Bis-5-alkylresorcinols from *Panopsis rubescens* that Inhibit DNA Polymerase β

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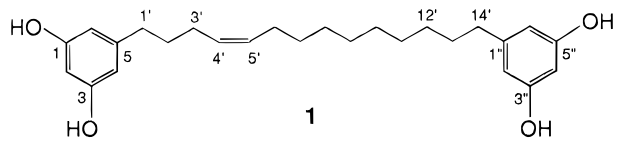
Bioassay-guided fractionation of *Panopsis rubescens*, using an assay to detect DNA polymerase β inhibition, led to the isolation of two new bis-5-alkylresorcinols (**1** and **2**), in addition to one known bis-5-alkylresorcinol (**3**). The structures of **1**−**3** were established as 1,3-dihydroxy-5-[14′-(3″,5″-dihydroxyphenyl)-*cis*-4′-tetradecenyl]benzene (**1**), 1,3-dihydroxy-5-[14′-(3″,5″-dihydroxyphenyl)-*cis*-7′-tetradecenyl]benzene (**2**), and 1,3-dihydroxy-5-[14′-(3″,5″-dihydroxyphenyl)tetradecenyl]benzene (**3**), respectively, by spectroscopic and chemical analyses. Compounds **1**−**3** exhibited potent inhibition of calf thymus DNA polymerase β, with IC_{50} values of 7.5, 6.5, and 5.8 μM, respectively.

Cellular DNA defense mechanisms exist to repair the DNA damage resulting from exogenous sources, including chemotherapeutic agents. This effect has been shown to reduce the potency of and promote resistance to DNA-damaging agents.[1] DNA polymerase β, one of the five known eukaryotic DNA polymerases, is believed to be responsible for repairing DNA damage after exposure to some anticancer drugs such as bleomycin and pepleomycin,[2] monofunctional DNA alkylating agents,[3] cisplatin,[4] and neocarzinostatin.[5] A recent study using isolated DNA polymerase β inhibitors demonstrated that inhibition of the enzyme was correlated with potentiation of bleomycin and cisplatin activity.[6] Although a few DNA polymerase β inhibitors have been reported, including the nucleotide dideoxy TTP,[7] sulfate- or sialic acid-containing glycolipids,[8] phospholipids,[9] flavonoids,[10] triterpenoids,[11] fatty acids, and fatty acid derivatives,[12] the search for more potent DNA polymerase β inhibitors is essential to secure agents that can be used to validate polymerase β as a therapeutically relevant antitumor target and potentially be employed for combination chemotherapy with DNA-damaging agents.

Some naturally occurring phenolic and catecholic principles[13] exhibit potent biological activities,[14,15] such as the skin-sensitization properties of poison oak and poison ivy, as well as antiviral activity and cytotoxic activity against human prostate cancer. 1,3-Dihydroxy-5-alkylbenzenes, known as resorcinols, occur naturally in the Proteaceae, Anacardiaceae, Myristicaceae, Ginkogoaceae, and Graminae families and show a wide variety of biological and biochemical activities, including fungicidal and bacteriocidal activities against pathogens[16] and DNA cleavage.[17] In our survey of crude plant extracts for DNA polymerase β inhibitors,[18] a methyl ethyl ketone extract of *Panopsis rubescens* (Pohl) Pittier det. S. McDaniel (Proteaceae) exhibited potent inhibition of DNA polymerase β (81% and 77% inhibition at 100 and 50 μg/mL, respectively). To date, there has been no report describing the chemical constituents of this plant. To permit isolation of the inhibitory principles, the crude extract was subjected to bioassay-guided fractionation using an assay sensitive to DNA polymerase β inhibition. Bioassay-guided fractionation of the methyl ethyl ketone extract of *P. rubescens* led to the isolation of three bis-5-alkylresorcinols, two of which have not been described previously. All three were found to be potent inhibitors of DNA polymerase β. Presently, we report the isolation and structure elucidation of these three bioactive principles, as well as their potency of inhibition of DNA polymerase β.

