described above. After the solution was chilled in an ice bath, 100 mg of sodium borohydride was slowly added, and the mixture was kept at 4 °C for 18 h. The solution was neutralized with Amberlite IR-120 (H^+) ion-exchange resin. The resin was removed by filtration and washed with water. The filtrate was evaporated to near dryness, and preparative TLC was used to purify the product. Two precoated 2-mm-thick silica gel plates, Sil G-200 with UV indicator (Machery-Nagel and Co., Germany), were used. The solvent system was 5:1:4 *n*-butyl alcohol–ethanol–water (upper phase). The main band at R_f 0.44, located with a UV lamp, was scraped off the plates and the gel was extracted with ethanol. Evaporation gave a residue that was dissolved in water, from which a white solid (**15**; 68 mg) was obtained, mp 173 °C (lit.[19] 176 °C). The IR spectrum was identical with the reference sample **15** supplied by Dr. Gilham. The mobilities on TLC were identical, as were the K_i values with adenosine deaminase (0.9 μM, 1 μM). Dialcohol **15** was also prepared by reduction of a sample of **14** that had been obtained earlier[7] from 9-(5-deoxy-β-D-*erythro*-pent-4-enofuranosyl)adenine, and it was identical with the other two preparations in every way.

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Novel Antitumor Nitrosoureas and Related Compounds and Their Reactions with DNA

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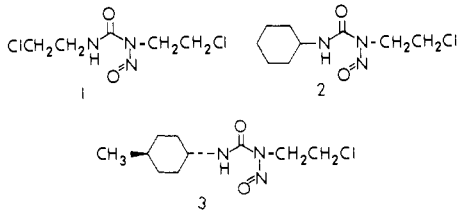
A number of nitrosoureas have been synthesized in order to examine those structural features which lead to optimum interstrand DNA cross-linking as this property may relate to their cytotoxicity and antitumor effects. The compounds include 2-chloroethyl trifluoromethanesulfonate, **5**, 2-chloroethyl *p*-nitrobenzenesulfonate, 2-chloroethyl *p*-toluenesulfonate, and 2-chloroethyl methanesulfonate. Of these compounds, **5**, bearing the most reactive leaving group, cross-links 11% of DNA after 6 h, presumably by way of an intermediate chloroethonium ion. A second group of compounds designed to favor low activation S_N2 displacement by biological nucleophiles included *N*-(2-chloroethyl)-*N*-nitrosoacetamide (**12**), 5-[3-(2-chloroethyl)triazenyl]imidazole-4-carboxamide (**13**), and ethyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**14**) which cross-link DNA to the extent of 44, 33, and 12% in 6 h, respectively. The extent of DNA cross-linking produced by ethyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**14**) is increased in the presence of thiol nucleophiles which favor decomposition to give the alkylating species. This result led to the design of 3,3′-bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl]propyl disulfide (**17**) which after reductive activation undergoes intramolecularly activated decomposition to an electrophile, leading to enhanced rates of DNA cross-linking. Compound **17** is active against leukemia in mice (T/C = 136). Based on the preceding analysis, novel nitrosoureas were synthesized which were designed to produce internally activated alkylating species. The lead compound, 1-[2-[(2-chloroethyl)thio]ethyl]-3-cyclohexyl-1-nitrosourea (**22**), produces 90% interstrand cross-linked DNA in 10 min under physiological conditions and in vivo a percent T/C value of 194 against leukemia L1210 in rodents. Comparison of the reactivity of compound **22** toward DNA with that of the modified nitrosoureas **30–32** supports the interpretation of the high reactivity of **22** being due to side-chain activation by the neighboring sulfur atom.

2-Haloethylnitrosoureas, including BCNU [*N,N*′-bis(2-chloroethyl)-*N*-nitrosourea (**1**)], CCNU [*N*-(2-chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea (**2**)], and MeCCNU [*N*-(2-chloroethyl)-*N*′-(4-methylcyclohexyl)-*N*-nitrosourea (**3**)], are of clinical value in the treatment of a range of



malignant diseases.[1-6] Previous papers in this series have examined various aspects of the chemistry of the nitrosoureas, including aqueous decomposition, alkylation and interstrand cross-linking of DNA (which appear to underlie their molecular mechanism of action), and DNA strand scission.[7-10] The results of several investigations suggest that alkylation and DNA cross-linking by nitrosoureas require the generation of a chloroethyl alkylating agent upon aqueous decomposition.[7,11-14] Chloroethylation of an appropriate base in DNA is followed by labilization of the C–Cl bond and a second alkylation to complete the

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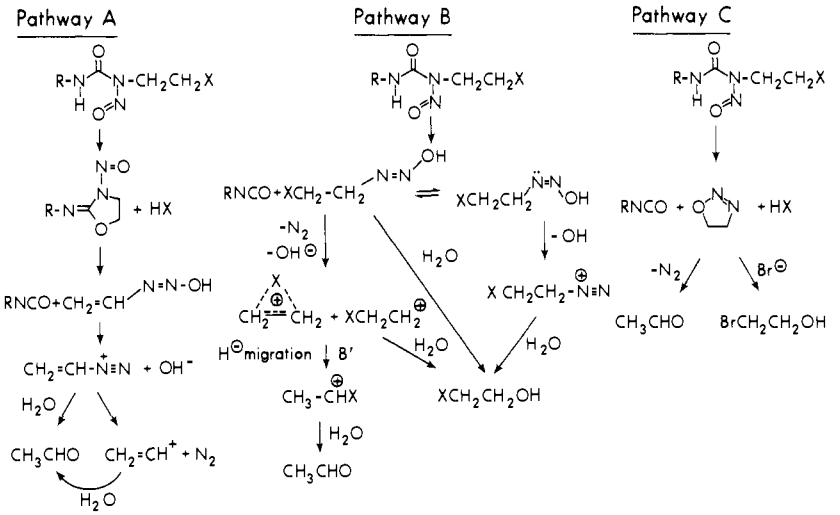
Scheme I



cross-link.[7,10,12] DNA interstrand cross-links are more likely to result in a lethal lesion to the cell than alkylation, since the former is more difficult to repair. These results prompted the present study of those structural features of nitrosoureas which promote DNA cross-linking, leading to the rational design of new antitumor nitrosoureas and further evidence bearing on their molecular mechanism of action. This does not require nor imply that the formation of interstrand cross-links in DNA is the only or principal mechanism of action of 2-haloethylnitrosoureas.

