N-(2-Chloroethyl)-*N*-nitrosoureas Covalently Bound to Nonionic and Monocationic Lexitropsin Dipeptides. Synthesis, DNA Affinity Binding Characteristics, and Reactions with ^{32}P-End-Labeled DNA†

Kevin M. Church, Richard L. Wurdeman, Yi Zhang, Fa-Xian Chen, and Barry Gold*

Eppley Institute for Research in Cancer and Allied Diseases and Department of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, Nebraska 68198-6805

Received January 11, 1990; Revised Manuscript Received April 4, 1990

ABSTRACT: The synthesis and characterization of a series of compounds that contain an *N*-alkyl-*N*-nitrosourea functionality linked to DNA minor groove binding bi- and tripeptides (lexitropsins or information-reading peptides) based on methylpyrrole-2-carboxamide subunits are described. The lexitropsins (lex) synthesized have either a 3-(dimethylamino)propyl or propyl substituent on the carboxyl terminus. The preferred DNA affinity binding sequences of these compounds were footprinted in ^{32}P-end-labeled restriction fragments with methidiumpropyl-EDTA·Fe(II), and in common with other structural analogues, e.g., distamycin and netropsin, these nitrosoureas recognize A-T-rich runs. The affinity binding of the compound with the dimethylamino terminus, which is ionized at near-neutral pH, appeared stronger than that observed for the neutral dipeptide. The sequence specificity for DNA alkylation by (2-chloroethyl)nitrosourea–lex dipeptides (Cl-ENU–lex), with neutral and charged carboxyl termini, using ^{32}P-end-labeled restriction fragments, was determined by the conversion of the adducted sites into single-strand breaks by sequential heating at neutral pH and exposure to base. The DNA cleavage sites were visualized by polyacrylamide gel electrophoresis and autoradiography. The alkylation of DNA by Cl-ENU–lex was compared to that by *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea (CCNU), which has no DNA affinity binding properties. While all the Cl-ENU compounds generate DNA breaks as a consequence of the formation of *N*7-alkylguanine, the Cl-ENU–lex compounds induced, in a time- and dose-dependent fashion, intense DNA cleavage bands at adenine, cytosine, and thymine residues associated with affinity binding sites. These non-G cleavages induced by Cl-ENU–lex were inhibited by the coaddition of distamycin at concentrations that did not affect G alkylation break sites. CCNU, even at much higher concentrations, does not generate any similar detectable lesions at non-G sites. Therefore, linking the Cl-ENU moiety to minor groove binders is a viable strategy to qualitatively and quantitatively control the delivery and release of the ultimate DNA alkylating agent in a sequence-dependent fashion.

N-(2-Chloroethyl)-*N'*-alkyl-*N*-nitrosoureas (Cl-ENU)[1] are some of the few clinically useful antineoplastic agents employed in the treatment of human brain malignancies (Levin & Wilson, 1976). The predominant position for DNA alkylation by Cl-ENU is at N7-G, a major groove site, with other monofunctional modifications also found at O^6-G, N3-C, N3-T, and the phosphodiester backbone (Ludlum et al., 1975; Gombar et al., 1980; Tong et al., 1982b; Carter et al., 1988). The *O*^{6}-(2-chloroethyl)-G adduct, which slowly cyclizes to *N*1,*O*^{6}-ethano-G and then undergoes nucleophilic attack by an N3-C on the complement strand, is the precursor for the cytotoxic interstrand cross-link between N1-G and N3-C. This cross-link is thought to be responsible for Cl-ENU anticancer activity (Tong et al., 1982a, 1983). In fact, one mechanism contributing to Cl-ENU tumor resistance involves the removal of the 2-chloroethyl group from the O^6-G position by *O*^{6}-alkylguanine alkyltransferase before it can cyclize (Erickson et al., 1980).

The chemical principle(s) responsible for alkylation of DNA by Cl-ENU, as well as by other related DNA alkylating agents, in terms of the nucleophilic base sites that are modified and the sequence specificity for these modifications is (are) a topic of current interest (Mattes et al., 1986a; Hartley et al., 1986; Carter et al., 1988; Buckley & Brent, 1988; Briscoe & Duarte, 1988; Wurdeman & Gold, 1988; Warpehoski & Hurley, 1988; Wurdeman et al., 1989). To gain an insight into the factors responsible for DNA adduction and to increase and alter the covalent modifications of DNA, a series of compounds were synthesized in which a Cl-ENU moiety is connected to minor groove recognizing lexitropsins[2] (lex). It is demonstrated that these compounds, because of their affinity binding properties, generate DNA adducts with a high degree of sequence specificity and, importantly, extensively modify

† This work was supported by USPHS Research Grant CA29088 and Core Grant CA36727 awarded by the National Cancer Institute and by American Cancer Society Center Grant ACS SIG-16. The support of K.M.C. by an Elizabeth Bruce Memorial Fellowship is gratefully acknowledged.

[1] Abbreviations: ANU, *N*-alkyl-*N*-nitrosourea; ANU–lex, *N*-alkyl-*N*-nitrosourea covalently linked to a lexitropsin molecule: bp, base pair; *N*-Cbz-β-Ala, *N*-carbobenzoxy-β-alanine; CCNU, *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea; CDI, 1,1-carbonyldiimidazole, Cl-ENU, *N*-(2-chloroethyl)-*N*-nitrosourea; DMF, *N*,*N*-dimethylformamide; DMS, dimethyl sulfate; EDTA, ethylenediaminetetraacetic acid; ENU, *N*-ethyl-*N*-nitrosourea, FAB-MS, fast-atom-bombardment mass spectrometry; HPLC, high-performance liquid chromatography; lex, lexitropsin (information-reading peptide); MNU, *N*-methyl-*N*-nitrosourea; *N*7-MeG, *N*7-methylguanine; THF, tetrahydrofuran; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

[2] The term lexitropsin is used to identify information-reading peptides (Kopka et al., 1985).

0006-2960/90/0429-6827$02.50/0

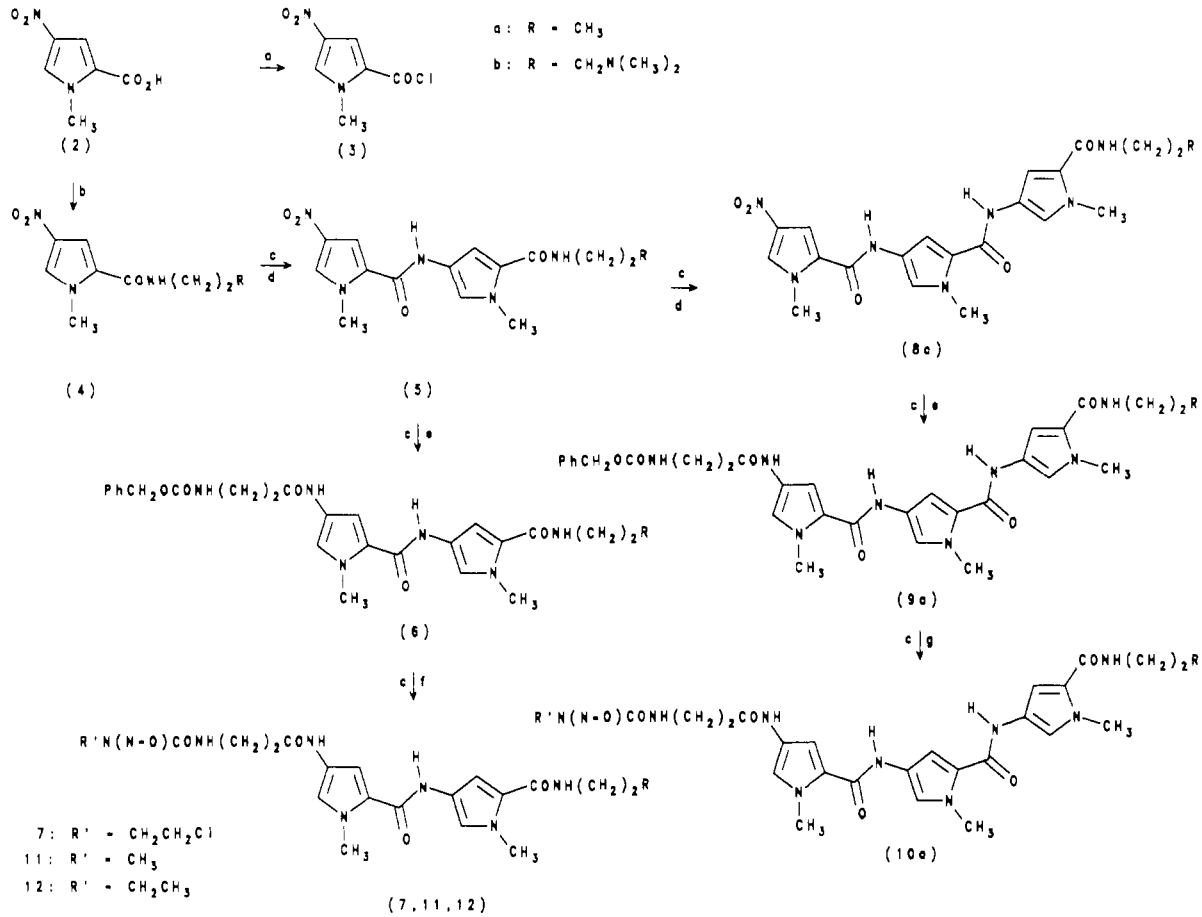

FIGURE 1: Syntheses of ANU–lex compounds: a, $SOCl_2$; b, $H_2N(CH_2)_2R$; c, H_2, Pd/C; d, compound **3**; e, *N*-Cbz-β-Ala; f, $ClCH_2CH_2N(N{=}O)CO_2X$, $CH_3N(N{=}O)CO_2X$, or $CH_3CH_2N(N{=}O)X$; g, $CH_3N(N{=}O)CO_2X$.

DNA at base sites not observed with the other Cl-ENU compounds.

Materials and Methods

All NMR experiments were performed on a Varian XL-300 with tetramethylsilane as the internal reference and Me_2SO-d_6 as the solvent. Homonuclear, heteronuclear, and phase-sensitive heteronuclear multiple-bond correlation spectroscopies were used to assist in NMR assignments. All NMR coupling constants (J) are reported in hertz.

Materials. THF and MeCN were dried over CaH_2 and distilled onto, and stored over, 3-Å molecular sieves. DMF was passed over neutral alumina and stored over 4-Å molecular sieves. EtOH was distilled from $Mg(OEt)_2$ onto 3-Å molecular sieves. Other solvents were of the highest available purity.

CCNU was synthesized from cyclohexylamine and *N*-(2-chloroethyl)-*N*-nitrosocarbamic acid *N'*-succinimidyl ester or pentafluorophenyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate (Martinez et al., 1982). *N*-Carbobenzoxy-β-alanine (*N*-Cbz-β-Ala) was prepared by the condensation of β-alanine and benzyl chloroformate. CDI was crystallized from anhydrous THF. Amines were dried over KOH. Restriction enzymes were purchased from New England Biolabs and IBI.

