Sesquiterpenes from *Maytenus jelskii* as Potential Cancer Chemopreventive Agents

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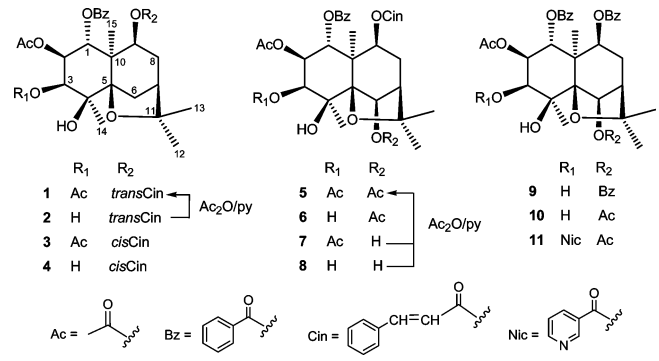
Seven new (**1**−**4** and **7**−**9**) sesquiterpenes with a dihydro-β-agarofuran skeleton, along with four known compounds (**5**, **6**, **10**, and **11**), have been isolated from the leaves of *Maytenus jelskii*. The structures of the new compounds were elucidated by means of spectroscopic data analysis, including 1D and 2D NMR techniques, and their absolute configurations were determined by circular dichroism and chemical correlations. The compounds have been tested for their inhibitory effects on Epstein−Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Compound **10** was found to be an effective antitumor-promoting agent and also showed a potent chemopreventive effect in an in vivo two-stage carcinogenesis model.

Species of the Celastraceae family have been used for centuries throughout South America and mainland China as insect repellents and insecticides in traditional agriculture and for the treatment of a plethora of medical aliments from stomach complaints and fever to rheumatoid arthritis and cancer.[1,2] Many of the medicinal properties associated with these species have now been attributed to a large family of highly oxygenated sesquiterpenoids, based on the tricyclic dihydroagarofuran skeleton, which are chemotaxonomic markers for the family.[3,4] Due to their structural diversity and range of biological activities, this intriguing class of natural products are considered as a "privileged structures".[5]

Cancer chemoprevention is defined as the use of specific natural and synthetic agents to reverse or suppress carcinogenesis and to prevent the development of invasive cancer.[6] In particular, the inhibition of the promotion stage of carcinogenesis is expected to be an efficient approach for cancer prevention. Natural products from plants and their synthetic derivatives play an important role in developing innovative agents to inhibit the onset of cancer. In fact, several classes of natural products are known to be potential chemopreventive agents.[7,8]

As part of our search for bioactive metabolites from Celastraceae species, we have previously reported sesquiterpenes as antitumor promoters from Celastraceae species.[9−11] In continuing research toward the discovery of naturally occurring cancer chemopreventive agents, we report herein on the isolation and structure elucidation of seven new sesquiterpenes (**1**−**4** and **7**−**9**), in addition to four known compounds (**5**, **6**, **10**, and **11**), from *Maytenus jelskii* Zahlbruchner. This species is a small shrub that grows in the forests of Bolivia, Peru, and Ecuador and whose chemical investigation has not been previously reported. The structures of the new compounds were determined by application of 1D and 2D NMR techniques, including COSY, HSQC, HMBC, and ROESY experiments. Absolute configurations were determined by CD studies and chemical correlations. The compounds have been tested for their antitumor-promoting effects on EBV-EA activation in Raji cells induced by the tumor promoter TPA (12-*O*-tetradecanoylphorbol-13-acetate) as a test for potential cancer chemopreventive effects. Compound **10**, the most active analogue in this in vitro assay, was further evaluated in an in vivo two-stage carcinogenesis model.



Results and Discussion

The EtOH extract of the leaves of *M. jelskii* was partitioned into a CH_2Cl_2−H_2O (1:1, v/v) solution. The CH_2Cl_2 fraction was subjected to multiple chromatographic steps, involving vacuum-liquid chromatography (VLC), medium-pressure liquid chromatography (MPLC), and preparative TLC on silica gel and Sephadex LH-20, to yield 11 sesquiterpene esters (**1**−**11**). The structures of the new compounds, **1**−**4** and **7**−**9**, were deduced as described below.

Compound **1** was obtained as a colorless lacquer and showed the molecular formula $C_{35}H_{40}O_{10}$ by analysis of its HREIMS (*m*/*z* 620.2639, calcd 620.2621). The IR absorption bands at 3524, 1746, 1731, and 1714 cm^{-1} indicated hydroxy and ester functions, and the UV spectrum revealed the occurrence of aromatic ring absorptions at 223 and 274 nm. The mass spectrum exhibited peaks attributable to the presence of methyl ($M^+ - 15$, *m*/*z* 605, CH_3), benzoate ($M^+ - 15 - \underline{122}$, *m*/*z* 483, C_6H_5COOH), cinnamate ($M^+ - 15 - \underline{148}$, *m*/*z* 457, C_8H_7COOH), and acetate ($M^+ - 15 - 148 - \underline{60}$, *m*/*z* 397, CH_3COOH) groups. This was confirmed by the ^1H and ^{13}C NMR spectra (Table 1), which included signals of 10 aromatic and two olefinic protons (δ_H 6.40−7.75), 12 aromatic and two olefinic carbons (δ_C 118.2−144.7), and two carboxyl groups at δ_C 164.9 and 165.9, assigned to a benzoyl and a cinnamoyl group. Signals of two acetyl groups [δ_H 1.77 (s), δ_C 20.9 (q), 170.2 (s) and δ_H 2.28 (s), δ_C 20.3 (q), 170.4 (s)] were also observed. When **1** was treated with acetic anhydride in pyridine, compound **1** was unaltered; this fact together with the presence of a singlet at δ_H 3.38, interchangeable with D_2O, confirmed the presence of a tertiary hydroxy group. In its ^1H NMR spectrum (Table 1) an ABX system was observed, with signals at δ 5.26 (J_{AB} = 2.8 Hz), 5.29 (J_{BA} = 2.8 Hz, J_{BX} = 11.0 Hz), and 6.24 (J_{XB} = 11.0 Hz), assignable to protons H-1ax, H-2ax, and H-3eq, respectively, and two methine

