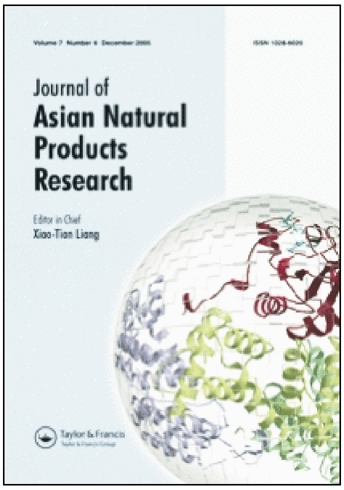

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

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To cite this Article Xiao, Qi , Zeng, Yan-Bo , Mei, Wen-Li , Zhao, You-Xing , Deng, Yuan-Yuan and Dai, Hao-Fu(2008) 'Cytotoxic prenylated xanthones from *Calophyllum inophyllum*', Journal of Asian Natural Products Research, 10: 10, 993 — 997

To link to this Article: DOI: 10.1080/10519990802240387
URL: http://dx.doi.org/10.1080/10519990802240387

Journal of Asian Natural Products Research
Vol. 10, No. 10, October 2008, 993–997

Cytotoxic prenylated xanthones from *Calophyllum inophyllum*

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(*Received 29 February 2008; final version received 5 May 2008*)

A new prenylated xanthone (**1**), named caloxanthone N, together with two known constituents, gerontoxanthone C (**2**) and 2-hydroxyxanthone (**3**), was isolated from the ethanolic extract of the twigs of *Calophyllum inophyllum*. Their structures were completely elucidated using a combination of 1D, 2D NMR techniques (COSY, HMQC, HMBC, and ROESY) and HR-ESI-MS analyses. Compounds **1** and **2** exhibited cytotoxicity against chronic myelogenous leukemia cell line (K562) with IC_{50} values of 7.2 and 6.3 $\mu g\,ml^{-1}$, respectively.

Keywords: *Calophyllum inophyllum*; cytotoxic activity; prenylated xanthone; caloxanthone N

1. Introduction

Calophyllum inophyllum Linn. (Clusiaceae) is an evergreen shrub widely distributed in tropical areas. In China, *C. inophyllum* is distributed in Hainan and Taiwan provinces, and is used in traditional Chinese folk medicine for the treatment of eye diseases, wounds, rheumatisms, and inflammations [1,2]. Since inophyllums B and P isolated by Ptail [3] showed strong activity against human immunodeficiency virus type 1, much attention has been paid to studies on the chemical components of *C. inophyllum*. These studies have revealed that, besides pyranocoumarins [4–9], *C. inophyllum* is also a rich source of xanthones [4,5,8], triterpenes [9,10], and flavonoids [11]. In our on-going search for cytotoxic agents from tropical medicinal plants in Hainan, the ethanolic extract of the twigs of *C. inophyllum* showed inhibitory activity toward human chronic myelogenous leukemia cell line (K562). Bioassay-guided fractionation led to the isolation of a new and two known xanthones, and their structures were unambiguously elucidated as caloxanthone N (**1**), gerontoxanthone C (**2**), and 2-hydroxyanthone (**3**) (Figure 1) by extensive spectroscopic analysis. Compounds **1** and **2** showed significant cytotoxicity against chronic myelogenous leukemia cell line (K562) *in vitro* by MTT method. In this paper, we report the isolation and identification of the cytotoxic principles from *C. inophyllum*.

2. Results and discussion

Compound **1** was obtained as yellow powder and reacted positively to the Gibbs and $FeCl_3$ reagent indicating the presence of a phenolic group. The $[M + Na]^+$ ion peak at *m/z* 449.1576 in HR-ESI-MS spectrum corresponded to the molecular formula $C_{24}H_{26}O_7$. This formula can also be validated through ^{1}H-NMR, ^{13}C-NMR, and DEPT spectra. The IR spectrum displayed free hydroxyl

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ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10519990802240387
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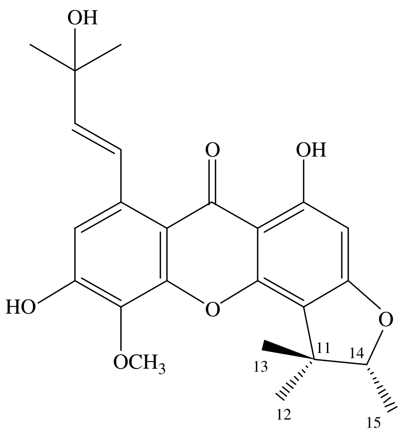

