Bioactive Pyranoxanthones from the Roots of *Calophyllum blancoi*

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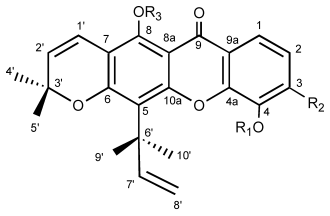
Phytochemical investigation of the roots of *Calophyllum blancoi* growing in Taiwan resulted in the isolation of three new pyranoxanthones, blancoxanthone (1), acetyl blancoxanthone (2) and 3-hydroxyblancoxanthone (3), in addition to two known pyranoxanthones, pyranojacaeubin (4) and caloxanthone (5). Structural characterization of the isolated compounds was determined by spectral analyses especially 2-D NMR. Biological study of the isolated xanthones revealed that blancoxanthone (1) exhibited significant anti-coronavirus activity.

Key words *Calophyllum blancoi*; Guttiferae; pyranoxanthones; blancoxanthone; antiviral activity

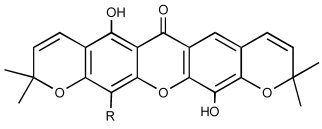
The genus *Calophyllum* (Guttiferae) is composed of about 130 species, mostly trees, confined to the warm humid tropics of the world.[1)] Some species are used in traditional medicine for their antimicrobial,[2)] analgesic,[3)] gastroprotective activities[4)] and many of them are acknowledged for the presence of pyranocoumarins which act as potent inhibitors of HIV-1 replication and cytopathicity.[5)] Besides, the genus is considered as a rich source of phenolic compounds and xanthone derivatives which possess antibacterial,[6,7)] antifungal,[1)] antiplatelet aggregation,[7)] immunomodulatory,[8)] cancer-chemopreventive[9)] as well as anti-HIV-1 virus[10)] and topoisomerase inhibitory activities.[11)] With the aim of discovering new bioactive compounds from the local flora, we have conducted a phytochemical study of the roots of *Calophyllum blancoi* Planchon growing in Taiwan. Three new pyranoxanthones, blancoxanthone (**1**), acetyl blancoxanthone (**2**) and 3-hydroxyblancoxanthone (**3**) in addition to two known pyranoxanthones, pyranojacaeubin (**4**) and caloxanthone (**5**) were isolated and characterized for the first time from an acetone extract of the roots of this species. The anti-coronavirus activity of the isolated compounds was evaluated.

