Mispyric Acid, a New Monocyclic Triterpenoid with a Novel Skeleton from *Mischocarpus pyriformis* that Inhibits DNA Polymerase β

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Abstract: Bioassay-guided fractionation using an assay to monitor DNA polymerase β inhibition led to the isolation of the inhibitor, mispyric acid (**1**), a monocyclic triterpenoid with a novel skeleton from *Mischocarpus pyriformis*. Its structure, including the relative stereochemistry, was established on the basis of HRMS and 1D and 2D NMR spectroscopic methods. Mispyric acid inhibited DNA polymerase β with an IC_{50} of 20 μM in the presence of bovine serum albumin (BSA) and 14 μM in the absence of BSA, consistent with the possibility that **1** may be of utility in vivo. This possibility was further supported by the finding that compound **1** successfully potentiated the cytotoxic action of bleomycin in cultured cells, reducing the number of viable cells by nearly 50% when employed at 50 μM concentration in the presence of an otherwise nontoxic (75 nM) concentration of bleomycin. Finally, the novel structural framework of **1** suggests that its biosynthesis may obtain via a novel biogenetic pathway.

Cell resistance to chemotherapeutic agents often limits the effectiveness of anticancer treatment. For some DNA damaging agents, resistance can result from the ability of the cell to repair the damaged DNA, which thereby reduces their effectiveness.[1] DNA polymerase β, an enzyme responsible for DNA repair after exposure to chemotherapeutic agents such as the bleomycins,[2] monofunctional DNA alkylating agents,[3] cisplatin,[4] and neocarzinostatin,[5] is a potential target for adjuvant antitumor therapy; selective inhibition of this enzyme by otherwise noncytotoxic agents might logically be expected to potentiate chemotherapeutic treatment, thus enhancing the efficacy of anticancer therapies. In fact, a recent study in our laboratory using isolated DNA polymerase β inhibitors revealed that inhibition of this enzyme function in cultured cells resulted in potentiation of bleomycin and cisplatin cytotoxicity.[6]

In our continuing search for naturally occurring DNA polymerase β inhibitors,[6−8] we found that a methyl ethyl ketone

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extract prepared from *Mischocarpus pyriformis* contained potent DNA polymerase β inhibitory activity. Subsequent bioassay-guided fractionation of the extract, using an assay to detect DNA polymerase β inhibition, led to the isolation of the polymerase β inhibitor mispyric acid (**1**), a new monocyclic triterpenoid
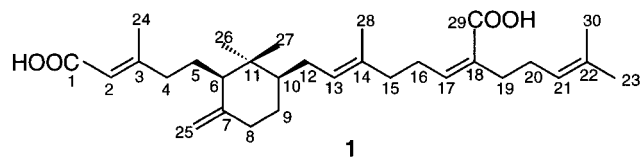
having a structurally novel skeleton. Herein, we describe the isolation of **1** through bioassay-guided fractionation, its structure elucidation, and potency as a DNA polymerase β inhibitor, as well as the potentiation of bleomycin cytotoxicity by **1** in cultured cells. The novel skeleton of **1** argues for its formation by a novel biogenetic pathway; a possible route is discussed.

Results and Discussion

As part of a survey of crude plant extracts for inhibition of DNA polymerase β, a methyl ethyl ketone extract prepared from the dried stem bark of *M. pyriformis* exhibited strong inhibitory activity toward DNA polymerase β (92% inhibition at 100 μg/mL; 83% inhibition at 50 μg/mL). The crude extract was still strongly active after passage through a polyamide 6S column to remove polyphenols; these tend to be strong, although nonspecific inhibitors of DNA binding enzymes. Accordingly, the extract was subjected to bioassay-guided fractionation to permit isolation and characterization of the principle(s) responsible for inhibition of DNA polymerase β.

