Chemistry of Natural Compounds, Vol. 45, No. 2, 2009

TWO NOVEL PHENOLIC COMPOUNDS FROM *Stenoloma chusanum* AND THEIR ANTIFUNGAL ACTIVITY

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UDC 547.56

*Two new phenolic compounds, 4-*O*-β-D-(6-*O*-gentisoylglucopyranosyl) vanillic acid (**1**), 2-*O*-β-D-(6-*O*-gentisoylglucopyranosyl) gentisic acid (**2**), together with three known compounds, vanillic acid (**3**), syringic acid (**4**), and gentisic acid (**5**), were isolated from the whole part of* Stenoloma chusanum *(L.) Ching. Structures of the two new compounds **1**, **2** were elucidated on the basis of spectroscopic methods, including two-dimensional NMR techniques and HR ESI-MS analysis. The compounds′ activities against* Candida albicans, Cryptococcus neoformans, Trichophyton rubrum, Trichophyton mentagrophytes, Microsporum canis, Epidermophyton floccosum, *and* Aspergillus niger *were determined, and the minimal inhibitory concentrations (MIC) were 25–100 μg/mL.*

Key words: *Stenoloma chusanum* (L.) Ching, phenolic compounds, antifungal activity, MIC.

Stenoloma chusanum (L.) Ching (a wild fern) belongs to Pteridophyta Lindsaeae and is distributed throughout the south of the Changjiang River in P. R. China. It is a folk medicine in some provinces of China, and is used to cure anemopyretic cold, hepatitis, dysentery, food poisoning, pesticide poisoning, burn injury, and incised wound [1]. Extracts of *S. chusanum* have antibacterial and antifungal effects [2, 3].

In our previous study, orientin and vitexin were obtained from *S. chusanum* [4]. This paper describes the isolation and structure elucidation of two new compounds from this plant. The antifungal activities of the two new compounds and three known phenolic acids were also evaluated *in vitro*.

Compound **1** was obtained as a white needle crystal (in MeOH–H_2O) with melting point (mp) 228.3–232.7°C. It showed a dark-blue color with ferric chloride reagent. Compound **1** appeared to have the molecular formula $C_{21}H_{22}O_{12}Na$ by HR ESI-TOF MS analysis (obsd 489.10035, calcd 489.09969), corresponding to the molecular formula $C_{21}H_{22}O_{12}$, which was further confirmed by the ^1H NMR, ^{13}C NMR, and DEPT data (Table 1).

The ^1H NMR spectral data of compound **1** contained the signals of one carboxyl at δ 12.65 (1H, br), two phenolic hydroxyls at 9.34 (1H, br) and 9.74 (1H, br), one methoxyl at 3.80 (3H, s), and six aromatic protons from 6.80 to 7.46. ^1H NMR and ^{13}C NMR spectra showed the signals of a *β*-D-glucose residue (Table 1).

The ^1H–^1H COSY spectrum of compound **1** displayed the correlation of the aromatic proton at δ 7.45 (1H, dd, J = 8.4, 1.9 Hz) with 7.16 (1H, d, J = 8.5 Hz). The HMBC spectrum showed the long-range correlation between the proton at 7.45 and the carbon at 112.7, which are connected with the proton at 7.46 (d, J = 1.9 Hz) according to the HSQC spectrum. Based on the above, it can be deduced that three protons at 7.45, 7.16, and 7.46 constituted one ABX system, and that H (7.16, d, J = 8.5 Hz) is *ortho*-coupled with H (7.45, dd, J = 8.4, 1.9 Hz), which is *meta*-coupled with H (7.46, d, J = 1.9 Hz).

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 0009-3130/09/4502-0182 ©2009 Springer Science+Business Media, Inc.

TABLE 1. Data of ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), and DEPT for Compounds **1** and **2** (DMSO-d_6, TMS, δ, ppm, J/Hz)