Results and Discussion

Dried twigs from *P. rubescens* were soaked successively with hexanes, methyl ethyl ketone, methanol, and water. The methyl ethyl ketone extract was found to inhibit polymerase β and 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_2Cl_2, and 9:1 MeOH−NH_4OH (Table 1). The final eluate contained polyphenols, which tend to be strong, albeit nonspecific, inhibitors of DNA polymerase β. The inhibitory 8:2 MeOH−CH_2Cl_2 fraction (93% inhibition of DNA polymerase β at 100 μg/mL; 88% inhibition at 50 μg/mL) was applied to a Sephadex LH-20 column and was subjected to a normal-phase elution scheme. The 1:1 CH_2Cl_2−acetone fraction from the Sephadex LH-20 column exhibited the strongest DNA polymerase β inhibitory activity and was fractionated further using a reversed-phase C_{18} open column. Three inhibitory fractions, including two 1:13:6 CH_2Cl_2−MeOH−H_2O fractions and one 1:15:5 CH_2Cl_2−MeOH−H_2O fraction, were combined and fractionated further using a reversed-phase C_8 open column. The 1:13:6 CH_2Cl_2−MeOH−H_2O fraction showed the most potent inhibitory activity against DNA polymerase β and was purified further using a C_{18} reversed-phase HPLC column to afford active compounds **1**−**3**.



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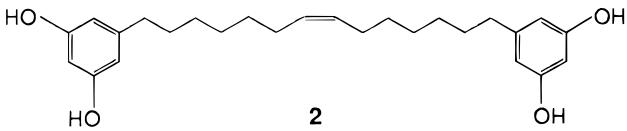

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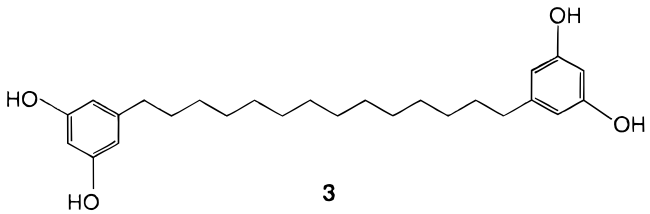

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Table 1. DNA Polymerase β Inhibition Data from Bioassay-guided Fractionation of a Methyl Ethyl Ketone Crude Extract of *Panopsis rubescens* (Percent Inhibition of Polymerase β)

column	% inhibition	
crude extract	81[a]	77[b]
Polyamide 6S		
8:2 MeOH−CH_2Cl_2	93[a]	88[b]
Sephadex LH-20		
1:1 CH_2Cl_2-acetone	93[a]	90[b]
C_{18} reversed phase		
1:13:6 CH_2Cl_2−MeOH−H_2O	95[a]	85[b]
C_8 reversed phase		
1:13:6 CH_2Cl_2−MeOH−H_2O	92[b]	77[c]
HPLC (C_{18})		
fraction 1 (**1**)	98[b]	51[c]
fraction 2 (**2**)	92[b]	44[c]
fraction 3 (**3**)	79[b]	50[c]

[a] 100 μg/mL. [b] 50 μg/mL. [c] 10 μg/mL.

