A New Biflavonoid from *Calophyllum panciflorum* with Antitumor-Promoting Activity

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A new biflavonoid named pancibiflavonol (**1**) was isolated from an EtOH extract of the stem bark of *Calophyllum panciflorum*, along with six known biflavonoids, and its structure was elucidated by spectroscopic methods. These biflavonoids all exhibited significant inhibitory activity against 12-*O*-tetradecanoylphorbol-13-acetate-induced Epstein−Barr virus early antigen activation in Raji cells.

In our previous paper[1] concerning studies of biologically active natural products from Papua New Guinea medicinal plants, the isolation of a novel dibenzofuran named calophyfuran and two new xanthones named pancixanthones A and B from *Calophyllum panciflorum* A. C. Smith (Guttiferae) and elucidation of their structural features were described. In further studies, a new biflavonoid named pancibiflavonol (**1**) was isolated from an EtOH extract of the stem bark of the same plant along with six known biflavonoids. In a primary screening test for antitumor-promoting agents, we found that some xanthones isolated from plants of the *Guttiferae* family showed potent inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein−Barr virus early antigen (EBV-EA) activation.[2] Furthermore, we reported the inhibitory effects of flavonoids[3,4] and biflavonoids[5] on EBV-EA activation in Raji cells induced by TPA.

This paper describes the isolation and structural characterization of pancibiflavonol (**1**) and the results of assays examining the inhibitory effects on TPA-induced EBV-EA activation of eight biflavonoids isolated from plants of the *Guttiferae* family found in a search for more active antitumor promoters.

The dried stem bark of *C. panciflorum* was extracted with EtOH under reflux. The EtOAc-soluble portion of the extract was fractionated by Si gel column chromatography with $^{i}Pr_2O$−acetone, acetone, CH_2Cl_2−MeOH, and MeOH, successively. The $^{i}Pr_2O$−acetone eluate was further subjected repeatedly to Si gel column chromatography and preparative TLC, and a new biflavonoid named pancibiflavanol (**1**) was found, along with six known biflavonoids (**2**−**7**).

Pancibiflavonol (**1**) was obtained as a yellow powder. The molecular formula was determined to be $C_{30}H_{20}O_{12}$ by HRFABMS. The UV spectrum showed absorption bands with a profile similar to that (λ_{max} 222, 290, 369 nm) of garcinianin (**2**).[6] The IR spectrum exhibited bands at ν_{max} 3317 and 1647 cm^{-1} due to hydroxy and conjugated carbonyl groups. The ^1H and ^{13}C NMR spectra of **1** at 23 °C (see Table 1) showed signals forming respective pairs (relative ratio; 2:1) as in **2**.[6] In the ^1H NMR spectrum at 149 °C (Figure 1), the paired signals changed to single signals, suggesting the presence of two atropisomers of **1**. In support of the view that two atropisomers were present, the ^1H NMR and H−H COSY spectra at 23 °C showed three ^1H singlets at δ 5.95 (5.96), 5.95 (6.05), and 6.17 (6.02); AB-type protons at δ 7.14 (7.07) and 6.39 (6.61) (each 2H, d, J = 8.4 Hz); and ABC-type protons at δ 7.65 (7.77) (1H, d, J = 1.5 Hz), 7.57 (6.95) (1H, dd, J = 8.4, 1.5 Hz), and 6.92 (6.44) (1H, d, J = 8.4 Hz), in addition to two chelated hydroxy signals at δ 12.34 (12.22) and 12.52 (12.48). The remaining 1H-doublets at δ 5.77 (5.61) and 4.82 (5.02) were attributable to protons H-2 and H-3, respectively, and the trans stereochemistry between these protons was indicated by large J values (12.1 Hz). The ^{13}C NMR spectrum (see Table 1) of **1** was similar to those of two known monomeric flavonoids, naringenin (**10**) and quercetin (**11**). This result suggested that pancibiflavonol has the structure of a C-3/C-8″ or C-3/C-6″-linked naringenin−quercetin dimer. The structure **1** was further confirmed by ^1H-detected heteronuclear multiple quantum coherence (HMQC) spectroscopy and ^1H-detected heteronuclear multiple bond connectivity (HMBC) spectroscopy. The existence of the linkage between C-3 in the upper naringenin unit and C-8″ in the lower quercetin unit was