Results and Discussion

The EI-MS of **1** revealed a molecular ion peak $[M]^+$ at *m/z* 378 corresponding to the molecular formula $C_{23}H_{22}O_5$, in accordance with the ^{13}C-NMR data. The UV spectrum revealed absorption at λ_{max} 240, 260, 285 and 350 nm suggestive of xanthones.[12)] The IR spectrum exhibited absorption bands for hydroxyl (3444 cm^{-1}), α,β-unsaturated chelated carbonyl (1649 cm^{-1}) and aromatic (1581 cm^{-1}) groups. In the NMR spectrum, correlations among the hydrogen and the chemical shifts of the corresponding carbons were established in the ^{1}H–^{1}H COSY and from HMQC spectra. The ^{1}H-NMR spectrum (Table 1) displayed signals for three mutually coupled aromatic protons at δ 7.70 (1H, dd, J=7.8, 1.3 Hz), 7.23 (1H, t, J=7.8 Hz), 7.27 (1H, dd, J=7.8, 1.3 Hz) as well as signals of a phenolic hydroxyl at δ 6.41 and a chelated hydroxyl at δ 13.44. The ^{13}C-NMR data (Table 2) demonstrated a carbonyl signal at δ 181.3 along with twelve aromatic carbon signals, five of them are oxygenated at δ 154.0, 145.3, 159.4 and 156.7 (double intensity). The fore-mentioned data were found to be in conformity with a penta-substituted xanthone.[12,13)] The chelated hydroxyl (δ 13.44) showed HMBC correlations to three quaternary carbon signals at δ 156.7 (C-8), 116.8 (C-8a) and 104.1 (C-7) indicating its attachment to C-8 of the xanthone skeleton. Furthermore, the HMBC correlations (Fig. 1) between H-3/C-4, C-4a; H-2/C-9a; H-1/C-3, C-9 indicated that the second hydroxyl group is attached to C-4 of the xanthone skeleton. The two-methyl singlet at δ 1.52 and the two endocyclic olefinic doublets at δ 6.78 and 5.63 (each 1H, d, J=9.9 Hz) were attributable to a 2,2-dimethylpyrene ring fused to a benzene ring. This was supported by the ^{13}C-NMR data which revealed two-methyl signal at δ 28.0, two olefinic methine carbons at δ 116.1 and 127.3 together with a quaternary carbon signal at δ 78.4. The two olefinic protons at δ 6.78 and 5.63 of the dimethylpyrene ring were correlated to a carbon signal at δ 104.1 (C-7) through ^2J and ^3J correlations, respectively, together with the correlation between H-1′ and C-8, indicating that the dimethyl pyrene ring was fused to the xanthone in a linear form.[12)] This was supported by HMBC correlations (Fig. 1) between H-5′/C-4′; H-4′/C-3′, C-2′; H-2′/C-1′; H-1′/C-6, C-8 confirming the attachment of the 2,2-dimethylpyrene moiety to C-6/C-7. On the other hand, the two-methyl singlet at δ 1.65 and the coupling pattern (*cis–trans*) between the olefinic proton signals at δ 6.70 (dd, J=17.2, 10.4 Hz, H-7′), δ 5.20 (d, J=17.2 Hz, H-8′a) and δ 5.05 (d, J=10.4 Hz, H-8′b) suggested the presence of a terminal olefin as a part of



1 R_1 = H, R_2 = H, R_3 = H
2 R_1 = Ac, R_2 = H, R_3 = H
3 R_1 = H, R_2 = OH, R_3 = H
6 R_1 = Ac, R_2 = H, R_3 = Ac



4 R = H
5 R = prenyl

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Table 1. ^{1}H-NMR Spectral Data ($CDCl_3$, 300 MHz) of Compounds **1**—**3**[a,b]

Position	1	2	3
1	7.70 dd (1.3, 7.8)	8.12 dd (1.1, 7.8)	7.59 d (8.7)
2	7.23 t (7.8)	7.34 t (7.8)	7.00 d (8.7)
3	7.27 dd (1.3, 7.8)	7.47 dd (1.1, 7.8)	
1′	6.78 d (9.9)	6.75 d (9.9)	6.69 d (9.9)
2′	5.63 d (9.9)	5.60 d (9.9)	5.70 d (9.9)
4′	1.52 s	1.47 s	1.49 s
5′	1.52 s	1.47 s	1.49 s
7′	6.70 dd (10.4, 17.2)	6.28 dd (10.3, 17.1)	6.50 dd (11.4, 17.5)
8′	5.05 d (10.4)	4.85 d (10.3)	4.87 d (11.4)
	5.20 d (17.2)	4.90 d (17.1)	5.04 d (17.5)
9′	1.65 s	1.69 s	1.74 s
10′	1.65 s	1.69 s	1.74 s
8-OH	13.44 s	13.49 s	13.91 s
4-OH	6.41 s		
4-OAc		2.41 s	

a) Assignment were determined using HMQC and HMBC techniques. *b*) Data in parentheses are coupling constants (*J*) in Hz.

1,1-dimethylallyl group.[14] The latter proton at δ 5.05 (H-8′b) showed HMBC correlations with the signals at δ 155.8 (C-7′), 41.3 (C-6′) while the six proton singlet at δ 1.65 (H-9′, 10′) showed correlations with quaternary carbon signals at δ 41.3 (C-6′) and 113.2 (C-5). The EI-MS exhibited the loss of a fragment ion corresponding to a dimethylallyl moiety at *m/z* 279 $[M-C_5H_9-2Me]^+$. Based on the previous discussion, the structure was designated as **1**, and it was named blancoxanthone.

