Functional Analogues of Bleomycin: DNA Cleavage by Bleomycin and Hemin–Intercalators

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ABSTRACT: New hemin–intercalators (Hem-G's) that cleave DNA were synthesized, on the basis of 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) as an intercalator moiety. Hem-G's, which possess an intramolecular ligand of the ferrous ion (a histidine or imidazole moiety), cleave DNA very efficiently and act at guanine–pyrimidine sequences preferentially. Bleomycin (BLM) also cleaved DNA with the same base-sequence selectivity shown by Hem-G's. The 5′-terminus of the DNA fragments cleaved by Hem-G's or by BLM is a phosphoryl group, while the 3′-terminus of the cleaved DNA fragments does not possess a 3′-phosphoryl group. There are more than three kinds of 5′-end ^{32}P-labeled DNA fragments, which can be substrates of terminal deoxynucleotidyl transferase (TdT). One of the 3′-termini of the cleaved DNA fragments is a 3′-hydroxy group. The mobility of the 3′-end ^{32}P-labeled DNA fragment cleaved by Hem-G's or by BLM corresponds to the removal of pyrimidine bases having guanine at the 5′-side. The mobility of one kind of the cleaved 5′-end ^{32}P-labeled DNA fragments corresponds to the removal of guanine having pyrimidine at the 3′-side, followed by 3′-dephosphorylation. We propose that there exist plural mechanisms for DNA cleavage by Hem-G's or by BLM. The deduced structures of the cleaved DNA fragments suggest that one of the mechanisms involves deletion of two nucleotide units from DNA.

There are many antitumor agents that have been established to cleave DNA. Bleomycin (BLM) is one of the most potent DNA-cleaving agents. BLM is known to degrade cellular DNA in a reaction requiring Fe(II) and O_2 as cofactors (Umezawa & Takita, 1980). Recent evidence strongly suggests that the ultimate agent of DNA damage is some form of activated reduced oxygen, produced as a consequence of oxidation of BLM-chelated Fe(II) to Fe(III) in a quaternary DNA–BLM–Fe–O_2 complex (Burger et al., 1983; Grollmann & Takeshita, 1980; Takeshita et al., 1978). BLM could be regarded as consisting of the following three parts: (i) DNA-recognizing part [bis(thiazole) moiety], (ii) Fe-chelating part (oligopeptide moiety), and (iii) sugar part. The structural requirement for DNA-cleaving ability of BLM is considered to be the bis(thiazole) moiety and the Fe-chelating part (Sugiura et al., 1983).

Recently, we reported the synthesis of porphyrin (Fe)–intercalators that possess strong DNA-cleaving ability (Hashimoto et al., 1983). The structures of the porphyrin (Fe)–intercalators were designed on the basis of a consideration of the function and the structure of BLM. The compounds consist of an intercalator moiety that recognizes the DNA molecule and a porphyrin (Fe) moiety that can activate oxygen. The base-sequence selectivity of the DNA cleavage with the porphyrin (Fe)–intercalators was shown to be very similar to that of BLM (Hashimoto et al., 1984c).

Similar methodology for designing DNA-cleaving agents has recently been reported. Dervan et al. reported that ethylenediaminetetraacetic acid (EDTA) covalently connected with molecules that possess affinity for DNA [such as methidium (Hertzberg & Dervan, 1982), distamycin analogues (Schultz et al., 1982; Schultz & Dervan, 1983; Taylor et al., 1984), and oligonucleotides (Dreyer & Dervan, 1985)] cleave DNA efficiently. Methidiumpropyl-EDTA cleaves DNA efficiently in a reaction dependent on ferrous ion and oxygen (Hertzberg & Dervan, 1982, 1984). It cleaves DNA in a relatively non-sequence-specific manner. Distamycin–EDTA's and penta-*N*-methylpyrrolecarboxamide–EDTA cleave DNA in the presence of ferrous ion at adenine–thymine-rich regions (Schultz et al., 1982; Schultz & Dervan, 1983; Taylor et al., 1984). Lown and Joshua (1982) reported that acridine–hemine also cleaves DNA.

In this paper, we report (i) the design and synthesis of new DNA-cleaving hemin–intercalators (Hem-G's) as functional analogues of BLM, (ii) the sequence selectivity of DNA cleavage by Hem-G's and by BLM, (iii) the terminal analysis of DNA fragments produced by Hem-G's and by BLM, and (iv) the mechanism of the DNA-cleaving reaction with Hem-G's and BLM (two nucleotide unit deletion mechanism).

Materials and Methods

Materials. Bleomycin (BLM) (sulfate) was purchased from Sigma Chemical Co. Plasmid DNA pBR322, T4 polynucleotide kinase, and Klenow fragment of DNA polymerase I were from Boehringer Mannheim. Other enzymes and nucleotides were from Wako Co. Labeled compounds were from Amersham.

Alkylamino-Glu-P-1 (**1**). 2-Amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) (1 equiv) was mixed with bromoalkylamine hydrobromide salt (2 equiv) and heated to the melting point (ca. 200 °C) for 30 min. The mixture was cooled, and then water was added. The resulting solution was washed with EtOAc, and then the pH of the aqueous phase was adjusted to 11–12 with KOH. The mixture was extracted with EtOAc, and the organic layer was dried over Na_2SO_4 and then evaporated. The residue was recrystallized from EtOH containing HBr to give alkylamino-Glu-P-1 as the HBr salt. **1a** gave a 64% yield. Anal. Calcd for $C_{13}H_{19}N_5OBr_2$: C, 37.08; H, 4.55; N, 16.63. Found: C, 37.07; H, 4.32; N, 16.51. **1b** gave an 82% yield and mp 283–288 °C. Anal. Calcd for $C_{14}H_{19}N_5Br_2$: C, 40.32; H, 4.59; N, 16.77. Found: C, 40.56; H, 4.67; N, 16.32. **1c** gave a 69% yield. Anal. Calcd for $C_{15}H_{21}N_5Br_2$: C, 41.78; H, 4.91; N, 16.24. Found: C, 41.66; H, 5.08; N, 15.95.