Isolation and Structure Elucidation. The methyl ethyl ketone extract of *M. pyriformis* was fractionated initially on a polyamide 6S column, which was washed successively with H_2O, 1:1 MeOH−H_2O, 8:2 MeOH−CH_2Cl_2, 1:1 MeOH−CH_2-

Table 1. NMR Data for Mispyric Acid (**1**) in CDCl$_3$[a]

position	δ_C (ppm)	δ_H (ppm)
1	172.1 (s)[b]	
2	114.8 (d)	5.69 (br s)
3	164.1 (s)	
4	40.2 (t)	2.12 (m)
5	23.0 (t)	1.63 (m); 1.73 (m)
6	53.3 (d)	1.64 (m)
7	148.5 (s)	
8	37.5 (t)	1.98 (m); 2.32 (m)
9	30.2 (t)	1.17 (m); 1.75 (m)
10	48.6 (d)	1.21 (m)
11	39.7 (s)	
12	29.1 (t)	1.63 (m); 2.15 (m)
13	125.3 (d)	5.14 (m)
14	134.5 (s)	
15	39.2 (t)	2.07 (m)
16	28.1 (t)	2.60 (q) ($J = 7.3$ Hz)
17	145.6 d	6.00 (t) ($J = 7.3$ Hz)
18	130.6 (s)	
19	34.5 (t)	2.31 (m)
20	27.9 (t)	2.26 (m)
21	123.4 (d)	5.13 (m)
22	132.4 (s)	
23	25.6 (q)	1.68 (br s)
24	19.2 (q)	2.18 (br s)
25	106.5 (t)	4.51 (br s), 4.68 (br s)
26	15.0 (q)	0.59 (s)
27	26.6 (q)	1.04 (s)
28	16.0 (q)	1.59 (br s)
29	173.4 (s)	
30	17.7 (q)	1.59 (br s)

[a] H NMR spectrum recorded at 500 MHz; ^{13}C NMR spectrum at 75 MHz. [b] Letters s, d, t, and q indicate, respectively, singlet, doublet, triplet, and quartet, as determined from the DEPT spectrum.

Cl$_2$, and 9:1 MeOH−NH$_4$OH. The 8:2 MeOH−CH$_2$Cl$_2$ fraction showed significant DNA polymerase β inhibitory activity (83% inhibition at 50 μg /mL) and was applied to a C$_{18}$ reversed-phase open column for further fractionation. The 9:1 MeOH−H$_2$O fraction from the C$_{18}$ column displayed the strongest inhibition of DNA polymerase β; this material was fractionated further using a C$_8$ reversed-phase open column. The active MeOH fraction from the C$_8$ column then provided mispyric acid (**1**) as a pure, inhibitory principle.

Mispyric acid (**1**) was obtained as a colorless, viscous oil having $[\alpha]^{20}_D$ + 12.5° (c 0.05, MeOH). Its molecular formula (C$_{30}$H$_{46}$O$_4$), indicating eight degrees of unsaturation, was established on the basis of the HREIMS spectrum (found, m/z 470.3399 [M]$^+$; calcd, 470.3396 for C$_{30}$H$_{46}$O$_4$). The ^{1}H NMR spectrum of **1** showed six olefinic proton signals at δ 6.00 (br t, $J = 7.3$ Hz), 5.69 (br s), 5.14 (m), 5.13 (m), 4.68 (br s), and 4.51 (br s), four methyl groups attached to double bonds at δ 2.18 (br s), 1.68 (br s), and 1.59 (2 × CH$_3$, br s), and two additional methyl groups at δ 1.04 (s) and 0.59 (s). The ^{13}C NMR and DEPT spectra suggested that **1** was a triterpenoid comprised of six methyl, 10 methylene, six methine, and eight quaternary carbons. The spectra also revealed that **1** had four trisubstituted double bonds, one gem disubstituted double bond [δ 148.5 (s) and 106.5 (t)], and two carboxyl groups [δ 173.4 (s) and 172.1 (s)]. All proton and carbon chemical shifts of **1** (Table 1) were measured through the ^{1}H−^{1}H and HMQC spectra. The ^{1}H−^{1}H COSY spectrum revealed the following spin−spin couplings: H-2 (δ 5.69) to CH$_3$-24 (δ 2.18) and H$_2$-4 (δ 2.12); Ha-25 (δ 4.68) to Hb-25 (δ 4.51) and H-6 (δ 1.64); Hb-25 to Ha-25 and H-6; H$_2$-8 (δ 2.32 and 1.98) to H$_2$-9 (δ 1.75 and 1.17); H-13 (δ 5.14) to H$_2$-12 (δ 2.15 and 1.63) and CH$_3$-28 (δ 1.59); H-17 (δ 6.00) to H$_2$-16 (δ 2.60); H$_2$-16 to H$_2$-15 (δ 2.07); H-21 (δ 5.13) to H$_2$-20 (δ 2.26), CH$_3$-23 (δ

