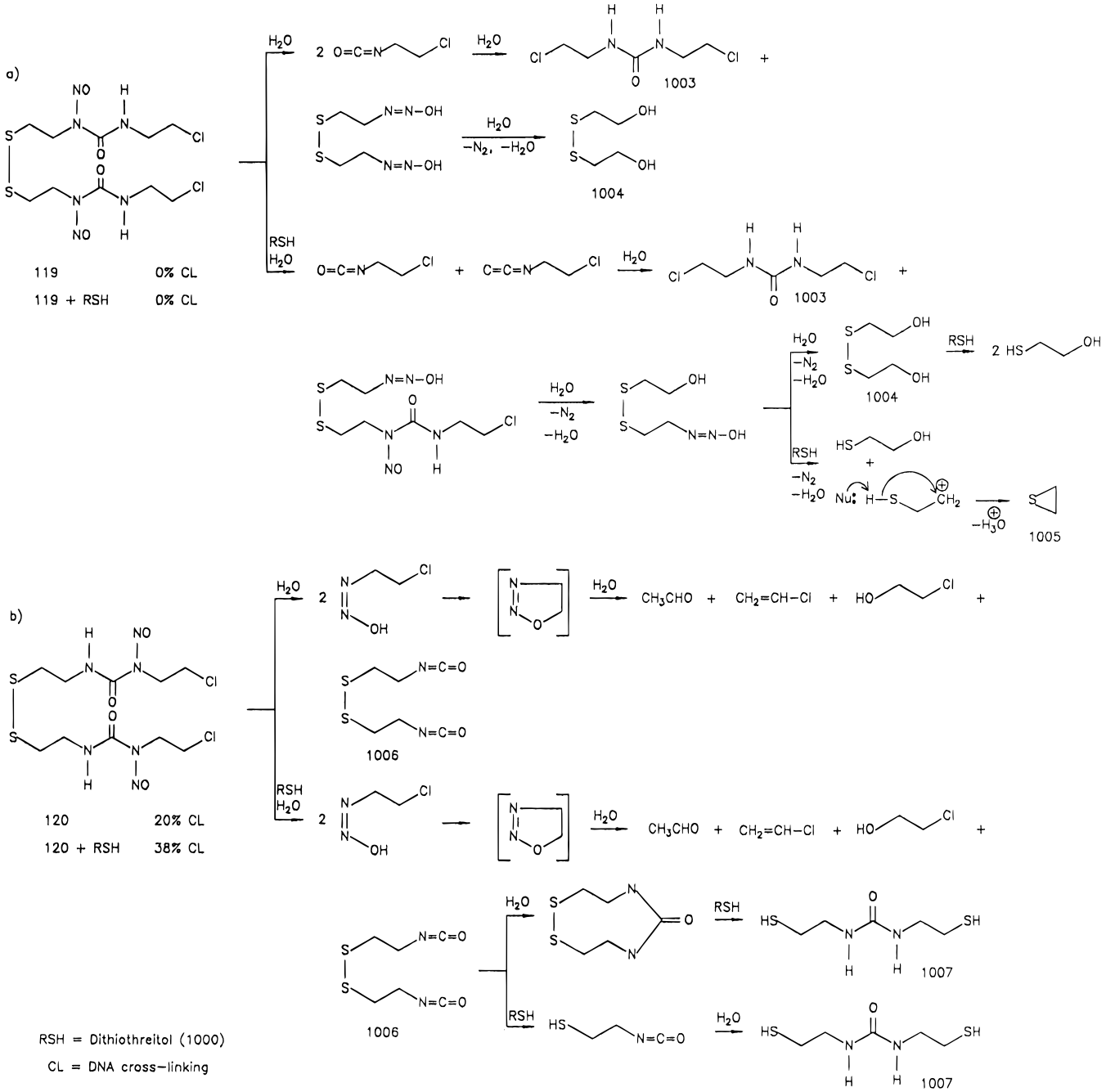


against the L1210 leukemia line in mice (Table 55). A mechanism which explains[704] the decomposition, alkylation and cross-linking of DNA is illustrated in Scheme 108c. Two general pathways of decomposition are possible, depending on whether the first step is the nitrosourea fragmentation to the diazohydroxide or the sulfur mustard cyclization to an episulfonium ring. In either case, one DNA molecule reacts with the diazohydroxide, and the other DNA molecule reacts with the episulfonium ring to form the cross-link. As a test for the mechanism the compounds **1009**−**1011** were synthesized and shown[704] to have a high DNA alkylation but no cross-linking capability (Table 55). All three analogs can undergo the initial nitrosourea fragmentation (Scheme 108c) to cyclohexyl isocyanate and the alkanediazohydroxide which can alkylate the DNA, but none of them can form a second electrophilic species, the episulfonium ion, capable of alkylating a second DNA and completing the cross-link. The neighboring group effect of oxygen in **1009** is less than that of sulfur and that of sulfur in **1010** is absent since the lone electron pair is bound by the S−O group. In the case of compound **1011** the hydroxyl group is a much weaker leaving group than the chlorine atom and cannot form the episulfonium ring even with the anchimeric assistance from sulfur. These analogs **1009**−**1011** also have a lower anticancer activity than compound **1008** (Table 55).[704]

Further support for the anchimeric-assisted decomposition mechanism of Scheme 108c was provided by a study[705] of the decomposition products of the *S*-CENU (**1008**) and *O*-CENU (**1009**). In Scheme 110a there is shown the product distribution of **1008** and **1009** and in Scheme 110b,c the proposed mechanisms for their decomposition.[705] In particular, the formation of the 2,3-dihydrothiophene **1012** from **1008** was considered as evidence for two sulfur neighboring group interactions to form two thiiranium intermediates (Scheme 110b), comparable to the interstrand cross-linking of DNA by sulfur participation at two sites.[705] In contrast, no 2,3-dihydrofuran was found for the decomposition of **1009** which cannot form two oxiranium intermediates **1013** (Scheme 110c) and, thus, cannot cross-link the DNA (Table 55). More evidence in support of this mechanism was provided[705] by an analysis of the products obtained in the decomposition of the specifically deuterated derivatives **1014**−**1017** (Chart 27). The research on the interaction of CENU drugs with thiol compounds was reviewed.[706]

Another modification of the decomposition of *N*-nitrosoureas is to delay the in vivo process by the use of disubstitution at the N3-position. These so-called "latent" compounds[10] have no hydrogen on the N3-nitrogen and thus cannot undergo the initial fragmentation reaction to an isocyanate and diazohydroxide by path A, Scheme 91. Such compounds require an enzymatic dealkylation before anticancer activity can be expressed (Scheme 111). A wide variety of such N3,N3-disubstituted analogs have been considered among the aliphatic (Table 3), ali-

Scheme 109

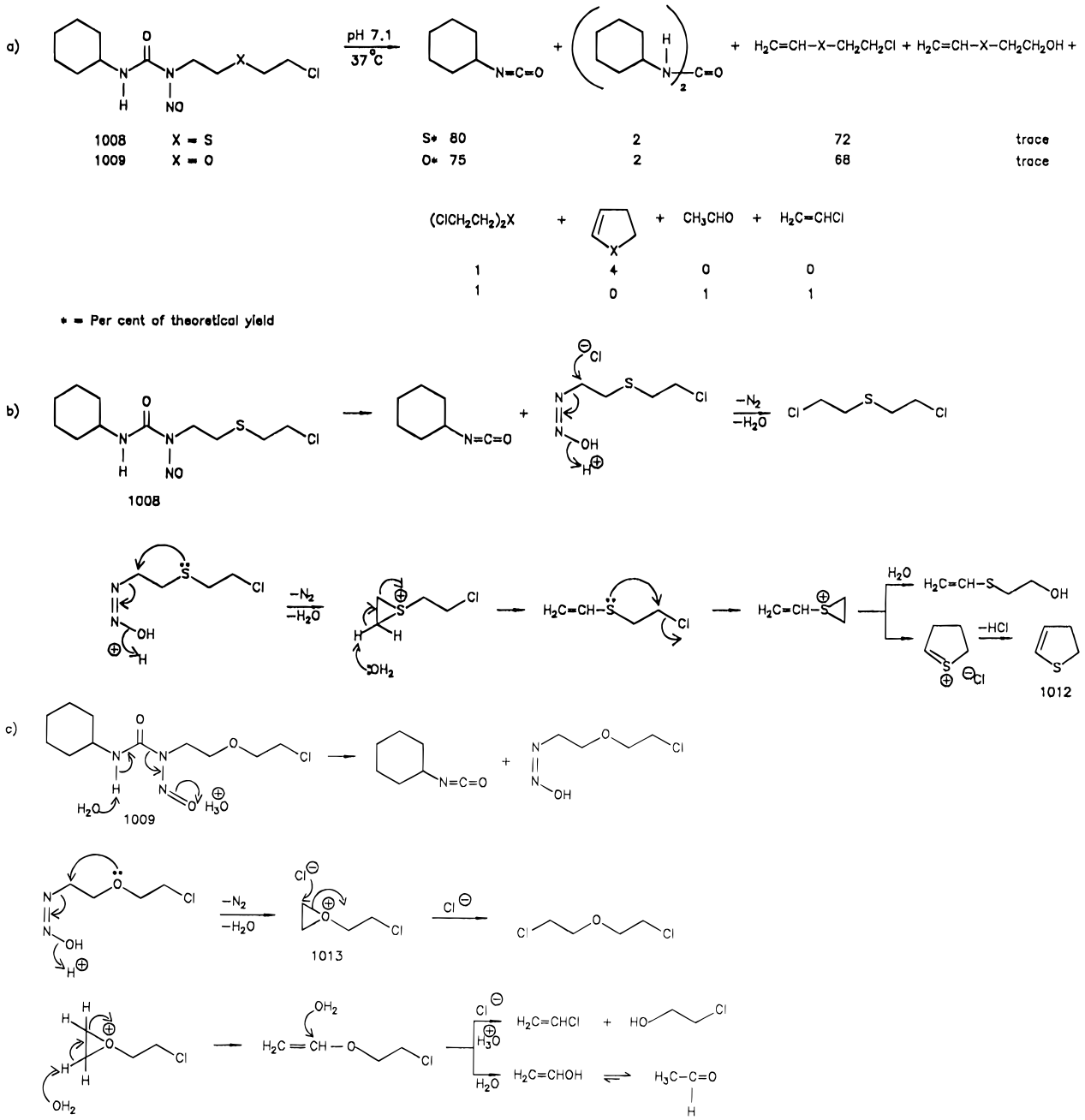


cyclic (Table 13), carbohydrate (Tables 21, 23, 24, 27, 29, and 31), and amino acid (Table 34) *N*-nitrosourea analogs discussed in this review. In general, these compounds possess higher anticancer activity, longer half-lives, and more favorable therapeutic ratios than the corresponding N3-monosubstituted CENU analogs. A number of these disubstituted analogs containing a β-hydroxyl group on an alkyl group attached to the N3-nitrogen can undergo an internal cyclization reaction to produce a cyclic urethane and the alkane diazohydroxide (Scheme 17b). Such compounds possess the dual functions of preventing the release of a toxic isocyanate moiety and anchimerically assisting in the release of the cytotoxic diazohydroxide. Examples of such analogs are the CENU analogs of aliphatics (**65**−**71**, Table 3), alicyclics (**191**−**196**, Table 13), monosaccharides (**418**−**422**, Table 21), (**440**−**462**, Table 23), (**463**−**483**, Table 24), (**519**−**523**, Table 27), (**533**−**540**, Table 29), and disaccharides (**553**−**565**, Table 31). The cyclization mechanism in Scheme 17 was proven for a number of these compounds by the isolation and characterization of the 2-imidazolone intermediates **198** and **199** in Scheme 31, **484** in Scheme 47b, **541** in Scheme 54b, and **566** in Scheme 57b.

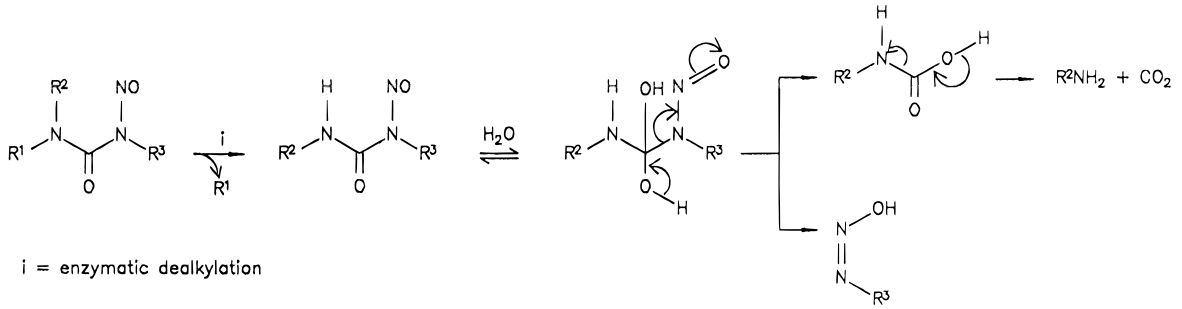
Such anchimeric assistance by an adjacent β-group is evident for many CENU carbohydrates, as proven by the structures of the six decomposition products **1018**−**1023** of chlorozotocin (**368**) obtained under physiological conditions (Scheme 112a).[707] Similar products were obtained[326] from the decomposition of streptozotocin (**367**). All of these compounds are weakly carbamoylating nitrosoureas.

Interestingly, two metabolites of the clinical drug ACNU (**311**) are cyclic ureas **1024a**,**b**, resulting from an S_N2' attack of the N3-nitrogen on the β-chlorine atom (Scheme 112b).[280,281]

Scheme 110



Scheme 111



X. Biochemical Reactions and Anticancer Activity

Early studies on the mechanism of action of alkylnitrosoureas (ANU) and chloroethylnitrosoureas (CENU) revealed that these drugs possessed both alkylating and carbamoylating activities.[72,708–710] The reaction of [^{14}C]2-chloroethyl-labeled CCNU **934** (Chart 26) with DNA yielded nucleotides from DNA, whereas that with the [^{14}C]cyclohexyl-labeled CCNU **939** yielded labeled proteins.[711] The former reaction was attributed to the alkylation of nucleic acids by either the $ClCH_2CH_2N{=}NOH$ or $ClCH_2CH_2^+$ species, and the latter to protein carbamoylation by the cyclohexyl isocyanate (pathway A, Scheme 91).

Scheme 112

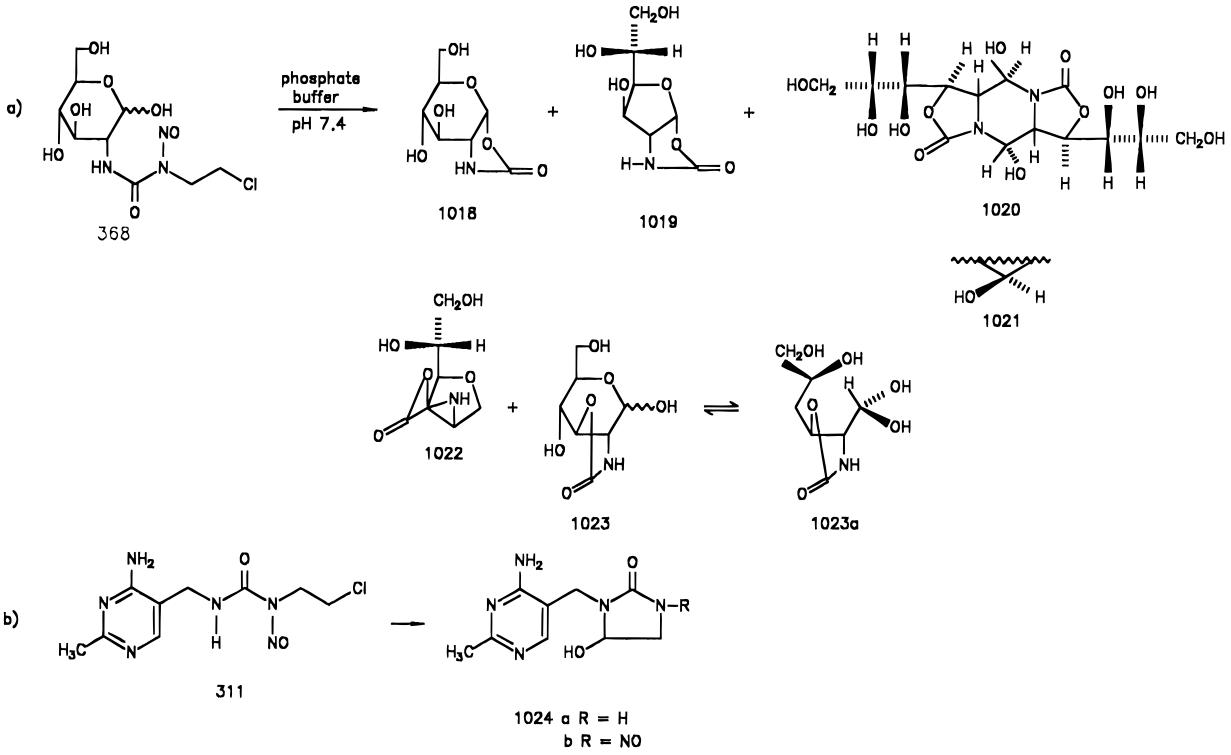
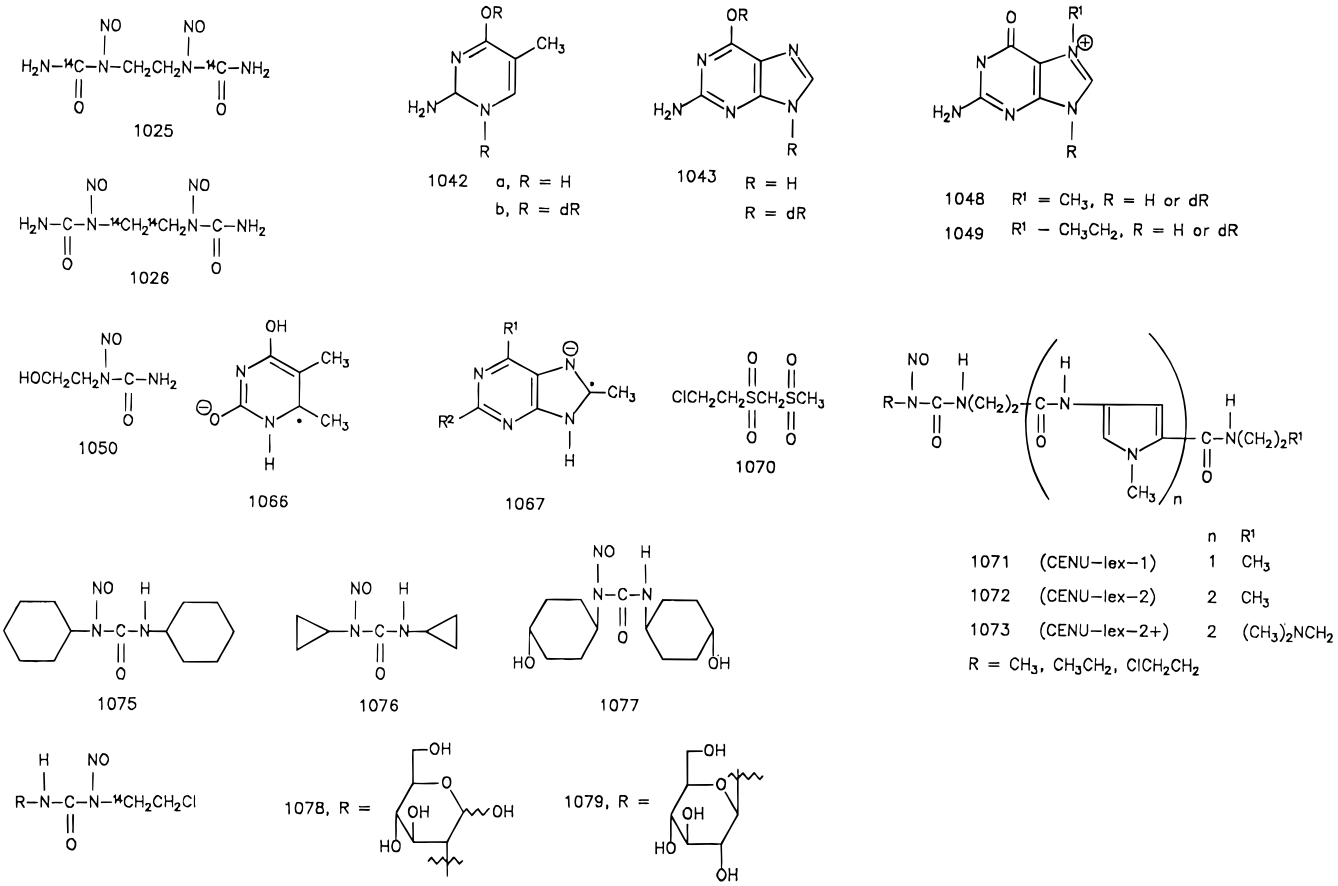


Chart 28. Structures for Section X



Similar results were obtained with ^{14}C-labeled BCNU (**21**)[712] and ACNU (**311**)[713] and EBNU (**90**).[141,142] A ^{14}C-labeled C═O group of EBNU (**1025**, Chart 28) was found[141] to be bound to cytoplasmic and nuclear proteins but not to nucleic acids of L1210 cells, whereas a ^{14}C-labeled 1,2-ethanediyl-EBNU (**1026**) was bound to both proteins and nucleic acids. The radiolabeling reactions of **1026** are alkylations, resulting from an intermediate ethanediylbis(diazohydroxide), HON═N(CH$_2$)$_2$N═NOH, while the radiolabeling reactions of **1025** are carbamoylations from the unstable carbamic acid intermediate H$_2$NC(═O)OH. The radioactivity of the compound **1025** bound to cytoplasmic protein was approximately double that

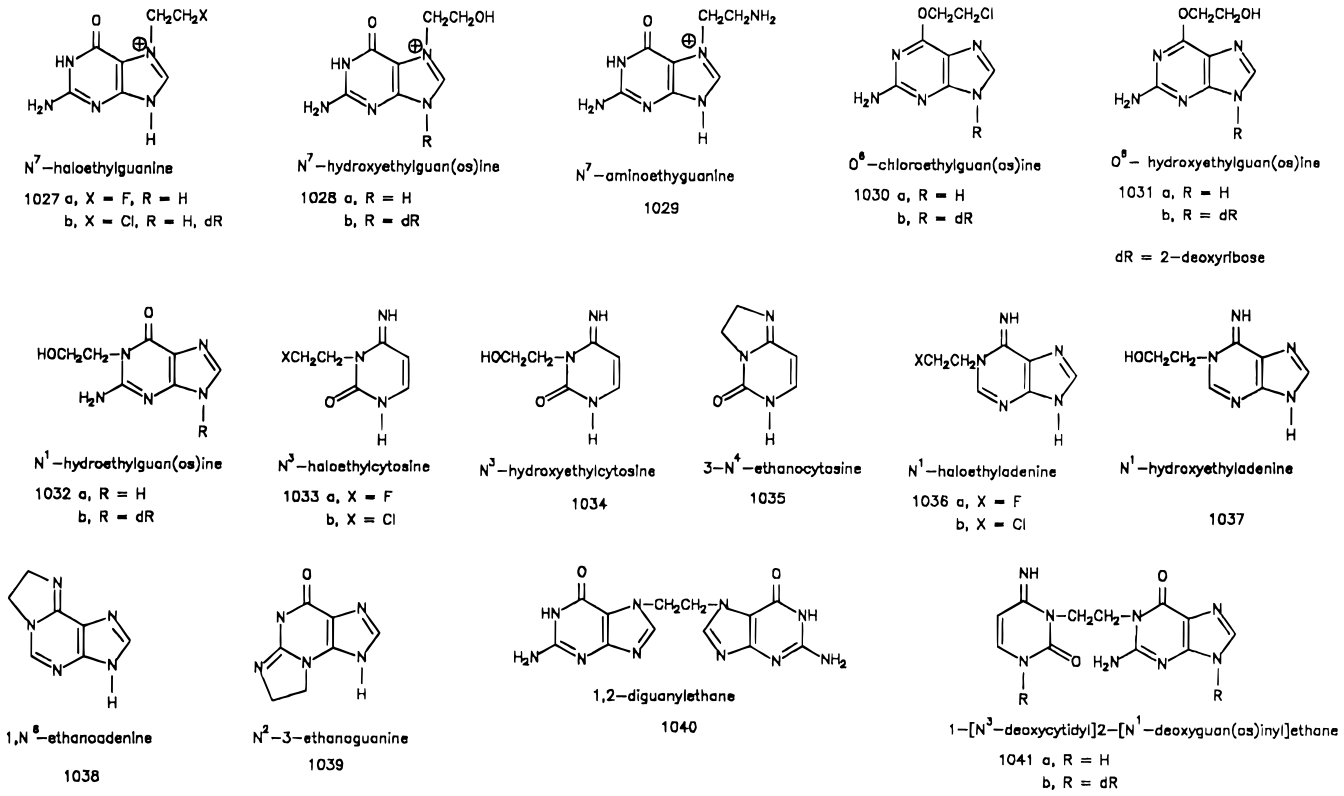


Figure 3. Alkylated bases obtained from reaction of CENUs with DNA.

of the [^{14}C]cyclohexyl-CCNU (**939**) because the reaction of **1025** can yield two carbamoylating species per molecule.[141] Although the in vitro alkylating activity of EBNU and CCNU were the same, the in vivo alkylating activity of **1026** involving the L1210 murine leukemia was much higher than that for CCNU.[141]

Among proteins the order of binding for **1025** was histone > cytochrome c > RNase A, albumin > γ-albumin, and for **1026**, histone > RNAse > albumin > cytochrome $c \gg \gamma$-globulin. Binding studies with synthetic polypeptides revealed[141] a large binding affinity of **1025** to polylysine up to 8 h and a lesser binding to polyhistidine. Compound **1026** was bound to protein and polypeptides to a much lower extent. Similar binding studies of **1026** with nucleic acids and synthetic nucleotides revealed[141] a strong preference for RNA over DNA and of poly G and poly *C* over poly A and poly U. However, CCNU (**137b**) was bound to poly G and poly *C* to the same extent,[711] BCNU (**33**) was bound to poly *C* almost 6 times greater than poly G,[712] and ACNU (**311**) was bound to DNA almost four times greater than RNA.[713] Thus each CENU analog has a different specificity for the binding of nucleic acids and polynucleotides.

A. DNA Alkylation

Alkylation of nucleic acids is considered[714,715] to be responsible for the cytotoxicity of the CENUs while the carbamoylation of proteins was the cause of the toxic side effects of these antitumor agents.[72,97] Reviews have been published concerning the alkylation of DNA by a variety of alkylating agents[714] and by *N*-nitrosoureas[715–721] in particular.