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HnG$_2$ (*n* = *2–4*). Hemin chloride (1 equiv) was dissolved in dry pyridine (ca. 20 mg/mL), and pivaloyl chloride (2.2 equiv) was added slowly. Then, **1** (2 equiv) was added, and the mixture was stirred at room temperature for 30 min. The reaction mixture was concentrated under reduced pressure and then separated by silica gel column chromatography (EtOAc–MeOH–NH_4OH). The fractions containing H*n*G$_2$ were evaporated, and the residue was reprecipitated from a mixture of pyridine and aqueous HCl. H2G$_2$ gave a 39% yield and Fab M^+ 1063 (calcd 1063.07). Anal. Calcd for $C_{60}H_{58}N_{14}O_2FeCl\cdot4HCl\cdot3H_2O$: C, 55.50; H, 5.28; N, 15.10. Found: C, 55.74; H, 4.89; N, 14.61. H3G$_2$ gave a 45% yield and Fab M^+ 1091 (calcd 1091.12). Anal. Calcd for $C_{62}H_{62}N_{14}O_2FeCl\cdot8HCl\cdot C_5H_5N$: *C*, 53.24; H, 5.00; N, 13.90. Found: C, 53.28; H, 4.97; N, 13.76. H4G$_2$ gave a 26% yield and Fab M^+ 1119 (calcd 1119.17). Anal. Calcd for $C_{64}H_{66}N_{14}O_2FeCl\cdot4HCl\cdot6H_2O$: C, 54.57; H, 5.86; N, 13.76. Found: C, 54.70; H, 5.33; N, 13.24.

HnG (*n* = *2–4*). H*n*G was prepared as described for the preparation of H*n*G$_2$ except that the amounts of added pivaloyl chloride and **1** were reduced to 1.1 and 0.75 equiv, respectively. H2G gave a 48% yield and Fab M^+ 839 (calcd 839.78). Anal. Calcd for $C_{47}H_{45}N_9O_3FeCl\cdot4HCl\cdot3H_2O$: C, 52.56; H, 5.16; N, 11.74. Found: C, 52.44; H, 4.92; N, 11.82. H3G gave a 28% yield and Fab M^+ 853 (calcd 853.83). Anal. Calcd for $C_{48}H_{47}N_9O_3FeCl\cdot2HCl\cdot H_2O$: C, 58.81; H, 5.25; N, 12.86. Found: C, 58.79; H, 5.10; N, 11.81. H4G gave a 26% yield and Fab M^+ 867 (calcd 867.85). Anal. Calcd for $C_{49}H_{49}N_9O_3FeCl\cdot2HCl\cdot2H_2O$: C, 58.14; H, 5.47; N, 12.45. Found: C, 57.48; H, 5.07; N, 11.52.

H4G-His and H4G-Im. H4G (1 equiv) was dissolved in dry pyridine (ca. 10 mg/mL) and pivaloyl chloride (1.2 equiv) was added. Then, histidine methyl ester (1.2 equiv) or (aminopropyl)imidazole (1.2 equiv) was added. The mixture was stirred at room temperature for 1 h and then separated by silica gel column chromatography (EtOAc–MeOH–NH_4OH). The fractions containing H4G-His or H4G-Im were evaporated, and the residue was reprecipitated from a mixture of pyridine and aqueous HCl. H4G-His gave a 42% yield and Fab M^+ 1019 (calcd 1019.02). Anal. Calcd for $C_{56}H_{58}N_{12}O_4FeCl\cdot6HCl\cdot C_5H_5N$: C, 56.70; H, 5.38; N, 14.09. Found: C, 56.54; H, 5.30; N, 14.52. H4G-Im gave a 36% yield and Fab M^+ 975 (calcd 975.01). Anal. Calcd for $C_{55}H_{58}N_{12}O_2FeCl\cdot8HCl\cdot6H_2O$: C, 46.34; H, 5.15; N, 11.93. Found: C, 46.01; H, 4.89; N, 11.85.

Reactions of pBR322 with Hem-G's and with BLM. Plasmid DNA pBR322 (0.5 μg) was dissolved in 9 μL of a reaction buffer [4 mM $Na_2S_2O_4$, 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.8), 50 mM NaCl]. To this solution, 1 μL of dimethylformamide (DMF) solution of Hem-G (0.1–1 mM) or 1 μL of aqueous solution of BLM containing 1 mM $FeCl_2$ was added. The mixture was incubated for 0.5–2 h at 20 or 37 °C. Then, the incubation mixture was mixed with 10 μL of sample loading buffer (pH 8.05, 50% glycerin, 100 mM Tris, 40 mM NaOAC, 36 mM NaCl, 0.01% bromophenol blue) (Maniatis et al., 1982). Finally, 5 μL of the mixture was analyzed by 0.7% agarose gel electrophoresis (120 V; pH 8.05, 50 mM Tris, 20 mM NaOAC, 18 mM NaCl; migration distance of bromophenol blue 5.0 cm). The gel was stained with 0.5 μg/mL ethidium bromide and then quantitated by densitometry.

Reactions of ^{32}P-End Labeled DNA with Hem-G's and with BLM. ^{32}P-End labeled DNA fragments of defined sequences were obtained from pBR322. A 375 base pair 5′-end labeled DNA fragment was prepared by the cleavage of the plasmid with *Eco*RI followed by removal of the 5′-phosphoryl groups with alkaline phosphatase (AP). The 5′-ends were labeled with [γ-^{32}P]ATP (5000 Ci/mmol) and T4 polynucleotide kinase (Maniatis et al., 1982). After the second digestion with *Bam*HI, the DNA was isolated from a 5% polyacrylamide gel (Maxam & Gilbert, 1980).

A 375 base pair 3′-end labeled DNA fragment was prepared by cleavage of the plasmid DNA with *Eco*RI and *Bam*HI, followed by enzymatic extention of the 3′-end with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP (3000 Ci/mmol) (Maniatis et al., 1982). The shorter DNA fragment was isolated from a 5% polyacrylamide gel (Maxam & Gilbert, 1980).