Table 2. IC$_{50}$ Values[a] and Kinetic Constants[b] for **1**

IC$_{50}$	K_{is} (DNA)	K_{ii} (DNA)	K_i ([^{3}H]TTP)
20 (BSA)[c] 14 (No BSA)	53	20	18

[a] Obtained from the DNA Polymerase β inhibition assay; reported as micromolar concentrations. [b] Reported as micromolar concentrations. [c] BSA, bovine serum albumin at 0.1 mg/mL.
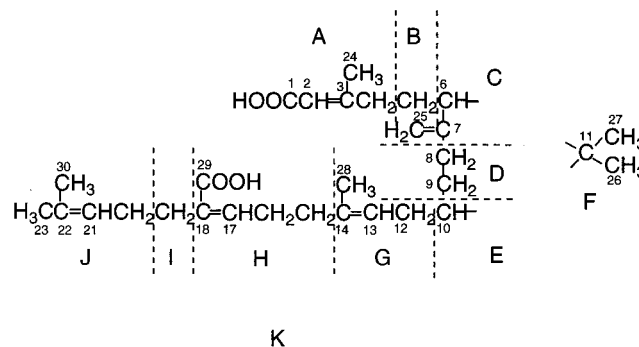
Figure 1. Ten partial structures (A−J) determined for mispyric acid (**1**), nine of which (A−E and G−J) were connected to give substructure K.
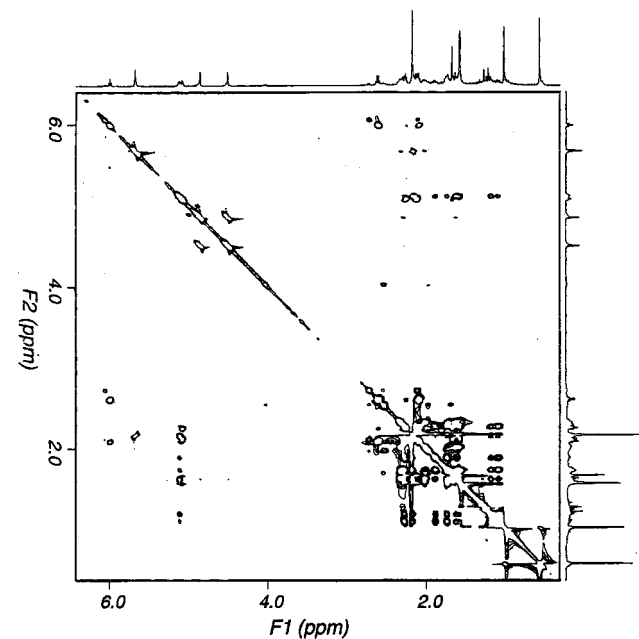
Figure 2. TOCSY spectrum of **1** recorded at 500 MHz in CDCl$_3$.