In order to ascertain the possible alkylation products of DNA, a number of studies[712,722–732] were devoted to the reactions of CENU analogs with synthetic polynucleotides and with DNA. Experimentally, the polynucleotides were incubated[733–735] with ^{14}C-labeled CENU and then digested to the monomer level so that modified nucleosides and heterobases could be separated and identified. Polynucleotides were hydrolyzed either with an aqueous 1 N hydrochloric acid solution at 100 °C for 30 min or with venom phosphodiesterase for 14 h at 37 °C. DNA was digested to its monomeric nucleosides with a combination of deoxyribonuclease I, venom phosphodiesterase, spleen phosphodiesterase, and bacterial alkaline phosphatase.[733–735] Modified purines were separated from DNA by a controlled depurination reaction without complete digestion, such as heating with an aqueous 25 mM sodium cacodylic buffer solution at pH 7 and 100 °C for 15 min. High-pressure liquid chromatography (HPLC) was used to separate and isolate the modified nucleosides from DNA and RNA.[715,716] Structures of the modified bases were determined by the combination of ultraviolet spectroscopy,[715,716] mass spectrometry of underivatized bases and their corresponding trimethylsilylated nucleoside derivatives,[714,715] and the synthesis of the modified bases.[723–725,727]

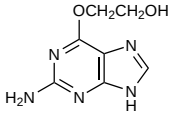
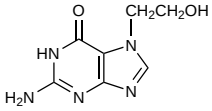
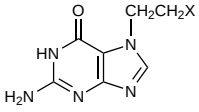
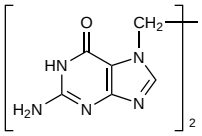
A number of modified heterobases of nucleotides have been determined[715–721] from studies of the CENUs with synthetic polynucleotides and DNA (Figure 3). The modified bases can be divided in two types i.e. monoalkylated **1027**–**1034**, **1036**, **1037** and dialkylated **1035**, **1038**–**1041** (Figure 3). The 2-haloethyl compounds **1033** and **1036** are capable of undergoing either intramolecular cyclization reactions to give derivatives **1035**[712,723] and **1038**[725] respectively, or a second alkylation reaction with another nucleoside, resulting in either interstrand or intrastrand cross-links. Syntheses of the bridged compound **1041**,[736,737] and compounds related to

Table 56. Distribution of Adducts Formed by Alkylation of Single- and Double-Stranded DNA in Vitro by *N*-Methyl-*N*-nitrosourea and Higher Homologues[a]

site of alkylation		alkylation (%)					
		single-stranded DNA		double-stranded DNA			
		MNU[b,c] (**25**)	ENU[b,c] (**27**)	MNU[b,c] (**25**)	ENU[b,c] (**27**)	PNU[d] (**28**)	BNU[e,f] (**30**)
adenine	N^1	2.8	2.0	0.7−1.3	0.2−0.3		
	N^3	2.6	1.2	8.0−9.0	2.8−5.6		
	N^7	1.8	0.6	0.8−2.0	0.3−0.6		13.2
guanine	N^3	0.4	0.5	0.6−1.9	0.6−1.6		
	O^6	3	7.0	5.9−8.2	7.8−9.5	9.3 (2.6)[g]	8.4 (2.5)[g]
	N^7	69	10.0	65−70	11−11.5	14.7 (1.6)[g]	12.8 (1.4)[g]
thymine	O^2		6.0	0.1−0.3	7.4−7.8		8.2
	N^3			0.1−0.3	0.8		0.1
	O^4		4.0	0.4−0.7	1.0−2.5		0.8
cytosine	O^2		5.5	0.1	2.7−2.8		
	N^3		1.7	0.1−0.6	0.2−0.6		
phosphate		10	65.0	12−17	57		43

[a] Table taken from ref 721. [b] Reference 739. [c] Reference 740. [d] Reference 685. [e] Reference 741. [f] Reference 742. [g] Data in parentheses represent the % yield of prop-2-yl or but-2-yl adducts.

Table 57. Distribution and Product Ratio at Alkylated Guanine by the Reaction of Calf Thymus DNA with Various *N*-(2-Haloethyl)-*N*-nitrosoureas[a]

compound no.		total identified products (%)						
		 **1031a**	 **1028a**	 **1027a,b**	 **1040**	**1027a,b/ 1028a**	**1031a/ 1028a**	ref(s)
33	BCNU	3.0	51	X = Cl: 42	4	0.59, 0.83	0.067	714,740
137a	FCNU	2.3	87	X = F: 10.7	nd[c]	0.12	0.026	714
137b	CCNU	3.0	60	X = Cl: 31	6	0.62, 0.50	0.055	714,740
329	HECNU	4.0	63	X = Cl: 24	9	0.38	0.063	740
329	HECNU in vivo[b]	nd[c]	38	57	5	1.50		741

[a] Table taken from references 721 and 744. [b] Rat kidney DNA 24 h after application of 0.1 mmol of HECNU/kg of body weight. [c] Not determined.

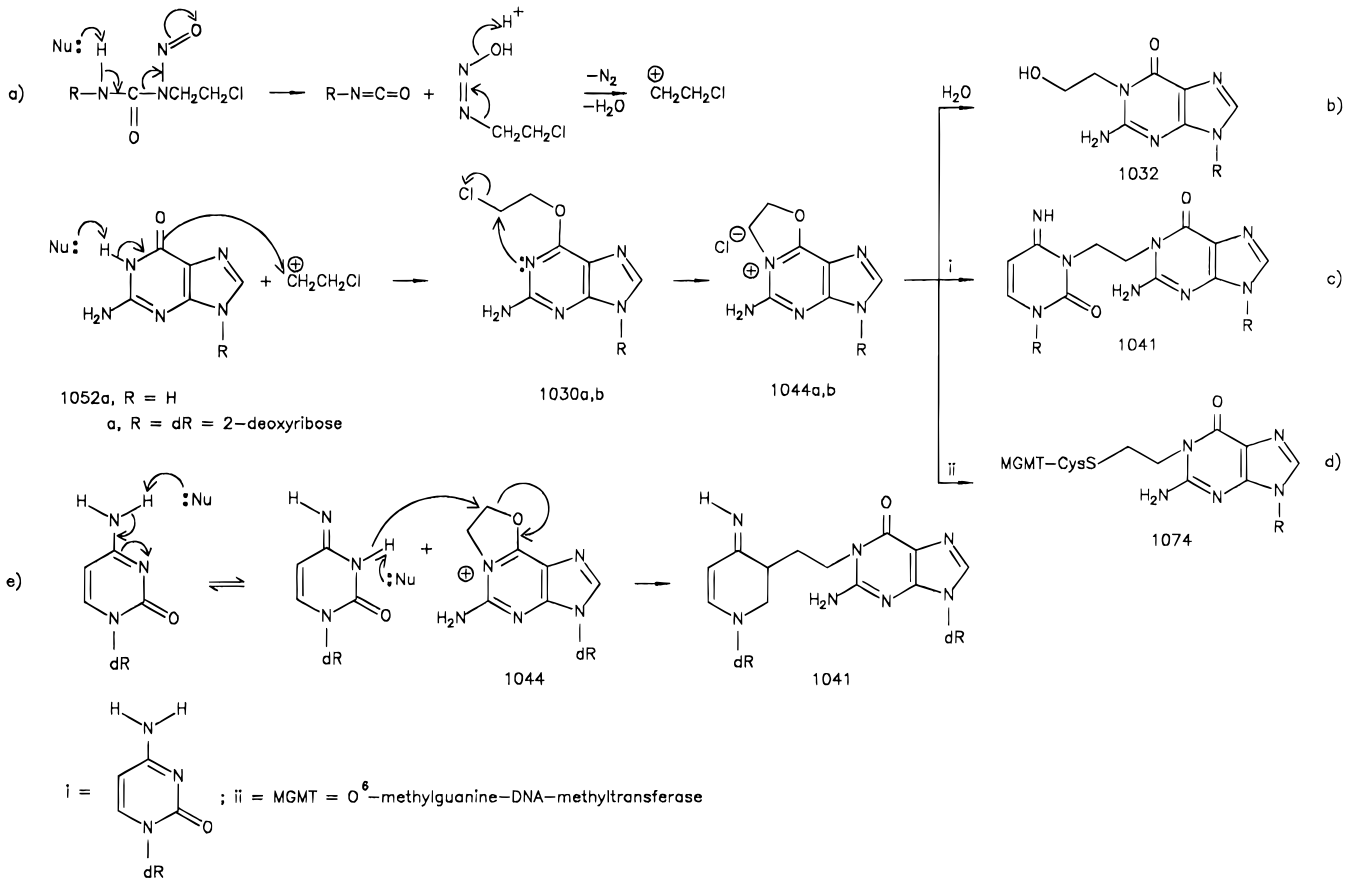
1041[738] have been reported. The compounds **1040**[728,729] and **1041**[730] were isolated from reactions of BCNU with nucleotides and DNA. The N2-3-ethanoguanine (**1039**) was isolated from CCNU-treated DNA.[732] Compound **1040** was considered[729] to be a model for DNA intrastrand cross-links and compound **1041** a model[730] for interstrand cross-links. Syntheses of **1041a** and **1041b** were achieved by the reactions of 2-deoxycytidine with 6-(2-fluoroethyl)-guanine[736] and 6-(2-fluoroethyl)-2-deoxyguanosine,[737] respectively.

Summaries of the DNA base alkylations by ANUs and CENUs are presented in Tables 56 and 57. As can be seen in Table 56 the *N*-methyl-*N*-nitrosourea (NMU, **25**) alkylates the N7-position of guanine predominantly in both single- and double-stranded DNA. The higher alkyl *N*-nitrosoureas, such as ENU (**27**), PNU (**28**), and BNU (**30**) alkylate the phosphodiester position predominantly, and also the oxygen atoms of guanosine, cytosine, and thymine.[685,739−742] Similar results were obtained[721] from reactions of MNU and ENU with mouse and rat liver DNA. The reaction of several different CENUs with calf thymus DNA yielded mainly the N7-alkylated guanines **1027a**,**b** and **1028a**, as shown in Table 57.[716,743,744] However, the ratio of the two N7-alkylated guanines varies greatly with the chemical structure of the CENU (Table 57). The phosphodiester moiety also is an alkylation site for CENUs.[745] MNU and ENU are known mutagens and the O4-alkyl-dT (**1042**) and O6-alkyl-G (**1043**) (Chart 28) are considered to be the protomutagenic lesions in DNA.[697,739,746−749]

The reaction of calf thymus DNA with *N*-[^{3}H](2-chloroethyl)-2-nitrosourea (**26**) was reported[737] to yield at least 13 DNA alkylation products, which were separated by HPLC and identified by comparison with standard compounds. The major products were N^7-(2-hydroxyethyl)deoxyguanosine **1028b** (4%), N^7-(2-chloroethyl)guanine **1027b** (14.6%), and phosphotriesters (1%). The minor alkylation products included the bridged compounds **1040** (3.2%) and **1041** (3.4%), and O^6-(2-hydroxyethyl)deoxyguanosine **1031b** (1.5%) and N^1-(2-hydroxyethyl)deoxyguanosine **1032b** (3.8%). The O6-$HOCH_2CH_2$-dG/N7-$HOCH_2CH_2$-dG ratio (**1031a**/**1028a**) produced with **27** was found[737] to be 10 times lower than that of the O6-$ClCH_2CH_2$−dG/N7-$ClCHCH_2$-dG ratio (**1030a**/**1027b**). Thus, the reactive intermediates leading to the formation of hydroxyethyl products were found[737] to be different from those leading to the chloroethyl products after reaction of DNA with CNU (**26**).

The formation of various polynucleotide adducts and DNA adducts resulting from reactions with *N*-nitrosoureas and the relative reactivity of the guanine N7 and O6 positions on these reactions has been the subject[714,721,750] of much research. The most nucleophilic site is considered[729] to be the N7-position of guanine as in compounds **1027**−**1029** (Figure 3). The hard and soft acid/base theory[696] was used to explain the differential *O*- and *N*-alkylation. Since exocyclic oxygen atoms are stronger bases than

Scheme 113

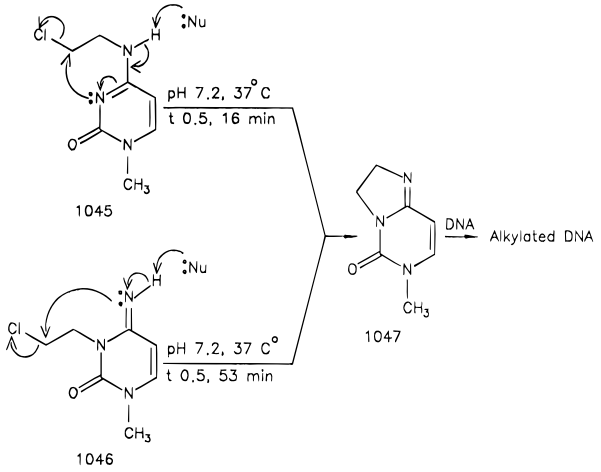


nitrogen atoms and, since electrophilic species generated from *N*-nitrosoureas are stronger acids than those generated from other alkylating agents, e.g. alkyl sulfonates, the former have a greater preference for reactions at O atoms. The longer and more branched the alkyl group, the stronger the acid character of its active species and the greater the preference for O sites[747] (Table 56). The difference in alkylation sites of electrophiles reflect differences in the fundamental electronic makeup of the reactive species, as calculated from gas-phase activation energies.[751] The unrearranged N7-alkylation products arise from an S_N2-type mechanism, whereas the partially rearranged O6-alkylation products arise from a combination of S_N2 and S_N1 processes (See also Scheme 105).[721,751]

Explanations have been proposed for the formation of the various polynucleotide adducts formed in CENU reactions. Thus, the model compound O6-(2-chloroethyl)guanine **1030a** was synthesized and shown[752] to be unstable, with a half-life of 17.8 min at pH 7.4 and 37 °C, rearranging to N^1-(2-hydroxyethyl)guanine **1032a**,[726] perhaps through the intermediate N^1,O^6-ethanoguanine **1044** (Scheme 113a,b). Chloroethyl isocyanate and chloroethylamine are generated from the *N*-(chloroethyl)-*N*-nitroso group attached to the N3-position of BCNU (**33**) and form the aminoethyl nucleosides, i.e. compound **1029** (Figure 3).[727] In contrast, the reaction of CCNU (**137b**) failed to yield such products, but rather chloroethyl and hydroxyethyl derivatives were obtained.[715]

The 2-hydroxyethyl analogs **1028**, **1031**, **1032**, **1034**, and **1037** are not formed in vitro by hydrolysis

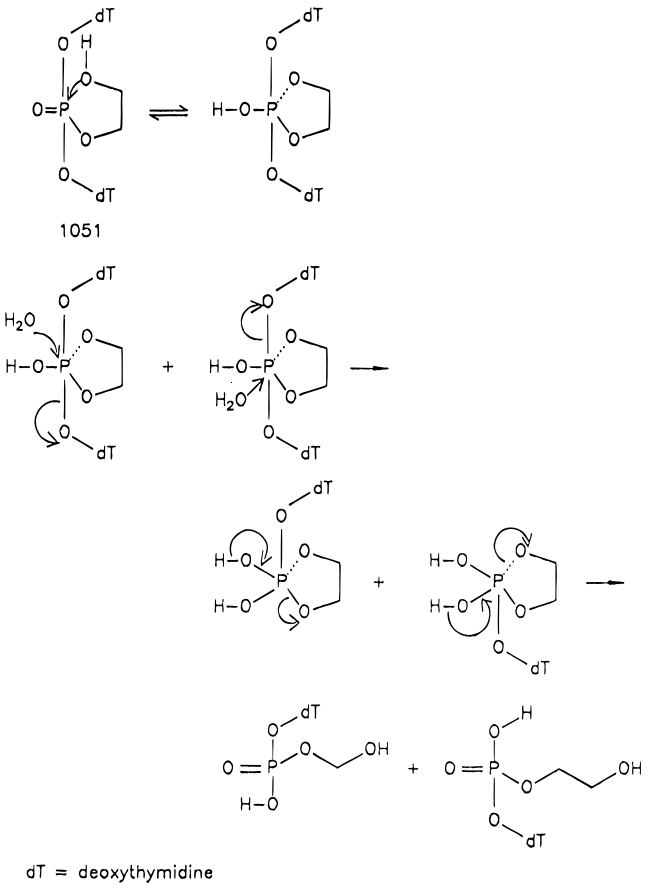
Scheme 114



of the corresponding 2-haloethyl compounds **1027**, **1030**, **1033**, and **1036**.[715,716,725] Rather the presence of the hydroxyethylated compounds was explained by the hydrolysis of an oxadiazolidine intermediate[680,712,721] (Scheme 91, pathway B). The in vitro reaction of CENUs with DNA favors the formation of N^7-(hydroxyethyl)guanine **1028a** over the N^7-(2-chloroethyl)guanine **1027b** (Table 57), whereas the in vivo reaction favors the formation of **1027**.[744]

Alternate sites proposed[753] for the chloroethylation of DNA at the N3- and N4-positions of cytidine were based on the known alkylation[714] of these cytidine positions by various alkylating agents. The reactions of model compounds **1045** and **1046** (Scheme 114) were shown[753] to cause alkylation of PM 2 CCC-DNA at a low level. Further, the cyclization of **1045** and **1046** to the ethanediyl analog **1047** was demon-

Scheme 115



strated[753] to be first order (Scheme 114). In these reactions no N3- and/or N4-hydroxyethylated cytidine derivatives were observed.[753]

The stabilities of the various DNA adducts is also an important factor. In general, 7-substituted guanosines are considerably less stable than the corresponding nonalkylated bases.[754−758] The effect of the N7-substituent on the stability of N7-alkylated guanine analogs **1027a**,**b**, **1028a**, **1029**, **1040**, **1048**, and **1049** (Figure 3, Chart 28) was studied.[754] Such compounds decompose by the imidazole ring opening and depurination reactions. A direct correlation was found[754] between the electron-withdrawing effects, expressed as the Taft substituent constant σ^*, of the alkyl substituent on the N7-position and the respective acid dissociation constants pK_a and chemical stabilities of the N7-alkylated guanosines.

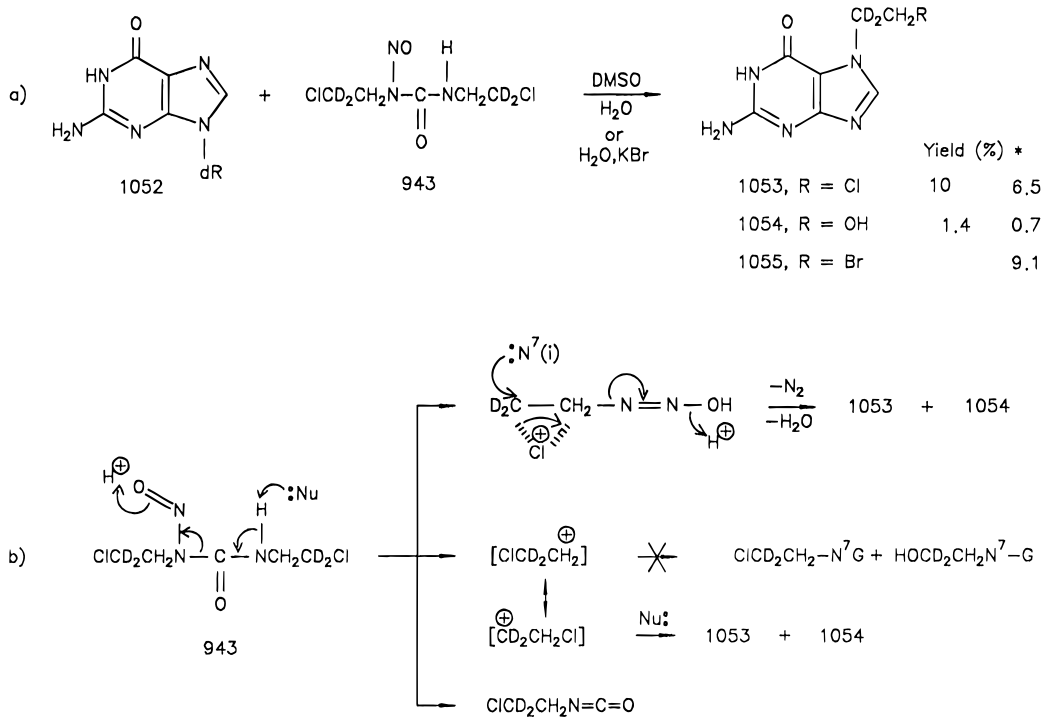
Alkyl adducts of the phosphate moiety of DNA are stable,[744] but the 2-hydroxyethyl adducts are relatively unstable. The mechanism of the reaction of 2-hydroxyethylated *N*-nitrosoureas with DNA was studied[759] on model compounds. Thus, the reactions of 3′- and 5′-deoxythymidine monophosphate with HENU (**1050**, Chart 28) and MNU (**25**) resulted in an almost complete alkylation. The methyl phosphate triester which was formed from MNU was highly stable at pH 12.5 and 7.0, with a half-life of 165 min and >3 days at the two pH values, while the corresponding hydroxyethyl phosphate triesters from **1050** were found[759] to be less stable with half-lives of <1 min and 60 min, respectively. The short half-life at strongly alkaline pH values for the hydroxyethylated deoxythymidines can be explained by a base-catalyzed formation of a dioxaphospholane ring **1051** (Scheme 115). Thus, it was hypothesized[745,759] that the hydroxyethylation of phosphodiesters in DNA by HENU (**1050**) would induce single-strand breaks of DNA according to the mechanism of Scheme 115. These breaks would be detected[745] by different kinetics than those obtained from the alkylations of nucleic bases and/or from repair processes.

Important evidence about the mechanism of in vivo alkylation of DNA was presented.[760−762] The presence of a 2-chloroethyl carbocation as the active alkylating species was disputed[760−762] by elegant experiments utilizing specifically deuterated CENU analogs. Thus, the reaction of the deoxyguanosine (**1052**) with 1,3-bis(2-chloro,2,2-dideuterioethyl)-1-nitrosourea (2,2,2′,2′-D_4-BCNU, **943**) yielded,[760] after depurination, the dideuterated products **1053** and **1054** as shown in Scheme 116a. In a separate experiment the addition of potassium bromide resulted in a product shift, with the formation of a N^7-(2-bromoethyl)guanine **1055** as the main product. The most important aspect of these experiments is that all of the products contain the two deuteriums on the carbon adjacent to the carbon bearing the halogen and hydroxyl moieties. An initial nucleophilic displacement at the C2-position could lead to a caged ion pair in which the chloride ion, bromide ion, or water would react at the C1-position to yield the observed products (Scheme 116b). A free carbocation intermediate would be expected[760] to yield a mixture of products containing deuterium on both carbons (Scheme 116b).

Additional important information was provided[761,762] by the reaction of 1,1,1′,1′-D_4-BFNU (**1056**) with deoxyguanosine (Scheme 117a). In this instance, the product structures **1057**−**1062** were determined by a combination of HPLC and tandem mass spectrometry (MS−MS). The absence of GuaN7-CD_2CH_2X products is evidence against a 2-fluoroethyl carbocation intermediate. Apparently the presence of an adjacent electron-withdrawing nitroso group weakens the C-F bond and makes it more susceptible to nucleophilic displacement[761] (Scheme 117b). An explanation for the observed products is afforded by the proposed[761] mechanism shown in Scheme 118a (also Scheme 106 in section IX). Thus, the reaction of the imidourea tautomer **1056** with water forms the tetrahedral intermediate **1063** which undergoes a cyclization reaction to either the 2-amino-1,3-oxazolidine, path A, or the 1,2,3-oxadiazolium ion, path B. Further reaction of these intermediates with the 2-deoxyguanosine would yield the dideuterated diazohydroxide **1064**.[761,762] At this point, the reaction of the intermediate **1064** with water and fluoride ion could yield either the dideuterated products **1057** and **1060** or undergo a loss of a deuterium from a short-lived diazoalkane **1065**, and subsequent reaction with either water or fluoride ion to yield the monodeuterated products **1058** and **1061** (Scheme 118b). The pathway A was favored[761] because it represents a lower energy state for the neutral intermediate **1064**.

A radical mechanism of DNA alkylation was indicated from molecular orbital calculations, by the MINDO/3 method, of DNA bases.[763] A positive correlation was demonstrated between the MO-derived net atomic charges on heteroatoms and superdelo-

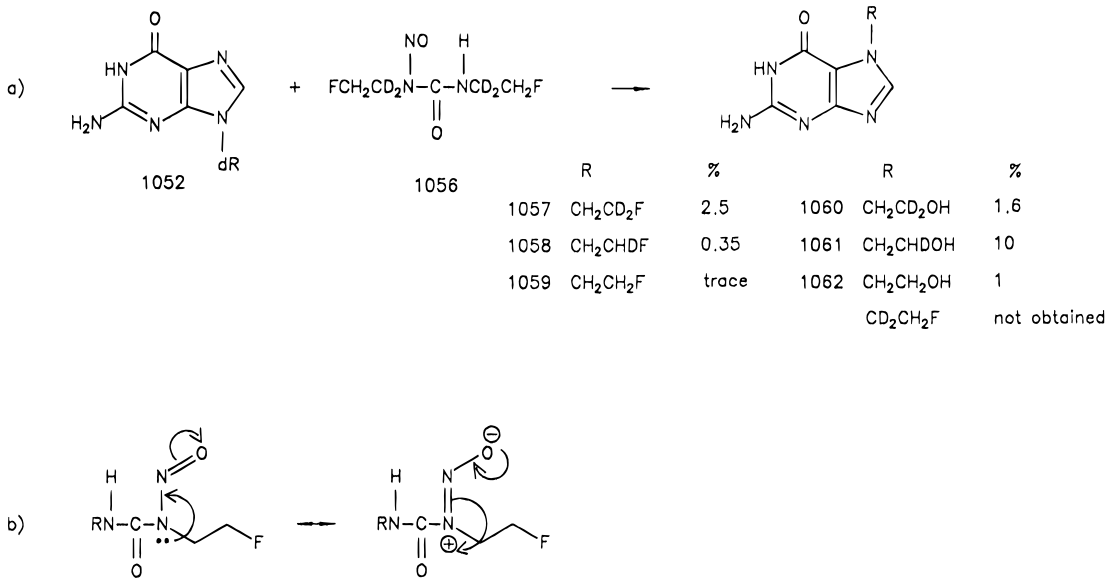
Scheme 116



dR = 2–deoxyribose; i = 1052

* = Product yields obtained after depurination reactions.

Scheme 117



dR = 2–deoxyribose

The structures and yields were obtained after a depurination reaction.