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Table 1. $^1H-^{13}C$ NMR (δ, $CDCl_3$, J in Hz in parentheses) Data of Compounds **1**−**4**

	1		**2**		**3**		**4**	
position	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]
1	6.24 d (11.0)	67.8 d	6.22 d (11.3)	68.1 d	6.19 d (10.9)	68.1 d	6.17 d (11.2)	68.3 d
2	5.29 dd (2.8, 11.0)	68.6 d	5.31 dd (2.8, 11.3)	70.7 d	5.27 dd (3.2, 10.9)	68.7 d	5.31 dd (2.8, 11.2)	70.7 d
3	5.26 d (2.8)	75.3 d	3.84 dd (2.8, 11.0)	77.0 d	5.25 d (3.2)	75.6 d	3.85 dd (2.8, 10.9)	77.2 d
4		69.7 s		70.9 s		69.9 s		70.8 s
5		89.5 s		92.0 s		89.7 s		92.1 s
6	1.86 d (12.2)	32.5 t	1.85 d (12.9)	32.1 t	1.86 m (12.2)	32.9 t	1.86 d (12.2)	32.1 t
	2.40 dd (3.8, 12.2)		2.52 m		2.40 m		2.53 m	
7	2.04 m	42.4 d	2.03 m	42.2 d	2.04 m	42.6 d	2.02[b] m	42.2 d
8	2.12 m	30.3 t	2.12 m	31.0 t	2.14 m	30.7 t	2.02[b] m	30.9 t
9	4.82 d (5.0)	73.0 d	4.82 d (5.2)	73.5 d	4.79 d (5.1)	72.5 d	4.84 d (5.4)	72.9 d
10		47.5 s		48.1 s		47.9 s		48.1 s
11		83.7 s		85.3 s		84.2 s		85.4 s
12	1.31 s	29.8 q	1.35 s	29.8 q	1.31 s	29.8 q	1.36 s	29.8 q
13	1.46[b] s	24.0[b] q	1.46 s	24.2 q	1.47 s	24.2 q	1.47 s	24.1 q
14	1.46[b] s	24.0[b] q	1.42 s	23.8 q	1.46 s	24.6 q	1.43 s	23.8 q
15	1.46[b] s	19.7 q	1.48 s	20.2 q	1.50 s	19.7 q	1.48 s	20.2 q

[a] Based on DEPT, HSQC, and HMBC experiments. [b] Overlapping signals.

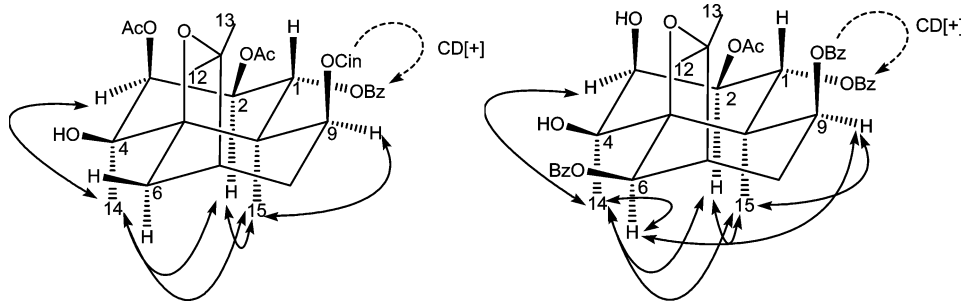

Figure 1. ROE effects (solid line) and CD exciton coupling (dashed line) for compounds **1** (left) and **9** (right).

protons at δ 4.82 (d, $J = 5.0$ Hz, H-9) and 2.04 (m, H-7). The geometry of the disubstituted double bond of the cinnamate moiety was determined as *trans* by the large vicinal coupling constant value of the olefinic proton signals at δ 6.40 (d, $J = 16.0$ Hz) and 7.48 (overlapping signal). A methyl group at δ 1.46 attached to a carbon at δ 69.7 bearing a hydroxy group (Me-14), and three methyls at δ 1.31 (Me-12) and 1.46 (Me-13 and Me-15) were confirmed from the ^{13}C NMR spectrum. All these data indicated that **1** is a polyester sesquiterpene with a 1,2,3,4,9-pentasubstituted dihydro-β-agarofuran skeleton.

The regiosubstitution of **1** was determined by a HMBC experiment, showing three-bond correlations between the carboxyl signals of the acetate groups at δ_C 170.2 and 170.4 and the resonances at δ_H 5.29 (H-2) and 5.26 (H-3), respectively. The carboxyl signal of the cinnamate group at δ_C 165.9 was correlated with the signal at δ_H 4.82 (H-9), whereas the carboxyl signal of the benzoate group at δ_C 164.9 was linked to the resonance at δ_H 6.24 (H-1). The relative configuration of **1** was established on the basis of the coupling constants and confirmed by a ROESY experiment. Therefore, the $J_{1,2}$ (11.0 Hz) and $J_{2,3}$ (2.8 Hz) values indicated a *trans* and *cis* relationship between H-1/H-2 and H-2/H-3, respectively. The ROESY experiment showed ROE effects of H-2 to Me-14 and Me-15, H-3 to Me-14, and H-9 to Me-15 (Figure 1).

