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Lignans from the fruits of *Forsythia suspensa*

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Abstract—Bioactivity-guided fractionation of the methanol extract of the fruits of *Forsythia suspensa* Vahl has led to the isolation of two new monoepoxylignans, forsythialan A (**1**) and B (**2**), together with a known tetrahydrofurofuran lignan, phillyrin (**3**). The structures of compounds **1** and **2** were elucidated by spectroscopic methods. The antioxidant activities of these lignans have been assessed by evaluating their protective effects against peroxynitrite-induced oxidative stress. The compounds **1** and **2** showed protective effects against renal epithelial cell injury by 3-morpholinosydnonimine (SIN-1), a peroxynitrite generator. The relatively stronger antioxidant activities of compounds **1** and **2** may be associated with the presence of aromatic hydroxy function.

Forsythia suspensa Vahl (Oleaceae) is a climbing plant, which is widely distributed in China, Korea and Japan.[1] The extract of the dried fruits has long been used in the Chinese, Korean and Japanese folk medicines to treat gonorrhea, erysipedas, inflammation, pharyngitis, pyrexia, tonsillitis, and ulcer.[2,3] Moreover, it has also been shown that the crude drug exhibits potential antibacterial, antiviral, choleretic and antipyretic effects.[4–7] Phytochemical studies on the plant have shown that phenolic compounds including lignans and flavonols, one class of the major components, are responsible for the various biological activities of the herb.[8]

Lignans, with carbon skeletons composed of C6–C3 units linked in various modes, are present in different terrestrial and marine organisms.[9] Tetrahydrofurofuran lignans are one of the largest groups of lignans[10] whose members are of special interest owing to their powerful antitumoral,[11] antiinflammatory,[12] antioxidant,[13] and insecticide properties,[14] including phosphodiesterase inhibition[15] and hypocholesterolemic activities in humans.[16] The biological activities of lignans can be modified by modulating the degree of oxidation at specific benzylic and arylic positions,[17] as observed in the cytochrome P 450-mediated oxidations in mammalian cells, where stable metabolites with aliphatic and aromatic hydroxylation and increased pharmacological properties are produced.[18]

In the present investigation, two new compounds, forsythialan A (**1**) and forsythialan B (**2**), were isolated and characterized from the fruits of *F. suspensa* along with one known compound, phillyrin (**3**) (Fig. 1). This is the first report of the occurrence of compounds **1** and **2** from a natural source. The complete ^{1}H and ^{13}C NMR spectral data for these compounds are also reported here for the first time. The effects of these compounds were evaluated on peroxynitrite ($ONOO^{-}$)-induced oxidative injury in LLC-PK_1 cells using 3-morpholinosydnonimine (SIN-1) to generate $ONOO^{-}$ in vitro.

The dried fruits of *F. suspensa* used in this study were purchased from the Beijing market in China and identified by Jian Cui, College of Life and Environmental Sciences, Central University for Nationalities, China. A voucher specimen is deposited at College of Life and Environmental Sciences, Central University for Nationalities, China.

The dried and powdered fruits (500 g) were refluxed twice with 80% methanol in H_2O. The combined extract was concentrated under reduced temperature (45 °C) and pressure, and freeze dried to give a powdered mass

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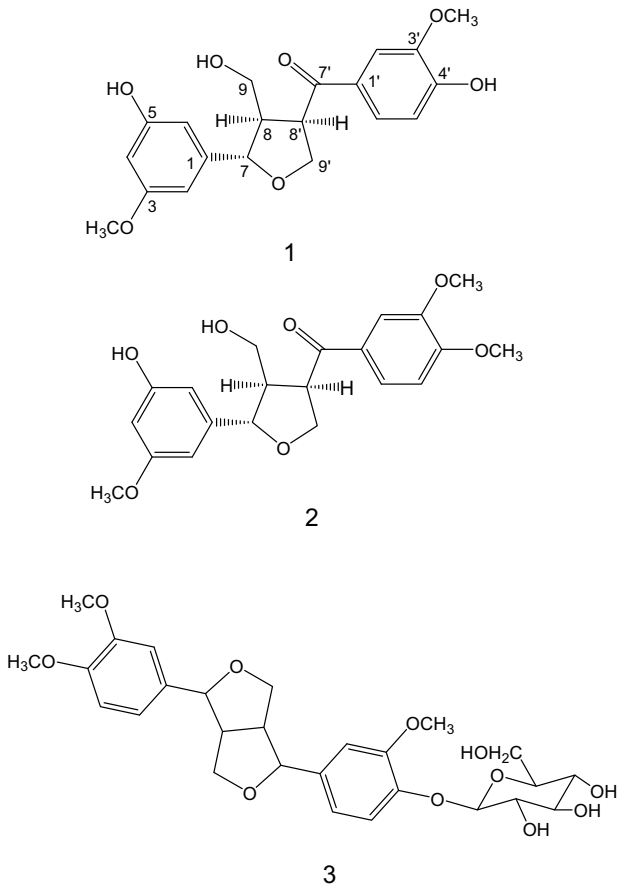

Figure 1. Chemical structures of lignans from *Forsythia suspensa* Vahl (Oleaceae).

