# Harbinatic Acid, a Novel and Potent DNA Polymerase β Inhibitor from *Hardwickia binata*

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Bioassay-guided fractionation of an active methyl ethyl ketone extract of *Hardwickia binata*, using an assay sensitive to DNA polymerase β inhibition, resulted in the isolation of a potent inhibitor. This proved to be a novel diterpenoid, which has been named harbinatic acid (**1**). The structure of **1** was established as 3α-*O*-*trans*-*p*-coumaroyl-7-labden-15-oic acid from spectroscopic analysis and by comparison with the published data for a structurally related compound. Compound **1** strongly inhibited calf thymus DNA polymerase β, with an $IC_{50}$ value of 2.9 μM.

Cellular DNA defense mechanisms, such as base excision repair, exist to reverse DNA damage resulting from either exogenous or endogenous sources.[1] DNA repair pathways and their constituent repair proteins have become important in cancer research because of their likely involvement in chemotherapeutic agent resistance.[2] One of the DNA repair enzymes identified as a potential target for anticancer drug therapy is DNA polymerase β, a 39-kDa gap-filling enzyme involved in base excision repair.[3−5] Its role in repairing damaged DNA, after exposure to some DNA damaging agents,[4−7] has been linked to increased drug resistance that must result in diminished efficacy of anticancer drug therapies.[8] Inhibition of this repair system by directly inhibiting the enzyme with a noncytotoxic agent might well potentiate chemotherapeutic treatment and possibly allow for lower doses of antitumor agents to be administered.

In our continuing survey of crude plant extracts to identify DNA polymerase β inhibitors,[9−14] a methyl ethyl ketone extract prepared from *Hardwickia binata* Roxb. (Leguminosae) exhibited potent inhibition of DNA polymerase β (82 and 66% inhibition at 100 and 50 μg/mL, respectively). The isolation of several chemical constituents, such as flavonoids, from the heartwood of *H. binata* has been reported.[15] To isolate the inhibitory principle(s), we used an assay sensitive to DNA polymerase β inhibition to guide the fractionation of the active extract. Bioassay-guided fractionation resulted in the isolation of a potent inhibitor, a novel diterpenoid that we have named harbinatic acid (**1**). This paper describes the isolation and structure elucidation of the bioactive principle, as well as its potency as an inhibitor of DNA polymerase β.

## Results and Discussion

The dried whole plant was soaked successively with hexanes, methyl ethyl ketone, methanol, and water. The methyl ethyl ketone extract was found to inhibit DNA polymerase β strongly in a primary screen. The active extract was fractionated first through a polyamide 6S column, which was washed 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 4:1 MeOH−$CH_2Cl_2$ fraction, which exhibited significant activity against DNA polymerase β, was subjected to chromatography on a Sephadex LH-20 column using a normal-phase elution scheme. The most strongly inhibitory fraction (1:1 $CH_2Cl_2$−$Me_2CO$) from the Sephadex LH-20 column was then fractionated using a reversed-phase $C_8$ open column. The most active fractions (17:3 and 1:0 MeOH−$H_2O$) from the $C_8$ open column were subjected to further fractionation on a cyano (CN) HPLC column, using a normal-phase elution scheme. Fraction 2 (a main peak) from the CN column showed the greatest inhibitory activity against DNA polymerase β. Purification of the active fraction using a reversed-phase $C_{18}$ HPLC column furnished the new inhibitor **1**.

Harbinatic acid (**1**) had a molecular formula of $C_{29}H_{40}O_5$ based on the $[M + H]^+$ ion observed at *m*/*z* 469.2966 in the HRMS. The IR spectrum of **1** displayed absorptions at 3600 (br, OH and COOH), 1738−1660 (acid and ester carbonyls), and 1600−1385 $cm^{-1}$ (four peaks, aromatic ring); the UV spectrum exhibited maxima at 214 and 291 nm (aromatic ring). Its $^1H$ NMR spectrum revealed an AA′BB′ doublet system at δ 6.84 (2H, d, $J$ = 8.4 Hz) and 7.45 (2H, d, $J$ = 8.4 Hz) and an AB doublet at δ 6.32 (1H, d, $J$ = 16.2 Hz) and 7.62 (1H, d, $J$ = 16.2 Hz). The $^{13}C$ NMR spectrum contained resonances assigned to an ester carbonyl carbon at δ 166.9 (s), two conjugated olefinic carbons at δ 143.9 (d) and 115.7 (d), an oxygen-substituted aromatic carbon at δ 157.4 (s), and other aromatic carbons at δ 129.6 (d), 126.8 (s), and 115.5 (d) (Table 1). These suggested the presence of a *p*-(*E*)-coumaroyloxy substituent in **1**. This was confirmed by the significant fragment ions in the EIMS spectrum at *m*/*z* 147 and 164, along with an ion peak at *m*/*z* 304 resulting from the loss of elements of *p*-coumaric acid from the molecular ion (Figure 1).