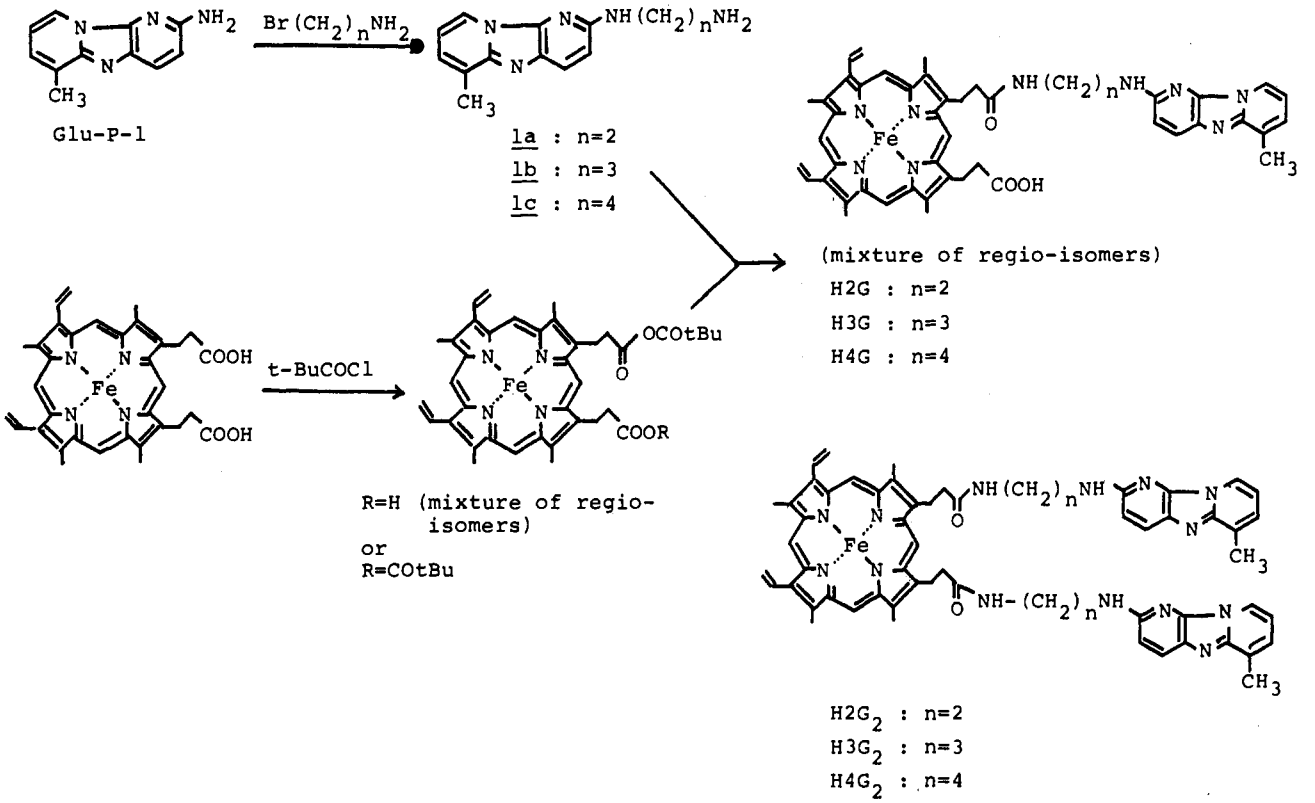
^{32}P-End labeled DNA (0.8 μCi) was mixed with 1 μg of calf thymus DNA and reacted with Hem-G's (100 μM final concentration) or with BLM (10 μM final concentration) as described above. The mixture was extracted with an equal volume of phenol saturated with water. The resulting DNA fragments were precipitated by addition of 3 volumes of EtOH to the aqueous phase. The DNA fragments thus obtained were analyzed by sequence-analyzing gel electrophoresis according to Maxam and Gilbert's protocol or resuspended in an appropriate buffer for enzymatic reactions for terminal analysis.

Analysis of DNA Termini by Gel Electrophoresis. The DNA product from the cleavage reactions was suspended in 100 μL of an appropriate buffer for enzymatic reactions. The presence of the 5′-phosphoryl group or 5′-hydroxy group was tested with alkaline phosphatase (AP) or T4 polynucleotide kinase. The enzymatic reaction mixture for AP contained 10 mM $MgCl_2$, 100 mM NaCl, 100 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol (DTT), 15 units of AP, and the cleaved DNA products. The enzymatic reaction mixture for T4 polynucleotide kinase for kination of the 5′-hydroxy group contained 100 mM Tris-HCl (pH 7.6), 20 mM $MgCl_2$, 10 mM DTT, 0.2 mM spermidine, 0.2 mM EDTA, 1 mM ATP, 9 units of T4 polynucleotide kinase, and the cleaved DNA products. The presence of the 3′-phosphoryl group or the 3′-hydroxy group was tested with terminal deoxynucleotidyl transferase (TdT) or T4 polynucleotide kinase (in the absence of ATP or ADP). The enzymatic reaction mixture for TdT contained 200 mM potassium cacodylate (pH 7.6), 2 mM $CoCl_2$, 1 mM DTT, 10 mM ATP, 10 mM GTP, 10 mM CTP, 10 mM UTP, 10 units of TdT, and the cleaved DNA products. The enzymatic reaction mixture for T4 polynucleotide kinase for 3′-dephosphorylation contained 500 mM imidazole hydrochloride (pH 6.6), 20 mM $MgCl_2$, 10 mM DTT, 1 μM spermidine, 0.1 μM EDTA, 9 units of T4 polynucleotide kinase, and the cleaved DNA products. Each mixture was incubated at 37 °C for 30 min, and then 100 μL of 0.3 M CH_3COONa was added. The solution was extracted with 200 μL of phenol saturated with water, followed by addition of 0.6 mL of EtOH to the aqueous phase. The resulting DNA precipitates were analyzed by 8% or 20% polyacrylamide denaturing gel electrophoresis according to the Maxam–Gilbert protocol (Maxam & Gilbert, 1980).

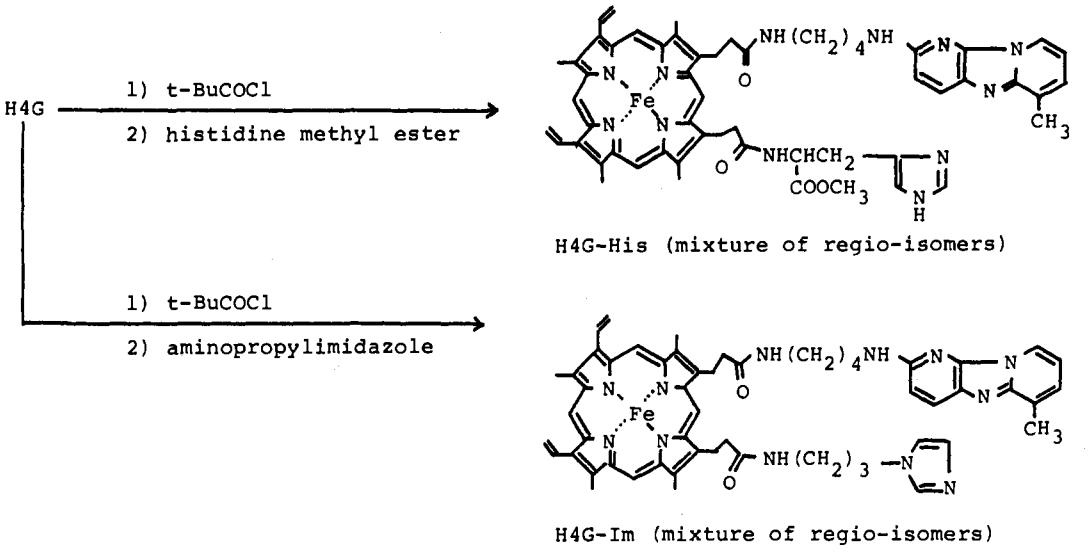
Results and Discussion

Design and Synthesis. We designed new Hem-G's as functional analogues of BLM on the basis of a consideration of the function and the structure of BLM. Hem-G's possess a DNA-recognizing intercalator moiety and a hemin group in the molecule. We used 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) as the intercalator moiety (Hashimoto et al., 1984b). Glu-P-1 is a mutagen isolated from a pyrolysate of L-glutamic acid (Yamamoto et al., 1978). The pathway of the chemical modification of DNA by Glu-P-1 was