(84.5 g). The crude extract was then re-dissolved in water and extracted twice with dichloromethane to yield 45.3 g of dichloromethane extract. An aliquot (24 g) of the dichloromethane extract was divided into 36 fractions (FS 1–FS 36) on silica gel column chromatography (Qingdao Chem., 200–400 mesh, 600 g) using $CHCl_3$–MeOH mixtures of increasing polarity [100:1 (FS 1–FS 5), 50:1 (FS 6–FS 15), 20:1 (FS 16–FS 20), 10:1 (FS 21–FS 25), 5:1 (FS 26–FS 30), 2:1 (FS 31–FS 32)]. Compound **1** (50 mg) was purified from fraction FS 12 (200 mg) by semi-preparation HPLC (RP-18, 250 × 10 mm, 10 μm) using MeOH–H_2O (50%, v/v). Compound **2** (15 mg) was obtained through HPLC separation (MeOH–H_2O, 30:70) from fraction FS 7. The compound **3** (98 mg) was isolated from fraction FS 24 by chromatography on silica gel ($CHCl_3$–MeOH, 15:1).

Compound **1** was isolated as colorless oil. In the positive mode ESIMS of **1**, quasimolecular ion peaks at *m/z* 375 $[M+H]^+$ and 397 $[M+Na]^+$ were observed, and HRESIMS analysis indicated a protonated ion peak at 375.14444 (Error: +1.51 ppm) which corresponded to the molecular formula $C_{20}H_{22}O_7$. The IR absorptions at 3394 and 1665 cm^{-1} showed the presence of a hydroxyl group and a carbonyl group conjugated with an aromatic ring in the furanoid lignan skeleton.

The ^1H NMR spectrum of **1** indicated the presence of two aromatic methoxy groups at δ 3.91 and 3.95 (each 3H, s), four geminal protons attached to carbon atoms bearing an oxygen functionality at δ 3.65 (1H, dd, J = 5.7, 10.9 Hz, H-9α), 3.77 (1H, dd, J = 4.4, 10.9 Hz, H-9β), 4.18 (1H, m, H-9′α), and 4.28 (1H, br d, J = 10.9 Hz, H-9′β), and two methines and one oxymethines at δ 2.89 (1H, m, H-8), 4.18 (1H, m, H-8′), and 4.67 (1H, d, J = 9.1 Hz, H-7). They were similar to those of vladinol D in the ^1H NMR data.[19] In the low-field region, six aromatic protons could be divided into two groups, with the first including two broad singlets at δ 7.02 (1H, s, H-2) and 6.87 (2H, s, H-4 and H-6), indicating a 1,3,5-trisubstituted benzene ring and the second showing an ABX coupling system assignable to a 1,3,4-trisubstituted benzene ring that appeared at δ 7.57 (1H, d, J = 1.9 Hz, H-2′), 7.56 (1H, dd, J = 1.9, 8.2 Hz, H-6′), and 6.96 (1H, d, J = 8.2 Hz, H-5′).

The ^{13}C and DEPT NMR spectra showed 20 signals including one carbonyl carbon signal (δ 197.99, C-7′), 12 carbons for two aromatic rings, 2 methylenes (δ 61.35 and 70.78, C-9 and C-9′), 3 methines (δ 83.85, 52.22 and 49.62, C-7, C-8 and C-8′), and two methoxy carbons (δ 55.97 and 56.07, OCH_3-3 and OCH_3-3′). These spectroscopic data were matched with the structure proposed for **1** being a monoepoxy-type lignan.[20]

