A New Acylated Oleanane Triterpenoid from *Couepia polyandra* that Inhibits the Lyase Activity of DNA Polymerase β

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Bioassay-directed fractionation of a *n*-hexane extract of *Couepia polyandra* using an assay to detect inhibitors of the lyase activity of DNA polymerase β resulted in the isolation of the new triterpene 3β,16β,23-triacetoxyolean-12-en-28-oic acid (**1**) and four known compounds, oleanolic acid, betulinic acid, stigmasterol, and β-sitosterol. The structure of the new compound was established on the basis of extensive 1D and 2D NMR spectroscopic interpretation. All five compounds inhibited DNA polymerase β lyase activity.

One of the most common mechanisms of action of currently used anticancer agents is that of DNA damage. This damage is subject to repair by various DNA repair pathways, and these pathways have been linked to resistance to various anticancer drugs.[1,2] It has been shown that the eukaryotic enzyme DNA polymerase β (pol β) can repair damage after exposure to DNA-damaging agents,[3] and one of our groups has shown that isolated DNA polymerase β inhibitors can potentiate the cytotoxic activity of bleomycin and cisplatin by inhibiting this repair.[4] The use of inhibitors of pol β as chemopotentiating agents in cancer treatment thus offers the prospect of achieving therapeutic responses at lower doses of DNA-damaging agents.

It has recently been shown that pol β also has an intrinsic dRP lyase activity.[5] DNA damage is normally repaired by base excision repair. This involves the steps of removal of the modified base using a DNA glycosylase, followed by binding of an apurinic/apyrimidinic endonuclease (AP endonuclease) to the AP site to hydrolyze the phosphodiester bond 5′ to the abasic site. The 5′ terminal deoxyribose phosphate (dRP) must then be removed before further repair can proceed, and it is this phosphate that is removed by the lyase activity of pol β.[5,6] Inhibitors of the lyase activity of pol β should thus also be potentiators of the cytotoxicity of DNA-damaging agents.

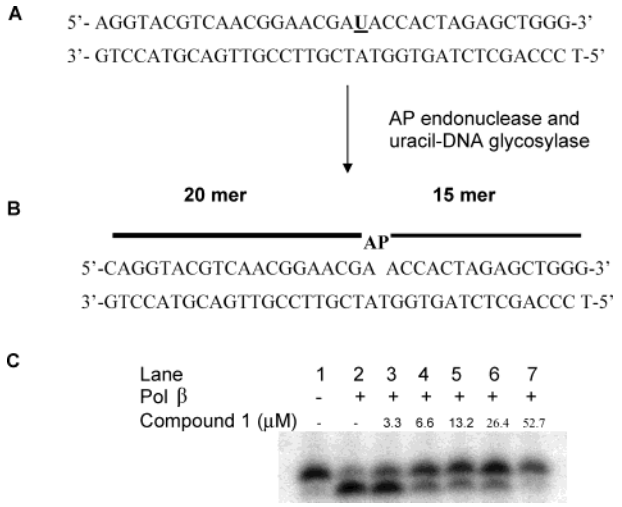

Figure 1. Bioassay for polymerase β lyase activity. (A) A 36-base oligodeoxyribonucleotide containing a uridine at position 21. (B) The oligodeoxyribonucleotide with an apurinic site. (C) A denaturing polyacrylamide gel of the oligodeoxyribonucleotide after incubation with polymerase β and various concentrations of **1**. The upper band consists of unrepaired oligodeoxyribonucleotide, indicating inhibition of polymerase β lyase activity.

Results and Discussion

In view of the successful isolation of naturally occurring inhibitors of the polymerase β activity of pol β,[4,7] and as a part of our continuing research to identify novel naturally occurring anticancer agents from both plants[8] and marine organisms,[9] it was decided to target the isolation of specific inhibitors of the lyase activity of this enzyme. A bioassay was thus developed for this activity. In brief, a 36-base oligodeoxyribonucleotide containing a uridine at position 21, labeled at its 3′-end with [α-^{32}P]ddATP, was used as the substrate (Figure 1A). After purification and annelation to its complementary strand, an AP site was created using AP endonuclease and uracil-DNA glycosylase (Figure 1B). dRP-excision activity was determined using this labeled DNA substrate, polymerase β, and various concentrations of test compounds. After incubation and product stabilization with $NaBH_4$, the reaction products were separated on a denaturing polyacrylamide gel and visualized by autoradiography. Pol β lyase inhibitory activity was indicated by the appearance of an upper band of unrepaired oligodeoxyribonucleotide (Figure 1C).