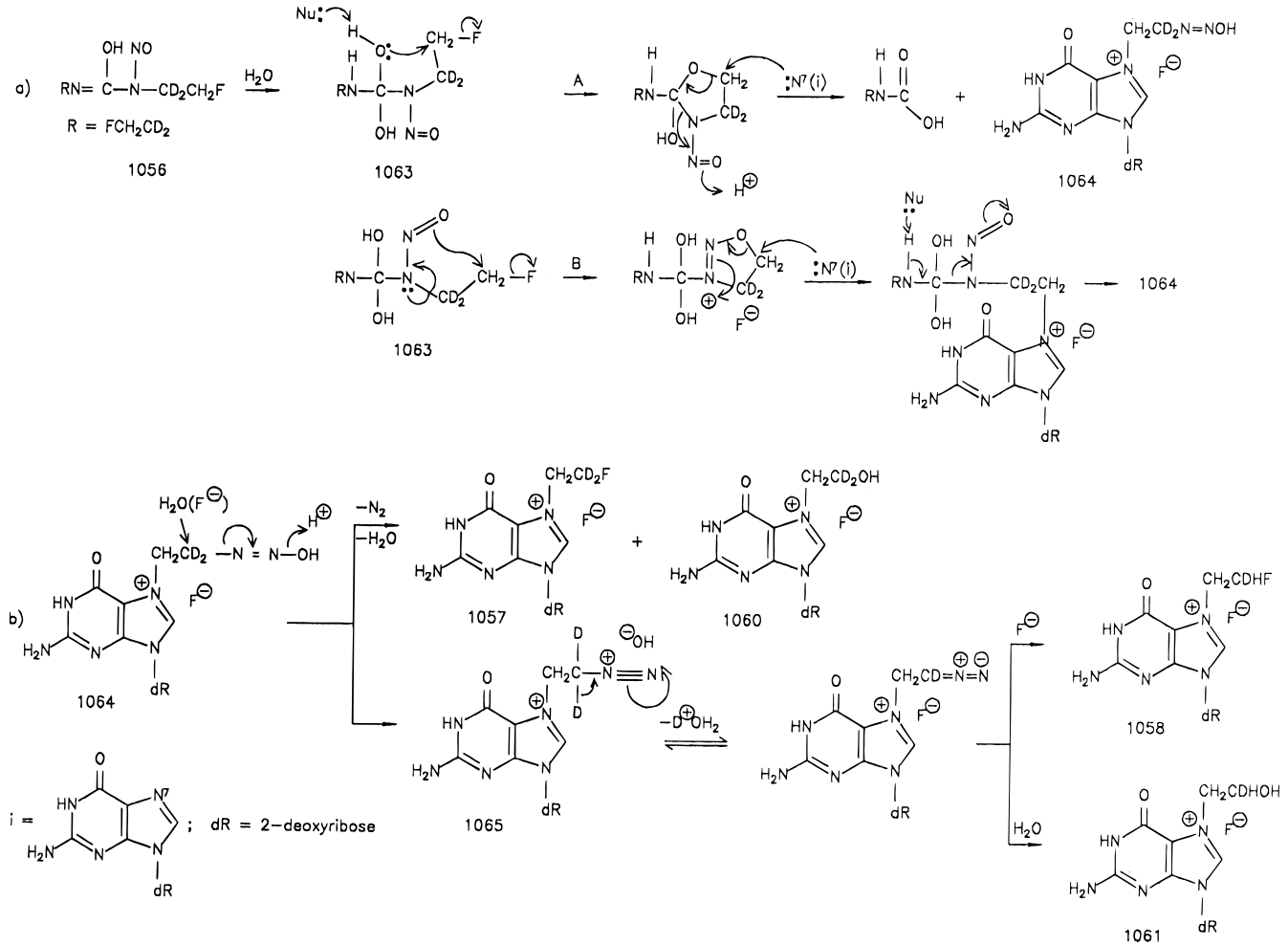
calizability and the percentage alkylation by MNU (**25**).[763] Radical products were formed in photoinduced reactions of DNA bases and MNU at different pH levels.[702] At pH 5–7 EPR evidence was presented[702] for the transfer of a methyl radical from MNU to thymine and purine bases, resulting in free radical anions **1066** and **1067**, respectively (Chart 28).

B. DNA Cross-Linking

Much attention has been devoted to the intrastrand and interstrand DNA–DNA cross-linking and the DNA–protein cross-linking reactions. Reviews of earlier work have been published.[764,765]

DNA interstrand and DNA–protein cross-linking have been determined[735] by the alkaline elution

Scheme 118



technique using the following procedure. The cells were labeled with 0.01 μCi/mL [2-^{14}C]thymidine for 24 h. After drug treatment and postinoculation, the cells were isolated and mixed with [^{3}H]thymidine-labeled L1210 cells which serve as an internal reference in the assay. The mixture of cells were exposed to 300 rads of γ-irradiation at 0 °C to introduce a known frequency of DNA single-strand breaks in the DNA. The cells then were deposited on a 2 μM pore polyvinyl chloride filter, lysed with 2% sodium dodecyl sulfate (SDS), either with or without proteinase K. An aqueous solution of 0.02 M EDTA tetrapropylammonium hydroxide, pH 12.1, was then pumped through the filter at a rate of 2 mL/h, and fractions were collected for radioactivity determination.[734]

Interstrand cross-links cause a reduction of the elution rate by increasing the effective size of the DNA strand so that the presence of these cross-links can be determined by following the reduction of the DNA elution rate.[764,765] DNA−protein cross-links also result in a reduction of the elution rate because the proteins adsorb to the filter and DNA strand linked to protein are thus retained on the filter. The addition of proteinase-K eliminates these DNA−protein associations. The difference between the assays with and without proteinase gives the amount of DNA−DNA interstrand cross-links (ICL). Cross-linking can be calculated[766−769] in terms of the "cross-link index" defined as $[(1 - R_0)/(1 - R_1)]^{0.5} - 1$, where R_0 and R_1 are the relative retention for untreated and drug-treated cells, respectively.[769] Relative retention is the fraction of [^{14}C]DNA remaining on the filter when 30% of the internal standard [^{3}H]DNA remain on the filter. The cross-link index was found[768,769] to be proportional to a drug concentration under standard conditions of cell treatment which is a proportional measure of DNA cross-linking both with DNA and proteins.

A basic two-step mechanism of DNA interstrand cross-linking by CENUs has been determined, and it was concluded[733,770] that a first step of about 1 h duration is a chloroethylation of a nucleophilic site on one strand of the DNA, followed by a slower step of about 2−8 h duration, involving a displacement of the chlorine atom by a nucleophilic site on the opposite strand, resulting in an 1,2-ethanediyl bridge between the strands (Scheme 113e). Consistent with this hypothesis were the following facts. Firstly, the reaction of 1-(2-fluoroethyl)-3-cyclohexyl-1-nitrosourea (FCNU, **137a**) produced a much lower number of cross-links than the 2-chloroethyl drug CCNU (**137b**), as expected from the lower reactivity of the fluorine atom as a leaving group.[733] Secondly, the reaction of *N*-nitrosoureas containing no 2-haloethyl moiety, such as *N*-methyl-*N*-nitrosourea (MNU, **25**) and streptozotocin (**367**) produced no detectable cross-linking.[733] Thirdly, the rate of the second step, i.e. the cross-link formation in the absence of the drug, was found[733] to be independent of the type of CENU which was employed in the initial incubation. Support for the second rate-determining step of the DNA

cross-linking mechanism was provided by observations of the following type. Thus BCNU (**33**) with a half-life of 79 min at pH 7.2 and at 37 °C[669] effected maximum DNA cross-links in vitro after 6−8 h.[733,770] The N3-unsubstituted analog CNU (**26**), with a half-life of 8 min under comparable conditions[669] effected maximum DNA cross-links in 4 h.[753] The fact that an increase in decomposition rate by a factor of 10 only increased the rate of cross-linking by a factor of ~2 supports the hypotheses that it is not the initial decomposition and alkylation of DNA bases by *N*-nitrosourea drugs but rather the rate of the second alkylation by the chloroethylated bases which determines the overall rate of DNA interstrand cross-linking.[704]

Examination of geometric models of a double-stranded DNA led to the prediction[733,765] that an interstrand cross-link could be formed most readily between guanine and cytosine heterobases. The isolation of the compounds **1035**, **1040**,[728,729] and **1041**[730] (Figure 3) after reactions between CENUs and DNA was considered as support for this hypothesis of a two-carbon bridge for either inter- or intrastrand cross-linking. A possible mechanism for the formation of **1041b** would entail production of the O^6-(2-chloroethyl)guanine **1030b**, followed by an intramolecular cyclization to N^1,O^6-ethanoguanine **1044b** and the S_N2 attack at the ethanediyl bridge of **1044b** by the N3 of deoxycytosine (Scheme 113c,e). The initial attack on the O6-position of guanine was supported[764,765] by the findings that cell lines which are able to remove O6-substituents from guanine are resistant to cross-link formation. Such cells would, presumably, remove a chloroethyl moiety from the O6-position before it could rearrange and lead to cross-link formation.

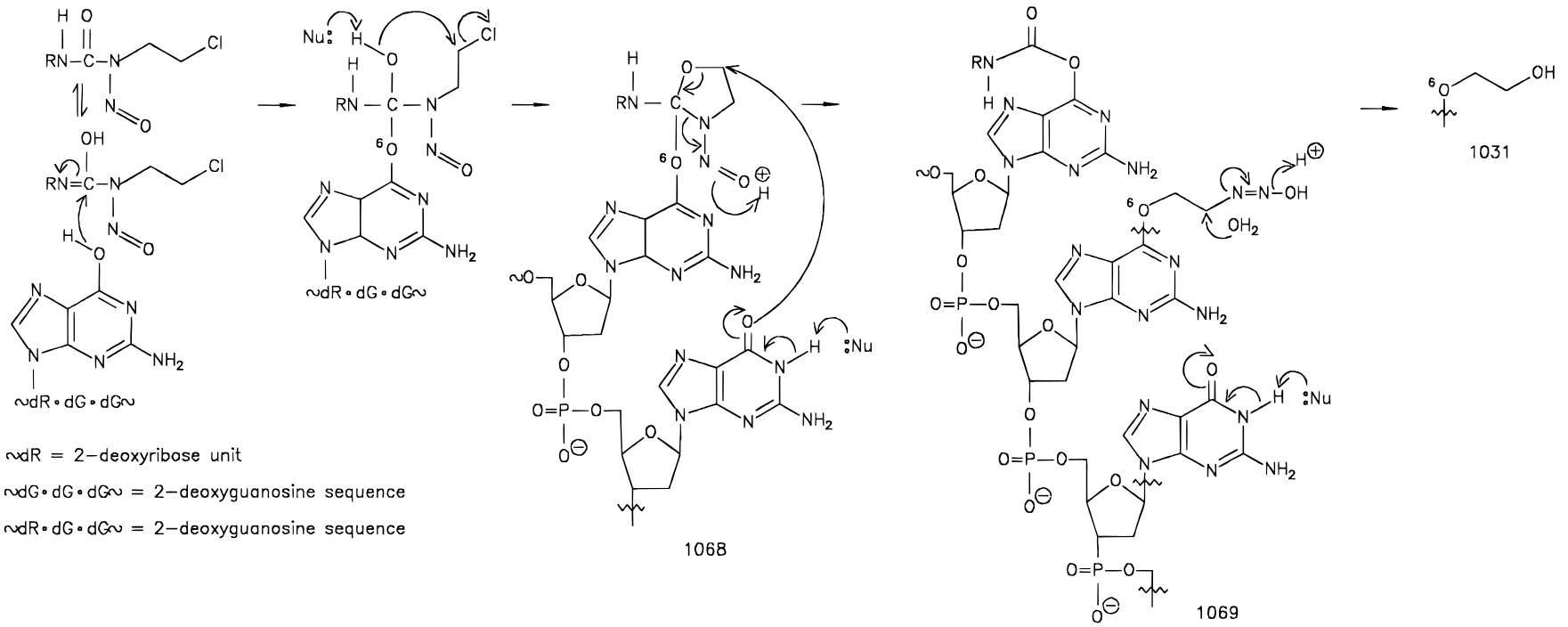
There are problems with the standard cross-linking mechanism.[771,772] First, the mechanism (Scheme 113c,e) requires second-order kinetics but the kinetics are actually either first order or pseudo first order, i.e. suggesting an intramolecular alkylation. Second, the rate-limiting step should be the rate of hydrolysis, whereas the rate-limiting step of CENUs at near neutral pH was found[704] to be the formation of a tetrahedral intermediate by addition of water to the imidourea form of the parent compound. Third, the formation of the carbocation intermediate in Scheme 113a would predict that the product ratios of CENU hydrolysis and DNA alkylation reactions should be the same and that DNA should be alkylated randomly without regard to base sequence. In fact, the hydrolysis of CENUs results in a ratio of chloroethanol, equivalent to chloroethylated DNA adducts, to acetaldehyde, equivalent to hydroxyethylated DNA adducts, of approximately 2:1, whereas the alkylation reaction of DNA with CENUs resulted in the ratio of hydroxyethylated guanines i.e. $HOCH_2CH_2$-N^7-G (**1028**) and $HOCH_2CH_2$-O^6-G (**1031**) to chloroethylated guanine $ClCH_2CH_2$-N^7-G, **1027b** of about 2:1. CENUs and ANUs were shown[773−775] to alkylate DNA regioselectively in contiguous assemblies of guanine in vitro and in vivo. This regioselectivity is consistent with an intermediate covalently bound to DNA at dG1 in a dG1dG2dN3 codon, where dN is any other base.[772] Fourth, a $ClCH_2CH_2$-O^6-G intermediate **1030b** or the N^1,O^6-ethanodiG intermediate **1044** (Scheme 113c,e) are not important because the major alkylated DNA product in vivo is $HOCH_2CH_2$-N^7-dG (**1028b**) while the $HOCH_2CH_2$-N^1-dG (**1032b**) is present in only trace amounts.[736,744,760] The $ClCH_2CH_2$-N^7-dG (**1027b**) is stable to hydrolysis and is not the precursor of $HOCH_2CH_2$-N^7-dG (**1028**).[724,725] Fifth, computer modeling studies[771] revealed that the cyclization of $ClCH_2CH_2$-O^6-dG (**1030b**) to **1044** (Scheme 113), is not possible in the DNA model, duplex B-oligo$(dGdC)_5$, because the Watson−Crick paired cytosine on the opposite strand blocks the **1030b** from assuming the proper geometry.

An alternate mechanism for DNA alkylation and subsequent cross-linking was proposed[771] which fits most of the experimental results (Scheme 119). In this case, the O6 of dG1 of the triplet codon attacks the imidourea to form a tetrahedral intermediate.[64,776] This intermediate can undergo intramolecular cyclization reactions to yield either a 2-amino-1,3-oxazolidine **1068**, or a 1,2,3-oxadiazolium ion (not shown) (Schemes 91 and 106). At this point the O6 of a neighboring guanine dG2 could react as a nucleophile, opening the rings and forming the corresponding (2-hydroxyazo)ethyl-O^6-dG **1069**. Subsequent reaction of the diazohydroxide **1069** with water would produce the $HOCH_2CH_2$-O^6-dG **1031**, whereas the reaction with nucleophiles either on the same strand or on opposite strands would produce either intra- or inter-cross-linked products (Scheme 119).[771] The previously discussed experiments[760−762] with deuterated CENUs (Schemes 116 and 117) lent support to such a mechanism by specifically eliminating free carbocations as reactive intermediates arising from decomposition reactions of the parent drugs. Factors such as DNA sequence[773−775,777] and ionic strength[778] may affect these reactive intermediates. The use of a GGG codon as the point of attack on the DNA strand was based on experiments[773−775,777] with CENU drugs resulting in preferential alkylation at the N7 of guanine located in the middle position of contiguous assemblies of guanine bases. The base sequence selectivity is explained[779] by the fact that the site of greatest electronegativity is the N7 position of guanines flanked by other guanines. An intermediate with a positive charge, such as the chloronium ion intermediates in Scheme 116b, would be expected to react preferentially at such sites on the DNA.[771,773] It should be recalled that such cyclic positively charged intermediates were proposed[678] in the chemical decomposition of CENUs (Scheme 94).

The presence of the 2-chloroethyldiazohydroxide intermediate was shown[773] to be crucial because the reaction of ethylnitrosourea (**27**) which decomposes to ethanediazohydroxide, gave no preferential alkylation of DNA bases. In the latter case the intermediate would not have a cationic character. The reaction of the alkylating agent $ClCH_2CH_2$-SOSO (**1070**, Chart 28), occurred predominantly by a S_N2 mechanism to yield only (2-chloroethyl)-N^7-guanine and displayed no regioselectively in the alkylation of the DNA bases.[773] Additional details about the base sequence specificity and neighboring group effects are discussed in a review.[775]

The DNA methylation mechanism[771] involving a nucleophilic attack of the O6 of guanine either on the carbonyl carbon of the urea form of MNU (**25**) or the

Scheme 119


≈dR = 2–deoxyribose unit
≈dG•dG•dG≈ = 2–deoxyguanosine sequence
≈dR•dG•dG≈ = 2–deoxyguanosine sequence
1068
1069
1031
Intrastrand Cross–linking
A Reaction within the same
single helix of DNA
Interstrand Cross–linking
A Reaction with another strand
of the double helix of DNA

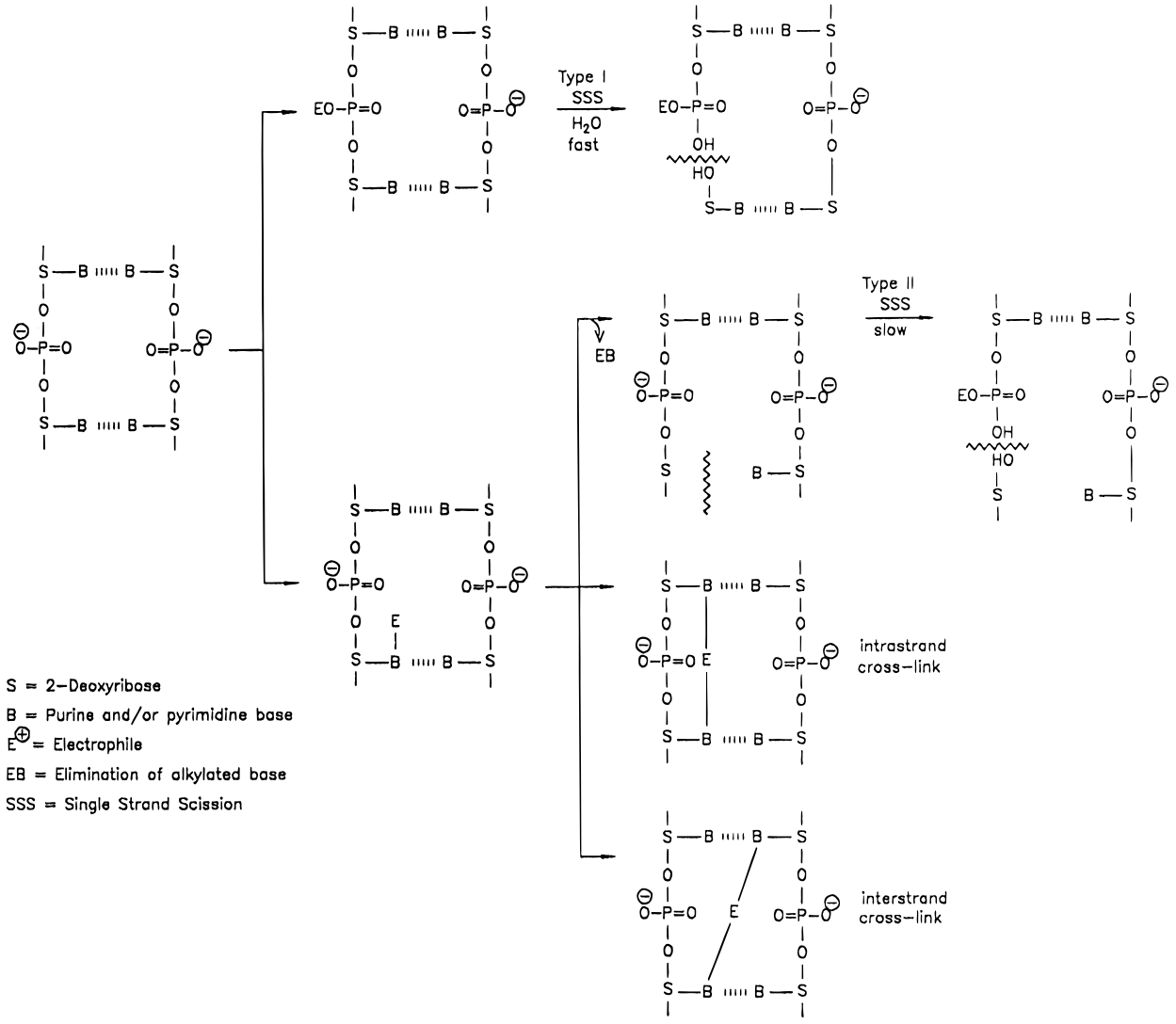
imino carbon of the isourea form of MNU, as outlined in Scheme 119, was challenged.[780] A free methyldiazonium ion $CH_3N_2^+$ was considered[780] to be the agent which is responsible for the alkylation of the DNA bases. Furthermore, the specificity of DNA base alkylation observed for MNU can be explained[780] by the enhanced reactivity of the N7-position of guanine toward the methyldiazonium ion because of neighboring structural factors. A study[780] of the alkylation patterns of DNA by the agents MNU (**25**), MNNG (**24**), *N*-nitroso(1-acetoxyethyl)methylamine, and diazomethane revealed the same qualitative and quantitative N^7-MeG pattern, which is consistent with the formation of a common DNA−methylation intermediate, i.e. the methyldiazonium ion. There was no preferential methylation of a 3′ guanine (G) within the G_2, G_3, and G_4 sites of a DNA restriction fragment which would have been predicted on the basis of the mechanism[771] shown in Scheme 119. The presence of inorganic cations or organic DNA affinity cations inhibited the alkylation of DNA by MNU independent of base sequence.[780] This result could be explained[780] by a binding of the cations to the phosphate backbone of the DNA and a concomitant shielding of the nucleophilic sites on the DNA bases from reaction with the positively charged methyldiazonium ion. Neither the inorganic or organic cations had any effect on the methylation of DNA by the uncharged alkylating agent dimethyl sulfate (DMS).[778] Steric effects were ruled out[780] as a cause of the N^7-MeG patterns produced by the methyldiazonium ion because the 5′G in the dG_3 and dG_4 sites are accessible to the bulkier DMS. An interesting finding in this study was that the average amount of N^7-MeG found within the dG_3 and dG_4 sites was about the same as that for an isolated G site within the DNA fragment. Thus, the observed preferential methylation of the poly dG sites would be the result of an electrophilic selectivity for certain guanine bases within those sites by the $CH_3N_2^+$ ion.[780] Fluctuations in the nucleophilicity at N^7-G sites within the DNA fragment could account for the 6−8-fold variation between methylation intensities at different guanine base sites.[780] The base-stacking geometries in the vicinity of the guanine bases could be an important factor in determining the alkylation frequency of the N7 sites in DNA. The dominant effect of the 5′ base on N^7-G methylation can be explained[780] by inspecting molecular models. Thus, in these models the N^7-G site in B-DNA is situated directly over the C4 and N1 of the 5′-purine and -pyrimidine rings, respectively. Base-stacking geometries can be used to explain[780] the fact that an adjacent cytosine (C) often causes a lowering of the alkylation at a 5′-G site because the 4-amino protons of C, which protrude into the major groove and bear a positive charge, are positioned over the N7 site of the 5′G.

A linked compound, containing the elements of MNU and methidium chloride, a DNA affinity compound, was synthesized and assayed [781] for its ability to deliver MNU (**25**) to DNA alkylation sites. In contrast to free MNU, the methylation of DNA by this linked compound was augmented in the presence of salts with increasing ionic strength. In this case all guanine sites were equally methylated. The DNA methylation by the MNU−methidium chloride linked compound probably results from a nonsequence specific intercalation of DNA by the methidium moiety at physiological ionic strength.[781] Under these conditions the methyldiazonium ion $CH_3N_2^+$ is rapidly generated, followed by a reaction with the adjacent guanine sites before it can diffuse away from its intercalated environment in the major groove of the DNA.[781]

Studies on the relation between cross-linking and the structures of CENUs were very instructive.[753,770] Thus, the reaction of BCNU (**33**) cross-linked 42% of DNA in 6 h at pH 7.2 and 37 °C, and the rate of cross-linking increased with increasing pH in the range of 4−10, in accordance with the suggested major mechanism of decomposition (Scheme 91, pathway A). The nature and position of the halogen atom is important.[753,770] Thus, the rate of cross-linking decreased in the order Cl ≫ Br > F > I. This order of halogen reactivity in cross-linking reactions is different than the order of leaving ability in S_N2 reactions, namely I > Br > Cl > F. The bromo- and iodoethyl analogs **36** and **38**, respectively, could undergo competing decomposition reactions in the plasma before reaching the cell nucleus, while the fluoroethyl analog **34** would be unreactive in the initial alkylation of the DNA base. Apparently, the chloroethyl analog **33** has an intermediate reactivity which prevents the competing decomposition reactions while still allowing the alkylation of the DNA bases followed by the formation of the interstrand cross-links.[704] Further, only one 2-haloethyl group is essential for the cross-linking by either CCNU (**137b**) or chlorozotocin (**368**), and the cross-linking was not observed when the halogen atom is replaced with either hydroxy or methoxy groups.[704] The position of the halogen atom is also crucial for the cross-linking reaction since it was shown[753,770] that the 2-chloroethyl drug BCNU (**33**) is an efficient cross-linking agent, whereas the 3-chloropropyl, 4-chlorobutyl, and 5-chloropentyl analogs, in spite of their alkylating capability of DNA, are ineffective cross-linking agents. Increasing the acidity of the N3 H by aryl substitution increased the cross-linking rate by increasing the rate of the first decomposition step.[770] A hydrogen atom on the N3 atom is required because the reaction of a disubstituted N3 analog, such as *N*-(2-chloroethyl)-*N*′,*N*′-dimethyl-*N*-nitrosourea (**941**) fails to form ICLs.[770] Substitution of the hydrogen by the methyl group in the 2-chloroethyl group, e.g. *N*-(2-chloropropyl)-*N*-nitrosourea (**41**), greatly reduced the extent of DNA cross-linking.[770] Cross-linking was not observed[767] either with methylnitrosourea (**25**) or streptozotocin (**367**) in the DNA of Chinese hamster cells. Furthermore, a 40 times greater concentration of **25** and **367** was required to produce a cytotoxicity equal to that of the (haloethyl)nitrosoureas.

An imperfect correlation was shown[770] between the percentage of DNA cross-linking and the in vitro and in vivo activity against L1210 leukemia. The exceptions included the 2-fluoro analog of CCNU (**137a**) with 6% cross-linking and 70−90% cures compared with CCNU (**137b**) with 37% cross-linking and 80−100% cures. The reaction of the N3 phenyl analog of BCNU (**208**) produced 36% cross-linking but the compound was inactive against the L1210 leukemia.[770] Similarly, the reaction of a specially de-

Scheme 120



signed[704] compound **1008** (section IX) produced 90% of cross-linking, but the compound was only moderately active against the L1210 leukemia.