C atom	Compound **1**			Compound **2**		
	δ_H	δ_C	DEPT	δ_H	δ_C	DEPT
1	-	124.2	C	-	124.8	C
2	7.46 (d, J = 1.9)	112.7	CH	-	146.1	C
3	-	148.3	C	7.13 (d, J = 8.5)	114.9	CH
4	-	150.0	C	7.27 (dd, J = 8.5, 2.1)	121.1	CH
5	7.16 (d, J = 8.5)	114.2	CH	-	148.8	C
6	7.45 (dd, J = 8.4, 1.9)	122.6	CH	7.35 (d, J = 2.1)	116.6	CH
7	-	166.8	C	-	166.8	C
3-OCH_3	3.80 s	55.5	CH_3	-	-	-
5-OH	-	-	-	8.95 br	-	-
7-COOH	12.65 br	-	-	12.52 br	-	-
1′	5.09 (d, J = 7.6)	99.3	CH	4.94 (d, J = 7.3)	100.8	CH
2′	3.34 m	73.0	CH	3.37 m	73.1	CH
3′	3.34 m	76.6	CH	3.37 m	75.7	CH
4′	3.29	69.7	CH	3.27	69.9	CH
5′	3.76	73.8	CH	3.76	73.9	CH
6′	4.18 (dd, J = 6.6, 11.9) 4.48 (dd, J = 1.8, 11.8)	63.4	CH_2	4.22 (dd, J = 6.9, 11.9) 4.52 (dd, J = 1.7, 11.9)	63.5	CH_2
Glucosyl-OH	5.20 br.s, 5.31 (d, J = 5.1), 5.36 br.s	-	-	5.20 (d, J = 4.7), 5.34 (d, J = 5.3), 5.41 (d, J = 3.9)	-	-
1″	-	120.4	C	-	120.5	C
2″	-	145.0	C	-	145.1	C
3″	6.80 (d, J = 8.3)	115.1	CH	6.82 (d, J = 8.3)	115.1	CH
4″	7.30 (dd, J = 8.3, 2.1)	121.7	CH	7.34 (dd, J = 7.8, 2.1)	121.7	CH
5″	-	150.4	C	-	150.5	C
6″	7.36 (d, J = 2.1)	116.3	CH	7.39 (d, J = 2.1)	116.3	CH
7″	-	165.4	C	-	165.4	C
2″-OH	9.74 br	-	-	9.77 br	-	-
5″-OH	9.34 br	-	-	9.34 br	-	-

Furthermore, the HSQC spectrum of compound **1** showed that the aromatic protons at 7.45, 7.16, and 7.46 are connected with the carbons at 122.6, 114.2 and 112.7, respectively. HMBC spectrum showed that the oxygenated sp^2 quaternary carbon at 150.0 is long-range correlated with protons at 7.16, 7.45, and 7.46, another oxygenated quaternary carbon at 148.3 correlated with protons at 7.46 and 7.16, and the quaternary carbon at 124.2 correlated with protons at 7.16 and 7.46. Those data suggested that six carbons at 122.6, 114.2, 112.7, 150.0, 148.3, and 124.2 existed in the same aromatic ring.

In addition, the HMBC spectrum of compound **1** showed that the carboxyl carbon at 166.8 is long-range correlated with the aromatic protons at 7.45 (dd, J = 8.4, 1.9 Hz) and 7.46 (d, J = 1.9 Hz), and the quaternary carbon at 148.3 is correlated with the methoxy protons at 3.80 (3H, s). So it can be reasoned that compound **1** contains a residue of 3-methoxy-4-hydroxy-benzoic acid, i.e., vanillic acid. Signals of protons and carbons of vanillic acid residue are assigned as in Table 1.

Likewise, the rest spectral signals of compound **1** established a gentisic acid residue based on the fact that the carbonyl carbon at 165.4 is long-range correlated with the aromatic proton at 7.36. Signals are assigned to the gentisic acid residue as in Table 1.

Because the HMBC spectrum of compound **1** showed the long-range correlation between the 4-$C_{\text{vanillic acid}}$ (δ 150.0) and 1′-H_{glucose} (δ 5.09), the vanillic acid residue must be located at the 1′-C position of the glucose residue. On the other hand, the long-range correlation of 7″-$C_{\text{gentisic acid}}$ (δ 165.4) with 6′-H_{glucose} (4.18, 4.48) implied that an ester bond formed between 7″-$C_{\text{gentisic acid}}$ and 6′-C_{glucose}. In the literature [5], compound **1** was named 4-*O*-*β*-D-(6-*O*-gentisoylglucosyl) vanillic acid.

TABLE 2. Antifungal Activity of Compounds **1–5**: Minimum Inhibitory Concentration, μg/mL

Fungi	Compound				
	1	**2**	**3**	**4**	**5**
Candida albicans	50	100	50	50	50
Cryptococcus neoformans	50	50	50	50	50
Trichophyton rubrum	50	-	50	50	50
T. mentagrophytes	100	-	100	100	100
Microsporum canis	25	100	25	50	50
Epidermophyton floccosum	50	-	25	25	50
Aspergillus niger	100	-	100	100	100

-: No effect at the highest test concentration of 100 μg/mL.

