

## STRUCTURAL ELUCIDATION OF A NEW FLAVOLIGNAN ACYLGLYCOSIDE FROM FALLEN NEEDLES OF *Pinus banksiana*

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A new flavolignan acylglycoside, namely phillygenin 4-O-(2''-trans-cinnamoyl)- $\alpha$ -L-rhamnopyranoside (**1**), along with a known phenolic acid (ellagic acid, **2**) and three known flavonol glycosides (kaempferol 3-O- $\beta$ -D-glucopyranoside (**3**), kaempferol 3-O- $\alpha$ -L-rhamnopyranoside (**4**), and quercetin 3-O- $\beta$ -D-glucopyranoside (**5**), was isolated and purified from the fallen needles of *Pinus banksiana* by repeated column chromatography. The structures of these secondary metabolites were mainly elucidated by the extensive use of 1D and 2D NMR experiments, together with IR, UV, and FAB MS spectra. Among the four known compounds, **2** and **4** have never been found in this conifer species previously.

**Keywords:** *Pinus banksiana*, Pinaceae, secondary metabolites, spectroscopic method, phillygenin 4-O-(2''-trans-cinnamoyl)- $\alpha$ -L-rhamnopyranoside.

*Pinus banksiana* Lam., a conifer species belonging to the family of Pinaceae, is widespread in Xiaoxing'an Ling, Daxing'an Ling, and Taibai Mountains of north China, northwest regions of America, and many forests of Canada [1–3]. Needles and barks of *P. banksiana* have long been used in Chinese folk medicine to treat and prevent cancer, cardiovascular and aging disorders, as well as to enhance immunity [4, 5]. Phytochemical investigations of *P. banksiana* have led to the isolation of several classes of secondary metabolites such as sesquiterpenes and flavonol glycosides [6–8]. In a previous study, we had reported the extraction and purification of *trans*-isoconiferin, caffeic acid, cedrusin, phillyrin, and phillyrin-6''- $\alpha$ -rhamnopyranoside from the branch wood of *P. banksiana* [2]. The current work describes the isolation and structural elucidation of a novel flavolignan acylglycoside, phillygenin 4-O-(2''-trans-cinnamoyl)- $\alpha$ -L-rhamnopyranoside (**1**) from the 70% acetone extract of the fallen needles of *P. banksiana*, together with four known compounds, ellagic acid (**2**), kaempferol 3-O- $\beta$ -D-glucopyranoside (**3**), kaempferol 3-O- $\alpha$ -L-rhamnopyranoside (**4**), and quercetin 3-O- $\beta$ -D-glucopyranoside (**5**). Compound **1** is a new flavolignan acylglycoside, and **2** and **4** are reported in this conifer species for the first time here.

Compound **1** was isolated as a white amorphous powder, with optical rotation  $[\alpha]_{\text{D}}^{20} -47.9^\circ$  (*c* 0.5; MeOH) and mp 199–201°C. The molecular formula of compound **1** was assigned to be C<sub>36</sub>H<sub>40</sub>O<sub>11</sub> on the basis of its positive FAB-MS spectrometric analysis (found [M + H]<sup>+</sup>, [M + K]<sup>+</sup>, and [M + Na]<sup>+</sup> ion peaks at *m/z* 649, 687, and 671, respectively).

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TABLE 1. NMR Spectroscopic Data for Compound **1** (DMSO- $d_6$ ,  $\delta$ , ppm, J/Hz)