The absolute configuration of **1** was resolved by the dibenzoate chirality method, an extension of the circular dichroism exciton chirality procedure.[12] The angle (73.0°) between the two chromophores (benzoate and cinnamate) was calculated by molecular mechanics calculations using the PC model.[13] Therefore, compound **1** was considered suitable for a CD study, showing a Davidoff-type split curve with a first positive Cotton effect at 269.8 nm ($\Delta\varepsilon$ +16.8) and a second negative effect at 227.4 nm ($\Delta\varepsilon$ −15.2), due to the couplings of the benzoate and cinnamate groups at C-1α and C-9β, respectively (Figure 1). Accordingly, the absolute configuration of **1** was established as (1*R*,2*S*,3*S*,4*S*,5*R*,7*R*,9*S*,10*R*)-2,3-diacetoxy-1-benzoyloxy-9-cinnamoyloxy-4-hydroxydihydro-β-agarofuran.

The HREIMS of compound **2** gave a molecular ion at *m*/*z* 578.2539, corresponding to the molecular formula $C_{33}H_{38}O_9$. Its ^1H and ^{13}C NMR data (Table 1) were closely related to those of **1**, except for the absence of the signals assigned to the acetyl group at C-3 and the shift of the signal corresponding to the H-3 proton from δ_H 5.26 in **1** to δ_H 3.84 in **2**. Detailed assignments of the ^1H and ^{13}C NMR data and the relative configuration were determined on the basis of COSY, HSQC, and HMBC experiments. The absolute configuration of **2** was established by chemical correlation. Thus, acetylation of **2** yielded a compound for which the spectroscopic data were identical to those of **1**.

Compounds **3** and **4** were closely related to **1** and **2**, respectively. Consequently, the stereostructure elucidation of these compounds was greatly aided by the comparison of their spectroscopic data. Even so, a complete set of 2D NMR spectra (COSY, HSQC, and HMBC) was acquired for each metabolite in order to gain the complete and unambiguous assignments of the ^1H and ^{13}C NMR resonances, as listed in Table 1. The same molecular formula and relative stereochemistry, previously assigned to **1** and **2** were also confirmed for **3** and **4**, respectively, by HREIMS and 2D NMR ROESY spectroscopy. Their NMR spectra (Table 1) revealed the absence of the *trans*-cinnamate double-bond signals and the presence of signals assignable to a *cis*-cinnamate double bond [δ_H 6.03 d, 6.92 d ($J = 12.5$ Hz) for **3**; δ_H 6.05 d, 6.96 d ($J = 12.6$ Hz) for **4**], suggesting an isomerization of the double bond at the cinnamate group. Since compounds **3** and **4** have the same basic polyhydroxy dihydro-β-agarofuran sesquiterpenoid core as **1**, their absolute configurations were proposed to be the same based on biogenetic grounds.

The structure and absolute configuration of compound **5** was elucidated on the basis of spectroscopic data analysis, including

Table 2. $^1H-^{13}C$ NMR (δ, $CDCl_3$, J in Hz in parentheses) Data of Compounds **7**−**9**

	7		**8**		**9**	
position	δ_H	δ_C[a]	δ_H	δ_C[a]	δ_H	δ_C[a]
1	6.24 d (11.3)	67.7 d	6.18 d (11.4)	67.8 d	6.29 d (11.3)	67.7 d
2	5.36 dd (3.1, 11.3)	68.1 d	5.33 dd (2.3, 11.4)	70.2 d	5.34 dd (2.6, 11.3)	70.3 d
3	5.24 d (3.1)	75.0 d	3.79 d (2.3)	76.8 d	3.81 d (2.6)	77.9 d
4		71.3 s		72.7[b] s		71.4 s
5		91.0 s		92.7 s		92.5 s
6	4.51 s	79.7 d	4.51 d (5.5)	80.0 d	5.76 s	80.7 d
7	2.20[b] m	49.1 d	2.19[b] m	48.9 d	2.34 t (2.5)	48.1 d
8	2.20[b] m	31.0 t	2.19[b] m	31.4 t	2.25 m	31.6 t
9	4.78 d (6.4)	72.4 d	4.76 d (6.4)	72.7[b] d	5.11 d (6.5)	72.9 d
10		49.8 s		50.4 s		51.7 s
11		84.9 s		86.4 s		86.3 s
12	1.60 s	30.2 q	1.61 s	29.8 q	1.60 s	30.0 q
13	1.55 s	26.4 q	1.54 s	26.3 q	1.53[b] s	25.9 q
14	1.82 s	24.0 q	1.77 s	23.7 q	1.53[b] s	24.0 q
15	1.56 s	20.4 q	1.59 s	20.8 q	1.67 s	20.8 q

[a] Based on DEPT, HSQC, and HMBC experiments. [b] Overlapping signals.

1D and 2D NMR experiments (COSY, ROESY, HSQC, and HMBC). This compound, described previously as a semisynthetic substance,[14] has been isolated from a natural source for the first time.