Acetylation of **1** was carried out using Py/Ac_2O to afford the diacetate **6** with an empirical formula $C_{27}H_{26}O_7$ as deduced from its MS data. The ^{1}H-NMR spectrum of **6** exhibited a downfield shift of H-1 (+0.39 ppm), H-2 (+0.07 ppm), H-3 (+0.15 ppm), H-2′ (+0.31 ppm) as well as an upfield shift of H-1′ (−0.29 ppm). In the meantime, the ^{13}C-NMR spectrum showed a downfield shift of C-3 (+8.2 ppm), C-4 (+2.5 ppm) in addition to an upfield shift of C-9 (−6.2 ppm) that proved the acetylation at C-4 and C-8.

Compound **2** had a molecular ion peak at *m/z* 420 as deduced from its EI-MS data and ^{13}C-NMR, 42 units more than that of **1**. The ^{1}H-NMR data of **2** (Table 1) showed three aromatic signals attributed to a xanthone skeleton (δ 8.12, 7.34, 7.47) with a chelated hydroxyl at δ 13.49, a dimethylpyrene ring (δ 6.75, 5.60, 1.47) as well as a 1,1-dimethylallyl group (δ 6.28, 4.90, 4.85, 1.69). The ^{13}C-NMR data (Table 2) and HMBC data were similar to those of **1** indicating the linear arrangement of the dimethylpyrene ring and the attachment of 1,1-dimethylallyl group to C-5. The appearance of a carbonyl signal at δ_C 168.9 together with a methyl proton singlet at δ_H 2.41, correlated to a methyl signal at δ_C 21.2 in the HMQC spectrum, strongly suggested that the hydroxyl group at C-4 was acetylated. This was supported by the absence of a proton signal assignable to C_4–OH as well as the relative downfield shift of H-1 ($\Delta\delta$ 0.42), H-2 ($\Delta\delta$ 0.11) and H-3 ($\Delta\delta$ 0.20). In addition, the HMBC data revealed correlations between the acetate singlet at δ_H 2.41 and the carbonyl at δ_C 168.9; the aromatic proton at δ_H 7.34 (H-2) and the oxygenated quaternary carbon signal at δ_C 154.6 (C-4) as well as another carbon at δ_C 103.9 (C-9a). Alkaline hydrolysis of **2** yielded blancoxanthone (**1**). Thus the structure was deduced as **2**.

Table 2. ^{13}C-NMR Spectral Data ($CDCl_3$, 75 MHz) of Compounds **1**—**3**[a]

C-atom	1	2	3
1	116.0 d	123.8 d	116.2 d
2	124.2 d	123.4 d	112.8 d
3	119.6 d	127.8 d	151.0 s
4	154.0 s	154.6 s	132.8 s
4a	145.3 s	144.7 s	146.0 s
5	113.2 s	112.7 s	113.6 s
6	159.4 s	158.0 s	158.8 s
7	104.1 s	105.2 s	104.9 s
8	156.7 s	157.8 s	155.2 s
8a	116.8 s	118.6 s	113.4 s
9	181.3 s	180.1 s	181.0 s
9a	103.6 s	103.9 s	102.9 s
10a	156.7 s	157.5 s	156.5 s
1′	116.1 d	115.6 d	115.5 d
2′	127.3 d	127.7 d	127.2 d
3′	78.4 s	78.4 s	78.2 s
4′	28.0 q	28.0 q	27.2 q
5′	28.0 q	28.0 q	27.2 q
6′	41.3 s	41.6 s	41.0 s
7′	155.8 d	150.0 d	152.0 d
8′	104.1 t	108.5 t	106.5 t
9′	28.3 q	29.9 q	29.1 q
10′	28.3 q	29.9 q	29.1 q
4-OAc		168.9 s	
		21.2 q	

a) Assignments were determined using DEPT, HMQC and HMBC techniques.

