Palbinone, a Novel Terpenoid from *Paeonia albiflora*; Potent Inhibitory Activity on 3α-Hydroxysteroid Dehydrogenase[1)]

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Palbinone, a novel terpenoid isolated from the roots of *Paeonia albiflora*, showed a strong inhibitory activity on the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)-linked 3α-hydroxysteroid dehydrogenase (3α-HSD) of rat liver cytosol. The structures of palbinone and a known compound, paeonilactone-B isolated from the active fraction of this plant were determined by the use of 2D NMR techniques (^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, ^{1}H–^{13}C long-range COSY, and HMBC).

Keywords palbinone; *Paeonia albiflora*; Paeoniaceae; 3α-HSD; NADPH; terpenoid; 2D NMR

Paeonia Radix, the roots of *Paeonia albiflora* PALLAS belonging to Paeoniaceae, is an important constituent of traditional Chinese medicines for the treatment of abdominal pain and syndromes such as stiffness of abdominal muscles.[2)] A number of physiologically active compounds such as paeoniflorin, oxypaeoniflorin, benzoylpaeoniflorin, albiflorin, paeoniflorigenone, and gallotanins have been isolated from this plant.[3)] The metabolism of these physiologically active compounds by human intestinal bacteria has been studied.[4)] So far as we know, however, there is no report about the inhibitory activity of this plant against the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)-linked 3α-hydroxysteroid dehydrogenase (3α-HSD). As a part of our search for biologically active substances among natural products, a number of crude drugs were screened for inhibitory activity against 3α-HSD. We found that the chloroform extract of roots of *Paeonia albiflora* PALLAS showed a strong inhibitory activity against 3α-HSD. The active fraction of this extract was separated with monitoring by *in vitro* bioassay,[5)] yielded a novel terpenoid designated as palbinone (**1**), together with a known compound, paeonilactone-B.[6)] Among these compounds, **1** was found to have a potent inhibitory activity against 3α-hydroxysteroid dehydrogenase. In the present paper, we wish to report the isolation and structure elucidation of palbinone (**1**), and its inhibitory activity on NADPH-linked 3α-hydroxysteroid dehydrogenase enzyme of rat liver cytosol.

Results and Discussion

Structure Elucidation of Palbinone Dried roots of *Paeonia albiflora* were pulverized and extracted with chloroform at room temperature. One of the fractions (fr. 4) obtained from the silica gel column chromatography of the chloroform extract showed a strong inhibitory activity against 3α-hydroxysteroid dehydrogenase. This active fraction was further purified by repeated silica gel column chromatography and preparative TLC to give palbinone (**1**) and paeonilactone-B.

Palbinone (**1**), red needles, mp 254—255 °C, $[\alpha]_D$ −223.8° ($CHCl_3$), showed ultraviolet (UV) absorptions at 237 and 387 nm (log ε: 3.2 and 3.0, respectively) and infrared (IR) absorptions at 3500 (OH), 1750 (ketone), 1690 (unsaturated ketone), and 1605 cm^{-1} (double bond, C=C–C=C–CO).[7)] These data suggested that **1** is an α, β, γ, δ, unsaturated cyclic ketone containing hydroxyl groups. It showed the molecular ion peak at *m/z* 358 in the electron impact mass spectrum (EIMS) and its molecular formula was determined to be $C_{22}H_{30}O_4$ (M^+ 358.2137, Calcd 358.2143) by high-resolution MS. The ^{1}H-NMR spectrum of **1** was analyzed by the application of ^{1}H–^{1}H chemical shift correlation spectroscopy (COSY),[8)] and indicated the presence of five *tert*-methyl groups (δ_H 0.80, 0.82, 0.93, 1.02, and 1.20), a hydroxy-bearing methine (δ_H 3.28), and a conjugated double bond (δ_H 6.40 and 6.90). The chemical shift values for these methyl groups were similar to those of common triterpenes but the number of methyl groups was less than that of usual triterpenes. On the other hand, the ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra of **1** exhibited signals due to two ketones (δ_C 180.89[9)] and 201.26), four carbons of two pairs of double bonds (δ_C 120.29, 141.57, 146.95, and 151.29), a hydroxy-bearing methine carbon (δ_C 78.64), five *tert*-methyl groups (δ_C 15.07, 18.32, 19.02, 19.44, and 27.79), four methylene carbons (δ_C 17.92, 26.72, 33.31, and 38.11), two methine carbons (δ_C 54.87 and 56.08), and four quaternary sp^3 carbons (δ_C 37.20, 38.96, 40.02, and 50.74). The ^{1}H- and ^{13}C-NMR data showed fewer methylene and methyl groups than usual terpenoids or steroids, but the chemical shift values for C-1 to C-10 and two methyl groups at C-28 and C-29 of **1** were similar to those of taraxasterol[10)] and dammarenediol-II.[11)] Hence, it was thought that the basic structure of rings A and B of **1** was similar to that of taraxasterol or dammarenediol-II type triterpenoids, but the total number of carbons was less than that of common terpenoids due to the lack of ring E or the side chain of ring D. Two pairs of double bonds, a ketonic group and a conjugated ketonic