1.68), and CH$_3$-30 (δ 1.59). Those observations allowed assignment of the partial structures A, C, D, G, H, and J (Figure 1). Further, analysis of the ^{1}H NMR, DEPT, and HMQC spectra provided partial structures B (δ_C 23.0, δ_H 1.63 and 1.73), E (δ_C 48.6, δ_{H2} 1.21), F (δ_C 39.7, δ_{CH3} 1.04 and 0.59), and I (δ_C 34.5, δ_{H2} 2.31). Due to the severely overlapped signals in the region δ 1.60−2.40 of the ^{1}H NMR spectrum, a detailed analysis of the spin−spin couplings of units B, E, and I via the ^{1}H−^{1}H COSY spectrum was not possible. Therefore, the relationship between these partial structures was examined via the ^{1}H−^{1}H total correlation spectroscopy (TOCSY) spectrum. The TOCSY spectrum (Figure 2) of **1** clearly exhibited the following correlations: H-2 with CH$_3$-24, H$_2$-4, and H$_2$-5; H-4 with H-5 and H-6; Ha-25 (δ 4.68) with H-6 and Ha-8 (δ 2.32); H-10 with H$_2$-8, H$_2$-9, and H$_2$-12; H-13 with H$_2$-9, H-10, H$_2$-12, and H$_2$-15; H-17 with H$_2$-15, H$_2$-16, and H$_2$-19; and H-21 with CH$_3$-23, CH$_3$-30, H$_2$-19, and H$_2$-20. Accordingly, substructure K

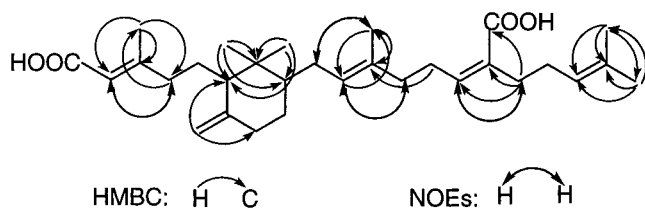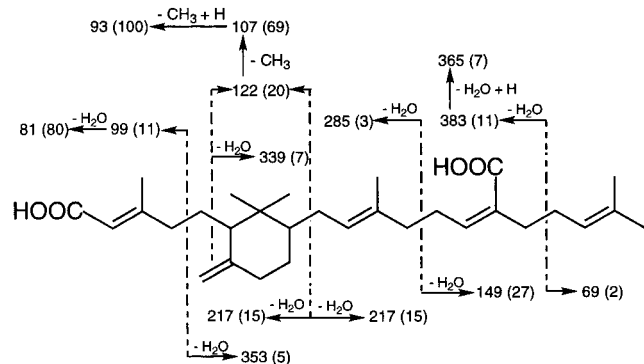**Figure 3.** Key HMBC and NOE correlations for **1**.

Figure 4. Fragment ions observed in the EIMS spectrum of **1**.

Figure 5. Possible pathway for the biosynthesis of **1**.

(connection of units A−E and G−J) was determined (Figure 1). The linkage of units F and K was determined from the HMBC experiment (Figure 3). The key correlations between CH$_3$-26 and C-10, C-11, and C-6, as well as correlations between CH$_3$-27 and C-6, C-11, and C-10, were observed in the HMBC spectrum of **1**. The NOEs between H-2 and H-4; H$_2$-12 and CH$_3$-28; H-13 and H$_2$-15; and H-17 and H-19 observed from the NOESY experiment indicated that the double bonds in the isoprenyl side chain of **1** all had trans configurations (Figure 3). Therefore, the structure was established as **1**; this structure was supported by the fragment peaks in the EI low-resolution mass spectrum (Figure 4).

Since the chemical shifts of Ha-12, H-6, and H$_2$-5 are poorly resolved, it was difficult to determine the relative configurations at positions 6 and 10 from the NOESY experiment. The relative stereochemistry could be determined from the chemical shifts of C-6 and C-10 because of the marked difference between the chemical shifts of the axial and equatorial substituted groups on the ring system. Compared with monocyclic triterpenoid **2**

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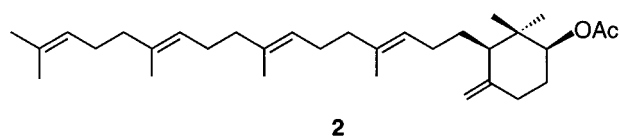
isolated from *Achillea odorata* L.,[9] the chemical shift of C-6 (δ 53.3) in **1** is close to that of the corresponding carbon (δ 51.4) in **2**. In contrast to C-6, the signal for C-10 appeared at slightly higher field (δ 48.6) since there was no β effect[9] from C-25 (Δ + 4.7). These observations indicated that the side chains on C-6 and C-10 have a *cis*-orientation, such that both can be equatorial. The absolute configurations are presently unknown.