Results and Discussion

Studies Related to Increasing the Efficiency of the Second Alkylation of the Cross-link. Nitrosoureas like BCNU (1) decompose under physiological conditions without enzymatic activation to give rise to isocyanates and 2-haloethyl cationic species, and the latter alkylate and cross-link DNA.[1,4,5] At least three decomposition pathways have been suggested (Scheme I). Evidence for the direct alkylation of nucleic acids includes the isolation from nitrosourea-treated poly(C) of nucleosides bearing additional two-carbon units[12,15] and the preparation from pyrimidines of 2-haloethyl-substituted species which exhibit alkylating ability toward DNA.[10] In addition, it was shown that the interstrand cross-linking reaction takes place by a distinct two-step process.[7] While the second alkylation necessary to complete the cross-link occurs at the carbon bearing the halogen, increasing its ability to act as a leaving group does not appear to increase the efficiency of cross-linking. Haloethylation of nitrogen atoms on, for example, cytosine in DNA would produce intermediate compounds which resemble nitrogen mustards.[12,15] The ability of these intermediates to produce a second alkylation completing the interstrand cross-link should parallel the alkylating ability of nitrogen mustards. The relative alkylating ability of chloro, bromo, and iodo nitrogen mustards has been reported previously[16] using a *p*-nitrobenzylpyridine test for alkylating ability. The relative values for Cl, Br, and I nitrogen mustards are 1.0, 18.6, and 20.2, respectively. By contrast, the extent of cross-linking produced by chloroethyl-, bromoethyl-, and iodoethylnitrosoureas is 43, 8, and <2%, respectively.

A plausible interpretation of this lack of correlation, at least for the case of the bromo and iodo substituents, is that as the group attached to the carbon which normally bears the chlorine atom in 2-chloroethylnitrosoureas increases in leaving ability, competitive decomposition pathways, e.g., involving the substituted 2-imino-*N*-nitrosooxazolidinone (pathway A, Scheme I) and/or the oxadiazoline (pathway C, Scheme I) and/or hydride migration, result in loss of the desired 2-substituted ethyl alkylating agent. Thus, attempts to increase reactivity of nitrosoureas toward their selected cell targets by merely incorporating groups of superior leaving ability are unlikely to be effective.

Thus, while chloride ion is not an exceptional leaving group,[17] its presence in therapeutically active 2-chloroethylnitrosoureas represents an effective compromise. For example, the leaving ability of Cl^- is not sufficiently high to favor exclusively the competing decomposition pathways involving oxadiazolines, imino-*N*-nitrosooxazolidinones, or hydride migrations which result in species other than the desired 2-chloroethyl alkylating species (see Scheme I). On the other hand, the ability of Cl^- to act as a leaving group is such that, after chloroethylation of an appropriate base, labilization of the chloride to substitution and production of interstrand cross-links are still permissable.

Studies Related to the Rate of Production of Chloroethyl Alkylating Species. Previous studies have shown that BCNU produces significant numbers of cross-links in DNA in vitro which reach a maximum after 6–8 h.[7,11] BCNU in a pH 7.2 aqueous buffered solution at 37 °C has a half-life of 79 min.[9] The rate of aqueous decomposition is increased significantly for the unsubstituted derivative 1-(2-chloroethyl)-1-nitrosourea (CNU, **5a**), which under comparable conditions has a half-life of 8 min.[9] CNU produces interstrand cross-links comparable with those of BCNU, which reach a maximum in 4 h.[10] The observation that an increase in the decomposition rate by a factor of 10 only increases the rate of cross-linking by approximately a factor of 2 supports the hypothesis that it is not the decomposition and initial alkylation but rather the rate of the second alkylation by the intermediate chloroethylated base which determines the rate of cross-linking.

Studies Related to Alternative Means of Generating Chloroethyl Alkylating Species. There is a substantial body of data to indicate that 2-chloroethylnitrosoureas owe their antitumor properties to the alkyl-

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Table I. Comparative Reactivities of 2-Chloroethyl and Related Derivatives toward Alkylation of DNA

leaving group ability, nucleophilic displacements in decreasing order:[a] no.	structure (R = $ClCH_2CH_2$-)	compound	rel rate of PM2-DNA alkylation (% alkylation by 5 mM drug in 180 min)
32	$HOCH_2CH_2SCH_2CH_2\overset{+}{N}\equiv N$[b]	1-[2-[(2-hydroxyethyl)thio]ethyl]-3-cyclohexyl-1-nitrosourea	96
31	$ClCH_2CH_2S(O)CH_2CH_2\overset{+}{N}\equiv N$[b]	2-chloroethyl 2-(3-cyclohexyl-1-nitrosoureido)ethyl sulfoxide	84
30	$ClCH_2CH_2OCH_2CH_2\overset{+}{N}\equiv N$[b]	1-[2-(2-chloroethoxy)ethyl]-3-cyclohexyl-1-nitrosourea	66
5a	$R-\overset{+}{N}\equiv N$[b]	1-(2-chloroethyl)-1-nitrosourea	70[c]
5b	$CH_3CH_2\overset{+}{N}\equiv N$	ethyl nitrosourea	53
6	$ROSO_2CF_3$	2-chloroethyl trifluoromethanesulfonate	40[d]
7	$ROSO_2C_6H_4NO_2$-*p*	2-chloroethyl *p*-nitrobenzenesulfonate	0
8	$ROSO_2C_6H_4CH_3$-*p*	2-chloroethyl *p*-toluenesulfonate	0
9	$ROSO_2CH_3$	2-chloroethyl methanesulfonate	0
10	$ROP(OR')_2$	tris(2-chloroethyl) phosphate	0

[a] See ref 23. [b] The diazonium ion or its equivalent as a leaving group is generated by aqueous decomposition of nitrosoureas.[1,4,5] [c] Compound 5a produces 37% interstrand cross-linking of λ-DNA at 32 °C in 6 h. [d] Compound 6 produces 11% interstrand cross-linking of λ-DNA at 37 °C in 6 h.

ating properties of 2-chloroethyl electrophiles produced from aqueous decomposition.[1-6] For this reason, we explored alternative means of generating 2-chloroethyl alkylating species.

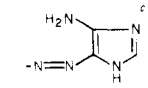
Initial experiments involved the chloroethyl species $ClCH_2CH_2$–L with leaving groups L other than the diazohydroxide or diazonium ion which result from the nitrosoureas. A series of compounds was prepared and their alkylating ability toward DNA compared with that of selected nitrosoureas as shown in Table I. In the series **5–10**, only the trifluoromethanesulfonate derivative **6**, other than the nitrosoureas, exhibited significant alkylation of PM2-CCC-DNA. This derivative also produced 11% cross-linking of DNA after a reaction time of 6 h [by comparison, CNU (**5a**) cross-links 37% λ-DNA in 6 h[10]]. It may be noted, in connection with the results summarized in Table I, that Baker and co-workers tested chloroethyl sulfonates, chloroethylnitrosoacetamides, and chloroethyl nitrosocarbamates closely related to **7**, **8** and **9** and found them to exhibit little or no antitumor activity.[22]

Reutov et al. have reported that the acetolysis of 2-chloroethyl *p*-nitrobenzenesulfonate (**7**) at elevated temperatures results in products which suggest some participation by a cyclic chloronium ion.[18,19] While this observation indicates that the nosylate **7** generates a reactive species which could lead to DNA cross-linking, no such activity was observed at pH 7.2 and 37 °C.