In addition to providing evidence for the *p*-(*E*)-coumaroyloxy moiety, the $^{13}C$ NMR spectrum of **1** contained 20 signals for carbon atoms of the terpenoid moiety (five methyl, six methylene, five methine, and four quaternary carbons) whose chemical shifts were characteristic of labdane-type diterpenoids.[16] Further, the $^1H$ and $^{13}C$ NMR data for **1** were similar to those of the known labdane diterpene 7-labden-3β,15-diol (**2**),[17] except for the resonances due to H-3, C-15 (and H-15), C-19,[18] and the *p*-(*E*)-coumaroyloxy moiety. The fact that H-3 appeared at δ 4.80 as a broad singlet in **1** rather than at δ 3.20 as a multiplet in **2** supported the assignment of the ester function in **1** at C-3, in the α-position. The assignments were supported by

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**Table 1.** $^{13}C$ NMR Data for Compounds **1** and **2** [$\delta$ (mult)][a]

| carbon | 1 | 2 |
|---|---|---|
| 1 | 31.7 (t) | 36.8 (t) |
| 2 | 24.1 (t) | 27.5 (t) |
| 3 | 78.0 (d) | 79.2 (d) |
| 4 | 36.2 (s) | 38.7 (s) |
| 5 | 44.5 (d) | 49.8 (d) |
| 6 | 22.4 (t) | 23.5 (t) |
| 7 | 121.8 (d) | 122.0 (d) |
| 8 | 135.0 (d) | 135.3 (d) |
| 9 | 54.6 (d) | 55.4 (d) |
| 10 | 36.1 (s) | 37.4 (s) |
| 11 | 22.9 (t) | 24.5 (t) |
| 12 | 38.8 (t) | 39.7 (t) |
| 13 | 31.6 (d) | 30.6 (d) |
| 14 | 40.8 (t) | 40.0 (t) |
| 15 | 178.2 (s) | 61.2 (t) |
| 16 | 21.5 (q) | 19.8 (t) |
| 17 | 21.6 (q) | 21.9 (q) |
| 18 | 27.3 (q) | 27.9 (q) |
| 19 | 19.4 (q) | 15.1 (q) |
| 20 | 13.1 (q) | 13.7 (q) |
| 1′ | 166.9 (s) | |
| 2′ | 115.7 (d) | |
| 3′ | 143.9 (d) | |
| 1″ | 126.8 (s) | |
| 2″, 6″ | 129.6 (d) | |
| 3″, 5″ | 115.5 (d) | |
| 4″ | 157.4 (s) | |

[a] $^{13}C$ NMR spectra were recorded in $CDCl_3$ + $CD_3OD$ at 75 MHz.

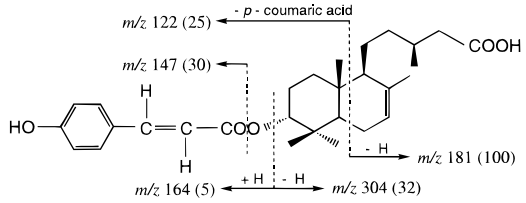
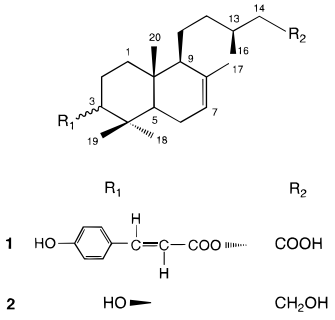



