(+)-Myristinins A and D from *Knema elegans*, which Inhibit DNA Polymerase β and Cleave DNA

Jing-Zhen Deng, Shelley R. Starck, Shisheng Li, and Sidney M. Hecht*

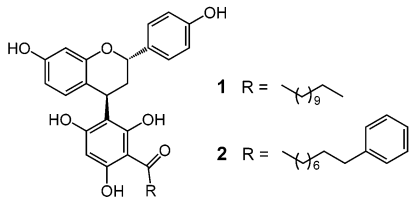
Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia 22903

Received May 23, 2005

A survey of crude plant extracts for DNA polymerase β inhibitors resulted in the identification of a methyl ethyl ketone extract prepared from *Knema elegans* that strongly inhibited the enzyme. Subsequent bioassay-guided fractionation of the extract, using an assay to monitor the activity of DNA polymerase β, led to the isolation of two potent inhibitors, (+)-myristinins A (**1**) and D (**2**), which are known flavans having unusual structures. (+)-Myristinins A and D exhibited IC_{50} values of 12 and 4.3 μM, respectively, as inhibitors of DNA polymerase β in the presence of bovine serum albumin (BSA), and 2.7 and 1.2 μM in the absence of BSA. As such, they are the most potent DNA polymerase β inhibitors reported to date. Compounds **1** and **2** potentiated the cytotoxicity of bleomycin toward cultured $P388D_1$ cells, reducing the number of viable cells by at least 30% when employed at 9 μM concentration for 6 h in the presence of an otherwise nontoxic concentration of bleomycin (75 nM). Principles **1** and **2** also induced strong Cu^{2+}-dependent DNA strand scission in a DNA cleavage assay. Accordingly, **1** and **2** exhibit two activities, namely, DNA polymerase β inhibition and DNA damage.

Currently utilized cancer chemotherapeutic agents include DNA-damaging agents such as bleomycin and cisplatin.[1] Cancer cells, however, can repair the damage inflicted to their DNA, thus reducing the effectiveness of damaging antitumor agents that function by this mechanism.[2−4] DNA polymerase β, an enzyme involved in base excision repair,[5] is responsible for repairing DNA damaged by exposure to chemotherapeutic agents such as monofunctional alkylating agents,[5a,6] cisplatin,[7] and bleomycin.[8,9] Because of its role in the repair of such lesions, DNA polymerase β is of interest as a target for identifying agents that can be used for adjuvant anticancer drug therapy. In fact, inhibition of this enzyme has been shown to result in potentiation of bleomycin and cisplatin cytotoxicity in cultured cells.[10] In principle, it should be possible to identify a single therapeutic agent capable of mediating DNA damage and blocking polymerase β-mediated repair. Our recent finding that naturally occurring bis-5-alkylresorcinols can exhibit both activities[11] encouraged us to search for novel and more potent agents having the same properties.

Members of the genus *Knema* Warb. (Myristicaceae) are tropical evergreen trees; some species are used to target cancers in Thai popular medicine.[12b] A few papers have reported the isolation of phenylalkyl phenol derivatives,[12−15] lignans,[13] isocoumarins,[14] flavans,[13b,15] and stilbenes[13b] from plants in this genus. An early report on the isolation of alkyl and phenylalkyl anacardic acids from the seed oil of *K. elegans* Warb. has appeared.[13a] Some phenylalkyl phenol derivatives from this genus exhibited significant toxicity toward three human tumor cell lines.[12b] In our continuing survey of plant extracts for DNA polymerase β inhibitors,[10,11,16] a methyl ethyl ketone extract prepared from the trunk wood of *K. elegans* exhibited potent inhibition of DNA polymerase β. Subsequent bioassay-guided fractionation of this extract resulted in the isolation of two potent polymerase β inhibitors, (+)-myristinins A (**1**) and D (**2**). (+)-Myristinins A and D are unusual naturally occurring flavans; members of this novel class of flavans have been isolated previously from *Myristica cinnamomea*[17] and *Horsfieldia amygdaline* (myristinin A).[18a] Further, the myristinins have been reported to inhibit phospholipase A2[18b] and cyclooxygenase-2 (COX-2)[17] and to possess antifungal activity.[17] Herein, we describe the isolation of **1** and **2** from *K. elegans* through bioassay-guided fractionation, their structure identification, activities as both DNA polymerase β inhibitors and DNA cleaving agents, and their ability to potentiate the cytotoxicity of bleomycin in cultured cells.



