Tiliabisflavan A, a New Flavan-3-ol Dimer from *Tilia amurensis* with Cytotoxic and Anti-Inflammatory Effects

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Received July 23, 2014, Accepted September 10, 2014, Published online January 5, 2015

Keywords: *Tilia amurensis*, Tiliaceae, Flavan-3-ol dimer, Cytotoxicity, Anti-inflammation

In the search for anticancer compounds from Korean medicinal plants, the methanolic extract from the trunk of *Tilia amurensis* Rupr. (Tiliaceae) was found to have significant cytotoxicity against A549 (lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma) in our screening test. *T. amurensis* is commonly known as "bee tree" and widely distributed in the mountainous areas of Korea, China, and Japan. *T. amurensis* has been used to treat various diseases as a Korean traditional medicine from ancient times.[1] Its flowers have been applied for alleviating a fever, and its leaves have also been traditionally used for analgesia and rheumatoid arthritis. Importantly, an extract of this tree has been used to treat stomach cancer without any side effects.[1] A recent pharmacological study revealed the potential of *T. amurensis* as an anticancer agent by evaluating the DNA topoisomerase inhibitory activity.[2] However, only a few constituents associated with the anticancer activity from *T. amurensis* have been reported. Recently, our phytochemical investigations on *T. amurensis* revealed the presence of bioactive lignan constituents with antitumor and anti-inflammatory activities.[3] Our continuing interest in further research on the bioactive constituents from *T. amurensis* led us to investigate bioactive metabolites of *T. amurensis* trunk. A bioassay-guided fractionation and chemical investigation of its MeOH extract resulted in the isolation and identification of four flavan-3-ols, including a new flavan-3-ol dimer, tiliabisflavan A (**1**) (Figure 1). The isolated compounds were evaluated for their antiproliferative activities against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines, as well as their inhibitory effects on NO production in a lipopolysaccharide (LPS)-activated BV-2 cell line.

Compound **1** was obtained as a colorless gum. The molecular formula of **1** [$[\alpha]_D^{25}$ +31.5° (*c* 0.35, MeOH)] was determined to be $C_{31}H_{28}O_{12}$ based on positive high-resolution electrospray ionization mass spectrometry (HR-ESIMS) data (*m/z* 615.1479 $[M + Na]^+$, calcd for $C_{31}H_{28}NaO_{12}$, 615.1478). The infrared (IR) spectrum showed absorption bands for hydroxyl group (3358 cm^{-1}) and aromatic rings (1614 and 1450 cm^{-1}). The structure of **1** was further elucidated by detailed analysis of ^1H and ^{13}C NMR chemical shifts and by heteronuclear multiple-quantum correlation (HMQC) and heteronuclear multiple-bond correlation (HMBC) experiments. The ^1H and ^{13}C NMR spectra of **1** were similar to those of **2** except for the particular signals assigned to methylene carbon [δ_H 3.88 (s); δ_C 15.5]. The assigned proton and carbon resonances of **1** in CD_3OD are summarized in Table 1. The ^1H, ^{13}C NMR, and HMQC spectra of **1** displayed the characteristic signals for the C-ring of flavan-3-ol [δ_H 4.75 (br s, H-2), 4.10 (br s, H-3), 2.82 (dd, J = 16.5, 4.5, H-4a), and 2.67 (dd, J = 16.5, 3.5, H-4b); δ_C 79.2 (C-2), 66.0 (C-3), and 27.7 (C-4)]. In addition, the 1,3,4-trisubstituted B ring signals [δ_H 6.94 (d, J = 1.5, H-2′), 6.72 (overlap, H-5′, H-6′); δ_C 144.7 (C-3′, C-4′), 130.5 (C-1′), 118.5 (C-6′), 114.7 (C-5′), and 114.2 (C-2′)] were observed, together with the A-ring carbon signals [δ_C 154.6 (C-5), 154.0 (C-7), 152.4 (C-9), 105.5 (C-8), 99.3 (C-10), and 95.6 (C-6)]. The NMR data were closely related to those of **2**[4–6]; however, instead of the two A ring aromatic methines (C-6, C-8) in **2**, the NMR spectra of **1** showed only one aromatic methine (δ_C 95.6, CH) for the A ring and an additional quaternary aromatic carbon (δ_C 105.5, C) as well as particular benzylic methylene signals [δ_H 3.88 (s); δ_C 15.5 (CH_2)]. This indicated that compound **1** is comprised symmetrically of two epicatechin units connecting C-6 and C-8 positions through a methylene bridge. In the HMBC spectrum (Figure 2), the CH_2 proton signal at δ_H 3.88 showed HMBC correlations with δ_C 154.0 (C-7), 152.4 (C-9), and 105.5 (C-8), which revealed that the two epicatechin units in **1** were linked between their C-8 position through a methylene bridge. The other partial structures of **1** were clearly elucidated by the interpretation of the ^1H–^1H correlation spectroscopy (COSY) and HMBC, and thus, the gross structure of **1** was established as shown in Figure 1 and named tiliabisflavan A.