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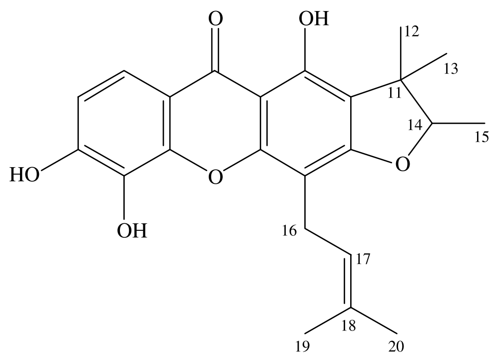

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Figure 1. Structures of compounds **1** and **2**.

(3473 cm^{-1}), chelated hydroxyl (3244 cm^{-1}), conjugated carbonyl (1648 cm^{-1}), and aromatic ring (1605, 1565, 1505, 1479 cm^{-1}) absorptions. These data, together with those obtained from the UV spectrum [λ (MeOH) 238, 249, 255, 280 sh, and 331 nm] were consistent with the presence of a xanthone skeleton [4,5]. In the ^{1}H-NMR spectrum (Table 1), a chelated hydroxyl group (δ 13.66), two aromatic proton singlets (δ 7.02 and 6.12), and one methoxyl (δ 3.98) were observed. The ^{1}H-NMR spectrum of **1** also

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data of **1** (acetone-d_6) and **2** (CD_3OD).

	1		**2**	
No.	δ_C	δ_H (Hz)	δ_C	δ_H (Hz)
1	165.5 (s)	13.66 (1H, s)	161.9 (s)	
2	94.2 (d)	6.12 (1H, s)	113.2 (s)	
3	166.7 (s)		165.4 (s)	
4	113.2 (s)		107.7 (s)	
5	135.2 (s)		134.1 (s)	
6	156.3 (s)		153.0 (s)	
7	112.8 (d)	7.02 (1H, s)	113.7 (d)	6.85 (1H, d, 8.7)
8	138.2 (s)		117.6 (d)	7.56 (1H, d, 8.7)
9	183.1 (s)		182.0 (s)	
4a	152.9 (s)		152.5 (s)	
8a	112.0 (s)		115.2 (s)	
9a	104.4 (s)		103.8 (s)	
10a	152.4 (s)		147.9 (s)	
11	44.5 (s)		45.3 (s)	
12	21.8 (q)	1.33 (3H, s)	21.5 (q)	1.76 (3H, s)
13	25.9 (q)	1.62 (3H, s)	25.9 (q)	1.32 (3H, s)
14	91.7 (d)	4.56 (1H, q, 6.5)	92.0 (d)	4.50 (1H, q, 6.5)
15	14.5 (q)	1.40 (3H, d, 6.4)	14.7 (q)	1.40 (3H, d, 6.6)
16	126.8 (d)	7.89 (1H, d, 15.9)	22.5 (t)	3.23 (2H, d, 7.3)
17	142.3 (d)	6.20 (1H, d, 15.9)	123.1 (d)	5.23 (1H, t, 7.3)
18	70.8 (s)		132.4 (s)	
19	30.6 (q)	1.40 (3H, s)	26.1 (q)	1.65 (3H, s)
20	30.6 (q)	1.40 (3H, s)	17.9 (q)	1.61 (3H, s)
5-OMe	62.0 (q)	3.98 (3H, s)		