Synthesis and Characterization (See Figure 1). (*A*) (*1-Methyl-4-nitropyrrole-2-carboxamido)propane* (**4a**). 1-Methyl-4-nitropyrrole-2-carboxylic acid (**2**) (Lown & Krowicki, 1985) (7.2 g, 40 mmol) and CDI (6.86 g, 40 mmol) were dissolved in 100 mL of dry DMF and stirred for 3 h. Then 4.2 mL (48 mmol) of *n*-propylamine was added and the solution stirred at room temperature for 24 h. The DMF was removed in vacuo at 60 °C and the residue triturated with 150 mL of H_2O, which resulted in the formation of a yellow-green precipitate. The solid was collected, washed twice with H_2O, and dried in vacuo to give the product: yield 65%; mp 142–143 °C; TLC (silica, Me_2CO/CH_2Cl_2, 1:19) R_f 0.72; IR (KBr disk) 3328, 3317, 2959, 1638, 1530, 1500, 1421, 1316 cm^{-1}; ^1H NMR δ 8.39 (t, 1 H, CONH), 8.12 (d, 1 H, pyrrole CH), 7.45 (d, 1 H, pyrrole CH), 3.93 (s, 3 H, NCH_3), 3.17 (q, 2 H, $CONHCH_2$), 1.52 (m, 2 H, CH_2CH_3), 0.89 (t, 3 H, J = 7.5, CH_2CH_3); ^{13}C NMR δ 159.77 (CONH), 133.78, 127.71, 126.56, 107.14, 40.42, 37.31, 22.29, 11.39.

(*B*) *3-(1-Methyl-4-nitropyrrole-2-carboxamido)-1-(N,N-dimethylamino)propane* (**4b**). This compound was prepared as described for **4a** except *N*,*N*-dimethyl-1,3-diaminopropane (4.4 mL, 35.2 mmol) was used instead of propylamine. Yield 69%; TLC (silica, NH_4OH/MeOH, 1:99) R_f 0.38; ^1H NMR δ 8.43 (t, 1 H, CONH), 8.12 (d, 1 H, pyrrole CH), 7.43 (d, 1 H, pyrrole CH), 3.94 (s, 3 H, NCH_3), 3.23 (q, 2 H, $CONHCH_2CH_2$), 2.54 [t, 2 H, J = 6.9, $CH_2N(CH_3)_2$], 2.15 [s, 6 H, $N(CH_3)_2$], 1.64 [m, 2 H, $CH_2CH_2N(CH_3)_2$]; ^{13}C NMR δ 159.73 (CONH), 133.78, 127.67, 126.55, 107.10, 56.76, 45.12, 37.33, 37.08, 27.01.

(*C*) *Synthesis of [1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]propane* (**5a**). Carboxylic acid **2** (2.42 g, 14 mmol) and 15 mL of $SOCl_2$ in 40 mL of dry THF were refluxed for 30 min. The excess $SOCl_2$ and THF were removed, and the crude acid chloride (**3**) was dried in vacuo. In a separate reaction, nitro compound **4a** (3.0 g, 14 mmol), 10% Pd/C (1 g), and 20 mL of cyclohexene in 60 mL of 95% EtOH were refluxed for 8 h. The solution was filtered and the catalyst washed with 200 mL of EtOH. The combined EtOH solutions were concentrated in vacuo, and the crude amine was taken up in 30 mL of THF, containing 6.1 mL (35 mmol) of diisopropylethylamine. This amine solution was slowly added to a cooled solution (−20 °C) of acid chloride **3** in 40 mL of MeCN. After the addition, the solution was

stirred at room temperature for 1 h and the solvents were removed in vacuo. The residue was triturated with H_2O to give a yellow precipitate. The solid was collected by filtration and sequentially washed with H_2O and MeOH to afford dipeptide **5a**: yield 83%; mp 241–243 °C, ^{1}H NMR δ 10.24 (s, 1 H, CONH), 8.18 (d, 1 H, pyrrole CH), 8.07 (t, 1 H, CON*H*CH_2), 7.59 (d, 1 H, pyrrole CH), 7.21 (d, 1 H, pyrrole CH), 6.86 (d, 1 H, pyrrole CH), 3.97 (s, 3 H, NCH_3), 3.83 (s, 3 H, NCH_3), 3.14 (q, 2 H, $CH_2CH_2CH_3$), 1.51 (m, 2 H, CH_2CH_3), 0.88 (t, 3 H, J = 7.2, CH_2CH_3); ^{13}C NMR δ 160.83 (CONH), 156.58 (CONH), 133.58, 127.85, 126.09, 123.20, 121.08, 117.65, 107.31, 103.78, 40.13, 37.32, 35.85, 22.49, 11.34.

(*D*) *Synthesis of 3-[1-Methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]-1-(N,N-dimethylamino)propane* (**5b**). Nitro compound **4b** (2.0 g, 0.79 mmol) was reduced to the corresponding amine in 4 h as described above for **4a**. The resulting amine was dissolved in 20 mL of DMF containing 2.5 g of diisopropylethylamine, and this solution was then slowly added to a cooled solution (−20 °C) of acid chloride **3** (ca. 0.79 mmol) in 20 mL of MeCN. After the addition, the solution was stirred at room temperature for 8 h. The solvents were removed in vacuo, and the residue was taken up in 3 mL of Me_2CO and flash chromatographed, initially with Me_2CO/MeOH (1:1), to remove unreacted starting materials, and then with 100% MeOH to afford the impure product. This material was crystallized from Me_2CO/MeOH (20:80) to give **5b**: yield 67%; mp 247–249 °C; TLC (silica, NH_4OH/MeOH, 1:49) R_f 0.30; ^{1}H NMR δ 10.25 (s, 1 H, CONH), 8.18 (d, 1 H, J = 2.4, pyrrole CH), 8.13 (t, 1 H, CONH), 7.60 (d, 1 H, J = 2.1, pyrrole CH), 7.22 (d, 1 H, J = 2.1, pyrrole CH), 6.84 (d, 1 H, J = 2.4, pyrrole CH), 3.97 (s, 3 H, NCH_3), 3.83 (s, 3 H, NCH_3), 3.21 (q, 2 H, CONHCH_2CH$_2$), 2.25 (t, 2 H, J = 7.8, CONHCH$_2$CH$_2$CH_2), 2.14 [s, 6 H, N(CH$_3$)$_2$], 1.62 (m, 2 H, CONHCH$_2$CH_2CH$_2$); ^{13}C NMR δ 161.06, 156.84, 133.79, 128.15, 126.31, 123.33, 121.34, 117.89, 107.52, 103.84, 57.07, 45.16, 37.43, 37.11, 35.98, 27.16.

(*E*) *[1-Methyl-4-[1-methyl-4-(N-carbobenzoxy-3-aminopropanamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]propane* (**6a**). Nitrodipyrrole peptide **5a** (3.0 g, 9 mmol) was reduced to the corresponding amine in 6 h as described above for the reduction of **4a**. The amine was taken up in 25 mL of dry DMF and then added to a solution containing *N*-Cbz-β-Ala (2.0 g, 9 mmol) and CDI (1.46 g, 9 mmol) in 75 mL of DMF that had been stirring for 3 h at room temperature. The resulting solution was then stirred under N_2 for 24 h. The solvent was removed in vacuo, and 200 mL of H_2O was added. The solid that formed was washed with H_2O and dried in vacuo. The residue was crystallized from Me_2CO/2-PrOH (1:4) to give the protected carbamate **6a**: yield 34%; mp 220–222 °C; IR (KBr disk) 3322, 2945, 1684, 1652, 1584, 1533, 1512 cm^{-1}; ^{1}H NMR δ 9.89 (s, 1 H, CONH), 9.86 (s, 1 H, CONH), 8.00 (t, 1 H, CON*H*CH$_2$), 7.35 (m, 6 H, phenyl and CONH), 7.18 (m, 2 H, pyrrole CH's), 6.86 (m, 2 H, pyrrole CH's), 5.02 (s, 2 H, PhCH_2), 3.83 (s, 3 H, NCH_3), 3.79 (s, 3 H, NCH_3), 3.29 (q, 2 H, CONHCH_2CHCONH), 3.13 (q, 2 H, NHCH_2CH$_2$CH$_3$), 2.44 (t, 2 H, J = 7.8, CONHCH$_2$CH_2CONH), 1.49 (m, 2 H, CH_2CH$_3$), 0.87 (t, 3 H, J = 7.2, CH$_2$CH_3); ^{13}C NMR δ 167.45, 161.22, 158.35, 156.05, 137.15, 128.31, 127.67, 123.06, 122.73, 121.96, 121.87, 118.11, 117.71, 104.10, 103.89, 65.17, 40.19, 37.16, 36.07, 35.88, 22.57, 11.41.

(*F*) *3-[1-Methyl-4-[1-methyl-4-(N-carbobenzoxy-3-aminopropanamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]-1-(N,N-dimethylamino)propane* (**6b**). Dipyrrole **5b** (1.11 g, 2.09 mmol) was reduced to the amine in 4 h as described above for **4a**. This amine derivative of **5b** was dissolved in 10 mL of DMF and added to the imidazolide derivative of *N*-Cbz-β-Ala (640 mg) and the resulting solution stirred at room temperature under N_2 for 16 h. The solvent was removed and the residue flash chromatographed with NH_4OH/MeOH (1:99) to give the protected carbamate: yield 31%; IR (KBr disk) 3300, 2946, 1685, 1636, 1584, 1534, 1261 cm^{-1}; ^{1}H NMR δ 9.89 (s, 1 H, CONH), 9.86 (s, 1 H, CONH), 8.07 (t, 1 H, CONH), 7.35 (s, 6 H, phenyl and NH), 7.18 (d, 1 H, pyrrole CH), 7.17 (d, 1 H, pyrrole CH), 6.86 (d, 1 H, pyrrole CH), 6.81 (d, 1 H, pyrrole CH), 5.02 (s, 2 H, PhCH_2), 3.82 (s, 3 H, NCH_3), 3.79 (s, 3 H, NCH_3), 3.28 (q, 2 H, CONHCH_2CH$_2$CONH), 3.18 [q, 2 H, CH_2CH$_2$N(CH$_3$)$_2$], 2.44 (t, 2 H, J = 6.9, CONHCH$_2$CH_2CONH), 2.24 [t, 2 H, J = 6.6, CH_2N(CH$_3$)$_2$], 2.13 [s, 6 H, N(CH$_3$)$_2$], 1.61 (m, 2 H, CH$_2$CH_2CH$_2$); ^{13}C NMR δ 167.36, 161.07, 158.26, 155.95, 137.09, 128.23, 127.59, 122.98, 122.69, 121.94, 121.80, 118.03, 117.64, 103.90, 103.84, 65.10, 57.02, 45.09, 37.08, 37.01, 35.94, 35.78, 27.09.

(*G*) *[1-Methyl-4-[1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]propane* (**8a**). Nitro compound **5a** (1.0 g, 3 mmol) was reduced to the corresponding amine in 6 h as described above for **4a**. The amine was taken up in 30 mL of DMF, containing 1.3 mL of diisopropylethylamine, and slowly added to a cooled (−20 °C) solution of acid chloride **3** in 30 mL of MeCN. After the addition, the solution was stirred at room temperature for 3 h. The solvent was then removed, and the resulting dark gummy material was triturated with 10 mL of MeOH and 150 mL of H_2O to afford a fine yellow-green precipitate. The precipitate was collected by filtration, washed with 100 mL of H_2O, and dried in vacuo to give tripeptide **8a**: yield 87%; ^{1}H NMR δ 10.31 (s, 1 H, CONH), 9.96 (s, 1 H, CONH), 8.19 (d, 1 H, pyrrole CH), 8.02 (t, 1 H, CONH), 7.60 (d, 1 H, pyrrole CH), 7.28 (d, 1 H, pyrrole CH), 7.20 (d, 1 H, pyrrole CH), 7.05 (d, 1 H, pyrrole CH), 6.87 (d, 1 H, pyrrole CH), 3.98 (s, 3 H, NCH_3), 3.87 (s, 3 H, NCH_3), 3.81 (s, 3 H, NCH_3), 3.13 (q, 2 H, $CH_2CH_2CH_3$), 1.50 (m, 2 H, CH_2CH_3), 0.88 (t, 3 H, J = 7.2, CH_2CH_3); ^{13}C NMR δ 160.88, 157.99, 156.58, 133.51, 127.90, 126.02, 122.84, 122.79, 121.73, 121.14, 118.31, 117.47, 107.33, 104.24, 103.88, 40.06, 37.33, 36.03, 35.77, 22.48, 11.33.