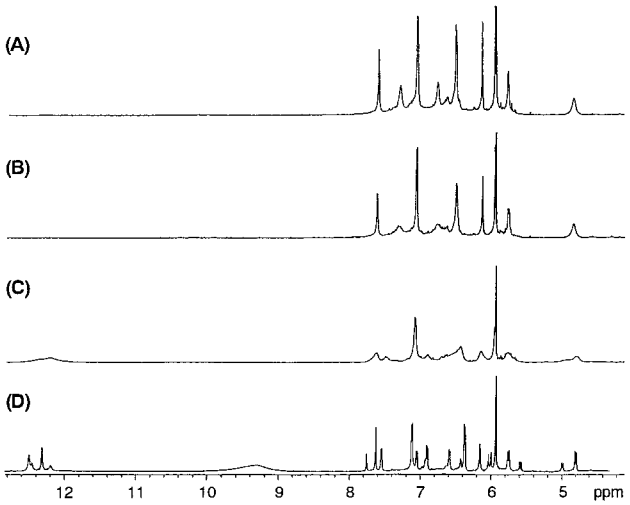

Figure 1. ^1H NMR spectra of **1** measured at 23 (**A**), 80 (**B**), 120 (**C**), and 149 °C (**D**).

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Table 1. ^{1}H and ^{13}C NMR Spectral Data of **1**, Naringenin (**10**), and Quercetin (**11**)[a]

	1				Naringenin (**10**)	Quercetin (**11**)
carbon	δ_H	δ_H (149 °C)	δ_C	δ_C (120 °C)	δ_C	δ_C
2	5.77, 5.61 (d, 12.1)	5.79 (br)	80.91, 81.71 (d)	80.85 (d)	78.41 (d)	
3	4.82, 5.02 (d, 12.1)	4.86 (br)	48.06, 47.17 (d)	47.67 (d)	41.95 (t)	
4			196.56 × 2 (s)	195.48 (s)	196.33 (s)	
4a			101.48 × 2 (s)	*b*	101.75 (s)	
5			163.81, 163.87 (s)	163.26 (s)	163.47 (s)	
5-OH	12.34, 12.22 (s)	*b*				
6	5.95, 6.05 (s)	5.97 (s)	96.25, 96.40 (d)	95.85 (d)	95.79 (d)	
7			166.91, 167.53 (s)	166.11 (s)	166.62 (s)	
8	5.95, 5.96 (s)	5.96 (s)	95.32 × 2 (d)	94.75 (d)	94.95 (d)	
8a			162.86, 162.78 (s)	162.37 (s)	162.91 (s)	
1′			128.81, 127.66 (s)	128.83 (s)	128.83 (s)	
2′,6′	7.14, 7.07 (2H, d, 8.4)	7.07 (2H, d, 8.1)	128.36 (128.53) (d)	127.60 (d)	128.30 (d)	
3′,5′	6.39, 6.61 (2H, d, 8.4)	6.52 (2H, d, 8.1)	114.40, 114.99 (d)	114.17 (d)	115.15 (d)	
4′			157.26, 157.59 (s)	156.91 (s)	157.71 (s)	
2″			145.03, 146.85 (s)	146.49 (s)		147.65 (s)
3″			135.09, 135.46 (s)	134.67 (s)		135.70 (s)
4″			175.38, 175.74 (s)	175.18 (s)		175.80 (s)
4″a			101.58 × 2 (s)	*b*		102.99 (s)
5″			159.67, 159.47 (s)	159.14 (s)		160.69 (s)
5″−OH	12.52, 12.48 (s)	*b*				
6″	6.17, 6.02 (s)	6.15 (s)	98.44, 97.67 (d)	97.55 (d)		98.16 (d)
7″			163.81, 163.87 (s)	163.26 (s)		163.84 (s)
8″			100.31, 99.58 (s)	101.26 (s)		93.33 (d)
8″a			154.20, 153.50 (s)	153.61 (s)		156.10 (s)
1‴			121.88 × 2 (s)	121.51 (s)		121.95 (s)
2‴	7.65, 7.77 (d, 1.5)	7.61 (br)	114.66, 116.28 (d)	114.66 (d)		115.04 (d)
3‴			144.96, 145.97 (s)	144.49 (s)		145.01 (s)
4‴			147.59, 147.51 (s)	147.12 (s)		146.75 (s)
5‴	6.92, 6.44 (d, 8.4)	6.78 (br)	115.72 × 2 (d)	114.98 (d)		115.58 (d)
6‴	7.57, 6.95 (dd, 8.4 & 1.5)	7.31 (br)	120.22, 118.18 (d)	115.05 (d)		119.96 (d)

[a] Values in (δ_H and δ_C) ppm. ^{1}H and ^{13}C NMR spectra were recorded at 600 and 150 MHz, respectively, in DMSO-d_6, 23 °C, unless otherwise stated. All signals correspond to 1H and were observed as a singlet, unless otherwise stated. Figures in parentheses are coupling constants (J) in Hertz (Hz). [b] No signal was detected due to overlapping or broadening of the signal.