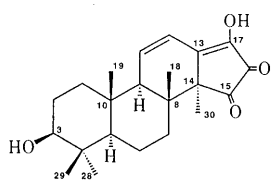

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Chart 1

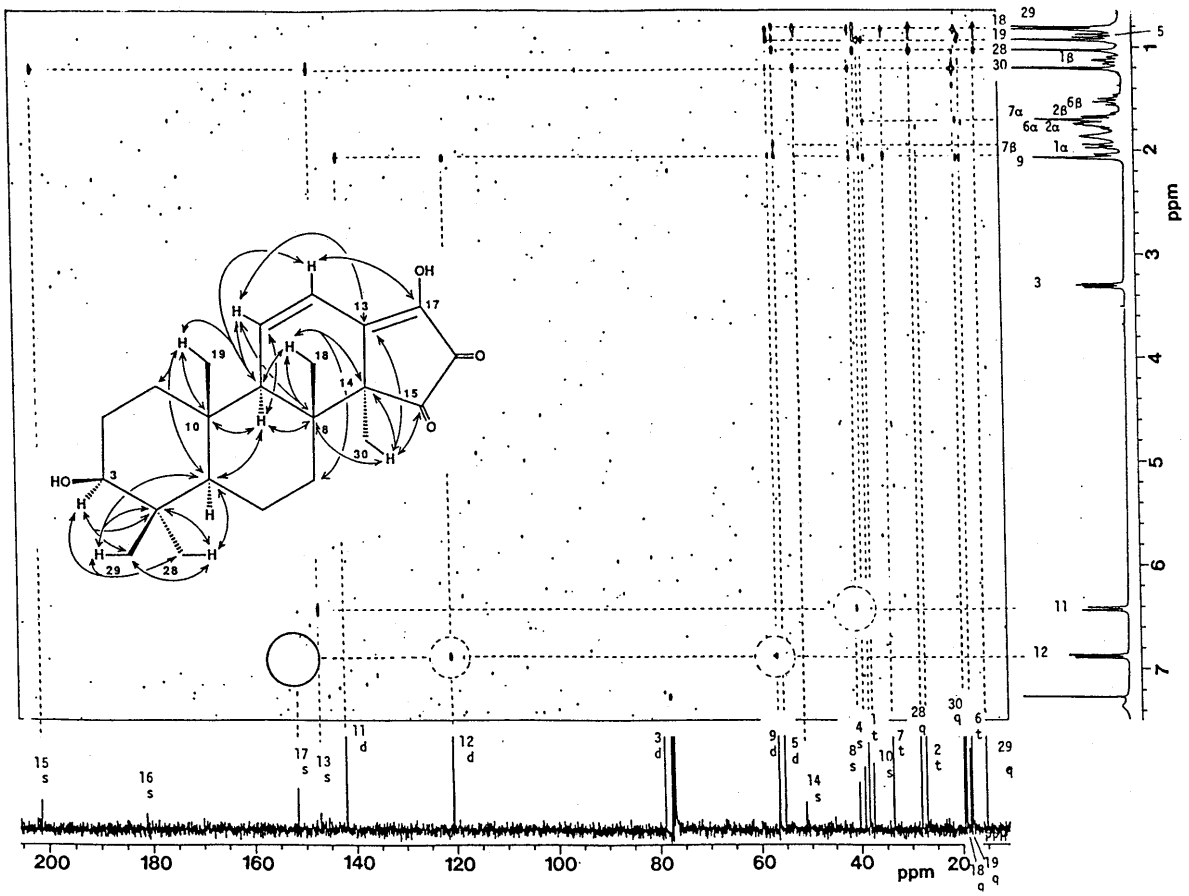

Fig. 1. ^{1}H–^{13}C Long-Range COSY Spectrum of Palbinone (**1**) in $CDCl_3$

The full circle indicates the expected correlation peak, which was not observed in this spectrum, but was observed in the HMBC spectrum