| C atom    | $\delta_H$                  | $\delta_C$ | DEPT            |
|-----------|-----------------------------|------------|-----------------|
| Aglycone  |                             |            |                 |
| 1         | –                           | 135.39     | qC              |
| 2         | 6.99 (1H, d, J = 2.1)       | 110.55     | CH              |
| 3         | –                           | 148.55     | qC              |
| 4         | –                           | 145.93     | qC              |
| 5         | 7.04 (1H, d, J = 8.0)       | 115.24     | CH              |
| 6         | 6.90 (1H, dd, J = 2.1, 8.0) | 118.18     | CH              |
| 7         | 4.34 (1H, d, J = 6.3)       | 86.68      | CH              |
| 8         | 2.80 (1H, m)                | 53.99      | CH              |
| 9         | 4.04 (1H, m); 3.68 (1H, m)  | 69.76      | CH <sub>2</sub> |
| 10-OMe    | 3.72 (3H, s)                | 55.46      | CH <sub>3</sub> |
| 1'        | –                           | 131.28     | qC              |
| 2'        | 6.96 (1H, d, J = 2.2)       | 109.59     | CH              |
| 3'        | –                           | 148.46     | qC              |
| 4'        | –                           | 147.66     | qC              |
| 5'        | 6.79 (1H, d, J = 7.8)       | 111.65     | CH              |
| 6'        | 6.87 (1H, dd, J = 2.2, 7.8) | 117.56     | CH              |
| 7'        | 4.76 (1H, d, J = 6.1)       | 81.25      | CH              |
| 8'        | 3.06 (1H, m)                | 49.39      | CH              |
| 9'        | 3.71 (1H, m); 3.18 (1H, m)  | 68.98      | CH <sub>2</sub> |
| 10'-OMe   | 3.70 (3H, s)                | 55.44      | CH <sub>3</sub> |
| 11'-OMe   | 3.73 (3H, s)                | 55.45      | CH <sub>3</sub> |
| Rhamnosyl |                             |            |                 |
| 1''       | 5.30 (1H, d, J = 1.6)       | 98.41      | CH              |
| 2''       | 5.29 (1H, dd, J = 1.6, 3.2) | 71.03      | CH              |
| 3''       | 3.58 (1H, dd, J = 3.2, 9.6) | 71.87      | CH              |
| 4''       | 3.10 (1H, m)                | 68.66      | CH              |
| 5''       | 3.18 (1H, m)                | 71.40      | CH              |
| 6''       | 0.89 (3H, d, J = 6.1)       | 17.58      | CH <sub>3</sub> |
| Cinnamoyl |                             |            |                 |
| 1'''      | –                           | 133.82     | qC              |
| 2'''/6''' | 7.59 (1H, dd, J = 2.0, 6.9) | 128.56     | CH              |
| 3'''/5''' | 7.26 (1H, m)                | 128.94     | CH              |
| 4'''      | 7.28 (1H, m)                | 130.65     | CH              |
| 7'''      | 7.49 (1H, d, J = 16.2)      | 145.08     | CH              |
| 8'''      | 6.55 (1H, d, J = 16.2)      | 117.92     | CH              |
| 9'''      | –                           | 165.56     | qC              |

In the TLC chromogenic reaction,  $R_f$  values of compound **1** were 0.57 and 0.19 in solvents A and B, respectively), when spraying with 10% sulfuric acid (in EtOH) solution as reagent followed by heating [9, 10]. Its IR spectrum displayed the presence of phenyl (1525 and 1635  $\text{cm}^{-1}$ ) and hydroxyl (3390  $\text{cm}^{-1}$ ) moieties. The UV spectrum of compound **1** showed absorptions at 285 nm and 235 nm, which suggested its bis-perhydrofuran unit [11, 12].

In the  $^1\text{H}$  NMR spectrum (Table 1), compound **1** presented two pairs of ABX style aromatic proton signals at  $\delta$  6.99 (1H, d, J = 2.1 Hz, H-2),  $\delta$  7.04 (1H, d, J = 8.0 Hz, H-5),  $\delta$  6.90 (1H, dd, J = 2.1, 8.0 Hz, H-6) and  $\delta$  6.96 (1H, d, J = 2.2 Hz, H-2'),  $\delta$  6.79 (1H, d, J = 7.8 Hz, H-5'),  $\delta$  6.87 (1H, dd, J = 2.2, 7.8 Hz, H-6'). Proton peaks for benzylic oxymethine H-7 and H-7' were found as two doublets at  $\delta$  4.34 (1H, J = 6.3 Hz) and 4.76 (1H, J = 6.1 Hz). Coupling signals at  $\delta$  2.80 (1H, m) and 3.06 (1H, m) were assignable to H-8 and H-8' of the *cis*-diequatorial substituted 2,6-diaryl-3,7-dioxabicyclo[3,3,0]octane [13], whereas the oxygen-containing methylene protons H-9 and H-9' were confirmed at  $\delta$  4.04, 3.68 (each 1H, m) and  $\delta$  3.71, 3.18 (each 1H, m) [14]. The three aromatic methoxyls were evidenced by their signals ( $\delta_H$  3.72 (3H, s) and  $\delta_C$  55.46 for 10-OMe), ( $\delta_H$  3.70 (3H, s) and  $\delta_C$  55.44 for 10'-OMe), and ( $\delta_H$  3.73 (3H, s) and  $\delta_C$  55.45 for 11'-OMe) in the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra, respectively. These clues together with its  $^{13}\text{C}$  NMR data (Table 1) indicated that the aglycone moiety of compound **1** could be a flavolignan named phillygenin [15, 16]. The downfield shifts  $\Delta\delta$  of approximately 1.11 and 3.14 ppm for C-3 and C-1, respectively, compared to phillygenin corresponded well with the linkage of one substituent at C-4 of its equatorial ring in phillygenin [15].