(*H*) *[1-Methyl-4-[1-methyl-4-[1-methyl-4-(N-carbobenzoxy-3-aminopropanamido)pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]propane* (**9a**). Nitro compound **8a** (2.0 g, 4.4 mmol) was reduced to the corresponding amine in 12 h as described above for the reduction of **4a**. The amine was dissolved in 20 mL of DMF and added to a solution containing the imidazolide derivative of *N*-Cbz-β-Ala (980 mg, 4.4 mmol) in 60 mL of DMF. The solution was stirred for 24 h under N_2. The DMF was then removed to afford a dark residue, which was triturated with 10 mL of MeOH and 150 mL of H_2O to give a precipitate that was collected, washed with H_2O, and dried in vacuo. The solid was then crystallized from Me_2CO. Yield 25%; IR (KBr disk) 3309, 2956, 1683, 1635, 1585, 1533, 1436, 1262 cm^{-1}; ^{1}H NMR δ 9.94 (s, 1 H, CONH), 9.92 (s, 2 H, 2 CONH), 8.02 (t, 1 H, CONH), 7.35 (m, 6 H, phenyl and CONH), 7.26 (d, 1 H, pyrrole CH), 7.19 (m, 2 H, 2 pyrrole CH's), 7.06 (d, 1 H, pyrrole CH), 6.91 (d, 1 H, pyrrole CH), 6.89 (d, 1 H, pyrrole CH), 5.03 (s, 2 H, PhCH_2), 3.86 (s, 3 H, NCH_3), 3.85 (s, 3 H, NCH_3), 3.81 (s, 3 H, NCH_3), 3.31 (q, 2 H, CONHCH_2CH$_2$CONH), 3.14 (q, 2 H, CONHCH_2CH$_2$CH$_3$),

2.46 (t, 2 H, J = 7.5, $CONHCH_2CH_2CONH$), 1.50 (m, 2 H, CH_2CH_3), 0.88 (t, 3 H, J = 7.5, CH_2CH_3).

(*I*) [*1-Methyl-4-[1-methyl-4-[3-[3'-(2-chloroethyl)-3'-nitrosoureido]propanamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]propane* (**7a**). **6a** (474 mg, 93 mmol) was deprotected by refluxing with 100 mg of 10% Pd/C and 9 mL of cyclohexene in 40 mL of 95% ethanol for 30 min. The solution was filtered and the catalyst washed with 100 mL of H_2O. This aqueous amine filtrate was washed twice with ether, concentrated, and dried overnight in vacuo. The amine was dissolved in 5 mL of DMF, cooled to 0 °C, and treated with 1.1 equiv of *N*-(2-chloroethyl)-*N*-nitrosocarbamic acid *N'*-succinimidyl ester or pentafluorophenyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate for 1 h and at room temperature for 2 h. The solution was diluted with 50 mL of EtOAc and washed three times with ice-cold 5% $NaHCO_3$. The organic layer was dried ($MgSO_4$) and reduced in volume to 2 mL. This solution was flash chromatographed with 100% EtOAc to remove unreacted starting material and then with 100% Me_2CO to elute the orange-yellow nitrosourea product. Yield 43%; TLC (silica, EtOAc) R_f 0.26; IR (KBr disk) 3298, 3126, 2961, 1728, 1642, 1528 cm^{-1}; ^1H NMR δ 9.98 (s, 1 H, CONH), 9.88 (s, 1 H, CONH), 8.82 (t, 1 H, urea NH), 8.01 (t, 1 H, pyrrole NH), 7.19 (s, 2 H, 2 pyrrole CH's), 6.89 (s, 1 H, pyrrole CH), 6.87 (s, 1 H, pyrrole CH), 4.12 (t, 2 H, J = 6.3, $ClCH_2CH_2$), 3.84 (s, 3 H, NCH_3), 3.81 (s, 3 H, NCH_3), 3.63 (m, 4 H, $ClCH_2CH_2$ and $CONHCH_2CH_2CONH$), 3.14 (q, 2 H, $CONHCH_2CH_2CH_3$), 2.63 (t, 2 H, J = 6.9, $CONHCH_2CH_2CONH$), 1.50 (m, 2 H, $CONHCH_2CH_2CH_3$), 0.87 (t, 3 H, J = 7.2, $CH_2CH_2CH_3$); ^{13}C NMR δ 167.48, 161.21, 158.33, 152.59, 123.09, 122.79, 121.97, 121.79, 118.14, 117.70, 104.11, 103.91, 40.05, 39.77, 37.06, 36.04, 35.83, 35.15, 22.55, 11.36; MS (FAB, Xe, 9 keV) m/z 509 (1, M), 480 (5, M + 1 − NO), 401 (11), 342 (23), 220 (50), 194 (19), 181 (11), 123 (72), 108 (24).

(*J*) *3-[1-Methyl-4-[1-methyl-4-[3-[3'-(2-chloroethyl)-3'-nitrosoureido]propanamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]-1-(N,N-dimethylamino)propane* (**7b**). The same method described for the preparation of **7a** was used except the amine, obtained by the reduction of **6b**, and the chloroethyl nitroso transfer reagent were stirred at 0 °C for 6 h. The residue obtained by concentrating this reaction solution was triturated with Me_2CO/Et_2O (1:9) to give an orange solid. The solid was reprecipitated twice from the same solvent system and dried in vacuo. Preparative TLC (silica, diisopropylethylamine/MeOH, 1:99) was used when the NMR indicated that the product required further purification. Yield 45%; ^1H NMR δ 10.04 (s, 1 H, CONH), 9.89 (s, 1 H, CONH), 8.82 (t, 1 H, CONH), 8.08 (t, 1 H, CONH), 7.19 (d, 2 H, pyrrole CH), 6.89 (d, 1 H, pyrrole CH), 6.83 (d, 1 H, pyrrole CH), 4.10 (t, 2 H, J = 6.3, $ClCH_2CH_2$), 3.83 (s, 3 H, NCH_3), 3.79 (s, 3 H, NCH_3), 3.65 (m, 4 H, $ClCH_2$ and $NNOCONHCH_2$), 3.18 (m, 2 H, $CONHCH_2CH_2CH_2$), 2.62 (t, 2 H, $CONHCH_2CH_2CONH$), 2.25 [t, 2 H, J = 7.2, $CH_2N(CH_3)_2$], 2.14 [s, 6 H, $N(CH_3)_2$], 1.61 (m, 2 H, $CH_2CH_2CH_2$); ^{13}C NMR δ 167.45, 161.38, 158.32, 152.54, 122.70, 122.62, 122.05, 121.76, 118.12, 117.97, 104.32, 103.97, 55.06, 42.62, 40.13, 37.02, 35.99, 35.88, 35.85, 35.07, 24.99; MS (FAB, Xe, 9 keV) m/z 553 (2, M + 1), 552 (3, M), 444 (7, M − $ClCH_2CH_2NNO$), 342 (13), 277 (3), 220 (37), 194 (15), 123 (94), 108 (53).

(*K*) [*1-Methyl-4-[1-methyl-4-[3-(3'-methyl-3'-nitrosoureido)propanamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]propane* (**11a**). The same methods described for the preparation of **7a** were used except the amine, obtained from the reduction of **6a**, and the *N*-methyl-*N*-nitrosocarbamic acid *N'*-succinimidyl ester transfer reagent were stirred at 0 °C for 1 h and for 2 h at room temperature. The solution was then diluted with 50 mL of EtOAc and washed three times with ice-cold 5% $NaHCO_3$. The organic layer was dried ($MgSO_4$) and then reduced in volume to 2 mL. This solution was flashed chromatographed with 100% EtOAc to remove unreacted starting material and then with 100% Me_2CO to give the nitrosourea product: yield 33%; TLC (silica, EtOAc) R_f 0.24; IR (KBr disk) 3313, 2961, 1700, 1637, 1528 cm^{-1}; UV ($CHCl_3$) 295 nm (ϵ 31 515); ^1H NMR δ 9.95 (s, 1 H, CONH), 9.86 (s, 1 H, CONH), 8.77 (t, 1 H, urea NH), 8.00 (t, 1 H, pyrrole CH), 7.18 (s, 2 H, 2 pyrrole CH's), 6.87 (d, 1 H, pyrrole CH), 6.85 (d, 1 H, pyrrole CH), 3.83 (s, 3 H, NCH_3), 3.79 (s, 3 H, NCH_3), 3.59 (q, 2 H, $CONHCH_2CH_2CONH$), 3.10 (m, 5 H, CH_3NNO and $CONHCH_2CH_2CH_3$), 2.61 (t, 2 H, J = 6.9, $CONHCH_2CH_2CONH$), 1.49 (m, 2 H, $CONHCH_2CH_2CH_3$), 0.87 (t, 3 H, J = 7.5, $CH_2CH_2CH_3$); ^{13}C NMR δ 167.18, 160.87, 157.99, 152.48, 122.81, 122.51, 121.69, 121.52, 117.87, 117.44, 103.85, 103.65, 40.04, 36.95, 35.95, 35.74, 35.09, 26.79, 22.46, 11.32; MS (FAB, Xe, 9 keV) m/z 461 (2, M + 1), 431 (4, M + 1 − NO), 401 (5), 342 (20), 220 (46), 194 (23), 123 (100), 108 (35).

(*L*) *3-[1-Methyl-4-[1-methyl-4-[3-(3'-ethyl-3'-nitrosoureido)propanamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]-1-(N,N-dimethylamino)propane* (**12b**). This compound was prepared by treating the amine obtained from the deprotection of **6b** with pentafluorophenyl *N*-ethyl-*N*-nitrosocarbamate for 6 h at 0 °C in DMF. The solvent was removed in vacuo at 30 °C and the resulting residue treated with Me_2CO/Et_2O (1:9) to obtain the orange nitrosourea product. The crude product was precipitated twice from the same solvent system. Yield 53%; IR (KBr disk) 3216, 2944, 1718, 1653, 1647, 1581, 1525, 1498, 1466, 1437, 1405 cm^{-1}; UV (MeOH) 297 nm (ϵ 23 100), 456 (ϵ 450); ^1H NMR (30 °C) δ 9.93 (s, 1 H, CONH), 9.84 (s, 1 H, CONH), 8.68 (t, 1 H, CONH), 8.06 (t, 1 H, CONH), 7.16 (d, 2 H, 2 pyrrole CH's), 6.87 (d, 1 H, pyrrole CH), 6.84 (d, 1 H, pyrrole CH), 3.82 (s, 3 H, NCH_3), 3.79 (s, 3 H, NCH_3), 3.77 (q, 2 H, J = 7.0, CH_2NNO), 3.58 [q, 2 H, $N(NO)CONHCH_2$], 3.20 [m, 2 H, $CH_2CH_2CH_2N(CH_3)_2$], 2.59 (t, 2 H, J = 7.1, $NNOCONHCH_2CH_2$), 2.44 [t, 2 H, J = 7.0, $CH_2N(CH_3)_2$], 2.29 [s, 6 H, $N(CH_3)_2$], 1.67 (m, 2 H, $CH_2CH_2CH_2$), 0.93 (t, 3 H, J = 7.4, CH_3CH_2NNO); ^{13}C NMR (30 °C) δ 167.48, 161.19, 158.29, 152.57, 122.89, 122.76, 121.99, 121.74, 118.06, 117.74, 104.06, 103.91, 56.51, 44.43, 36.93, 36.67, 35.95, 35.80, 35.14, 34.45, 26.52, 11.77.