Results and Discussion

Isolation and Structure Identification. A methyl ethyl ketone extract prepared from the trunk wood of *K. elegans* was found to strongly inhibit DNA polymerase β (93% inhibition at 100 μg/mL; 84% inhibition at 50 μg/mL). The crude extract retained its inhibitory activity after passage through a polyamide 6S column to remove polyphenols. Accordingly, the extract was subjected to bioassay-guided fractionation, using an assay to detect inhibition of DNA polymerase β.

The extract was fractionated initially on a polyamide 6S column to remove polyphenols; the 4:1 CH_2Cl_2−MeOH fraction from this column had strong DNA polymerase β inhibitory activity. This fraction was subjected to further fractionation on a Sephadex LH-20 column using a normal-phase elution scheme. The most strongly active fraction (1:1 CH_2Cl_2−Me_2CO) from the Sephadex LH-20 column was then fractionated using a reversed-phase C_{18} open column to provide one highly active fraction (1:15:4 CH_2Cl_2−MeOH−H_2O). Further fractionation of this material on a reversed-phase C_8 open column again gave a strongly active fraction (1:13:6 CH_2Cl_2−MeOH−H_2O). Isolation and

* To whom correspondence should be addressed. Tel: (434) 924-3906. Fax: (434) 924-7856. E-mail: sidhecht@virginia.edu.

10.1021/np058064g CCC: $30.25
Published on Web 11/28/2005

purification of this fraction using a reversed-phase C_{18} HPLC column afforded the strongly active principles **1** and **2**.

Compound **1** was obtained as a colorless amorphous solid having $[\alpha]_D^{22}$ +45° (*c* 0.13, MeOH). Its molecular formula $C_{33}H_{40}O_7$ was established on the basis of the LC/HRESI-FTMS spectrum (found *m/z* 549.2850 $[M + H]^+$; calcd for $C_{33}H_{41}O_7$ *m/z* 549.2855). The ^1H NMR spectrum suggested the presence of five phenolic hydroxyl groups in **1** (δ_H 14.29, 10.56, 10.17, 9.30, and 8.98, each s or br s); this was further supported by five acetate methyl signals at δ_H ~2.20 in the ^1H NMR spectrum of the penta-*O*-acetyl derivative and by the pseudomolecular ion of the penta-*O*-acetate at $[M + H]^+$ in the ESIMS spectrum. The ^{13}C NMR spectra indicated that **1** had 18 aromatic carbons (δ_C 94.52−164.35) comprised of three substituted benzene rings. Analysis of the proton−proton coupling system at δ_H 2.07−5.37 allowed the assignment of **1** as a 4-substituted flavan. Further, an alkylcarbonyl side chain in **1** was deduced from the ^1H and ^{13}C NMR spectra [δ_H 1.15−1.30 (16 H, m), 0.84 (3 H, t, *J* = 7.2 Hz, Me) and δ_C 14.00, δ_C 205.48 (s, C=O)]. Through analyses and comparison with the reported NMR and MS data,[17] compound **1** was identified as (+)-myristinin A. The structure and absolute configuration of (+)-myristinin A have been confirmed by total synthesis.[19]