The effect of electrophiles from CENUs on cross-linking was explained[753] as shown in Scheme 120. Alkylation of the internucleotide phosphate linkages resulted in a fast type I single-strand scission (SSS).[745] Alkylation of the bases resulted in depurination followed by a slower type II SSS, involving either the hydrolysis of apurinic sites or further intramolecular and intermolecular alkylation by the chloroethylated bases.[745] Intermolecular alkylations produced either inter- or intrastrand DNA cross-links.[745,753]

The effect of DNA single-strand scission on interstrand cross-linking was studied[745] by incubating λ-DNA at pH 7.2 and 37 °C with an equimolar mixture of *N*′-(cyclohexyl)-*N*-(2-hydroxyethyl)-*N*-nitrosourea (CHNU, **969**, Scheme 99) and BCNU (**33**), both known agents of single-strand scissions. Addition of 5 mM CHNU to 5 mM BCNU caused a sharp drop in the extent of cross-linking by BCNU probably because the CHNU decomposition resulted in DNA strand scission through phosphate alkylation.[745]

Additional insights into DNA SSS was provided[217,218] by a study of DNA reactions with adriamycin analogs **248** and **249**. Alkaline elution filter assays of the DNA from L1210 cells treated with **248** and **249** revealed[217,218] a damage from protein-associated SSS and DNA−protein cross-links, i.e. a DNA intercalation effect, and also DNA SSS and DNA−DNA cross-links, i.e. a DNA alkylation effect. However, the interaction of adriamycin with DNA produced only DNA lesions associated with intercalation. The intercalation of DNA was established by the inhibition of the DNA topoisomerisase II-catalyzed unwinding of plasmid PC15 supercoiled DNA.[217,218] The alkylation of DNA by **248** appears to proceed by the established two-step mechanism for other CENU compounds (Schemes 113 and 119).

Another interesting area of DNA cross-linking research involves the design of drugs that will form cross-links in the minor groove of the DNA double helix. A number of alkyl- and (2-chloroethyl)nitrosourea moieties have been chemically linked[782−785] to DNA minor groove binding di- and tripeptides, such as lexitropsin (lex), an "information peptide"[786] based on methylpyrrole-2-carboxamide subunits. These compounds **1071**−**1073** (Chart 28) "force" the reactions between nucleophilic centers in the minor groove and the reactive intermediates generated from the hydrolysis of CENUs. The main reasons for synthesizing these compounds were, firstly, to increase the overall number of DNA lesions by CENUs, and, secondly, to obtain a different type of interstrand

cross-link in the minor groove of DNA which would be impervious to the DNA repair mechanisms of major groove lesions found in many resistant tumor cells.[783] The sequence specificity for alkylation, as observed in the ^{32}P-labeled restriction fragments, was mainly for adenine (A) and only those adenines associated with lexitropsin binding sites.[782] The alkylation of the N7- position of guanine by CENU-lex-2 **1072**, which is the major alkylation site for major groove reactions by CENUs, occurred to a much lesser extent than the alkylation at the adenine in the minor groove.[782] This adenine alkylation could be effectively inhibited[787] by the minor groove binding antibiotic distamycin A which acts as a competitor of **1072** for A-T rich minor groove binding sites. The inhibitory doses (ID_{50}) of all three CENU-lex peptides **1071**−**1073** for sensitive L1210 cells were essentially equivalent.[782] This result indicates that factors other than affinity binding properties are involved in these minor DNA groove alkylations. Of the two dipeptides, CENU-lex-2$^+$ **1073**, an ionized species at physiological pH, was the stronger equilibrium binder, but the neutral dipeptide CENU-lex-2 **1072**, was the more powerful minor groove alkylation agent.[782] The biological evaluation of these CENU-lex peptides revealed[783] that they are somewhat less cytotoxic than their CENU counterparts against the CCNU-sensitive 9L rat brain tumor cell line. A reason for this finding is that the minor groove *N*-alkyladenine adduct fails to form stable DNA cross-links as compared with the major groove O^6-(2-chloroethyl)-deoxyguanosine adduct **1030b** (Scheme 113). The N^3-(2-chloroethyl)adenine adducts may be too unstable to form effective cross-links and the N^3-(2-hydroxyethyl)adenine adducts cannot form cross-links but rather single strand breaks via apurinic sites[783] (type II SSS, Scheme 120). However, the CENU-lex dipeptides were more cytotoxic in a 9L cell line which had no DNA repair enzyme (AT).[783] In vitro studies[783] revealed that the reactions of compounds **1071**−**1073** produced lower levels of sister chromatid exchanges (SCE) than CENUs in CENU-sensitive 9L cells but the same level of SCEs as CENU drugs in CENU-resistant 9L-2 cells. Minor groove binding represents a "detoxification" pathway for the CENU-lexs, since this lesion is formed at the expense of the cytotoxic major groove interstrand cross-links.[783]

In order to put this discussion in perspective it should be noted that the extent of interstrand cross-linked DNA produced by reactions of CENUs is much lower than that of other bifunctional alkylating agents under comparable conditions.[753] For example, the percentage of cross-linked DNA produced by mitomycin *C* at 1.2×10^{-4} M was found to be 84% compared to 5% for CCNU (**137b**) at 2.2×10^{-4} M. Even at a concentration of 65×10^{-4} M the interaction of CCNU with DNA produced only 47% of cross-linking.[753] These results support the idea that the CENUs, such as CCNU (**137b**), are more discriminating in their base-pair positions on the DNA double strands.[773−775] Additionally, the ratio of hydrolyzed drug to bound drug was 3271 ± 148 for CCNU at 2.2×10^{-4} M concentration and 42 for mitomycin *C* at 1.2×10^{-4} M.[753] Thus, CCNU reacts more readily with water and binds less to DNA than mitomycin C.

C. DNA Repair

The longer period of 2−8 h between DNA-monoadduct formation and subsequent reaction to form DNA interstrand crosslinks[733] affords an opportunity for the cell to repair the DNA damage and restore the normal condition. In particular, alkylation of the O6-position of guanine is thought[697,714,739] to be particularly mutagenic and carcinogenic because it can cause a shift in the base pairing of DNA. Normal, i.e. healthy, cells can eliminate these alkylated DNA products by means of the repair enzyme O^6-methylguanine-DNA-methyltransferase (MGMT). MGMT has been purified from bacterial cells,[788] rat liver,[789] and human cells.[790] This enzyme is believed[791−793] to act as a protein acceptor, irreversibly transferring the O^6-alkyl group to a cysteine residue within its structure, thus inactivating the enzyme and restoring the guanine to its unmodified form. The alkyltransferase (AT) enzyme undergoes a suicide reaction with its substrate—O^6-alkylguanine or O^6-alkylguanosine. The reaction is unusual in being stoichoimetric, each AT enzyme can repair only a single O^6-alkylG residue. The isolated bacterial enzyme prevents the cross-linking reaction of purified DNA in vitro with BCNU.[794] The enzyme, O^6-alkylguanine-DNA-alkyltransferase (GAT, AT), isolated from extracts of human leukemic lymphoblasts, prevented the cross-linking reaction of DNA in vitro with a variety of CENUs.[795] More specifically, the formation of the bis adduct, 1-(3-deoxycytidyl)-2-(1-deoxyguanosinyl)-ethane (**1041**) was prevented by the AT enzyme in rat liver.[796] Evidence was presented[797] in support of the reaction of the GAT enzyme with the DNA cross-link intermediate N^1,O^6-ethanoguanine **1044b**. This reaction would remove this important cross-linking intermediate of CENU drugs. Recently, the linkage between DNA and MGMT was shown[798] to be 1-(guan-1-yl)-2-(cystein-*S*-yl)ethane (**1074**) (Scheme 113d). A DNA repair activity was found[799] in an extract of a *Escherichia coli* strain which transferred a chloroethyl group from a chloroethyl phosphotriester isomer of a poly (dT) poly(dA) copolymer to the bacterial protein. The chemical, biological and clinical aspects of the AT enzyme were discussed in reviews.[800−802]

The DNA repair is obviously important in the reduction of the mutagenic and carcinogenic potential of various chemicals. It was hypothesized[803−805] that a lack of efficient repair of O^4-alkylthymine (**1042**) and O^6-alkylguanine (**1043**) DNA adducts is responsible for the carcinogenicity of nitrosoureas and nitrosamines. Specially bred transgenic mice were raised[806,807] to express a chimeric gene consisting of the inducible *P*-enolpyruvate carboxykinase (GTP) promoter which is linked to the bacterial O^6-alkylguanine-DNA-alkyltransferase (*ada*) gene. The administration of MNU (**25**) to these mice resulted[807] in a three times faster repair of the O^6-methylguanine-DNA adducts than in nontransgenic mice. This result is in agreement with the greater level of GAT enzyme in the transgenic mice. In the ada^- mice the depletion of hepatic alkyltransferase occurred at lower doses of carcinogen, and the recovery of alkyltransferase activity occurred later than in the ada^+ mice.[807]

The foregoing discussion identifies the continuing dilemma in the use of DNA alkylation drugs, such as *N*-nitrosoureas. Thus, although the repair of DNA alkylated products reduces the mutagenic and carcinogenic potential of the drugs, this same DNA repair removes the possibility of DNA interstrand cross-links, a crucial aspect of the cytotoxicity against cancer cells. To further complicate the matter it was observed[808−810] that some cells derived from the human cell pool are deficient in the elimination and repair of such lesions. The repair-deficient cell lines when treated with methylating agents, such as *N*-methyl-*N*-nitrosourea (NMU, **25**) and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG, **24**), were found[809] to have reduced rates of removal of O^6-methylguanines from their DNA. These human cell lines can be divided into methylation repair deficient type Mer^- and methylation repair sufficient type Mer^+.[809] The removal of other alkyl groups beside methyl from the O6-position of guanine led to the realization[811] that it is the change in the tautomeric structure of the guanine ring after O6-alkylation which is the recognition feature for the enzyme. Therefore, cell lines with an efficient O6-alkylguanine repair system Mer^+ could also remove O^6-(chloroethyl)guanine monoadducts rapidly enough to prevent the subsequent formation of cross-links.[811−813]

Support for this DNA repair mechanism came from experiments[811] in which 13 human cell lines, derived from various types of cancers, were reacted with CENUs for 1 h, then washed and incubated in the absence of drug for 6 h in order to allow a delayed interstrand cross-linking. A determination of the DNA interstrand cross-links and the DNA−protein cross-links by alkaline elution techniques, using two methods,[734,735] revealed an inverse relationship between DNA interstrand cross-links and methylating repair capacity, i.e. six Mer^- strains clearly formed more cross-links in the presence of CENUs than five of the seven Mer^+ lines.[811] The correlation between cytotoxicity and the cross-link index was excellent.[811] The DNA−protein cross-linking was not significantly different between Mer^+ and Mer^- cell lines, and this result supports the existence of DNA−DNA interstrand cross-links (ICL) as the site of the repair differences. On the basis of the foregoing it was hypothesized[811] that only certain tumor cell lines (Mer^-) would exhibit sensitivity to CENU drugs and this repair-deficient parameter could be used as an in vitro screen during the search for new nitrosourea compounds with antitumor activity. These correlations between Mer phenotype and cytotoxicity were shown[814] not to extend to another type of antitumor agent, such as dichlorodiammine platinum(II) (Cisplatin), which is also involved in cross-linking of DNA.

The CENU-resistant rat brain tumor line 9L-2, compared with the CENU sensitive 9L cell line, was shown[815] to have a 50% reduced level of DNA interstrand cross-links, reduced sister chromatid exchanges (SCE), and a 20% reduced level of O^6-alkylguanine but the same levels of N^7-CH_3-G and N^3-CH_3-A after treatment with MNU (**25**). Resistant cells have a higher cellular level of the MGMT enzyme, increased repair of O^6-methylguanine, and 50% fewer DNA ICL than the sensitive cells.[816] Similar results were found[817] for the interaction of sensitive and resistant human glioma cells with *N*-(2-chloroethyl)-*N*-nitrosourea (CNU, **26**). Thus, the finding that resistant cells contained much less of the cross-linked adduct **1041** and of the DNA minor bases can be explained[817] by the higher levels of the AT enzyme in these cells. However, the relationship between AT enzyme activity and DNA repair activity in cells is an imperfect one. In a study of the sensitivity of five human testis and five bladder cancer cell lines to MNU (**25**) and mitozolomide there was found[818] no direct correlation between the level of AT activity and the relative resistance of the cell lines to the nitrosourea drugs. Hence, it was concluded[818] that resistance to nitrosourea drugs can be caused by other factors beside DNA repair enzyme levels, and that, at high levels of expression, the AT fails to confer a resistance in proportion to its activity.

Analogous results were obtained for human cancer cell lines. Thus, normal (IMR-90) and simian virus 40 transformed (VA-13) human embryo cells were treated[812] with several CENUs, i.e. BCNU (**33**), CCNU (**137b**), CNU (**26**), and CZT (**368**). All of the analogs were more toxic to the transformed VA-13 cells because of a dose-dependent DNA ICL which was not detected in the normal IMR-90 cells. A DNA−protein cross-linking was present to at least an equal extent in both cell types, and therefore, the lack of DNA−DNA cross-linking in the IMR-90 cells could not be attributed either to a lack of drug uptake or to increased chemical inactivation of intracellular drug. The conclusion[812] was reached that no DNA cross-links were formed in the normal cells, i.e. the removal of DNA chloroethyl adducts occurred prior to their conversion to ICL. The DNA−protein cross-links, arising from an initial chloroethylation of protein sites followed by reaction with DNA sites, would not be affected by the DNA repair mechanisms. Differences in DNA repair between the cell types were much greater in the presence of the noncarbamoylating CENUs, CNU and CZT, than the carbamoylating CENUs, BCNU and CCNU.[812] In this case the carbamoylating CENUs can inhibit the single-strand rejoining step of DNA repair and the processing of RNA transcripts.[812]

Pretreatment of Mer^+ cells with MNNG (**24**) inhibited the DNA cross-link repair process and allowed a subsequent treatment with CENUs to produce a synergistic increase of 3−4 log cell kill.[819,820] An interpretation[819,820] of this pretreatment was that MNNG produced enough O^6-methylguanine lesions to deplete all of the repair capacity of the cells. Consequently, on subsequent treatment with a drug that produced O^6-(chloroethyl)guanine, no repair capability was available to remove the O6 lesions, and hence these lesions were converted to ICLs which were lethal to the cell.[814,819,820] Different human cell strains, pretreated with MNNG (**24**), showed different abilities to repair O^6-methylguanine lesions caused by BCNU (**33**)[821] and HECNU (**329**).[822] Analogously, the pretreatment of CENU resistant cells with streptozotocin (SZT, **367**) resulted[821] in a depletion of the MGMT enzyme, an increased BCNU-induced DNA ICL, and a 2−3 log enhancement of BCNU cytotoxicity in vitro. In addition, there was no induction of expression of MGMT-mRNA which

also would lead to an enhancement of cytotoxicity.[821]

Purified human AT enzyme was inactivated by pretreatment with several methylating and alkylating CENU agents.[823] Alkyltransferase activity in cells was shown to be depleted by exposure to other substrates for the enzyme, such as O^6-methylguanine[824−830] and O^6-benzylguanine.[831] Thus, although O^6-methylguanine was shown[832] to increase the cytotoxicity of O6-alkylating drugs in vitro, there was no enhancement of their activity in vivo. In contrast, the presence of O^6-benzylguanine caused a rapid and large depletion of cellular alkyltransferase activity.[831] O^6-Benzylguanine was shown[833−837] to increase the sensitivity of colon cancer cells (HT29), malignant glioma cells (SF767), and melanoma cells (M19-mel) to the cytotoxic effects of a number of chloroethylating and methylating agents. The enhancement of the sensitivity was greater in cell lines with high AT activity, and no enhancement was observed in cell lines lacking this protein.[833] Prior administration of O^6-benzylguanine before the addition of BCNU (**33**) resulted in an enhancement of the killing of the cancer cells because of an increase in DNA cross-linking.[835] The finding that an addition of O^6-benzylguanine after BCNU administration failed to increase the cytotoxicity of BCNU was interpreted[835] to mean that some of the cancer cell lines can undergo very rapid repair of cross-link precursors.

The ability of O^6-alkyl adducts to sensitize cells to nitrosoureas should depend[825] on the rate of formation of O^6-alkylguanine and the kinetics of three different reactions which take place differently in different cell types: (1) the repair of O^6-alkylguanine adducts, (2) the generation of cross-links from chloroethyl adducts in the case of chloroethylating agents, and (3) the rate of synthesis of new alkyltransferase enzymes.

The MNNG pretreatment of Mer^+ cells failed to affect the cell kill of other DNA alkylating agents, such as L-phenylalanine mustard (L-PAM), nitrogen mustard (HN-2) and 4-*S*-(propionic acid)sulfidocyclophosphamide (CC-2) and the cross-linking drug *cis*-diamminedichloroplatinum(II) (Cisplatin).[814] The explanation[814] for this difference was that these agents produced either N7-alkylated or substituted guanines which are not susceptible to the O6-alkylated guanine repair enzyme, as is the case for CENU drugs. A further explanation for the difference in DNA repair of DNA lesions caused by CENUs and by other alkylating agents would be the location of the alkylated bases on the DNA strand. Thus, while alkylations of DNA by CENUs occur at three or more contiguous guanine moieties,[773] the guanine alkylations by other agents, such as nitrogen mustards, occur in a more random pattern.[773,775] The corresponding DNA repair enzymes could have a specificity based on the sequence of bases. Thus, the AT enzymes repairing the CENU-caused DNA lesions might be unable to repair other lesions in different base sequences, such as those caused by nitrogen mustards.[773] It was reported that O^6-methylguanine adducts in different sequences are repaired[838] at different rates and are not converted[839] to mutations in *E. coli* with equal efficiencies.

D. Carbamoylation

The hydrolysis of nitrosoureas produces an isocyanate from the N3 side of the molecule (Scheme 91, pathway A).[73,840] The liberated isocyanate can carbamoylate protein −NH and −SH groups and may thus cause the toxicity effects of nitrosoureas. The relative alkylating and carbamoylating activities of nitrosoureas are discussed in section VIII.[97,841] Carbamoylating reactions of nitrosoureas have been reviewed.[73,840] The strong carbamoylating CENUs, such as BCNU (33) and CCNU (**137b**), have been studied for their effects as inhibitors of macromolecular synthesis,[842−844] RNA processing,[845,846] DNA polymerase II,[847] repair of X-ray induced single strand breaks in DNA,[844,848−852] production of DNA strand breaks,[848,853] cross-linking of DNA,[733] alterations in the pattern of de novo purine biosynthesis,[854] and selective modification of one class of histones.[855] The active carbamoylating agent, 2-chloroethyl isocyanate, has been shown to inhibit chymotrypsin,[856] tubulin,[857] glutathione reductase,[858] and cellular esterases.[859] The strongly carbamoylating BCNU and CCNU inhibited the incorporation of labeled precursors into nucleic acids and proteins during a 1 h drug exposure, while the weak carbamoylators CZT (**368**) and CNU (**26**) failed to inhibit the incorporation.[860] Thus, these inhibitions are not essential to the cytotoxicity of CENU drugs.

E. Carbamoylation and Repair of Drug and X-ray Irradiation-Induced DNA Lesions

Early studies[844] with BCNU (**33**) revealed that it is an inhibitor of the repair of DNA strand breaks resulting from X-ray irradiation of L1210 leukemia cells. In this case 2-chloroethyl isocyanate, the carbamoylating species produced by the decomposition of BCNU, was more effective than BCNU in interfering with the ligase stage of the excision repair of DNA strand breaks caused by X-ray irradiation.[844] The 2-chloroethyl isocyanate inhibited[849] the rejoining of single-strand breaks, mediated by the DNA ligase enzyme, but not the production of these breaks. Hence, if the carbamoylation moiety of CENUs can inhibit enzymes involved in the repair of DNA strand breaks then it should be possible to increase both the cytotoxicity of such drugs[765] and the effect of X-ray irradiation of cancer cells.[765,860,861] This synergistic effect would be in addition to the prevention of DNA intrastrand cross-linking (ICL) by the inhibition of the alkyltransferase (AT) enzyme. The weak carbamoylating CENU analog SZT (**367**) and the structurally similar but strong carbamoylating analog GANU (**432**) produced similar levels of DNA single-strand breaks (SSB) in L1210 cells, but the SSBs persisted as least twice as long in the GANU-treated cells.[850] This result is consistent with a carbamoylation-induced inhibition of the rejoining of SSBs. Likewise, the weak carbamoylating CENU analog CZT (**368**) did not inhibit the rejoining of X-ray-induced SSBs.[851]

The role of carbamoylating CENUs as effective synergists for the X-ray irradiation of tumors was explored[862] in studies with the bis-cyclohexyl congener DCNU (**1075**, Chart 28) a nonalkylating but strong carbamoylating nitrosourea.[863] It was known

that DCNU is both an inhibitor of DNA repair[851] and a radiation synergist.[852] Furthermore, compound **1075** was inactive[6] against the L1210 leukemia. This fact supports the strong relationship between alkylation and cytotoxicity. A comparison of DCNU with its alkylating analog CCNU (**137b**) revealed[862] that both compounds decompose to the same isocyanate but have very different half-lives at pH 7.4, i.e. 5.6 min for DCNU and 52.5 min for CCNU. A series of analogs of **1075** were synthesized and studied[862] with regard to their half-life, alkylating activity, and carbamoylating activity. The bis-cyclopropyl congener **1076** exhibited the best combination for a radiosynergist of shortest half-life, i.e. 2.5 min at pH 7.4 and 37 °C, and highest carbamoylating activity, i.e. 208% of CCNU activity. The bis(*trans*-4-hydroxycyclohexyl) congener (BHCNU, **1077**, Chart 28) possessed the best combination of increased water solubility and high carbamoylating activity, i.e. 90% of CCNU activity.[862] L1210 and HeLa cells, exposed to the carbamoylating CENUs, such as BCNU (**33**) and CCNU (**137b**) were reported[864] to show no inhibition of DNA lesions caused by the X-ray irradiation.

The synergistic effect of carbamoylation on drug cytotoxicity is further complicated by the question of how normal cells survive reactions with anticancer drugs such as CENUs. Although normal Mer^+ cells remove alkyl groups from the O6-position of guanine and prevent the interstrand cross-linking, these cells can nevertheless be killed by alkylation at other sites. These alkylations at sites other than guanine O6 are probably repaired by an excision, a general mechanism for the repair of a variety of DNA lesions in normal human cells.[865] Since the carbamoylation reactions produced by CENUs can inhibit the strand-rejoining step in DNA excision repair[844,849] this interference with excision repair could decrease the survival of Mer^+ cells more than that of Mer^- cells.[865] To test this possibility normal human embryo cells of type IMR-90 were incubated[866] with several CENUs for 1 h, washed free of the drug and irradiated with 1000 rad of X-rays, followed by a repair period of 30 min in the drug-containing medium. The controls were subjected to the same 1 h of initial incubation but not followed by X-ray irradiation. In these experiments the reaction of the lower carbamoylating CENUs, such as CNU (**26**), PCNU (**257**), and *cis*-2-OH CCNU (**184**) at concentrations of 25−100 μM, produced little or no effect on the DNA single-strand breaks frequency, caused either by drug alone or by drug and X-rays.[866] In contrast, the presence of high carbamoylating CENUs, such as BCNU (**33**), CCNU (**137b**), and *trans*-4-OH CCNU (**189**) resulted in strong increases in single-strand break frequency at doses of 25−100 μM.[866] At the same time, the survival of the normal IMR-90 cells treated with the low carbamoylators, **26**, **184**, and **257**, for 2 h was much greater than the survival time of cells treated with the high carbamoylators **33**, **137b**, and **189**.[866] On the basis of this work, the conclusion[812,866] was reached that high carbamoylating nitrosoureas should be avoided in clinical practice because the difference in cytotoxicity toward the normal Mer^+ cells and the cancerous Mer^- cells is narrowed. A reason for this result is that the Mer^+ cells are more sensitive to the high carbamoylators because of the use of excision for DNA repair.[813] Since human cells use excision repair more than rodent cells[867,868] it is understandable why nitrosoureas, which have high activities against rodent tumors, are often less effective in combating human cancers. However, the use of low carbamoylating CENUs, such as CZT (**368**),[869] PCNU (**257**),[870] and ACNU (**311**)[278,279] have also produced disappointing clinical results. A suggestion was made[866] that hydroxylated CENU analogs would provide a better test of the benefit of removing carbamoylating activity. Hence, the monohydroxylated metabolites of CCNU (**184**−**189**) have been studied extensively and are discussed in sections VIII, XI.A, and XI.D.

F. Carbamoylation and Myelosuppression

The reaction of *N*-nitrosourea drugs with biological components of the bone marrow results in an often serious side-effect known as myelotoxicity or myelosuppression. Ideally, an effective anticancer drug should react only with the cancer cells and not with normal cells, such as the bone marrow cells. Various studies[654,871−873] were undertaken to compare the extent of DNA cross-linking, single-strand breaks, and DNA repair in bone marrow and cancer cells caused by nitrosourea drugs.

The extent of DNA adduct repair in L1210 leukemia cells and murine bone marrow was determined[654,871] after treatment with 2-chloroethyl-2-^{14}C labeled CCNU (934), CZT (**1078**) and GANU (**1079**, Chart 28). CCNU and GANU are relatively high carbamoylators while CZT is a very weak carbamoylator. If carbamoylation is an important mechanism for inhibiting DNA repair through DNA ligase inhibition, then the low carbamoylating CZT should allow unhindered repair. After treatment of L1210 cells with the ^{14}C-labeled CZT (**1078**) a 40% removal of the [^{14}C]chloroethyl moiety from DNA occurred within the first 6 h and a 52% removal at 18 h during a subsequent drug-free incubation of the cells.[871] The initial decrease in ^{14}C activity after 6 h would constitute a repair of the DNA monoadduct, which was formed most likely via the chloroethylation of the guanine O6-position.[871] The shallow shape of the repair curve for CZT at the longer times of 12 to 18 h indicated that the repair of the interstrand cross-links is slower than the repair of the DNA monoadducts.[871] In contrast, the higher carbamoylating ^{14}C-labeled GANU (**1078**) and CCNU (**934**) both inhibited the removal of [^{14}C]chloroethyl adducts from leukemia L1210 DNA up to 12 h. The same pattern of ^{14}C-label removal by the three drugs occurred with murine bone marrow DNA.[871] Since no correlation was shown between carbamoylation, the extent of DNA alkylation and the lack of myelotoxicity for the two nonmyelosuppressive carbohydrate CENU analogs GANU (**432**) and CZT (**368**) it was concluded[871] that the DNA repair and carbamoylating activity are not essential for marrow-sparing characteristics of glucose-linked nitrosoureas.