Compounds **1** and **2** were obtained as colorless solids and compound **3** as colorless microcrystals. The high-resolution fast-atom bombardment mass spectra (FABMS) of **1** and **2** indicated that both had the same molecular formula ($C_{26}H_{36}O_4$). Compound **1** had an ^{1}H NMR spectrum with resonances at δ 1.20 (12H, m), 1.65 (4H, m), 1.97 (4H, m), 2.40 (4 H, m), 5.30 (2H, qn, J = 9.0, 4.5 Hz), 6.05 (2H, br s), and 6.15 (4H, br s); the ^{1}H NMR spectrum of **2** was quite similar. The ^{13}C NMR spectra of **1** indicated the presence of aromatic, oxygen-substituted aromatic, olefinic, and alkane carbons. Analysis of the ^{1}H and ^{13}C NMR spectra, as well as the MS data, suggested that **1** and **2** were bis-5-alkylresorcinol derivatives with a double bond in the alkane chain.[17a] The configurations of the double bonds were determined from the coupling constants between the olefinic protons and by the chemical shifts for vinylic carbons.[19] The two olefinic proton signals for H-4′ and H-5′ at δ 5.30 appeared in the ^{1}H NMR spectrum of **1** as a quintet because their chemical shift values were quite close; the coupling value measured, $J_{4',5'}$ = 9.0 Hz, indicated a *Z* geometry in **1**. The *Z* configuration was confirmed by the chemical shifts of C-3′ and C-6′ at δ 26.73 and 26.45, respectively, compared with the corresponding chemical shifts of δ 31−34 that would be expected for the corresponding *E* configuration.[19] The ^{1}H NMR spectrum of **2** had a signal for the olefinic protons at δ 5.32 as a triplet (J = 4.5 Hz) resulting from coupling with the allylic protons; no coupling between the olefinic protons was observed. This implied a possible symmetric structure for **2**. The *Z* geometry of the double bond in **2** was subsequently established on the basis of the chemical shift at δ 26.83 for the allylic carbons C-6′ and C-9′.[19,20] The MS of **1** and **2** indicated that both had the same molecular weight (412), suggesting that the alkene must have a chain length of 14 carbon atoms. However, the exact positions of the double bonds could not be determined on the basis of the NMR data. The positions were established through ozonolysis of the respective tetra-*O*-acetates, followed by analysis of chemical ionization mass spectra (CIMS) of the crude aldehydic product mixtures.[21]

Acetylation of **1** and **2**, followed by ozonolysis in CS_2 at −78 °C, afforded the crude aldehydic product mixtures from **1** and **2**. These mixtures were subjected directly to CIMS analysis (Scheme 1). The CIMS of the reaction mixture derived from **1** showed two strong pseudomolecular ion peaks at *m*/*z* 349 (attributed to **1b**) and 265 (**1a**) for aldehydes having M_r values of 348 and 264, indicating that oxidative cleavage had occurred between carbon atoms 4′

Scheme 1. Chemical Ionization Mass Spectroscopic Analysis of the Ozonolysis Products of Tetra-*O*-acetates of **1** and **2**

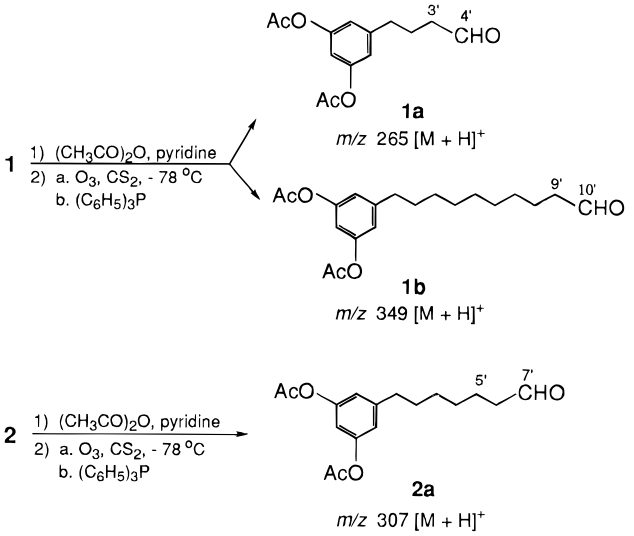

Table 2. IC_{50} Values for Bis-5-alkylresorcinols from *Panopsis rubescens*

compound	IC_{50} (μM)
1	7.5
2	6.5
3	5.8

and 5′ of the 14-carbon chain. Therefore, the structure of **1** was established as 1,3-dihydroxy-5-[14′-(3″,5″-dihydroxyphenyl)-*cis*-4′-tetradecenyl] benzene. Subsequently, a single strong pseudomolecular ion peak was observed at *m*/*z* 307 (**2a**) in the CIMS of the reaction mixture derived from **2** for the formed aldehyde, corresponding to M_r 306. This indicated that the olefin in **2** was located between carbons 7′ and 8′ in the 14-carbon chain, consistent with the observation of a single aldehyde for this symmetric compound. Therefore, the structure of **2** was established as 1,3-dihydroxy-5-[14′-(3″,5″-dihydroxyphenyl)-*cis*-7′-tetradecenyl]benzene. Compounds **1** and **2** are new bis-5-alkylresorcinol derivatives.

