DNA Polymerase β Lyase Inhibitors from *Maytenus putterlickoides*

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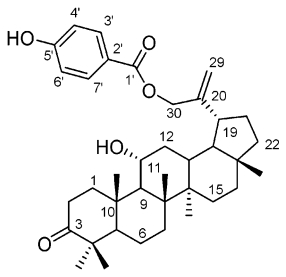
During a survey of plant secondary metabolites for DNA polymerase β lyase inhibitors, we found that a crude methyl ethyl ketone extract prepared from *Maytenus putterlickoides* showed strong inhibition of the lyase activity of DNA polymerase β in an in vitro assay. Bioassay-guided fractionation of the extract, using an in vitro assay, resulted in the discovery of a new active principle, 30-(4′-hydroxybenzoyloxy)-11α-hydroxylupane-20(29)-en-3-one (**1**), as well as a known compound, (−)-epicatechin (**2**). Compounds **1** and **2** exhibited DNA polymerase β lyase inhibitory activity with IC_{50} values of 62.8 and 18.5 μM, respectively. Compound **2** was capable of potentiating the action of the monofunctional methylating agent methyl methanesulfonate in cultured human cancer cells, consistent with the possible utility of inhibitors of this type in vivo.

The search for new anticancer agents from natural sources continues to be a productive strategy for the discovery of new therapeutic agents; currently used strategies emphasize mechanism-based methods involving discrete cellular or molecular targets.[1] One of the most important classes of antitumor drugs are DNA-damaging agents.[2] However, the toxicity of these agents generally limits treatment and can preclude their use altogether. Further, cancer cells contain mechanisms that repair the damage inflicted to their DNA, thus lessening the potency of, and promoting resistance to, DNA-damaging agents.[3−5] These systems include base excision repair (BER), nuclear excision repair (NER), and DNA mismatch repair (MMR).[6,7] The DNA BER pathway is responsible for the repair of damaged DNA after exposure to some DNA-damaging agents such as bleomycin (BLM),[8] monofunctional DNA alkylating agents,[9] cisplatin,[10] and neocarzinostatin.[11] DNA polymerase β, one of the five known eukaryotic DNA polymerases, is one of the key enzymes involved in BER.[12] It is the smallest eukaryotic polymerase and contains two functional domains: an N-terminal 8-kDa domain which has a 5′ 2-deoxyribose phosphate (dRP) excision activity and a C-terminal 31-kDa polymerase domain.[13] It has been reported that both the polymerase (DNA resynthesis) and 5′-dRP lyase activities of DNA polymerase β are essential for the mammalian single nucleotide patch BER. The lyase-mediated transformation is the key rate-limiting step.[14] DNA polymerase β may be regarded as a promising target for adjuvant anticancer therapy, since selective inhibition of the lyase activity of this enzyme by otherwise noncytotoxic agents might be expected to sensitize cancer cells to DNA-damaging agents and thus potentiate their action.

During our survey of plant secondary metabolites for DNA polymerase β lyase inhibitors, a methyl ethyl ketone extract prepared from *Maytenus putterlickoides* exhibited potent inhibition of the lyase activity of DNA polymerase β. Fractionation of the extract was guided by an in vitro assay sensitive to lyase inhibition. Bioassay-guided fractionation resulted in the isolation of a new inhibitor, 30-(4′-hydroxybenzoyloxy)-11α-hydroxylupane-20(29)-en-3-one (**1**), as well as a known compound, (−)-epicatechin (**2**). This report describes the bioassay-guided isolation of inhibitors **1** and **2** and the determination of their structures and potencies as DNA polymerase β lyase inhibitors.