(*M*) [*1-Methyl-4-[1-methyl-4-[1-methyl-4-[3-(3'-methyl-3'-nitrosoureido)propanamido]pyrrole-2-carboxamido]-pyrrole-2-carboxamido]pyrrole-2-carboxamido]propane* (**10a**). The *N*-Cbz compound **9a** (0.44 g, 0.69 mmol) was deprotected by refluxing with 300 mg of 10% Pd/C and 8 mL of cyclohexene in 30 mL of 95% EtOH for 5 h. The solution was then filtered and the catalyst washed with 100 mL of 0.2 N HCl. The combined filtrate and acidic washings were extracted twice with Et_2O, neutralized with NaOH, and concentrated in vacuo. DMF (5 mL) was added to the residue, and the resulting mixture was filtered to remove salts. The DMF filtrate was cooled (0 °C) and treated with *N*-methyl-*N*-nitrosocarbamic acid *N'*-succinimidyl ester (490 mg, 0.76 mmol) for 1 h and at room temperature for 2 h. EtOAc (100 mL) was added and the organic layer washed three times with ice-cold 5% $NaHCO_3$. The organic layer was dried

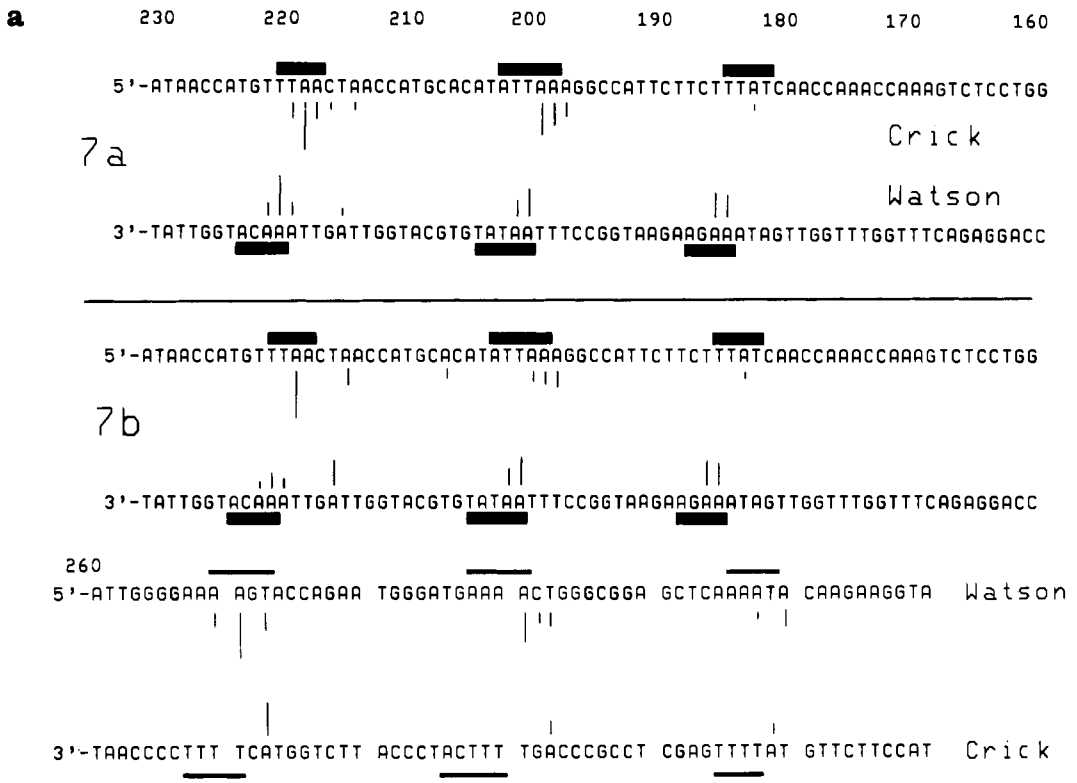

FIGURE 2: Sequence of 85 and 576 bp restriction fragments. (a) 85 bp fragment: The ANU–lex affinity regions are indicated by thick horizontal bars, and non-G cleavage sites for **7a** and **7b** are denoted by vertical lines (|). Line length indicates relative band intensities. (b) 576 bp fragment: The ANU–lex affinity regions are indicated by thick horizontal bars, and non-G cleavage sites for **7a** are denoted by vertical lines (|). Line length indicates relative band intensities.

($MgSO_4$) and reduced in volume to 2 mL. This solution was flash chromatographed with 100% EtOAc to remove starting material and then with MeOH/EtOAc (1:9) to elute the nitrosourea. Yield 19%; UV (MeOH) 298 nm (ϵ 29 800); ^{1}H NMR δ 9.96 (s, 1 H, CONH), 9.93 (s, 1 H, CONH), 9.89 (s, 1 H, CONH), 8.78 (t, 1 H, CONH), 8.00 (t, 1 H, CONH), 7.25 (d, 1 H, pyrrole CH), 7.18 (d, 2 H, 2 pyrrole CH's), 7.04 (d, 1 H, pyrrole CH), 6.90 (d, 1 H, pyrrole CH), 6.87 (d, 1 H, pyrrole CH), 3.85 (s, 3 H, NCH_3), 3.84 (s, 3 H, NCH_3), 3.80 (s, 3 H, NCH_3), 3.59 (q, 2 H, $CONHCH_2CH_2CONH$), 3.10 [m, 5 H, $CH_3N(NO)$ and $CONHCH_2CH_2CH_3$], 2.61 (t, 2 H, J = 7.2, $CONHCH_2CH_2CONH$), 1.49 (m, 2 H, $CONHCH_2CH_2CH_3$), 0.87 (t, 3 H, J = 7.2, $CONHCH_2CH_2CH_3$); ^{13}C NMR δ 167.49, 161.17, 158.38, 158.35, 152.75, 123.05, 122.73, 122.02, 121.75, 118.37, 118.12, 117.61, 104.62, 104.07, 103.93, 40.12, 37.02, 35.97, 35.78, 35.15, 26.84, 22.49, 11.33; MS (FAB, Xe, 9 keV) m/z 582 (1, M), 553 (2, M + 1 − NO), 523 (1), 342 (14), 245 (11), 220 (29), 194 (18), 177 (16), 123 (94), 108 (34).

Stability Studies. A 1 mM solution of **7(a,b)**, **10a**, **11a**, or **12b** in 50 mM sodium phosphate buffer (pH 8.0) containing varying amounts of a MeCN or Me_2SO was maintained at 37 °C through >7 half-lives. Aliquots were periodically removed, and the amount of the unreacted nitrosourea was determined by reverse-phase HPLC [column, Waters NovaPak C_{18}, 4 μm; solvent, MeCN/H_2O (pH 5.0); detection, 254 and 298 nm]. Good pseudo-first-order rate plots for **7a**, **7b**, **10a**, **11a**, and **12b** were obtained ($r^2 > 0.998$) with calculated $t_{1/2}$ of 24, 30, 119, 89, and 89 min, respectively. The $t_{1/2}$ values of and *N*-methyl-*N'*-cyclohexyl-*N*-nitrosourea, MNU, and CCNU under identical conditions were determined to be 80, 3, and 25 min, respectively.

During the hydrolysis of **7a**, a less retentive HPLC peak appeared whose intensity increased as the peak for parent nitrosourea decreased. This component was collected by preparative HPLC (column, Partisil ODS-3; solvent, MeCN/H_2O, 1:3; flow rate, 3 mL/min; detection, 254 nm) and the residue submitted to FAB-MS analysis; m/z (% intensity) 401 (12, M + 1), 400 (7, M), 342 (14, M − $NHCH_2CH_2CH_3$).

Preparation of ^{32}P-End-Labeled DNA Restriction Fragments. 5'-^{32}P-End-labeled DNA restriction fragments were prepared from a 3220 bp DNA clone containing the promotor region of the coat protein gene of the canine parvovirus (Rhode, 1985) by initial endonuclease restriction with *Nco*I, followed by sequential treatment with calf intestine alkaline phosphatase, phosphorylation with T4 kinase in the presence of [γ-^{32}P]ATP and *Hin*dIII digestion (Maxam & Gilbert, 1980; Wurdeman & et al., 1989). The 85 and 576 bp 5'-^{32}P-end-labeled fragments (Figure 2) were purified by electrophoresis on a 5% polyacrylamide gel and isolated by electroelution. The corresponding 3'-^{32}P-end-labeled fragments were prepared from the same *Nco*I restriction digest using the Klenow fragment of DNA polymerase I (Sanger & Coulson, 1980) and [α-^{32}P]GTP and nonlabeled bases followed by *Hin*dIII restriction and polyacrylamide gel purification.

DNA Footprinting. Because of the hydrolytic instability of **7(a,b)**, it was not feasible to determine their DNA binding specificities. However, the *N*-methyl- and *N*-ethylnitrosoureas, **11a**, **12b**, and **10a**, have $t_{1/2}$'s of >2 h at 37 °C in pH 7.0 buffer, and they were used in the DNA footprinting studies with MPE·Fe(II) (Van Dyke et al., 1982; Hertzberg & Dervan, 1984). The distamycin or ANU–lex was preincubated with the ^{32}P-end-labeled restriction fragment and sonicated calf thymus DNA (83 μM final concentration) for 30 min at 25 °C in pH 7.4 buffer. Freshly prepared MPE·Fe(II) (10 μM final concentration) was added followed by dithiothreitol (100 μM final concentration) and the solution incubated at 37 °C for 20 min, at which time the reaction was stopped by precipitation of the DNA with the addition of NaOAc and

cold EtOH and centrifugation. The DNA pellet was washed with cold 70% EtOH, dried, and dissolved in loading buffer [80% deionized formamide, 50 mM Tris–borate (pH 8.3), 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue]. The DNA samples were denatured at 90 °C for 1.5 min and then cooled in ice–H_2O prior to electrophoresis at 65 W on a 12% polyacrylamide gel containing 7.8 M urea and Tris–EDTA borate buffer (pH 8.3). The gel was exposed to Kodak X-OMAT AR film at −70 °C and the resulting autoradiogram analyzed by using a Shimadzu CS-9000 scanning densitometer.

Reactions of ANU with ^{32}P-End-Labeled DNA Fragments. The restriction fragment (80 000–100 000 cpm) and sonicated calf thymus DNA (final concentration 83 μM) were dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing the desired concentration of NaCl or cationic DNA affinity binder. This DNA solution was incubated at 37 °C for 2 h, unless stated otherwise, with a freshly prepared solution of ANU. The reactions were terminated by cooling and precipitation of the DNA with NaOAc and EtOH. The DNA was washed with cold 70% EtOH and dried in vacuo.

Generation and Analysis of DNA Strand Breaks. Strand breaks in the reacted DNA were generated by either of the following: (1) neutral thermal hydrolysis—alkylated DNA was heated at 90 °C for 15 min, to depurinate/depyrimidinate thermally labile adducts, precipitated with NaOAc and EtOH, washed with cold EtOH, and then treated with hot piperidine to convert the abasic sites into single-strand breaks (Lawley & Brookes, 1973); or (2) Maxam–Gilbert G-specific reaction—alkylated DNA was precipitated and washed and then directly treated with 1 M piperidine at 90 °C for 20 min to preferentially convert *N*7-alkyl-G into single-strand breaks (Maxam & Gilbert, 1980). In both cases the piperidine was removed in vacuo. In control experiments no alkylating agent was added. The dried DNA was suspended in loading buffer and denatured at 90 °C for 1.5 min and then cooled in ice. The DNA was placed into wells on top of a 12% polyacrylamide (7.8 M urea) denaturing gel which was run at 65 W. The standard G and/or G + A reaction lanes (Maxam & Gilbert, 1980) were included as sequence markers. The gel was then exposed to Kodak X-OMAT AR film at −70 °C and the resulting autoradiogram analyzed using a Shimadzu CS-9000 scanning densitometer.