Scheme I



Scheme II



established (Hashimoto et al., 1982, 1984a), and the intercalative ability of Glu-P-1 was also established by flow dichroism measurements (Imamura et al., 1980a) and an unwinding experiment (Imamura et al., 1980b). The association constant of Glu-P-1 with DNA was calculated to be about 10^3 M^{-1}, and the unwinding angle was 20–25°. The conformation of the intercalated complex of Glu-P-1 and DNA was simulated by the use of the Giglio function (K. Yamaguchi et al., unpublished results; Giglio, 1969). The covalent binding of the activated Glu-P-1 to DNA is sequence-selective (Hashimoto et al., 1984a). Thus, Glu-P-1 recognizes DNA on the basis of its intercalative ability. Covalent linking of Glu-P-1 with hemin gives Hem-G's, as shown in Scheme I (H2G, H3G, H4G, $H2G_2$, $H3G_2$, and $H4G_2$). Hemin is considered to have the ability to activate oxygen molecule, which could attack DNA and cause DNA cleavage. We also desinged a structure that possesses a histidine moiety or an imidazole moiety that could act as the fifth ligand of the ferrous ion in hemin (H4G-His and H4G-Im, in Scheme II).

Synthesis of Hem-G's (H*n*G, n = 2–4; H*n*G_2, n = 2–4) was performed as shown in Scheme I. Glu-P-1 was N-alkylated with bromoalkylamine to give alkylamino-Glu-P-1 (**1**) in yields of 40–70%. Compound **1** could be also prepared from 2-bromo-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole and the corresponding diaminoalkane. Thus, the site of N-alkylation was confirmed. Condensation of **1** with hemin chloride *tert*-butyl ester (which was prepared from hemin chloride and pivaloyl chloride) gave Hem-G's. Esterification on hemin with 1 equiv of pivaloyl chloride followed by condensation with **1** gave hemin–monointercalators (H2G, H3G, and H4G) as a mixture

Table I: Percentage of Closed Circular DNA That Was Converted to Open Circular DNA and Linear DNA[a]

drug	concn (μM)	%[b]	drug	concn (μM)	%[b]
none		9	H4G + histidine[f]	100	41
$FeCl_2$	100	8	H4G + imidazole[f]	100	43
hemin	100	10	H4G-His	10	36
$H2G_2$	100	39	H4G-His	25	50
H2G	100	41	H4G-His	50	49
$H3G_2$	100	40	H4G-His	100	64
H3G	100	40	H4G-His	100[c]	10
$H4G_2$	10	20	H4G-His	100[d]	30
$H4G_2$	100	41	H4G-His	100[e]	55
$H4G_2$	100[c]	10	H4G-Im	10	47
$H4G_2$	100[d]	15	H4G-Im	25	46
$H4G_2$	100[e]	37	H4G-Im	50	50
H4G	10	15	H4G-Im	100	56
H4G	25	24	H4G-Im	100[c]	10
H4G	50	47	H4G-Im	100[d]	27
H4G	100	42	H4G-Im	100[e]	58
H4G	100[c]	10	BLM[g]	10	64
H4G	100[d]	26	BLM	50	78
H4G	100[e]	34			

[a] The reaction mixture (10 μL) contained final concentrations of 200 μM nucleotides of pBR322 DNA, 4 mM $Na_2S_2O_4$, 10 mM Tris-HCl (pH 7.8), and 50 mM NaCl. The reaction mixture was saturated with air and incubated at 20 °C for 1 h. Incubation at 37 °C increased the percentage of cleaved DNA. [b] The percentage was calculated from the ratio of closed circular DNA to open circular and linear DNA. [c] $Na_2S_2O_4$ was not added. [d] The reaction was continued for 30 min. [e] The reaction was continued for 2 h. [f] Concentration of the additive (histidine or imidazole) was 1 mM. [g] $FeCl_2$ was added (100 μM).

of regioisomers of the amide bond. We used the mixture in the experiments described below without separation of the regioisomers. Esterification of hemin with 2 equiv of pivaloyl chloride followed by condensation with **1** gave hemin–bis intercalators ($H2G_2$, $H3G_2$, and $H4G_2$) in yields of 25–45%. Condensation of the *tert*-butyl ester of H4G with histidine methyl ester or (aminopropyl)imidazole gave H4G-His or H4G-Im, respectively, in 30–50% yield (Scheme II). The products were purified by silica gel column chromatography and reprecipitation from a mixture of pyridine and aqueous hydrochloric acid. The structure of these Hem-G's was deduced from the Fab mass spectra, UV spectra, and elemental analysis data. The purity of these products was checked by high-performance liquid chromatography (ODS/CH_3CN, Polygosil CN/CH_2Cl_2, and Polygosil NH_2/CH_2Cl_2), thin-layer chromatography (silica gel/EtOAc–MeOH–NH_4OH), and gel electrophoresis (0.7% agarose). These Hem-G's are stable compounds and are scarcely soluble in water, alcohol, and other low-boiling point organic solvents. They are soluble in pyridine, DMF, and dimethyl sulfoxide (Me_2SO).

DNA-Cleaving Ability of Hem-G's. The cleavage of DNA was followed by monitoring the conversion of supercoiled closed circular pBR322 DNA to open circular DNA and linear DNA (analyzed by agarose gel electrophoresis and quantitated by staining with 0.5 μg/mL ethidium bromide for 30 min and densitometry; Table I). Hem-G's possess strong DNA-cleaving ability in the presence of sodium hydrosulfite. Ferrous chloride alone or hemin itself, which lacks a DNA-recognition moiety, showed no effective cleavage of DNA. Presumably, sodium hydrosulfite acts as a reducing agent and regenerates Fe(II) from Fe(III) to produce a continuous source of the active metal ion. Reduction of Fe(III) to Fe(II) could be monitored by following the change of the UV spectrum of Hem-G's in the presence of sodium hydrosulfite (red shifts of the Soret band and Q-band by 5–20 nm). When the DNA-cleaving reaction with Hem-G's was performed at 20 °C, the ratio of the cleaved DNA (open circular and linear forms of DNA vs. closed circular form of DNA) was almost maximum within 1 h.

Very little effect of chain length (n) of the methylene spacers between the Glu-P-1 moiety and the hemin moiety was apparent. Introduction of a second intercalator moiety also had little effect on the DNA-cleaving ability: the efficiencies of DNA cleavage by HnG (n = 2–4) and HnG$_2$ (n = 2–4) are almost the same. However, H4G-His and H4G-Im showed extremely strong DNA-cleaving ability: the efficiency is slightly lower than that of BLM. Presumably, the histidine moiety or imidazole moiety of the compounds (H4G-His and H4G-Im) acts as the fifth intramolecular ligand of the ferrous ion of the porphyrin ring. Addition of histidine or imidazole to the incubation mixture of H4G and DNA had no effect (no enhancement) on the DNA-cleaving ability of H4G. The histidine or imidazole moiety is required to be linked covalently to H4G.