Chloroethylnitrosoureas appear to alkylate by an S_N1 or low activation energy S_N2 process.[1,7,13] Therefore, a second series of compounds was prepared which could result in 2-chloroethylcarbonium ions, 2-chloroethyldiazonium ions, or similar alkylating species by such mechanisms. This series included 1-(2-chloroethyl)-3-nitro-1-nitrosoguanidine (**11**),[20] *N*-(2-chloroethyl)-*N*-nitrosoacetamide (**12**), 5-[3-(2-chloroethyl)triazenyl]imidazole-4-carboxamide (**13**),[21] ethyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**14**), and *N*-(2-chloroethyl)-*N*-nitroso-*p*-toluenesulfonamide (**15**).[22]

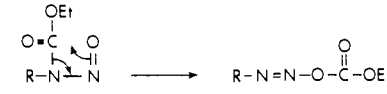
Table II. Polarographic Behavior and Relative Rates of Aqueous Decomposition of 2-Chloroethyl Derivatives

$ClCH_2CH_2\overset{NO}{\overset{|}{N}}-R$

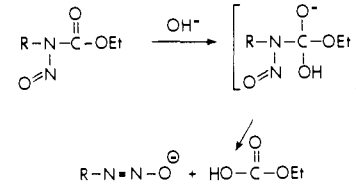
no.	R	$E_{1/2}$, 1	$E_{1/2}$, 2	$t_{1/2}$, min[d]
5a	$-CONH_2$	−0.752	−1.010	8 ± 2
11	$-C(NH)NHNO_2$[b]	−0.543	−1.142	stable
12	$-COCH_3$[b]	−0.652	−1.152	51 ± 5
13[a]		−0.998		5 ± 2
14	$-COOCH_2CH_3$	−0.653	−1.073	81 ± 8
15	$-SO_2C_6H_4CH_3$-*p*[b]	−0.038	−1.042	stable

[a] For structure 13 substitute H for NO. [b] 5% ethanol. [c] 5% dimethyl sulfoxide. [d] Time required to effect 50% decomposition.

Scheme II



Scheme III



These compounds were assayed for their aqueous stability and ability to produce DNA interstrand cross-links. Polarographic analysis was used to determine the stabilities of these derivatives at 37 °C in aqueous solution buffered to pH 7.1 (Table II). The ability to produce DNA interstrand cross-links was determined by the ethidium fluorescence assay and compared with their antileukemic

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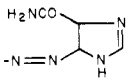
(22) K. A. Hyde, E. Acton, W. A. Skinner, L. Goodman, J. Greenberg, and B. R. Baker, *J. Med. Pharm. Chem.*, **5**, 1 (1962).

Table III. Antitumor Activity of 2-Chloroethyl Derivatives against Leukemia 1210[a]

$ClCH_2CH_2\overset{NO}{\overset{|}{N}}-R$

no.	R	% interstrand DNA cross-linking at 6 h[b]	dose, (mg/kg)/day	change in wt (g) day 0 to 5, treated/control	survivors on day 5	mean survival time/days, treated/control	% T/C	ref
5a	$-CONH_2$	37	1.3	−2.9/0.3	6/6	25.3/9.6	263	22
11	$-C(NH)NHNO_2$	0	20	−0.8/0.6	6/6	11.3/7.1	159	36
12	$-COCH_3$	44						
13[c]		33	6	−1.6/0.4	6/6	22.4/9.9	226	21
14	$-COOCH_2CH_3$	12	0.9	−1.4/0.4	6/6	15.6/11.1	140	22
15	$-SO_2C_6H_4CH_3$-*p*	0	1	−0.4/0.0	6/6	12.3/10.5	117	22
22		90	32	−1.5/0.5	6/6	16.7/8.6	194	*d*
30		0	200	−4.4/1.4	6/6	14.9/9.8	152	*d*
31		0	100	−2.2/1.4	6/6	15.4/9.7	159	*d*
32		0	200	−1.1/1.4	6/6	16.4/16.9	110	*d*

[a] Assays for activity against lymphoid leukemia L1210 performed according to specifications established by the Cancer Chemotherapy National Service Center.[37] Suspensions of the compounds in saline and Tween 80 were administered intraperitoneally within 5 min of preparation of suspensions. L1210 cells (10^5) were implanted intraperitoneally in mice on day 0. Average weight change of treated mice minus average weight change of control mice by doses. Compounds 5a and 13 are curative for L1210. [b] This term means the percentage in a given sample of the DNA molecules that contain at least one interstrand cross-link. [c] For structure 13 substitute H for NO. [d] Present work.

Table IV. Effects of Inter- and Intramolecular Nucleophilic Catalyzed Decomposition of Nitroso Compounds on Their DNA Cross-linking Properties

no.	compd	% interstrand cross-linking at 6 h[a]
14	5 mM ethyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate	12
	+ 15 mM 2-mercaptoethanol	24
	+ 15 mM dithiothreitol	28
17	2.5 mM 3,3′-bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl]propyl disulfide	8
	+ 15 mM dithiothreitol	25
	+ 25 mM sodium dithionite	21

[a] Reactions performed in 40 mM potassium phosphate buffer, pH 7.2, at 37 °C (see Experimental Section). As defined, this gives the percentage in a given sample of the DNA molecules that contain at least one interstrand link.

activities (Table III). Neither the nitroguanidine nor the *p*-toluenesulfonamide derivatives show the ability to produce DNA cross-links, which is in accord with their observed stability in aqueous buffer.

We next attempted to modify a derivative which exhibits a low extent of DNA cross-linking in an attempt to increase its cross-linking efficiency. The derivative chosen for modification was ethyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**14**).

March has suggested[23] that the first step in the decomposition of similar nitrosocarbamates is a 1,3-nitrogen to oxygen rearrangement (Scheme II). Nitrosocarbamate decomposition is base catalyzed which Smith has suggested[24] involves a nucleophilic attack by hydroxide ion at the carbonyl liberating a carbonic ester and a diazotate (Scheme III). While a slow 1,3-nitrogen to oxygen shift may explain the decomposition of ethyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**14**) under physiological conditions, increasing the possibility of nucleophilic attack at the carbonyl group in a pH 7.2 buffered solution should increase the rate of decomposition to give the 2-chloroethyl cation and subsequent cross-linking.