Further support for these contentions came from incubation studies[871] of BCNU (**33**), CZT (**368**), HECNU (**329**), and acetamido CNU (**598**) with rat bone marrow followed by a 600 rad dose of X-ray irradiation, resulting in a controlled level of DNA

single-strand breaks. The administration of either the weak-carbamoylating CZT (**368**) or the strong carbamoylating BCNU (**33**) resulted in an initial low level of cross-links which slowly decreased to a negligible levels by 48 h. In contrast, the administration of the weak carbamoylating drugs, HECNU (**329**) and acetamido-CNU (**598**), resulted in an increase of DNA cross-links in the bone marrow up to 24 h after drug injection, followed by a sharp decrease at 48 h with some residual cross-linking. Thus, the time pattern of cross-link formation differed according to the CENU studied, but the final low level of DNA cross-linking for all the drugs led to the conclusion[872] that a DNA repair process was operating regardless of the different carbamoylation potentials among these four drugs. The profile of DNA single-strand breaks (SSB) during the same time period showed a somewhat complementary course.[872] Thus, the administration of the four drugs caused, after 6 h, an initial low level of SSB, followed by a decrease at 24 h, a sharp increase at 36 h, and a subsequent decrease at 48 h. The origin of the SSB could not be interpreted with certainty, but they could be attributable to gaps left after excision repair[872] and not the action of the drugs themselves on DNA. The disappearance of the cross-links was the result of a true DNA repair process and not to a decrease in cell viability. Since out of the four drugs only CZT (**368**) is nonmyelosuppressive, it is apparent that the amounts of ICL and SSB and rates of DNA repair cannot fully explain the level of bone marrow toxicity. A valid conclusion[319] from this work is that healthy murine bone marrow has the capacity to remove DNA lesions, as detected by alkaline elution, by one or more repair processes, and it makes little difference whether the drugs are carbamoylators.

The different DNA interstrand cross-linking (ICL) levels caused by the four drugs could not be explained in simple terms.[872] Thus, the acute toxicities of the drugs were not directly correlated[872] with ICL formation since HECNU (**329**), the least toxic drug with a $LD_{50} = 128$ μmol/kg, caused the same high level of ICL as the more toxic acetamido-CNU (**598**) with a $LD_{50} = 87$ μmol/kg. Differences in the passive membrane permeability were not important for ICL formation because both the hydrophilic CZT (**368**) and the lipophilic BCNU (**33**) were equally inefficient in causing DNA cross-links.[872] No direct correlation existed[873] between acute toxicity and myelosuppression since CZT and acetamido-CNU (**598**), with nearly equal LD_{50} values of 91 and 98 μmol/kg, caused a weak and a strong myelosuppression, respectively, as measured by the depletion of granulocyte-committed (CFU-C) and pluripotent (CFU-S) stem cell activities. However, a correlation existed[873] between a higher level of DNA-ICL and a greater degree of myelosuppression for the more active anticancer drugs, HECNU (**329**) and acetamido-CNU (**598**). The ability of the stem cells to recover from either a single or multiple doses of CENUs may be more relevant for the long-term chronic toxicity as e.g. HECNU (**329**) exhibited a lower chronic toxicity than BCNU (**33**) and CZT (**368**) when administered by repeated injections.[5,296]

G. Carbamoylation and Cytotoxicity

While alkylation is considered to be the chief mode of cytotoxicity of nitrosoureas toward many tumor cell lines such as leukemia L1210, the suggestion was made[874] that the carbamoylating activity may be more important against other cell lines. For example, a TLX5 lymphoma, made resistant to either BCNU (**33**) or dimethyltriazene in vivo, was resistant in vitro to both BCNU and CCNU and cross-resistant to their respective chloroethyl and cyclohexyl isocyanates.[874] However, the L1210 leukemia cell line which was made resistant to BCNU in vivo was also resistant in vitro against BCNU and CCNU but was not cross-resistant to cyclohexyl isocyanate derived from CCNU and only slightly resistant to chloroethyl isocyanate derived from BCNU. Consequently, the TLX5 lymphoma, which is naturally resistant to alkylating agents of the (2-chloroethyl)amino type,[342] may be sensitive in vivo to nitrosoureas because of the intracellular release of isocyanate.[874] The cross-resistance between the isocyanates and nitrosoureas could neither be explained by differences in the cellular uptake of the nitrosoureas-sensitive and nitrosoureas-resistant lines nor by an elevation of nonprotein thiols.[874] On the basis of the foregoing, a low carbamoylating CENU, such as CZT (**368**) would be predicted to produce little cytotoxicity toward the TLX5 tumor line, but in practice CZT was found[874] to have comparable anticancer activities against both the L1210 and TLX5 cell lines. This apparent contradiction could be explained[875] if one assumes that the slow in vitro carbamoylation of lysine by CZT fails to correlate with its in vivo carbamoylations of cellular constituents. Therefore, the suggestion was made[874] that compounds capable of releasing isocyanate, but not a haloalkanediazohydroxide, should be synthesized and tested as potentially more effective anticancer agents.

H. Cell-Cycle Specificity

Another important aspect of the biological activity of nitrosoureas is their cytotoxicity against the different cell-cycle positions.[876] In early studies it was shown that synchronized Don *C* cells,[343] lymphocytes,[877] and 9L rat brain tumor cells[878] were less sensitive to BCNU in the S phase than in other phases. A detailed study was reported[879] of the effect of BCNU on the progression of cultured H-Ep2 cells through the cell cycle. On the basis of progression to the metaphase, cells initially in the last half of G_2 phase were not inhibited, whereas cells in the first part of G_2, in S, and in G_1 phases were either delayed or prevented from progression through the cycle with continuous exposure to BCNU. On exposure to BCNU, the labeling of cells initially in the S phase, was partially inhibited, and the progression into the S phase of cells initially in the first half of G_1 phase was inhibited or delayed.[879]

The cell cycle response patterns of a representative group of nitrosoureas, such as CCNU (**137b**), MeCCNU (**138b**), BCNU (**33**), CZT (**368**), ENU (**27**), and BHCNU (**1077**), was reported.[880,881] These drugs represent a mixture of chemical reactivities, such as alkylation, cross-linking, and carbamoylation, and their responses are shown in Table 58. It was

Table 58. Cell Phase Specificity of Nitrosoureas[856]

compound no.		relative phase sensitivity[a]	chemical reactivity[b]
137b	CCNU	G_2-M > G_1 > S	A, C-L, C
138b	MeCCNU	$G_1 \geq G_2$-M > S	A, C-L, C
33	BCNU	G_2-M > G_1 > S	A, C-L, C
257	PCNU	G_2-M ≥ G_1 > S	A, C-L, C
368	CZT	G_2-M > G_1 > S	A, C-L
27	ENU	S > G_2-M ≥ G_1	A, C
1077	BHCNU	G_2-M > G_1 > S	C

[a] Phases of the cell cycle: G_1 = preparatory phase for DNA synthesis, but no DNA synthesis occurring; G_2 = preparatory phase for the mitosis; M = mitosis; S = DNA synthesis phase. [b] A = alkylation, C-L = cross-linking, C = carbamoylation.

found[880] that the CENUs **137b**, **138b**, **33**, and **368** were more cytotoxic to cells in the G_1 and G_2-M cell phases than in the other phases. Apparently, the formation of cross-links varies with the position of the cell cycle. Because the conformation of DNA changes in each cell-cycle phase,[882] steric factors may control the reaction of chemical intermediates with DNA bases and the formation of cross-links. Thus, the relative resistance of S phase cells to alkylation by CENUs could be caused by chromatin folding[880] on the basis of the observation[883] that the number of O^6-ethylguanine adducts formed in cells treated with ENU (**27**) decreased as the folding level of chromatin increased. Further, the changes in DNA conformation could affect the number of cross-links after treatment with a CENU. Thus, the cytotoxic effects of BCNU (**33**) and the number of cross-links formed in 9L cells were both increased after intracellular polyamine depletion,[884] a process known to alter the conformation of DNA.[885] A treatment of cells with a polyamine synthesis inhibitor, such as α-(difluoromethyl)ornithine (DFMO), resulted[886] in no change of the number of initial DNA alkylation products in cells treated with MeCCNU (**138b**). On the basis of this result, it can be assumed[886] that conformational changes of DNA affect only the cross-link formation. The cytoxicities of BCNU (**33**) and MeCCNU (**138b**) were enhanced against rat brain tumors by a prior administration of DFMO.[886−892] The addition of putrescine resulted[886,893] in a restoration of the normal intracellular polyamine content and a reversal of the potentiation of BCNU and MeCCNU cytotoxicity. It was shown[886] that the polyamine depletion by DFMO mediated no reduction in the activity of several DNA repair enzymes. Therefore, the enhanced cytotoxicity of the nitrosourea drugs was not the result of an impaired repair of the alkylated bases in the DNA but rather the inhibition of ornithine decarboxylase by DFMO and the resulting depletion of the intracellular polyamines.[886,893] In a further study it was demonstrated[894] that a prior administration of DFMO resulted in the sensitization of the repair-proficient Mer^+ tumor cells to BCNU but not a sensitization of the repair-deficient Mer^- cells. There were no BCNU-induced interstrand cross-links (ICL) in either the polyamine depleted or control Mer^+ cells. This result was believed to suggest[894] that sites other than DNA cross-links were responsible for the DFMO potentiation of BCNU cytotoxicity. Finally, it was hypothesized[894] that the sites of action of nitrosourea drugs with Mer^+ and Mer^- tumor cells are different, i.e. the Mer^+ are affected by polyamine depletion and the Mer^- cells by ICL formation.

These cell-cycle phase responses of the CENUs are probably not caused by differences in the passive drug diffusion through the cell membrane[880] since there was little difference[895] among the passive diffusions of urea, mannitol and vincristine across the membrane of S49 lymphoma cells that had been enriched in various phases of the cell cycle. Furthermore, the duration of each cell cycle phase is not related to the observed sensitivity patterns of the different nitrosoureas (Table 58).[880] Thus, the entire cell cycle for the 9L rat brain tumor cells is 20 h, with the S and G_1 phases both equal to 8 h and the G_2-M phase equal to 4 h. Since both the S and G_1 phases are of the same duration, the relative resistance of S phase cells to the CENU drugs is not attributable to an extended phase duration that would allow repair of DNA damage.[880]

The reaction of the low carbamoylating CZT (**368**) occurred preferentially in the G_1 and G_2-M cell phases just as in the case of the high carbamoylating CENUs (Table 58). Hence, the presence or absence of an isocyanate product is not important for the specificity.[880] The drug ENU (**27**) can carbamoylate and alkylate but not cross-link DNA, so in this case the monoalkylated adducts of DNA bases when incorporated into polynucleotides could result in base mispairing and lethal mutations during the S phase,[896] resulting in the S phase sensitivity to this drug. It was not known whether the ENU alkylations involve either the DNA precursor pool or the DNA itself in the S phase. However, it is believed[897] that MNU (**25**) may have a major effect on DNA precursors. The compound BHCNU (**1077**) can react only as a carbamoylator and, thus, probably has little or no effect on DNA.[880] Rather this compound was shown[898] to cause changes in plasma and nuclear membranes, a dose-dependent inhibition of glutathione reductase, and a reduction of mitotic spindle formation, the latter reaction supporting[896] the increased sensitivity of G_2-M phase cells toward BHCNU.

Rat liver cells in the DNA-synthesizing S phase appeared to have a higher level of MGMT activity than cells in other phases as judged[899] by the lower O^6-methylguanine/N^7-methylguanine ratio in the DNA-synthesizing cells. After treatment with *N*-methyl-*N*-nitrosourea (**25**) and dimethylnitrosamine the alkylated guanine base ratio was constant in G_1 cells, decreased by 15% in 30% S phase cells, and decreased by 40% in 80% S phase cells. It was concluded[899] that the elimination of O^6-methylguanine during the S phase could protect the DNA synthesis from base-mispairings and/or from hypomethylation at G-C sites.

I. Reactions with Nuclear Components

The reactions of CENUs with various components of the cell nucleus must be considered in any discussion of the biological reactions of these drugs. It is possible that the site of drug interaction within the nucleus might be either equally or more important than the type of lesions of DNA, RNA, protein, and carbamoylation and alkylation reactions.[900,901]

The relative levels of myelosuppression of CENUs was investigated with this problem in mind.[901] It was possible to demonstrate, using the techniques of ECTHAM-cellulose chromatography or DNAase II

digestion, followed by solubilization in magnesium buffer, that transcriptional chromatin acts as a preferential target for nitrosourea interaction. The transcriptional activity, as measured by the uptake of a pulse of [^{3}H]uridine, and the 2-[^{14}C]chloroethyl CZT (**1078**, Chart 28) binding were both localized within certain later-eluting fractions. Stimulation of the transcriptional activity with sodium butyrate increased the drug binding about 2-fold, while maintaining the increase within the transcriptional fraction of the chromatin.[900,901] The extended structural configuration of transcriptional chromatin was believed to be a target for drugs' attack.[901]

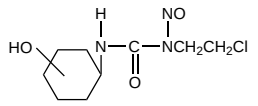
The nonmyelosuppression of a CENU drug such as CZT (**368**) was explored[901] by the incubation of 2-[^{14}C]-chloroethyl CZT (**1078**) with L1210 cells and murine bone marrow cells followed by a digestion of the mixture with micrococcal nuclease (MCN) to cleave the linker DNA between the nucleosomes. The digestion of bulk chromatin in the L1210 cells was greater than the concomitant release of ^{14}C-labeled chromatin. In this case the alkylation occurred at enzyme-inaccessible DNA associated with the nucleosome core particle. A corresponding incubation of **1078** with the murine bone marrow resulted in a slightly greater nuclease digestion of the alkylated chromatin than the bulk chromatin. This result was interpreted[901] to suggest a preferential alkylation of the "exposed" linker DNA. The same incubations with the myelosuppressive 2-[^{14}C]chloroethyl CCNU **934**, at the same concentrations, resulted in no differentiation, as the core particles of both L1210 and murine bond marrow were alkylated to the same degree.[902] The myelosuppressive ACNU (**311**) was found[903] to be preferentially bound to the DNA associated with the core histones in both L1210 and murine bone marrow cells, whereas the weakly myelosuppressive ^{14}C-labeled GANU (**1079**), like CZT (**1078**), was bound preferentially to linker DNA in the bone marrow and the core DNA in L1210. If the enzyme-accessible DNA lesions of the linker DNA in bone marrow were repaired in preference to the enzyme-inaccessible DNA lesions of the core particles in the L1210 leukemia cells,[904,905] then the nonmyelosuppression of CZT (**368**) and GANU (**432**) could be explained.[901]

Similar results for CCNU and CZT were obtained with human bone marrow[906] and HeLa cells.[907] The overall levels of alkylation of the bone marrow was the same for both compounds. However, a different picture emerged when the bone marrow was separated into 14 fractions by centrifugal elutriation (fractionation).[906] After a 2 h incubation with ^{14}C-labeled CZT (**1078**) and CCNU (**934**, Chart 26), the CCNU was found mainly in three fractions that contained lymphocytes, monocytes, and normoblasts while the bound CZT was found mainly in fractions containing mature and immature myeloid cells and the highest CFU-GM activity. An analysis[906] of the subpopulations of bone marrow cells revealed similar degrees of DNA strand breakage and DNA-protein cross-linking, as measured by alkaline elution of pooled fractions of elutriated bone marrow. DNA interstrand cross-links (ICL) were not found[906] after incubation with either drug. The most significant finding of the study[906] was the difference in the site of drug alkylation by CZT and CCNU in bone marrow chromatin. Endonuclease digestions of the chromatin with MCN, DNase I, and DNase II/Mg^{2+} demonstrated that specific regions of chromatin were alkylated by the two drugs. Thus, CCNU was preferentially bound to the transcriptionally active regions of chromatin, whereas CZT was predominantly bound to the transcriptionally inactive regions. It was hypothesized[906] that the lethal damage to human bone marrow is caused by *N*-nitrosourea drugs which alkylate targets within transcriptionally active regions of chromatin.

Nuclear histone and non-histone proteins were not substantially alkylated by CENUs at physiological concentrations.[907] Carbamoylation of nuclear proteins, especially H2b, occurred with MNU (**25**) and of non-histone protein with CNU (**26**).[908] Nuclear RNA accounted for 20% of the total nuclear alkylation, but it was stated[900,901] that these alkylated RNA's contributed little to the cytotoxic properties of the nitrosoureas because of their rapid duplication and rates of turnover. The H1-histone protein in L1210 and the non-histone fraction in HeLa cells were selectively carbamoylated by CCNU (**137b**).[908] Pretreatment of log-phase HeLa cells with 5.0 mM sodium butyrate resulted in histone modifications and an increase in transcriptional activity of chromatin.[907] As a result, the cellular uptake of both CZT and CCNU, the chromatin alkylation, and the histone protein carbamoylation were increased.[907] The preferential binding of labeled EBNU **1025** and **1026** (Chart 28) to poly-lysine and poly-histidine, as well as the selective binding of **1026** to poly-arginine, might be responsible for the modification of basic proteins in nuclear chromatin.[141,142]

The non-histone protein nuclear matrix is associated with replicating chromatin[909] and specific DNA−protein attachment sites may be necessary for normal DNA synthesis.[900,901] The nuclear matrix protein−DNA reassociation properties after treatment of the nuclear DNA with either CZT (**368**) or CCNU (**137b**) were determined by the use of a nitrocellulose filter assay.[900,901] When DNA was labeled with either 2-[^{14}C]-chloroethyl CZT (**1078**) or 2-[^{14}C]-chloroethyl CCNU (**934**) the alkylated DNA failed to reassociate with the matrix proteins and was not retained on the nitrocellulose filter.[900,901] Since A-T rich regions of DNA have been shown to associate preferentially with matrix proteins,[910] it is possible that the nitrosoureas alkylate these base sequences, preventing recognition of the binding proteins. If these protein−DNA associations are critical to replications,[911] then the drug-induced interference, either during or just prior to the S phase, may have cytotoxic potential.[900,901] Inhibition of DNA synthesis would occur because either the DNA or chromatin fiber would be prevented from associating with the nuclear matrix.[900,901] The appearance of chromatin under the electron microscope was radically changed by treatment with steroids and/or nuclear-reacting drugs.[912−914] The nuclear matrix may exert a chromatin-organizing function that permits fluidity while maintaining some restrictive influence on chromatin arrangement.

Table 59. Hydroxy Metabolites of CCNU (137b) in Rat Liver Microsomes, Rat Plasma, and Humans



		rat liver microsomes[a–c]						
		control		phenobarbital[f]		rat plasma[c]		humans[d]
compound no.		*a*	*b,c*	*a*	*b,c*	control	phenobarbital[f]	
184	*cis*-2	nd[e]	nd[e]					
185	*trans*-2	14	nd[e]	3		8		
186	*cis*-3	trace	30	3.3	16	4		
187	*trans*-3	31	39	11	13	23	21	
188	*cis*-4	54	21	77	67	54	62	50
189	*trans*-4	3	9	5	5	8		50

[a] Reference 195. [b] Reference 194. [c] Reference 915. [d] Reference 196. [e] Not detected. [f] Induced with phenobarbital.

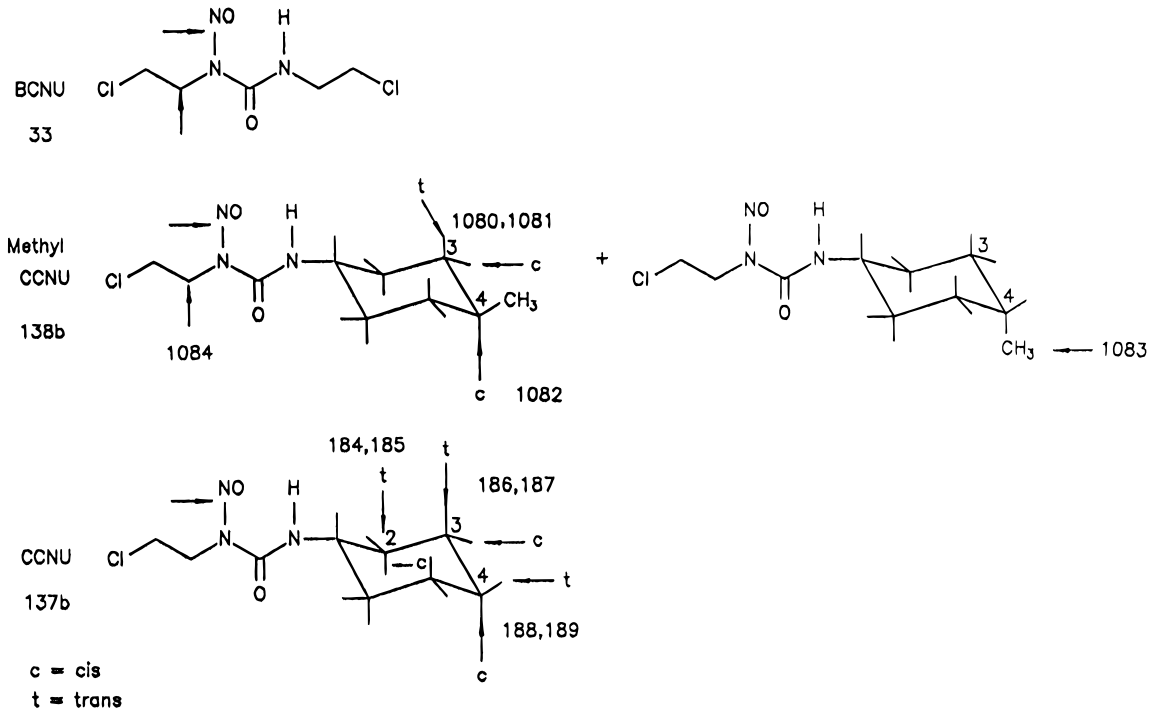


Figure 4. Sites of cytochrome P-450-dependent hydroxylation and denitrosation.

XI. Metabolism, Pharmacology, Toxicology, Mutagenicity, and Carcinogenicity

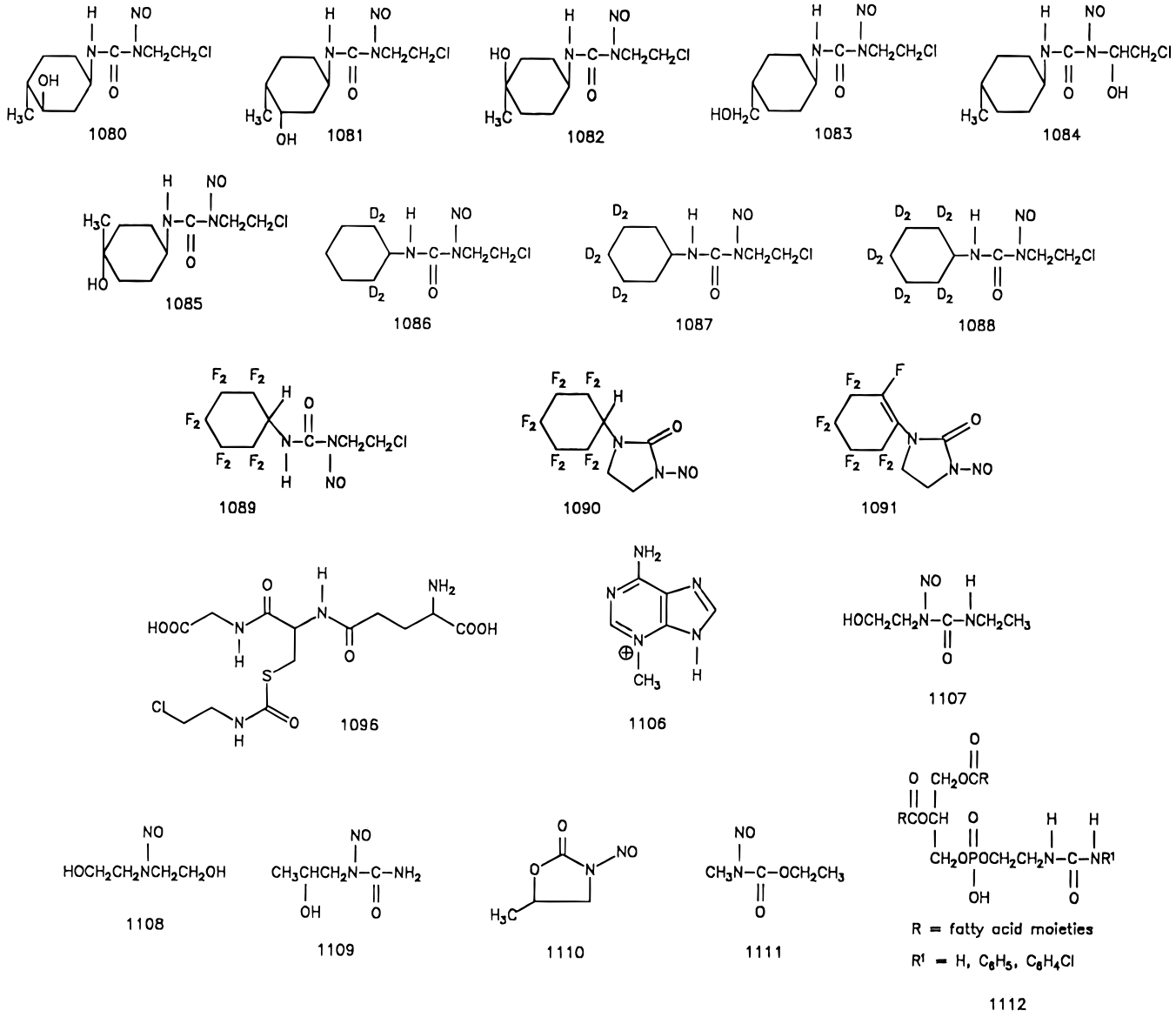
A. Metabolism

The overall anticancer activity of (2-chloroethyl)nitrosoureas (CENUs) is dependent not only on the biochemical decomposition mechanisms occurring within the tumor cells but also on their metabolism, protein-binding catalyzed degradations in the plasma and lipid partitioning among different organs and tissues. In this section will be summarized some of the key points involved with these other factors. The reader is directed to various reviews[3,12,21–27,194] which cover these topics in detail.

Some metabolic reactions result in the formation of more active intermediates. An important example of such reactions is the monooxygenation of the cyclohexane ring of CCNU (**137b**) resulting in a number of hydroxylated products **184**−**189**. These products are formed by the cytochrome P450-catalyzed oxidation both in vitro with rat liver microsomes[24,191–195,915,916] and in vivo in rats and humans.[196] The structures and relative percentages of the hydroxylated products, as well as the effect of phenobarbital on the product distribution, are shown in Table 59. The identity of the hydroxylated CCNU metabolites was provided by a comparison of the NMR and MS spectra with those of authentic standards.[194,198] Thus, after iv administration of CCNU[196] only a 1:1 mixture of the *cis*-4- and *trans*-4-hydroxy metabolites was found in the human plasma. Studies of the oxygenation of CCNU by purified cytochrome P-450 isozymes a−c revealed[917] a stereoselective oxidation that resulted in the formation of *cis*-4-hydroxy-CCNU (**188**) by isozymes a and b, and the *cis*-4-hydroxy- (**188**) and *trans*-3-hydroxy- (**187**) analogs by isozyme c. The rate of metabolic hydroxylation exceeded the rate of decomposition, hence, it was concluded[6,192,198] that the hydroxylated metabolites are the immediate precursors of the biologically active species. The synthesis and anticancer activity of the six possible monohydroxy CCNU metabolites are discussed in section VII.A.3 while the chemical and biological properties of these metabolites is covered in section VIII.