**Figure 1.** EIMS fragments for **1**.

the $^{13}C$ NMR spectral data. The $^{13}C$ NMR exhibited signals at $\delta$ 31.7 (t), 24.1 (t), 36.2 (s), and 44.5 (d) corresponding to C-1, C-2, C-4, and C-5, respectively. Compared with the corresponding $\delta$ values of the carbons for **2**, the C-1, C-2, C-4, and C-5 resonances were shifted upfield by 2.50−5.30 ppm owing to the strong shielding effect of the 3-α-axial substituent. The signal at $\delta$ 178.2 due to C-15 in the $^{13}C$ NMR spectrum and the absorptions at 1738−1660 $cm^{-1}$ in the IR spectrum indicated the presence of a carboxyl group at C-15 in **1** instead of the methylenol in **2**. All assignments for **1** were confirmed by the $^1H-^1H$ COSY, $^{13}C-^1H$ COSY, and NOESY spectra. Therefore, the structure of **1** was established as 3α-*O*-*trans*-*p*-coumaroyl-7-labden-15-oic acid.




In the DNA polymerase $\beta$ assay, harbinatic acid (**1**) showed potent inhibition of DNA polymerase $\beta$, with an $IC_{50}$ of 4.7 $\mu$M in the presence of bovine serum albumin (BSA) and 2.9 $\mu$M in the absence of BSA. The lack of significant binding of **1** to serum albumin should be of potential advantage in vivo, inasmuch as binding to serum albumin would likely diminish the ability of this agent to effect inhibition of DNA polymerase $\beta$. Relative to some other classes of polymerase $\beta$ inhibitors reported recently,[13,14] **1** has the advantage of being more rigid structurally, which should facilitate an understanding of those structural features required for polymerase $\beta$ inhibitory activity.

Labdane-type diterpenoids are often found in the resinous exudates of plants, particularly those that grow in semi-arid regions.[19] Some labdane lactones have exhibited significant cytotoxicity toward a number of human cancer lines,[20] as well as anti-mutagenic activity,[21] larvicidal activity,[22] insect antifeedant properties,[23] and other biological activities.[24] Some extracts containing labdanes have been used for medicinal and cosmetic purposes.[25] Compound **1** is the first example of a labdane attached to a *p*-(*E*)-coumaroyloxy moiety. It is also the first diterpenoid found to exhibit potent activity as an inhibitor of DNA polymerase $\beta$.

## Experimental Section

**General Experimental Procedures.** Polyamide 6S (a product of Riedel-de Haen, Germany) was obtained from Crescent Chemical. Sephadex LH-20 (Pharmacia; 40 $\mu$m) was purchased from Sigma Chemicals. Silica RP $C_8$ and RP $C_{18}$ (32−60 $\mu$m) were obtained from ICN Pharmaceuticals. The Econosil CN HPLC column (250 × 10 mm, 10 $\mu$m) was from Alltech; the Kromasil reversed-phase $C_{18}$ HPLC column (250 × 10 mm, 5 $\mu$m) was obtained from Higgins Analytical Inc. Optical rotations were measured on a Perkin−Elmer 243 B polarimeter. UV and IR spectral measurements were made on Perkin−Elmer Lambda Array 3840 and Perkin−Elmer 1600 Series FTIR spectrophotometers, respectively. $^1H$ and $^{13}C$ NMR spectra were recorded on General Electric GN-300 or QE-300 NMR spectrometers. Low resolution CIMS and EIMS mass spectra were recorded on a Finnigan MAT 4600 mass spectrometer. HPLC/HRMS were recorded on a New Star T70 FT/MS mass spectrometer at SmithKline Beecham Pharmaceuticals. Unlabeled dNTPs and calf thymus DNA were purchased from Sigma Chemicals; [$^3H$]TTP was from ICN Pharmaceuticals. DEAE-cellulose paper (DE-81) was obtained from Whatman.

**Plant Material.** *Hardwickia binata* was collected in India in October 1972. Voucher specimen Shah 293 is preserved at the United States Arboretum Herbarium (NA), Washington, DC.