The full NMR assignments and connectivities of **1** were determined by ^1H–^1H COSY, HSQC, and HMBC spectroscopic data analysis. The ^1H–^1H COSY spectrum showed three spin systems: (i) H-7 → H-8 → H2-9; (ii) H-8′ → H2-9′, and (iii) H-8 → H-8′, which connected (i) and (ii). The HMQC spectrum revealed that the proton at δ 4.67 (H-7) is attached to the carbon at δ 83.85 (C-7), and the HMBC spectrum showed that H-7 was correlated to C-8, C-8′, and C-9′, suggesting that a tetrahydrofuran unit was formed by ring closure involving an oxygen atom bridged to C-7 and C-9′. Furthermore, the HMBC correlations of H-7 to C-1, C-2, and C-6 established that the tetrahydrofuran unit was attached to the 1,3,5-trisubstituted benzene ring at C-1. The HMBC spectrum showed that carbonyl C-7′ was correlated to H-2′, H-6′, H-8′, and C-9′, suggesting that C-7′ is connected to the tetrahydrofuran ring and the 1,3,4-trisubstituted benzene ring through a C-1′ → C-7′ → C-8′ linkage.

The relative configuration of **1** was established from the NOESY spectrum. The NOESY spectrum displayed correlations between H-8 and H-8′, and H-7 and H-9 but was not observed between H-7 and H-8. This led to the assignment of *trans* and *cis* orientions for H-7/H-8 and H-8/H-8′, respectively (Fig. 2). Consequently, these data as well as the optical rotation ($[\alpha]_D^{25}$ −7.2°) established the structure of **1** as 7S, 8R, 8′R, which was similar to that of magnone A.[21] Compound **1** represents the first monoepoxy-type lignan bearing a 1,3,5-trisubstituted phenyl ring with a carbonyl group. Accordingly, compound **1** was proposed to be a new lignan and has been accorded the trivial name forsythialan A.[22]

The ^1H and ^{13}C NMR spectroscopic properties for compound **2** were similar to those of **1**, except for the peaks

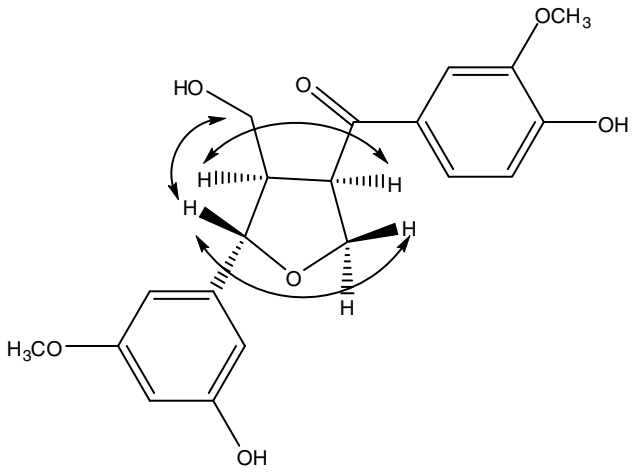

Figure 2. NOESY connectivities of compound **1**.

related to the second aromatic ring. The chemical shifts of C-4′ (δ 153.71), C-3′ (δ 149.27) and C-5′ (δ 110.15) revealed that there is a methoxy group on C-4′. Comparing the ^{1}H NMR data of **2** with those of **1**, the configurations of chiral centers were established as 7S, 8R, and 8′R.

Additionally, **2** exhibited the $[M+H]^+$ ion peak at *m*/*z* 389 in the ESIMS, which differed from that of **1** by 14 amu, corresponding to a methoxyl (+31 amu) instead of hydroxyl group (−17 amu). HRESIMS analysis indicated a protonated ion peak at 389.1582 (Error: −3.19 ppm) which corresponded to the molecular formula $C_{21}H_{24}O_7$. Consequently, compound **2** was established as forsythialan B.[23]

The ^{1}H and ^{13}C NMR assignments for compounds **1** and **2** are listed in Table 1. The key NOESY correlations of **1** are shown in Figure 2. Only hydrogen atoms binding to the chiral carbons are shown for clarity.

The structures of the known compound **3** were identified by comparing their spectroscopic data (ESIMS, ^{1}H and ^{13}C NMR) with those of reported values and found to be phillyrin.[24]

To investigate the protective effect from oxidative damage, we employed the SIN-1-induced cellular oxidative model.[25] Although the oxidative stress induced by 0.5 mM SIN-1 caused cell death of about 58% viability, treating cells with compound **1** or **2** markedly reverted the cell death in a dose-dependent manner (Fig. 3). On the other hand, compound **3** did not show any significant protective effect against $ONOO^-$-induced cell death. Compounds **1** and **2** showed a stronger activity than vitamin C, a well-known antioxidant. Significant protective effects were achieved starting from 10 μM. With the concentration of 50 μM, the viability of cells treated with compounds **1** and **2** was 95% and 85%, respectively. Compounds **1** and **2** alone did not affect viability in untreated cells (data not shown).