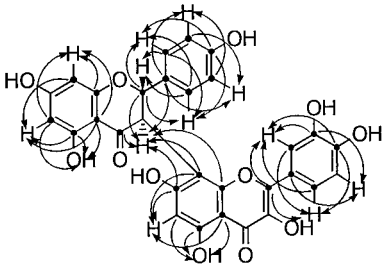


Figure 2. C−H long-range correlations in the HMBC spectrum of **1**.

indicated by the presence of a lone singlet proton at δ_H 6.17 (6.02) (H-6″) in the lower quercetin part. The carbon linked of the lone singlet has the significant C−H three-bond correlation between a hydrogen-bonded proton at δ_H 12.52 (12.48) (5″-OH) and the C−H three-bond correlation between one of the trans coupled protons at δ_H 4.82 (5.02) and the carbon signals at δ_C 154.20 (153.50) (C-8″a) and 163.81 (163.87) (C-7″). Consequently, the remaining two singlets at δ 5.95 (6.05) and 5.95 (5.96) were assignable to the protons of C-6 and -8 on the upper naringenin unit, respectively. Other C−H long-range correlations observed are shown by arrows in Figure 2. On the basis of these results, the structure of pancibiflavonol is proposed to be **1**. This is the second example of flavanone−flavonol biflavonoids in nature.[6]

Other biflavonoids isolated from the EtOAc-soluble portion of the EtOH extract were identified as garcinianin (**2**),[6] GB-2 (**3**),[7,8] GB-1 (**4**),[7,8] GB-2a (**5**),[9,10] GB-1a (**6**)[7,8,11], and GD-IV (**7**)[12] by comparisons of ^{1}H and ^{13}C NMR,[10] IR, UV, and MS spectra with spectroscopic data reported in the literature.[6−14]

Eight biflavonoids (**1**−**6**, **8**, and **9**) isolated from plants of the *Guttiferae* family were tested for antitumor-promoting activity in a short-term in vitro assay of TPA-induced EBV-EA activation in Raji cells. Talbotaflavone (**8**)[15,16] and morelloflavone (**9**)[15] were isolated from *Garcinia dulcis* (Roxb.) Kurz. collected in the Central Province of Papua New Guinea. Their inhibitory effect on activation of the virus and their effect on the viability of Raji cells are shown in Table 2.

All of the test compounds inhibited EBV activation, even at 1 × 10^2 mol ratio, and showed a significant inhibitory effect at high concentrations (1 × 10^3 mol ratio). However, all compounds showed weak cytotoxicity in assays of Raji cells, even at 1 × 10^3 mol ratio. Six of the biflavonoids, but not GB-2 (**3**) and GB-1a (**6**), showed weak inhibitory activity even at 1 × 10 mol ratio/TPA (4.3−11.3%). Among these compounds, garcinianin (**2**) and talbotaflavone (**8**) showed a significant inhibitory effect on EBV-EA activation (100% inhibition of activation at 1 × 10^3 mol ratio/TPA, and 10.4−11.3% inhibition of activation at 1 × 10 mol ratio/TPA).