**Extraction and Isolation.** The dried whole plant was extracted successively with hexane, methyl ethyl ketone, MeOH, and $H_2O$. The methyl ethyl ketone crude extract was found to exhibit inhibition of DNA polymerase $\beta$ (82% inhibition at 100 $\mu$g/mL). The crude extract continued to have significant activity after passage through a polyamide 6S column to remove polyphenols. Therefore, this extract was chosen for bioassay-guided fractionation. A total of 1.35 g of the crude extract prepared from *H. binata* was used for bioassay-guided fractionation; a typical set of experiments is described below. The crude extract (290 mg) was applied to a 15-g polyamide 6S column that was washed 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 4:1 MeOH−$CH_2Cl_2$ fraction (83 mg) exhibited significant inhibition of DNA polymerase $\beta$ (84% inhibition at 100 $\mu$g/mL) and was fractionated further on a Sephadex LH-20 column, which was eluted successively with hexane, 1:1 hexane−$CH_2Cl_2$, $CH_2Cl_2$, 1:1 $CH_2Cl_2$−$Me_2CO$, $Me_2CO$, and MeOH. The 1:1 $CH_2Cl_2$−$Me_2CO$ fraction (33 mg), which displayed the strongest inhibitory activity (40% inhibition at 10 $\mu$g/mL), was subjected to fractionation on a reversed-phase $C_8$ open column; this column was washed

successively with 1:9, 3:7, 5:5, 13:7, 15:5, 17:3, and 1:0 MeOH−$H_2O$. The most active fractions (17:3 and 1:0 MeOH−$H_2O$, 8.5 mg) were fractionated further on a CN HPLC column (250 × 10 mm, 10 $\mu$m), using a gradient elution of 3:1→1:1 hexane−EtOAc (containing 20% MeOH in EtOAc) over a period of 40 min at a flow rate of 4.0 mL/min (monitoring at 235 nm). The most active fraction (2.4 mg) was obtained as a main peak having a retention time of 8.8 min. Purification of the active principle was completed using a reversed-phase $C_{18}$ HPLC column (250 × 10 mm, 5 $\mu$m); elution was accomplished with a linear gradient from 30% aqueous $CH_3CN$ to 5% aqueous $CH_3CN$ over a period of 50 min at a flow rate of 4.0 mL/min (detection at 235 nm) to afford pure **1** (0.9 mg).

**Harbinatic acid (1):** obtained as amorphous powder; 5.8 mg total; $[\alpha]^{22}_D$ −11° (*c* 0.18, MeOH); UV (MeOH) $\lambda_{max}$ (log $\epsilon$) 214 (4.89), 291 (4.58) nm; IR (KBr) $\nu_{max}$ 3600 (br), 1718, 1660, 1600, 1580, 1410, 1380, 1360, 1210 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz) $\delta$ 7.62 (1H, d, $J$ = 16.2 Hz, H-3′), 7.45 (2H, d, $J$ = 8.4 Hz, H-2″ and H-6″), 6.84 (2H, d, $J$ = 8.4 Hz, H-3″ and H-5″), 6.32 (1H, d, $J$ = 16.2 Hz, H-2′), 5.42 (1H, br s, H-7), 4.80 (1H, br s, H-3), 1.56 (3H, s, Me-17), 1.00 (3H, s, Me-19), 0.99 (3H, d, $J$ = 6.2 Hz, Me-16), 0.90 (3H, s, Me-18), 0.82 (3H, s, Me-20); $^{13}C$ NMR (see Table 1); negative CIMS *m*/*z* 468 $[M]^+$ (100); positive CIMS *m*/*z* 305 (20), 284 (15), 183 (5), 155 (8), 127 (35), 115 (45), 73 (70), 65 (100); EIMS *m*/*z* 304 (32), 283 (30), 181 (100), 165 (5), 164 (5), 147 (30), 91 (35); LC/HRESI−FTMS *m*/*z* 468.2880 (calcd for $C_{29}H_{40}O_5$, 468.2876).

**DNA Polymerase $\beta$ Inhibition Assay.** After dissolving the crude extract samples or fractions in 1:1 DMSO−MeOH, 6 $\mu$L of the sample and 4 $\mu$L of rat DNA polymerase $\beta$[26] (6.9 units, 48,000 units/mg) were added to 50 $\mu$L of the standard reaction mixture, which 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 $\mu$M dNTPs, 0.04 Ci/mmol [$^3$H]TTP, and 0.25 mg/mL of activated calf thymus DNA. After incubation at 37 °C for 60 min, the radioactive DNA product was collected on DEAE-cellulose paper filters (DE-81) and dried. The radioactive filters were washed successively with 0.4 M $K_2HPO_4$ and 95% EtOH, and the radioactivity was determined.

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