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Phytochemical Studies on Meliaceous Plants. VIII.[1] Structures and Inhibitory Effects on Epstein–Barr Virus Activation of Triterpenoids from Leaves of *Chisocheton macrophyllus* KING

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A 24-epimeric mixture of a new triterpenoid, 24-hydroxydammara-20,25-dien-3-one (1), and two known triterpenoids, moronic acid (2) and betulonic acid (3), were isolated from leaves of *Chisocheton macrophyllus* KING, and the inhibitory effects of these triterpenoids on Epstein–Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate were tested.

Keywords *Chisocheton macrophyllus*; Epstein–Barr virus; triterpenoid; antitumor promoter; Meliaceae; leaf

In a continuation of our phytochemical studies on the constituents of meliaceous plants,[1] we have now isolated a 24-epimeric mixture of new triterpenoid, 24-hydroxydammara-20,25-dien-3-one (**1**), and two known triterpenoids, moronic acid (**2**)[2] and betulonic acid (**3**),[3] from leaves of *Chisocheton macrophyllus* KING (Meliaceae). In this paper, we describe the structure elucidation of compound **1** and the inhibitory effects of these triterpenoids on Epstein–Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a strong tumor promoter.[4] In the preliminary screening test of some MeOH extracts from meliaceous plants, the MeOH extract of leaves of *C. macrophyllus* was found to show significant inhibitory effects on EBV-EA activation (Table I). To isolate the active component, the MeOH extract was suspended in H_2O and the suspension was extracted successively with AcOEt and *n*-BuOH. Each extract was subjected to short term *in vitro* assay (Table I).

As shown in the table, the AcOEt extract obtained from the original MeOH extract was more inhibitory than the *n*-BuOH and H_2O extracts. The AcOEt extract was further purified by silica gel column and high performance liquid chromatography to isolate three triterpenoids, **1**, **2**, and **3** (0.02, 1.80, and 0.02% from the MeOH extract, respectively), together with β-sitosterol. Compounds **2** and **3** were identified as moronic acid[2] and betulonic acid[3] by comparison of their physical properties with the respective reported data.

The new triterpenoid (**1**), mp 154—155 °C, $[\alpha]_D$ +82.6° ($CHCl_3$) showed a carbonyl (1695 cm^{-1}) absorption in the IR spectrum. Electron impact (EI) MS and accurate mass spectroscopic data showed **1** to possess the molecular formula, $C_{30}H_{48}O_2$. The mass spectral fragmentation pattern of **1** was characteristic of a dammarane skeleton.[5] Namely, **1** gave a significant fragment ion at *m/z* 205.160 ($C_{14}H_{21}O$) due to cleavage of the C-ring of the dammarane skeleton and showed a fragment at *m/z* 125.097 ($C_8H_{13}O$) ascribed to the side chain moiety as a result of C-17/20 bond fission. The ^{1}H-NMR spectrum of **1** showed signals at δ 4.09 (1H, t, J=6.2 Hz) due to a proton geminal to a hydroxyl group. In addition, **1** showed signals characteristic of four olefinic protons at δ 4.74, 4.77 (1H each, each br s) and at δ 4.86, 4.96 (1H each, each br s) as well as a vinyl methyl signal at δ 1.74 (3H, s). In the nuclear Overhauser effect correlation spectroscopy (NOESY) experiments on **1**, significant NOE cross peaks between a vinyl methyl and two olefinic protons (δ 4.86, 4.96) and between a vinyl methyl and a proton geminal to a hydroxyl group were observed. The results indicated that **1** has a 20,25-dien-24-ol moiety as the side chain. This structure was also confirmed by

TABLE I. Inhibitory Effects of Extracts from Leaves of *C. macrophyllus* on TPA-Induced EBV-EA Activation[a]

Sample	Concentration[b]		
	100	10	1
MeOH extract	0[c] (0)[d]	12.5 (60)	51.7 (>80)
AcOEt extract	0 (0)	29.4 (60)	72.5 (>80)
n-BuOH extract	0 (0)	82.3 (70)	100.0 (>80)
H_2O extract	0 (70)	81.3 (>80)	100.0 (>80)

a) Positive control (100%) on the basis of the activation by TPA at 20 ng/ml (=32 pM). *b*) μg/ml. *c*) Values represent percentages relative to the positive control. *d*) Values in parentheses are viability (percent) of Raji cells; in this screening test, the cell viability required for the judgement of inhibitory effect was more than 60%.