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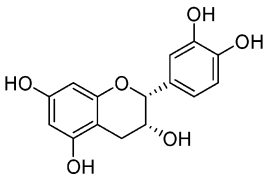

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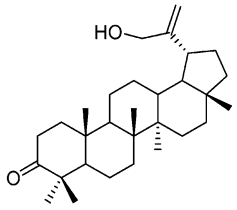

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Twigs of *M. putterlickoides* were soaked successively with hexanes, methyl ethyl ketone, methanol, and water at room temperature. The methyl ethyl ketone extract (MEK) strongly inhibited the dRP lyase activity of DNA polymerase β and was fractionated initially on a polyamide 6S column; the column was eluted successively with H_2O, 1:1 MeOH−H_2O, 4:1 MeOH−CH_2Cl_2, 1:1 MeOH−CH_2Cl_2, and 9:1 MeOH−NH_4OH. The final eluate strongly inhibited the enzyme activity, presumably because this fraction contained polyphenols, which tend to be strong and nonspecific inhibitors of DNA polymerase β. The 4:1 MeOH−CH_2Cl_2 fraction also had significant DNA polymerase β lyase inhibitory activity and was applied to a Sephadex LH-20 column for further fractionation, employing a reversed-phase elution scheme. The 6:4 MeOH−H_2O fraction from the Sephadex LH-20 column, which exhibited the strongest activity, was fractionated further on a C_{18} reversed-phase open column, resulting in two active fractions (4:6 MeOH−H_2O and MeOH). The MeOH fraction was purified further using a diol column to afford the new compound **1**.

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Table 1. ^{1}H and ^{13}C NMR Spectral Data for **1** in $CDCl_3$

position	δ_H	δ_C
1	1.90 o,[a] 1.42 o	39.0
2	2.48 m	34.4
3		219.0
4		38.4
5	1.35 o	55.1
6	1.38 o, 1.60 o	19.9
7	1.23 o, 1.51 o	34.5
8		42.3
9	1.39 o	55.0
10		37.4
11	3.91 ddd (11.0, 10.0, 5.0)	70.7
12	1.65 o, 1.32 o	27.7
13	1.70 o	37.7
14		42.6
15	1.10 o, 1.68 o	27.6
16	1.36 o, 1.53 o	35.6
17		43.3
18	1.37 o	47.9
19	2.39 m	47.8
20		150.4
21	1.32 o, 2.15 m	30.6
22	1.26 o, 1.35 o	40.0
23	1.10 s	24.0
24	1.07 s	21.0
25	0.91 s	16.9
26	1.08 s	17.1
27	0.93 s	14.6
28	0.80 s	18.3
29	4.61 d (2.0), 4.72 d (2.0)	110.2
30	4.22 br s	68.4
1′		168.0
2′		132.7
3′	7.71 d (9.0)	129.0
4′	7.53 d (9.0)	131.1
5′		150.4
6′	7.53 d (9.0)	131.1
7′	7.71 d (9.0)	129.0

[a] Overlapped signals (appearing as multiplets) are indicated by "o".

Purification of the 4:6 MeOH−H_2O fraction also using a diol column yielded compound **2**.