The high-resolution FABMS of **3** provided the molecular formula $C_{26}H_{38}O_4$, which indicated the presence of two more hydrogen atoms in **3** than in **1** or **2**. The ^{1}H NMR spectrum of **3** was quite similar to those of **1** and **2**, with the exception that no olefinic proton signal (at ~δ 5.30) nor allylic proton signal (at ~δ 2.40) was present. This indicated that **3** was simply the saturated analogue of **1** and **2**. Therefore, the structure of **3** was established as 1,3-dihydroxy-5-[14′-(3″,5″-dihydroxyphenyl)tetradecenyl]-benzene, a natural product that has been reported previously.[17]

The first step in the bioassay-guided fractionation employed a polyamide 6S column. This was used to separate polyphenols, which would likely bind indiscriminately to cellular constituents instead of selectively inhibiting polymerase β. A C_{18} reversed-phase HPLC column was employed successfully for the separation of the structurally similar and air-sensitive bis-5-alkylresorcinols **1**, **2**, and **3**.

Compounds **1**−**3** displayed potent inhibitory activity against rat DNA polymerase β; their IC_{50} values were 7.5, 6.5, and 5.8 μM, respectively (Table 2), in the presence of bovine serum albumin (BSA), as determined under the assay conditions described in the Experimental Section. It is interesting that, unlike many of the polymerase β inhibitors reported to date,[9,11,12,18] compounds **1**−**3** lack any carboxylate functionality.

In regard to their biological activities, this is the first report in which alkylresorcinols have been shown to inhibit a mammalian DNA polymerase β; to our knowledge they exhibit the strongest inhibition of any of the reported naturally occurring DNA polymerase β inhibitors. Further, it is interesting that these alkylresorcinols both cleave DNA[17] and inhibit the repair of DNA damage by polymerase β. It seems plausible that these two activities could function synergistically to potentiate the destruction of cancer cells.

Experimental Section

General Experimental Procedures. Polyamide 6S (a product of Riedel-de Haen, Germany) was obtained from Crescent Chemical Co. Lipophilic Sephadex LH-20 (Pharmacia; 40 μm) was purchased from Sigma Chemicals. Silica RP C_8 and RP C_{18} (32−60 μm) chromatographic supports were obtained from ICN Biomedical Research Products. A Kromasil C_{18} column (250 × 10 mm, 5 μm) for HPLC was obtained from Higgins Analytical Inc. ^{1}H and ^{13}C NMR spectra were recorded on GN-300 or QE-300 NMR spectrometers. Low-resolution chemical ionization (CI) and electron-impact (EI) mass spectra were recorded on a Finnigan MAT 4600 mass spectrometer. High-resolution mass spectra were taken on a VG ZAB-SE mass spectrometer. Unlabeled dNTPs and calf thymus DNA were purchased from Sigma Chemicals; [^{3}H]TTP was from ICN Pharmaceuticals. DEAE−cellulose paper (DE-81) was purchased from Whatman.

Plant Material. Twigs of *P. rubescens* were collected in Peru in April 1976 in inudated shoreline forest along Rio Nancy from Bellavista to Quebrada de Morropon. Voucher specimen Rimachi 2211 is preserved at the Institute for Botanical Exploration, Mississippi State University.