Compound **7** gave the molecular formula $C_{35}H_{40}O_{11}$ (HREIMS), and its IR spectrum indicated the presence of ester and hydroxy groups. The ^1H and ^{13}C NMR spectra (Table 2) combined with the mass data were used to establish its structure as that of a dihydro-β-agarofuran sesquiterpene polyester containing two acetates, one benzoate, one cinnamate, and two hydroxy groups. The ^1H NMR data of **7** were similar to that of **1**, except for the presence of an oxymethine proton at δ_H 4.51 replacing methylene resonances at δ_H 1.86 (d, $J = 12.2$ Hz) and 2.40 (dd, $J = 3.8$, 12.2 Hz), suggesting that this compound possesses an additional hydroxy group. The position of the hydroxy group at C-6 was confirmed by $^1H-^1H$ COSY and HMBC correlations. The regiosubstitution of **7** was established by a HMBC experiment, showing three-bond correlations between H-2 (δ_H 5.36) and H-3 (δ_H 5.24) with the carboxylic carbons of the two acetate groups at δ_C 170.1 and 170.3, respectively, and couplings of H-1 (δ_H 6.24) with the carboxylic carbon of the benzoate (δ_C 164.8) and H-9 (δ_H 4.78) with the carboxylic carbon of the cinnamate (δ_C 165.6) group. The ROESY experiment enabled the relative configuration of the hydroxy and ester groups to be determined as 1α, 2β, 3β, 4β, 6β, and 9β. The structure of compound **8** was elucidated by spectroscopic methods, including HREIMS, IR, UV, and NMR data (Table 2) and comparison with those of **7**. Thus, the ^1H NMR spectrum of **8** indicated that it is the 3-deacetyl derivative of **7**, since the signal corresponding to the H-3 proton shifted from δ 5.24 to δ 3.79, and the signals for the acetyl group at δ_H 2.32 (s), δ_C 20.9, and δ_C 170.3 were not observed. Detailed assignments of the ^1H and ^{13}C NMR signals and the regiosubstitution and relative configuration were based on 2D NMR experiments. The absolute configurations of **7** and **8** were determined by chemical correlations. Thus, when compounds **7** and **8** were acetylated, the previously described compound **5** was obtained, for which the absolute configuration has been established by CD studies.[14]

Compound **9** was obtained as a colorless lacquer. Its molecular formula was established as $C_{38}H_{40}O_{11}$ by HREIMS and its ^1H and ^{13}C NMR data (Table 2). These values indicated that compound **9** was a 1,2,3,4,6,9-hexasubstituted dihydro-β-agarofuran sesquiterpene. The regiosubstitution was established by the long-range $^1H-^{13}C$ HMBC couplings observed between the H-1 (δ_H 6.29), H-6 (δ_H 5.76), and H-9 (δ_H 5.11) proton resonances and the carboxyl signals of the benzoate groups at δ_C 164.6, 166.1, and 165.3, respectively, whereas H-2 (δ_H 5.34) was coupled to the carboxyl signal of the acetate group (δ_C 170.5). The ROESY experiment enabled the relative configuration as 1α, 2β, 3β, 4β, 6β, and 9β. Its absolute configuration was established as (1*R*,2*S*,3*S*,4*S*,5*S*,6*R*,7*R*,9*S*,10*R*)-2-acetoxy-1,6,9-tribenzoyloxy-3,4-dihydroxydihydro-β-agarofuran by CD studies (Figure 1), showing a curve with a first Cotton effect at 237.0 ($\Delta\varepsilon = +18.2$) and a second one at 219.8 ($\Delta\varepsilon = -5.1$).

Table 3. Relative Ratio of Epstein-Barr Virus Early Antigen Activation in the Presence of Compounds **1**, **2**, and **5**−**11**[a]

	concentration (mol ratio/TPA)[b]			
compound	1000	500	100	10
1	11.8 (60)[c]	53.2	86.3	100
2	10.9 (60)	52.0	86.2	100
5	8.9 (60)	39.6	87.4	100
6	9.3 (60)	50.1	84.3	100
7	10.1 (60)	51.3	85.2	100
8	5.3 (60)	35.2	85.2	100
9	13.7 (60)	54.2	87.1	100
10	0.0 (60)	33.2	77.5	98.6
11	7.5 (60)	36.6	87.6	100
β-carotene	8.6 (70)	33.3	82.1	100

[a] Values represent percentages relative to the positive control value (100%) ($n = 3$). [b] TPA concentration was 20 ng/mL (32 pmol/mL). [c] Values in parentheses represent viability percentages of Raji cells.

The known sesquiterpenes **5**,[14] **6**,[14] **10**,[15] and **11**[16] were identified from their spectroscopic data upon comparison with values reported in the literature. The isolated compounds have the basic polyhydroxy dihydro-β-agarofuran sesquiterpenoid core of 6-deoxymagellanol[15] for **1**−**4** and magellanol[15] for **5**−**11**.

The antitumor-promoting effect of dihydro-β-agarofuran sesquiterpenes[9,10] previously reported prompted us to examine the purified constituents from *M. jelskii* as potential chemopreventive agents. Thus, the inhibitory effects of compounds **1**, **2**, and **5**−**11** on EBV-EA activation induced by the tumor promoter TPA in Raji cells, conducted as a primary screening test in the search for cancer-chemopreventive agents,[17] were assessed (Table 3). Compounds **3** and **4** could not be assessed due to the small amounts obtained. The evaluated compounds preserved a high viability of Raji cells (60% at 1000 mol ratio/TPA), indicating that their cytotoxicity seems to be moderate against these cells. All the compounds tested showed some degree of inhibitory effect on EBV activation. Compound **8**, with an inhibition value of 94.7% at a 10^3 mol ratio/TPA, was also more active than β-carotene, which has been intensively studied in cancer chemoprevention using animal models.[8] Moreover, compound **10** exhibited a remarkable biological effect, proving to be more potent than the control at all the concentrations assessed.