group were assigned to rings C and D. One of the double bond carbons was observed at low field (δ_C 151.29) in the ^{13}C-NMR spectrum due to bonding with oxygen. Two protons at low field (δ_H 6.40 and 6.90), coupled with $J=10.0$ Hz, suggested that one pair of double bond carbons gave doublet signals while the other pair of double bond carbons gave singlets, in accordance with the DEPT spectrum. Thus, the structure for palbinone was tentatively suggested to be **1**, having an enolic 1,2-dione function in ring D and another double bond in ring C.

Then, we measured the ^{1}H-detected heteronuclear multiple-bond multiple-quantum coherence (HMBC) spectrum[12] of **1** in order to confirm the assumed structure (**1**). As shown in Fig. 1, the ^{13}C-signals at δ_C 201.26 (C-15) and δ_C 146.95 (C-13) showed long-range correlations with the ^{1}H-signals at δ_H 1.20 (30-H_3) and δ_H 1.20 (30-H_3) and 6.40 (11-H), respectively. In turn, the ^{13}C-signal at δ_C 56.08 (C-9) was correlated with the ^{1}H-signals at δ_H 0.82 (18-H_3), 0.93 (19-H_3), 6.40 (11-H), and 6.90 (12-H), and the signal at δ_C 37.20 (C-10) was correlated with the ^{1}H-signals at δ_H 0.93 (19-H_3) and 2.07 (9-H). Some other significant long-range correlations are shown by arrows (Fig. 1), and all support the planar structure proposed for palbinone.

The relative stereochemisty of **1** was determined on the basis of the coupling constants of the proton signals and the results of NOE experiments. The 3-H proton at δ_H 3.28 was found to be dd with coupling constants $J=12.5$ and 5.5 Hz, and the 5-H proton was also found to be dd with coupling constants $J=12.0$ and 2.0 Hz. These facts suggested that 3-H and 5-H should be in axial positions in the chair conformation of ring A, and this was supported by the NOE experiment. Irradiation of the signals at 29-H_3 and 18-H_3 caused increases of signal intensity of the 19-, 28-, 2β-, and 6β-protons and the 19-, 6β-, and 7β-protons, respectively, and irradiation of 19-H_3 and 28-H_3 enhanced the signal intensity of the 29-, 18-, 6β-, 2β-, and 11-protons and the 29-, 5-, 6α-, and 3-protons, respectively. Also, irradiation of 30-H_3 gave NOE enhancement of the 7α- and 9-protons (Fig. 2b—f). On the basis of these data the structure of palbinone was concluded to be **1**.

The Inhibitory Activity of Palbinone NADPH-linked 3α-hydroxysteroid dehydrogenase reduces various ketosteroids and, therefore, plays an important role in the metabolism of steroid hormones. This enzyme is inhibited by many nonsteroidal and steroidal anti-inflammatory drugs. A good correlation was also found between the logarithm of the concentrations of these drugs required to inhibit 3α-HSD and the human anti-inflammatory dose.[5] The IC_{50} value (50%-inhibitory concentration) of palbinone (**1**) against 3α-HSD was 0.046 μM, while IC_{50} for indomethacin was 3.2 μM under similar assay conditions (Fig. 3). Palbinone (**1**) is thus a more potent inhibitor than indomethacin, which had been the strongest known inhibitor of 3α-HSD.

The structure of palbinone is similar to that of substrate 5α-androstan-17β-ol-3-one, but the Lineweaver–Burk plot indicated that palbinone is a non-competitive inhibitor of the NADPH-linked reduction of this substrate (Fig. 4).