Compound **1** was obtained as colorless needles. Its molecular formula ($C_{37}H_{52}O_5$), requiring 12 degrees of unsaturation, was established on the basis of the HREIMS spectrum (found, *m*/*z* 576.3820; calcd for $C_{37}H_{52}O_5$, 576.3815). Compound **1** responded positively to a Liebermann-Burchard test for triterpenoids. Further evidence from ^{1}H NMR, ^{13}C NMR, DEPT, and MS suggested that **1** had a lupane skeleton. The ^{1}H NMR spectrum showed an AA′BB′ spin system at δ 7.71 (2H, d, J = 9.0 Hz) and 7.53 (2H, d, J = 9.0 Hz), suggesting the presence of a 1,4-disubstituted aromatic ring. It also contained two terminal olefinic proton signals at δ 4.72 (1H, d, J = 2.0 Hz) and 4.61 (1H, d, J = 2.0 Hz), one hydroxymethylene signal at δ 4.22 (2H, m), one hydroxymethine signal at δ 3.91 (1H, ddd), and six methyl group signals at δ 0.80 (3H, s) 0.91 (3H, s), 0.93 (3H, s), 1.07 (3H, s), 1.08 (3H, s), and 1.10 (3H, s). The ^{13}C NMR spectrum contained resonances assigned to an ester carbonyl carbon at δ 168.0 (s), an oxygen-substituted aromatic carbon at δ 150.4, and other aromatic carbons at δ 132.7, 131.1, and 129.0 (Table 1). These suggested the presence of a *p*-hydroxybenzoyl substituent in **1**. In addition, the ^{13}C NMR spectrum of **1** contained 30 signals for carbon atoms of the triterpenoid moiety (six methyls, 11 methylenes, six methines, and seven quaternary carbons). The ^{1}H and ^{13}C NMR data for **1** were similar to those of the known lupane triterpene 30-hydroxylupane-20(29)-en-3-one (**3**),[15] except for the resonances due to C-11 (H-11) and C-30 (H-30), and for the *p*-hydroxybenzoyl moiety. The protons at C-30 in **1** appeared at δ 4.22 (2H, br s) as opposed to δ 3.16 in **3**, thus supporting the assignment of the ester in **1** at C-30. These assignments were unambiguously confirmed by the HMBC spectrum, which showed cross-peaks between H_2-30 and C-1′, C-20, and C-29 (Figure 1). The ^{13}C NMR exhibited signals at δ 55.0, 70.7, and 27.7 corresponding to C-9, C-11, and C-12, respectively. Compared with the corresponding δ values of the carbons in **3**, the C-9, C-11, and C-12 resonances were relatively deshielded, suggesting the presence of a hydroxyl group at C-11. In the NOESY spectrum of **1**, the proton signal for H-11 correlated with H_3-25 and H_3-26. Thus, the hydroxyl group at C-11 had the α configuration. All assignments for **1** were confirmed by the ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra. Therefore, the structure of **1** was established as 30-(4′-hydroxybenzoyloxy)-11α-hydroxylupane-20-(29)-en-3-one. Compound **1** is the first example of a lupane attached to a *p*-hydroxybenzoyl moiety.

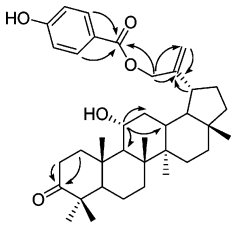

Figure 1. Selected HMBC correlations of **1**.

Table 2. Potentiation of MMS Cytotoxity in A549 Cells by Compounds **1** and **2**[a]

	viable cells (% of control)		
compound	compound alone[a]	MMS[b]	compound[b] + MMS[c]
1	100 ± 0.56	100 ± 1.06	100 ± 0.59
2	100 ± 0.54	100 ± 1.11	86.6 ± 0.38

[a] The results are from three experiments. [b] Compounds **1** and **2** were employed at 200 μM concentration. [c] MMS was employed at 62.5 μM concentration.

Compound **2** was identified as (−)-epicatechin by comparison with literature values (including mp, ^{1}H and ^{13}C NMR, and MS data).[16]

Compounds **1** and **2** inhibited the lyase activity of DNA polymerase β in a concentration-dependent manner; they had IC_{50} values of 62.8 and 18.5 μM, respectively. Although a series of triterpenoids has been reported to inhibit the polymerase (DNA resynthesis) activity of DNA polymerase β,[17] compound **1** is the first lupane-type triterpenoid found to inhibit the lyase activity.

Compounds **1** and **2** were tested further for their ability to potentiate the action of the monofunctional methylating agent methyl methanesulfonate (MMS) in reducing the numbers of viable cultured mammalian cells. Accordingly, A549 cells (human lung carcinoma) were incubated for 48 h in the presence of MMS alone, compound **1** or **2** alone, and the two in combination. As shown in Table 2, MMS and inhibitors were nontoxic to the cultured cells when employed at 62.5 and 200 μM concentrations, respectively. However, in the presence of both MMS and compound **2**, the number of viable cells was reduced to 86.6% of that found in the untreated control. Thus, compound **2** was capable of potentiating the action of MMS in a cultured human cancer cell line. Compound **1** did not potentiate MMS cytotoxicity in A549 cells.