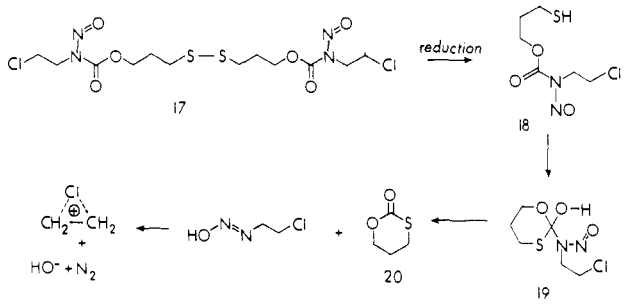
To increase the nucleophilic character of the reaction mixture, DNA interstrand cross-linking for ethyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (**14**) was determined in the presence of 2-mercaptoethanol or dithiothreitol. In both experiments it was conceivable that the thiol compound would compete favorably with the DNA for the chloroethyl alkylating moiety. In the event, in both experiments, as shown in Table IV, the extent of DNA cross-linking increased significantly.

Encouraged by these results, a compound was now designed which could result in similar but intramolecular nucleophilic activation of the nitrosocarbamate to produce the desired alkylating species. The desired modified nitrosocarbamate is shown in Scheme IV.

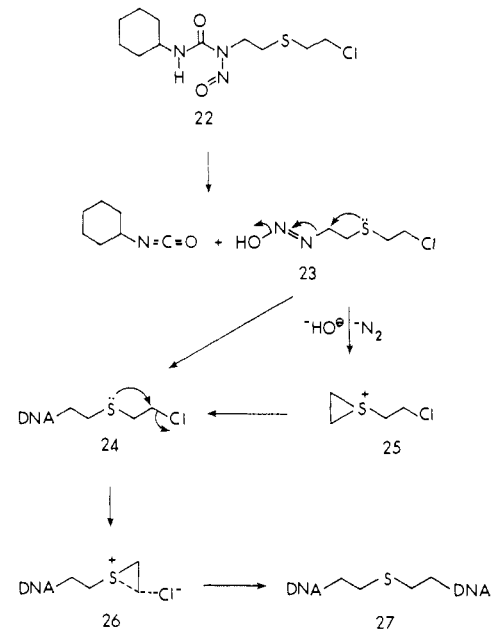
Reaction of 3-hydroxypropyl disulfide with 2-chloroethyl isocyanate afforded the biscarbamate **16**. Nitrosation with sodium nitrite and HCl resulted in 3,3′-bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl]propyl disulfide (**17**). Reduction of the disulfide **17** in situ could produce the thiol derivative **18**, which can interact intramolecularly or intermolecularly with the carbamate carbonyl. Intramolecular attack would result in the intermediate **19** shown in Scheme IV. Proton transfer in the intermediate yields 2-chloroethyl diazohydroxide and 1,3-oxathian-2-one (**20**). The results of the ethidium fluorimetric assay for DNA interstrand cross-linking by this compound are shown in Table IV. It may be seen that addition of either reductant increased the extent of cross-linking compared with the

(23) J. A. March, in "Advanced Organic Chemistry: Reactions, Mechanisms and Structure", McGraw-Hill, New York, 1977, p 956.
(24) P. A. S. Smith, "Open Chain Nitrogen Compounds", Vol. 2, W. A. Benjamin, New York, 1966, p 256.

Scheme IV



Scheme V

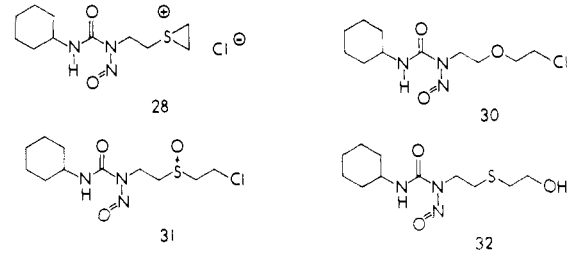


parent disulfide. To confirm that some of the reduced disulfide undergoes intramolecular cyclization during the release of the chloroethyl alkylating agent, an attempt was made to isolate the 1,3-oxathian-2-one. A solution of 3,3′-bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl]propyl disulfide (**17**) was allowed to decompose in a 200 mM, pH 7.2, potassium phosphate buffer at 37 °C in the presence of sodium dithionite. Extraction of the aqueous solution with ether gave a mixture of compounds. Gas chromatographic separation combined with high-resolution mass spectral analysis confirmed the presence of a compound with the molecular formula corresponding to the 1,3-oxathian-2-one. Compound **17** is active against standard leukemia, T/C = 136 on a 1–9 day schedule in mice (Table III).

Studies Related to Nitrosoureas Which Produce Internally Activated Alkylating Species. With the discovery that 2-chloroethylnitrosoureas exhibit high activity in the leukemia L1210 test system, a wide variety of derivatives has been synthesized for biological testing.[1,3,4,6] Replacement of the chlorine atom with halogens other than fluorine, as well as chain lengthening or chain branching, has generally resulted in a lowering or loss of activity.[1,3,4] Consequently, most of the synthetic work in this area has been concentrated on the modification of the substituent which produces the isocyanate moiety upon decomposition by pathway B which, however, is not directly involved in the expression of antitumor properties. A different approach was made to modify the alkylating portion of the molecule to increase the extent of DNA alkylation and cross-linking. The rationale of this approach is to incorporate selective anchimeric assistance in the electrophile released by decomposition pathway B (Scheme I).

This type of compound incorporated five design features: (i) The compound should inhibit decomposition pathways involving either the nitrosooxazolidinone (Scheme I, pathway A) or oxadiazoline (Scheme I, pathway B), thus more efficiently producing an electrophilic species capable of cross-linking. (ii) In accord with point (i), the substituted group should be chloride as a compromise in leaving ability between S_N2 reactivity toward DNA nucleophiles and low reactivity in cyclization. (iii) The intermediate carbonium ion should be stabilized so as to promote its formation while permitting subsequent attack by biological nucleophiles. (iv) Substitution of the chloride necessary for the second alkylation to complete the cross-link should be activated by the drug itself so that cross-linking will not be as dependent on the initial site of alkylation. (v) The distance between the two alkylation sites can be greater than the two-carbon link provided by 2-chloroethylnitrosoureas in case the latter interaction is too restrictive.