showed two methyl singlets (δ 1.40, 6H) and two *trans*-olefinic protons (δ 7.89 and 6.20, each 1H, d, $J = 15.9$ Hz), which suggested the presence of a γ,γ-dimethylallyl chain. Two methyl singlets (δ 1.33 and 1.62, each 3H), a methyl doublet (δ 1.40, 3H, d, $J = 6.4$ Hz), and one-proton quartet (δ 4.56, 1H, q, $J = 6.5$ Hz) suggested the presence of an α,α,β-trimethyl-dihydrofuran ring. A combination of the $^1H-^1H$ COSY and HSQC experiments permitted the assignment of all of the protonated carbons (Table 1). It remained to establish the positions of the substituents on the xanthone skeleton. In the HMBC spectrum (Figure 2), the proton of chelated hydroxyl group (δ 13.66) was correlated to three carbons C-9a (δ 104.4), C-2 (δ 94.2), and C-1 (δ 165.5), which suggested that the chelated hydroxyl group was located at C-1. Aromatic carbons with an oxygen function were observed at C-1 (δ 165.5), C-3 (δ 166.7), and C-4a (δ 152.9) in the ^{13}C NMR spectrum, which indicated that this aromatic ring was a phloroglucinol ring [12]. Therefore, the α,α,β-trimethyldihydrofuran ring was clearly fused at C-4 through an oxygen at C-3 position. This result was further confirmed by the long-range correlations of H-12 (δ 1.33) and H-13 (δ 1.62) with C-4 (δ 113.2) and H-14 (δ 4.56) with C-3 (δ 166.7) in the HMBC spectrum. The methoxyl group was

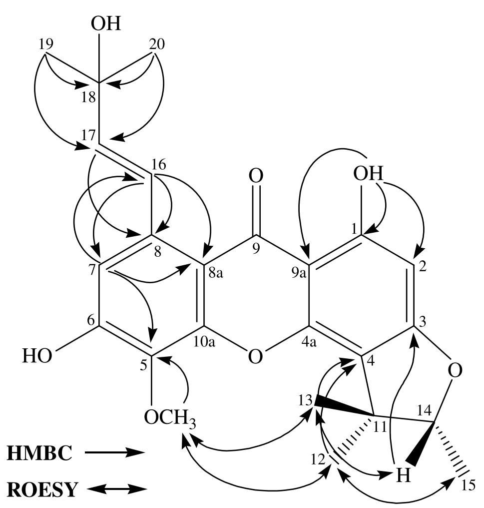

Figure 2. Key HMBC and ROESY correlations of compound **1**.

deduced to be located at C-5 position by ROESY experiment revealing the cross-peaks from the methoxyl (δ 3.98) to H-12 (δ 1.33) and H-13 (δ 1.62). The positions of the γ,γ-dimethylallyl moiety and the remaining phenolic hydroxyl group were established as follows. In the HMBC spectrum (Figure 2), one of the olefinic proton H-16 (δ 7.89) was correlated to three aromatic carbons C-8a (δ 112.0), C-7 (δ 112.8), and C-8 (δ 138.2). The resonance of C-8 (δ 138.2) also gave cross-peak with the other olefinic proton H-17 (δ 6.20). These results demonstrated clearly that the γ,γ-dimethylallyl moiety was located at C-8. The cross-peaks from the aromatic proton (δ 7.02) to C-8a (δ 112.0), C-16 (δ 126.8), and C-5 (δ 135.2) in the HMBC spectrum indicated that the aromatic proton (δ 7.02) was assigned to be at C-7 position. The downfield shifts of C-6 (δ 156.3) and C-18 (δ 70.8) revealed that these two carbons should be substituted by hydroxyl group. The ROESY experiment showed that H-14 (δ 4.56) correlated with H-13 (δ 1.62), while not with H-12 (δ 1.33). This result indicated that when H-14 and CH_3-13 were assigned β-orientation, CH_3-12 and CH_3-15 were in α-orientation. On the basis of the above results, the structure of compound **1** was thus elucidated as **1**, named caloxanthone N.

The structures of compounds **2** and **3** were identified to be gerontoxanthone C (**2**) [13] and 2-hydroxy-xanthone (**3**) [14] by comparing their corresponding spectroscopic properties (NMR and MS) with the values reported in the literatures.

Compounds **1**–**3** were evaluated for their cytotoxic activity against chronic myelogenous leukemia cell line (K562) using the MTT method. Compounds **1** and **2** showed cytotoxic activity with IC_{50} values of 7.2 and 6.3 $\mu g\,ml^{-1}$, respectively, while compound **3** was inactive ($>20\,\mu g\,ml^{-1}$).