Time Course. In these experiments the DNA was incubated with drug for the specified time but was not precipitated prior to the neutral thermal hydrolysis. Therefore, any unreacted ANU was present during the 15-min exposure to 90 °C. In certain cases, the ANU was incubated in buffer for 120 min prior to the addition of DNA.

Results

Synthesis. The preparation of the ANU–lex compounds (Figure 1), which is based on the syntheses of netropsin and distamycin (Lown & Krowicki, 1985), allows for the elaboration of the unstable alkylnitrosoureido functionality in the final step and provides flexibility in altering the *N*-alkyl nitroso substituent. In general, the yield of *N*-Cbz-protected amine **6** from **2** was ~17%. The conversion of the primary amine derived from **6** to the nitrosourea was achieved by nitroso acyl transfer reagents (Martinez et al., 1982) in 40–50% yield. The noncharged ANU–lex derivatives were routinely purified by flash chromatography. This was not possible with **7b** or **12b**, and repeated precipitation from Me_2CO–Et_2O was used to remove side products. While this generally gave pure product, as judged by NMR and/or HPLC, further purification by preparative silica TLC was sometimes performed. It was found that the amount of ethyldiisopropylamine (1% in 99% MeOH) used to deactivate the silica was critical in obtaining separation without significant decomposition. Higher percentages of Hunig's base or less bulky amines caused substantial degradation of nitrosourea product. The ^{1}H and ^{13}C NMR spectra of **10a** were completely assigned by NMR, including phase-sensitive heteronuclear multiple-bond correlation experiments (Williamson et al., 1989). The FAB-MS for the ANU compound showed weak parent ions along with M − HNO and M − RN_2OH fragmentations that are characteristic of high molecular weight 1,3-dialkylnitrosoureas (Konakahara et al., 1988).

In summary, **7a**, **7b**, **11a**, and **12b** were synthesized from **2** in 6–8% yield. The yield for the tripeptide **10a** was ~3%.

Stability Studies. The hydrolytic stability of the ANU–lex derivatives were evaluated at 37 °C in 50 mM sodium phosphate buffer (pH 8.0) using HPLC to monitor the loss of ANU starting material. Linear pseudo-first-order plots ($r^2 > 0.999$) for the hydrolysis of **7** were obtained with a calculated $t_{1/2}$ of ~25 min. The *N*-methyl and *N*-ethyl analogues, as anticipated, are more stable (Garrett et al., 1965). The measured $t_{1/2}$'s indicate that the hydrolysis of the nitrosourea is not influenced by the dipeptide structure. The $t_{1/2}$'s for *N*-methyl-*N'*-cyclohexyl-*N*-nitrosourea, CCNU, and MNU are 80, 25, and 3 min, respectively, under the same conditions.

It was observed that as the disappearance of starting material progressed, a more rapidly eluting peak appeared. This peak was collected, and on the basis of its FAB-MS, an isocyanate structure is tentatively assigned. Isocyanates are formed from the base-catalyzed hydrolysis of 1,3-dialkylnitrosoureas (Werner, 1919; Boivin & Boivin, 1951; Snyder & Stock, 1980).

DNA Footprinting. The DNA footprinting experiments require an initial 30-min incubation of ligand with DNA at room temperature and then a 15-min reaction time in the presence of the MPE·Fe(II) at 37 °C (Van Dyke et al., 1982; Hertzberg & Dervan, 1984). Because of the relative hydrolytic instability of **7**, it was not possible to determine the binding specificity of these compounds. Therefore, the more stable *N*-methyl and *N*-ethyl analogues **11a**, **12b**, and **10a** were used. Assuming that the rates of hydrolysis are not accelerated by DNA binding, there will be ≤10% decomposition of these ANU–lex compounds to isocyanate during the incubation period. Still, the data reflect the binding of a mixture, and it is not possible to determine the contribution that the isocyanate and other possible hydrolysis products have on the footprinting patterns.

The nonionic ANU–lex compounds, **11a** and **10a**, are both marginal inhibitors of DNA cleavage by MPE·Fe (data not shown), while the protection by the charged compound (**12b**) is clear. A slight shift in the sites protected from MPE cleavage by **12b**, relative to distamycin, indicates that the introduction of a nitrosourea moiety onto the dipeptide causes some qualitative change in the molecule's affinity binding properties. As with distamycin, the compounds show specificity for A-T-rich regions. The staggered protection on the Watson and Crick strands afforded by ligand against MPE·Fe(II) degradation, which is mediated by a poorly diffusible oxidizing species, has been previously noted (Dervan, 1986) and is a consequence of the spatial arrangement of the bases across the B-DNA minor groove (Figure 2).

DNA Alkylation. Before discussing the alkylation of the restriction fragments, it is important to point out a minor discrepancy between the initially assigned sequence of the canine parvovirus (CPV-b) derived plasmid (Rhode, 1985) and our own Maxam–Gilbert sequencing data. We have assigned

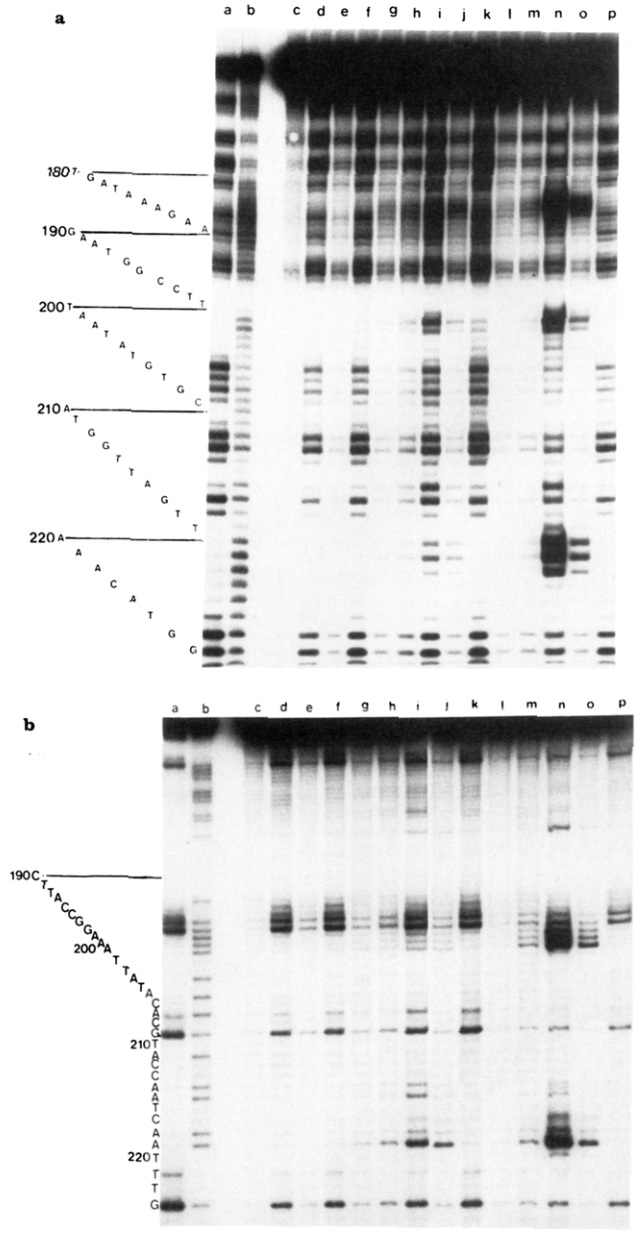

FIGURE 3: Dose–response for cleavage of 85 bp fragment by **7**. (a) Crick strand: lane a, Maxam–Gilbert G; lane b, Maxam–Gilbert G + A; lane c, control; lane d, 500 μM CCNU; lane e, 500 μM CCNU + 100 mM NaCl; lane f, 500 μM CCNU + 100 μM distamycin; lanes g–i, 50, 100, and 500 μM **7b**, respectively; lane j, 500 μM **7b** + 100 mM NaCl; lane k, 500 μM **7b** + 100 μM distamycin; lanes l–n, 50, 100, and 500 μM **7a**, respectively; lane o, 500 μM **7a** + 100 mM NaCl; lane p, 500 μM **7a** + 100 μM distamycin. (b) Watson Strand; lane a, Maxam–Gilbert G; lane b, Maxam–Gilbert G + A; lane c, control; lane d, 500 μM CCNU; lane e, 500 μM CCNU + 100 mM NaCl; lane f, 500 μM CCNU + 100 μM distamycin; lanes g–i, 50, 100, and 500 μM **7b**, respectively; lane j, 500 μM **7b** + 100 mM NaCl; lane k, 500 μM **7b** + 100 μM distamycin; lanes l–n, 50, 100, and 500 μM **7a**, respectively; lane o, 500 μM **7a** + 100 mM NaCl; lane p, 500 μM **7a** + 100 μM distamycin.

position 221 in the 85 bp restriction fragment (Figure 2, Crick strand), which is in a lex binding domain, as a T on the basis of Maxam–Gilbert G, G + A, *T*, and T + C sequencing reactions (data not shown). Originally this base was characterized as a C (Rhode, 1985), although a more recent report of the entire genome of a canine parvovirus strain (CPV-N) is consistent with our data (Reed et al., 1988).

The cleavage patterns in the DNA restriction fragments by CCNU, **7a**, and **7b**, after sequential neutral thermal hydrolysis and piperidine-mediated cleavage at apurinic/apyrimidinic

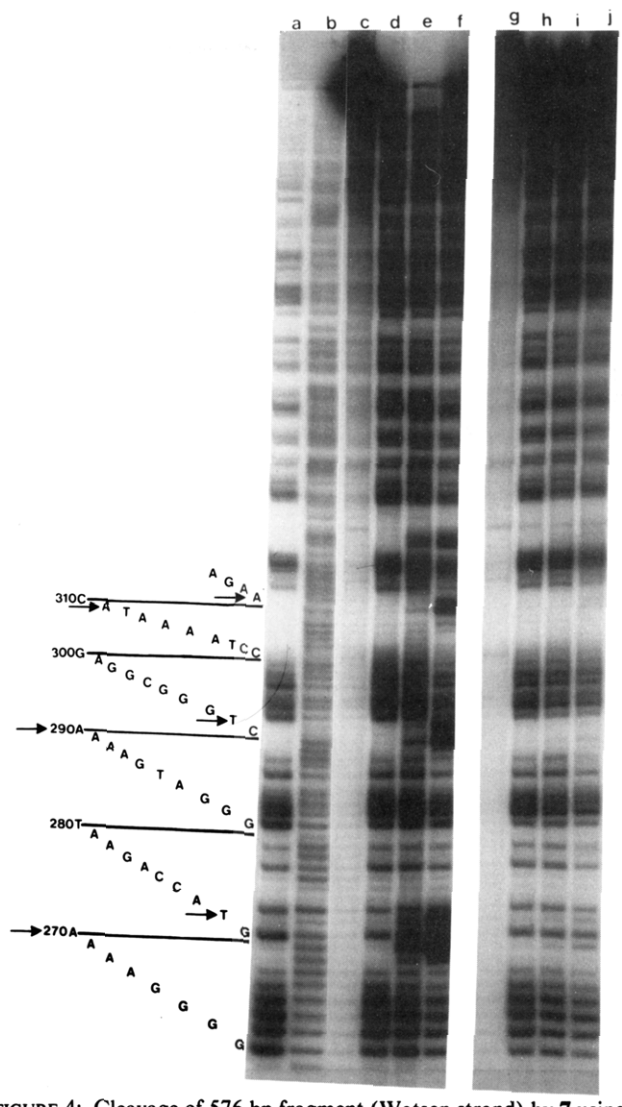

FIGURE 4: Cleavage of 576 bp fragment (Watson strand) by **7** using neutral thermal or direct piperidine cleavage: lane a, Maxam–Gilbert G; lane b, Maxam–Gilbert G + A; lanes c–f, neutral thermal treatment followed by piperidine; lanes g–j, hot piperidine treatment; lanes c and g, control; lanes d and h, 500 μM CCNU; lanes e and i, 500 μM **7b**; lanes f and j, 500 μM **7a**.

sites, was compared at 37 °C in pH 8.0 buffer. DNA cleavage by CCNU, **7a**, and **7b** shows definite dose responses (Figure 3). CCNU produces an alkylation pattern basically restricted to G sites and is similar to that previously reported using direct piperidine treatment (Hartley et al., 1986). The alkylation by CCNU is strongly inhibited by NaCl but only marginally affected by the addition of 100 μM distamycin. It should be noted that neither NaCl nor distamycin qualitatively alters the DNA cleavage patterns induced by CCNU.