Methyl-CCNU (**138b**) undergoes a more complex monooxidation of the cyclohexyl ring to give **1080**−**1082**, the 4-methyl group to give **1083** and the urea side chain to give **1084** (Figure 4, Chart 29).[193,918] An unusual metabolite **1085**, containing the *trans*-4-

Chart 29. Structures for Section XI

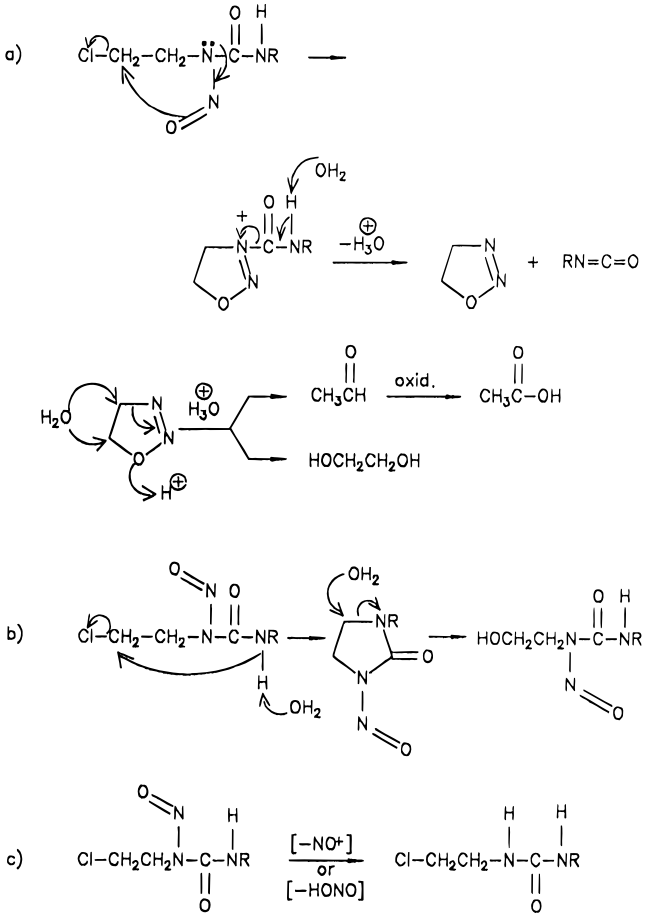


hydroxy-*cis*-4-methylcyclohexyl ring, resulted from a cytochrome P450-dependent homolytic hydrogen abstraction to give a free radical intermediate.[919] Addition of a hydrogen atom to the opposite side of the ring would yield the inverted C4 configuration.[919]

Metabolism-directed design[920] was used in attempts to control the metabolic hydroxylation of CCNU. Analogs of CCNU (**137b**) were synthesized[921] with the cyclohexyl ring either partly deuterated (2,2′,6,6′-d_4, **1086**, 3,3′,4,4′,5,5′,-d_6, **1087**) or wholly deuterated (2,2′,3,3′,4,4′,5,5′,6,6′-d_{10}, **1088**) (Chart 29) at positions susceptible to the metabolic hydroxylation. After incubation with rat liver microsomes the total yield of hydroxy derivatives from CCNU-d_{10} (**1088**) was 74% of that from CCNU but the relative proportions of the various 2-, 3-, and 4-hydroxy metabolites were similar.[921] However, the biooxidation of CCNU-d_4 (**1086**) yielded much less of the *trans*-2-OH CCNU (**185**) than from CCNU, i.e. 0.2% as compared to 2.3%, whereas the yield of **185** was greater, i.e. 17%, for the CCNU-d_6 analog (**1087**). Thus the presence of deuterium at various ring positions caused a "metabolic switching" effect of ring hydroxylation to the non-deuterated sites.[921] CCNU and its deuterated analogs had similar activities against the TLX-5 lymphoma in mice, albeit at different dose levels.[921] The blockage of the metabolic hydroxylation of CCNU was effected[922] by the use of the decafluorocyclohexyl analog FCCNU (**1089**). Administration of FCCNU to rats intracerebrally inoculated with L1210 leukemia produced a high toxicity in the dose range of 30−140 mg/kg while no anticancer activity was observed at doses below the toxic range.[922] This result strongly supports the contention[192,198] that the hydroxylated CCNU analogs are the active forms of the drug in vivo. At pH 7.4 and room temperature, the compound **1089** decomposed to the 3-nitrosoimazolidin-2-ones **1090** and **1091**.[922] The presence of the fluorine atoms would be expected to increase the lipophilicity, and, thus, change the pharmacokinetic patterns of FCCNU, relative to CCNU.[922]

Another example of a metabolic activation is the disulfide-bisCENU analogs CNCC **119**−**121** (Table 6, section VII.A.2). An in vivo reduction of the disulfide bond to the thiol groups, and a subsequent oxidation of the thiol moiety, produced the biologically active sulfoxide (**125**) and sulfone (**126**) metabolites (Table 7).[163]

Scheme 121



Metabolic routes which deactivate *N*-nitrosoureas include dechlorination and *N*-denitrosation.[27] The major metabolic pathway, dechlorination, requires cytosol and cytochrome P450 involvement for maximal activity (Scheme 121a,b). Evidence for the intermediates has been provided by studies of [^{14}C]-ACNU (**311**)[923,924] and [^{14}C]fotemustine (**134**)[181,925] drugs.

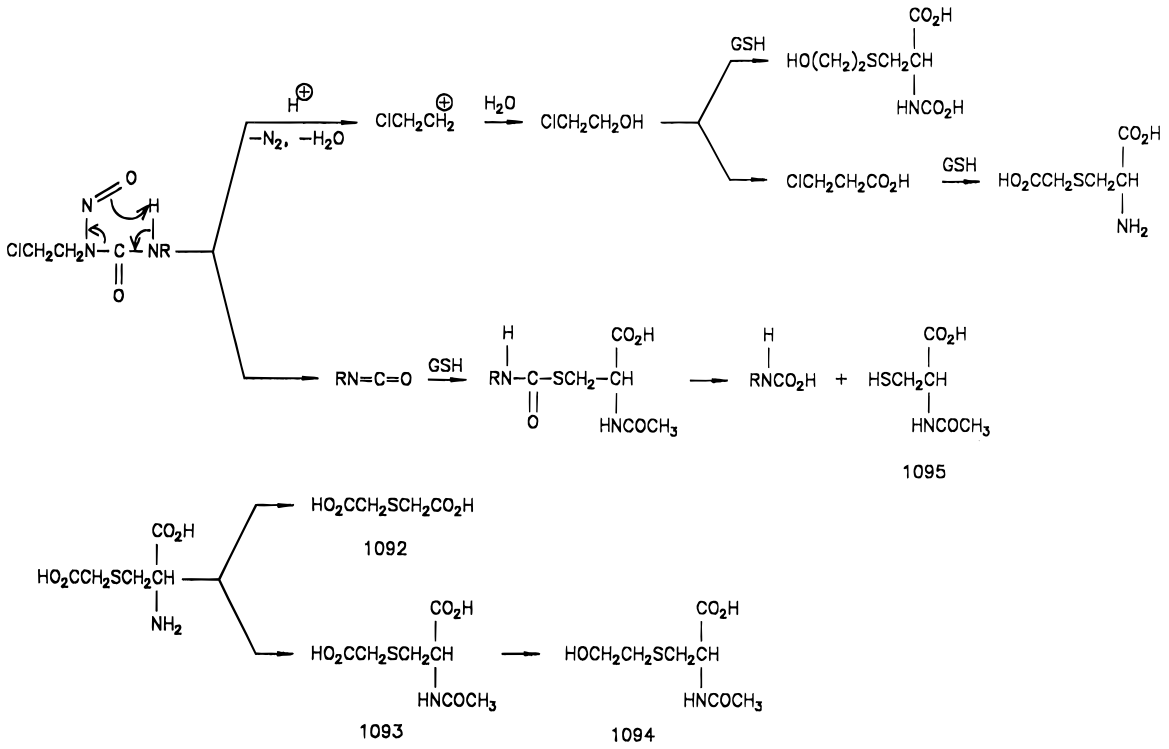
The denitrosation reaction (Scheme 121c) is mediated by the microsomal cytochrome P450 enzymes and NADPH cytochrome P450 reductase[926] under aerobic and anaerobic conditions. BCNU (**33**) undergoes denitrosation which is catalyzed by liver microsomes in the presence of NADPH.[193] Further studies have shown[927] that the microsomal denitrosation of BCNU (**33**) can be catalyzed by two independent enzyme systems, the cytochrome P450 monooxygenases and glutathione S-transferase. The suggestion was made[927] that the cytotoxicity of BCNU could be improved by a differential inhibition of these enzyme systems. Cytochrome P450 forms a nitroso complex during denitrosation of CCNU.[27] The loss of the nitroso group in BCNU (**33**), CCNU (**137b**), and PCNU (**257**) caused[928] a considerable reduction in the activity, and a systematic toxicity to rats with these drugs. This pathway is inducible by phenobarbital, causing a complete loss of activity and toxicity for BCNU.[928] A cytosol denitrosation can also occur, albeit at a slower rate and the reaction is catalyzed in the rat liver cytosol by a glutathione-dependent enzyme.[929,930] An interesting finding[930] was that the μ, but not the π nor the α, isozymes of glutathione transferase catalyzed the denitrosation of BCNU. The μ isozyme was shown[931] to have genetic polymorphism, with 40% of the human population having a μ isozyme deficiency. The implication of this work[930] is that a certain proportion of the population could show a resistance to (chloroethyl)nitrosoureas, such as BCNU, either because of the absence of the μ isozyme of glutathione transferase or some abnormal ratio of the different isozymes.

The reduced glutathione (GSH) content of 9L rat brain tumor cells, which are sensitive to BCNU (**33**) but resistant to nitrogen mustards, was found[932] to be twice as high as that found in the BCNU-resistant 9L-2 tumor cell line. The depletion of the GSH content in the 9L cells with buthionine sulfoximine had no significant effect on the cytotoxicity of BCNU, CCNU, and MeCCNU but the depletion enhanced the cytotoxicity of nitrogen mustards against both tumor cell lines.[932] Incubation of BCNU with GSH either alone or with glutathione transferase had no effect on BCNU cytotoxicity. However, a pretreatment of 9L cells with GSH significantly protected the cells against nitrogen mustard cytotoxicity. Although the elevated GSH and glutathione transferase activity could not be correlated with tumor cell resistance to BCNU and other nitrosourea drugs, it might be invoked as a mechanism for the resistance to nitrogen mustards in the 9L cell line.[932]

The alkylating and carbamoylating intermediates from the chemical decomposition of CENUs could be removed by glutathione (GSH) in reactions catalyzed by glutathione transferase.[27] As shown in Scheme 122, important metabolites obtained from the 2-chloroethyldiazohydroxide are 4-thiodiacetic acid (**1092**), *N*-acetyl(carboxymethyl)cysteine (**1093**) and *N*-acetyl-(hydroxyethyl)cysteine (**1094**), while the metabolite obtained from the isocyanate intermediate is *N*-acetylcysteine (NAC, **1095**).[27]

Because the isocyanate portion of nitrosoureas is considered largely responsible for the toxicity of these drugs,[97] much attention has been devoted to the metabolism of this group. The in vivo release of 2-chloroethyl isocyanate from BCNU has been demonstrated[933] in the rat by the identification of the GSH conjugate in bile and the NAC adduct in urine. These *S*-linked conjugates inhibited rat liver glutathione reductase in vitro at essentially the same molar concentration as BCNU (**33**).[933] A BCNU−GSH conjugate, *S*-[(2-chloroethyl)carbamoyl]glutathione (SCCG, **1096**, Chart 29) undergoes a reaction involving the transfer of an aminoethyl group to the N7-position of guanosine.[934] The resulting N^7-(aminoethyl)guanosine adduct readily undergoes depurination which results in the DNA damage.[934] The ^{14}C-labeled isocyanate portion of CCNU (**939**) was biotransformed[644] to the following urinary metabolites in rats (50% of the total isolate): 55% of 3-*trans*, 3-*cis*, 4-*cis* and 4-*trans*-hydroxycyclohexylamines and 30% cyclohexylamine. Similarly, experiments with ^{14}C-labeled MeCCNU (**138b**) yielded[644,918] the metabolites (66% of total) in decreasing order: *cis*-3-hydroxyl-*trans*-4-methylcyclohexylamine, *trans*-4-methylcyclohexylamine, *trans*-4-(hydroxymethyl)-cyclohexylamine and *trans*-3-hydroxy-*trans*-4-methylcyclohexylamine. The carbamoylated *S*-linked conjugates of CCNU in rats and humans have been identified[935] by liquid chromatography-chemical ion-

Scheme 122



ization mass spectrometry (LC/MS) and LC/MS/MS. In rats the 4-hydroxycyclohexyl, 3-hydroxycyclohexyl, and cyclohexyl isocyanate components were identified[935] as their GSH conjugates in bile, and their NAC conjugates in urine. In humans the urinary metabolites were the NAC conjugate of the 4-hydroxycyclohexyl and 3-hydroxycyclohexyl isocyanates.[935] Three distinct *N*-acetylcysteine−MeCCNU adducts were isolated from rat liver microsomes after a treatment with MeCCNU (**138b**).[936]

A study[653] of the inactivation of purified yeast glutathione reductase (GSSG-R) by chloroethyl nitrosoureas revealed that CCNU (**137b**), MeCCNU (**138b**), BCNU (**33**), and *trans*-4-hydroxy-CCNU (**189**), strongly inhibit, and ACNU (**311**) weakly inhibit, the reductase while the analogs *cis*-2-hydroxy-CCNU (**184**), CZT (**368**), and GANU (**432**) caused no inactivation of the enzyme at the half-life of each compound. The strong inhibitors are also high carbamoylators, with the exception of ACNU, and the noninhibitors are low carbamoylators, with the exception of GANU.[653] In order to prove that the isocyanate was the reactive moiety, the ^{14}C-labeled cyclohexyl CCNU (**939**) and the ^{14}C-labeled 2-chloroethyl BCNU (**21**) were incubated with the GSSG-reductase in the absence and presence of NADPH.[653] The inactivation, in the presence of NADPH, followed the same time course with concurrent ^{14}C labeling of the reductase. In the absence of NADPH there was no inhibition of the enzyme and very low ^{14}C labeling.[653] Furthermore, the amount of ^{14}C bound per mole of nitrosourea-incubated enzyme was stoichiometric, so that it is possible that these isocyanates could serve as active site probes of GSSG reductase.[653] These conjugates may contribute to the toxic effect of the parent nitrosoureas. Such toxicities were evident in patients who received a single iv injection of BCNU (**33**) and developed an irreversible inhibition of erythrocyte glutathione reductase (GSSG-R) within minutes.[38,858] This inhibitory effect could be reproduced with in vitro enzyme assays and was not found[858] among 19 additional erythrocytes enzymes. The erythrocytic GSSG-R inhibition by BCNU resulted[858] in a lower level of GSH, and to increased oxidative hemolysis, particularly, in glucose-6-phosphate dehydrogenase (G-6-PD)-deficient patients. A resupply of GSSG-R depended on the capacity of the bone marrow to release new erythrocytes during the drug-free intervals. The BCNU inhibition was shown[858] to occur also in human leukocytes and platelets and in all mouse organs tested.

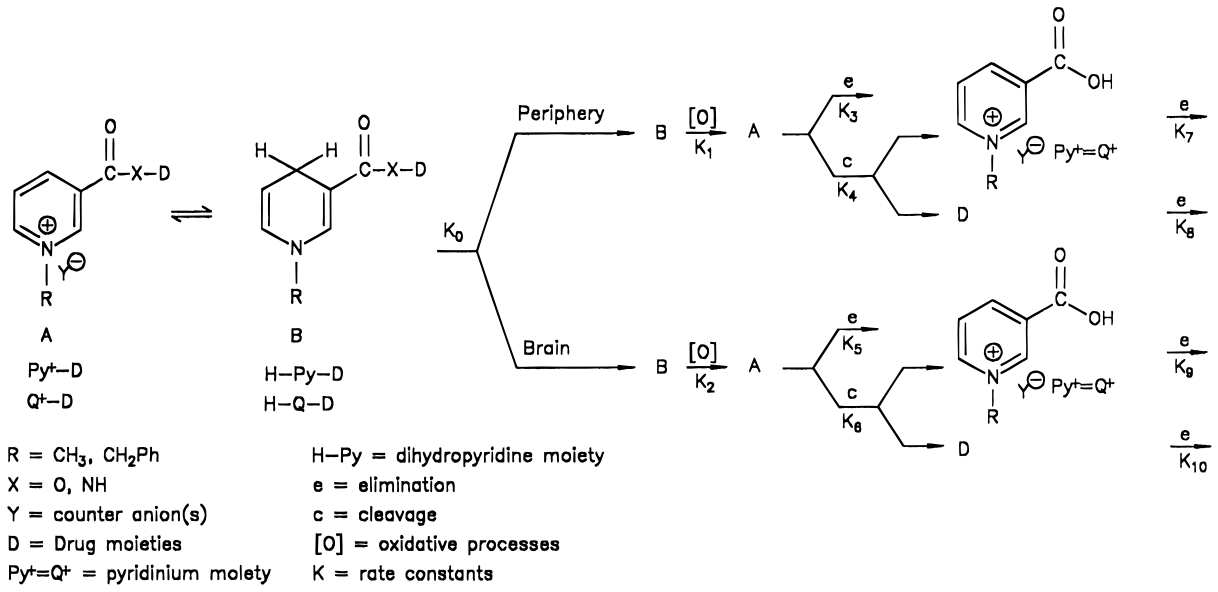
Table 60. Stability of Selected CENU Analogs in Buffer and Serum[a]

		$t_{1/2}$,[b] min			
no.	compound	aqueous buffer	serum	log P[c]	metabolism[d]
26	CNU	5			
33	BCNU	49	14	+1.5	D
137b	CCNU	53	34	+2.8	H
138b	MeCCNU	60	22	+3.3	D/H
257	PCNU	26	27	+0.37	D
311	ACNU	34−75		+0.09	
368	CZT	48		−1.02	

[a] Table taken from refs 3 and 25. [b] Half-life measured in phosphate buffer, pH 7.4, at 37 °C. [c] Partition coefficient: P = [compound in 1-octanol]/[compound in water]. [d] D = denitrosation, H = hydroxylation.

Another important aspect of CENU metabolism is their degradation[25,937] by protein-binding catalyzed reactions in the serum. The rate of degradation of certain nitrosoureas in serum are higher than in aqueous buffer at the same pH and temperature (Table 60).[937] An exception is the nitrosourea PCNU (**257**) which was shown[937] not to undergo the serum protein-catalyzed reactions, as evidenced by its identical half-life in both buffer and in serum. The protein-catalyzed degradation of lipophilic nitrosoureas can be inhibited by serum lipoproteins.[938] This

Scheme 123



inhibition was explained[938] by a partitioning of the lipophilic CENUs into the hydrophobic core region of the lipoproteins where the CENUs are chemically stable and not free to undergo either protein binding or water-mediated reactions. Thus, individual variations of patients's serum lipoprotein concentrations, and, hence, the partitioning, could represent a significant factor which may affect the tissue distribution and pharmacokinetics of lipophilic drugs.[938]

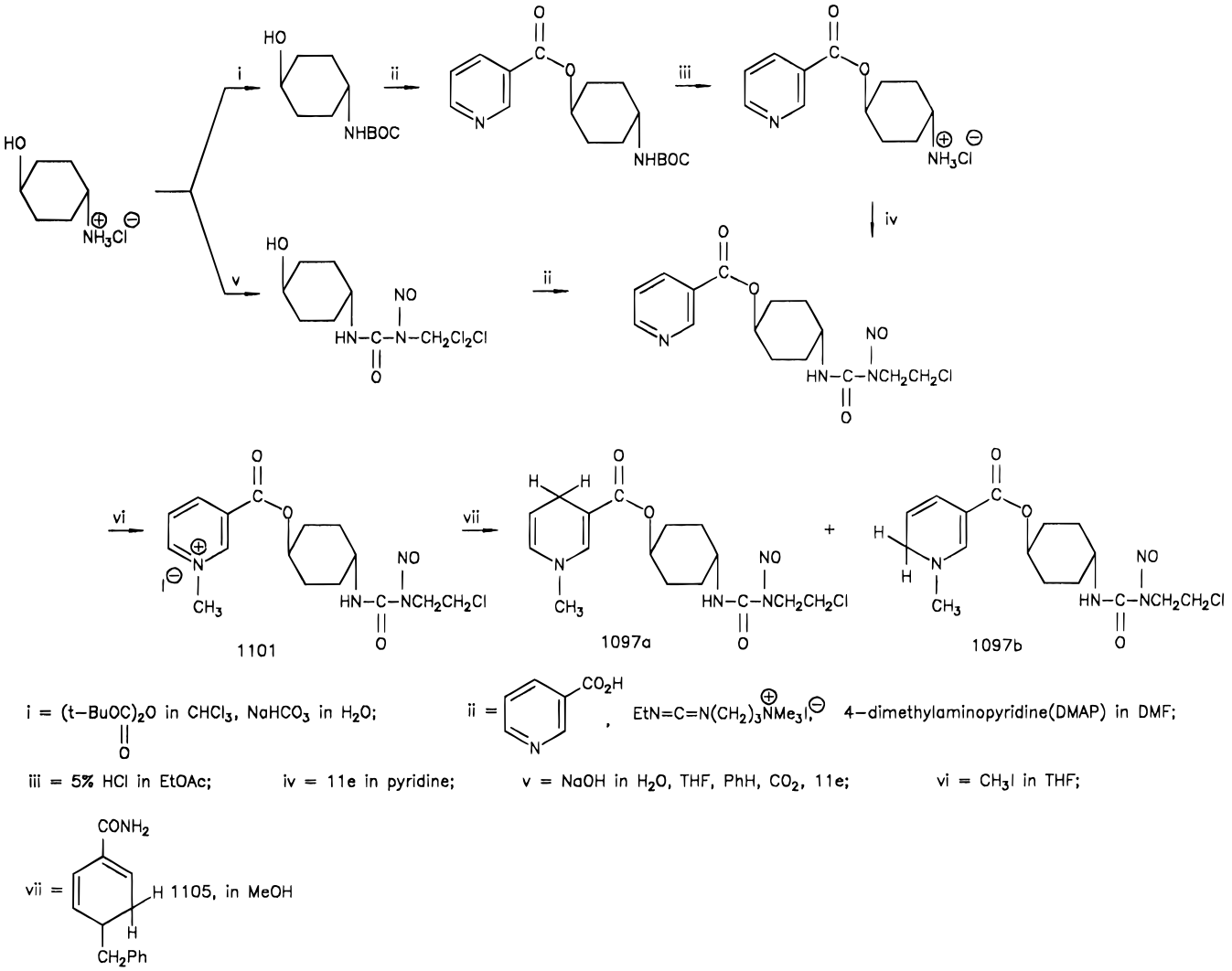
B. Pharmacology

General reviews of the pharmacology of nitrosoureas have been published.[3,12,23,25] Hence, only specialized topics will be discussed in this section. References to the pharmacological studies of specific compounds are included in several parts of section VII.

A concerted effort has been devoted to the design of drug analogs which will cross the blood−brain barrier (BBB), and which will not affect other organs or tissues. The ideal drug for crossing the BBB would have a combination of high lipophilicity, low ionization at physiological pH, and low plasma protein affinity.[109] Administration of a lipophilic nitrosourea, such as CCNU (**137b**), results in an initial high cerebrospinal fluid (CSF)/plasma ratio. However, high concentrations are not attained in the cancer cells because of intracellular and transcapillary exchanges during the drug's movement to the brain and cancer cells as well as losses across the cancer cell capillaries.[939] An intracellular "lock-in" mechanism is needed to prevent such drug losses. One such approach involves the design of a pyridinium salt−dihydropyridine redox system.[939−942] The process is outlined in a somewhat simplified form in Scheme 123. A drug (D moiety) is attached to either a methyl or benzyl nicotinic acid or amide, forming the pyridinium salt quaternized species Py^+-D (Q^+-D) (A), which then is reduced to the lipophilic dihydropyridine analog H−Py−D (HQ−D) (B). The B species is the so-called chemical delivery system (CDS) or a (pro)n drug.[939] After the B conjugate is injected into the blood stream it is distributed in the general circulation and also enters the brain via the BBB. The B species is then reoxidized enzymatically by a NAD/NADH system to the original A form. The A species is also formed in the periphery but it is rapidly eliminated from the general circulation before cleavage, i.e. $k_3 \gg k_4$, whereas it is locked in the brain because the large ionic species cannot easily cross the BBB. The trapped quaternary−drug conjugate A then is slowly hydrolyzed to the quaternary carrier Q^+ and the active drug D (Scheme 123). Ideally the carrier Q^+ is rapidly eliminated from the brain, i.e. $k_9 > k_6$, and the drug D is normally metabolized and eliminated (k_{10}). Thus, the overall process results in a preferential release of the active drug D in the brain. The rapid elimination of the A species from the general circulation $k_3 > k_7$, k_8, ensures a greatly reduced incidence of toxic side effects.[939]

The syntheses of the dihydropyridine conjugates of *trans*-4-OH-CCNU **1097**[943] and of HECNU **1098**−**1100**[944] are illustrated in Schemes 124 and 125, respectively. The crucial step in all of the reaction sequences is the selective reduction, by a direct hydride transfer, of the pyridinium compounds **1101**−**1104** to the corresponding dihydropyridine compounds **1097**−**1100** with 1-benzyl-1,2-dihydroisonicotinamide (**1105**) without a concomitant reduction of the *N*-nitroso moiety.[945] Reduction of the *trans*-4-OH-CCNU conjugate **1101** was reported[944] to yield a 80:20 mixture of the 1,4- and 1,2-dihydropyridine species **1097a** and **1097b**, respectively. Support for the proposed mechanism of action came from several experiments.[943] First, the relative log *P* values of the drug, *trans*-4-OH-CCNU (**189**), the oxidized conjugate (**1101**), and the reduced conjugate (**1097**) are +0.99, −1.30, and +2.53, respectively. Thus, the dihydropyridine conjugate **1097**, which must pass through the BBB, is the most lipophilic species and the pyridinium salt conjugate **1101**, which must be locked-in the brain as well as be eliminated rapidly from the periphery, is the most hydrophilic species.[943] Secondly, in vitro stability studies revealed[943] that the reduced B form **1097** is more stable, i.e. has longer half-life and a lower pseudo-first-order hydrolysis rate constant, in rat blood than in rat brain homogenate. This result is in agreement with the observed rapid transformation in the brain of **1097**