Principle **2** was isolated as a colorless amorphous solid having $[\alpha]_D^{22}$ +85° (*c* 0.10, MeOH) and had the molecular formula $C_{36}H_{38}O_7$ based on the LC/HRESI-FTMS spectrum (found *m/z* 583.2688 $[M + H]^+$; calcd for $C_{36}H_{39}O_7$ *m/z* 583.2693). The ^1H and ^{13}C NMR spectra of **2** were very close to those of **1**. Relative to compound **1**, principle **2** had five additional aromatic protons at δ_H 7.10−7.26 (m), but no terminal methyl group at δ_H ~0.84 (δ_C ~14.00). The observation and combined analyses of the mass spectra indicated that **2** contained an additional benzene ring at the end of the alkyl carbonyl substituent. On the basis of a comparison with the reported NMR and MS data,[17] compound **2** was identified as (+)-myristinin D. It may be noted that the CD spectra of **1** and **2** also support the assigned absolute configuration.[20]

Potency of DNA Polymerase *β* Inhibition. In the DNA polymerase β inhibition assay, compound **1** had an IC_{50} value of 12 μM in the presence of bovine serum albumin (BSA) and 2.7 μM in the absence of BSA. Compound **2** was somewhat more active, with IC_{50} values of 4.3 μM in the presence of BSA and 1.2 μM in its absence.

Kinetic analysis revealed a noncompetitive pattern of inhibition for **1** with respect to the DNA template-primer (K_i = 11 μM); the strength of association between **1** and DNA polymerase β, and **1** and the enzyme−DNA complex, was comparable. When the DNA template-primer concentration remained constant and the dNTP concentrations were varied, a mixed pattern of inhibition was observed (K_{is} = 8.2 μM and K_{ii} = 4.3 μM). Compound **2** was also a pure noncompetitive inhibitor with respect to the DNA template-primer and exhibited a nearly identical K_i value (12 μM). However, when dNTP concentrations were varied, the pattern of inhibition was mixed (K_{is} = 23 μM and K_{ii} = 0.8 μM). Clearly, compound **2** bound most tightly to the enzyme−DNA−dNTP tertiary complex, as evidenced by the submicromolar kinetic constant with respect to dNTPs.

Potency of DNA Cleavage. Compounds **1** and **2** were found to cleave DNA in the presence of Cu(II) (Figure 1). As shown in the figure, relaxation of supercoiled plasmid DNA was dose-dependent for both compounds and readily detectable over the full concentration range tested (50 μM to 100 nM) after a 1 h incubation period. Interestingly, incubation for time periods of several days produced steadily increasing amounts of DNA cleavage such that cleavage could be detected even when **1** or **2** was present only at 10 pM concentration. It may be noted that under these conditions the concentration of DNA plasmid greatly exceeded that of **1** or **2**, arguing that each myristinin molecule must promote the formation of multiple DNA breaks.

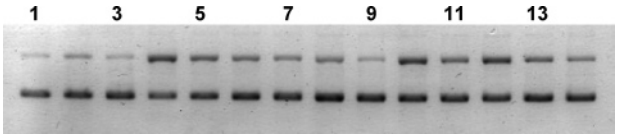


Figure 1. DNA strand scission by compounds **1** and **2** in the presence of 20 μM Cu^{2+}, measured after agarose gel electrophoresis. Lane 1, supercoiled pSP64 plasmid DNA alone; lane 2, pSP64 plasmid DNA + 20 μM Cu^{2+}; lane 3, 50 μM **1**; lanes 4−8, 20 μM Cu^{2+} + 50, 10, 2, 0.5, and 0.1 μM **1**, respectively; lane 9, 50 μM **2**; lanes 10−14, 20 μM Cu^{2+} + 50, 10, 2, 0.5, and 0.1 μM **2**, respectively.