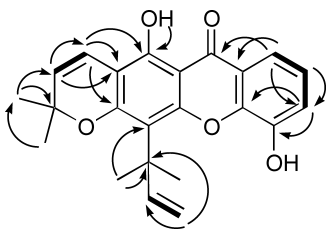

Fig. 1. Key HMBC (Arrows) and COSY (Bold Lines) Correlations of **1**

The ^{1}H-NMR of **3** (Table 1) indicated the presence of the 2,2-dimethylpyrene ring and 1,1-dimethylallyl group similar to those of **1**. The xanthone moiety was represented by only two *o*-coupled aromatic proton signals at δ 7.59 and 7.00 (each 1H, d, J=8.7 Hz) assigned to H-1 and H-2, respectively, instead of three signals in case of **1**. The relative upfield shift of the latter proton (H-2) suggested that it is *o*-located to a hydroxyl group. The ^{13}C-NMR of **3** (Table 2) proved a structure similar to **1**, but the xanthone skeleton possessed an additional oxygenated quaternary carbon signal at δ_C 151.0 and only two upfield aromatic CH signals at δ_C 116.2 and 112.8 were observed, indicating the extra-hydroxyl in the xanthone ring. This was confirmed by EI-MS that showed that the molecular ion peak of **3** at *m/z* 394. Comparing the ^{13}C-NMR data of **3** with that of **1** (Table 2), it was found that they are basically similar except for the significant downfield shift of C-3 (+31.4 ppm) suggesting hydroxylation at C-3, associated with the upfield shifts of both C-2 and C-4 due to *o*-effect of a hydroxyl group at C-3. The HMBC data showed correlations between the aromatic signal at δ_H 7.59 (H-1) and carbon signals at δ_C 181.0 (C-9) and 151.0 (C-3) while the relatively upfield aromatic signal at δ_H 7.00 (H-2)

was correlated to δ_C 102.9 (C-9a) and 132.8 (C-4). Other HMBC data were similar to those of **1**. Thus the structure of **3** was established 3-hydroxyblancoxanthone.

During the course of fractionation, pyranojacareubin **4**[15] and caloxanthone **5**[12] were isolated and identified through direct comparison of their data with the published values. The isolated pyranoxanthones were tested against coronavirus *in vitro*. The antiviral concentration of 50% effectiveness (EC_{50}) was defined as the concentration which achieved 50% inhibition of virus-induced cytopathic effects. The results showed that blancoxanthone (**1**) and pyranojacareubin (**4**) exhibited viral inhibition at 3 and 15 μg/ml respectively. This experiment suggested that compound **1** might be a potential candidate in the treatment of coronavirus infection.

Experimental

Optical rotations were recorded on a JASCO DIP-1000 polarimeter. IR and UV spectra were measured on Hitachi T-2001 and Hitachi U-3210 spectrophotometers, respectively. EI-MS and FAB-MS were measured on a VG Quattro 5022 mass spectrometer. The ^{1}H-, ^{13}C-NMR, COSY, HMQC, HMBC, and NOESY spectra were run on a Bruker Avance FT-300 spectrometer. The chemical shifts are given in δ (ppm) and coupling constants in Hz. Si gel 60 (Merck) was used for column chromatography, and precoated silica gel plates (Merck, Kieselgel 60 F-254, 1 mm) were used for preparative TLC.

Plant Material Roots of *Calophyllum blancoi* were collected from Taiwan, in June 2002, and was identified by one of the authors (Y. C. Shen). A voucher specimen was deposited in the Institute of Marine Resources, National Sun Yat-sen University.