The first compound synthesized based on these design features was 1-[2-[(2-chloroethyl)thio]ethyl]-3-cyclohexyl-1-nitrosourea (**22**), prepared via the corresponding urea **21**. This nitrosourea derivative produces 90% cross-linked λ-DNA in 10 min at pH 7.2 and 37 °C. Compound **22** also showed a significant antileukemic effect of T/C = 194 against L1210 (see Table III). Decomposition, alkylation, and cross-linking by **22** can be envisaged to occur as outlined in Scheme V. Initial DNA alkylation can occur by S_N2 reaction with the diazohydroxide **23** or by alkylation of the sulfonium ion **25** produced by S_N1 elimination of nitrogen and hydroxide. The second alkylation may result from the reactive sulfur half-mustard **24**, presumably through the episulfonium ion **26**.[25] Conversely, due to labilization of the C–Cl bond (e.g., in the formation of the sulfonium ion **28**), the initial alkylation



may result from the sulfur half-mustard portion of the molecule, followed by subsequent decomposition and alkylation by the nitrosourea already attached to its cell target. This interpretation of the in vivo and in vitro activity of **22** was tested by the synthesis and an examination of the reactivity of the modified structures **30**, **31**, and **32**. Whereas both **30** and **31** readily alkylate PM2-DNA (to the extent of 66 and 85% in 3 h, respectively; see Table I), neither compound is able to cross-link DNA. The neighboring group effect of oxygen is less than that of sulfur[26,27] and that of the sulfur atom in **31** is removed, since the lone pair is bound up by the S–O group. Consequently, whereas the nitrosourea moiety in both **30** and **31** can undergo unassisted decomosition to generate an

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electrophile as in pathway B (Scheme I) and therefore alkylate DNA, the required anchimeric assistance to activate the second site, thereby completing the cross-link, is not available. This may be reflected in their observed lower in vivo activity against standard leukemia (T/C of 152 and 159, respectively, see Table III) compared with **22** of T/C = 194.

The chemical rationale for the reactivity of **22** was further tested with compound **32**. This compound, like **30** and **31**, readily alkylates DNA under physiological conditions, 96% in 60 min (Table I), but fails to cross-link DNA. In this case, whereas anchimeric assistance is available from the sulfur to permit initial alkylation, the leaving ability of the -OH group on the alkylated nucleoside is not sufficient, even with assistance from the sulfur atom, to permit completion of the interstrand cross-link. It may be noted that, in contrast to **22**, **30**, and **31**, compound **32** was found to be inactive against leukemia in mice.

The synthesis and reactions with DNA of further examples of "side-chain activated" nitrosoureas like **22** will be reported in a subsequent paper.

Experimental Section

Throughout this work, melting points were determined on a Fisher-Johns apparatus and are uncorrected. The IR spectra were recorded on a Nicolet 7199 FT spectrophotometer, and only the principal, sharply defined peaks are reported. The NMR spectra were recorded on Bruker 200 and 400 and Varian HA-100 analytical spectrometers. The spectra were measured on approximately 10–15% (w/v) solutions in appropriate deuterated solvents with tetramethylsilane as standard. Line positions are reported in parts per million from the reference. Mass spectra were determined on an Associated Electrical Industries MS-9 double-focusing high-resolution mass spectrometer. The ionization energy, in general, was 70 eV. Peak measurements were made by comparison with perfluorotributylamine at a resolving power of 15000. Kieselgel DF-5 (Camag, Switzerland) and Eastman Kodak precoated sheets were used for thin-layer chromatography. Microanalyses were carried out by D. Mahlow of this department. In the workup procedures reported for the various syntheses described, solvents were removed with a rotary evaporator under reduced pressure unless otherwise stated.

Materials. Ethidium bromide was purchased from Sigma Chemical Co., St Louis, MO., and λ-DNA (M_r 31 × 10^6) was obtained from Miles Biochemicals, Miles Laboratories, Inc., Elkhart, Indiana. PM2-CCC (covalently closed circular) 87% CCC-DNA was obtained as described previously.[28]

2-Chloroethyl *p*-toluenesulfonate (**8**) was purchased from Eastman, and 2-chloroethyl methanesulfonate (**9**) and tris(2-chloroethyl) phosphate (**10**) were from Aldrich. Compounds prepared in this laboratory are described in the following paragraphs.

The N_2O_4 used in this work was prepared by condensation of nitrogen dioxide in an appropriate flask. Oxygen was bubbled through the liquid to oxidize nitrous oxide impurities. The liquid was then distilled from P_2O_5 and stored in a sealed container at −78 °C when not in use.

2-Chloroethyl Trifluoromethanesulfonate (6). A mixture of trifluoromethanesulfonic anhydride (**4**; 6.5 g, 23 mmol) and 2-chloroethanol (1.85 g, 23 mmol) was carefully protected from moisture and heated at 60–70 °C for 30 min. The reaction mixture was cooled and then fractionated under reduced pressure, with the main fraction distilling at 58–59 °C (14 mm). The resulting 2-chloroethyl trifluoromethanesulfonate is extremely water sensitive and fumes readily in moist air: ^{1}H NMR (neat) δ 3.26 (t, 2 H, CH_2), 4.21 (t, 2 H, CH_2); IR ν_{max} (film) 1410, 1140 (SO_2), 1200 cm^{-1} (C–F). MS calcd for $C_3H_4ClF_3O_3S$: 176.9833 (M^+ − Cl), 162.9676 (M^+ − CH_2Cl), 142.9562 (M^+ − CF_3). Found: 176.9863, 162.9676, 142.9566.

2-Chloroethyl *p*-Nitrobenzenesulfonate (7). This compound was prepared according to the method of Reutov et al.[18] and isolated as white needles from benzene–cyclohexane: yield 2.4 g (78%); mp 100–101 °C, lit.[18] mp 102–103 °C; ^{1}H NMR ($CDCl_3$) δ 3.7 (t, 2 H, CH_2), 4.4 (t, 2 H, CH_2), 8.1 (d, 2 H, Ar H), 8.4 (d, 2 H, Ar H).

***N*-(2-Chloroethyl)-*N'*-nitro-*N*-nitrosoguanidine (11).** This compound was prepared according to the method of McKay and Milks:[20] yield 320 mg (61%); mp 94–96 °C, lit.[20] 96 °C; ^{1}H NMR (Me_2SO-d_6) δ 3.5 (t, 2 H, CH_2), 4.2 (t, 2 H, CH_2), 7.9 (s, 1 H, exchangeable), 8.6 (s, 1 H, exchangeable).

***N*-(2-Chloroethyl)-*N*-nitrosoacetamide (12).** To *N*-(2-chloroethyl)acetamide (1.5 g, 12 mmol)[29] in 20 mL of ether containing a suspension of sodium bicarbonate (1.7 g, 20 mmol) at −30 °C was added 2.0 g (20 mmol) of N_2O_4 in 5 mL of ether dropwise with a syringe. The mixture stirred for 1.5 h at less than −20 °C and was then poured into 50 mL of 10% sodium bicarbonate. The ether layer was washed twice with water and dried ($MgSO_4$), and the solvent was removed, resulting in a dark yellow oil: yield 1.2 g (66%): ^{1}H NMR ($CDCl_3$) δ 2.80 (s, 3 H, CH_3), 3.45 (t, 2 H, CH_2), 4.10 (t, 2 H, CH_2); IR ν_{max} ($CHCl_3$) 1730 (C=O), 1510 (N=O) cm^{-1}. Anal. ($C_4H_7N_2O_2Cl$) C, H, N. M_r 150.0197; MS 150.0201.