Effective cleavage of DNA requires an intercalator moiety that acts as a binding (recognition) site of DNA, as well as an intramolecular ligand such as a histidine or imidazole moiety. The hemin–intercalators, which possess strong DNA-cleaving ability (especially H4G-His and H4G-Im), bind to DNA through the intercalator moiety (Glu-P-1 skeleton). At the bound site of the DNA helix, activation of molecular oxygen by the hemin moiety results in the cleavage of the DNA, though the nature of the activated oxygen species is not yet known. Similar mechanisms were proposed for derivatives of EDTA by Hertzberg and Dervan (1982, 1984), for acridine–hemin by Lown and Joshua (1982), and for BLM by various authors (Burger et al., 1981; Grollman & Takeshita, 1980; Sugiura et al., 1983; Takeshita et al., 1978; Umezawa & Takita, 1980).

Sequence-Selective DNA Cleavage with Hem-G's. As shown in Table I, the synthesized hemin–intercalators (Hem-G's) cleave DNA efficiently. The cleaving abilities of H4G-His and of H4G-Im are comparable to (more than half of) that of BLM. BLM is known to cleave DNA at guanine–pyrimidine sequences preferentially (D'Andrea & Haseltine, 1978; Mirabelli et al., 1982; Sugiura et al., 1983; Takeshita & Grollman, 1978). By analysis of the products obtained from the DNA-cleaving reaction of BLM, it was found that the activated oxygen species attack position 4′ of deoxycytidine or deoxythymidine in DNA (Burger et al., 1980; Giloni et al., 1981), and the cleaved sites have a 5′-phosphoryl group and a 3′-phosphoglycolic acid group (Burger et al., 1980, 1981; Giloni et al., 1981; Grollman, & Takeshita, 1980; Umezawa & Takita, 1980). To investigate the reaction mechanism of DNA cleavage by Hem-G's and by BLM, we analyzed (a) the base-sequence specificity of the cleavage reactions and (b) the terminal structure of the cleaved fragments. In this section, we describe the base-sequence selectivity of DNA cleavage by Hem-G's and by BLM.

Sequence specificity of the DNA-cleaving reactions with Hem-G's and with BLM was investigated by the use of end-labeled DNA fragments and base-sequence-analyzing gel electrophoresis (Maxam & Gilbert, 1980). End-labeled DNA fragments were prepared from pBR322. 3′-End ^{32}P-labeled DNA fragment was prepared from the smaller DNA fragment (base numbers 1–375) obtained by the restricted hydrolysis of pBR322 with *Eco*RI and *Bam*HI. The smaller DNA fragment was ^{32}P-labeled with [^{32}P]dATP and the Klenow fragment of DNA polymerase I (Maniatis et al., 1982). 5′-End ^{32}P-labeled DNA fragment was also prepared from pBR322. Plasmid DNA pBR322 was hydrolyzed with *Eco*RI and ^{32}P-labeled with [^{32}P]ATP and T_4 polynucleotide kinase. Then, the labeled DNA was hydrolyzed with *Bam*HI. The

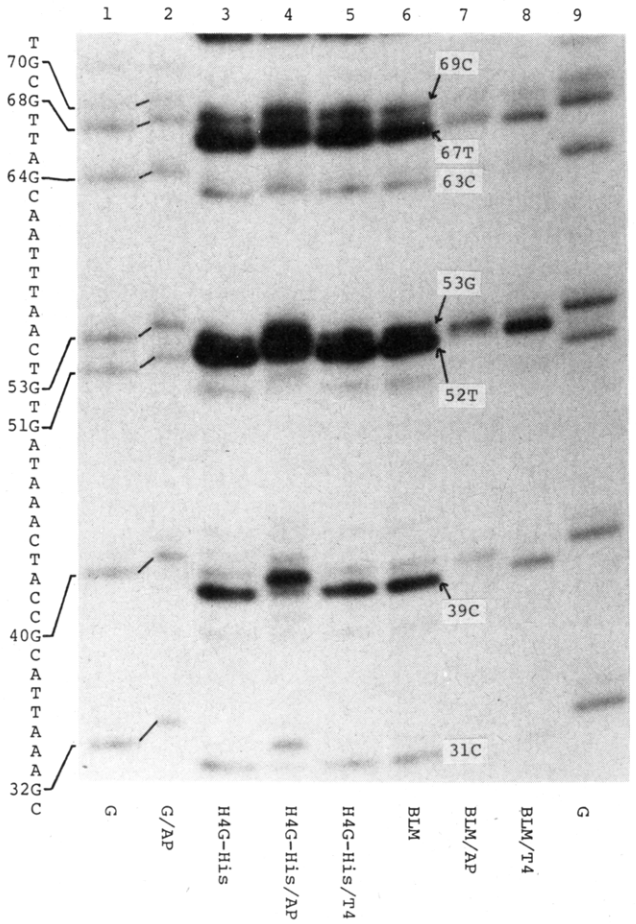


FIGURE 1: Analysis of DNA fragments of 3′-end ^{32}P-labeled DNA by 8% polyacrylamide denaturing gel electrophoresis. (Lanes 1 and 9) DNA was cleaved by $(CH_3O)_2SO_2$ followed by treatment with piperidine (G reaction). (Lane 2) DNA was treated as described for the G reaction followed by incubation with alkaline phosphatase (AP). (Lane 3) DNA was cleaved by H4G-His. (Lane 4) DNA was cleaved by H4G-His and then treated with AP. (Lane 5) DNA was cleaved by H4G-His and then treated with T4 polynucleotide kinase (T4) in the presence of ATP. (Lane 6) DNA was cleaved by BLM. (Lane 7) DNA was cleaved by BLM and then treated with AP. (Lane 8) DNA was cleaved by BLM and then treated with T4 and ATP.

resulting DNA fragments were separated by 5% polyacrylamide gel electrophoresis, and the shorter DNA fragment (base numbers 1–375) was used for the following reactions. The end-labeled DNA fragments were incubated with Hem-G's or with BLM. The incubation mixture was analyzed by high-resolution gel electrophoresis as described by Maxam and Gilbert (1980). The gels were autoradiographed and monitored by densitometry. The results of autoradiography are shown in Figures 1 and 2 (results for H4G-His and for BLM). The results for H*n*G (n = 2–4), H*n*G$_2$ (n = 2–4), and H4G-Im (data not shown) were similar to that for H4G-His. The ladder spots of DNA cleaved by all the Hem-G's and by BLM showed similar patterns.