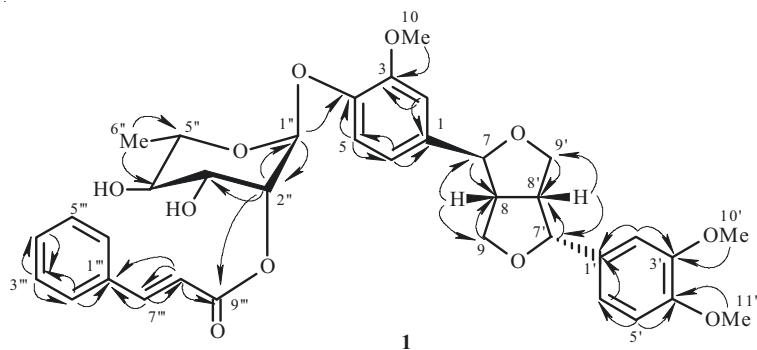


Fig. 1. Key HMBC correlations observed in compound **1**.

In the  $^1\text{H}$  NMR spectrum of compound **1**, an anomeric proton observed at  $\delta$  5.30 (1H, d,  $J = 1.6$  Hz, H-1'') and one secondary methyl moiety at  $\delta$  0.89 (3H, d,  $J = 6.1$  Hz, H-6''), along with the remaining sugar peaks ranging between  $\delta$  5.29 and 3.10 revealed the presence of one  $\alpha$ -L-rhamnosyl residue [9]. This conclusion was also supported by the corresponding  $^{13}\text{C}$  NMR signals appearing at  $\delta$  98.41 (C-1'') and 17.58 (C-6''). Relative to the spectral data of an authentic rhamnosyl unit, the sharp downfield of H-2'' and C-2'' to  $\delta$  5.29 and 71.03, respectively, indicated that the hydroxyl of the rhamnosyl 2'' position was replaced [17, 18].

The presence of a *trans*-cinnamoyl unit in compound **1** was revealed by its  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra: a monosubstituted benzene ring was confirmed by the symmetrical  $^1\text{H}$  NMR signals at  $\delta$  7.59 (1H each, dd,  $J = 2.0, 6.9$  Hz, H-2'''/6'''), 7.26 (1H each, m, H-3'''/5'''), and 7.28 (1H, m, H-4''') and  $^{13}\text{C}$  NMR peaks at  $\delta$  128.56 (C-2'''/6'''), 128.94 (C-3'''/5'''), 133.82 (C-1''') and 130.65 (1H, m, H-4''') [18, 19]. In addition, the olefinic proton signals characteristically resonated at  $\delta$  7.49 (1H, d, H-7''') and 6.55 (1H, d, H-8''') with a low coupling constant of  $J = 16.2$  Hz, suggesting the *trans*-configuration for the cinnamoyl unit [17–19].

In the H $\rightarrow$ C couplings of the HMBC spectrum of compound **1**, correlations were observed between anomeric H-1''  $\delta$  5.30 (1H, d,  $J = 1.6$  Hz) of the rhamnose and C-4 ( $\delta$  145.93) of the aglycone, and between H-2''  $\delta$  5.29 (1H, dd,  $J = 1.6, 3.2$  Hz) of the sugar and C-9''' ( $\delta$  165.56) of the cinnamoyl residue, as demonstrated in Fig. 1, verifying that the rhamnosyl moiety is attached to C-4 of phillygenin, and the *trans*-cinnamoyl unit is linked to C-2'' of the rhamnosyl sugar. The DEPT spectrum data, as shown in Table 1, classified 36 carbon signals of compound **1** into two methylenes ( $\text{CH}_2$ ), 22 methines ( $\text{CH}$ ), four methyls ( $\text{CH}_3$ ) and eight quaternary carbons (qC), with each signal agreeing well with the corresponding carbons in phillygenin 4-*O*-(2''-*trans*-cinnamoyl)- $\alpha$ -L-rhamnopyranoside.

Thus, compound **1** was established as phillygenin 4-*O*-(2''-*trans*-cinnamoyl)- $\alpha$ -L-rhamnopyranoside, which is a new flavolignan acylglycoside isolated and elucidated for the first time here.

The chemical structures of the four known compounds, ellagic acid (**2**), kaempferol 3-*O*- $\beta$ -D-glucopyranoside (**3**), kaempferol 3-*O*- $\alpha$ -L-rhamnopyranoside (**4**), and quercetin 3-*O*- $\beta$ -D-glucopyranoside (**5**), were determined mainly by comparing their MS and NMR data with those in previously published literature [20–22]. To the best of our knowledge, compounds **2** and **4** have never been reported from *P. banksiana* previously.