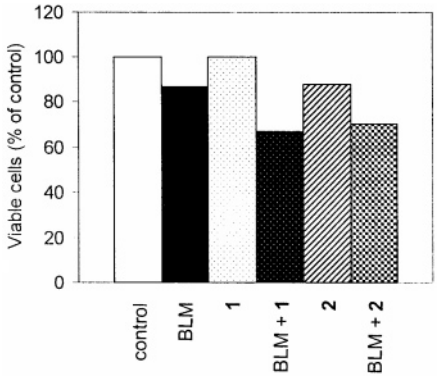


Figure 2. Potentiation of bleomycin cytotoxicity by **1** and **2**. Cells were treated as described for 6 h. Viability was assessed by trypan blue exclusion. White bar, control, no treatment; black bar, 0.075 μM BLM; black dots, 9 μM **1**; white dots, 0.075 μM BLM + 9 μM **1**; striped, 9 μM **2**; checkered, 0.075 μM BLM + 9 μM **2**.

Table 1. IC_{50} Values[a] and Kinetic Constants[a] for Compounds **1** and **2**

	IC_{50}				
compound	BSA	no BSA	K_i[b]	K_{is}[c]	K_{ii}[c]
1	12	2.7	11	8.2	4.3
2	4.3	1.2	12	23	0.8

[a] Concentrations are in μM. The standard error was shown to be <±5% of the values determined. [b] With respect to the DNA template-primer. [c] With respect to dNTPs.

Potentiation of Bleomycin Cytotoxicity in Cultured Cells. Since DNA polymerase β has been implicated in the repair of DNA damage induced by bleomycin[8,9] and other chemically used DNA-damaging agents, and likely contributes to diminishing the effectiveness of such agents, inhibitors **1** and **2** were tested on cultured $P388D_1$ cells for their ability to block DNA repair, thereby potentiating the cytotoxic effects of bleomycin. Figure 2 shows that when **1** and **2** were tested alone (9 μM), the number of viable cells was only slightly less than in the untreated control. However, co-administration of a nontoxic concentration of bleomycin (75 nM) resulted in reduction in the number of viable cells by at least 30%.

(+)-Myristinins A (**1**) and D (**2**) are members of a unique class of naturally occurring flavans. Their ability to mediate DNA damage at exceptionally low concentrations and to inhibit the repair of that damage suggests their potential utility in a new strategy for antitumor therapy.

Experimental Section

General Experimental Procedures. Polyamide 6S (Riedel-de Haen, Germany, pour density 0.25 g/mL) for column chromatography was purchased from Crescent Chemical Co., Inc. Lipophilic Sephadex LH-20 (a product of Pharmacia Inc., Sweden, bead size 25−100 μm) was from Sigma Chemicals. Reversed-phase C_8 (32−63 μm) and C_{18} (32−63 μm) resins were obtained from ICN Pharmaceuticals. A Kromasil reversed-phase C_{18} HPLC column (250 × 10 mm, 5 μm) was from Higgins Analytical Inc. Optical rotations were measured on a Perkin-Elmer 243 B polarimeter. The UV spectroscopic measurements were made on a Perkin-Elmer Lambda Array 3840 spectrophotometer. The CD spectra were measured on a JASCO CD J-720 spectropolarimeter. The ^{1}H and ^{13}C NMR spectra were recorded using DMSO-d_6 as an internal standard on a Varian-Unity Inova 500/51 spectrometer at 500 (for ^{1}H) and 125 MHz (for ^{13}C), respectively. Low-resolution chemical ionization (CI) (methane) mass spectra were obtained on a Finnigan MAT 4600 mass spectrometer and high-resolution ESI/FT mass spectra were recorded on a New Star T70 FT/MS spectrometer at Glaxo SmithKline Beecham Pharmaceuticals. For the DNA polymerase β inhibition assay and DNA cleavage assay, compounds **1** and **2** were dissolved in 1:1 DMSO−MeOH and in 100% DMSO for the cell culture studies, with a final DMSO concentration of 0.25% in each incubation medium. Recombinant rat liver DNA polymerase β was prepared as described previously.[21] Antibiotic antimycotic solution, Hank's balanced salt solution, Dulbecco's modified Eagle's medium containing 4500 mg glucose/L, unlabeled dNTPs, and calf thymus DNA were purchased from Sigma Chemicals; calf thymus DNA was activated by the method described previously.[22] [^{3}H]dTTP was purchased from ICN Pharmaceuticals. DEAE-cellulose paper (DE-81) was from Whatman. The P388D$_1$ cell line (mouse, lymphoid neoplasm) was purchased from American Type Culture Collection. Trypan blue dye and donor horse serum were from Gibco BRL. Blenoxane, the clinically used mixture of bleomycins consisting predominantly of BLM A_2 and BLM B_2, was a gift from Bristol Laboratories. Agarose gels were quantified for percent DNA cleavage utilizing Molecular Dynamics ImageQuant version 5.0 software.