The relative configurations of C-2 and C-3 in **1** were determined to be *cis* for (2*R*,3*R*) or (2*S*,3*S*) configuration [δ_C 79.2 (C-2)/66.0 (C-3) and $J_{3,4a}$ = 4.5 Hz, $J_{3,4b}$ = 3.5 Hz for **1**] on the basis of the comparison with the chemical shifts of C-2/C-3 [δ_C 79.9/67.5 for (+)-epicatechin (**2**) and δ_C 82.6/68.7 for (+)-catechin (**3**)] and the coupling constants of H-3/H-4

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($J_{3,4a}$ = 4.5 Hz, $J_{3,4b}$ = 2.5 Hz for **2** and $J_{3,4a}$ = 5.5 Hz, $J_{3,4b}$ = 8.5 Hz for **3**).[4–8] The measured optical rotation for **1**, $[\alpha]_D^{25}$ +31.5°, supported a (2*S*,3*S*) absolute configuration.[6] The absolute configuration of **1** was also confirmed by its circular dichroism (CD) data showing a positive cotton effect (λ_{max} 288 nm, $\Delta\varepsilon$ +1.3).[9] Thus, the absolute configuration of **1** was established to be 2*S* and 3*S* as shown in Figure 1. To the best of our knowledge, although the stereoisomers of **1** were already reported,[10,11] this is the first report of the 2*S*,3*S* form.

The known compounds were identified as (+)-epicatechin (**2**),[4–6] (+)-catechin (**3**),[7,8] and (+)-gallocatechin (**4**)[7,8] by the comparison of their spectroscopic and physical data with those previously reported. The known compounds **3** and **4** are reported here for the first time from this plant.

In this study, the isolated compounds **1–4** from *T. amurensis* were evaluated for their cytotoxic activities against A549, SK-OV-3, SK-MEL-2, and HCT-15 human tumor cell lines using the sulforhodamine B (SRB) bioassay.[12] The results (Table 2) showed that all the tested isolates (**1–4**) had cytotoxicity against the tested cell lines with IC_{50} values of 5.68–29.95 μM. In particular, flavan-3-ol dimer, compound **1**, exhibited the strongest cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines [IC_{50} (**1**): 13.37, 14.61, 5.68, and 11.03 μM, respectively]. Despite having the same skeleton for **1–4**, the differences in activity may be associated with the alteration of the hydroxyl group in these molecules, which is related to the pro-oxidant effect. The effect may cause different potency in cytotoxicity of the active compounds by influencing inhibition of cell viability, induction of apoptosis and necrosis, and cell cycle arrest in cancer cells through electron-transfer reactions leading to moderate formation of reactive oxygen species (ROS).[13]

Next, anti-inflammatory activities of **1–4** were also evaluated by measurement of the produced NO levels in LPS-activated BV-2. As shown in Table 3, compounds **2–4** presented IC_{50} values between 26.11 and 30.26 μM. Compound **1** exhibited the highest inhibitory activity. It significantly inhibited NO levels with an IC_{50} value of 4.96 μM, which is more effective than N^G-monomethyl-L-arginine (NMMA), a well-known NO synthase inhibitor. These results indicate that the new flavan-3-ol dimer, tiliabisflavan A (**1**), can exert anti-inflammatory activity in activated microglia. In this study, compounds **2–4** did not reduce the viability of BV-2 cells. Although compound **1** also did not influence

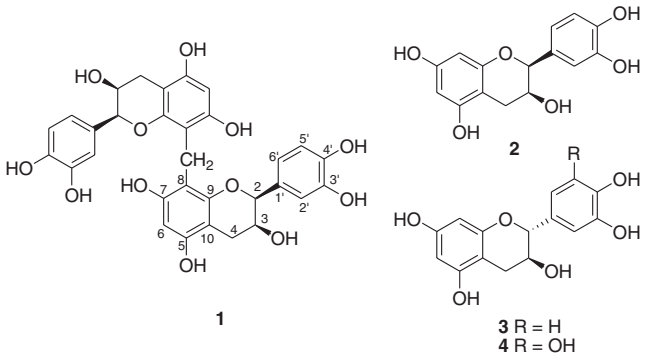

Figure 1. Chemical structures of compounds **1–4**.

Table 1. ^{1}H and ^{13}C NMR data of compound **1** in CD_3OD[a].

	1	
Position	δ_H	δ_C
2	4.75 br s	79.2
3	4.10 br s	66.0
4	2.82 dd (16.5, 4.5) 2.67 dd (16.5, 3.5)	27.7
5		154.6
6	5.95 br s	95.6
7		154.0
8		105.5
9		152.4
10		99.3
1′		130.5
2′	6.94 d (1.5)	114.2
3′		144.7
4′		144.7
5′	6.72 overlap	114.7
6′	6.72 overlap	118.5
-CH_2-	3.88 s	15.5

[a] ^{1}H and ^{13}C NMR data were recorded at 500 and 125 MHz, respectively. Coupling constants (in Hz) are given in parentheses.