On the basis of the results of the in vitro assay, we investigated the effect of compound **10** on mouse skin papillomas in a two-stage carcinogenesis test, using 7,12-dimethylbenz[*a*]anthracene (DMBA) as initiator and TPA as promoter. This model is used

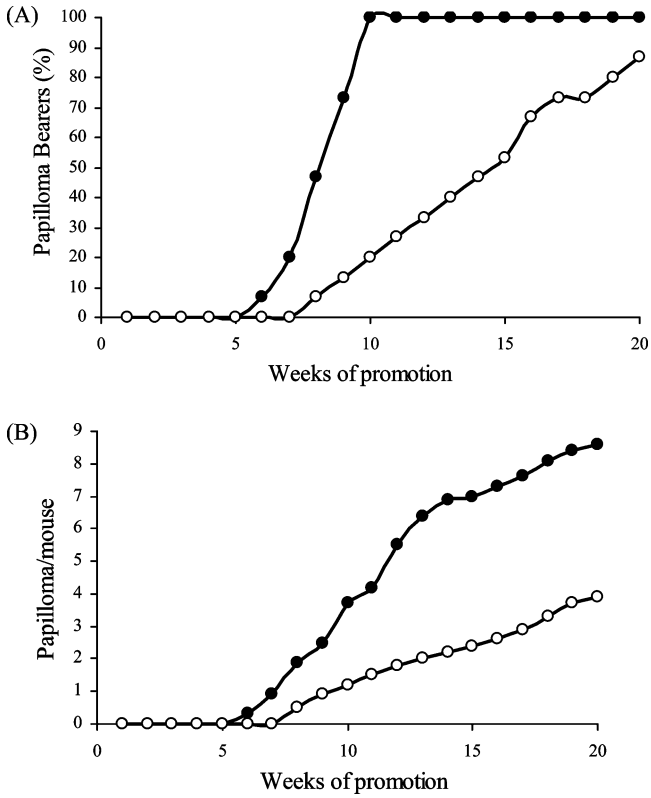

Figure 2. Inhibition of TPA-induced tumor promotion by multiple application of **10**. All mice were initiated with DMBA (390 nmol) and promoted with 1.7 nmol of TPA, given twice weekly starting 1 week after initiation. (A) Percentage of mice bearing papillomas. (B) Average number of papillomas per mouse. (●) Control (TPA alone); (○) TPA + 85 nmol of **10**. After 20 weeks of promotion, the group treated with **10** was significantly different from the positive control ($p < 0.05$) on papillomas per mouse.

since mouse skin proves to be a unique target to differentiate between the effect of a test agent as either an anti-initiator or an anti-promoter under appropriate experimental conditions,[18] in which retinoic acid, a well-known inhibitor of tumor promotion, is used as a positive control.[19] As shown in Figure 2A, papilloma-bearing mice in the positive control group treated with DMBA (390 nmol) and TPA (1.7 nmol, twice/week) were noted as early as week 6, with the percentage of papilloma-bearing mice increasing rapidly, reaching 100% at week 10. In contrast, the percentage of papilloma-bearing mice in the group treated with **10** (85 nmol) along with DMBA/TPA was 20% at week 10 and 53.3% at week 15, increasing to 90% at week 20. These tumor-inhibitory effects were also seen as a reduction in the number of papillomas in the compound **10**-treatment group. Thus, as demonstrated in Figure 2B, mice treated with compound **10** developed 3.9 papillomas/mice by week 20, while the positive control group reached 8.6 papillomas/mice at the end of the experiment. These results showed that when **10** was applied before each TPA treatment, the formation of papillomas on mouse skin was remarkably delayed and the number of papillomas was significantly reduced.

A preliminary structure−activity relationship study, based on the results of the in vitro assays, showed the following trends. The replacement of an acetate by a benzoate group on C-6 decreased the activity (22.5% in **10** vs 12.9% in **9**). In addition, the results indicated that a substituent at C-9 on the core skeleton is relevant for the activity, and the presence of a cinnamate group has a detrimental effect on activity with respect to the presence of a benzoate group (**6** vs **10**, 15.7% and 22.5% of inhibition). In general, we can conclude that the most active compounds from this series have a magellanol-type basic polyhydroxylated core.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 automatic polarimeter in $CHCl_3$ at 25 °C, and the $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. CD spectra were recorded in MeCN on a JASCO J-600 spectropolarimeter. UV spectra were obtained in absolute EtOH on a JASCO V-560 instrument. IR (film) spectra were measured in $CHCl_3$ on a Bruker IFS 55 spectrophotometer. ^{1}H and ^{13}C NMR spectra were performed on a Bruker Avance 400 at 400 and 100 MHz, respectively, and chemical shifts are shown in δ (ppm) with TMS as an internal reference. EIMS and HREIMS were recorded on a Micromass Autospec spectrometer. Silica gel 60 (particle size 15−40 and 63−200 μm) for column chromatography, silica gel 60 F_{254} for TLC, and nanosilica gel 60 F_{25} for preparative high-performance TLC were purchased from Macherey-Nagel, and Sephadex LH-20 was obtained from Pharmacia Biotech. Compounds used for CD were purified by high-performance TLC and eluted with a mixture of *n*-hexane−EtOAc (6:4).

Plant Material. *Maytenus jelskii* was collected in the Urubamba Province, Cuzco, Perú, in December 2006. A voucher specimen (CUZ 29845) was identified by Professor Alfredo Tupayachi Herrera and deposited at the Herbarium of Missouri Botanical Garden, St. Louis, MO.