Plant Materials. Trunk wood of *Knema elegans* (Warb.) (PR80701) was collected in Thailand in 1975 and was supplied by the Medicinal Plant Resources Laboraotry, USDA, Beltsville, MD, where a voucher specimen is preserved.

Extraction and Isolation. Trunk wood of *K. elegans* Warb. was extracted successively with hexanes, methyl ethyl ketone, MeOH, and H_2O. The crude methyl ethyl ketone extract was found to exhibit strong inhibition of DNA polymerase β (93% inhibition at 100 μg/mL) and continued to exhibit potent inhibitory activity after passage through a polyamide 6S column to remove polyphenols. Accordingly, this extract was subjected to bioassay-guided fractionation. A total of 975 mg of the methyl ethyl ketone crude extract was used for bioassay-guided fractionation; a typical set of experiments is described below. The crude extract (260 mg) was applied to a 15 g polyamide 6S column, which was washed successively with H_2O, 1:1 MeOH−H_2O, 4:1 CH_2Cl_2−MeOH, 1:1 CH_2Cl_2−MeOH, and 9:1 MeOH−NH_4OH (200 mL/fraction). The 4:1 CH_2Cl_2−MeOH fraction (205 mg) exhibited strong inhibition of DNA polymerase β (95% inhibition at 100 μg/mL; 88% inhibition at 50 μg/mL). This fraction was applied to a 20 g Sephadex LH-20 column, which was eluted successively with hexanes, 1:1 hexanes−CH_2Cl_2, CH_2Cl_2, 1:1 CH_2Cl_2−Me_2CO, Me_2CO, and MeOH (250 mL/fraction). The Me_2CO fraction (72 mg) displayed the strongest inhibitory activity (93% inhibition at 50 μg/mL) and was subjected further to fractionation on an 18 g reversed-phase C_{18} open column. This column was washed successively with H_2O and then 0:3:7, 0:5:5, 1:13:6, 1:15:4, 1:17:2, and 1:19:0 CH_2Cl_2−MeOH−H_2O (150 mL/fraction). The most active fraction (1:15:4 CH_2Cl_2−MeOH−H_2O, 41 mg) was applied to a 15 g reversed-phase C_8 open column. Washing of this column with H_2O and then successively with 0:3:7, 0:5:5, 1:11:8, 1:13:6, 1:15:4, and 1:17:2 CH_2Cl_2−MeOH−H_2O (120 mL/fraction) provided the strongest inhibitory fraction (1:13:6 CH_2Cl_2−MeOH−H_2O, 15 mg). This fraction was then applied to a reversed-phase C_{18} HPLC column (250 × 10 mm, 5 μm), using a gradient elution of 65:35 → 95:5 MeCN−H_2O over a period of 60 min at a flow rate of 3.0 mL/min (monitoring at 280 nm) to afford compounds **1** (3.4 mg) and **2** (2.7 mg) in that order.