A hexane extract of the stem bark of *Couepia polyandra* (Kunth) Rose (Chrysobalanaceae) was selected for bioassay-guided fractionation on the basis of its strong inhibitory activity and the absence of any reported phytochemistry on the plant. Initial liquid−liquid partition of the crude extract indicated that the activity was equally distributed between the hexane and $CHCl_3$ fractions of hexane-aqueous MeOH and $CHCl_3$-aqueous MeOH partitions, respectively. The hexane and $CHCl_3$ fractions had similar ^{1}H NMR spectroscopic and silica gel TLC characteristics and were thus combined. Chromatography over MCI gel followed by reversed-phase preparative TLC yielded the new active triterpenoid 3β,16β,23-triacetoxyolean-12-en-28-oic acid (**1**) in addition to four known compounds, which were identified as oleanolic acid,[10] betulinic acid,[11] stigmasterol,[12] and

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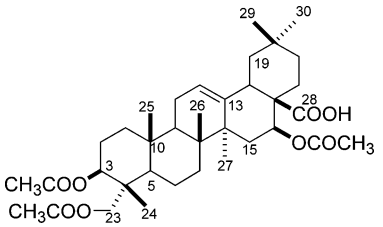
β-sitosterol,[13] by comparison of their spectral data with values reported in the literature.



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Compound **1** was obtained as an optically active viscous liquid, $[\alpha]_D$ +21.6° (*c* 0.56, $CHCl_3$), and was shown to have the molecular formula $C_{36}H_{54}O_8$ by HRFABMS, ^{13}C NMR, and APT spectral data. It gave a positive Liebermann-Burchard test for triterpenoids. Characteristic IR absorption bands were observed at 1732 and 1723 cm^{-1}, indicating the presence of carbonyl groups in its structure. The mass fragments observed at *m*/*z* 554, 494, and 434 in its EIMS indicated the presence of three acetate groups in its structure. The ^1H NMR spectrum showed the presence of six methyl singlets at δ 0.75, 0.87, 0.90, 0.92, 1.08, and 1.12, an oxymethine proton at δ 5.14 (dd, J = 10.6, 4.8 Hz), an olefinic proton at δ 5.27 as a triplet (J = 3.4 Hz), nine methylenes, three methines, and an acetyl methyl singlet at δ 2.08. These observations suggested the presence of a 3β-acetylated pentacyclic triterpenoid skeleton in **1**. The ^1H NMR spectrum also showed the presence of a primary and an additional secondary acetate group [δ 3.84 (1H, d, J = 11.6 Hz), 3.57 (1H, d, J = 11.9 Hz), and 2.02 (3H, s) and δ 5.07 (1H, dd, J = 10.4, 2.2 Hz), and δ 1.98 (3H, s)].