The cell membrane rigidity and permeability were also assessed by measuring the LDH release.[26] Figure 4 shows the effects of compounds **1** and **2** on LDH release induced by $ONOO^-$ in LLC-PK$_1$ cells at different concentrations. Treatment with SIN-1 caused an increase in LDH release into the medium, 131% of control. However, pretreatment with compound **1** or **2** exerted efficient inhibitory activity on LDH release triggered by SIN-1 in LLC-PK$_1$ cell system. Even the lowest concentration of

Table 1. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) data of compounds **1** and **2** in $CDCl_3$ (δ ppm)

Number	Forsythialan A (**1**)			Forsythialan B (**2**)		
	^{1}H	^{13}C	HMBC	^{1}H	^{13}C	HMBC
1		132.3			132.32	
2	7.02, s	110.04	1, 3, 4, 6, 7	7.03, s	108.97	1, 3, 4, 6, 7
3		146.84			146.85	
4	6.87, s	114.06	2, 3, 5, 6	6.88, s	114.04	2, 3, 5, 6
5		145.57			145.62	
6	6.87, s	120.08	1, 2, 4, 5, 7	6.88, s	120.11	1, 2, 4, 5, 7
7	4.67, d, *J* = 9.1	83.85	1, 2, 6, 8, 9, 8′, 9′	4.68, d, *J* = 9.1	83.88	1, 2, 6, 8, 9, 8′, 9′
8	2.89, m	52.22	8′	2.91, m	52.25	8′
9	α 3.65, dd, *J* = 5.6, 10.9	61.35	7, 8, 8′	α 3.67, dd, *J* = 5.6, 10.9	61.42	7, 8, 8′
	β 3.77, dd, *J* = 4.4, 10.9			β 3.78, dd, *J* = 4.4, 10.9		
1′		129.42			129.79	
2′	7.57, d, *J* = 2.0	110.39	1′, 3′, 4′, 6′, 7′	7.58, d, *J* = 2.0	110.67	1′, 3′, 4′, 6′, 7′
3′		146.85			149.27	
4′		150.84			153.71	
5′	6.96, d, *J* = 8.2	114.07	3′, 4′, 6′, 7′,	6.92, d, *J* = 8.3	110.15	3′, 4′, 6′, 7′,
6′	7.56, dd, *J* = 2.0, 8.2	123.81	1′, 2′, 4′, 5′, 7′	7.61, dd, *J* = 2.0, 8.3	123.16	1′, 2′, 4′, 5′, 7′
7′		197.99			197.96	
8′	4.18, m	49.62	7, 8, 9, 9′	4.19, m	49.66	7, 8, 9, 9′
9′	α 4.18, m	70.78	7, 8, 7′, 8′	α 4.19, m	70.8	7, 8, 7′, 8′
	β 4.28, bd, *J* = 11.0			β 4.29, bd, *J* = 11.0		
OCH_3-3	3.91, s	56.07	3	3.93, s	56.01	3
OCH_3-3′	3.95, s	55.96	3′	3.95, s	55.99	3′
OCH_3-4				3.97, s	56.12	4′

Assignments were confirmed by DEPT, 1H–1H COSY, NOESY, and HMBC spectra.

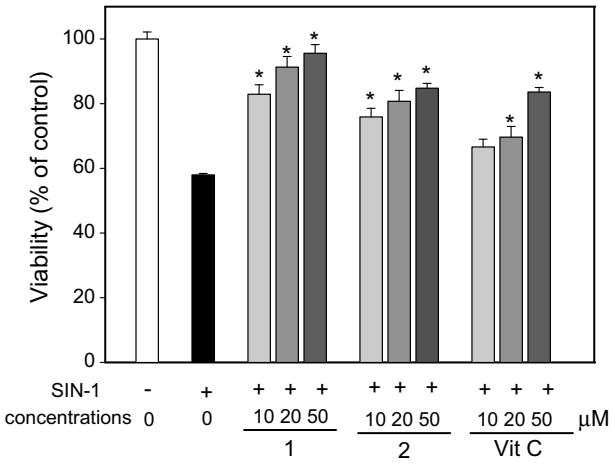

Figure 3. Protective effects of compounds **1** and **2** on $ONOO^-$-induced cell death. Cells were preincubated with test compound at various concentrations for 1 h, and then treated with 0.5 mM SIN-1 for 24 h. Cell viability was measured using MTT reduction assay. Values are means ± SEM ($n = 3$). $^*P < 0.01$ significantly different from the group treated with SIN-1 alone.