(+)-Myristinin A (1): colorless amorphous solid; $[\alpha]_D^{22}$ +45° (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ϵ) 236 (5.22), 286 (4.89) nm; CD (MeOH) $\Delta\epsilon$ (nm) +1.0 (214), +3.4 (238), and −0.6 (288); ^{1}H and ^{13}C NMR data were consistent with those of myristinin A;[17] negative CIMS *m/z* (rel %) 548 [M]$^-$ (70), 438 (50), 428 (3), 365 (2), 308 (100), 240 (5), 120 (2), and 110 (3); positive CIMS *m/z* 549 [M + H]$^+$; ESI-FTMS *m/z* 571 [M + Na]$^+$, 467, 461 [438 + Na]$^+$, 428, 413, 365, 240, 120, and 111; LC/LRESI/FTMS *m/z* 549 [M + H]$^+$, 439, 429, 418, and 411; LC/HRESI/FTMS *m/z* 549.2850 [M + H]$^+$ (calcd for $C_{33}H_{41}O_7$, 549.2855).

Acetylation of 1. To a cooled (0−5 °C) solution of 1.8 mg (3.5 μmol) of **1** in 600 μL of dry pyridine was added 280 μL (302 mg, 2.9 mmol) of acetic anhydride under argon. The reaction mixture was stirred under argon at 25 °C for 1.5 h, followed by the addition of an ice chip and subsequent extraction with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O, dried over $MgSO_4$, and concentrated under diminished pressure; the residue was subjected to purification on a reversed-phase C_{18} HPLC column (250 × 10 mm, 5 μm), employing a gradient elution of 85:15 → 100:0 MeCN−H_2O over a period of 25 min at a flow rate of 3.0 mL/min (detection at 280 nm) to afford a penta-*O*-acetyl derivative of **1** as an oily product: yield 2.2 mg (86%); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.88 (3H, t, J = 7.3 Hz), 1.26 (16H, m), 1.58 (2H, m), 2.25 (3H, s), 2.27 (6H, s), 2.28 (6H, s), 2.71 (2H, t, J = 7.0 Hz), 2.33 (2H, m), 3.81 (1H, dd, J = 9.5, 4.8 Hz), 5.61 (1H, t, J = 3.0 Hz), 6.47 (1H, dd, J = 8.1, 2.0 Hz), 6.68 (1H, d, J = 8.1 Hz), 6.78 (1H, d, J = 2.0 Hz), 7.00 (1H, s), 7.05 (2H, d, J = 8.2 Hz) and 7.28 (2H, d, J = 8.2 Hz); LC/LRESI/FTMS *m/z* 759 [M + H]$^+$, 717, 675, 597, 577, 565, 555, 539, 497, and 195.

(+)-Myristinin D (2): colorless amorphous solid; $[\alpha]_D^{22}$ +85° (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ϵ) 236 (5.12), 286 (4.65) nm; CD (MeOH) $\Delta\epsilon$ (nm) +0.3 (212), +0.7 (237), and −0.3 (290); ^{1}H and ^{13}C NMR data were identical with those of myristinin D;[17] negative CIMS *m/z* (rel %) 582 [M]$^-$ (65), 472 (30), 365 (12), 342 (100), 240 (20), 120 (5), and 110 (10); positive CIMS *m/z* 583 [M + H]$^+$; ESI-FTMS *m/z* 605 [M + Na]$^+$, 541, 468, 495 [472 + Na]$^+$, 468, 462, 413, 365, 335, 240, 120, and 111; LC/LRESI/FTMS *m/z* 583 [M + H]$^+$, 473, 463, and 447; LC/HRESI/FTMS *m/z* 583.2688 [M + H]$^+$ (calcd for $C_{36}H_{39}O_7$, 583.2693).