TABLE II. ^{13}C-NMR Spectral Data for **1** (100.5 MHz, $CDCl_3$, δ_C, ppm from TMS)

C-1	39.98	C-16	29.17
C-2	34.12	C-17	45.48, 45.64
C-3	218.17	C-18	15.86[a]
C-4	47.62	C-19	15.36[a]
C-5	55.39	C-20	152.52
C-6	19.69	C-21	107.64
C-7	34.76	C-22	31.30, 31.35
C-8	40.39	C-23	33.59, 33.68
C-9	50.30	C-24	75.71, 75.74
C-10	36.95	C-25	147.57, 147.60
C-11	21.92	C-26	111.04, 111.10
C-12	25.00	C-27	17.61, 17.65
C-13	47.43, 47.48	C-28	26.78
C-14	49.45	C-29	21.04
C-15	30.53	C-30	16.08[a]

a) Assignments in each column may be interchanged.

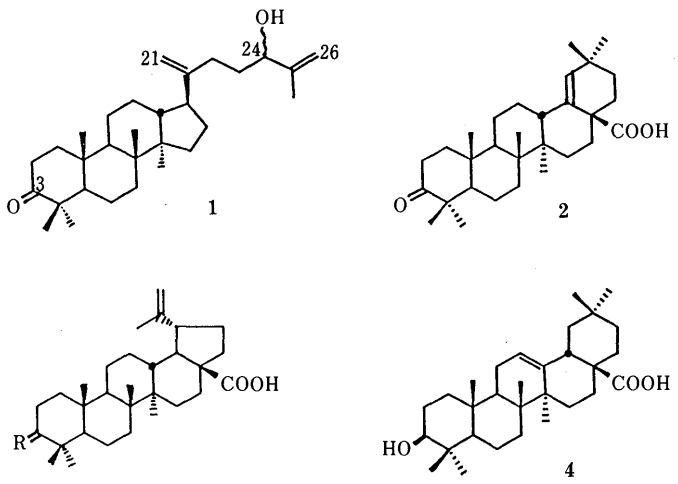

Fig. 1

TABLE III. Inhibitory Effects of Triterpenoids (**1**—**5**) on TPA Induced EBV-EA Activation[a]

Compound	Concentration[b]			
	1000	500	100	10
Compound **1** (**1**)	53.7[c] (>80)[d]	85.5 (>80)	100.0 (>80)	100.0 (>80)
Moronic acid (**2**)	0.0 (60)	29.6 (>80)	66.8 (>80)	90.2 (>80)
Betulonic acid (**3**)	0.0 (50)	21.7 (70)	45.9 (>80)	80.6 (>80)
Oleanolic acid (**4**)[e]	30.0 (60)	—	80.0 (>80)	100.0 (>80)
Betulic acid (**5**)[e]	80.0 (>80)	—	100.0 (>80)	100.0 (>80)

a) Positive control (100%) on the basis of the activation by TPA at 20 ng/ml (=32 pM). *b*) Mol ratio/TPA (20 ng/ml). *c*) Values represent relative percentages to the positive control and averages of three experiments. *d*) Values in parentheses are viability (percent) of Raji cells; in this screening test, the cell viability required for the judgment of inhibitory effect was more than 60%. *e*) Ref. 10*a*.

^{1}H–^{1}H correlation spectroscopy (COSY) and by the ^{13}C-NMR spectrum (Table II). The ^{13}C-NMR spectrum of compound **1** showed splitting of the signals of certain carbons, *i.e.*, C-13,17 and C-22-27. The splitting of these carbons is consistent with the presence of a mixture of C-24 epimers.[6] Based on the combined evidence, the structure of **1** is defined as a 24-epimeric mixture of 24-hydroxy-dammara-20,25-dien-3-one.

Compounds **1**, **2**, and **3** isolated from *C. macrophyllus*[8] and other triterpenoids (**4** and **5**) were tested utilizing the short-term *in vitro* assay of EBV-EA activation. Their inhibitory effects on EBV-EA activation and viability of Raji cells are shown in Table III. As shown in the table, moronic acid (**2**) (major triterpenoid) showed strong inhibitory effects at ratios of 500 and 100 mol triterpenoid/1 mol TPA without seriously affecting the viability of Raji cells. Betulonic acid (**3**) showed almost the same effects as **2**. Inhibitory activities of **2** and **3** were more potent than those of previously tested oleanolic acid (**4**)[10a] and betulic acid (**5**),[10a] while compound **1** showed no remarkable activity compared to those of **2** and **3**. The present *in vitro* assay results strongly suggested that the inhibitory effect of the MeOH extract of *C. macrophyllus* on EBV-EA activation may be due largely to the effects of compound **2** (and also **3**). The results also suggested that these triterpenoids[10] may be valuable inhibitors of tumor promoters *in vivo*, because many compounds that inhibit EBV-EA induction by TPA have been shown to act as inhibitors of tumor promotion *in vivo*.[11] On the basis of the results of this *in vitro* assay, we are currently examining the inhibitory effect of **2** on two stage carcinogenesis in mice.