Although monocyclic triterpenoids have been isolated from *Achillea odorata* L,[9] *Polypodiodes formosana*,[10] and several species of the genus *Iris*,[11,12] compound **1** is a monocyclic triterpenoid with a novel skeleton. From a biogenetic perspective, monocyclic triterpenes have been proposed to form as a
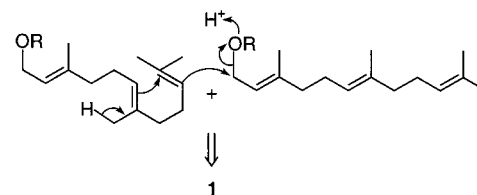
result of direct cyclization of squalene oxide[9] or by cleavage of bicyclic triterpenes.[12] However, compound **1** is presumably derived from two farnesyl units by direct coupling and cyclization (Figure 5). This would constitute a novel pathway for the biosynthesis of monocyclic triterpenes.

Potency of DNA Polymerase β Inhibition. In the DNA polymerase β assay, mispyric acid had an IC$_{50}$ value of 20 μM in the presence of bovine serum albumin (BSA) and 14 μM in the absence of BSA. While the potency of mispyric acid as a DNA polymerase β inhibitor is no better than that of the anacardic acids identified previously,[6] the activity of mispyric acid is largely unaffected by serum albumin, suggesting the greater potential of agents of this type for use in vivo. The ring and double bonds in mispyric acid also impose sufficient conformational restraints to facilitate definition of those structural elements essential for the expression of polymerase β inhibitory activity. Kinetic analysis of enzyme inhibition revealed that **1** was a mixed inhibitor with respect to activated DNA (K_{is} = 53 μM; K_{ii} = 20 μM) and was uncompetitive with respect to TTP (K_i = 18 μM). Since there is a strict order of substrate addition to DNA polymerase β the DNA template-primer followed by the addition of a nucleotide,[13] association of mispyric acid with DNA polymerase β, occurred to the greatest extent when activated DNA was bound to the enzyme active site or when the enzyme was primed for the nucleotidyl transfer reaction with both substrates in their respective sites.

Potentiation of Bleomycin Cytotoxicity in Cultured Cells. Bleomycin is known to induce single-strand DNA breaks, as well as double-strand breaks and alkali-labile lesions.[14,15] DNA polymerase β, a 39 kDa gap-filling enzyme involved in base-excision repair,[3a,16] has been implicated in the repair of single-strand breaks induced by bleomycin and other DNA damaging antitumor agents.[1−5] As shown in Figure 6, mispyric acid was not cytotoxic to cultured CCL46 cells, a mouse lymphoid neoplasm, when employed at 50 μM concentration. However,

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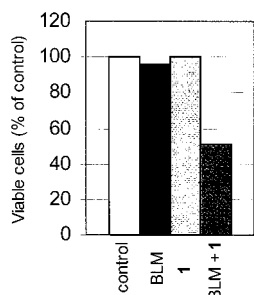
Figure 6. Potentiation of bleomycin cytotoxicity by mispyric acid (**1**). Cells were treated as described for 6 h. Viability was assessed by trypan blue exclusion: white bar, control, no treatment; black bar, 0.075 μM BLM; light gray bar, 50 μM **1**; dark gray bar, 0.075 μM BLM + 50 μM **1**.

when used concurrently with bleomycin, the latter of which was also present at a nontoxic (75 nM) concentration, a reduction in the number of viable cells of nearly 50% was realized. Thus mispyric acid shows excellent properties in potentiating the action of bleomycin in this cultured cell line, as would be predicted for an inhibitor of DNA polymerase β. This finding is consistent with a recent study in our laboratory which showed that several isolated DNA polymerase β inhibitors related to anacardic acid potentiated the cytotoxicity of antitumor agents such as bleomycin and cisplatin, and inhibited unscheduled DNA synthesis induced by these agents.[6] In addition, the recent finding that overexpression of DNA polymerase β reduced the sensitivity of CHO cells to several DNA damaging agents[17] supports the suggestion that the enzyme constitutes an excellent target for enhancing the cytotoxicity of currently used DNA damaging agents.