(−)-Epicatechin (**2**) has thus far exhibited diverse biological activities including antioxidant activity,[18] superoxide radical scavenging activity,[19] antiprotozoal activity,[20] protein tyrosine phosphatase inhibitory activity,[21] and

other biological activities.[22] In regard to these biological activities, this is the first report in which (−)-epicatechin has been shown to inhibit a mammalian DNA polymerase β lyase activity and potentiate the cytotoxicity of MMS.

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_{18} resin was obtained from ICN Biochemical Pharmaceuticals (Costa Mesa, CA). LiChroprep Diol chromatographic support was obtained from EM Separations Technology (Gibbstown, NJ). All fractionations were carried out in open columns. ^{1}H and ^{13}C NMR, DEPT, and 2D-NMR (^{1}H−^{1}H COSY, HMQC, HMBC, NOESY) spectra were taken on a Varian 500 NMR spectrometer. HREIMS were recorded on a JEOL AX-505H mass spectrometer. Unlabeled dNTPs and calf thymus DNA were purchased from Sigma Chemicals; [^{3}H]dTTP was from ICN Pharmaceuticals. [α-^{32}P]ddATP (3000 Ci/mmol) was obtained from ICN Biomedicals, Inc. AP endonuclease was from Trevigen, Inc. Uracil-DNA glycosylase was obtained from New England Biolabs, Inc. Synthetic oligodeoxyribonucleotides were purchased from Integrated DNA Technologies, Inc. Distilled, deionized water (Milli-Q system) was employed for all aqueous manipulations. The human lung carcinoma cell line A549 was purchased from American Type Culture Collection (ATCC).

Plant Material. Twigs of *Maytenus putterlickoides* (Exell and Mendonca) (PR 34250) were collected in Kenya in 1973 and were supplied by the Medicinal Plant Resources Laboratory, USDA, Beltsville, MD, where voucher specimens are preserved.

Extraction and Isolation. The MEK extract of *M. putterlickoides* showed significant lyase inhibitory activity of DNA polymerase β and was fractionated initially using a 35 g polyamide 6S column. To the column (60 × 1.5 cm) was applied 790 mg of the extract. The column was eluted successively with H_2O, 1:1 MeOH−H_2O, 4:1 MeOH−CH_2Cl_2, 1:1 MeOH−CH_2Cl_2, and then 9:1 MeOH−NH_4OH (200 mL fractions). The 4:1 MeOH−CH_2Cl_2 fraction (250 mg) strongly inhibited the lyase activity of DNA polymerase β; this material was fractionated further on a 15 g Sephadex LH-20 column, which was washed successively with 2:8 MeOH−H_2O, 4:6 MeOH−H_2O, 6:4 MeOH−H_2O, 8:2 MeOH−H_2O, MeOH, and then Me_2CO (200 mL fractions). The 6:4 MeOH−H_2O (90.9 mg) fraction, which exhibited the strongest activity, was fractionated further on a 10 g C_{18} reversed-phase open column, which was eluted successively with 2:8 MeOH−H_2O, 4:6 MeOH−H_2O, 6:4 MeOH−H_2O, 8:2 MeOH−H_2O, MeOH, and then Me_2CO (100 mL fractions). Two active fractions (4:6 MeOH−H_2O and MeOH) were obtained. The MeOH fraction (9.8 mg) was fractionated further using a 5 g diol column. The column was washed successively with 7:3 hexanes−CH_2Cl_2, 5:5 hexanes−CH_2Cl_2, 2:8 hexanes−CH_2Cl_2, CH_2Cl_2, 99:1 CH_2Cl_2−MeOH, and then MeOH (80 mL fractions). The 5:5 hexanes−CH_2Cl_2 fraction afforded pure compound **1** (2.2 mg). The 4:6 MeOH−H_2O fraction (17.6 mg) was fractionated further using a 5 g diol column; elution was with 99:1 CH_2Cl_2−MeOH, 98:2 CH_2Cl_2−MeOH, 95:5 CH_2Cl_2−MeOH, 90:10 CH_2Cl_2−MeOH, and MeOH. The 99:1 CH_2Cl_2−MeOH fraction afforded pure product **2** (3.2 mg).