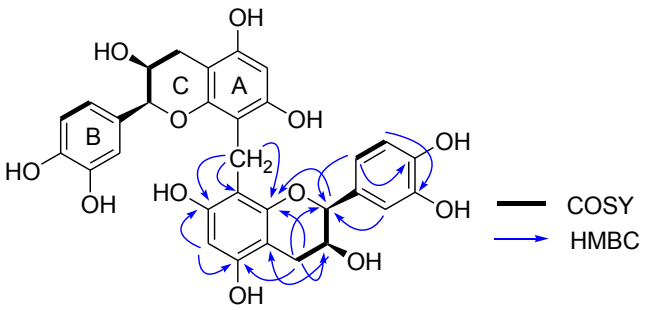

Figure 2. Selected 2-D NMR correlations for **1**.

Table 2. Cytotoxic activities of compounds (**1–4**) from *T. amurensis*.

	IC_{50} (μM)[a]			
Compounds	A549	SK-OV-3	SK-MEL-2	HCT-15
1	13.37	14.61	5.68	11.03
2	>30.0	>30.0	20.55	28.27
3	29.95	15.96	17.12	22.33
4	15.68	23.33	24.24	>30.0
Doxorubicin[b]	0.001	0.003	0.002	0.081

[a] IC_{50} value of compounds against each cancer cell line, which is defined as the concentration (μM) that caused 50% inhibition of cell growth *in-vitro*; values are means of triplicate determinations.
[b] Doxorubicin as a positive control.

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Table 3. Inhibitory effects of compounds (**1–4**) from *T. amurensis* on NO production in LPS-activated BV-2 cells.

Compounds	IC_{50} (μM)[a]	Cell viability[b]
1	4.96	89.6 ± 4.6*
2	29.08	99.5 ± 2.2
3	26.11	99.0 ± 3.1
4	30.26	99.4 ± 5.2
NMMA[c]	14.77	102.6 ± 3.0

[a] IC_{50} value of compounds was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells.
[b] Cell viability at 20 μM was expressed as a percentage (%) of the LPS only treatment group. The results are averages of three independent experiments, and the data are expressed as mean ± SD.
[c] NMMA as a positive control.
*p-value < 0.05.

the viability of BV-2 at IC_{50} (data not shown); it had little cell toxicity at concentration of 20 μM. We suggest that the difference in effects on NO production and cell viability between compounds **1** and **2–4** may be associated with their molecular structures. Compound **1** is a flavan-3-ol dimer, while **2–4** are monomeric compounds. Previous studies also reported that anti-inflammatory and antioxidant activities of flavonoids were modified by the degree of polymerization.[14,15]

Experimental

Plant Material. The trunk of *T. amurensis* was collected at Hwacheon-Myun, Hongcheon city, Gangwon-do, Korea, in March 2010. Samples of plant material were identified by one of the authors (K.R.L.). A voucher specimen (SKKU 2010-03) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and Isolation. *T. amurensis* trunks (4 kg, air-dried weight) were extracted twice with 80% aqueous MeOH (2 × 4 h) under reflux. The extract was then filtered, concentrated under vacuum, and partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, yielding 12.5 g, 40.4 g, 11.0 g, and 69.3 g residues, respectively. Each fraction was evaluated for its cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines using SRB bioassay. The EtOAc-soluble fraction, which showed the most significant cytotoxicity, was subjected to fractionation with a silica gel column chromatography using a gradient of increasing MeOH in $CHCl_3$ from 10 to 50% to give nine fractions (E1–E9). Fraction E7 was subjected to column chromatography on a Sephadex LH-20 with MeOH, and further purified by semipreparative reverse-phase high-performance liquid chromatography (HPLC) (250 mm × 10 mm i.d., 10 μm, Econosil RP-18 column, flow rate; 2 mL/min, 35% MeOH) to give compound **1** (5 mg). The other compounds **2** (27 mg), **3** (3 mg), and **4** (6 mg) were isolated by the purification using reverse-phase HPLC with 70% MeOH from the fraction E5.

Tiliabisflavan A (1). Colorless gum; $[\alpha]_D^{25}$ +31.5° (*c* 0.35, MeOH); UV (MeOH) λ_{max} (log ε) 282 (2.5), 235 (3.8) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$) 288 (+1.3), 240 (+0.7); IR (KBr) ν_{max} 3358, 2947, 2834, 2495, 2238, 2072, 1614, 1450, 1121, 1032, 981, 674 cm^{-1}; ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; ESIMS (positive-ion mode) *m/z*: 615 $[M + Na]^+$; HR-ESIMS (positive-ion mode) *m/z*: 615.1479 $[M + Na]^+$ (calcd. for $C_{31}H_{28}NaO_{12}$, 615.1478).

Acknowledgments. We thank Dr E. J. Bang, Dr S. G. Kim, and Dr J. J. Seo of the Korea Basic Science Institute for their aid in the NMR and MS spectral measurements. This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A5A2A28671860).

Supporting Information. 1-D and 2-D NMR spectra of **1**, the general experimental procedures, and bioassay protocol are available as Supporting Information.

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