5-[3-(2-Chloroethyl)triazenyl]imidazole-4-carboxamide (13). This compound was prepared according to the method of Shealy et al.[21] and isolated as an off white solid: yield 45 mg (~50%); mp 111–113 °C, lit.[21] 114 °C; IR ν_{max} (Nujol) 3450, 3050, 1635, 1585, 1420.

Ethyl *N*-(2-Chloroethyl)-*N*-nitrosocarbamate (14). To ethyl *N*-(2-chloroethyl)carbamate (1.5 g, 8 mmol)[30] in 20 mL of ether containing a suspension of sodium bicarbonate (1.7 g, 20 mmol) at −30 °C was added N_2O_4 (2.0 g, 20 mmol) in 5 mL of ether dropwise with a syringe. The mixture stirred for 1.5 h at less than −20 °C and was then poured into 50 mL of 10% sodium bicarbonate. The ether layer was washed twice with water and dried ($MgSO_4$), and the solvent was removed, resulting in a pale yellow oil: yield 1.16 g (66%); ^{1}H NMR ($CDCl_3$) δ 1.45 (t, 3 H, CH_3), 3.45 (t, 2 H, CH_2), 4.1 (t, 2 H, CH_2), 4.55 (q, 2 H, CH_2); IR ν_{max} ($CHCl_3$) 1750 (C=O), 1520 (N=O) cm^{-1}. Anal. ($C_5H_9N_2O_3Cl$) C, H, N. M_r 180.0302; MS 180.0309

***N*-(2-Chloroethyl)-*N*-nitroso-*p*-toluenesulfonamide (15).** This compound was prepared according to the method of Goodman et al.:[22] yield 240 mg (52%); mp 49–50 °C, lit.[22] 49–50 °C; ^{1}H NMR ($CDCl_3$) δ 2.4 (s, 3 H, CH_3), 3.4 (t, 2 H, CH_2), 4.0 (t, 2 H, CH_2), 7.3 (d, 2 H, Ar H), 7.8 (d, 2 H, Ar H).

3,3'-Bis[*N*-(2-Chloroethyl)carbamoyl]propyl Disulfide (16). 3-Hydroxypropyl disulfide[31] (2.8 g, 15.4 mmol) and 2-chloroethyl isocyanate (3.25 g, 30.8 mmol) were refluxed in ether (75 mL) for 24 h. Most of the ether was removed in vacuo and the residual oil solidified on standing. The solid was collected and washed several times with a little ice-cold ether. Recrystallization from chloroform–petroleum ether gave 3,3'-bis[*N*-(2-chloroethyl)carbamoyl]propyl disulfide (**16**) as a colorless solid: yield 4.2 g (70%); mp 82–84 °C; ^{1}H NMR ($CDCl_3$) δ 2.03 (quintet, 4 H, J = 7 Hz, 2-CH_2), 2.75 (t, 4 H, J = 7 Hz, 2-CH_2S), 3.46–3.66 (m, 8 H, 2 × NCH_2CH_2Cl), 4.18 (t, 4 H, J = 7 Hz, 2-CH_2O); IR ν_{max} ($CHCl_3$) 3500 (NH), 1735 cm^{-1} (CO). Anal. ($C_{12}H_{22}Cl_2N_2O_4S_2$) C, H, N, Cl, S. M_r 392.0390; MS 392.0389.

3,3'-Bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl]propyl Disulfide (17). To a stirred mixture of 3,3'-bis[*N*-(2-chloroethyl)carbamoyl]propyl disulfide (**16**; 0.196 g, 0.5 mmol) in chloroform (10 mL) and 1 N hydrochloric acid (25 mL) at 0 °C was added sodium nitrite (0.7 g, 10 mmol) in portions over a period of 3 h. The mixture was stirred at 0 °C for a further 3 h. The chloroform layer was removed, dried (Na_2SO_4), and evaporated in vacuo. Preparative layer chromatography of the residual oil on silica gel ($CHCl_3$) gave the dinitroso compound, yield 0.175 g (78%), as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 2.24 (quintet, 4 H, J = 7 Hz, 2 × CH_2), 2.82 (t, 4 H, J = 7 Hz, 2 × -CH_2S), 3.47 (t,

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Table V. Mass Spectral Data for Compound 20

m/e measured	m/e calcd	rel intensity	fragment
118.0085	118.0089	1.05	$C_4H_6O_2S$ (M^+)
74.0196	74.0190	100.00	C_3H_6S ($M^+ - CO_2$)
58.0435	58.0418	13.03	C_3H_6O ($M^+ - COS$)

4 H, J = 7 Hz, 2 × CH_2Cl), 4.10 (t, 4 H, J = 7 Hz, 2 × CH_2O), 4.62 (t, 4 H, J = 7 Hz, 2 × $-CH_2N$); IR ν_{max} ($CHCl_3$) 1760 (C=O), 1510 cm^{-1} (NO). Anal. ($C_{12}H_{20}Cl_2N_4O_6S_2$) C, H, N, Cl. M_r 450.0201; MS 450.0209.

Detection of 1,3-Oxathian-2-one (20). A 3.0-mL solution was prepared containing 150 mM 3,3′-bis[*N*-(2-chloroethyl)-*N*-nitrosocarbamoyl]propyl disulfide, 200 mM potassium phosphate (pH 7.3), and 0.5 M sodium dithionite in a 20% acetonitrile aqueous solution which was incubated at 37 °C for 6 h. Extraction of the aqueous mixture with ether, drying ($MgSO_4$), and removal of the solvent resulted in approximately 5 mg of a colorless liquid. Although not analytically pure, the mass spectral characteristics are as shown in Table V.

1-[2-[(2-Chloroethyl)thio]ethyl]-3-cyclohexyl-1-nitrosourea (22). Compound **22** was prepared as a yellow oil as described elsewhere.[7]

1-[2-(2-Chloroethoxy)ethyl]-3-cyclohexyl-1-nitrosourea (30). Diethylene glycolamine (1.0 g, 10 mmol) in 50 mL of 1,2-dichloroethane was saturated with HCl. The solution was cooled and thionyl chloride (2.0 mL, 27 mmol) was added. The mixture was slowly warmed to room temperature and then heated at 60 °C for 1 h. Cooling of the solution and addition of 25 mL of ether resulted in a white solid, which could be recrystallized from ethanol/ether. The white crystals were hygroscopic and difficult to prepare in analytically pure form, but the recrystallized 2-(2-chloroethoxy)ethylamine hydrochloride was found suitable for the next step.

