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Abstract

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Isolation, linobiflavonoid, novel, biflavonoid, from, *Linostoma pauciflorum*, Griff, CMMB

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MANUSCRIPT

Isolation of Linobiflavonoid, a Novel Biflavonoid from *Linostoma pauciflorum* Griff.

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AbSTRACT

A novel biflavonoid, that we have named linobiflavonoid, and the known biscoumarin ether, daphnoretin, were isolated from the root extracts of *Linostoma pauciflorum* Griff. The structure of linobiflavonoid was determined from interpretation of its NMR spectroscopic data and from a comparison of this data with those of known biflavonoids and biflavones. The known flavones, 5,4'-dihydroxy-7,3',5'-trimethoxyflavone and 5,4'-dihydroxy-7-methoxyflavone along with stigmasterol were isolated from the vines of the same plant. 4'-Dihydroxy-7,3',5'-trimethoxyflavone was active against mycobacterium tuberculosis (MIC 3.13 μ M) and KB-oral cavity cancer (IC₅₀ 17.41 μ M).

Keywords: Biflavonoid, linobiflavonoid, *Linostoma pauciflorum* Griff., 5,4'-dihydroxy-7,3',5'-trimethoxyflavone, 5,4'-dihydroxy-7-methoxyflavone, daphnoretin, stigmasterol.

1. Introduction

Linostoma pauciflorum Griff. (Thymelaeaceae) is a shrub or small tree which is widespread in the southern part of Thailand. This plant has several uses. For example, a dried

mixture of the ground root and urine is wrapped in the leaves of *Senna alata* (L.) Roxb. and then smoked like a cigarette to relieve the symptoms of nasal polyps. The whole plant is chopped and scattered over ponds to poison fish while the water extracts of the plant material are used as an insecticide and sprayed on plants (Upho, 2005). In this paper we report the isolation and structure determination of a novel biflavonoid **1**, that we have named linobiflavonoid, and the known biscoumarin ether, daphnoretin, from the root extracts of *L. pauciflorum* G. The known flavones, and 5,4'-dihydroxy-7-methoxyflavone along with stigmasterol were isolated from the vines of the same plant. The activities of 5,4'-dihydroxy-7,3',5'-trimethoxyflavone against mycobacterium tuberculosis and KB-oral cavity cancer are also reported.

Figure1.

2. Results and discussion

The vines and the roots of *L. pauciflorum* G. were collected from Khounkanun district, Phuttalung province, Thailand. The MeOH extract of the dried roots was concentrated *in vacuo* and the resulting residue was subjected to successive separations by column chromatography. This resulted in the isolation of pure samples of the novel biflavonoid **1**, that we have named linobiflavonoid based on its botanical origins, and the known natural product daphnoretin (Cordell, 1984; Zhang et al., 2008), . Using a similar protocol the MeOH extracts of the vines yielded pure samples of the known flavones,⁴ **2** and **3** and stigmasterol **5**.⁵ Compounds **2-5** were identified by comparisons of their NMR and MS data with those reported in the literature.

Compound **1**, was a brown amorphous powder, its molecular formula was determined to be C₃₀H₂₀O₁₀ from its HRMS (CI⁺) spectrum. In the ¹H-NMR spectrum of compound **1**, two *para*-disubstituted aromatic rings, ring A' ($J_{2'',3''}$ and $J_{5'',6''} = 8.4$ Hz) and ring C ($J_{2',3'}$ and $J_{5',6'} = 8.6$ Hz) and two tetrasubstituted aromatic rings, rings A and C', were identified. In the latter two rings the two attached protons had a *meta* relationship ($J_{6,8} = 1.9$ Hz and $J_{5',7'} = 1.2$ Hz). Resonances for two methine groups (broad singlets at $\delta = 5.70$ and 3.50) and a pair of diastereotopic benzylic methylene protons (CH_aH_b, $\delta = 3.30$ and 3.17, $J_{ab} = 14.0$ Hz) represented the remaining protons. **WERE THE OH RESONANCES SEEN IN CDCI3??**

HSQC experiments allowed correlations between these protons and their attached carbons. The $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectroscopic data for rings A, B, C were similar to those of the parent flavone naringenin,⁶ and those of the mono-flavone moiety of the 3,3'-biflavanone isoikokianin.⁷ While the $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectroscopic data of the 2-substituted-2-benzylbenzofuranyl moiety of **1** (rings A', B' and C' and CH_aH_b) matched closely with those of this same moiety present in the biflavonoids isolated from *Berchemia zeyheri*.⁸ HMBC experiments showed correlations between δ 5.70 (H-2) and δ 90.7 (C-2'') and δ 3.49 (H-3) and δ 90.7 (C-2'') which were used to assign the C-3/C-2'' linkage between the flavone and 2-benzylbenzofuranyl moieties. The relative stereochemistry at the C-2/C-3 position was established to be *cis* on the basis of the very small vicinal coupling constant for $J_{2,3}$ (both H-2 and H-3 were broad singlets). In the literature $J_{2,3}$ is about 0 Hz for *cis* flavones^{9,10} and 5.7-12.2 Hz for *trans* flavones.¹¹⁻¹⁵ The configuration of C-2'' relative to that of H-2 and H-3 could not be determined.

The activities of the flavone **2** against mycobacterium tuberculosis and KB-oral cavity cancer were determined. Flavone **2** had a MIC value of 3.13 μM against mycobacterium tuberculosis and an IC_{50} value of 17.41 μM against KB-oral cavity cancer.