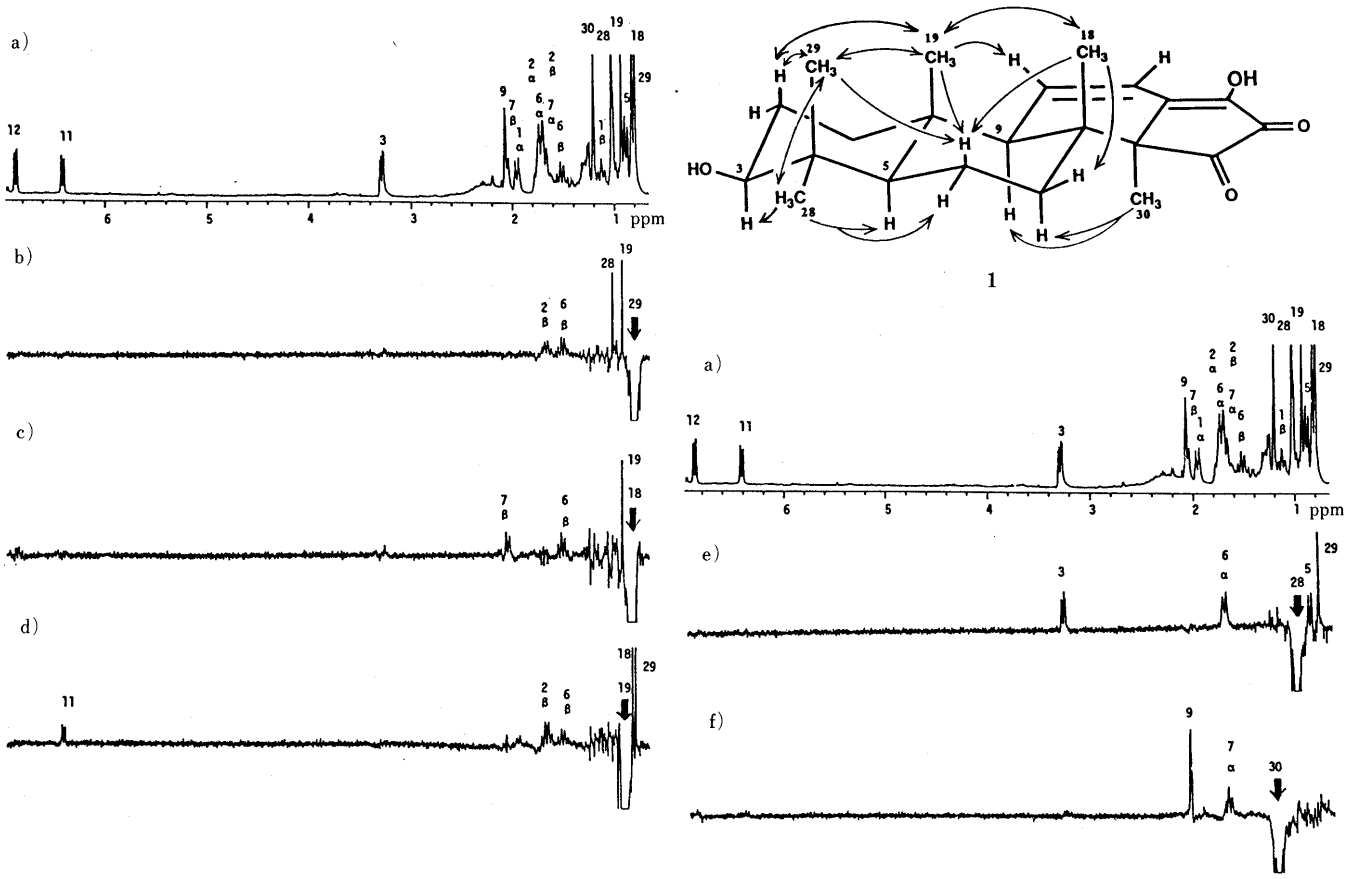

Fig. 2. Normal ^{1}H-NMR and NOE Difference Spectra of Palbinone (**1**)

a) Normal spectrum. b—f) NOE difference spectra on irradiation at δ 0.80, 0.82, 0.93, 1.02, and 1.20, respectively.