Extraction and Isolation The dried roots of *Calophyllum blancoi* (2 kg) were chopped and extracted three times with acetone (3×2 l) then the extract was concentrated under vacuum to yield a crude extract (73 g). The acetone extract was partitioned between EtOAc/water (1 : 1) to furnish ethyl acetate extract (31 g) which was subjected to flash column (Si gel, 350 g) using hexane to hexane/EtOAc (1 : 1) for elution to yield fractions A and B. Fraction A (1.15 g) was further separated on column packed with Sephadex LH-20 using CH_2Cl_2/MeOH (2 : 1) as a solvent to give **1** (10 mg) and fraction C. Fraction B (201 mg) was also subjected to flash column (Si gel, 350 g) using hexane/EtOAc (6 : 1) to (1 : 1) for elution followed separation on Sephadex LH-20 using MeOH to yield **2** (2.7 mg) and **3** (6 mg). Fraction C was further separated on silica gel preparative TLC using hexane/CH_2Cl_2/CH_3CN (10 : 20 : 1) to afford **4** (21 mg) and **5** (29 mg).

Blancoxanthone (**1**): Yellowish powder; UV (MeOH) λ_{max} 240, 260, 285, 350 nm; IR (CH_2Cl_2) ν_{max} 3444 (OH), 2926, 2854 (CH), 1649 (conj. C=O), 1581, 1010, 968, 887, 737; FAB-MS *m/z* 379 $[M+H]^+$; EI-MS *m/z* 378 $[M]^+$, 361 $[M-OH]^+$, 333 $[M-CO-OH]^+$, 279 $[M-C_5H_9-2Me]^+$, 77 (benzene ring), 69 $[C_5H_9]^+$, $C_{23}H_{22}O_5$; ^{1}H-NMR (300 MHz, $CDCl_3$): Table 1; ^{13}C-NMR (75 MHz, $CDCl_3$): Table 2.

Acetylation of **1**: **1** (6 mg) was treated with Ac_2O/Py (1 : 1) and left at room temperature for 24 h. After work out the product gave 4 mg of blancoxanthone-4,8-di-*O*-acetate **6**. IR (CH_2Cl_2) ν_{max} 2973 (CH), 1749 (C=O of acetate), 1653 (conj. C=O), 1595, 1262, 1191, 1083, 765; FAB-MS *m/z* 463 $[M+H]^+$, EI-MS *m/z* (rel. int.) 462 $[M]^+$, 445 $[M-CH_3]^+$ (100), 405 $[M-CH_3CO-CH_3]^+$, $C_{27}H_{26}O_7$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.09 (1H, dd, *J*=7.8, 1.3 Hz, H-1), 7.30 (1H, t, *J*=7.8 Hz, H-2), 7.42 (1H, d, *J*=7.8 Hz, H-3), 6.49 (1H, d, *J*=9.9 Hz, H-1′), 5.74 (1H, d, *J*=9.9 Hz, H-2′), 1.48 (6H, s, H-4′, 5′), 6.32 (1H, dd, *J*=17.2, 10.4 Hz, H-7′), 4.91 (1H, d, *J*=17.2 Hz, Ha-8′), 4.86 (1H, d, *J*=10.4 Hz, Hb-8′), 1.72 (6H, s, H-9′, 10′), 2.51 and 2.41 (each 3H, s, 2×Ac); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 123.8 (C-1), 123.4 (C-2), 127.8 (C-3), 156.5 (C-4), 144.5 (C-4a), 112.7 (C-5), 157.5 (C-6), 109.3 (C-7), 157.6 (C-8), 118.6 (C-8a), 175.1 (C-9), 104.3 (C-9a), 156.4 (C-10a), 115.7 (C-1′), 127.8 (C-2′), 78.4 (C-3′), 28.0 (C-4′, C-5′), 41.6 (C-6′), 150.0 (C-7′), 108.5 (C-8′), 29.9 (C-9′, C-10′), 21.2 (2×Ac), 169.0 (CH_3-<u>C</u>O), 169.9 (CH_3-<u>C</u>O).