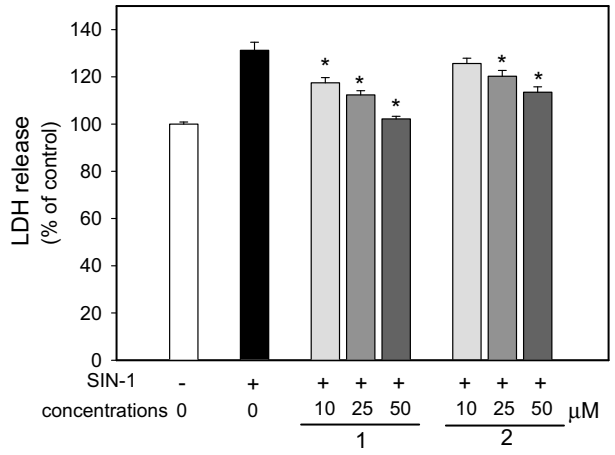

Figure 4. Effects of compounds **1** and **2** on LDH release induced by $ONOO^-$ in LLC-PK_1 cells. LLC-PK_1 cells were treated with 0.5 mM SIN-1 in the absence or presence of test compound at various concentrations for 24 h. LDH leakage in culture medium was measured with a colorimetric LDH assay kit. Values are means ± SEM ($n = 3$). $^*P < 0.05$ significantly different from the cells treated with SIN-1 alone.

compound **1** (10 μM) significantly reduced the LDH leakage, and at 50 μM, compound **1** reversed the LDH release induced by SIN-1 to almost the control level.

These results demonstrated that compounds **1** and **2** exhibited antioxidant effects against $ONOO^-$-induced oxidative stress. The hydroxy substituents on the phenyl moieties may contribute to the antioxidant activities of these epoxylignans.

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22. *Forsythialan A (**1**):* Colorless oil; $[\alpha]_D^{25}$ −7.2 (*c* 0.56, chloroform); IR ($CHCl_3$) υ_{max} 3394, 2962, 1665, 1591, 1517, 1464, 1428, 1261, 1163, 1030, 799, 756 cm^{-1}; ^1H (300 MHz) and ^{13}C (75 MHz) NMR data, see Table 1; ESIMS (positive mode) *m/z* 397 $[M+Na]^+$; HRESIMS *m/z*: 375.1444 $[M+H]^+$ (Calcd for $C_{20}H_{22}O_7H$: 375.1438) and 397.1264 $[M+Na]^+$ (Calcd for $C_{20}H_{22}O_7Na$: 397.1258).
23. *Forsythialan B (**2**):* Colorless oil; $[\alpha]_D^{25}$ −21.5 (*c* 0.93, chloroform); IR ($CHCl_3$) υ_{max} 3429, 2931, 1669, 1595, 1517, 1464, 1421, 1267, 1161, 1022, 818, 757 cm^{-1}; ^1H (300 MHz) and ^{13}C (75 MHz) NMR data, see Table 1; ESIMS (positive mode) *m/z* 397 $[M+Na]^+$; HRESIMS *m/z*: 389.1582 $[M+H]^+$ (Calcd for $C_{21}H_{24}O_7H$: 389.1595).
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25. Commercially available LLC-PK_1 cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 incubator with 5% FBS-supplemented DMEM/F-12 medium. After confluence had been reached, the cells were seeded into 96-well plates at a density of 2×10^4 cells per well and incubated for 2 h. At the end of the incubation period, cells were pretreated with test compound and incubated for 1 h. After the pretreatment period, SIN-1 (final concentration, 0.5 mM) was added to culture medium and incubated for 24 h. Controls were not treated with

SIN-1 or test compound. The proper concentration of SIN-1 and the incubation time were determined by the preliminary experiment. MTT solution (final concentration, 500 μg/ml) was added to each well. After incubation for 4 h at 37 °C, the MTT solution was removed from the medium. The resultant formazan crystals in the renal cells were solubilized with 100 μL of dimethylsulfoxide. The absorbance of each well was then read at 560 nm using a microplate reader (model SPECTRAmax 340PC, Molecular Devices, Sunnyvale, CA, USA).

26. Leakage of LDH from cells was measured using an LDH assay kit (CytoTox 96, Promega) according to the manufacturer's procedure. After 24 h of incubation with SIN-1 and/or test compound, plates were centrifuged and aliquots of culture media were transferred to fresh 96-well plates. The activity of LDH in the aliquots was assayed by the conversion of tetrazolium salt into a red formazan product. The absorbance was recorded in a microplate reader at 492 nm. The extent of LDH leakage was expressed as the percentages of the absorbance of control.