Extraction and Isolation. The dried leaves (790 g) of *M. jelskii* were sliced into chips and extracted with EtOH in a Soxhlet apparatus for 48 h. Evaporation of the solvent under reduced pressure provided 171.1 g of crude extract, which was partitioned into a CH_2Cl_2−H_2O (1:1, v/v) solution. The CH_2Cl_2 (61.2 g) fraction was submitted to VLC on a silica gel column, using mixtures of *n*-hexane−EtOAc of increasing polarity as eluents, to afford nine fractions (I−IX). Fraction VII was chromatographed on Sephadex LH-20, using as eluent *n*-hexane−$CHCl_3$−MeOH (2:1:1), to provide five fractions (A−E). Three fractions (B−D) were purified further by silica gel flash column chromatography, eluted in a step gradient manner with CH_2Cl_2−Me_2CO (from 10:1 to 7:3), and further purified by preparative TLC with *n*-hexane−1,4-dioxane (6:4), *n*-hexane−Et_2O (2:8), and CH_2Cl_2−Me_2CO (8:2) to give the new compounds **1** (7.6 mg), **2** (6.5 mg) **3** (1.3 mg), **4** (1.4 mg), **7** (9.2 mg), **8** (10.8 mg), and **9** (21.8 mg), in addition to the known compounds **5**, **6**, **10**, and **11**.

(1*R*,2*S*,3*S*,4*S*,5*R*,7*R*,9*S*,10*R*)-2,3-Diacetoxy-1-benzoyloxy-9-*trans*-cinnamoyloxy-4-hydroxydihydro-β-agarofuran (1): colorless lacquer; $[\alpha]^{25}_D$ +42.4 (*c* 3.7, $CHCl_3$); CD λ_{ext} (MeCN) 269.8 ($\Delta\varepsilon$ = +16.8), 241.4 ($\Delta\varepsilon$ = 0), 227.4 ($\Delta\varepsilon$ = −15.2) nm; UV λ_{max} (EtOH) (log ε) 274 (4.3), 223 (4.4) nm; IR ν_{max} (film) 3524, 2960, 2926, 2854, 1746, 1731, 1714, 1636, 1224, 1140, 1024, 756, 712 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.77 (3H, s, OAc-2), 2.28 (3H, s, OAc-3), 3.38 (1H, s, OH-4), 6.40 (1H, d, *J* = 16.0 Hz, OCin), OBz, OCin [7.30 (2H, m), 7.43 (4H, m), 7.48 (3H, m), 7.75 (2H, d, *J* = 7.2 Hz)], for other signals, see Table 1; ^{13}C NMR ($CDCl_3$) δ 20.3 (q, OAc-3), 20.9 (q, OAc-2), 118.2 (d, OCin), OBz, OCin [127.9 (2 × d), 128.0 (2 × d), 128.6 (2 × d), 129.1 (2 × d), 129.7 (s), 129.9 (d), 132.7 (d), 134.4 (s)], 144.7 (d, OCin), 164.9 (s, OBz-1), 165.9 (s, OCin-9), 170.2 (s, OAc-2), 170.4 (s, OAc-3), for other signals, see Table 1; EIMS *m*/*z* 620 [M]$^+$ (3), 605 (14), 579 (1), 483 (1), 457 (21), 397 (4), 248 (11), 131 (100), 105 (89); HREIMS *m*/*z* 620.2639 [M]$^+$ (calcd for C_{35} H_{40} O_{10}, 620.2621).

(1*R*,2*S*,3*S*,4*S*,5*R*,7*R*,9*S*,10*R*)-2-Acetoxy-1-benzoyloxy-9-*trans*-cinnamoyloxy-3,4-dihydroxydihydro-β-agarofuran (2): colorless lacquer; $[\alpha]^{25}_D$ +112.7 (*c* 1.6, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 278 (4.8), 223 (4.9) nm; IR ν_{max} (film) 3474, 2931, 1740, 1733, 1716, 1639, 1277, 1241, 1169, 757, 714 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.88 (3H, s, OAc-2), 3.38 (1H, s, OH-4), 3.55 (1H, d, *J* = 11.0 Hz, OH-3), 6.49 (1H, d, *J* = 16.0 Hz, OCin), OBz, OCin [7.31 (2H, m), 7.45 (5H, m), 7.58 (2H, m), 7.78 (2H, d, *J* = 7.2 Hz)], for other signals, see Table 1;^{13}C NMR ($CDCl_3$) δ 20.8 (q, OAc-2), 118.6 (d, OCin), OBz, OCin [127.7 (4 × d), 128.8 (2 × d), 129.3 (2 × d), 129.8 (s), 130.2 (d), 132.9 (d), 134.6 (s)], 144.8 (d, OCin), 165.0 (s, OBz-1), 166.1 (s, OCin-9), 170.6 (s, OAc-2), for other signals, see Table 1; EIMS *m*/*z* 578 [M$^+$] (22), 563 (4), 545 (4), 503 (1), 456 (1), 430 (4), 396 (2), 265 (4), 248 (6), 131 (100), 105 (97); HREIMS *m*/*z* 578.2539 [M$^+$] (calcd for $C_{33}H_{38}O_9$, 578.2516).

(1*R*,2*S*,3*S*,4*S*,5*R*,7*R*,9*S*,10*R*)-2,3-Diacetoxy-1-benzoyloxy-9-*cis*-cinnamoyloxy-4-hydroxydihydro-β-garofuran (3): colorless lacquer; $[\alpha]^{25}_D$ +20.4 (*c* 0.3, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 276 (3.1), 224 (3.4) nm; IR ν_{max} (film) 3521, 2928, 2857, 1735, 1635, 1454, 1372, 1279, 1144, 1027, 761, 716 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.78 (3H, s,