The majority of the dark spots of 3′-end ^{32}P-labeled DNA cleaved with H4G-His or with BLM correspond exactly to the release of pyrimidine bases, cytosine (C) and thymine (T). The nucleotide at the 5′-side of all the "released" C and T that gave the dark ladder spots was guanylic acid (Figure 1). In other words, the majority of the dark ladder spots correspond to the ladder spots of the guanine (G) lane of Maxam and Gilbert's protocol (1980) with one nucleotide unit less, and the nucleotide at the 3′-side of the G was C or T.

Similarly, the majority of the dark ladder spots of 5′-end ^{32}P-labeled DNA cleaved with H4G-His or with BLM appears to correspond nearly (not exactly: vide infra) to the release of C and T. The 5′-side of the released C and T that gave the dark ladder spots was guanylic acid (Figure 2). In other words, the dark ladder spots correspond to the Maxam and Gilbert's G lane (G is degraded) (Maxam & Gilbert, 1980) but with slower mobility (by about one nucleotide unit).

Thus, the results suggest that Hem-G's preferentially cleave DNA at the sites of G-C and G-T sequences. Other sequences are also cleaved, but less frequently. The sites of DNA cleaved by Hem-G's are illustrated in Figure 3. The order of cleaving probability is G-C = G-T $\gg$ A-T > G-A > A-C > G-G = A-G > C-T. The base-sequence selectivity of DNA cleavage by Hem-G's is very similar to that of BLM (Figures 1 and 2). This sequence-selective cleavage of DNA with BLM is essentially the same as that reported by other authors (D'-Andrea & Haseltine, 1978; Mirabelli et al., 1982; Sugiura et al., 1983; Takeshita et al., 1978).

Terminal Analysis of DNA Fragments of DNA Cleaved by Hem-G's and by BLM. As mentioned above, the 3′-fragments of DNA cleaved by Hem-G's or by BLM correspond exactly to the release of C or T on high-resolution gel electrophoresis. On the other hand, the mobilities of the 5′-fragments of DNA cleaved by these compounds correspond to the release of G with decreased mobility by 0.8–1 nucleotide unit. This decreased mobility of the 5′-fragments of the cleaved DNA is due to the structure of the 3′-terminus, which is different from that of DNA fragments produced by Maxam and Gilbert's protocol (1980).

In this case, there considered to be a loss of negative charge as a result of 3′-dephosphorylation or neutralization of the terminal phosphoryl group. In fact, a terminal phosphoryl group is known to increase the mobility of a DNA fragment relative to a fragment with a terminal hydroxy group (Tapper & Clayton, 1981). In our electrophoretic system, the difference of mobility between a DNA with a terminal phosphoryl group and a DNA with a terminal hydroxy group corresponds to 0.8–1 nucleotide unit (Figures 1 and 2). The nature of the terminus of the DNA fragments cleaved by Hem-G's and by BLM was examined by comparing the electrophoretic mobilities with those of known DNA cleavage products on high-resolution gel electrophoresis.

3′-End ^{32}P-labeled DNA fragment was treated with Hem-G's and with BLM and analyzed by high-resolution gel electrophoresis [results for H4G-His and BLM are shown; the results for H*n*G (n = 2–4), H*n*G$_2$ (n = 2–4), and H4G-Im were essentially the same as those for H4G-His]. The mobilities of the resulting DNA fragments were compared to those produced by the G reaction as in Maxam and Gilbert's protocol (1980). Both the G reaction and BLM are reported to produce 5′-termini that are phosphorylated (Burger et al., 1980, 1981; Giloni et al., 1981; Grollman & Takeshita, 1980; Maxam & Gilbert, 1980; Sugiura et al., 1983; Takeshita et al., 1978). Figure 1 (lane 3) demonstrates that H4G-His produces oligonucleotides that comigrate with those from BLM (lane 6), suggesting the presence of phosphoryl groups on the 5′-termini. Further evidence for this was obtained by treatment of these DNA products with alkaline phosphatase (AP), which removes 5′-phosphoryl groups from DNA substrates. Figure 1 (lane 2) demonstrates that AP treatment of the DNA products of the G reaction results in a decrease of the electrophoretic mobility by 0.8–1 nucleotide unit. AP treatment of the DNA products from the H4G-His cleavage reaction (Figure 1, lane 4) and from the BLM cleavage reaction (lane 7) produces the same shifts in electrophoretic mobility. In addition, treatment with T4 polynucleotide kinase in the