Scheme 124

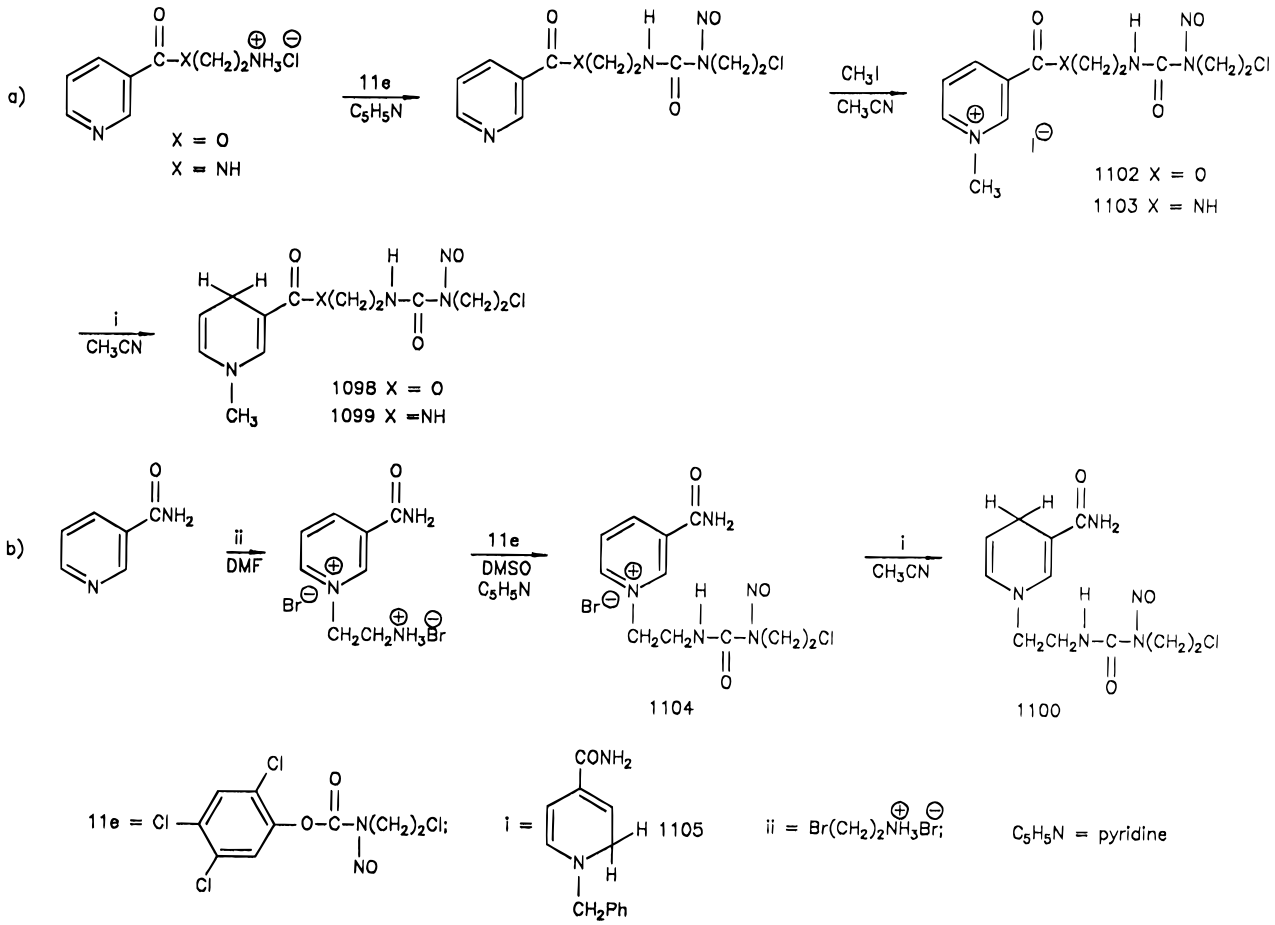


to the oxidized form **1101**, $k_2 > k_1$. The reduced species **1097** also is less stable in blood than in a pH 7.4 buffer, indicating a susceptibility of **1097** to enzymatic attack by blood enzymes.[943] The oxidized form **1101** was found[943] to be relatively stable in both rat whole blood and the 20% rat brain homogenate. This result supports the idea of a sustained release of the active drug D by hydrolysis of the ester bond of **1101**. Thirdly, in vivo tissue distribution studies revealed[943] that the free drug **189** reached a peak concentration in blood, brain, lung, and kidney within 15 min, but after 90 min was undetectable in the brain and lungs, probably the result of metabolism and lipophilicity. Thus, the free drug can cross the BBB but can also exit the brain easily, i.e. the brain/blood ratio is near unity. However, the iv administration of the dihydropyridine conjugate **1097** resulted[943] in a peak concentration of the oxidized conjugate **1101** in the tissues within 15 min, but more importantly, the brain/blood ratio was increasing over 90 min, indicating an accumulation of the conjugate **1101** in the brain with time. After iv administration of the reduced conjugate **1097**, the time-concentration profile for the release of the drug **189** revealed[943] a low but sustained level from 1 to 4 h, $k_6 > k_5$, whereas the iv administration of the free drug resulted in a sharp drop in brain concentration within 1 h. The fact that no buildup of the drug **189** occurred in the brain after injection of **1097** is attributable[943] to a rapid clearance of **189** from the brain as the result of excretion and metabolism. The half-life of **189** in the brain was estimated[943] to be 10 min as compared to a half-life of 65 min for the drug−conjugate **1101**, $k_{10} > k_6$, Scheme 123.

Further studies of the pyridinium−dihydropyridine redox system for 4-OH-CCNU (**189**) and HECNU (**329**) were reported.[944] In general, the dihydropyridine conjugates **1097**−**1099** were less stable in rat blood and in a 20% rat brain homogenate than the corresponding pyridinium salt conjugates **1101**−**1103**. However, the dihydropyridine−HECNU conjugate **1100** was more stable in rat blood and only slightly less stable in rat brain than the corresponding oxidized conjugate **1104**.[944] This result seems to indicate[319] that stable nonhydrolyzable compounds would not be acceptable candidates for an effective brain "lock-in" mechanism.

In order to overcome the low water solubility of the 4-OH-CCNU-CDS **1097** two aqueous-based formulations derived from (hydroxypropyl)-β-cyclodextrin (HPBCD) and Tween 80 were compared[946] with DMSO in terms of stability and tissue biodistribution. The two aqueous formulations produced a 250−400-fold increase in water solubility. After a single iv injection of the reduced conjugate **1097** into rats the concentrations of the oxidized conjugate **1101** in the

Scheme 125



brain and lung were higher for the DMSO solution than for the aqueous-based formulations after 2 h, but the brain/lung ratio was 3.71 with the cyclodextrin formulation and 2.65 for the DMSO solution, hence, a selective brain enhancement of 139%.[946] The Tween-80 system was less effective than the HPBCD system.[946]

Long-term storage stabilization of the *N*-nitrosoureas BCNU (**33**) and CCNU (**137b**) was effected[947] by formulation of stable cycloamylose clathrates. The encapsulation of the drugs CZT (**368**), RFCNU (**437**), and CNCC (**119**−**121**) in liposomes resulted in an improvement in activity compared to the free drugs.

The enhancement effect of 2-nitroimidazoles, such as misonidazole MISO (**291**), on the cytotoxicity of CENU drugs is not the result[948] of alterations in the pharmacokinetics of these drugs.

C. Toxicology

The subacute and chronic toxicities of CENUs have been reviewed.[28,29] In addition, toxicity effects have been discussed in sections of other reviews.[5,10,112,296,949] The major acute toxicities are myelosuppression and hepatotoxicity.[29] Myelosupression is discussed in sections VIII, X.F, and X.I of this review. A single, nonlethal dose of CCNU (**137b**), was shown[950] to reduce microsomal drug metabolism, including levels of cytochrome(s) P450, by 40−60% for up to 6 weeks. Studies[951] on the cytotoxicity and cellular kinetics of rat liver cells were used to explain the difference between the hepatoxic nitrosoureas carmustine (BCNU, **33**), and lomustine (CCNU, **137b**), and the nonhepatoxic nitrosourea fotemustine (**134**). Renal toxicity (nephrotoxicity) is a major acute toxic side effect of streptozotocin (SZT, **367**).[28,29] However, kidney toxicity is not a major problem for related CENU drugs, such as BCNU (**33**), CCNU (**137b**), and CZT (**368**).

Chronic toxicities of CCNU (**137b**), MeCCNU (**138b**), morpholino-CNU (**279**), CZT (**368**), BCNU (**33**), HECNU (**329**), and HECNU-MS (**333**) were compared.[112,296] The order of decreasing toxicity was BCNU > CZT > morpholino-CNU > HECNU-MS > HECNU > CCNU > MeCCNU. The acetamido CNU (**598**) was more toxic than HECNU but less toxic than CZT or BCNU.[5] The differences between the acute toxicity LD_{50} and the long-term toxicity after repeated iv injections are shown for several CENUs in Table 61. The two compounds BCNU and HECNU have very similar acute toxicities, as judged by their respective LD_{50} values of 117 and 128 μmol/kg, whereas after repeated iv injections the BCNU was four times more toxic than HECNU.[5] It was difficult to correlate[5] this difference in toxicities with biological parameters, but the most toxic compound BCNU was the strongest inhibitor of glutathione reductase and caused a low level of DNA cross-linking.

D. Mutagenicity and Carcinogenicity

1. Basic Concepts

The alkylation of DNA by alkylating agents, such as *N*-nitrosoureas, can lead to secondary cancers, such as acute nonlymphocytic leukemias.[952−954] It has been determined[714,739,746−749,955] that alkylation of

Table 61. Long-Term Toxicity of CENU Analogs[5]

no.	compound	LD_{50},[a] μmol/kg	long-term toxicity,[b] mmol/m^2	DNA cross-linking potential in vivo[c] (rad-equiv)	glutathione reductase inhibition,[d] min
33	BCNU	117	0.71	34	20
329	HECNU	128	2.89	156	60
598	acetamido CNU	95	1.80	155	∞
368	CZT	87	1.22	24	∞

[a] Lethal dose = single ip-administered dose resulting in the death of 50% of the untreated rats; observation on day 60. [b] Median total dose resulting in the first significant decrease of median survival time when compared with that of untreated controls. [c] After 24 h in bone marrow of rats. [d] Human glutathione reductase; time required for 50% inhibition.

the oxygen atoms of the bases, in particular, the O6 of guanine and O4 of thymine, lead to DNA mutations. The nitrosoureas containing small alkyl groups, e.g. methylnitrosourea (**25**) and ethylnitrosourea (**27**), are particularly mutagenic.[721,750,956−959] The alkylation of these oxygen atoms leads to changes in the Watson−Crick pairing of the DNA double helix which results in abnormal base pairs and point mutations. In particular, the O6-RG base (**1043**), where R = alkyl groups, results in a G:T → A:T transition, whereas the O4-RT base (**1042**), results in a A:T → G:C transition.[714,739] The N^7,N^9-dialkylated guanines were not mutagenic lesions as formerly thought,[958] but such bases are unstable and subject to imidazole ring opening[755−757] and depurination[758] reactions which can lead to single-strand scission. Minor miscoding pathways can result from the presence of 3-alkylpyrimidines and 1-alkylpurines while apurinic and apyrimidinic reactions result from the presence of 3-alkyl- and 7-alkylpurines in the DNA structure.[721] Reviews of the DNA adducts of mutagens and carcinogens have been published.[714,721,739,747,750,959]

The suspected lethal DNA lesion, 3-methyladenine (**1106**, Chart 29), and the protomutagenic O^6-methylguanine (**1043**, R = CH_3) and O^4-methylthymidine (**1042**, R = CH_3) (Chart 28) lesions are removed by repair enzymes (section X).[960−962] As is discussed in section X.C, the repair of O^6-methylguanine adducts involves the transfer of the methyl group to a cysteine residue of an alkyl acceptor transferase enzyme, AAT, which becomes inactivated in the process.[963] Such a direct transfer usually involves no error in DNA coding since the DNA is converted to its original condition without replacing part of the DNA strand. The repair enzymes, methylguanine-methyltransferase (MMT), and 3-methyladenine-DNA glycosylase II are induced after exposure to the alkylating agents and help to protect the cell from transformation to a cancerous state.[745] Different organs and the same organ in different species show wide variations in their susceptibilities to alkylating agents which alkylate the O6-position of guanine.[964] The high susceptibility of certain organs, such as rat brain, to induction of cancer by *N*-nitroso-*N*-alkylureas, ANUs, appeared to be based on a corresponding low ability to remove O^6-alkylguanines (**1043**) from the DNA.[965,966] However, a dependence of cancer induction on a low level of DNA lesion repair is not absolute since mouse brain which has a low capacity to remove O^6-alkyl-G in vivo, fails to show a high sensitivity to ANU drugs.[967] Further, the level of AAT in organs of both the rat and mouse was not the reason for the large difference in susceptibility to ANUs between the rat brain and the mouse brain.[803] The DNA repair capability in organs more susceptible to carcinogenesis, such as rat brain, was not lower than that in organs less susceptible to carcinogenesis, such as mouse brain.[967]

The mutagenic potential of a variety of alkylating agents was not correlated[960] with their carcinogenic (cancer-causing) potential or their electrophilicities. No obvious proportionality was found[968] between the carcinogenicity, as measured by the carcinogenic dose TD_{50}, and the mutagenicity in *Salmonella* strains TA 1535 and TA 100. For example, ENU (**27**) was the most potent carcinogen of a group of nine alkylating agents (low TD_{50} value) but was only a weak mutagen. Conversely, glycidaldehyde was highly mutagenic but was one of the weakest carcinogens (high TD_{50} value). The ratio of N7/O6 alkyl guanines in native DNA in vitro was shown[968] to have a positive correlation with the mean of the TD_{50} values. Furthermore, for induction of thymomas (thymus cancer) in a given strain of mice, the sole quantitative measure that was positively correlated with the carcinogenicity of a series of alkylation agents was the extent of alkylation of the guanine O6-oxygen in the target DNA,[969,970] but various strains differed greatly in their overall sensitivity.[971,972]

A relationship was shown[721] between DNA adduct formation at the cellular level and mutagenic and carcinogenic changes at the tissue level. Thus, the bacteria *E. coli* was exposed to MNU (**25**) and ENU (**27**) and the type and frequency of specific DNA base changes in mutants were compared[973] to the concentration of O^6-alkyl-dG (**1043**) and O^4-alkyl-dT (**1042**) in the DNA of the xanthine guanine phosphoribosyltransferase (gpt) gene. It was found[973] that MNU induced 100% G:C → A:T transitions, whereas ENU induced 73% G:C → A:T transitions, 21% A:T → G:C transitions, and 6% transversions. The predominance of point mutations is consistent with simple base pair mismatches induced by specific DNA adducts known to be generated by these alkylating agents.[739] The DNA alkylation ratios **1042**/**1043** was 0.014 for MNU and 0.28 for ENU. These results are consistent with the A:T → G:C transition after ENU exposure but not after MNU exposure.[973] Thus, the relative ratios of protomutagenic lesions in DNA are important in determining the actual base pair changes induced by compounds.[974]

Mutagenesis by both MNU and ENU exhibited a DNA sequence specificity, with 82% of the MNU- and 71% of the ENU-induced GC → AT mutations occurring at the middle guanines of the sequence 5′-GG(A or T)-3′.[973] This sequence specificity could be attributed to a preferential alkylation of bases,[975,976] local repair deficiencies,[977] and/or alterations in mispairing capabilities because of the local DNA environment.[978] In particular, the preference of alkyla-

tion reactions by MNU and ENU for contiguous patterns of guanines in DNA indicates that there are localized "hot spots" for mutagenic and/or carcinogenic changes.[975,976]

In order to study more thoroughly the formation of mutagenic "hot spots" on the *E. coli* xanthine-guanine phosphoribosyltransferase gene, four oligodeoxyribonucleotides of sequence 5′-CCG^{1}TG^{2}G^{3}G^4-ATAGGGCTG-3′ were synthesized[979] A radiolabeled tag, 1′,2′-[^{3}H]deoxyguanosine was located at one of the four indicated sites, 1, 2, 3, or 4. After incubation of the deoxynucleotides with methylnitrosourea (MNU, **25**), it was found[979] that there was a greater amount of O^6-metG at positions 3 and 4 than at 1 and 2, and that this uneven distribution of alkylated bases existed only in the double-stranded form of the oligomers. On the basis of these results it was concluded[979] that the secondary structure is an important factor. The relative formation of O^6-metG at positions 3 and 4 were 3.52 and 2.92, respectively, but the corresponding number of MNU-induced mutations at those sites were 0 and 10. Therefore, it was concluded[979] that there is no perfect correlation of O^6-metG formation with mutation frequency at the labeled sites 1−4. Hence, other factors besides sequence-dependent binding, such as site-specific repair and polymerase fidelity, must be considered[979] in understanding the mechanism of mutagenesis by chemical mutagens such as MNU.

The mutational frequency of the clinical drug BCNU (**33**) was analyzed[980] at the adenine phosphoribosyltransferase gene of a Chinese hamster ovary cell line. The predominant BCNU-induced mutations were G:C → T:A transversions (51%) while the expected G:C → A:T inversions from miscoding of O^6-alkylguanine comprised only 16% of the recovered mutations. This particular cell line is deficient in the GAT repair enzyme and it should be particularly susceptible to mutations caused by alkylations at the O6-position of guanine in DNA. However, the simple miscoding of O^6-alkylguanine for thymine is not the predominant mechanism for mutation in these cells. Of particular interest was the finding[980] that two "hot spots" for BCNU-induced G:C → T:A transversions contained different surrounding base sequences but similar helix parameters, when analyzed by an application of Calladine's Rules. These observations[980] of the DNA helix influence on base alkylations and substitutions leading to mutagenesis parallel recent findings[780] concerning the specificity of DNA base alkylations by various clinical nitrosourea drugs.

Some studies with oncogenes revealed[981−984] a direct relationship between DNA adduct formation, DNA mutations and subsequent neoplastic transformations of cells. For example, each of the Ha-ras-1 oncogenes present in MNU-induced mammary carcinoma became activated by G → A transitions in the second nucleotide of codon 12.[981−983] Furthermore, in MNU-induced rat kidney mesenchymal cancers, the K-ras gene is activated also by a G → A transition at codon 12.[983] A G → A transition of the K-ras gene at either codons 12 or 13 was observed[984] in NMU-induced thymic lymphomas in mice. No such mutations were observed[985] in Ha-ras-1 oncogenes present in mammary carcinomas induced by 7,12-dimethylbenz(*a*)anthracene (DMBA), a carcinogen that fails to induce specific G → A transitions. Thus, the malignant activation of the Ha-ras-1 locus in NMU-induced mammary carcinomas must be the direct result of the mutagenic effect of NMU. Efficient induction of mammary carcinomas can be readily achieved by a single dose of NMU.[984] Because of the relatively rapid decomposition of NMU under physiological conditions its mutagenic effect must occur within hours following its administration.[983] A proposal was made[983] that the malignant activation of the Ha-ras-1 oncogene by the NMU-induced G → A mutations must be concomitant with the initiation of the carcinogenic process. Ras oncogenes cannot transform normal rodent embryo cells unless they acquire the capability of self-proliferation, either spontaneously by carcinogen treatment or by transfection of other oncogenes, such as c-myc, adenovirus Ela, or even H-ras, when linked to strong transcriptional enhancers.[983]

2. Comparative Mutagenic and Carcinogenic Properties

A number of studies have dealt with the comparative mutagenic and carcinogenic properties of ANUs and CENUs under in vitro and in vivo conditions. The relative mutagenicity of *N*-methyl- and *N*-(2-chloroethyl)nitrosoureas were reported.[38] All of the compounds, with the exception of the pyrimidine-methyl analog ACNU (**311**), were increasingly mutagenic after incubation with induced rat liver microsomes. Importantly, no correlation was found between the in vitro alkylating activity and the mutagenic potential of these compounds.[38] It was not possible to use mutagenicity as a predictor of anticancer activity in the L1210 screening system.[38]

A comparison[986] was made among the mutagenicity, anticancer activity and chemical properties of short-chain nitrosoureas and nitrosoamides. The two sets of compounds were not only chemically similar, but had also similar lipophilicities, half-lives, and decompositions to a diazohydroxide intermediate. However, while the nitrosoureas were active as anticancer agents, they were less mutagenic in the Ames test and more toxic than the corresponding nitrosoamides which were inactive as antileukemic agents.[986] The nitrosoureas and nitrosoamides with long chains were less mutagenic than the compounds with short chains against the *Salmonella typhimurium* strains. However, this relationship was reversed in other test systems.[986] The major structural difference between the two sets of compounds is attributed to the fact that the nitrosoureas also decompose to an isocyanate. One can speculate[319] that since the isocyanate moiety normally reacts with proteins, whereas the diazohydroxide intermediate reacts with DNA, RNA, and proteins, and since the production of abnormal DNA would lead to mutation, the nitrosoamides, on a molar basis, would be more mutagenic. Other possibilities offered[986] for the difference in mutagenic potential were various decomposition sites in the cancer cells, since nitrosourea and nitrosoamide decompositions are initiated by proton abstraction and proton addition, respectively, and different membrane transport sites in cancer cells, normal cells and bacteria.

The mutagenicity of *N*-methyl-*N*′-aryl-*N*-nitrosoureas (**998**) were explored.[39,987] No correlations were

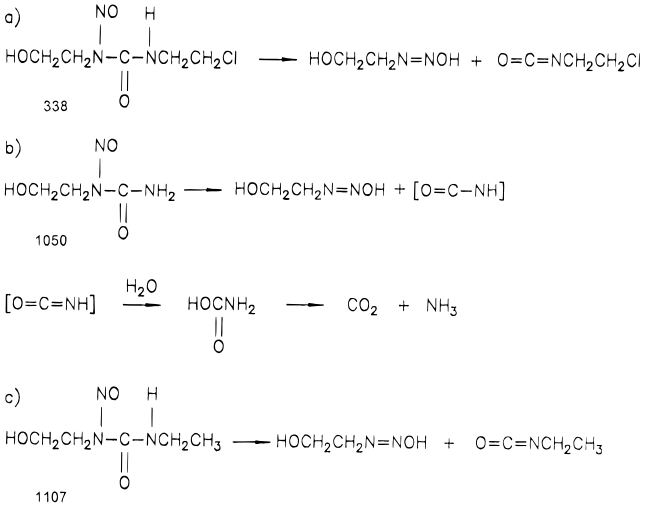
found[39] between the mutagenicity of these compounds and their carcinogenicity. The administration of arylnitrosoureas and *N*-methyl-*N*-nitrosourea (**25**) resulted[39] in different cancers in test animals.

The carcinogenic activity of seven representative CENUs was determined after repeated iv injections to rats.[5,112] No common target organs for lesions were found for the various CENUs although the nerve and lung tissue were most commonly affected by neoplastic lesions.[5,112,988] BCNU (**33**) and morpholino-CNU (**279**) were the most carcinogenic and HECNU (**329**) and HECNU-MS (**333**) were the least carcinogenic compounds.[5,112] Long-term experiments involving ip injections in SD rats once a week for up to 52 weeks were revealing.[5,112] The BCNU-treated rats with a median survival time (MST) of 359 days, exhibited[989] a definite dose-related local carcinogenic effect, with malignant neoplasms in the intraperitoneal cavity while the HECNU-treated rats, with a MST of 648 days, showed no such local carcinogenic effect.[14,989−992] The CENU-carbohydrate chlorozotocin (**368**), which has a high activity against the L1210 leukemia and no bone marrow suppression, was a very potent carcinogen in such a long-term ip inoculation schedule.[5,990] An important conclusion[112] from this work is that chronic toxicity and carcinogenicity studies can be used to distinguish between compounds which have similar anticancer activities and acute toxicities, and that these biological parameters should be determined before subjecting new active compounds to clinical trials.

Further information about the relation of structure and carcinogenicity was obtained from studies of various hydroxyalkyl CENU analogs. The N3-nitroso analogs, 1-nitroso-1-(2-hydroxyethyl)-3-(2-chloroethyl)urea (**338**) and 1-nitroso-1-(2-hydroxypropyl)-3-(2-chloroethyl)urea (**339**) were much less active as anticancer agents than the isomeric N1-nitroso analogs **329** (HECNU) and **337** (Chart 11).[298,299] The analogs **338** and **339** exhibited[298] both low toxicity and high mutagenicity and carcinogenicity. Isomers **338** and **339** caused a 14-fold higher rate of DNA single strand breaks in fetal hamster lung cells than isomers **329** and **327**. The lower activities of **338** and **339** can be related[298] to the formation of hydroxyethylated DNA by the decomposition mechanisms of Schemes 115 and 119. These intermediates cannot undergo interstrand cross-linking with adjacent DNA strands. In contrast, the administration of the chloroethyl isomers **329** and **327** caused[298] an 11-fold higher amount of DNA cross-links than either **338** or **339**. In this case, the intermediate 2-chloroethylated DNA can undergo cross-linking reactions according to the mechanisms of Schemes 113c or 119. A conclusion[298] from this work was that the DNA cross-linking is related to anticancer activity, while the DNA single strand breaks are related to mutagenicity and carcinogenicity but not to anticancer activity.

In earlier studies[993−995] the N-nitroso compound, 1-(2-hydroxyethyl)-1-nitrosourea (HENU, **1050**) was found to be more carcinogenic than 1-ethyl-1-nitrosourea (ENU, **27**) in inducing thymic lymphomas in mice. However, the toxicity, i.e. LD_{50} values, of both **1050** and **27** was about the same.[993] The greater carcinogenicity of **1050** than that of **27** was explained

Scheme 126



by an analysis of the pattern of DNA alkylation products formed from the two compounds.[993] The hydroxyethylated O6- and N7-positions of guanine and the diphosphate ester, arising from **1050**, would be expected to be less stable than the corresponding ethyl DNA adducts arising from **27** (section X.A). In particular, the hydroxyethylation of phosphodiesters in DNA by **1050** would induce single-strand breaks, according to the mechanism outlined in Scheme 115.

A comparison of the DNA hydroxylation among the hydroxyethyl compounds **338**, **1050**, and **1107** was instructive.[996,997] Thus, the compounds **338** and **1107** were found to be carcinogenic inducers of kidney cancers in rats, whereas **1050** was not hepatocarcinogenic. However, compound **1050** caused the greatest extent of DNA hydroxyethylation to give O^6-(hydroxyethyl)-dG (**1031**) in all organs, with the highest effect of 37.2 μmol/mol dG in the liver, while compounds **338** and **1107** caused little DNA hydroxyethylation of 1.4−3.3 μmol/mol dG in all organs.[997] Thus, the hepatocarcinogenicity of **338** and **1107** cannot be explained[997] on the basis of their hydroxyethylating reactions with cellular DNA in vivo, even though it could be assumed that all three compounds decompose to the same *N*-hydroxyethyldiazohydroxide intermediate (Scheme 126).