OAc-2), 2.25 (3H, s, OAc-3), 3.39 (1H, s, OH-4), 6.03 (1H, d, J = 12.5 Hz, OCin), 6.92 (1H, d, J = 12.5 Hz, OCin), OBz, OCin [7.13 (2H, d, J = 7.7 Hz), 7.31 (4H, m), 7.48 (2H, m), 7.83 (2H, d, J = 7.7 Hz)], for other signals, see Table 1; ^{13}C NMR ($CDCl_3$) δ 20.7 (q, OAc-2), 21.4 (q, OAc-3), 119.9 (d, OCin), OBz, OCin [128.1 (2 × d), 128.9 (2 × d), 129.8 (2 × d), 130.3 (2 × d), 131.5 (s), 131.9 (d), 133.1 (d), 134.7 (s)], 144.8 (d, OCin), 164.7 (s, OCin-9), 165.4 (s, OBz-1), 170.6 (s, OAc-2), 171.0 (s, OAc-3), for other signals, see Table 1; EIMS m/z 620 [M^+] (4), 605 (11), 515 (1), 470 (4), 457 (27), 454 (5), 396 (7), 370 (1), 307 (3), 265 (4), 248 (13), 173 (4), 131 (93), 105 (100); HREIMS m/z 620.2625 [M^+] (calcd for C_{35} H_{40} O_{10}, 620.2621).

Acetylation of 2. A mixture of acetic anhydride (2 drops), triethylamine (4 drops), compound **2** (3 mg), and 4-(dimethylamino)pyridine in dichloromethane (2 mL) was stirred at room temperature for 16 h. The reaction was quenched by the addition of ethanol (0.5 mL), followed by stirring for 30 min at room temperature. The mixture was evaporated to dryness, and the residue was purified by preparative TLC, using *n*-hexane−EtOAc (1:1), to give compound **1** (2.6 mg).

(1*R*,2*S*,3*S*,4*S*,5*R*,7*R*,9*S*,10*R*)-2-Acetoxy-1-benzoyloxy-9-*cis*-cinnamoyloxy-3,4-dihydroxydihydro-β-agarofuran (4): colorless lacquer; $[\alpha]^{25}_D$ +20.7 (c 0.2, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 275 (3.7), 224 (4.0) nm; IR ν_{max} (film) 3515, 2927, 2857, 1732, 1632, 1604, 1451, 1384, 1278, 1165, 765, 715 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.92 (3H, s, OAc-2), 3.38 (1H, s, OH-4), 3.53 (1H, d, J = 10.9 Hz, OH-3), 6.05 (1H, d, J = 12.6 Hz, OCin), 6.96 (1H, d, J = 12.6 Hz, OCin), OBz, OCin [7.16 (2H, d, J = 7,7 Hz), 7.33 (4H, m), 7.50 (2H, m), 7.87 (2H, d, J = 8.3 Hz), for other signals, see Table 1; ^{13}C NMR ($CDCl_3$) δ 20.9 (q, OAc-2), 119.8 (d, OCin), OBz, OCin [127.7 (2 × d), 128.4 (2 × d), 128.9 (d), 129.5 (2 × d), 129.9 (2 × d), 130.2 (s), 133.0 (d), 134.5 (s)], 144.6 (d, OCin), 164.6 (s, OCin-9), 165.1 (s, OBz-1), 170.6 (s, OAc-2), for other signals, see Table 1; EIMS m/z 578 [M^+] (15), 552 (10), 545 (4), 430 (6), 370 (2), 322 (6), 279 (2), 248 (7), 149 (25), 131 (22), 105 (100), 57 (27); HREIMS m/z 578.2498 [M^+] (calcd for $C_{33}H_{38}O_9$, 578.2516).

(1*R*,2*S*,3*S*,4*S*,5*S*,6*R*,7*R*,9*S*,10*R*)-2,3-Diacetoxy-1-benzoyloxy-9-*trans*-cinnamoyloxy-4,6-dihydroxydihydro-β-agarofuran (7): colorless lacquer; $[\alpha]^{25}_D$ +111.5 (c 0.9, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 280 (4.3), 224 (4.4) nm; IR ν_{max} (film) 3501, 3474, 2963, 2931, 1749, 1733, 1716, 1637, 1260, 1114, 1019, 759, 715 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.81 (3H, s, OAc-2), 2.32 (3H, s, OAc-3), 3.99 (1H, br s, OH-4), 4.54 (1H, br s, OH-6), 6.40 (1H, d, J = 16.0 Hz, OCin), OBz, OCin [7.29 (2H, m), 7.45 (5H, m), 7.55 (2H, m), 7.74 (2H, d, J = 7.2 Hz)], for other signals, see Table 2; ^{13}C NMR ($CDCl_3$) δ 20.3 (q, OAc-2), 20.9 (q, OAc-3), 117.9 (d, OCin-9), OBz, OCin [128.0 (2 × d), 128.1 (2 × d), 128.6 (2 × d), 128.8 (s), 129.1 (2 × d), 129.5 (s), 130.0 (d), 132.8 (d), 134.3 (s)], 145.0 (d, OCin-9), 164.8 (s, OBz-1), 165.6 (s, OCin-9), 170.1 (s, OAc-2), 170.3 (s, OAc-3), for other signals, see Table 2; EIMS m/z 636 [M^+] (1), 621 (3), 603 (19), 561 (2), 488 (2), 481 (1), 473 (7), 428 (1), 356 (2), 306 (1), 264 (3), 131, (100), 105 (81); HREIMS m/z 636.2597 [M^+] (calcd for $C_{35}H_{40}O_{11}$, 636.2571).