The capacity of tumor cells for repairing the damage[18] inflicted by DNA damaging antitumor agents such as bleomycin and cisplatin results in a limitation in the antitumor efficacy that can be realized by such agents. The identification of potent and specific agents, such as mispyric acid, capable of inhibiting DNA repair may contribute importantly to the realization of improved antitumor efficacy.

Experimental Section

General Methods. The ^{13}C NMR spectrum was recorded using tetramethylsilane (TMS) as an internal standard on a General Electric GE-300 spectrometer operating at 75 MHz. ^{1}H$-^1$H COSY, HMQC, HMBC, and NOESY spectra were measured on a Varian UnityInova 300/50 spectrometer at 300 MHz. The ^{1}H NMR and ^{1}H$-^1$H TOCSY spectra were recorded on a Varian-UnityInova 500/51 spectrometer at 500 MHz. Chemical shift values (δ) are given in parts per million relative to TMS. *J* values are given in Hertz. The following abbreviations are used to denote the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Electron impact (EI) mass spectral data were obtained on a Finnigan MAT 4600 mass spectrometer; a HPLC/high-resolution electrospray ionization (LC/HRESI) mass spectrum was recorded on a New Star T70 FT/MS spectrometer at SmithKline Beecham Pharmaceuticals. All fractionations were carried out in open columns. All reagents were of the best available commercial quality and were used without further purification. Polyamide 6S (a product of 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 μ) was obtained from Sigma Chemicals. Reversed-phase C-18 (32−63 μm) and C-8 (32−63 μm) resins were from ICN Pharmaceuticals. For the DNA polymerase β inhibition assay and the kinetic studies, active fractions from *Mischocarpus pyriformis* and pure

compound **1** were dissolved in 1:1 dimethyl sulfoxide (DMSO)-methanol. Compound **1** was dissolved in 100% DMSO for the cell culture studies, with a final DMSO concentration of 0.25% in the media for each experiment. Recombinant rat liver DNA polymerase β was a gift from Xiangyang Wang and Hongge Wang, prepared as described previously.[19] 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.[20] [^{3}H]TTP was purchased from ICN Pharmaceuticals. DEAE-cellulose paper (DE−81) was from Whatman. The CCL 46 cell line P388D[1] (derived from a 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 several bleomycins,[21] was a gift from Bristol Myers Squibb.

Extraction and Isolation. *Mischocarpus pyriformis* was collected in Australia in October, 1979. The dried plant material (stem bark) was soaked successively with hexanes, methyl ethyl ketone, MeOH, and water. The methyl ethyl ketone extract strongly inhibited DNA polymerase β (92% inhibition at 100 μg/mL and 83% inhibition at 50 μg/mL) and was fractionated first using a 21 g polyamide 6S column. To the column (40 × 3 cm) was applied 747 mg of the extract. The column was washed successively with H$_2$O, 1:1 MeOH−H$_2$O, 8:2 MeOH−CH$_2$Cl$_2$, 1:1 MeOH−CH$_2$Cl$_2$, and then 9:1 MeOH−NH$_4$OH to afford three active fractions, that is, the 8:2 MeOH−CH$_2$Cl$_2$, 1:1 MeOH−CH$_2$Cl$_2$, and 9:1 MeOH−NH$_4$OH fractions. The 1:1 MeOH−CH$_2$Cl$_2$ and 9:1 MeOH−NH$_4$OH fractions were the most polar and likely contained polyphenols. The 8:2 MeOH−CH$_2$Cl$_2$ fraction (281 mg) was fractionated further using a 12 g C$_{18}$ column (40 × 1.5 cm). Elution with 20% MeOH, 40% MeOH, 60% MeOH, 70% MeOH, 80% MeOH, 90% MeOH, MeOH, and then acetone afforded fractions 1−8. Active fraction 6 from the C-18 column (32 mg) contained the most strongly inhibitory material and was separated further using a 4 g C$_8$ column (30 × 1 cm) which was washed successively with 30% MeOH, 50% MeOH, 70% MeOH, 90% MeOH, MeOH, and acetone. The active MeOH fraction (7.7 mg) proved to contain a single, pure compound which was named mispyric acid (**1**).