Extraction and Isolation. The dried plant material was soaked successively with hexanes, methyl ethyl ketone, methanol, and water. The methyl ethyl ketone extract showed significant inhibition of DNA polymerase β (81% inhibition at 100 μg/mL). Since the crude extract continued to show significant activity (93% inhibition at 100 μg/mL) after passage through a polyamide 6S column, it was chosen for further bioassay-guided fractionation. In a typical experiment, a 15-g polyamide 6S column was loaded with 382 mg of the crude extract. The column was washed successively with H_2O, 1:1 MeOH−H_2O, 8:2 MeOH−CH_2Cl_2, 1:1 MeOH−CH_2Cl_2, and 9:1 MeOH−NH_4OH (200-mL fractions). The 8:2 MeOH−CH_2Cl_2 fraction (189 mg) exhibited significant inhibition of DNA polymerase β (93% inhibition at 100 μg/mL) and was fractionated further on a 20-g Sephadex LH-20 column, which was washed successively with hexane, 1:1 hexane−CH_2Cl_2, CH_2Cl_2, 1:1 CH_2Cl_2−acetone, acetone, and MeOH (300-mL fractions). The 1:1 CH_2Cl_2−acetone fraction (containing 60 mg of product) was the most active and was applied to a 15-g C_{18} column. This column was washed with water, then with increasing amounts of methanol in water, and finally with a ternary system (initially 1:11:8 CH_2Cl_2−MeOH−H_2O) to which increasing proportions of MeOH were added. The most active fractions, containing a total of 24 mg of material, eluted primarily with 1:13:6 CH_2Cl_2−MeOH−H_2O and were combined and applied to an 8-g C_8 column that employed an elution scheme similar to the C_{18} column described above. The most active fraction (12 mg; 92% inhibition at 50 μg/mL) eluted with 1:13:6 CH_2Cl_2−MeOH−H_2O and was fractionated on a 5 μm C_{18} reversed-phase HPLC column (250 × 10 mm), which was washed with a linear gradient of 4:1 → 19:1 CH_3CN−H_2O over a period of 50 min at a flow rate of 4.0 mL/min (monitoring at 280 nm), affording pure compounds **1**−**3** in order of elution from this column.

Compound 1: colorless solid; 5.2 mg; ^{1}H NMR ($CDCl_3$ + CD_3OD, 300 MHz) δ 1.21 (12H, m), 1.65 (4H, m), 1.97 (4H, m), 2.40 (4H, m), 5.30 (2H, qn, J = 9.0, 4.5 Hz), 6.06 (2H, br s), 6.15 (4H, br s); ^{13}C NMR ($CDCl_3$ + CD_3OD, 75 MHz) δ 26.45, 26.73 (C-3′, C-6′), 28.75, 28.83, 29.00, 29.05, 29.17, 29.20 (C-7′, C-8′, C-9′, C-10′, C-11′, C-12′), 30.69, 30.73 (C-2′, C-13′), 35.11, 35.51 (C-1′, C-14′), 99.37, 99.45 (C-2, C-4″), 106.71, 106.79 (C-4, C-6, C-2″, C-6″), 128.89, 129.97 (C-4′, C-5′), 144.91, 145.28 (C-5, C-1″), 156.97, 157.00 (C-1, C-3, C-3′, C-5″); HRFABMS m/z 413.2689 [M + H]$^+$ (calcd for $C_{26}H_{37}O_4$, 413.2692).

Compound 2: colorless solid; 1.2 mg; ^{1}H NMR ($CDCl_3$ + CD_3OD, 300 MHz) δ 1.20 (12H, m), 1.66 (4H, m), 1.98 (4H, m), 2.41 (4H, m), 5.32 (2H, t, J = 4.5 Hz), 6.05 (2H, br s), 6.14 (4H, br s); ^{13}C NMR ($CDCl_3$ + CD_3OD, 75 MHz) δ 26.83 (C-6′, C-9′), 28.88, 29.26, 29.29 (C-3′, C-4′, C-5′), 30.78 (C-2′, C-13′), 35.67 (C-1′, C-14′), 99.52 (C-2, C-4″), 106.66 (C-4, C-6, C-2″, C-6″), 128.98 (C-7′, C-8′), 145.35 (C-5, C-1″), 157.01 (C-1, C-3′, C-5″); HRFABMS m/z 413.2698 [M + H]$^+$ (calcd for $C_{26}H_{37}O_4$, 413.2692).