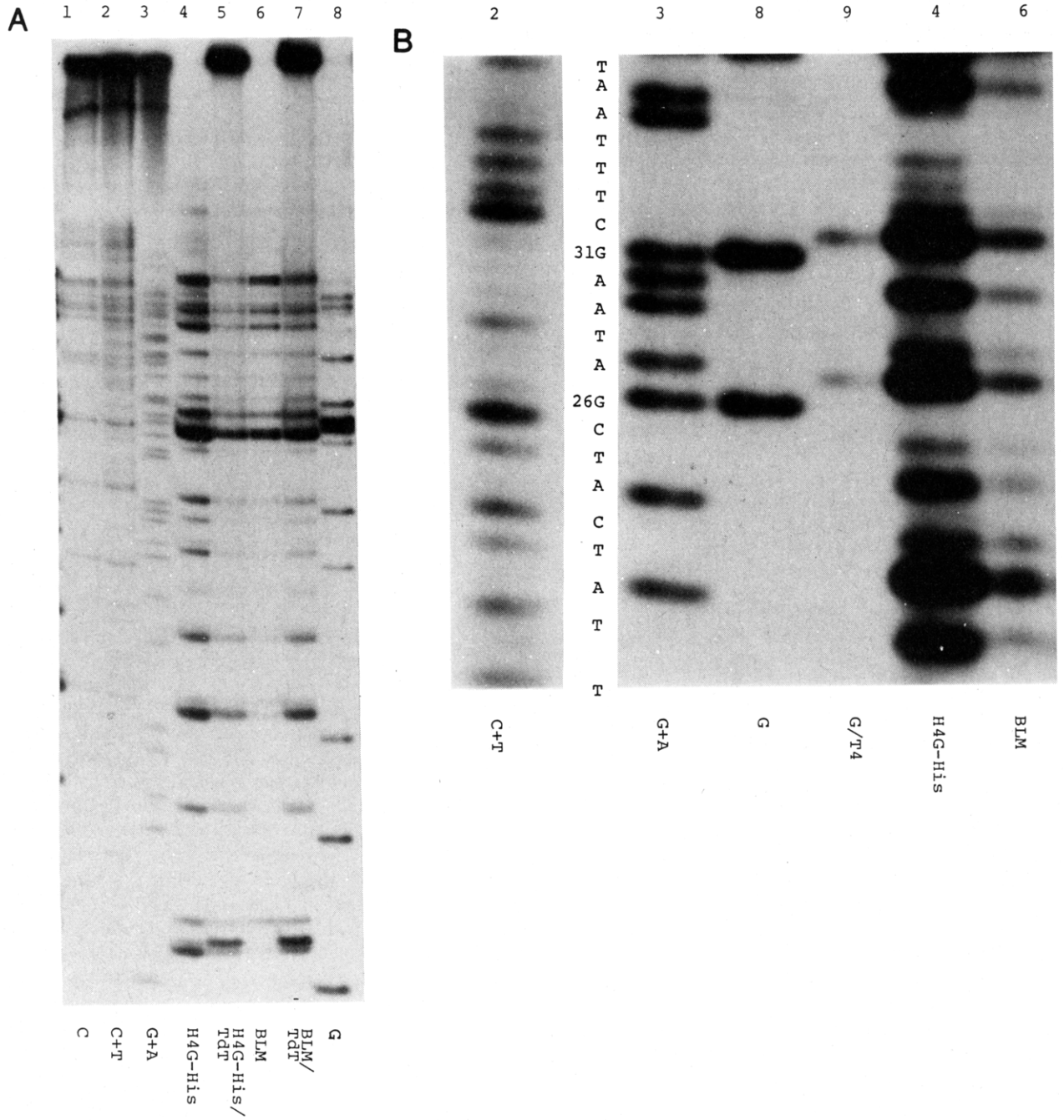


FIGURE 2: Analysis of DNA fragments of 5′-end ^{32}P-labeled DNA by (A) 20% and (B) 8% polyacrylamide denaturing gel electrophoresis. (Lane 1) DNA was cleaved by the method described by Maxam and Gilbert (C lane). (Lane 2) C+T lane. (Lane 3) G+A lane. (Lane 4) DNA was cleaved by H4G-His. (Lane 5) DNA was cleaved by H4G-His and then incubated with TdT in the presence of 5′-nucleotides. (Lane 6) DNA was cleaved by BLM. (Lane 7) DNA was cleaved by BLM and then treated with TdT and 5′-nucleotides. (Lane 8) G lane [DNA was cleaved by the method of Maxam and Gilbert (G reaction)]. (Lane 9) DNA was cleaved by G reaction followed by treatment with T4 polynucleotide kinase in the absence of ATP or ADP.

presence of 5′-ATP had no effect on the mobility of DNA fragments by H4G-His (lane 5) or BLM (lane 8). The results suggest that the cleavage of DNA with Hem-G's results in retention of the 5′-phosphoryl group. Similar results were reported in the case of methidiumpropyl-EDTA by Hertzberg and Dervan (1984).

The reactions of H4G-His or BLM with 5′-end ^{32}P-labeled DNA were carefully analyzed by high-resolution gel electrophoresis. Three major DNA products were distinguishable (Figure 2A, lanes 4 and 6). These products are not distinguishable on denaturing 8% polyacrylamide gel electrophoresis (Figure 2B). The mobility of these products is not exactly the same as that of any of the DNA fragments produced by Maxam and Gilbert's sequencing protocol. On the other hand, the mobilities of the DNA fragments from Maxam and Gilbert's G reaction followed by T4 polynucleotide kinase treatment (Figure 2B, lane 9) are exactly the same as those of the DNA fragments cleaved by H4G-His (lane 4) and BLM (lane 6). T4 polynucleotide kinase has been shown to be effective as a 3′-phosphatase in the absence of ATP or ADP (Cameron & Uhlenbeck, 1980). The results suggest that the termini of the DNA fragments cleaved by H4G-His and by BLM, at least in part, are 3′-hydroxy groups. This view is also supported by the fact that treatment of the DNA fragments cleaved by H4G-His or by BLM with terminal deoxynucleotidyl transferase (TdT) results in high molecular weight oligonucleotides (Figure 2A, lanes 5 and 7).

From the results mentioned above, we reached the following

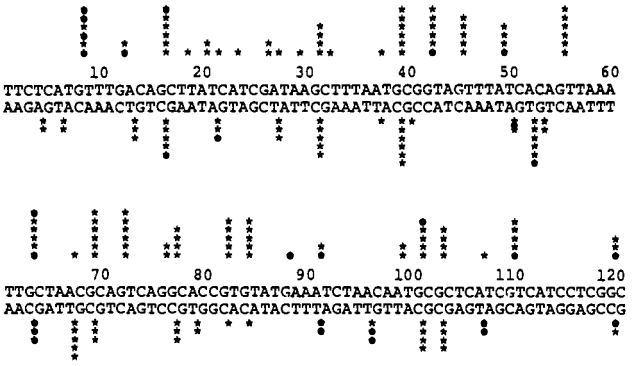


FIGURE 3: Illustration of sites preferentially cleaved by Hem-G's and BLM. (*) Relative probability of the cleavage by H4G-His. The pattern of DNA cleavage by H4G-His is essentially the same as those by BLM and the other Hem-G's. The probability was estimated from 8% gels for base numbers 25–120 and from 20% gels for base numbers 1–50.

conclusions. (i) Hem-G's and BLM cleave DNA at G-C (5′ → 3′) and G-T (5′ → 3′) sequences preferentially. (ii) The 3′-DNA fragments of DNA cleaved by Hem-G's or by BLM correspond to the products from the release of "C" or "T" having G at the 5′-side and possessing 5′-phosphorylated termini. (iii) One kind of the 5′-DNA fragments of DNA cleaved by Hem-G's or by BLM corresponds to the products from the release of "G" neighboring C or T at the 3′-side, followed by the 3′-dephosphorylation. Conclusions ii and iii mean that two nucleotide units (G-C or G-T) are deleted from DNA by treatment with Hem-G's and by treatment with BLM (Figure 4).

In the case of methidiumpropyl-EDTA, Hertzberg and Dervan (1984) reported that the 3′-termini of the cleaved products are phosphoryl and phosphoglycolic acid groups. Phosphoglycolic acid groups are also proposed to be the 3′-termini of the DNA fragments cleaved with BLM (Burger et al., 1980, 1981; Giloni et al., 1981; Grollman & Takeshita, 1980; Maxam & Gilbert, 1980; Sugiura et al., 1983; Takeshita et al., 1978). Henner et al. (1983) reported that a terminal phosphoglycolic acid group slightly increases the mobility of the DNA relative to a DNA with a terminal phosphoryl group. Therefore, the origin of the glycolic acid moiety of the 3′-terminus of DNA fragments cleaved by BLM, if our hypothesis is correct, should be a pyrimidine nucleotide moiety. One of the 5′-fragments of DNA cleaved by Hem-G's or by BLM, which is not a substrate of TdT (Figure 2A), might be an oligonucleotide having a phosphoglycolic acid group.