## EXPERIMENTAL

**General Experimental Procedures.** UV spectra were measured in MeOH on a Jenway 6405 spectrophotometer. IR experiments were done in KBr on a PerkinElmer BX FT-IR spectrometer. NMR spectra were recorded in DMSO- $d_6$  with TMS as an internal standard on a Bruker Avance DPX 400 spectrometer at frequencies of 400 and 100 MHz for  $^1\text{H}$  and  $^{13}\text{C}$  NMR, respectively. Optical rotation data were determined by a Jasco DIP-1000 digital polarimeter. An Electro Thermal 9100 apparatus was used to obtain the compounds' melting points (mp). Sephadex LH-20 (Sigma, USA) and silica gel (Shanghai Anyan Biological Company, China) were used for column chromatography. BS-100A fraction collectors were used to collect the fraction eluent. In TLC, DC-Plastikfolien Cellulose F (Merck Co.) plates were used with *t*-BuOH–AcOH– $\text{H}_2\text{O}$  (3:1:1, solvent A) and AcOH– $\text{H}_2\text{O}$  (3:47, solvent B) as developing solvents. Spots were detected on TLC under UV light (365 and 254 nm) or by heating after spraying with 1%  $\text{FeCl}_3$  in EtOH.

**Plant Material.** Fallen needles of *P. banksiana* were collected in Jiageda Qi, Daxin'an District, Heilongjiang Province, P. R. China, in October 2012. The plant materials were identified by Prof. Dan Wang at the Institute of the Chemical Industry of Forest Products, P.R. China. A voucher specimen (No. 201210008) has been deposited in the Herbarium of the Tianjin Key Laboratory of Pulp and Paper, College of Papermaking Science and Technology, Tianjin University of Science and Technology, P. R. China.

**Extraction and Isolation.** A 5.62 g portion of the fallen needles of *P. banksiana* was dried and finely powdered, then extracted in 70% acetone for more than 3 days. After evaporation of the aqueous acetone under vacuum, the dark brown residue was suspended in water and sequentially fractionated in separation funnels with *n*-hexane, CHCl<sub>3</sub>, and EtOAc, then freeze dried to afford EtOAc soluble fraction (ESF) powders. A portion of the resulting ESF (32.91 g) was separated by column chromatography over silica gel using a gradient of CHCl<sub>3</sub>-MeOH (99:1→49:1→1:1) as washing eluent to give five fractions (ESF<sub>1</sub>-ESF<sub>5</sub>) according to their TLC profiles. Fraction ESF<sub>3</sub> was further subjected to silica gel column chromatography with CHCl<sub>3</sub>-H<sub>2</sub>O-MeOH (40:9:1→20:4:1→1:1:1) to yield six fractions (ESF<sub>31</sub>-ESF<sub>36</sub>). Fraction ESF<sub>35</sub> was then loaded on a chromatographic column packed with Sephadex LH-20 using MeOH-H<sub>2</sub>O (3:1) as eluting solvent to give four fractions (ESF<sub>351</sub>-ESF<sub>354</sub>). Fraction ESF<sub>353</sub> was further subjected to Sephadex LH-20 column chromatography using *n*-hexane-EtOH (2:1, 1:2) and MeOH-H<sub>2</sub>O (1:1, 1:4) as eluting solvents to obtain 39 mg of compound **1** and 62 mg of compound **2** as amorphous powders. Fraction ESF<sub>33</sub> was also subjected to Sephadex LH-20 column chromatography with MeOH-H<sub>2</sub>O (2:1, 1:2, 1:5) and *n*-hexane-EtOH (3:1, 1:1, 1:4) as mobile solvents to yield yellowish amorphous compounds **3** (71 mg), **4** (28 mg), and **5** (46 mg).

**Phillygenin 4-O-(2''-trans-Cinnamoyl)- $\alpha$ -L-rhamnopyranoside (1).** White amorphous powder, mp 199–201°C,  $[\alpha]_D^{20}$  -47.9° (*c* 0.5, MeOH). IR (KBr,  $\nu_{\max}$ , cm<sup>-1</sup>): 3390 (hydroxyl group), 1635 and 1525 (phenyl group). UV (MeOH,  $\lambda_{\max}$ , nm): 235, 285.  $R_f$  0.57 (solvent A) and 0.19 (solvent B). Positive FAB-MS  $m/z$  [M + H]<sup>+</sup> at 649, [M + K]<sup>+</sup> at 687, and [M + Na]<sup>+</sup> at 671, indicating a molecular weight of 648 calculated for C<sub>36</sub>H<sub>40</sub>O<sub>11</sub>. For <sup>1</sup>H (400 MHz) and <sup>13</sup>C (100 MHz) NMR spectroscopic data in DMSO-d<sub>6</sub>, see Table 1.

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