Compound 3: colorless microcrystals; 2.9 mg; ^{1}H NMR ($CDCl_3$ + CD_3OD, 300 MHz) δ 1.18 (20H, m), 1.45 (4H, m), 1.66 (4H, m), 2.38 (4H, t, J = 7 Hz), 6.05 (2H, br s), and 6.14 (4H, br s); HRFABMS m/z 415.2842 [M + H]$^+$ (calcd for $C_{26}H_{39}O_4$, 415.2848).

Acetylation of 1. To a cooled (0−5 °C) solution of 1.2 mg (3.5 μmol) of **1** in 500 μL of dry pyridine was added dropwise 200 μL (216 mg, 2.1 mmol) of acetic anhydride dropwise under argon. The reaction mixture was stirred under argon at 25 °C for 2.5 h, followed by the addition of an ice chip and subsequent extraction with $CHCl_3$. The chloroform layer was washed with water, dried over $MgSO_4$, and concentrated under diminished pressure to afford the tetra-*O*-acetate of **1** as an oily product: yield 1.4 mg (83%); ^{1}H NMR ($CDCl_3$, 300 MHz), δ 1.25 (12H, m), 1.60 (4H, m), 2.02 (4H, m), 2.23 (12H, s), 2.41 (4H, m), 5.38 (2H, m), 6.67 (2H, br s), and 6.79 (4H, br s); positive ion CIMS m/z 581 [M + H]$^+$.

Acetylation of 2. To a cooled (0 °C) solution of 0.5 mg (1.4 μmol) of **2** in 500 μL of dry pyridine was added 200 μL (216 mg, 2.1 mmol) of acetic anhydride under argon. The reaction mixture was stirred under argon at 25 °C for 2.5 h, followed by the addition of an ice chip and subsequent extraction with $CHCl_3$. The chloroform layer was washed with water, dried over $MgSO_4$, and concentrated under diminished pressure to afford the tetra-*O*-acetate of **2** as an oily product: yield 0.6 mg (82%); ^{1}H NMR ($CDCl_3$, 300 MHz), δ 1.23 (12H, m), 1.58 (4H, m), 2.01 (4H, m), 2.22 (12H, s), 2.39 (4H, m), 5.39 (2H, t, J = 4.5 Hz), 6.68 (2H, br s), 6.80 (4H, br s); positive CIMS m/z 581 [M + H]$^+$.

Ozonolysis of the Tetra-*O*-acetate of 1. A cooled (−78 °C) solution containing 1.4 mg (3.2 μmol) of **1** in 230 μL of CS_2 was saturated with O_3. Excess O_3 was removed with a stream of argon followed by treatment with 1.0 mg (3.8 μmol) of solid triphenylphosphine. The reaction mixture was allowed to warm to room temperature and was subjected to CI mass spectrometric analysis directly: positive ion CIMS m/z 349 [M + H]$^+$ and 265 [M + H]$^+$.

Ozonolysis of the Tetra-*O*-acetate of 2. A cooled (−78 °C) solution containing 0.6 mg (1.4 μmol) of **2** in 230 μL of CS_2 was saturated with O_3. Excess O_3 was removed with a stream of argon followed by treatment with 1.0 mg (3.8 μmol) of solid triphenylphosphine. The reaction mixture was allowed to warm to room temperature and was subjected to CI mass spectrometric analysis directly: positive ion CIMS m/z 307 [M + H]$^+$.

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, [^{3}H]TTP (0.04 Ci/mmol), and 0.25 mg/mL of activated calf thymus DNA. After dissolving the crude extract samples or fractions in 50% DMSO−MeOH, 6 μL of the sample and 4 μL of rat DNA polymerase β[6,22] (6.9 units, 48 000 units/mg) were added to 50 μL of the standard reaction mixture; incubation was carried out at at 37 °C for 60 min. The radioactive DNA product was collected on DEAE-cellulose paper (DE-81), dried, and washed successively with 0.4 M K_2HPO_4, pH 9.4, and 95% ethanol. Radioactivity was measured using a liquid scintillation counter.

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