TABLE I. ^{1}H- (400 MHz) and ^{13}C- (100 MHz) NMR Data for Palbinone (**1**) in $CDCl_3$

Position	δ_H[a,b]	δ_C[c,d]	Position	δ_H[a,b]	δ_C[c,d]
1α	1.95 dt	38.11 t	9	2.07 brs	56.08 d
	(13.0, 4.0)		10	—	37.20 s
β	1.13 td		11	6.40 dd	141.57 d
	(13.0, 4.5)			(10.0, 2.5)	
2α	1.75 m	26.72 t	12	6.90 dd	120.29 d
β	1.66 m			(10.0, 3.2)	
3	3.28 dd	78.64 d	13	—	146.95 s
	(12.5, 5.5)		14	—	50.74 s
4	—	38.96 s	15	—	201.26 s
5	0.89 dd	54.87 d	16	—	180.89 s
	(12.0, 2.0)		17	—	151.29 s
6α	1.72 m	17.92 t	18	0.82 s	19.02 q
β	1.51 ddd		19	0.93 s	18.32 q
	(14.0, 11.5, 4.0)		28	1.02 s	27.79 q
7α	1.70 m	33.31 t	29	0.80 s	15.07 q
β	2.06 m		30	1.20 s	19.44 q
8	—	40.02 s			

δ value in ppm. *a*) Coupling constants in Hz. *b*) ^{1}H–^{1}H correlation spectra were measured. *c*) The multiplicities of carbon signals were determined by means of the DEPT method, and are indicated as s, d, t, and q. *d*) ^{1}H–^{13}C COSY, ^{1}H–^{13}C long-range COSY, and HMBC spectra were measured.

In conclusion, a constituent isolated from *Paeonia albiflora*, palbinone (**1**) seems to be the strongest inhibitor of 3α-HSD, so far found. This compound may have potent anti-inflamatory properties.

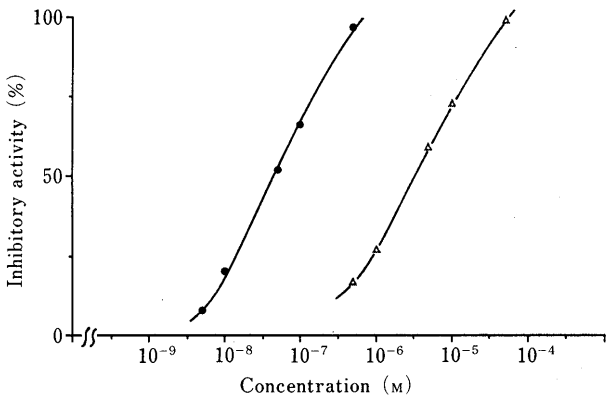

Fig. 3. Inhibition of NADPH-Dependent Substrate-Reduction Activity by Palbinone (**1**) and Indomethacin

●, palbinone; △, indomethacin.

Experimental

General Methods Melting points were determined with a Kofler-type apparatus and are uncorrected. Optical rotation was measured in chloroform solution on a JASCO DIP-4 automatic polarimeter at 25 °C. UV spectra were taken with a Shimadzu 210A UV spectrometer in methanol solution and IR spectra with a JASCO IRA-2 spectrometer in $CHCl_3$ solution. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL-GX 400 spectrometer in $CDCl_3$ solutions with tetramethylsilane as an internal standard, and chemical shifts are recorded in δ values. ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, and ^{1}H–^{13}C long-range COSY, and HMBC were obtained with the usual pulse sequence and data processing was performed with the standard JEOL software. MS and high-resolution MS were obtained with a JEOL JMS DX-300 spectrometer (ionization voltage, 70 eV; accelerating voltage, 3 kV) using a direct inlet system. Column chromatography was done with Wakogel C-200. Preparative TLC was carried out on Merck Kieselgel GF_{254} plates developed with $CHCl_3$–acetone (17 : 3) and spots were detected under UV light. Solutions were concen-

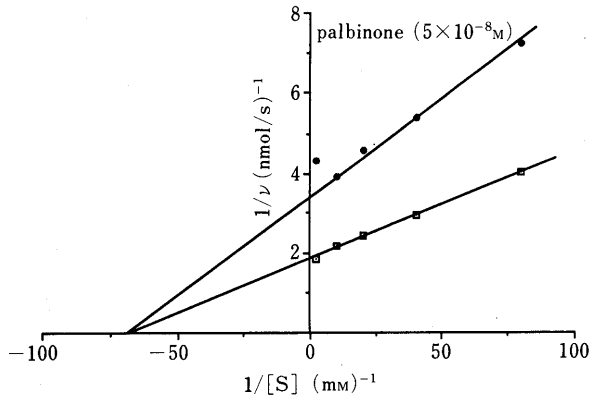

Fig. 4. Non-competitive Inhibition by Palbinone of NADPH-Dependent Substrate (5α-Androstan-17β-ol-3-one)-Reducing Activity of 3α-HSD

[S]: concentration of substrate.

trated under reduced pressure at a temperature below 40 °C.