Both the cationic and neutral derivatives of **7** also induce fragmentations similar to CCNU at G residues (Figure 3). The band intensities at G sites are stronger for the charged derivative **7b**. However, there are additional bands observed near footprinted DNA sites recognized by **10a**, **11a**, and **12b**, the hydrolytically stable analogues of **7**. In contrast to G cleavage sites, it is apparent that the intensities of these "new" bands at A, T, and C residues are more intense for the neutral analogue **7a**. In addition, these non-G cleavage sites are not observed using G-lane chemistry (Figure 4). The comigration of all of the bands generated by **7** with the Maxam–Gilbert markers suggests that strand breaks induced by these nitrosoureas yield phosphate termini. It is important to point out

that at ligand/DNA ratios of 0.6:1, 1.2:1, and 6:1 the relative band intensities, for G vs non-G sites, are unchanged.

The 85 bp restriction fragment has ANU–lex protection (binding) sites, as indicated by the MPE·Fe(II) footprinting, at 5′-T_{221} TAA, A_{203} TTAA, and T_{185} TAT on the Crick strand (Figure 2a). The non-G bands are associated with these affinity binding regions (Figure 3). For **7b** there are additional bands at A_{215} and A_{214} (Crick strand) further away from the affinity binding site. The relative intensities of these two cleavage sites is the major difference between **7a** and **7b** (Figure 2a). At the lex binding region between A_{203} and A_{199}, the most intense band generated by **7a** is at A_{200}. While **7b** cleaves at the same three A's, the intensities of the bands are essentially equivalent. On the Watson strand, A_{201} and the 3′-A are alkylation sites for both **7a** and **7b**. There is an additional cleavage at A_{183} (Crick strand) in the TTAT sequence. The Watson strand shows strong cleavage at A_{186} and A_{185} (Figure 3b). All of these bands are inhibited by distamycin and only moderately reduced by 100 mM NaCl.

The resolved affinity binding sites in the 576 bp fragment (Watson strand) identified by MPE·Fe footprinting are at A_{271} AGT, A_{293} AAA, and A_{314} AAT (Figure 2b). Non-G cleavage bands occur at A_{270} (most intense), T_{272}, A_{269}, and A_{273} on the Watson strand (Figure 4) and A_{272} on the Crick strand (data not shown). As mentioned above, these A and T cleavage sites are not as sensitive to the inhibitory effect of NaCl as the G sites. Most importantly, all of these non-G cleavages are strongly inhibited by the coaddition of 100 μM distamycin, a concentration that has little effect on G alkylation. Finally, as in the smaller fragment, the neutral compound **7a** is more effective at alkylating these non-G sites and less effective at alkylating G than **7b**.

Time Course. To corroborate the involvement of an affinity binding process, CCNU and **7** were incubated with the restriction fragment for 15, 30, 60, 120, and 240 min at 37 °C and then heated at 90 °C for 15 min to depurinate any thermal labile sites. At the higher temperature, the ligand·DNA complex will dissociate, and any product derived from an affinity mechanism will be effectively quenched. Concomitantly, the base-catalyzed hydrolysis of the "free" **7** will be driven to completion at the elevated temperature. Note that the DNA exposed to ANU is not precipitated and washed prior to the neutral thermal hydrolysis. The results (Figure 5a) confirm these predictions; only non-G sites associated with the cognate sequences of **7** show a time dependency. The band intensities at all G sites are independent of the time of incubation at 37 °C since noncomplexed compound is rapidly hydrolyzed to alkanediazonium ion at the elevated temperatures used to generate the abasic sites. These results indicate that under conditions not conducive to affinity binding, CCNU, **7a**, and **7b** alkylate DNA at G to a similar extent.

These data also address the rate of hydrolysis of **7** while it is complexed to DNA as compared to that in solution. The densitometric analysis of the gel data (Figure 5b) indicates that the alkylation of A_{219} by **7b** plateaus somewhere between 120 and 240 min, which represents ~5–10 half-lives on the basis of the $t_{1/2}$ in solution. In contrast, the alkylation at A_{219} and A_{220} by **7a** approaches a maximum level by 30–60 min (~2 half-lives).

The potential role of the isocyanate decomposition product was evaluated by preincubating **7** for 120 min in buffer prior to the addition of DNA. Under these conditions **7** would be 95% hydrolyzed by the time the [^{32}P]DNA is added. The results (Figure 5a, lanes a, h, and n) show that isocyanate is not responsible for the non-G cleavages. Interestingly, the data

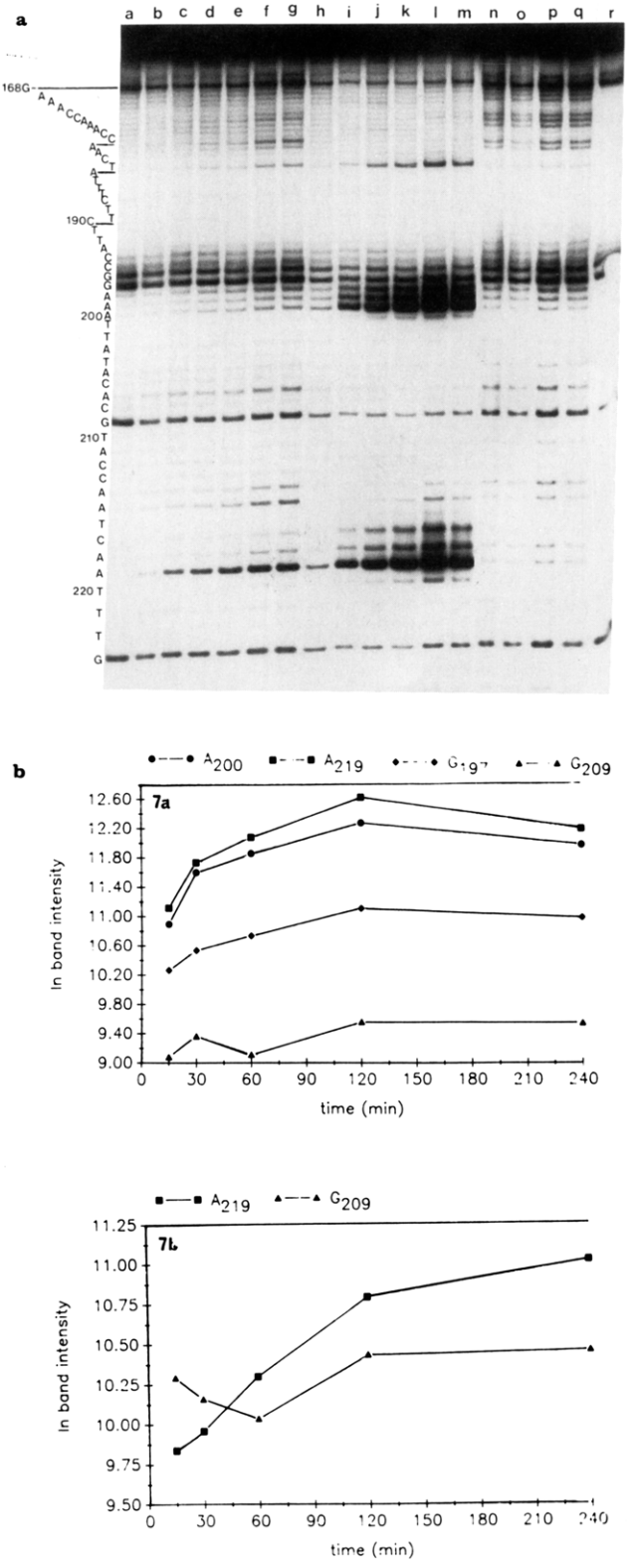

FIGURE 5: (a) Time course for cleavage of 85 bp fragment (Crick strand) by **7** using neutral thermal treatment followed by piperidine (except for lane r): lane a, 500 μM CCNU at 240-min incubation; lane b, 500 μM **7b** preincubated for 120 min in buffer in the absence of DNA, followed by 60-min incubation in the presence of DNA; lanes c–g, 500 μM **7b** at 15-, 30-, 60-, 120-, and 240-min incubation, respectively; lane h, 500 μM **7a** preincubated for 120 min in buffer in the absence of DNA, followed by 60-min incubation in the presence of DNA; lanes i–m, 500 μM **7a** at 15-, 30-, 60-, 120-, and 240-min incubation, respectively; lanes n–r, 120-min incubation with MNU; lane n, 500 μM MNU; lane o, 250 μM MNU; lane p, 1000 μM MNU; lane q, 1000 μM MNU + 100 μM distamycin; lane r, 1000 μM MNU using G-lane chemistry. (b) Densitometric analysis of lanes c–g (**7b**) and i–m (**7a**).

suggest that the respective isocyanates or some other "stable" alkylating agent can contribute to the G-cleavage bands under the conditions employed since the intensities of the G-bands are not decreased upon preincubation in the absence of DNA target, regardless of the Cl-ENU used.

Discussion

Affinity Binding. The DNA affinity binding of the *N*-methylpyrrolecarboxamide-based lexitropsins, e.g., distamycin and netropsin, has been characterized by single-crystal X-ray diffraction (Kopka et al., 1985; Col et al., 1987), NMR (Patel, 1982; Patel & Shapiro, 1985; Pelton & Wemmer, 1988), footprinting (Van Dyke et al., 1982; Lane et al., 1983; Portugal & Waring, 1987), sequence-specific DNA cleavers (Schultz et al., 1982; Taylor et al., 1984), and calorimetry (Marky & Breslauer, 1987b). The consensus of this literature is that the concave face of the peptide binds in the minor groove at A-T-rich sites via H-bonds and van der Waals contacts. Previous lexitropsins based on the *N*-methylpyrrolecarboxamide monomer have at least one cationic group, e.g., guanidine, amidine, or tertiary amine, at the carboxyl end of the peptide. Netropsin is a dicationic dipeptide with guanidinium and amidine ends. The electrostatic interaction of the ionized terminus with DNA contributes chiefly to the binding entropy and is generally thought to play no role in the sequence specificity. However, sequence-related changes in hydration of the minor groove can possibly cause entropic interactions to show sequence specificity (Breslauer et al., 1987).

The nitrosourea-linked lexitropsins also bind at A-T-rich runs but recognize somewhat different sequences relative to distamycin (data not shown). The data also indicate that the nitrosoureas are less efficient at protecting their binding sites from the MPE·Fe(II) generated damage. This could be a function of the strong dipole associated with the nitrosourea moiety. The neutral analogues **10a** and **11a** are weak binders, presumably because there is no electrostatic contribution to the affinity binding.