The ^{13}C NMR values for all the carbons were assigned on the basis of APT, HMQC, and HMBC spectral data (Table 1), which indicated the presence of six sp^3 methyls, 10 sp^3 methylenes, five sp^3 methines, six sp^3 quaternary carbons, one sp^2 methine carbon, one sp^2 quaternary carbon, and one carboxylic acid group. The above ^1H and ^{13}C NMR spectral data suggested that compound **1** was a 3β-acetoxyoan-12-en-28-oic acid derivative.[14] The 3β-substituted olean-12-en-28-oic acid skeleton in **1** was further supported by the COSY (H-1/H-2; H-2/H-3; H-5/H-6; H-6/H-7; H-9/H-11; H-11/H-12; H-14/H-15; H-15/H-16; H-18/H-19; H-21/H-22) and HMBC (H-3/C-1, C-2, C-4; H-5/C-3, C-4, C-6; H-6/C-5, C-7, C-8, C-10; H-9/C-8, C-10, C-11, C-12; H-12/C-9, C-11, C-13, C-14; H-16/C-14, C-15, C-17, C-18, C-22, C-28; H-19/C-18, C-20, C-21, C-22, C-28) correlations. The presence of six methyl singlets and a primary acetate group indicated that one of the alkyl groups in **1** must be in the form of an acetoxymethylene group. The three sets of fragment ions in the mass spectrum at *m*/*z* 374/240, 388/226, and 460/154 suggested the placement of the primary acetate group at either C-23 or C-24 and the additional secondary acetate group at C-16. The position of this secondary acetate group at C-16 was further supported by the key HMBC correlations: H-16/C-14, C-15, C-17, C-18, C-22, C-28; H-18/C-13, C-14, C-16, C-19, C-20, C-22, C-28; and H-22/C-16, C-17, C-18, C-20, C-28. A careful comparison of the ^{13}C NMR values of **1** with those of C-3/C-23 and C-3/C-24 diols,[14] after adjusting the latter values for acylation, indicated the placement of the primary acetate group at the C-23 position. This was supported by the NOESY spectrum of **1**, which showed correlations between the two C-23 methylene protons at δ 3.84 and 3.57 and the C-3 methine proton at δ 5.14 and the C-5 methine proton at δ 0.98. The appearance of the C-16 oxymethine proton at δ 5.07 (dd, J = 10.4, 2.2 Hz) suggested the β orientation of its acetate group, and this was supported by the NOESY spectrum of **1**, in which the oxymethine proton was correlated to the C-27 methyl singlet at δ 1.08. On the basis of the above spectral data, compound **1** was assigned as 3β,16β,23-triacetoxyolean-12-en-28-oic acid.

Table 1. NMR Data for Compound **1** ($CDCl_3$)[a]

position	^1H	^{13}C
1	1.82 m, 0.94 m	38.0
2	2.28 m, 1.38 m	23.7
3	5.14 dd (10.6, 4.8)	74.9
4		41.7[b]
5	0.98 m	47.5
6	1.46 m, 0.96 m	18.0
7	1.56 m, 1.12 m	33.2
8		39.3
9	1.42 m	47.8[c]
10		41.1[b]
11	2.14 m, 1.62 m	23.7
12	5.27 t (3.4)	122.2
13		143.8
14		42.0[b]
15	1.94 m, 1.24 m	36.3
16	5.07 dd (10.4, 2.2)	69.8
17		47.6[c]
18	2.81 dd (14.6, 3.4)	41.9
19	2.02 m, 1.08 m	47.7[c]
20		30.8
21	1.66 m, 1.28 m	33.8
22	2.10 m, 1.62 m	25.9
23	3.84 d (11.6), 3.57 d (11.9)	65.3
24	1.12 s	32.5
25	0.92 s	13.9
26	0.75 s	17.0[d]
27	1.08 s	17.1[d]
28		180.1
29	0.87 s	32.3
30	0.90 s	23.5
3-O*CO*CH_3		170.5[e]
3-OCO*CH_3*	2.08 s	20.9[f]
16-O*CO*CH_3		170.6[e]
16-OCO*CH_3*	2.02 s	21.0[f]
23-O*CO*CH_3		170.9[b]
23-OCO*CH_3*	1.98 s	21.1[f]

[a] Assignments made on the basis of COSY, HMQC, and HMBC and comparison with the literature data.[7] [b–f] Values having the same superscript in the column are interchangeable.

Table 2. IC_{50} of Polymerase β Lyase Inhibition of Compounds Isolated from *Coupeia polyandra*[a]

compound	IC_{50} (μM)
1	13.0
oleanolic acid	8.8
betulinic acid	43.6
stigmasterol	43.4
β-sitosterol	26.3

[a] Data are the mean of three determinations.

All the isolated compounds were tested for inhibition of DNA polymerase β lyase activity. As shown in Table 2, the IC_{50} values ranged from 8.8 to 43.6 μM, with oleanolic acid having the greatest activity.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter. IR (KBr) and UV (MeOH) spectra were measured on MIDAC M-series FTIR and Shimadzu UV-1201 spectrophotometers, respectively. NMR spectra were obtained on a JEOL Eclipse 500 spectrometer. HRFABMS were obtained on a JEOL HX-110 instrument. Chemical shifts are given in ppm (δ) with TMS (tetramethylsilane) as internal reference and coupling constants (J) in Hz.

Plant Material. Stem bark of *Couepia polyandra* (Kunth) Rose (Chrysobalanaceae) was collected in April 1980 in Jalisco, Mexico (E0813), and given collection number PR-53952. Herbarium vouchers are deposited in the herbarium of the U.S. Department of Agriculture in Beltsville, MD.