To the hydrochloride salt (500 mg, 3 mmol) and cyclohexyl isocyanate (400 mg, 3 mmol) in 50 mL of chloroform at 0 °C was added triethylamine (300 mg, 3 mmol) during a 30-min period. After stirring for 2 h at 0 °C, the solution was warmed to room temperature and stirred for another 2 h. Removal of the solvent resulted in a white solid, which was suspended in water and stirred to remove hydrochloride salts. The remaining white solid **29** was filtered and taken up in chloroform, dried, and crystallized by adding petroleum ether: yield 480 mg (65%); mp 82–84 °C; ^1H NMR ($CDCl_3$) δ 1.0–2.1 (m, 10 H, CH_2), 3.3–3.8 (m, 9 H, CH_2), 4.5 (d, 1 H, exchangeable), 4.8 (t, 1 H, exchangeable); IR ν_{max} ($CHCl_3$) 3200 (N-H), 1630 (C=O) cm^{-1}. Anal. ($C_{11}H_{21}ClN_2O_2$) C, H, N. M_r 248.1291; MS 248.1305.

To the urea (100 mg, 0.4 mmol) in 1 mL of 98% formic acid at 0 °C was added during 20 min sodium nitrite (100 mg, 1.4 mmol). After the addition, the mixture was stirred for 1 h at 0 °C and then 5 mL of H_2O was cautiously added. The aqueous mixture was extracted with chloroform, washed, and dried ($MgSO_4$), and the chloroform was removed to yield 60 mg (54%) of a yellow oil, **30**, which was difficult to purify: ^1H NMR ($CDCl_3$) δ 1.0–2.2 (m, 10 H, CH_2), 3.5 (m, 6 H, CH_2), 3.9 (m, 1 H, CH), 4.1 (t, 2 H, CH_2); IR ν_{max} ($CHCl_3$) 3400 (N-H), 1720 (C=O), 1520 (N=O) cm^{-1}. Anal. ($C_{11}H_{20}ClN_3O_3$) C, H, N.

Although an acceptable measurement could be made for the parent compound, the high-resolution mass spectrum was characterized by a tendency to undergo proton transfer to form cyclohexyl isocyanate and the appropriate diazohydroxide. For mass spectral data, see Table VI.

2-Chloroethyl 2-(3-Cyclohexyl-1-nitrosoureido)ethyl Sulfoxide (31). To a stirred solution of 1-[2-[(2-chloroethyl)-thio]ethyl]-3-cyclohexylurea (**21**; 1.323 g, 5 mmol) in chloroform (50 mL) at 0 °C was added *m*-chloroperbenzoic acid (0.889 g, 5.15 mmol) in portions over a period of 5 min. The mixture was stirred at 0 °C for another 30 min. The solution was washed with 10% sodium bicarbonate solution (25 mL), dried (Na_2SO_4), and evaporated in vacuo. The resulting oil solidified on standing. Recrystallization from ethyl acetate–petroleum ether gave the sulfoxide as an off-white solid: yield 1.233 g (88%); mp 127–128 °C; IR ($CHCl_3$) 3430, 3420 (NH), 1667 cm^{-1} (C=O); ^1H NMR ($CDCl_3$) δ 1.04–1.96 (m, 10, 5 × $-CH_2-$), 2.84–3.24 (m, 4, 2 × $-CH_2S$), 3.47 (m, 1, -CHN), 3.6–3.81 (m, 2, $-CH_2N$), 3.86–4.0 (m, 2, $-CH_2Cl$), 4.70, 5.23 (2 br s, 2, 2 × -NH-). Anal. ($C_{11}H_{20}N_3SO_3Cl$) C, H, N, S, Cl.

Table VI.

m/e measured	m/e calcd	% rel intensity	fragment
277.1191	277.1193	1	M^+
150.0350	150.0352	24	$ClCH_2CH_2OCH_2CH_2N_2OH^+$
125.0822	125.0840	2	$C_6H_{11}NCO^+$
109.0207	109.0235	31	$ClCH_2CH_2OCH_2CH_2^+$
93.0100	93.0107	21	$ClCH_2CH_2OCH_2^+$
83.0873	83.0861	100	$C_6H_{11}^+$

To a stirred solution of the above sulfoxide (0.840 g, 3 mmol) in 97% formic acid (7 mL) at 0 °C was added sodium nitrite (0.828 g, 12 mmol) in portions over a period of 3 h. The mixture was kept at 0 °C for 16 h, pured into ice-cold water (20 mL), and extracted with chloroform (3 × 20 mL). The extracts were dried (Na_2SO_4) and evaporated in vacuo to get compound **31** as a white solid: yield 750 mg (chloroform–ether) (81%); mp 41–42 °C; ^1H NMR δ ($CDCl_3$) 1.16–2.10 (m, 10 H, 5 × $-CH_2-$), 2.88 (t, 2 H, J = 7 Hz, $-CH_2S$), 3.14 (m, 2 H, $-CH_2S-$), 3.82–4.0 (m, 3 H, $-CH_2Cl$, -CHN), 4.18–4.36 (m, 2 H, $-CH_2N$), 6.81 (d, 1 H, J = 7 Hz, NH); IR ($CHCl_3$) 3430 (NH), 1730 (C=O), 1485 cm^{-1} (N=O).

1-[2-[(2-Hydroxyethyl)thio]ethyl]-3-cyclohexyl-1-nitrosourea (32). A solution of cyclohexyl isocyanate (1.875 g, 15 mmol) and 2-[(2-hydroxyethyl)thio]ethylamine (1.815 g, 15 mmol) in dichloromethane (20 mL) was stirred at room temperature for 16 h. The solvent was removed in vacuo. Trituration of the resulting oil with ethyl acetate–ether gave 1-[2-[(2-hydroxyethyl)thio]ethyl]-3-cyclohexylurea as a colorless solid: yield 3.57 g (92%); mp 71–72 °C; IR ($CHCl_3$) 3470 (NH), 3400 (OH), 1665 cm^{-1} (C=O); ^1H NMR ($CDCl_3$) δ 1.04–1.96 (m, 10, 5 × $-CH_2-$), 2.69, 2.75 (2 t, 4, J = 6 Hz, 2 × $-CH_2S$), 3.11 (br s, 1, -OH), 3.39 (q, 2, J = 6.5 Hz, $-CH_2N$), 3.49 (m, 1, -CHN), 3.76 (t, 2, J = 6 Hz, $-CH_2O$), 4.67 (d, 1, J = 6 Hz, CHNH), 5.14 (t, 1, J = 6 Hz, CH_2NH). Anal. ($C_{11}H_{22}N_2O_2S$) C, H, N, S.