An important difference among these three compounds is the structure of the isocyanate decomposition products. The intermediate derived from **1050** is unstable, resulting in the formation of carbamic acid which further decomposes to carbon dioxide and ammonia (Scheme 126). The isocyanates formed from **338** and **1107** are relatively stable and could carbamoylate proteins and/or DNA in the cells. Such cellular changes could result in their carcinogenic effects. The liver organ specificity could be attributed[319] to the nature of the alkyl portion of the isocyanate. Such organoleptism has been found[998] in the related *N*-nitrosamines. Thus, the hepatic carcinogen *N*-nitrosodiethanolamine (NDELA, **1108**), also caused a low amount of DNA hydroxyethylation in the liver tissue.[999−1001]

The mechanisms of the aqueous decomposition at pH 7.4 for the carcinogenic *N*-(hydroxyalkyl)-*N*-nitrosoureas **1050** and **1109,** and for the cyclic carbamates *N*-nitrosooxazolidones **957** and **1110** (Chart 29) were determined.[1002] The decomposition

products for **1050** and **1109** included aldehydes, acetone, glycols, and epoxides while the compounds **957** and **1110** yielded mainly glycols and cyclic carbonates. It was demonstrated[1002] that the (hydroxyalkyl)nitrosoureas **1050** and **1109** failed to decompose either to the cyclic carbamates **957** and **1110** or to their cyclic carbonate decomposition products under the physiological conditions. Furthermore, the aqueous decomposition of the *N*,*N*′-dialkyl-*N*-nitrosoureas **338** and **339** gave comparable products and yields to those obtained from the *N*-monoalkyl-*N*-nitrosoureas **1050** and **1109**.[1002]

Compound **1050** was shown[1003] to induce a variety of tumors in rats with a majority located in the lung and forestomach, compound **1109** induced chiefly lymphomas, leukemias, and forestomach tumors, while the application of cyclic carbamates **957** and **1110** resulted in forestomach tumors almost exclusively. It was suggested[1002] that the administration of compounds **957** and **1110** exclusively resulted in forestomach tumors because of the presence of esterases near the intragastrical application site which would cause a rapid decomposition. The half-lives of compounds **1050**, **1109**, **957**, and **1110** were 11, 35, 50, and 90 min, respectively, and hence the compounds that cause forestomach tumors predominantly are the most stable.[1002] In addition, the decomposition products from the two epoxides, acetaldehyde, propylene oxide, and ethylene oxide, are known to cause tumors, and in particular, forestomach tumors.[1002] Some factors which were cited[1002] for the differences in target organs of the (hydroxyalkyl)-nitrosoureas **1050** and **1109** were differences in molecular size, lipophilicities, and decomposition products.

The subject of organotropism of various *N*-nitroso compounds, *N*-nitrosoamines, *N*-nitrosourethanes, and *N*-nitrosoureas, was addressed.[1004] All of these compounds have a multipotent carcinogenic effect, namely, the neoplastic lesions are found in organs and tissues remote from the point of administration.[1004] The route of administration plays a role in the organotropism of nitrosoamides and nitrosoureas but not of that of nitrosoamines.[1005] Thus, *N*-nitroso-*N*-methylurea (**25**) and *N*-nitroso-*N*-methylurethane (**1111**) were found to produce squamous cell carcinomas of the forestomach after oral application, but **25** caused also brain tumors and **1111** lung tumors after intravenous administration.[1005] The administration of nitrosoamides transplacentally to pregnant animals resulted in different tumors in the offspring, depending on the animal species as well as on different strains within the same species.[1004] The methylation of DNA was fairly constant among all tissues, but the response of the various tissues was extremely variable. In rats, methylation and ethylation of DNA resulted[1006] in cancers of the kidney and brain, whereas in young adult mice a cancer of the thymus occurred.[1007]

Strongly carcinogenic *N*-nitrosoureas were shown[1008] to react with phosphatidyl ethanolamine (PE), but not other membrane phospholipids, in chicken erythrocyte ghosts and rat kidney cells while weak carcinogenic analogs failed to react. The reaction involves the carbamoylation of the amine group of PE by the isocyanate generated from the *N*-nitrosoureas, yielding compounds of general structure **1112**.

Table 62. The Transformation of Normal to Cancer Cells

1.	increase in nutrient transport, such as, glucose, amino acids, and phosphate
2.	higher rates of glycolysis, i.e., conversion of glucose to lactic acid
3.	increase in levels of proteins in and on surfaces of cancer cells
4.	decrease of the large external transformation sensitive glycoprotein (LETS, fibronectin) on cell surfaces
5.	appearance of incomplete surface carbohydrates on glycoproteins and glycolipids
6.	lowering of surface glycosyl transferase activity
7.	decrease of intracellular pH below 7
8.	mobility of surface receptor sites
9.	increase of agglutinability of cells mediated by lectins
10.	appearance of embryonic (fetal) antigens
11.	appearance of tumor-specific antigens
12.	alteration of cell adhesion
13.	decrease in contact inhibition
14.	decrease in chalones (glycoproteins) or response to chalones; effect on mitosis, cofactors: hydrocortisone or adrenalin
15.	decrease of c-AMP with concomitant increase of c-GMP
16.	decrease of DNA synthesis and cell division, stimulated by hormones, e.g. cortisol, and mediated by calcium ions and c-AMP
17.	appearance of unpolymerized cytoskeletal structures, dependence on pH
18.	genetic alterations—activations or repressions
19.	metabolic alterations
20.	metastasis

XII. Assessment of Present Status of Cancer Diseases and Suggestions for Future Explorations

Extensive investigations of various aspects of cancers during the past 40 years have generated a wealth of information concerning these diseases. Nevertheless, many key questions remain unanswered, and the cancer diseases remain the second highest cause of death after cardiovascular diseases. Although it is, generally, agreed that cancers are caused by mutations of the DNA, whereby are also gravely affected many other intracellular and cell surface functions (Table 62),[1009,1010] difficulties persist in understanding the factors which trigger the DNA mutations leading to transformations of healthy to cancerous cells. In particular, there is a lack of well-founded knowledge about the "latent" period of cancers in vivo before they can be diagnostically detected. It is believed that some cancers can be genetically transmitted in the form of oncogenes, or they could be induced by certain types of viruses, such as HIV, radiation, certain environmental factors, excessive smoking, dietary factors and other unknown agents. Another unsolved question remains whether some anticancer drugs, while curing the primary cancer, may, at the same time, initiate the formation of a secondary neoplastic growth in vivo. Although the onset of transformations of healthy to cancer cells in vivo are not fully understood, many differences between those cells have been known[1009,1010] for some time.

It is somewhat surprising that in spite of a large number of *N*-nitrosoureas anticancer drugs which

have been discovered in the past decades, only four drugs, i.e. carmustine (BCNU, **33**), lomustine (CCNU, **137b**), semustine (MeCCNU, **138b**), and streptozotocin (SZT, **367**), have been used in clinical oncology in the United States and that all these drugs were adopted in clinical practice at an early stage of the search for this type of anticancer agents, probably because the regulatory procedures were less cumbersome and less stringent than at the present time. To date, no prophylactic vaccine is available against any of the cancers, and the gene and immunotherapies are in their infancies. However, at present, there is a vigorous effort by major pharmaceutical companies to pursue these unconventional approaches for the development of new anticancer drugs. In summary, the meager output of new anticancer drugs, including *N*-nitrosoureas, for clinical oncology, in spite of a large pool of potential drugs which possess high activities and low toxicities, is somewhat puzzling. The low success rate could be attributed to a number of factors, such as delineated from a to h.

a. Poor selectivity by drugs between healthy and cancerous cells.

b. Plasma-mediated decomposition reactions of drugs before they reach the DNA.

c. High repair success rate by enzymes of DNA lesions in cancer cells.

d. Toxic side effects caused by interactions either of drugs or their key metabolites with healthy cells.

e. Alkylation and carbamoylation reactions of certain enzymes causing their deactivation.

f. Transport of drugs and metabolites and their permeation across the membrane in vivo.

g. A general failure to realize that synthetic manipulation of potential drugs always will be accompanied by changes in lipophilicity of the derivatives which, unwittingly, will result in transport and permeation changes across cell membranes.

h. Other unknown factors.

The lack of specificity for cancer cells in certain organs and tissues was tackled by attaching the *N*-nitrosourea moiety to carbohydrates, amino acids, and steroids. Thus, the D-glucose containing drug streptozotocin (**367**) was shown to have a specific activity against insulinomas, a cancer of the B-cells of the pancreas (section VI.C). The diabetogenic side effect of this drug can be suppressed by a coadministration of nicotinamide. Similarly, various steroid linked *N*-nitrosoureas were shown to have specific binding capabilities toward estrogen receptors, and, consequently, increased toxicity against hormone-dependent mammary carcinomas (sections VII.E and VII.I). Certain L-amino acid amide derivatives of *N*-nitrosoureas were shown to have high activities against leukemia cell lines, whereas the amino acid analogs were less active. Of course, the presence of these carrier moieties also changes the overall lipophilicities of the drugs, usually resulting either in less hydrophobic or even hydrophilic properties. Thus, it is expected that the amino acid amide derivatives would be transported more readily across the cell membranes than the ionized carboxylic acid derivatives. However, only a few of these drugs with carrier and/or receptor groups were used in clinical evaluations against human cancers, and none were adopted in clinical oncology, except SZT (**367**).

The plasma-mediated decomposition reactions of *N*-nitrosourea drugs might be prevented by the design of prodrugs which would be stable in plasma but would be transformed intracellularly to active DNA alkylating species. The N3,N3-disubstituted *N*-nitrosoureas were found to require an enzymatic dealkylation to give the cytotoxic N3-monosubstituted analogs which can then undergo a number of decomposition modes either to the alkyldiazonium ions or the other alkylation species. A number of such compounds cited in this review have been found to have superior anticancer activities and therapeutic indices as compared to the corresponding N3-monosubstituted analogs. Another type of *N*-nitrosourea drugs which requires metabolic activation are the CNCC compounds **119**−**121** which undergo in vivo reductions of a disulfide group, followed by oxidations of the resultant thiol groups (section VII.A.2). Another intracellular transformation is exemplified by the use of the pyridinium−dihydropyridine redox system which allows the passage of the covalently bound *N*-nitrosourea moiety across the blood−brain barrier and provides a method of "locking in" the drugs within the brain (section XI.B). However, to date, none of these potentially promising approaches have been adopted in clinical oncology. A prodrug which would undergo an intracellular transformation to the *N*-nitrosourea function, analogous to the enzyme-mediated activations of cyclophosphamide, has not been reported to date.

A major obstacle to the in vivo activity of the CENU drugs are the repair mechanisms of enzymes in the Mer^+ cells which remove the DNA lesions either by a general excision repair or by a transfer of alkylated DNA bases and DNA−DNA cross-links to alkyltransferase enzymes (section X.C). A part of this problem can be remedied by an initial administration of *N*-nitrosourea drugs, such as MNU (**25**) or SZT (**367**), which would cause an exhaustion of the DNA repair enzyme activity. A subsequent administration of a second CENU drug with high DNA alkylating and interstrand cross-linking capability would result in a much greater cytotoxicity against cancer cells (section X.C). Compounds which contain both the MNU and CENU moieties attached to a seconucleotide (section VII.F) have the potential for both interfering with the cellular DNA repair capability and forming cytotoxic DNA lesions. Further pursuits in this area would be of interest.

Another area of current interest includes the design of drugs specific for the minor groove of DNA and for specific DNA base sequences in oncogenes (section X.B). As was discussed in section X.A, the interactions of *N*-nitrosoureas with synthetic polynucleotides and native DNA result in alkylation of contiguous guanine bases, whereas other DNA alkylating agents, such as nitrogen mustards, were found to result in random DNA alkylation patterns. If oncogenes have DNA base sequences different from those of normal or untransformed genes, and if these base sequences undergo alkylation by *N*-nitrosoureas, then there is potential for the development of new drugs with cancer cell specificity.

The competition between the carcinogenic and carcinostatic properties of these drugs must be better understood. Thus, the same DNA repair mecha-

nisms which govern the removal of the DNA lesions in healthy cells, and therefore protect these cells from lethal mutations and ultimate neoplastic transformations, are also involved in the removal of the same lesions in cancer cells caused by drugs, thereby greatly reducing their effectiveness.

An important area will remain the specialized synthesis of drugs containing the so-called "reporter" markers, such as radioactive isotopes, whose presence can be conveniently ascertained using appropriate commercial instrumentation. Such labeled compounds can be advantageously used in mechanistic, metabolic, and diagnostic work, and, in particular, in studies concerning the transport, biodistribution, and persistence of drugs in various tissues and organs.

The aminoxyl (nitroxyl) radicals are another type of reporter moiety which has been extensively used for the past 30 years. The nitroxyl moiety can be much more readily introduced into a variety of compounds than radioactive species. The nitroxyl (spin)-labeled compounds can be followed in vitro and in vivo by electron paramagnetic resonance spectroscopy (EPR) and magnetic resonance imaging (MRI). Unlike the radioisotopes, the nitroxyl moieties result in considerable change of the lipophilicity of the parent compound, usually imparting a more hydrophilic property to a hydrophobic parent drug, and a less hydrophobic property to a highly hydrophilic parent drug. It is believed that this change in lipophilicity affects also the rate of permeation of drugs through cell membrane, and, hence, enhances the selectivity of the drugs toward cancer cells. Furthermore, it was found on several occasions that aminoxyl-labeled drugs are less toxic and often more active than the original parent drug, i.e. they have a higher therapeutic index (section VII.G). Since it has been found on numerous occasions that the paramagnetic nitroxyl compounds can be used as contrast enhancing agents in MRI, it can be hypothesized that spin-labeled anticancer drugs could be monitored during a chemotherapeutic regimen by MRI provided that computer-assisted data enhancements could be achieved in order to follow the very low anticancer drug concentrations within the living environment. Similarly, anticancer drugs containing other species with a spin, such as fluorine, phosphorus, and complexed transition metal and lanthanide moieties could be explored for their biodistribution in organs of animal models and even humans as a function of time after administration and the mode of administration using MRI. Such in vivo studies would provide a much more valuable assessment of the anticancer drugs' effectiveness and toxicity than the in vitro studies with cultures and isolated DNA.

In spite of the fact that a number of studies have been conducted in vitro concerning the interactions of *N*-nitrosoureas with DNA, and the products of such interaction elucidated, the transport and cell membrane permeation in vivo have been neglected probably because of considerable difficulties. Of course, it would be highly desirable to devise experimental conditions for the exploration of such transport and permeation using suitable labeled drugs in animal models, and even in humans. It is inconceivable that the differences between healthy and corresponding cancerous cells (Table 62)[1009,1010] would not be reflected in the rate of transport and permeation of drugs through cell membranes, and would have no effect on the observed selectivity of drugs for the cancerous DNA. Among the changes which occur during the transformation of healthy to cancerous cells the lipophilicity appears to be the dominant parameter. Over the years, the correlation of this parameter with anticancer activities has been used in a number of SAR and QSAR studies in order to systematically search for more effective drugs. These methods are somewhat imperfect because a correlation is used between the in vivo anticancer activity and the in vitro partition coefficient expressed as log *P* parameter, whereby the assumption is made that the level of increase in life span reflects a selectivity of drugs caused by the difference in the rate of transport and membrane permeation of the drug across the cancerous cell as compared to healthy cells. In spite of this simplistic hypothesis these correlations revealed that, generally, within a given structural series and with certain types of leukemias, the highest cytotoxicity was found to correlate with small negative and positive log *P* values, i.e. the effectiveness of drugs improved with "well-balanced" lipophilicity properties. However, with other cancer lines, the optimum might be located at different log *P* values. Hence, in designing new anticancer agents it will be necessary to establish this optimum.

Interestingly, none of the clinically used *N*-nitrosourea drugs, i.e. SZT (**367**) log $P = -1.45$, CCNU (**137b**) log $P = 2.83$, MeCCNU (**138b**) log $P = 3.30$, and BCNU (**33**) log $P = 1.53$ were found in the vicinity of that optimum when tested against leukemias, whereas several more recent potential drugs, such as the HECNU (**329**) log $P = 0.3$, acetamido-CNU (**598**) log $P = -0.2$, and MCNU (**531**) log $P = -0.7$, were in the optimum range, and exhibited high anticancer properties. Similarly, a number of nitroxyl-labeled *N*-nitrosourea drugs had lower log *P* values than the clinical drugs when tested against the P388 and L1210 leukemias.

A cautionary note for future studies on the correlations of lipophilicities of drugs with their anticancer activities came from investigations of *N*-nitrosourea decompositions in cell cultures in vitro (section VIII). The lipophilic *N*-nitrosoureas can permeate some cell membranes rapidly so that extracellular and intracellular concentrations reach an equilibrium in a short period of time. A dose function ΔC was devised which represented the concentration of the active alkylating species, such as 2-chloroethyldiazonium ion, and not the concentration of the parent drug within the intracellular compartment. When the ED_{50} ΔC values of structurally different lipophilic *N*-nitrosoureas were plotted against the corresponding log *P* values a straight line was obtained, whereas the same plot of concentrations of parent drugs against the log *P* values resulted in hyperbolic curves with maxima of anticancer activities at log *P* values ranging from 0.5 to 0.6. On the basis of these results it was concluded that for lipophilic *N*-nitrosoureas the anticancer activities in vitro are not affected by either the structure of the N3-substituent, the lipophilicity or the carbamoylating activity. Rather, the biodistribution of these

compounds might be more important than their structure in determining cytotoxicity. However, such in vitro results often are poor indicators of real events under in vivo conditions. Furthermore, the hydrophilic *N*-nitrosoureas, such as CZT (**368**), migrate across cell membranes more slowly than the hydrophobic analogs, and, consequently, could undergo extracellular decomposition reactions. Nevertheless, if the permeation of lipophilic *N*-nitrosourea drugs across cell membranes is not a decisive event, then the fate of drugs within the cell compartment becomes of paramount importance.Thus, the drug must undergo in the cancer cell, as compared to the healthy cell, a selective transformation to give active DNA alkylating species which should preferentially migrate to the cell nucleus, and cause DNA lesions by alkylation and cross-linking reactions. One experimental approach to attain this goal would be a covalent attachment of the *N*-nitrosourea drug to a nucleoside carrier moiety which could modify the genome, and cause lethal mutations. Unfortunately, attempts to use such nucleoside−*N*-nitrosourea analogs have yielded disappointing results (section VII.F).

XIII. Summary

This review was composed on the basis of a thorough perusal of nearly 1000 communications, including other review articles which were shorter and narrower in scope. The review contains a comprehensive, critical assembly of syntheses and anticancer activity data, complemented by topics on mechanisms and various biological aspects of a wide range of *N*-nitrosourea-type compounds covering a period of the past three decades, i.e. from the beginning of a systematic search for anticancer drugs of this class to the present. To date, no review as unique in scope is available. The large number of *N*-nitrosourea analogs with anticancer activities can be conveniently grouped into four major "generation" classes on the basis of their historical context, i.e. the logical evolution of theoretical and experimental approaches to the design of these compounds. The First Generation compounds are comprised of lipophilic aliphatic, alicyclic, aromatic, and heterocyclic analogs. The Second Generation compounds include the hydroxyalkyl and hydroxyalicyclic analogs which have more balanced hydrophilic/hydrophobic properties, as compared to the preceding class of compounds. The Third Generation compounds are considered to be those analogs which contain the so-called carrier groups, such as carbohydrates, amino acids, peptides, nucleotides, and steroids that are believed to provide enhanced transport properties through cell membranes and/or possess interactive capabilities toward specific receptors located on cell surfaces. The Fourth Generation, a more recent class of compounds, is comprised of conjugates, such as carbohydrate−amino acids, steroid−amino acids, and carbohydrate and amino acid analogs containing aminoxyl (nitroxyl) moieties. Depending on structure, these conjugates have a wide range of hydrophilic and hydrophobic properties, concomitant with various levels of anticancer activities. Comparisons of anticancer activities of potential drugs of various generations are attempted from literature figures reported from in vivo evaluations with lymphocytic P388 and lymphoid L1210 leukemias. The results are collected in tables. Following these topics, a section is devoted to structure−activity relationships among different structural classes of compounds. Then, the mechanisms of decompositions of *N*-nitrosoureas in aqueous media at acidic, basic, and physiological pH conditions are discussed in detail. A logical extension of these topics leads to discussions of interactions of *N*-nitrosoureas with cellular components, such as DNA. Included are alkylation and cross-linking reactions, repair of DNA lesions, carbamoylation and its effect on toxicity, myelosuppression, and the effect of nuclear components on the overall cytotoxicity. Finally, the important aspects of metabolism, pharmacology, toxicology, and carcinogenicity are discussed, since these factors affect the overall anticancer activity of *N*-nitrosourea drugs.

XIV. Addenda

This section includes overlooked references as well as papers that have been published during the period of May 1995 to September 1996.

Section I. Several books and treatises[1011−1014] are available on the oncology of anticancer drugs, including the *N*-nitrosoureas. Reviews[1015−1017] of the antitumor activity of nitrosourea drugs have been published.

Section VII.A.2b. A series of N1-[propargyl] derivatives, including 1,3-bis(2-propynyl)-1-nitrosourea (BPNU) were screened[1018] for anticancer activity in the NCI's primary antitumor drug screen. BPNU was shown[1018] to have a level of cytotoxic activity comparable to BCNU (**33**), CCNU (**137b**), and MeCCNU (**138b**) and, in particular, a marked specificity toward leukemic cells.

Section VII.A.2b. The sulfoxide drug **125** was tested[1019] against one murine and two human metastatic melanoma cell lines. The human melanomas were much more resistant to **125** than the murine melanoma. This result could be correlated with a lack of DNA cross-links and a higher GAT enzyme activity in the human cancer cells. A question was raised[1019] concerning the exact relationship between the Mer phenotype of cultured cancer cells and that of cancer cells surgically removed from cancer patients. It is possible that the original Mer phenotype could be modified by cell culture conditions.[1019]

Section VII.A.5b. Nitrosourea derivatives of sulfamerazine and sulfamethizole[1020] and of 2,2-dimethyl-1,3-dioxanes[1021] were reported.

Section VII.D.1. Interest has continued in the synthesis of amino acid derivatives of nitrosourea drugs. A review on these derivatives has appeared.[1017] A series of α,ω-diaminocarboxylic acid CENUs were synthesized and tested[1022] for anticancer activity. The compounds with $n = 4$ were reported[1022] to have the greatest biological activity. In an extension of previous work[497] the synthesis and anti-HIV activity screening of a series of N1-substituted ureas and nitrosourea derivatives of diamino acids ornithine and lysine was reported.[1023] The structures of related compounds are shown in Chart 17. Interestingly, the ureas were more cytotoxic than the corresponding nitrosoureas. A screening of these compounds in the NCI's primary antitumor drug screen revealed[1024] that the *N*1-methyl and *N*1-(2-chloroethyl)urea analogs were more selective for central nervous system cancer cell lines, and the N^1-

allylurea derivatives were more selective for lung cancer cell lines. The *N*1-propargylureas were not selective for any of the 60 human cell lines tested.

Section VII.E. Several cholesterol analogs with the CENU group substituted at the 6-position were synthesized and screened[1025] for in vitro activity against the L1210 leukemia cell line. Some of these compounds were reported[1025] to be more cytotoxic than BCNU **(33)** in this screening. Similar cholesterol analogs **723**−**725**, with the CENU moiety substituted at the 2-position, were essentially inactive against the L1210 leukemia.[224, 518] A steroid−amino conjugate, the estradiol 6-ester **921**, exhibited[628,631] a very low activity against a hormone-dependent MNU-induced rat mammary carcinoma. A report was published[1026] concerning the testing of steroid CENU analogs against a human meningioma cell line. The cytotoxicity of the steroid-linked nitrosoureas was superior in comparison to the unlinked components. Similar results have been obtained for CENU derivatives of steroid−amino acid conjugates against rat leukemia and mammary carcinoma lines, as discussed in section VII.I of this review.

Section VII.F. The enhancing effect of thymidine **(735)** on the cytotoxicity of BCNU **(33)** and 3′CTNU **(730)** could be explained[1027] by an enhanced uptake of **33** and an enhanced alkylation of DNA by both **33** and **730**. However, the DNA repair capacity of the cancer cells was not reduced by the presence of **735**. Additional research on seco-nucleosides containing the elements of the antimetabolite FU and the anticancer CENU moiety were published.[1028,1029] The ultimate goal of this research was to demonstrate an anticancer activity dependent on the hydrolytic release of 5-FU. The acid-catalyzed hydrolysis resulting in release of 5-FU from these seco-nucleoside proceeds in the following order of reactivity: MeO > MeS and FU^3 > 5-FU. Furthermore, it was known[549] that 5-FU seco-nucleotides with nitrogen or oxygen functional groups separated from the pyrimidine by a three-carbon chain are more readily hydrolyzed than their homologs with a two-carbon chain. On the basis of these findings, it was decided[1028] to synthesize several analogs containing the CENU moiety on a three-carbon side chain. These compounds were synthesized[1028] by modifications of the procedures which were used for the synthesis of the two-carbon side chain analogs. The three-carbon chain compounds were screened[1028] against two MAC tumor lines, a mammary carcinoma (Mca) and colon 38 adenocarcinoma. Analogous structure−activity relationships were found for the three-carbon side chain compounds as were found for the two-carbon side chain analogs. The latter are discussed in section VII.F of this review. The differences among the analogs with regard to molecular structure, water solubility, vehicle of administration, and cancer cell lines makes the interpretation of the screening results difficult. A $5\text{-}FU^3$-CENU, which was the most readily hydrolyzed under acid conditions of all the 5-FU seco-nucleotides studied, was shown[1029] not to be cleaved to 5-FU under in vivo conditions. Thus, it was concluded[1029] that the anticancer activity was predominantly attributable to the presence of the CENU moiety in these compounds. The 5-FU analog was more cytotoxic[1029] in both in vitro and in vivo screens against three murine colon tumor cell lines than the corresponding unsubstituted uracil analog. However, pharmacokinetic studies revealed that the improved activity was not attributable to the release of 5-FU.

Section X.C. Further studies have been reported on the role of inhibitors of the DNA repair enzymes, MGMT and GAT, in increasing the anticancer activity of the CENU class of drugs. Various O^6-benzylguanines have proven[1030] to be good inhibitors of these transferase enzymes in the presence of BCNU (**33**), resulting in an increased activity of **33** against prostate, breast, colon, and lung tumor cell lines. The prior administration of certain O^6-fluorobenzylguanines to cancer cells caused[1031] a considerable reduction in the level of the MGMT enzyme and a corresponding increase in the cytotoxicity of the drug ACNU (**311**). Analogously, the prior administration of O^6-methyl-2′-deoxyguanosine greatly reduced[1032] the level of the GAT enzyme in cancer cells and resulted in an increased cytotoxicity of ACNU (**311**) against the L1210 leukemia and B16 melanoma in mice.

Section XI.D. The mutations of genes caused by the nitrosourea drugs continues to be an area of study.[1033] This work has important implications in the understanding of how secondary cancers, such as leukemias, can result from the administration of these drugs. The frequency of mutations in the supF gene in African green monkey kidney cells (CV1) caused by the administration of CCNU (**137b**) was reported.[1033] The majority of the induced mutations were GC-targeted base pair substitutions. The occurrence of the GC → AT transition in the gene structure was concentration dependent. The induced mutations were nonrandomly located in sites of much greater frequency known as "hot spots". Either a common "hot spot" or "hot spots" exclusive for each CCNU concentration were generated. These results were interpreted[1033] to be consistent with the hypothesis that O^6-alkylguanine is responsible for the majority of GC-targeted mutations while O^4-alkylthymine and/or N^3-alkyladenine are probably responsible for AT-targeted mutations. Also, it is possible that the efficiency of the repair mechanism(s) involved in the removal of O^6-alkylguanine is influenced by the neighboring DNA sequence.[1033] A histoautoradiographical method was used to detect the DNA repair synthesis in different organs of mice after an administration of methylnitrosourea (MNU, **25**) by gavage into the stomach.[1034] It was believed[1034] that these results could be correlated with an organ-specific development of tumors in mice after oral application of MNU.

XV. References

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