3. Experimental

3.1 General experimental procedures

Melting point determinations were obtained on a Kofler hot stage apparatus, and are uncorrected. Optical rotation was measured at room temperature using a J-20C polarimeter. The UV spectra were measured on a Shimadzu UV-210A spectrometer. The IR spectra were measured on a Bio-Tad FTS 135 instrument, as KBr pellets. The ^{1}H, ^{13}C, and 2D NMR spectra were run on Bruker AM-400 and DRX-500 spectrometers, using TMS as an internal standard. The FAB-MS spectra were measured with a VG Auto Spectrometer. Column chromatography was performed with silica gel (Marine Chemical Industry Factory, Qingdao, China) and Sephadex LH-20 (Merck, Darmstadt, Germany). TLC was performed with silica gel 60 F254 (Merck) and developed by spraying with 10% H_2SO_4 followed by heating.

3.2 Plant material

The twigs of *Calophyllum inophyllum* L. used in this research were collected in Wenchang county, Hainan province, China, in May 2006, and authenticated by associate researcher Zheng-Fu Dai (Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou, China). The voucher specimen (No. 20060508) is deposited in the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences.

3.3 Extraction and isolation

The dried and crushed twigs of *C. inophyllum* (19.9 kg) were extracted with 95% EtOH thrice at room temperature. After the removal of EtOH by evaporation, the EtOH extract was suspended in water (6.0 l) and successively partitioned with petroleum ether to give Petro-soluble extract (290.0 g) and an aqueous residue. The aqueous residue was subjected to chromatography on D-101 resin with H_2O, a gradient of 50, 95, and 100% MeOH as the eluents, to afford four fractions. The 50, 95, and 100% MeOH eluents were collected and concentrated *in vacuo* to yield 65.0, 245.0, and 70.0 g residue, respectively.

The Petro-soluble extract (290.0 g) was applied to a silica gel (200–300 mesh) column packed in $CHCl_3$. The column was then eluted in gradient elution with $CHCl_3$–acetone to afford 16 fractions. The active fraction (fraction 16, 44.0 g) was then subjected to repeated column chromatography over silica gel using Pet–EtOAc as eluent and further separated by column chromatography over Sephadex LH-20 using 95% EtOH as eluent to afford **1** (24.7 mg). The 95% MeOH fraction (245.0 g) was subjected to vacuum liquid chromatography over silica gel, eluting with gradient elution $CHCl_3$–MeOH to afford 10 fractions. The active fraction (fraction 3, 5.7 g) was then subjected to repeated column chromatography over silica gel using Pet–acetone as eluent and further separated by column chromatography over Sephadex LH-20 using 95% EtOH as eluent to afford **2** (15.6 mg) and **3** (10.5 mg).

3.3.1 *Caloxanthone N (**1**)*

Yellow needles from EtOH (95%), 24.7 mg, mp 179–181°C, $[\alpha]_D^{17} + 59.32$ (*c* 1.2, acetone); UV (MeOH) λ_{max} ($\log \varepsilon$): 238 (2.06), 249 (2.05), 255 (2.60), 280 (sh) and 331 (2.32) nm; IR (KBr) ν_{max}: 3473, 3244, 2976, 1648, 1605, 1565, 1505, 1479, 1411, 1380, 1272, 1129, 1062, 1002, 960, 868, 823 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR spectral data: see Table 1; HR-FAB-MS: *m/z* 449.1576 $[M + Na]^+$ (calcd for $C_{24}H_{26}O_7Na$, 449.1571).

3.3.2 *Gerontoxanthone C (**2**)*

Pale yellow needles from EtOH (95%), 15.6 mg, ^{1}H-NMR and ^{13}C-NMR spectral data were consistent with the literature [13].

3.3.3 *2-Hydroxyxanthone (**3**)*

Yellow needles from EtOH (95%), 10.5 mg. ^{1}H-NMR and ^{13}C-NMR spectral data were consistent with the literature [14].

3.4 Bioassay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed according to the previously reported method [15]. The inhibition rates (IR%) were calculated using OD mean values from $IR\% = (OD_{control} - OD_{sample})/OD_{control}$. The IC_{50} value, which was defined as the concentration of sample needed to reduce a 50% of absorbance relative to the vehicle-treated control, was determined using the Bliss method. The same experiment was repeated independently thrice to obtain a mean IC_{50} and its SD.

Acknowledgements

This research was financially supported by National Basic Research Program of China (2007CB116306) and the Science and Technology Foundation of Chinese Academy of Agricultural Sciences (RKY0421).

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