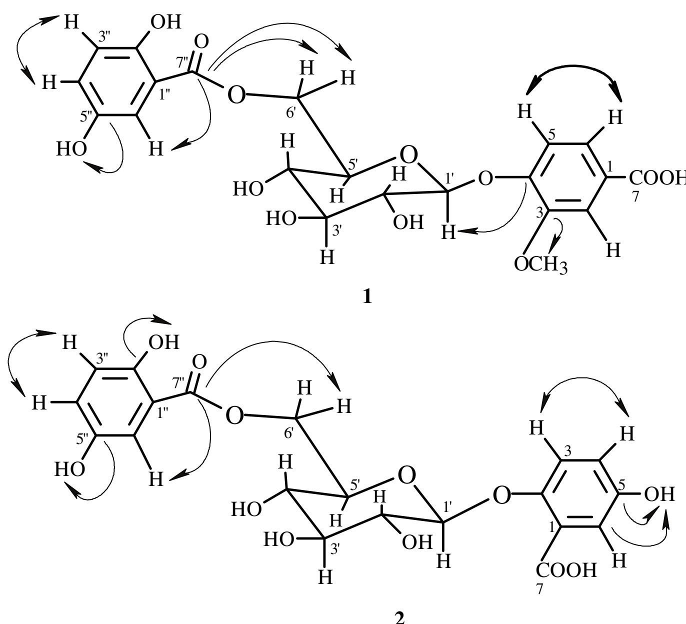

Fig. 1. Significant HMBC (→) and ^{1}H-^{1}H COSY (↔) correlations for compounds **1** and **2**.

The structure of compound **1** is displayed in Fig. 1.

Compound **2**, a white amorphous solid (in MeOH–H_2O), displayed a dark-blue color with ferric chloride reagent. Compound **2** revealed *quasi*-molecular ion peaks at *m/z* 451.1 [M-H]$^-$ and 475.0 [M+Na]$^+$ in the API-ES and appeared to have the molecular formula $C_{20}H_{20}O_{12}Na$ by HR ESI-TOF MS analysis (obsd 475.08470, calcd 475.08595). Its mp was 247.6–249.0°C.

The data of ^{1}H NMR, ^{13}C NMR, and DEPT for compound **2** in DMSO-d_6 are displayed in Table 1. Those signals were assigned according to the ^{1}H–^{1}H COSY, HSQC, and HMBC experiments.

The ^{1}H NMR spectrum of compound **2** showed one signal of carboxyl at δ 12.52 (1H, br), three signals of phenolic hydroxyl at 8.95 (1H, br), 9.34 (1H, br), and 9.77 (1H, br), and six signals of aromatic protons from 6.82 to 7.39. The ^{1}H NMR and ^{13}C NMR spectrum of compound **2** also displayed the signals of a *β*-D-glucose residue (Table 1).

The ^{1}H–^{1}H COSY spectrum of compound **2** revealed the correlation of the aromatic proton at δ 7.13 (d, J = 8.5 Hz) with the proton at 7.27 (dd, J = 8.5, 2.1 Hz), which are connected with the carbon at 121.1 according to HSQC spectrum. The carbon at 121.1 is correlated with the proton at 7.35 (1H, d, J = 2.1 Hz) from the evidence offered by HMBC spectrum. It can be deduced that the three aromatic protons at 7.13, 7.27, and 7.35 composed one ABX system. Also, the three aromatic protons at 7.13, 7.27, and 7.35 are connected with carbons at 114.9, 121.1, and 116.6, respectively, based on the data of the HSQC spectrum. Furthermore, it was shown in the HMBC spectrum of compound **2** that the carbonyl carbon at 166.8 is long-range correlated with the aromatic protons at 7.35 (d, J = 2.1 Hz) and 7.27 (dd, J = 8.5, 2.1 Hz), the quaternary carbon at 124.8 is

correlated with the aromatic proton at 7.13, the oxygenated quaternary carbon at 146.1 is correlated with the aromatic protons at 7.13 (d, J = 8.5 Hz) and 7.35 (d, J = 2.1 Hz), another oxygenated quaternary carbon at 148.8 is correlated with the aromatic protons at 7.27 and 7.35, as well as the phenolic hydroxyl proton at 8.95. The data described above suggested that a gentisic acid residue existed in compound **2**. Signals of protons and carbons of the gentisic acid residue are listed in Table 1.