CONCLUSIONS

New hemin–intercalators (Hem-G's) that cleave DNA were designed and synthesized. Among the synthesized Hem-G's, H4G-His and H4G-Im showed extremely strong DNA-cleaving ability. The Glu-P-1 moiety, which recognizes DNA by intercalation, and a functional group that can act as an intramolecular ligand of the ferrous ion in hemin are required for strong DNA-cleaving ability.

These Hem-G's cleave DNA at G-C and G-T sequences preferentially. The base-sequence selectivity of DNA cleavage by Hem-G's is almost exactly the same as that by BLM. The 5′-terminus of the DNA cleaved by Hem-G's or by BLM is a 5′-phosphoryl group, while the 3′-terminus of the DNA fragments cleaved by Hem-G's or by BLM consists of three (or more) groups, one of which is a 3′-hydroxy group. The analysis of nucleotide number (electrophoretic mobility) suggests that two bases (one is guanine and the other is a pyrimidine base) are eliminated by Hem-G's and by BLM (Figure 4).

In conclusion, the synthesized Hem-G's are functional analogues of BLM. In fact, some of the synthesized Hem-G's showed antitumor activity with a spectrum similar to that of BLM (T. Sasaki et al., unpublished results). The possible usefulness of these Hem-G's as novel BLM substrates for clinical use in cancer chemotherapy and further details of the

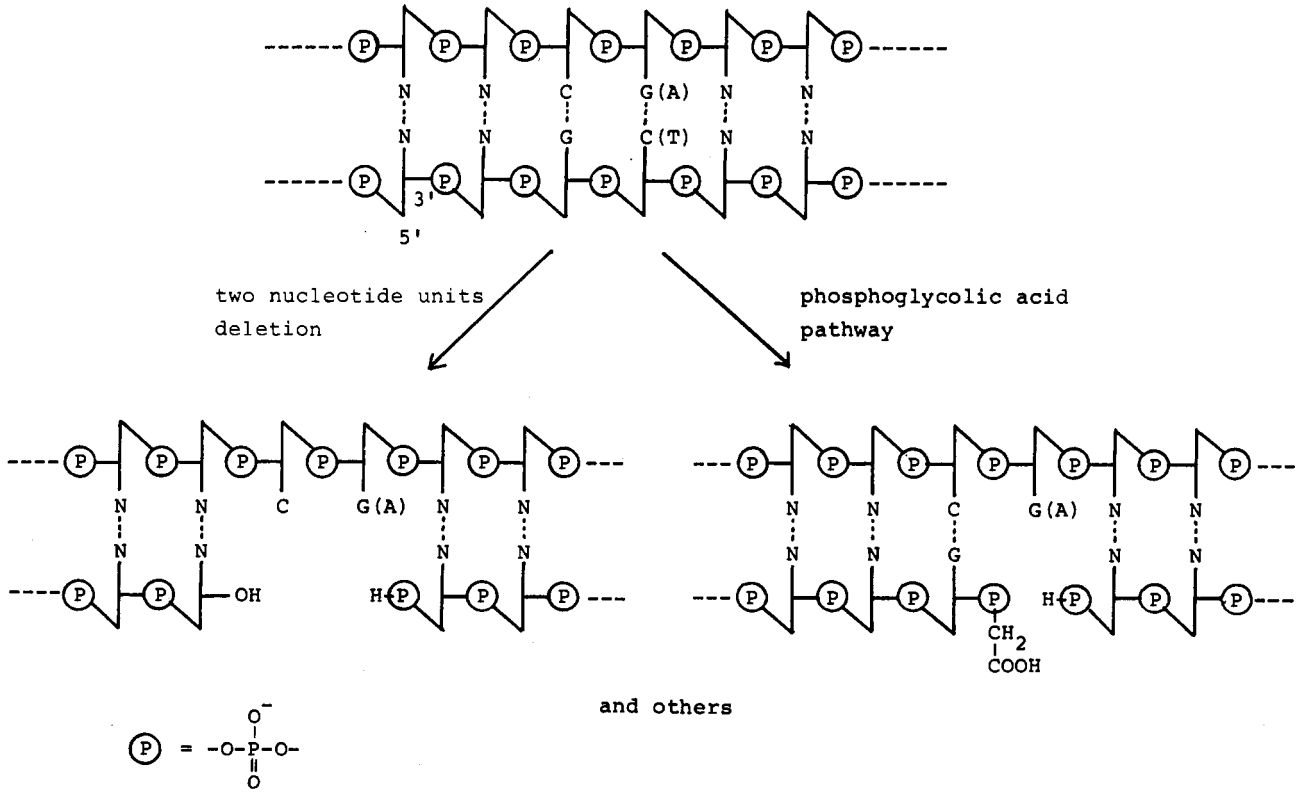


FIGURE 4: DNA cleavage by Hem-G's and by BLM.

molecular mechanism of the DNA-cleaving reactions are under investigation.

Registry No. 1a, 103498-87-9; **1b**, 94815-64-2; **1c**, 103498-88-0; H2G (isomer 1), 103479-01-2; H2G (isomer 1)·4HCl, 103478-84-8; H2G (isomer 2), 94811-53-7; H2G (isomer 2)·4HCl, 103478-87-1; $H2G_2$, 94790-74-6; $H2G_2$·4HCl, 103478-90-6; H3G (isomer 1), 103479-02-3; H3G (isomer 1)·2HCl, 103478-85-9; H3G (isomer 2), 94790-75-7; H3G (isomer 2)·2HCl, 103478-88-2; $H3G_2$, 94811-54-8; $H3G_2$·8HCl, 103478-91-7; H4G (isomer 1), 103479-03-4; H4G (isomer 1)·2HCl, 103478-86-0; H4G (isomer 2), 103479-04-5; H4G (isomer 2)·2HCl, 103478-89-3; $H4G_2$, 94790-72-4; $H4G_2$·4HCl, 103478-92-8; H4G-His (isomer 1), 103478-97-3; H4G-His (isomer 1)·6HCl, 103478-93-9; H4G-His (isomer 2), 103478-98-4; H4G-His (isomer 2)·6HCl, 103478-94-0; H4G-Im (isomer 1), 103478-99-5; H4G-Im (isomer 1)·8HCl, 103478-95-1; H4G-Im (isomer 2), 103479-00-1; H4G-Im (isomer 2)·8HCl, 103478-96-2; BLM, 11056-06-7; Glu-P-1, 67730-11-4; $Br(CH_2)_2NH_2$·HBr, 2576-47-8; $Br(CH_2)_3NH_2$·HBr, 5003-71-4; $Br(CH_2)_4NH_2$·HBr, 24566-81-2; *t*-BuCOCl, 3282-30-2; L-His, 1499-46-3; hemin, 16009-13-5; (aminopropyl)imidazole, 5036-48-6.

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