Non-Guanine Alkylation Reactions. The hydrolytic decomposition of *N*,*N*′-dialkyl-*N*-nitrosoureas yields an alkyl isocyanate and an alkanediazotate, the latter being rapidly converted to the alkanediazotic acid by protonation at physiological pH (Streitweiser, 1957; White & Woodcock, 1968; Friedman, 1970; Moss, 1974; Kirmse, 1976). Depending on the structure of the *N*-alkyl substituent, the diazotic acid ionizes to an alkanediazonium ion and/or a nitrogen-separated ion pair (White & Woodcock, 1968; Moss, 1974; Gold et al., 1984; Smith et al., 1985; Church & Gold, 1988). These carbocationic-like species are responsible for the observed in vivo and in vitro alkylation of DNA by *N*-nitroso compounds (Kriek & Emmelot, 1963; O'Connor et al., 1972; Magee et al., 1975; Singer, 1976; Beranek et al., 1980). The chemistry of Cl-ENU is somewhat more complicated as both 2-chloroethyl and 2-hydroxyethyl adducts are isolated (Ludlum et al., 1975; Gombar et al., 1980; Ludlum, 1987; Carter et al., 1988). It has been proposed that the 2-hydroxyethylation products are formed from 1,2,3-oxadiazoline that is derived from the intramolecular cyclization of the initial 2-chloroethanediazotate intermediate, while the 2-chloroethyl products come directly from the diazonium ion or related species (Weinkam & Lin, 1979; Lown & Chauhan, 1982; Lown et al., 1886). In no case has a Cl-ENU analogue been reported to modify A sites in polynucleotides, even when poly(A) was used as the target (Ludlum et al., 1975). Chloroethylation at N1-A has been reported in the reaction of adenosine with *N*,*N*′-bis(chloroethyl)-*N*-nitrosourea (Tong & Ludlum, 1979). The relative scarcity of modification at A by Cl-ENU's is somewhat surprising since alkylation of DNA by MNU and ENU yields a significant amount (~10%) of *N*3-alkyl-A (Singer et al., 1978; Swenson & Lawley, 1978). Alkylation at N3-T and N3-C are relatively minor processes (Ludlum et al., 1975; Gombar et al., 1980; Carter et al., 1988).

The pattern and relative intensities of alkylation sites generated by **7a** and **7b** differ somewhat. **7a** has an obvious bias toward alkylation at one end of each of its binding regions, while this effect is less pronounced with **7b**. It is assumed that the nitrosourea moiety is selectively oriented within each binding region and upon hydrolysis the alkanediazonium ion, which reacts at near diffusion-controlled rates (White et al., 1975; Gold & Linder, 1979; White et al., 1981), alkylates adjacent nucleophilic sites. This orientational preference may reflect either structural features of the dipeptide ligand, including the carboxyl terminus, or some negative interaction between the nitrosourea and minor groove atoms. Since the ANU–lex compounds are all weaker binders than distamycin, no favorable interaction between the nitrosourea functionality and DNA is likely.

The pattern of non-G alkylation at individual lex binding domains may result from diffusion of the electrophilic intermediate within the minor groove and/or slippage of the dipeptide within the binding site. The equivalent cleavage at A_{200}–A_{198} and the strong selectivity for reaction at A_{219} vs A_{218} with **7b** is most easily reconciled with ligand slippage, although some diffusion of the diazonium ion is expected. The cleavage pattern for **7a**, which is different in that the equivalent reaction in the A_{200}–A_{198} run is not observed, may also be explained by heterogeneous affinity binding that delivers alkanediazonium ion at different concentrations to different sites.

The binding alignment of DNA-cleaving lex compounds bearing an EDTA·Fe(II) functionality has been previously studied (Taylor et al., 1984; Schultz & Dervan, 1984). The general observation is that the predominant orientation situates the charged dialkylamino group attached to the carboxyl terminus of the polypeptide near the 5′-end of the T-rich strand in the affinity binding site (Taylor et al., 1984). This binding geometry prevails over the opposite binding mode by >2:1. In somewhat related work, the reaction of an *N*-(bromoacetyl)-lex with a restriction fragment has been reported (Baker & Dervan, 1985, 1989). This tripeptide, which is also based on *N*-methylpyrrolecarboxamide monomer and bears a dimethylamino group on the carboxyl terminus, gave one strong (5′-T<u>A</u>AAC) and one weak (5′-<u>A</u>AATT) thermally labile site at a single A within two of its affinity binding domains. Cleavage at these sites is consistent with the orientation predicted from the results of the DNA-cleaving lexitropsins; the charged dimethylamino group will be located toward the 5′-end of the T-rich strand. NMR data for the binding of distamycin to an 5′-AAATT site shows a 1.5:1 preference for this alignment (Pelton & Wemmer, 1988). Similar studies with 5′-AAAT and 5′-TTTTT sites indicate an even stronger preference for a similar orientation (Schultz & Dervan, 1984; Taylor et al., 1984; Wemmer, unpublished data). By analogy, **7b** should complex with DNA so that the nitrosourea moiety is located near A_{224} on the Watson strand (Figure 2). If **7b** does adopt this mode of binding, it certainly is not productive in terms of DNA alkylation. On the basis of only the alkylation pattern, the main orientation that yields DNA strand breaks places the formation of the alkanediazonium ion near A_{219} on the Crick strand. Due to the structure of B-DNA, this would also situate the reactive intermediate near the A_{221} on the Watson strand (Figure 2). In fact, A_{221} is the strongest band in the Watson strand (Figure

3b). Similarly, **7b** should bind so that the nitrosourea is located near A_{267} in the 576 bp fragment and point toward the $G_{263-266}$ run. If **7b** does adopt this mode of binding, it does not afford DNA alkylation. Again, on the basis of only the alkylation pattern, the main orientation that yields DNA strand breaks places the alkanediazonium ion somewhere between A_{270} and T_{272} on the Watson strand and near A_{272} on the Crick strand (Figure 2). Obviously, attempts to relate the binding orientation of **7b** with its nitrosourea functionality to lexitropsins with chelated metal complexes may be irrelevant. The results do indicate the potential risk in predicting orientations of ligands within binding sites.

At ligand/DNA ratios >1, which were used in this study, the binding sites may accommodate two lexitropsins in a parallel head-to-tail sandwich complex (Pelton & Wemmer, 1989). Thus, a second lex molecule could populate the least preferred orientation that places the nitrosourea near the bases that are cleaved. This is an unlikely scenario since it implies that only the second Cl-ENU–lex hydrolyzes to diazonium ion.

An alternative explanation for the data is that the nucleophilic reactivities of the different A residues in the restriction fragments may vary as a function of sequence and that the reactivities of the A's, rather than ligand orientation, drive the observed alkylation pattern. On the basis of sequencing gel experiments with MNU (Figure 5a, lanes n–q), methylation at N3-A shows no strong sequence dependence. Therefore, the observed alkylation pattern, presumably at N3-A, produced by **7** is probably not a result of intrinsic nucleophilic differences in N3-A.

Guanine Alkylation Reactions. As noted earlier, alkyl isocyanates are products formed in the hydrolysis of N,N′-disubstituted nitrosourea, e.g., **7**. While this byproduct may show some weak DNA alkylating activity, it cannot be responsible for the sequence-specific non-G bands in the sequencing gels because partial hydrolysis of **7** to the isocyanate, prior to adding the DNA, significantly reduces DNA cleavage at the A, T, and C residues associated with ANU–lex binding sites (Figure 5a). It was assumed that the bands derived from cleavages at G would involve the same alkylating agent that is responsible for the non-G cleavages. *However, preincubation of the Cl-ENU's in pH 8.0 Tris buffer at 37 °C for 120 min before adding the ^{32}P-labeled restriction fragment had no effect on the intensities of the G-bands.* Since the Cl-ENU's should be ~95% hydrolyzed under these conditions, a "stable" product must be produced that alkylates the DNA. It is clear that the G-cleavages are not an artifact of heating at 90 °C during the neutral thermal hydrolysis procedure since precipitation and washing the DNA prior to the neutral thermal hydrolysis had no effect on the intensities of the G-bands (Chen and Gold, unpublished results). Indeed, no similar stable alkylating agent is formed from the hydrolysis of MNU or *N*-methyl-*N*′-cyclohexyl-*N*-nitrosourea (Chen and Gold, unpublished results). The latter compound hydrolyzes to cyclohexyl isocyanate, and this provides additional evidence that the G-cleavage bands from CCNU and Cl-ENU–lex are not derived from the reaction of an isocyanate with DNA. These results provide strong evidence that Cl-ENU can alkylate G via intermediates other than alkanediazonium ions or related carbocation species, at least under some of the reactions conditions employed.

A model to describe the relationship between affinity binding and DNA alkylation is proposed (Figure 6). The distamycin inhibition of the alkylation at non-G residues by **7** and the proximity of the non-G cleavage sites to footprinted binding regions are consistent with this mechanism (upper half). The

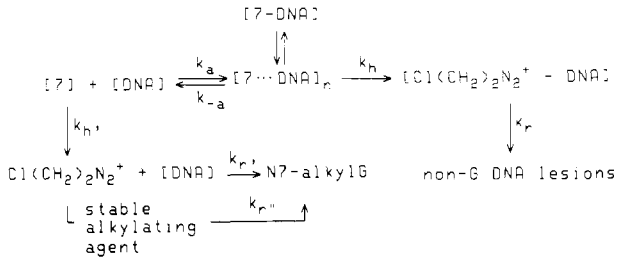

FIGURE 6: Scheme of pathways involved in DNA alkylation of **7**: k_a and k_{-a} are on–off rates of ligand; k_h and k_h' are rates of hydrolysis of affinity bound and nonbound **7**; k_r and k_r' are the respective rates of DNA modifications by alkylating agent derived from affinity bound and nonbound **7**; k_r'' is the rate of DNA modifications generated from the "stable" alkylating agent derived from hydrolysis of **7**.

footprinting data indicate that k_a/k_{-a} is larger for **7b** than for **7a**. Since the observed k_h' in the absence of DNA is essentially the same for both compounds, the formation of non-G lesions should be higher for **7b** and the relative yield of *N*7-alkyl-G, formed by a nonaffinity binding mechanism, higher for **7a**. The opposite is observed. The following are assumed: (1) the alkylation at non-G sites by both **7a** and **7b** involves the same electrophilic intermediate; (2) k_r and k_r' are equivalent for both compounds; and (3) the rate of hydrolysis of unbound drug (k_h') is the same for **7a** and **7b**. Therefore, the data imply that the k_h'/k_h ratio differs for **7a** and **7b**. This explanation is not unreasonable because the stronger association of **7b** with DNA could restrict base attack on the nitrosourea. Molecular modeling indicates that the nitrosourea group is relatively inaccessible upon tight complexation with DNA. This explanation is also consistent with the observed time course study (Figure 5a) that shows a rate of DNA alkylation for **7b** that is slower than predicted. Thus, it may be that the generation of reactive electrophile (alkanediazonium ion) from **7** occurs only after partial release of the dipeptide from the floor of the minor groove. This resulting loosely associated compound then hydrolyzes to the diazonium ion that alkylates the adjacent nucleophilic DNA atoms. This would explain why **11a** does not alkylate DNA at non-G sites and why N7-G alkylation by **11a** is enhanced in the presence of distamycin (data not shown). As commented on earlier, the hydrolysis of **11a** is relatively slow in the absence of DNA. According to the proposed scheme (Figure 6), the rate of hydrolysis of **11a** must be somewhat competitive with its migration out of the minor groove. If this is not the case and tightly bound **11a**–DNA complex does not hydrolyze, then no affinity binding derived alkylation will be observed. However, the addition of distamycin displaces **11a** from its affinity binding site, and its subsequent hydrolysis in solution yields methanediazonium ion. Accordingly, the methylation pattern generated by **11a**, which is restricted to G, is the same as that for MNU and is strongly inhibited by salt and other DNA affinity binding cations.