The remainder signals in the ^{1}H NMR and ^{13}C NMR spectra of compound **2** were almost the same as those of the gentisic acid residue in compound **1**. The signals are shown in Table 1 too.

As the HMBC spectrum of compound **2** showed the long-range correlation between the 5-C (δ 148.8) and the phenolic hydroxyl proton at 8.95, it can be established that $5\text{-C}_{\text{gentisic acid}}$ is connected with a hydroxyl and that the glucoside bond is located between the $2\text{-C}_{\text{gentisic acid}}$ and the $1'\text{-C}_{\text{glucose}}$. Moreover, the long-range correlation between $7''\text{-C}_{\text{gentisic acid}}$ (165.4) and $6'\text{-H}_{\text{glucose}}$ (4.22) argue for an ester bond formed between $7''\text{-C}_{\text{gentisic acid}}$ and $6'\text{-C}_{\text{glucose}}$. Compound **2** was then concluded to be 2-*O*-*β*-D-(6-*O*-gentisoylglucosyl) gentisic acid.

Three known compounds, vanillic acid (**3**), syringic acid (**4**), and gentisic acid (**5**), were also isolated from *S. chusanum.* They were identified by comparison of the spectral data (^{1}H NMR, ^{13}C NMR and MS) with the literature.

The antifungal activities of compounds **1–5** were evaluated *in vitro* against fungi strains. Results were expressed in the minimum inhibitory concentration (MIC) (Table 2).

Compound **1** was the glucoside of compound **3**. It was found that compound **1** had less activity against *E. floccosum* than compound **3**. Their MIC was 50 and 25 μg/mL, respectively, though they had the same activities against the other six fungi (MIC were 25–100 μg/mL). Compound **2** was the glucoside of compound **5**. The inhibitory activity of compound **2** against the six fungi were all lower than (or equal to) that of compound **5**.

Ma *et al.* reported that simple phenolic constituents were found to be responsible for the fungitoxic activity, but their corresponding glycosides were not active against the fungus used in their experiment [6]. Perry *et al.* also showed that arbutin was inactive, but hydroquinone was antimicrobial and cytotoxic [7]. The lower antifungal activities of compounds **1** and **2** compared with **3** and **5** in our experiment were probably due to the formation of the glycosides of simple phenolic acid. It is reasonable to propose that simple fungitoxic phenolic compounds are released from the corresponding glycosides through the enzymatic hydrolysis caused by microbial invasion or herbivore attack on foliage [8], or due to the isolation procedures.

Three simple phenolic acids, namely compound **3–5**, had the same activity against *Candida albicans, Cryptococcus neoformans, Trichophyton rubrum, T. mentagrophytes*, and *Aspergillus niger*. The MIC values were 50–100 μg/mL. Compound **3** had greater potential activity (MIC 25 μg/mL) than **4** and **5** against *Microsporum canis*. Compounds **3** and **4** had greater activity (MIC 25 μg/mL) than **5** against *Epidermophyton floccosum*.

Friedman *et al*. employed different strains of *E. coli* and other bacteria to evaluate the bactericidal activities of 34 benzoic acids and 35 benzaldehydes and compared their activity-structure relationships [9]. Their results revealed that compounds with mixed OH- and OCH_3-groups exhibited variable results, i.e., in some cases OCH_3-groups enhanced activity and in other cases they did not. In our experiments, compound **4** had one more OCH_3 than compound **3**; the antifungal activity of compound **4** against *M. canis* was less than compound **3**, though they had the same activity against the other fungi.

EXPERIMENTAL

General Experimental Procedures. Melting points were measured on a Buchi Melting Point B-540 instrument and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker-400 instrument with TMS as internal standard. ESI-MS was obtained using an Autospec-UltimaETOF instrument. High-resolution (HR) ESI-TOF MS was obtained by Mariner Mass Spectrum. Polyamide (100–200 mesh) (Qingdao Ocean Chemical Group Co. of China), LH-20 (Pharmacia Biotech), and Lichroprep RP-18 (40–63 μm) (Merck) were used for column chromatography.