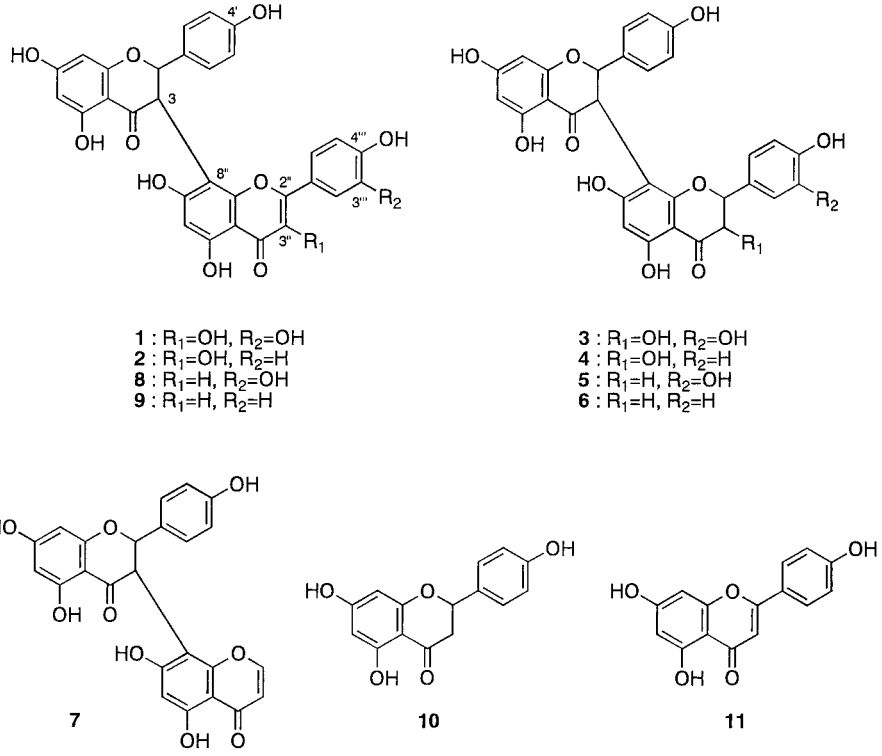
We wish to describe here the structure−activity relationships of the eight bisflavonoids isolated in this study. The characteristic structural feature of these bisflavonoids is the presence of a naringenin moiety as a common flavanone unit in the upper structural part, which is linked at C-3 with the lower flavonoid part at C-8″. These bisflavonoids can be classified into four types according to the presence

Table 2. Inhibitory Effects of Biflavonoids on TPA-Induced EBV-EA Activation[a]

	EBV-EA-positive cells (% viability)			
	compound concentration (mol ratio/32 pmol TPA)			
compound	1000	500	100	10
panciflavanone (**1**)	22.1 ± 0.9 (70)	50.2 ± 2.0 (>80)	75.0 ± 1.2 (>80)	94.4 ± 0.8 (>80)
garcinianin (**2**)	0.0 ± 0.3 (70)	31.4 ± 1.9 (>80)	69.2 ± 1.8 (>80)	89.4 ± 0.8 (>80)
GB-2 (**3**)	28.7 ± 1.0 (70)	63.9 ± 2.5 (>80)	90.3 ± 1.0 (>80)	100.0 ± 0.2 (>80)
GB-1 (**4**)	25.3 ± 0.4 (70)	61.0 ± 2.1 (>80)	88.2 ± 1.3 (>80)	95.7 ± 0.3 (>80)
GB-2a (**5**)	0.0 ± 0.6 (70)	42.5 ± 2.3 (>80)	71.3 ± 1.8 (>80)	90.6 ± 0.9 (>80)
GB-1a (**6**)	19.4 ± 0.7 (70)	48.2 ± 2.3 (>80)	82.9 ± 1.0 (>80)	100.0 ± 0.3 (>80)
talbotaflavone (**8**)	0.0 ± 0.3 (70)	30.0 ± 2.1 (>80)	69.5 ± 1.5 (>80)	88.7 ± 0.8 (>80)
morelloflavone (**9**)	0.0 ± 0.2 (70)	40.1 ± 2.5 (>80)	72.6 ± 1.2 (>80)	91.2 ± 0.9 (>80)

[a] Mole ratio/TPA (32 pmol = 20 ng/mL), 1000 mol ratio = 32 nmol, 500 mol ratio = 16 nmol, 100 mol ratio = 3.2 nmol, and 10 mol ratio = 0.32 nmol. Values are EBV-EA activation (%) ±S.D. in the presence of the test compound relative to the positive control (100%). Values in parentheses represent the viability (%) of Raji cells measured through trypan blue staining. At least 60% viability of Raji cells 2 days after treatment with compounds is required for accurate result.[17]

Scheme 1. Structures of Biflavonoids



(or absence) of a double bond at 2″ and a hydroxy group at 3″ in the lower part of the structure: (a) flavanone−flavonol (**1** and **2**), (b) flavanone−flavone (**8** and **9**), (c) flavanone−flavanol (**3** and **4**), and (d) flavanone−flavanone (**5** and **6**). Among these bisflavonoids, **2** and **8** showed stronger inhibitory activity than the others in assays of EBV-EA activation. Our results indicate that bisflavonoids having a double bond at C-2″ have more potent inhibitory activity than those lacking a double bond at C-2″, and the presence of an enolic hydroxy group at C-3″ is not essential for the activity.

In conclusion, it appears that garcinianin (**2**) and talbotaflavone (**8**) might be valuable antitumor promoters. Initiation−promotion tests examining the activity of these compounds in vivo are now in progress.