To a stirred mixture of the urea (0.246 g, 1 mmol), chloroform (6 mL) and 1 N hydrochloric acid (15 mL) at 0 °C was added sodium nitrite (0.7 g, 10 mmol) in portions over a period of 3 h. The mixture was kept at 0 °C for another 16 h. Usual workup and preparative layer chromatography on silica gel (chloroform) gave the nitrosourea as an oil: yield 190 mg (69%); IR ($CHCl_3$) 3460 (N-H), 1732 (C=O), 1485 cm^{-1} (N=O); ^1H NMR ($CDCl_3$) δ 1.08–2.1 (m, 10, 5 × $-CH_2-$), 2.54, 2.76 (2 t, 4, J = 6 Hz, 2 × $-CH_2S$), 3.74 (t, 2, J = 6 Hz, $-CH_2O$), 3.84 (m, 1, -CHN), 4.02 (t, 2, J = 6 Hz, $-CH_2N$), 6.80 (d, 1, J = 6 Hz, NH). Anal. ($C_{11}H_{21}N_3O_3S$) C, H, N, S.

Polarographic Determination of Decomposition Rates for Nitrosoureas. The Princeton Applied Research (PAR) Model 174A polarograph and 9300-9301 polarographic cell were used in a three-electrode configuration, which included an aqueous saturated calomel reference electrode (SCE), to which all potentials in this paper are relative, a platinum counter electrode, and a dropping mercury electrode (DME) with a controlled 2-s drop time. The temperature in the cell was maintained at 37.5 ± 0.2 °C by circulation of thermostatted water unless otherwise indicated. The resulting curves were recorded on a Houston 200 X-Y recorder. The sample solutions were buffered at pH 7.1 with 0.01 M potassium phosphate buffer in 0.01 M KCl supporting electrolyte. The pH value of the sample solutions were measured with an Accumet Model 520 pH meter before each run.

For compounds which showed extremely low solubility in aqueous solution, 5% acetonitrile aqueous solution was used; in some cases, differential pulse polarography of the aqueous solution was sufficiently sensitive and this was used whenever possible. All solutions were deaerated with purified nitrogen for 10 min before a run and blanketed with it during the run. Rates of decomposition of nitrosoureas were determined by measuring the rate of decrease of the amplitude of the polarographic wave as a function of time.[9]

Fluorescence Assay for Determining CLC Sequences in DNA Produced by Nitrosoureas. All measurements were performed on a G. K. Turner and Associates Model 430 spec-

trofluorometer equipped with a cooling fan to minimize fluctuations in the xenon lamp source. Wavelength calibration was performed as described in the manual for the instrument. One-centimeter-square cuvettes were used. The excitation wavelength was 525 nm and the emission wavelength was 600 nm. The 100× scale of medium sensitivity was generally used, and water was circulated between the cell compartment and a thermally regulated bath at 22 °C. The fluorometric method to detect CLC (covalently linked complementary) sequences in λ-DNA has been described. Cross-linking of DNA creates a nucleation site which allows renaturation of λ-DNA after heat denaturation (96 °C/3 min) and rapid cooling and thus provides intercalation sites for ethidium.[33,34] That this assay procedure detects the formation of CLC-DNA as a result of a chemical cross-linking event has been confirmed by experiments with the enzyme S_1-endonuclease.[34] This enzyme specifically cleaves single-stranded DNA and is essentially inactive on duplex DNA; therefore, it distinguishes DNA which is renaturable by virtue of a chemical cross-link and DNA which separates into single strands on heating. A 20-μL aliquot was taken at intervals from the reaction mixture (50 mM potassium phosphate, pH 7.2; 1.0 A_{260} unit of λ-DNA; 5 mM nitrosourea; total volume 200 μL) at 37 °C and added to the standard assay mixture (which was 20 mM potassium phosphate, pH 11.8; 0.4 mM EDTA; and 0.5 μg/mL of ethidium). The fluorescence after the heating and cooling cycle compared with control times 100 gives the percentage of CLC-DNA, i.e., DNA molecules containing at least one cross-link in a sample. For a standard set of conditions (i.e., type and concentration of DNA, pH, ionic strength, and the temperature), the accuracy of the CLC assay is determined by the precision of the fluorescence readings. Overall accuracy of the CLC assay is estimated at ±2%.

Fluorescence Determination of Alkylation of PM2-CCC-DNA by Nitrosoureas. A 20-μL aliquot was taken at intervals from the reaction mixture [50 mM potassium phosphate, pH 7.2; 1.2 A_{260} units of PM2-CCC-DNA (90% CCC); 5 mM nitrosourea or other alkylating agent in a total volume of 200 μL at 37 °C] and added to the standard assay mixture (which was 20 mM potassium phosphate, pH 11.8; 0.4 mM EDTA; and 0.5 μg/mL of ethidium). The fluorescence after heating at 96 °C/4 min, followed by rapid cooling, was compared with the initial value.

Under these conditions, unreacted PM2-CCC-DNA returns to register after heat denaturation because of topological constraints. Alkylated PM2-CCC-DNA shows a decrease in fluorescence because of thermally induced depurination followed by alkaline strand scission of the apurinic site in the assay medium.[35] The ratio of the decrease in fluorescence (after the heating and cooling cycle) to that of the control is a measure of the extent of alkylation. In a control experiment it was shown that none of the components interfered with the ethidium fluorescence. Provided no single-strand cleavage of PM2-CCC-DNA is observed (detected by the characteristic rise in fluorescence before the heating/cooling cycle), this technique can be used to measure levels of alkylation not readily observed using λ-DNA.

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Potential Prophylactic Antitumor Activity of Retinylidene 1,3-Diketones

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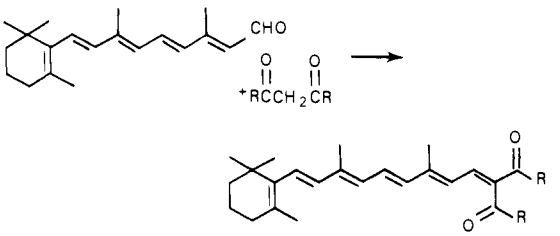
Treatment of *all-trans*-retinal with a series of 1,3-diketones using Knoevenagel conditions gave the expected condensation products. These retinylidene 1,3-diketones were characterized and their biological activities in the hamster tracheal organ culture test measured. It was found that the cyclohexane-1,3-dione derivatives are highly active in this in vitro assay, while other 1,3-diketones are less active. Retinylidenedimedone has been chosen for further evaluation.

The search for retinoids with improved systemic tolerance over that of retinal, retinol, or retinoic acid has focused attention on derivatives of retionic acid with a modified end group.[1,3] The finding that the known condensation product of retinal with acetylacetone[2] had substantial activity in the tracheal organ culture test[3] prompted a chemical effort to synthesize a variety of condensation products of retinal with aliphatic and alicyclic 1,3-diketones. The preparation of these retinylidene diketones,[4] their physical properties, and their biological

Scheme I



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