Mispyric acid (**1**): colorless, viscous oil; [α]$^{20}_{\mathrm{D}}$ + 12.5° (*c* 0.05, MeOH); ^{1}H NMR and ^{13}C NMR spectra (see Table 1); EIMS *m/z* (rel.%) 470 [M]$^+$ (4), 469 (8), 452 (10), 434 (12), 383 (11), 366 (9), 365 (7), 353 (5), 339 (7), 287 (10), 285 (3), 283 (11), 273 (15), 217 (15), 203 (18), 149 (27), 122 (20), 107 (69), 99 (11), 93 (100), 81 (80), and 69 (2); LC/HRESI−FT MS, *m/z* 470.3399 [M]$^+$ (C$_{30}$H$_{46}$O$_4$ requires 470.3396).

DNA Polymerase β Inhibition Assay. The standard reaction mixture for DNA polymerase β contained 62.5 mM 2-amino-2-methyl-1,3-propanediol buffer, pH 8.6, 10 mM MgCl$_2$, 1 mM DTT, 0.1 mg/mL bovine serum albumin, 6.25 μM dNTPs, 0.04 Ci/mmol [^{3}H]TTP, and 0.25 mg/mL activated calf thymus DNA. After dissolving the extract samples or fractions in 1:1 DMSO-MeOH, 6 μL of the sample and 4 μL of rat DNA polymerase β (6.9 units, 48,000 units/mg) were added to 50 μL of the standard reaction mixture and incubated at 37 °C for 60 min. Then the radioactive DNA product was collected on DEAE-cellulose paper filters and dried. The filters were washed successively with 0.4 M K$_2$HPO$_4$ (pH 9.4) and 95% ethanol and then dried and used for radioactivity determination. For the kinetic studies, inhibitor constants (K_i, K_{is}, and K_{ii}) were obtained using the same assay except

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that incubation was for 20 min. For all kinetic analyses, the samples were placed on ice immediately before application to DE−81 paper disks. Additionally, either dNTP concentrations were varied [25, 12.5, 6.25, 3.12, and 1.56 μM ([^{3}H]TTP was added in proportion, such that its specific activity remained constant)] or activated DNA concentration was varied [200, 100, 50, 25, and 12.5 μM (nucleotide concentration)].

Potentiation of Bleomycin Cytotoxicity in the Cultured Cells. P388D[1] cells were maintained as suspension cultures in 90% (v/v) Dulbecco's modified Eagle's medium containing 4500 mg glucose/L, 10% (v/v) donor horse serum, and 100 IU penicillin, 0.1 mg strepto-mycin, and 0.25 μg amphotericin B per milliliter at 37 °C in a 5% CO_2 in air atmosphere. To 12.5 mL tissue culture flasks was added 5 mL of a cell suspension containing ∼5 × 10^5 cells/mL; this was incubated for 1 h to stabilize the cells. Assays were carried out in 6 mL incubation volumes containing the indicated amounts of compound dissolved in DMSO and blenoxane dissolved in media. Cultures were incubated at 37 °C in a 5% CO_2 in air atmosphere for 6 h followed by determination of cell viability using trypan blue dye.

Supporting Information Available: Procedures for the extraction and isolation of mispyric acid, DNA polymerase β inhibition assay; ^{1}H NMR, ^{13}C NMR, ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra in CDCl$_3$ for **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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