DNA Lesions. The adducts that are responsible for the thermally labile sites have not been structurally characterized. The strong intensity of the A cleavage sites under neutral thermal conditions is consistent with a minor groove modified *N*3-alkyl-A. Conversion of the *N*3-alkyl-A lesion, by heating at neutral pH, to an apurinic site that is subsequently converted into a s-s break with 5′- and 3′-phosphate termini by hot piperidine treatment is compatible with the electrophoretic mobility of the bands. The weak intensity of these bands by use of piperidine, without prior depurination, eliminates an *N*7-alkyl-A structure (Mattes et al., 1986b). The possibility that phosphotriesters (Carter et al., 1988) are responsible for the DNA cleavage is remote since fragments with two distinct termini and different electrophoretic mobilities would be

generated upon hydrolysis. In fact, the 2-hydroxyethyl phosphotriester lesion is unstable and would generate "spontaneous" s-s breaks (Müller et al., 1987). No breaks are observed in DNA not subjected to elevated temperature (data not shown). No evidence for multiple termini is observed.

O^2-Alkyl-C modifications are thermally unstable products that rapidly depyrimidinate at 100 °C; $t_{1/2} < 5$ min (Singer et al., 1978). It is tempting to propose that the bands at C are a consequence of alkylating the minor groove O^2-position. Previously, alkylation at N3-C has been reported in low yield from Cl-ENU (Gombar et al., 1980; Carter et al., 1988), and this type of substitution does make the *N*-glycosyl bond more labile to hydrolysis (Beranek et al., 1980; MacFarland et al., 1990).

The nature of the lesions at T that yield DNA breaks is also speculative. An O^2-alkyl-T adduct is reasonable because the O^2 atom points into the minor groove site, and at 100 °C in pH 7.0 buffer, O^2-ethyl-T has a half-life in DNA of 2.7 h (Singer et al., 1978). O^2-Ethyl-T is also a major product of the reaction of DNA with ENU (~7% of the total ethylated adducts) (Singer, 1976). Previous reports on the alkylation at N3-T by Cl-ENU suggest that this modification is not thermally labile. Additional characterization of all the lesions is planned.

Conclusions

The strong inhibition by distamycin and sequence-selective alkylation pattern demonstrate that the affinity binding properties of **7** are responsible for the non-G lesions near affinity binding domains. The failure of CCNU to yield any detectable lesions at A, T, and C in the absence or presence of distamycin indicates that the high concentration of alkylating agent in the minor groove, because of affinity binding processes, "forces" reactions that normally do not occur to any significant extent. Thus, a major factor responsible for the site(s) of DNA modification by alkylating agents (major vs minor groove) may be associated with the relative concentrations of parent compound and/or its rate of hydrolysis in the two grooves. It is not clear whether this effect reflects the relative hydrophobicity, ionic strength, or microenvironmental pH's of the two grooves. The utility of these novel alkylating agents to serve as probes of the biological role of different DNA lesions and as chemotherapeutic agents is presently being studied.

Acknowledgments

We are grateful to Dr. Dan Williamson for helping in the design of the phase-sensitive heteronuclear bond-correlation NMR experiments and to Dr. Phillip Issenberg and David Cassada for the mass spectroscopy data. The parvovirus plasmid was originally provided by Dr. Solon Rhode. We thank Dr. David Wemmer for providing unpublished NMR data and Dr. Peter Dervan for the MPE. We acknowledge a grant from BioDesign Inc. which made available the Biograf software used in the molecular modeling work.

References

Baker, B. F., & Dervan, P. B. (1985) *J. Am. Chem. Soc. 107*, 8266–8268.

Baker, B. F., & Dervan, P. B. (1989) *J. Am. Chem. Soc. 111*, 2700–2712.

Beranek, D. T., Weis, C. C., & Swenson, D. H. (1980) *Carcinogenesis 1*, 595–606.

Boivin, J. L., & Boivin, P. A. (1951) *Can. J. Chem. 29*, 478–481.

Breslauer, K. J., Remeta, D. P., Chou, W.-Y., Ferrante, R., Curry, J., Zaunczkowski, D., Synder, J. G., & Marky, L. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8922–8926.

Briscoe, W. T., & Duarte, S. P. (1988) *Biochem. Pharmacol. 37*, 1061–1066.

Buckley, N., & Brent, T. P. (1988) *J. Am. Chem. Soc. 110*, 7520–7529.

Carter, C. A., Kirk, M. C., & Ludlum, D. B. (1988) *Nucleic Acids Res. 16*, 5661–5672.

Church, K. M., & Gold, B. (1988) *Chem. Res. Toxicol. 1*, 269–273.

Coll, M., Frederick, C. A., Wang, A. H.-J., & Rich, A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8385–8389.

D'Andrea, A. D., & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4120–4124.

Dervan, P. B. (1986) *Science (Washington, D.C.) 232*, 464–471.

Erickson, L. C., Laurent, G., Sharkey, N. A., & Kohn, K. W. (1980) *Nature (London) 288*, 727–729.

Friedman, L. (1970) in *Carbonium Ions* (Olah, G. A., & Schleyer, P. v. R., Eds.) Vol. II, pp 655–713, Wiley-Interscience, New York.

Garrett, E. R., Goto, S., & Stubbins, J. F. (1965) *J. Pharm. Sci. 54*, 119–123.

Gold, B., & Linder, W. (1979) *J. Am. Chem. Soc. 101*, 6722–6723.

Gold, B., Despande, A., Linder, W., & Hines, L. (1984) *J. Am. Chem. Soc. 106*, 2072–2077.

Gombar, C. T., Tong, W. P., & Ludlum, D. B. (1980) *Biochem. Pharmacol. 29*, 2639–2643.

Hartley, J. A., Gibson, Kohn, K. W., & Mattes, W. B. (1986) *Cancer Res. 46*, 1943–1947.

Hertzberg, R. P., & Dervan, P. B. (1984) *Biochemistry 23*, 3934–3945.

In Prestayko, A. W., Crooke, S. T., Baker, L. H., Carter, S. K., & Schein, P. S. (1981) *Nitrosoureas: Current Status and New Developments*, Academic Press, New York.

Kirmse, W. (1976) *Angew. Chem. 88*, 273–283.

Konakahara, T., Wurdeman, R. L., & Gold, B. (1988) *Biochemistry 27*, 8606–8613.

Kopka, M. L., Yoon, C., Goodsell, D., Pjura, P., & Dickerson, R. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1376–1380.

Kriek, E., & Emmelot, P. (1963) *Biochemistry 2*, 733–740.

Kriek, E., & Emmelot, P. (1964) *Biochim. Biophys. Acta 91*, 59–66.

Lane, M. J., Dobrowiak, J. C., & Vournakis, J. N. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3260–3264.

Levin, V. A., & Wilson, C. B. (1976) *Cancer Treat. Rep. 60*, 719–724.

Lown, J. W., & Chauhan, S. M. S. (1982) *J. Org. Chem. 47*, 851–856.

Lown, J. W., & Krowicki, K. (1985) *J. Org. Chem. 50*, 3774–3779.

Lown, J. W., Koganty, R. R., Bhat, U. G., Sapse, A.-M., & Allen, E. B. (1986) *Drugs Exp. Clin. Res. 12*, 463–473.

Ludlum, D. B. (1987) *Pharm. Ther. 34*, 145–153.

Ludlum, D. B., Kramer, B. S., Wang, J., & Fenselau, C. (1975) *Biochemistry 14*, 5480–5485.

MacFarland, J. G., Kirk, M. C., & Ludlum, D. B. (1990) *Biochem. Pharmacol. 39*, 33–36.

Magee, P. N., Nicoll, J. W., Pegg, A. E., & Swann, P. F. (1975) *Biochem. Soc. Trans. 1975*, 62–65.

Marky, L. A., & Breslauer, K. J. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8922–8926.

Marky, L. A., & Breslauer, K. J. (1987b) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4359–4363.

Martinez, J., Oiry, J., Imbach, J. L., & Winternitz, F. (1982) *J. Med. Chem. 25*, 178–182.
Mattes, W. B., Hartley, J. A., & Kohn, K. W. (1986a) *Nucleic Acids Res. 14*, 2971–2987.
Mattes, W. B., Hartley, J. A., & Kohn, K. W. (1986b) *Biochim. Biophys. Acta 868*, 71–76.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.
Moss, R. A. (1974) *Acc. Chem. Res. 7*, 421–427.
O'Connor, P. J., Capps, M. J., Lawley, P. D., & Shah, S. A. (1972) *Biochem. J. 129*, 519–528.
Patel, D. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6424–6428.
Patel, D. J., & Shapiro, L. (1986) *J. Biol. Chem. 261*, 1230–1240.
Pelton, J. G., & Wemmer, D. E. (1988) *Biochemistry 27*, 8088–8096.
Pelton, J. G., & Wemmer, D. E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5723–5727.
Portugal, J., & Waring, M. J. (1987) *FEBS Lett. 225*, 195–200.
Reed, A. P., Jones, E. V., & Miller, T. J. (1988) *J. Virol. 62*, 266–276.
Rhode, S. L., III (1985) *J. Virol. 54*, 630–633.
Schultz, P. G., Taylor, J. S., & Dervan, P. B. (1982) *J. Am. Chem. Soc. 104*, 6861–6863.
Singer, B. (1976) *Nature (London) 264*, 333–339.
Singer, B., Kröger, M., & Carrano, M. (1978) *Biochemistry 17*, 1246–1250.
Smith, R. H., Jr., Koepke, S. R., Tondeur, Y., Denlinger, C. L., & Michejda, C. J. (1985) *J. Chem. Soc., Chem. Commun. 1985*, 936–937.
Snyder, J. K., & Stock, L. M. (1980) *J. Org. Chem. 45*, 1990–1999.
Streitweiser, A., Jr. (1957) *J. Org. Chem. 22*, 861–869.
Swenson, D. H., & Lawley, P. D. (1978) *Biochem. J. 171*, 575–587.
Taylor, J. S., Schultz, P. G., & Dervan, P. B. (1984) *Tetrahedron 40*, 457–465.
Tong, W. P., & Ludlum, D. B. (1979) *Biochem. Pharmacol. 28*, 1175–1179.
Tong, W. P., Kohn, K. W., & Ludlum, D. B. (1982a) *Cancer Res. 42*, 3102–3105.
Tong, W. P., Kirk, M. C., & Ludlum, D. B. (1982b) *Cancer Res. 42*, 4460–4464.
Tong, W. P., Kirk, M. C., & Ludlum, D. B. (1983) *Biochem. Pharmacol. 32*, 2011–2015.
Van Dyke, M. W., Hertzberg, R. P., & Dervan, P. B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5470–5474.
Warpehoski, M. A., & Hurley, L. H. (1988) *Chem. Res. Toxicol. 1*, 315–333.
Weinkam, R. J., & Lin, H.-S. (1979) *J. Med. Chem. 22*, 1193–1198.
Werner, E. A. (1919) *J. Chem. Soc. 115*, 1093–1102.
White, E. H., & Woodcock, D. J. (1968) in *The Chemistry of the Amino Group* (Patai, S., Ed.) pp 440–483, Wiley-Interscience, New York.
White, E. H., Roswell, D. F., Politzer, I. R., & Branchini, B. R. (1975) *J. Am. Chem. Soc. 97*, 2290–2291.
White, E. H., Jelinski, L. W., Politzer, I. R., Branchini, B., & Roswell, D. F. (1981) *J. Am. Chem. Soc. 103*, 4231–4239.
Williamson, D. S., Smith, R. A., Nagel, D. L., & Cohen, S. M. (1989) *J. Magn. Reson. 82*, 605–612.
Wurdeman, R. L., & Gold, B. (1988) *Chem. Res. Toxicol. 1*, 146–147.
Wurdeman, R. L., Church, K. M., & Gold, B. (1989) *J. Am. Chem. Soc. 111*, 6408–6412.