Acetylblancoxanthone (**2**): Yellowish powder; IR (CH_2Cl_2) ν_{max} 3443 (OH), 2925, 2853 (CH), 1731 (acetate), 1649 (conj. C=O), 1624 (double bond), 1582, 1010, 967, 887, 737; FAB-MS *m/z* 421 $[M+H]^+$; EI-MS *m/z* (rel. int.) 420 $[M]^+$, 405 $[M-Me]^+$ (100), 392 $[M-CO]^+$, 390 $[M-2Me]^+$, 377 $[M-CH_3CO]^+$, 362 $[M-CH_3CO-Me]^+$, 77 (benzene ring), 69 $[C_5H_9]^+$, $C_{25}H_{24}O_6$; ^{1}H-NMR (300 MHz, $CDCl_3$): Table 1; ^{13}C-NMR (75 MHz, $CDCl_3$): Table 2.

Alkaline Hydrolysis of Acetylblancoxanthone (**2**): Hydrolysis (0.5 M NaOH, 0.5 ml; room temp.) of **2** (3 mg) and usual work-up yielded a product identical with compound **1** (1 mg).

3-Hydroxyblancoxanthone (**3**): Yellowish powder; FAB-MS *m/z* 394 $[M]^+$, 393 $[M-H]^+$; EI-MS *m/z* 394 $[M]^+$, 364 $[M-2Me]^+$, 351 $[M-CO-Me]^+$, 336 $[M-CO-2Me]^+$, 325 $[M-C_5H_9]^+$, 295 $[M-C_5H_9-2Me]^+$, 77 (benzene ring), 69 $[C_5H_9]^+$, $C_{23}H_{22}O_6$; ^{1}H-NMR (300 MHz, $CDCl_3$): Table 1; ^{13}C-NMR (75 MHz, $CDCl_3$): Table 2.

Viruses and Cells Human lung fibroblast (MRC-5) was used to provide target cells for virus infection in the XTT assay. It was grown in DMEM medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin G, 100 mg/l streptomycin and 0.25 mg/l amphotericin B. In the antiviral assay, the medium was supplemented with 3% FCS and the above mentioned antibiotics. The strain of Human coronavirus 229E (HCoV 229E) was obtained from American Type Culture Collection (ATCC), Rockville, U.S.A. Virus titers were determined by cytopathic effect in MRC-5 cells and were expressed as 50% tissue culture infective dose ($TCID_{50}$) per ml.

Cytotoxicity The MRC-5 cells were seeded onto a 96-well plate at a concentration of 1.0×10^5 cells per ml and a volume of 90 μl per well. Different concentrations of compound or DMSO (negative control) were applied to culture wells in triplicate. After incubation at 37 °C with 5% CO_2 for 4 d, a mixture of 0.1 ml PMS and 1 mg/ml XTT was added to each well with a volume of 50 μl for 3 h incubation. The absorbances were determined with an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 492 nm and a reference wavelength of 690 nm. Data were calculated as percentage of inhibition using the following formula: Inhibition %=[100−(At/As)×100]%. At and As refer to the absorbance of the test substances and the solvent control, respectively.

Antiviral Assay Using XTT Method[16] The antiviral activity of tested compounds against HCoV 229E was evaluated by the XTT method. MRC-5 cells were seeded onto 96-well plates with a concentration of 1.0×10^5 cells/ml and a volume of 70 μl per well. After incubation at 34 °C with 5% CO_2 over night, 20 μl test virus was added and incubated for another 2 h. Different concentrations of test substances, negative control or positive control (actinomycin D, IC_{50} 0.02 μg/ml) were then added to culture wells in triplicate. After incubation at 34 °C with 5% CO_2 for 4 d, the XTT test was carried out as previously described. The percent protection was calculated as (Atv−Acv)/(Acd−Acv)×100%. Atv indicates the absorbance of the test compounds with virus infected cells. Acv and Acd indicates the absorbance of the virus control and the absorbance of the cell control, respectively. The number of virus used in each experiment was based on infected target cells of 100 $TCID_{50}$ of virus to produce 50% XTT formazan products as in uninfected control cells.

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