Acetylation of 2. The same procedure as above was employed in this acetylation. To a cooled solution of 2.2 mg (3.8 μmol) of **2** in 650 μL of dry pyridine was added 300 μL (323 mg, 3.2 mmol) of acetic anhydride under argon. The reaction and then purification provided a penta-*O*-acetyl derivative of **2** as an oily product: yield 2.5 mg (85%); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.23−1.41 (8H, m), 1.56 (4H, m), 2.25 (3H, s), 2.27 (6H, s), 2.28 (6H, s), 2.33 (2H, m), 2.59 (2H, m), 2.73 (2H, t, J = 7.0 Hz), 3.83 (1H, dd, J = 9.5, 4.8 Hz), 5.62 (1H, t, J = 3.0 Hz), 6.48 (1H, dd, J = 8.1, 2.0 Hz), 6.66 (1H, d, J = 8.1 Hz), 6.76 (1H, d, J = 2.0 Hz), 7.01 (1H, s), 7.02 (2H, d, J = 8.2 Hz), and 7.29 (2H, d, J = 8.2 Hz); LC/LRESI/FTMS *m/z* 793 [M + H]$^+$, 751, 709, 631, 599, 589, 531, and 195.

DNA Polymerase β Inhibition Assay. To 50 μL of 62.5 mM 2-amino-2-methyl-1,3-propanediol buffer (pH 8.6) containing 10 mM $MgCl_2$, 1 mM DTT, 100 μg/mL BSA, 6.25 μM dNTPs including 0.04 Ci/mmol [^{3}H]dTTP and 0.25 mg/mL activated calf thymus DNA was added 6 μL of a solution containing a test compound and 4 μL of recombinant rat DNA polymerase β preparation (6.9 units, 4.8 × 10^4 units/mg). After incubation at 37 °C for 60 min, the radiolabeled DNA product was collected on DEAE-cellulose paper (DE-81), dried, and rinsed successively with 0.4 M K_2HPO_4, pH 9.4, and 95% EtOH for radioactivity determination. For the kinetic studies, inhibitor

constants (K_i, K_{is}, and K_{ii}) were obtained using the same assay, except incubation was for 20 min and the samples were placed on ice immediately before application to DE-81 paper. Additionally, the activated DNA concentrations were varied from 200, 100, 50, 25, to 12.5 μM (nucleotide concentration) or dNTP concentrations were varied from 25, 12.5, 6.25, 3.12, to 1.56 μM ([^{3}H]dTTP was added in proportion such that its specific activity remained constant).

DNA Cleavage Assay. Cleavage of supercoiled pSP64 plasmid DNA was carried out in 25 μL reaction mixtures (total volume) containing 600 ng of DNA and the indicated amounts of $CuCl_2$ and compounds **1** and **2** in 10 mM Tris-HCl, pH 7.2. The reactions were incubated at 37 °C for 1 h, terminated by the addition of 5 μL of 0.125% bromophenol blue in 30% glycerol, and applied to a 1.0% agarose gel containing 0.7 μg/mL ethidium bromide. The gel was run in 89 mM Tris with 8.9 mM H_3BO_3 and 2.0 mM Na_2-EDTA at 125 V for 2.5 h.

Bleomycin Cytotoxicity Assay in Cell Culture. P388D$_1$ cells were maintained as suspension cultures in 90% (v/v) Dulbecco's modified Eagle's medium containing 4.5 mg glucose/L, 10% (v/v) donor horse serum, and 100 IU penicillin, 0.1 mg streptomycin, and 0.25 μg amphotericin B per mL at 37 °C in a 5% CO_2 in air atmosphere. To 12.5 mL tissue culture flasks was added 5 mL of cell suspension containing $\sim 5 \times 10^5$ cells/mL; this was incubated for 1 h to stabilize the cells. Assays were carried out in 6 mL reaction cultures containing the desired amounts of test compound and blenoxane dissolved in media. Cultures were incubated at 37 °C in a 5% CO_2 in air atmosphere for 6 h followed by cell viability determination using trypan blue dye.

Acknowledgment. We thank Drs. M. E. Hemling and C. F. Ijames, GlaxoSmithKline, for the measurement of all ESIMS spectra. Recombinant rat liver DNA polymerase β was a gift from Dr. X. Wang and H. Wang. This work was supported at University of Virginia by NIH Research Grant CA50771, awarded by the National Cancer Institute.

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NP058064G