Bioassay Crude 3α-hydroxysteroid dehydrogenase was obtained from the supernatant fraction of the homogenate of rat liver according to the method of Penning.[2] The inhibitory effect was evaluated at 25 °C in 1 M potassium phosphate buffer (pH 6.0), 9 mM NADPH, 10 mM substrate (5α-dihydrotestosterone for inhibitory activity and 5α-androstan-17β-ol-3-one to obtain the Lineweaver–Burk plot) and 20 μl of 3α-hydroxysteroid dehydrogenase solution in a total volume of 1.0 ml. The reference blank to correct for nonspecific reduction of NADPH contained all the above components except the substrate. The reaction was initiated by the addition of 3α-hydroxysteroid dehydrogenase and the rate of NADPH oxidation was followed by recording the decrease in absorbance at 340 nm. The effect of putative inhibitors on the enzyme activity was determined by including in the reaction mixture 10 μl of a test compound at different concentrations. The inhibitory activity was expressed as the rate of A_{340} nm change due to utilization of NADPH. The sample was dissolved in acetonitrile, which was found to have no effect on the enzyme activity at below 1% concentration. The IC_{50} values were obtained from linear regression lines as the final concentration of inhibitors required to provide 50% inhibition.

Chemicals NADPH was obtained from Kohjin Co., Ltd. Tokyo, and indomethacin, 5α-dihydrotestosterone and 5α-androstan-17β-ol-3-one were from Sigma Chemical Co. Other chemicals were of analytical grade.

Extraction and Separation of the Constituents of *Paeonia albiflora* Pallas Dried roots (5.0 kg) of *Paeonia albiflora* was pulverized and extracted three times with $CHCl_3$ (3 × 16 l) at room temperature by soaking with $CHCl_3$ for one day in each extraction to give a $CHCl_3$ extract (50 g) after evaporation of the solvent under reduced pressure. The $CHCl_3$ extract was chromatographed on a silica gel (1.5 kg) column eluting first with $CHCl_3$ and then with an increasing amount of MeOH in $CHCl_3$ to give five fractions [fr. 1 ($CHCl_3$) 14.2 g, fr. 2 (2% MeOH–$CHCl_3$) 16.3 g, fr. 3 (5% MeOH–$CHCl_3$) 1.2 g, fr. 4 (10% MeOH–$CHCl_3$) 0.9 g, and fr. 5 (20% MeOH–$CHCl_3$) 10.8 g]. Fraction 4 showed inhibitory activity against 3α-hydroxysteroid dehydrogenase, and it was further purified by silica gel column chromatography followed by preparative TLC run three times with the solvent system $CHCl_3$–acetone (17 : 3) to give palbinone (**1**) (87 mg) and paeonilactone-B (155 mg).

Palbinone (**1**): Red needles (ether–hexane), mp 254—255 °C, $[\alpha]_D$ −223.8° ($CHCl_3$). MS *m/z*: 358 (M^+), 340, 325, 297, 231, 220, 207, 189 (base peak). HR-MS: Found: 358.2137. Calcd for $C_{22}H_{30}O_4$ (M^+): 358.2143. UV λ_{max} nm (log ε): 237 (3.2), 387 (3.0). IR ν_{max} cm^{-1}: 3500, 1750, 1700, 1690, 1605. ^1H- and ^{13}C-NMR: Table I.

Paeonilactone-B: Colorless needles, mp 88—89 °C. MS *m/z*: 196 (M^+). ^1H-NMR ($CDCl_3$) δ: 1.40 (3H, s, 10-H_3), 1.98 (1H, dd, *J* = 14, 9 Hz, 2β-H), 2.51 (1H, dd, *J* = 14, 6 Hz, 2α-H), 2.78 (1H, dd, *J* = 16, 4 Hz, 5β-H), 2.97 (1H, dd, *J* = 16, 8 Hz, 5α-H), 3.69 (1H, m, 4-H), 5.01 (1H, m, 3-H), 5.70 (1H, d, *J* = 3 Hz, 9-H), and 6.36 (1H, d, *J* = 3 Hz, 9-H). The structure of this compound was determined by comparing the spectral data with those of an authentic sample.[6]

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