(1*R*,2*S*,3*S*,4*S*,5*S*,6*R*,7*R*,9*S*,10*R*)-2-Acetoxy-1-benzoyloxy-9-*trans*-cinnamoyloxy-3,4,6-trihydroxydihydro-β-agarofuran (8): colorless lacquer; $[\alpha]^{25}_D$ +96.5 (c 0.6, $CHCl_3$); UV λ_{max} (EtOH) (log ε) 280 (4.5), 223 (4.6) nm; IR ν_{max} (film) 3447, 2925, 2854, 1733, 1637, 1273, 1163, 1111, 979, 756, 713 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.91 (3H, s, OAc-2), 4.20 (1H, s, OH-4), 5.15 (1H, d, J = 5.5 Hz, OH-6), 6.45 (1H, d, J = 16.0 Hz, OCin), OBz, OCin [7.30 (2H, m), 7.41 (5H, m), 7.54 (2H, m), 7.75 (2H, d, J = 7.1 Hz)], for other signals, see Table 2; ^{13}C NMR ($CDCl_3$) δ 20.5 (q, OAc), 118.0 (d, OCin), OBz, OCin [127.5 (4 × d), 128.0 (2 × d), 128.6 (2 × d), 129.5 (s), 130.1 (d), 132.9 (d), 134.3 (s)], 144.9 (d, OCin), 164.7 (s, OBz), 165.6 (s, OCin), 170.2 (s, OAc), for other signals, see Table 2; EIMS m/z 594 [M^+] (1), 579 (6), 561 (5), 534 (1), 519 (1), 428 (2), 412 (3), 314 (5), 264 (3), 149 (7), 131 (100), 105 (66); HREIMS m/z 594.2458 [M^+] (calcd for $C_{33}H_{38}O_{10}$, 594.2465).

Acetylation of 7 and 8. When compounds **7** (3.8 mg) and **8** (3.2 mg) were acetylated under the same conditions already described for compound **2**, compound **5** (3.0 and 2.9 mg, respectively) was obtained.

(1*R*,2*S*,3*S*,4*S*,5*S*,6*R*,7*R*,9*S*,10*R*)-2-Acetoxy-1,6,9-tribenzoyloxy-3,4-dihydroxydihydro-β-agarofuran (9): colorless lacquer; $[\alpha]^{25}_D$ +57.0 (c 2.2, $CHCl_3$); CD λ_{ext} (MeCN) 237.0 ($\Delta\varepsilon$ = +18.2), 226.0 ($\Delta\varepsilon$ = 0), 219.8 ($\Delta\varepsilon$ = −5.1) nm; UV λ_{max} (EtOH) (log ε) 223 (4.3) nm; IR ν_{max} (film) 3526, 2926, 2855, 1732, 1715, 1277, 1111, 1025, 757, 710 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.89 (3H, s, OAc-2), 3.91 (1H, s, OH-4), 3 × OBz [7.28 (2H, m), 7.44 (5H, m), 7.55 (4H, m), 7.96 (2H, d, J = 7.2 Hz), 8.25 (2H, d, J = 7.1 Hz)], for other signals, see Table 2; ^{13}C NMR ($CDCl_3$) δ 23.9 (q, OAc-2), 3 × OBz [128.0 (2 × d), 128.2 (2 × d), 128.7 (2 × d), 129.2 (2 × d), 129.4 (s), 129.5 (s), 129.6 (s), 130.2 (2 × d), 130.3 (2 × d), 132.9 (d), 133.1 (d), 133.5 (d)], 164.6 (s, OBz-1), 165.3 (s, OBz-9), 166.1 (s, OBz-6), 170.5 (s, OAc-2), for other signals, see Table 2; EIMS m/z 657 [$M - CH_3$]$^+$ (1), 639 (2), 612 (2), 532 (1), 490 (2), 264 (1), 105 (100); HREIMS m/z 657.2321 [$M - CH_3$]$^+$ (calcd for $C_{37}H_{37}O_{11}$, 657.2336).

In Vitro Epstein−Barr Virus Early Antigen Induction Assay. EBV genome-carrying lymphoblastoid cells (Raji cells, derived from Burkitt's lymphoma) were cultivated in 10% fetal bovine serum (FBS) in RPMI-1640 medium (Nissui). Spontaneous activation of EBV-EA in the subline Raji cells was less than 0.1%. The inhibition of EBV-EA activation was assayed using Raji cells (virus nonproducer type) as described previously.[20] The indicator cells were incubated for 48 h at 37 °C in 1 mL of a medium containing *n*-butyric acid (4 mmol), TPA (32 pmol) in dimethylsulfoxide (DMSO) as inducer, and various amounts of test compounds in 5 μL of DMSO. Smears were made from the cell suspensions, and the activated cells that were stained by EBV-EA positive serum from nasopharyngeal carcinoma patients were detected by an indirect immunofluorescence technique. In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) present was recorded. Triplicate assays were performed for each compound. The average EBV-EA induction of the test compounds was expressed as a relative ratio to the control experiment (100%), which was carried out with only *n*-butyric acid (4 mmol) plus TPA (32 pmol). EBV-EA induction was around 35%. The viability of treated Raji cells was assayed by the Trypan Blue staining method.

In Vivo Two-Stage Mouse Skin Carcinogenesis Test. The animals (specific pathogen-free female ICR mice, 6 weeks old) were divided into three experimental groups of 15 mice each. The back of each mouse was shaved with surgical clippers, and each mouse was topically treated with DMBA (100 μg, 390 nmol) in acetone (0.1 mL) as an initiation treatment. One week after initiation with DMBA, papilloma formation was promoted by the application of TPA (1 μg, 1.7 nmol) in acetone (0.1 mL) twice a week. Groups II and III received a topical application of compound **10** (85 nmol) in acetone (0.1 mL) 1 h before each promotion treatment, respectively. The incidence of papillomas was observed weekly for 20 weeks, and the percentage of mice bearing papillomas and the average number of papillomas per mouse were recorded. In our experiment, only typical papillomas larger than about 1 mm in diameter were counted in each case. Details of the in vivo two-stage carcinogenesis test have been reported previously.[19]

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for compounds **1**−**4** and **7**−**9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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