Six genus and seven species of fungi were employed in this experiment. They were obtained from China Committee of Culture Collection for Microorganisms (CCCCM): *Candida albicans* (CCCCM ID C.1a)*, Cryptococcus neoformans* (CCCCM ID D. 2w)*, Trichophyton rubrum* (CCCCM ID T.1a)*, Trichophyton mentagrophytes* (CCCCM ID T.1a)*, Microsporum canis* (CCCCM ID M.3d)*, Epidermophyton floccosum* (CCCCM ID E.1a), and *Aspergillus niger* (CCCCM ID A.3).

Plant Material. The plants were collected in Fuyang County, Zhejiang Province, China in November 2003 and were identified as *Stenoloma chusanum* (L.) Ching by Professor R. L. Guo at the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences. A voucher specimen (No. 542997) was deposited in the Herbarium of the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing, China.

Extraction and Isolation. Air-dried whole plant of *S. chusanum* (10 kg) was powdered and soaked with 70% ethanol at room temperature (15°C) for 7 days. After evaporating the ethanol, the extract was defatted with petroleum ether (60–90°C). Then the H_2O-soluble portion was chromatographed on a polyamide column (42 cm × 6 cm) using ethanol–H_2O (0–95%) as eluent. A total of 53 fractions was collected (500–600 mL each). Combined with TLC detection [polyamide-coated plastic film with MeOH–H_2O (4:1)], 8 pooled fractions (A-H) were given: tubes 1–8 fr A, 9–15 fr B, 16–20 fr C, 21–31 fr D, 32–37 fr E, 38–44 fr F, 45–49 fr G, 50–53 Fr H.

Fraction B was concentrated to 70 mL and then purified by LH20 gel filtration (60 cm × 2.0 cm) eluting with increasing concentrations of MeOH in H_2O (0–80%). A precipitate appeared in a part of the 40% MeOH–H_2O eluent. The precipitation was separated from the mother solution; then the solid was dissolved with H_2O and chromatographed on RP-18 column (15 cm × 1.6 cm) eluting with MeOH–H_2O (0–100%, volume of each 100 mL). Compound **3** (3 mg, elution volume 50 mL) and compound **4** (8 mg, elution volume 50 mL) were obtained in 20% eluent successively. The mother solution was purified by RP-18 column chromatography as in the above-mentioned method and yielded compound **5** (5 mg, elution volume 50 mL) in H_2O eluent.

Fraction C was concentrated to 30 mL and then purified by LH20 (60 cm × 2.0 cm) gel filtration using the same method described as in Fraction B. Compound **1** (8 mg, elution volume 100 mL) appeared in a part of the 70% MeOH–H_2O.

Fraction D was concentrated to 70 mL, 25 mL was taken out and purified by RP-18 column (15 cm × 1.6 cm) chromatography eluting with MeOH–H_2O to give compound **2** (10 mg, elution volume 30 mL).

Antifungal Susceptibility Testing. The minimum inhibitory concentration (MIC) was determined by the test tube dilution method on Sabouraud dextrose agar (SDA) slants contained no antibiotic. The five compounds were dissolved in ethanol and twofold dilution of the samples was carried out starting from the concentration of 1000 μg/mL (5% of ethanol). Series of sample solutions were mixed with SDA at the ratio of 1:9. The final concentrations of samples in the medium were 100, 50, 25, 12.5, 6.25, 3.125, 1.6, 0.8, 0.4, 0.2, 0.1, and 0.0 μg/mL. MIC was visually observed after 7 days of incubation at 26°C. The amount of inoculated fungi is $0.1–1.0 \times 10^6$ cfu/mL. Basal medium was used as reference simultaneously. When fungi developed in the basal medium, the treatment-developed fungi were marked "+" and those without fungi were marked "–". The minimum concentration of the treatment in which no fungi developed was the MIC and the unit was expressed as μg/mL.

Compound **1** (white needle crystal): mp 228.3–232.7°C. HR ESI-TOF MS (*m/z*): obsd 489.10035, calcd 489.09969 $[M+Na]^+$.

Compound **2** (white amorphous): mp 247.6–249.0°C. API-ES-MS (*m/z*): 451.1 $[M-H]^-$, 475.0 $[M+Na]^+$; HR ESI-TOF MS (*m/z*): obsd 475.08470, calcd 475.08595) $[M+Na]^+$.

ACKNOWLEDGMENT

The authors would like to thank Professor Yong-nian Shen in the Institute of Dermatology, Chinese Academy of Medical Sciences & Peking Union Medical College for assistance in antifungal susceptibility testing.

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