Experimental Section

General Experimental Procedures. Melting points were measured using a micromelting point hot-stage apparatus (Yanagimoto). ^{1}H and ^{13}C NMR, HMQC, and HMBC (J = 8 Hz) spectra were recorded using an A-400 or A-600 (JEOL) spectrometer, in Me_2CO-d_6. Chemical shifts are shown as δ values (ppm), with tetramethylsilane (TMS) as an internal reference. EIMS and HRMS analyses were performed using a Hitachi M-80 spectrometer equipped with a direct inlet system. FABMS and HRFABMS analyses were performed using a JMS−HX-110 spectrometer. UV spectra were recorded using a UVIDEC-610C double-beam spectrophotometer (JASCO) with the sample dissolved in MeOH; IR spectra were recorded using an IR-230 (JASCO) with the sample dissolved in $CHCl_3$; and optical rotations were recorded using a DIP-370 (JASCO) with the sample dissolved in $CHCl_3$ at 25 °C. Preparative TLC was performed using Kieselgel 60 F_{254} (Merck).

Extraction and Isolation. The plant material used in this study, *Calophyllum panciflorum* A. C. Smith (Guttiferae), was collected in the Central Province of Papua New Guinea. A voucher specimen was deposited at the herbarium of the University of Papua New Guinea. Dried stem bark (600 g) was extracted with EtOH under reflux. The EtOH extract was evaporated under reduced pressure resulting in an oily residue, which was fractionated with hexane, CH_2Cl_2, EtOAc, CH_2Cl_2−MeOH (3:1), and MeOH, successively. The EtOAc-soluble portion was further subjected to Si gel chromatography eluted with ^{i}Pr$_2$O−acetone (4:1, 7:3, 1:1, 3:7), acetone, CH_2Cl_2−MeOH (3:1), and MeOH, successively. Each fraction was

further subjected repeatedly to Si gel column chromatography and preparative TLC using appropriate combinations of hexane, benzene, $CHCl_3$, $^iPr_2O$, EtOAc, acetone, and MeOH as developing solvents to obtain the following components. From the $^iPr_2O$−acetone (4:1) eluate, a novel dibenzofuran and two new xanthones were isolated, along with four known xanthones, as described in our previous paper.[1] From the $^iPr_2O$−acetone (7:3) eluate; GD IV (**7**) (2.3 mg) and GB-1a (**6**) (84 mg) were obtained. From the $^iPr_2O$−acetone (1:1) eluate; pancibiflavonol (**1**) (30.1 mg), garcinianin (**2**) (12.6 mg), GB-1a (**6**) (29.1 mg), GB-1 (**4**) (328 mg), and GB-2a (**5**) (11.0 mg) were isolated. From the $^iPr_2O$−acetone (3:7) eluate, GB-2 (**3**) (815 mg) was obtained.

Pancibiflavonol (1): yellow powder. $[\alpha]_D = 0°$ (*c* 0.084, MeOH); CD (MeOH, 200−400 nm) no absorption; UV (MeOH) λ_{max} (log ϵ) 228 (4.41), 260 (4.20), 276 (4.21), 289 (4.20), 333 (4.00), 375 (4.02) nm; IR ($CHCl_3$) ν_{max} 3317 (br), 1647 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 342 (9), 314 (5), 287 (5), 270 (9), 126 (63); HRFabMS *m*/*z* $[M + H]^+$ 573.1048 ($C_{30}H_{21}O_{12}$ requires 573.1033).

Biological Assay. The inhibition of EBV-EA activation was assayed using Raji cells (EBV genome-carrying human lymphoblastoid cells; EBV nonproducer type), cultivated in 10% fetal bovine serum RPMI 1640 medium (Nakalai tesque). The indicator cells (Raji) (1×10^6 /ml) were suspended in 1 mL of medium containing *n*-butyric acid (4 mM, as inducer), 32 pmol of TPA (20 ng/mL in DMSO), and a known amount of test compound and were incubated at 37 °C in a CO_2 incubator. After 48 h, the cell suspensions were centrifuged at 1000 rpm for 10 min, and the supernatant was removed. The pelleted cells were suspended in 0.1 mL of phosphate buffer solution, and this cell suspension was used to prepare smears. The activated cells were detected by a conventional indirect immunofluorescence technique on the basis of their reactivity with high titer EBV-EA positive sera from nasopharyngeal carcinoma patients. In each assay, at least 500 cells were counted, and each of the experiments was repeated three times. The average extent of EA induction was compared with that in positive control experiments with *n*-butyric acid plus TPA, in which the extent of EA induction was ordinarily around 30%. Results were expressed as mean ±S.D. From previous observations, the measurement of extent of activation is not reliable if viability is less than 60%.[17]

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