In conclusion, we have isolated a novel biflavonoid **1**, with a C-3/C-2'' linkage between a 3-flavonyl and a 2-(2-benzyl)benzofuranyl moiety, and two known flavones **2** and **3** together with daphnoretin **4** and stigmasterol **5** from the vines and roots of *Linostoma pauciflorum* Griff. (Thymelaeaceae). Compounds 3-5 have been isolated from *Daphne* and *Wikstroemia* plants that also belong to the family Thymelaeaceae.^{4,16,17}

General experimental

TLC was performed using aluminium backed Merck F₂₅₄ sorbent silica gel. Compounds were detected under a 254 nm ultraviolet lamp. Purification of compounds by flash column chromatography (FCC) was achieved using Merck flash silica gel (40-63 μm). Optical rotations were measured using a 1 cm cell, in a Jasco DIP-370 digital polarimeter. Eight to ten measurements were taken and the average was used to calculate the specific rotation. Low resolution mass spectra were obtained either on a Shimadzu GC mass spectrometer (EI and CI) or a Waters LCZ single quadropole (ESI). High-resolution mass spectra were obtained either on a VG Autospec mass spectrometer (EI and CI) or a Waters QTOF (ESI). HRMS (exact masses) were used in lieu of elemental analysis and TLC analysis and ^1H and ^{13}C NMR spectroscopy were used as criteria for purity. ^1H and ^{13}C NMR spectra were recorded

on Varian Unity-300 (300 MHz ^1H , 75 MHz ^{13}C) or a Bruker (400 MHz ^1H , 100 MHz ^{13}C) or a Varian INOVA-500 (500 MHz ^1H , 125 MHz ^{13}C) spectrometer in deuteriochloroform (CDCl_3), unless otherwise specified. NMR assignments were based on COSY, DEPT, HSQC and HMBC experiments.

Extraction and Isolation

Plant materials. The vines and the roots of *Linostoma pauciflorum* Griff. were collected in May 2009 from Khounkanun district, Phuttalung province, Thailand, and identified as a *Linostoma pauciflorum* Griff. by Assist. Prof. Ubonwan Upho (Taksin University, Thailand). A voucher specimen has been deposited in the Forest Herbarium: BKF, Bangkok, Thailand (BKF. 125572).

Roots: The air-dried roots (1.27 Kg) were chopped into small pieces and extracted with 10 L MeOH. The MeOH extracts was concentrated *in vacuo*. The residue was treated with CH_2Cl_2 and the soluble part was then concentrated *in vacuo* to give a residue (10.90 g), which was subjected to column chromatography (CC) on silica gel. The column was eluted successively using gradient elution from 5: 95 to 100:0 of EtOAc/hexane followed by gradient elution with MeOH/EtOAc from 5: 95 to 100:0 and finally elution with CH_2Cl_2 :MeOH:H $_2\text{O}$ (6:4:1) to obtain 10 fractions. Fraction 9 (2.67 g) was a brown solid. After CH_2Cl_2 was added, the insoluble part was collected by filtration to give daphnoretin (**4**) (157.9 mg) as a white powder. The mother liquor was concentrated *in vacuo* to give a residue (2.50 g), which was re-chromatographed on silica gel with gradient elution with CH_2Cl_2 /MeOH/H $_2\text{O}$ from 35:3:1 to 10:3:1 of and then on silica gel 60 RP-18 using H $_2\text{O}$ /MeOH (1:5) to obtain compound **1** (7.4 mg) as a brown solid. **1**: $[\alpha]_{\text{D}}^{25}$ -108.7 (*c* 0.47, acetone). MS (CI +ve) *m/z* 565 ($\text{M}+\text{Na}^+$, 100%). HRMS (CI +ve) calculated for $\text{C}_{30}\text{H}_{22}\text{O}_{10}\text{Na}$ ($\text{M}+\text{Na}^+$) 565.1111, found 565.1101. ^1H and ^{13}C -NMR data for **1** are shown in Table 1.

Vines: The air-dried vines (1.27 Kg) were chopped into small pieces and extracted with 10 L MeOH. The MeOH extracts was concentrated *in vacuo*. The residue was treated with CH_2Cl_2 and the soluble part was then concentrated *in vacuo* to give a residue (21.00 g), which was subjected to quick column chromatography (QCC) on silica gel using gradient elution with EtOAc/petrol from 5:95 to 100:0, and then with MeOH/EtOAc from 5:95 to 100:0 to obtain 9 fractions. Fraction 3 (2.20 g) was a pale yellow solid, which was recrystallized from CHCl_3 /MeOH to obtain stigmasterol (**5**) (120.0 mg) as white shining crystals. Fraction 5

(1.15 g) was a brown gum, which was subjected to CC on silica gel eluted first with CH₂Cl₂ followed by gradient elution with EtOAc/CH₂Cl₂ (1:4) to obtain 5,4'-dihydroxy-7,3',5'-trimethoxyflavone (**2**) (22.7 mg). The above CH₂Cl₂ elutes (37.4 mg) were further separated by PTLC with 20% EtOAc/petrol (1:5) to afford 5,4'-dihydroxy-7-methoxyflavone (**3**) (3.1 mg).

Table 1. ¹H (*J*, Hz) and ¹³C NMR data compound **1** in CD₃OD

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