30-(4′-Hydroxybenzoyloxy)-11α-hydroxylupane-20(29)-en-3-one (1): colorless needles; mp 162−164 °C; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), see Table 1; positive ESIMS *m/z* 577 $[M + H]^+$; HREIMS *m/z* 576.3820 (calcd for $C_{37}H_{52}O_5$, 576.3815).

(−)-Epicatechin (2): $C_{15}H_{14}O_6$; mp 239−242 °C; positive ESIMS *m/z* 291 $[M + H]^+$; structure identified by direct comparison with ^{1}H and ^{13}C NMR and MS data reported previously.[16]

DNA Polymerase β Lyase Inhibition Assay. Preparation of the DNA Substrate. A 36-nucleotide oligodeoxyribonucleotide, which contained uridine at position 21, was labeled at its 3′-end using terminal deoxynucleotidyltransferase + [α-^{32}P]ddATP. The product was subjected to 20% denaturing polyacrylamide gel electrophoresis for purification. The band of interest was visualized by autoradiography and excised from the gel. After removal by the "crush and soak" method,[23] the DNA substrate was annealed to its complementary strand by heating to 70 °C for 3 min, followed by slow cooling to 25 °C.

dRP Excision Assay. To 200 μL of 354 nM [α-^{32}P]-labeled double-stranded oligodeoxynucleotide with uridine at position 21, 10 mM Hepes-KOH, pH 7.4, 5 mM $MgCl_2$, 50 mM KCl, and 10 mg/mL bovine serum albumin were added 2.4 units of uracil-DNA glycosylase and 3 units of AP endonuclease. After incubation at 37 °C for 20 min, an apurinic (AP) site was created in the [α-^{32}P]-labeled double-stranded oligodeoxynucleotide. Then the test samples (crude extracts, fractions, or compounds **1** and **2**, dissolved in DMSO) and 0.172 unit of rat DNA polymerase β were added to 5 μL of the above reaction mixture that contained the DNA substrate containing an AP site at position 21. After incubation for 30 min at room temperature, the reaction was terminated by the addition of 0.5 M $NaBH_4$ to a final concentration of 50 mM, and the reaction mixture was incubated for 10 min at room temperature. The reaction products were further incubated for 20 min at 70−80 °C and then were separated on a 20% denaturing polyacrylamide gel and visualized by autoradiography. Gels were visualized and quantified using a Molecular Dynamics PhosphorImager model 450.

Potentiation of the Action of MMS on Cultured Cells. The A549 cells were maintained in Kaighn's modification of Ham's F12 medium (F12K) with 2 mM L-glutamine supplemented with 1.5 g/L sodium bicarbonate and 10% fetal bovine serum at 37 °C in a 5% CO_2 in air atmosphere. Cytotoxicity was determined by MTT assay.[24] Two hundred microliters of culture samples containing approximately 1.0×10^4 of A549 cells were placed in each well of 96-well culture plates and treated with the appropriate concentration of DMSO (the control) or compound **1** or **2** and MMS. The cultures were incubated at 37 °C for 48 h in a 5% CO_2 in air atmosphere. After removal of the culture medium and addition of 15 μL of MTT (5 mg/mL) to each well, the samples were incubated for an additional 4 h at 37 °C in a 5% CO_2 atmosphere. Two hundred microliters of DMSO was added to each well. The OD_{570} value was obtained from a microplate reader. The results were expressed as "percent growth inhibition" according to the formula [(Nc − Ne)/Nc] × 100%, where Nc was the OD_{570} value counted in the control culture and Ne was the OD_{570} value in the treated culture.

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