Extract Preparation. The plant samples were dried, ground, soaked with *n*-hexane, and evaporated to give the dried hexane extract.

Extraction and Isolation. The crude hexane extract (0.45 g) was suspended in aqueous MeOH (MeOH−H_2O, 9:1, 50 mL) and extracted with three 50 mL portions of *n*-hexane The aqueous layer was then diluted to 60% MeOH (v/v) with H_2O and extracted with three 50 mL portions of $CHCl_3$. The *n*-hexane and $CHCl_3$ extracts were found to be equally active and were combined on the basis of their similar nature on TLC and their ^{1}H NMR spectra. The combined residue (0.41 g) was fractionated over MCI gel using MeOH−H_2O (75:25 → 100:0) to furnish 14 fractions (A−N), of which fractions B, H−I, and K−L were fractionated further on the basis of their activity and ^{1}H NMR spectra. Fraction B on reversed-phase preparative TLC (MeOH−H_2O, 75:35) yielded betulinic acid (2.2 mg). Similarly, fraction H on reversed-phase preparative TLC (MeOH−H_2O, 80:20) afforded stigmasterol (4.6 mg). Fraction I on reversed-phase preparative TLC (MeOH−H_2O, 80:20) yielded the new triterpene **1** (1.8 mg). Fractions K and L on reversed-phase preparative TLC with mobile phases MeOH−H_2O (85:15) and MeOH−H_2O (90:10) afforded β-sitosterol (2.6 mg) and oleanolic acid (2.4 mg), respectively. The four known compounds were identified by comparison of their spectral data with literature values.[10−13]

3β,16β,23-Triacetoxyolean-12-en-28-oic acid (1): colorless viscous liquid; $[\alpha]_D$ +21.6° (*c* 0.56, $CHCl_3$); UV (MeOH) λ_{max} 214 nm (ϵ 12 860); IR ν_{max} 2960, 1732, 1723, 1450, 1325, 1125, 1065 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m*/*z* (rel int) 614 [M^+] (6), 554 (14), 494 (12), 460 (28), 434 (21), 388 (21), 374 (23), 359 (18), 289 (23), 240 (15), 235 (10), 226 (32), 201 (100), 164 (13), 154 (28), 123 (12), 91 (46); HRFABMS *m*/*z* 615.3884 [M + H]$^+$ (calcd for $C_{36}H_{55}O_8$, 615.3897).

3′-End Labeling and Apurinic Site Preparation. A 36-base oligodeoxyribonucleotide containing a uridine (Figure 1A) at position 21 was labeled at its 3′-end with terminal deoxynucleotidyltransferase using [α-^{32}P]ddATP. The product was then purified using a 20% denaturing polyacrylamide gel. The band of interest was visualized by autoradiography and excised from the gel. After removal by the "crush and soak " method, these oligodeoxyribonucleotides were then annealed to their complementary strands by heating the solution at 70 °C for 3 min, followed by slow cooling to 25 °C. An AP site (Figure 1B) was created in a reaction mixture (200 μL) that contained 354 nM [α-^{32}P]-labeled double-stranded oligodeoxynucleotide containing a uridine at position 21 in 10 mM Hepes-KOH, pH 7.4 (200 μL total volume), containing 50 mM KCl, 5 mM $MgCl_2$, 10 mg/mL BSA, 3 units AP endonuclease, and 2.4 units uracil-DNA glycosylase. After incubation at 37 °C for 20 min, the [α-^{32}P]-labeled double-stranded oligodeoxynucleotide containing an AP site at position 21 was ready for dRP-excision assay.

dRP-Excision Assay. dRP-excision activity was determined using a reaction mixture (5 μL) that contained 354 nM [α-^{32}P]-labeled DNA substrate containing an AP site at position 21, 0.172 U polymerase β, and various concentrations of test compound. After incubation at room temperature for 30 min, the reaction was terminated, and the product (Figure 1B) was stabilized by the addition of 0.5 M $NaBH_4$ to a final concentration of 50 mM and then incubated at room temperature for 10 min. After additional incubation at 75 °C for 20 min the reaction products were separated on a 20% denaturing polyacrylamide gel and visualized by autoradiography (Figure 1C).

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