Experimental

The instruments used to obtain melting points, optical rotations, and IR, ^{1}H-NMR (400 MHz), ^{13}C-NMR (100.5 MHz), and mass spectra were the same as described in our previous paper.[1] Melting points are uncorrected. Optical rotations were measured for solutions in $CHCl_3$. EI-MS and accurate MS data were obtained at 30 eV. For silica gel column chromatography, Merck HF-254 was used and for thin layer chromatography, precoated silica gel plates (Merck HF-254) were used. Preparative HPLC was carried out on a Waters instrument with an M 6000A pump, a U6K septumless injector, a series R-401 differential refractometer and a silica-packed column (Tosoh, TSK-gel silica-150; 7.8 mm × 30 cm) with hexane–AcOEt as the eluant.

Plant Material and Extraction Leaves of *C. macrophyllus* were collected at the Bogor Botanical Garden (Java, Indonesia) in 1990 and a voucher specimens have been deposited in the herbarium of Setsunan University (Faculty of Pharmaceutical Sciences). The crushed leaves (786 g) of *C. macrophyllus* were extracted with MeOH (15 l × 2) and the solvent was removed *in vacuo*. The MeOH extract (61 g) was suspended in H_2O and the aqueous suspension was extracted with AcOEt (500 ml × 4) and *n*-BuOH (500 ml × 4), successively. Each layer was evaporated *in vacuo* to give extracts (AcOEt 37.9 g, *n*-BuOH 9.9 g, and H_2O 12.8 g). The results of the bioassay of each extract are given in Table I.

Isolation of Compounds 1—3 The AcOEt extract (37.0 g) was purified by silica gel column chromatography (solvent hexane–AcOEt) to afford 9 fractions. These fractions were subjected to the assay. Purification of the active fractions was carried out by silica gel column chromatography and/or HPLC to afford **1** (14 mg), **2** (1100 mg), and **3** (15 mg) along with β-sitosterol (700 mg). The 24-epimeric mixture of 24-hydroxydammara-20,25-dien-3-one (**1**), mp 154—155 °C (hexane), $[\alpha]_D^{20}$ +82.6° (c=0.26). IR ($CHCl_3$) cm^{-1}: 3450, 2950, 1695, 1635, 1450, 1385, 1080, 900. EI- and accurate MS *m/z* (%): 440.366 (M^+, Calcd for $C_{30}H_{48}O_2$ 440.365, 4), 422.354 (M^+-H_2O, Calcd for $C_{30}H_{46}O$ 422.355, 21), 341 (8), 315 [M^+ − side chain ($C_8H_{13}O$), 13], 313 (19), 245 (24), 205.160 (Calcd for $C_{14}H_{21}O^{5)}$ 205.159, 67), 125.097 [Calcd for $C_8H_{13}O$ (side chain), 125.097, 100], 108 (86), 95 (77), 81 (33). ^{1}H-NMR ($CDCl_3$) δ 0.88, 0.95, 1.01 (3H each, each s), 1.04 (3H, s, 29-H_3), 1.09 (3H, s, 28-H_3), 1.74 (3H, s, 27-H_3), 2.47 (2H, m, 2-H_2), 4.09 (1H, t, J=6.2 Hz, 24-H), 4.74, 4.77 (1H each, br s, 21-H_2), 4.86 4.96 (1H each, br s, 26-H_2). ^{13}C-NMR: given in Table II. Compound **2**, mp 212—213 °C (ref. 2 222 °C), $[\alpha]_D^{20}$ +33.1° (c=1.0) [ref. 2 +29.0° (c=0.41)]. The melting point, optical rotation, and IR, EI-MS, ^{1}H- and ^{13}C-NMR spectra of **2** were consistent with the published data for moronic acid.[2] Compound **3**, mp 243—246 °C (ref. 3*b* 236—238 °C), $[\alpha]_D^{25}$ +29.3° (c=0.13) [ref. 3*b* +30.0° (c=0.32)]. The melting point, optical rotation, and IR, EI-MS, ^{1}H- and ^{13}C-NMR spectra of **3** were consistent with the published data for betulonic acid.[3]

Biological Activities The inhibition of EBV-EA activation was assayed by using Raji cells (virus non-producer), EBV genome-carrying human lymphoblastoid cells, which were cultivated in 8% FBS RPMI 1640 medium (Nissui). The indicator cells (Raji) (1 × 10^6/ml) were incubated at 37 °C for 48 h in 1 ml of the medium containing *n*-butyric acid (4 mM, inducer), 2 μl of TPA (20 ng/ml in dimethyl sulfoxide (DMSO) and a known amount of test compound in DMSO. Smears were made from the cell suspension. The activated cells were stained by using the high titer EBV-EA positive sera technique. In each assay, at least 500 cells were counted and the experiments were repeated twice. The average EA induction was compared with that of positive control experiments with *n*-butyric acid (4 mM) and TPA (20 ng/ml), in which EA induction was usually around 30%.

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