MRC Letters

Magnetic Resonance in Chemistry

Received: 24 January 2010 Revised: 24 April 2010 Accepted: 27 April 2010 Published online in Wiley Interscience: 27 May 2010

(www.interscience.com) DOI 10.1002/mrc.2623

Structure determination of selaginellins G and H from *Selaginella pulvinata* by NMR spectroscopy

Yuan Cao,[a] Ji-Jun Chen,[b] Ning-Hua Tan,[b] Yong-Ping Wu,[c] Jie Yang[a] and Qiang Wang[a]*

Selaginellins G (1) and H (2), two new selaginellin derivatives, were isolated from the whole plant of *Selaginella pulvinata*. Their structures were elucidated, and complete assignments of the ^{1}H and ^{13}C NMR spectroscopic data were achieved by 1D and 2D NMR experiments (HSQC, HMBC, COSY and ROESY). Compound 1 displayed good antifungal activity against *Candida albicans* with an IC_{50} value of 5.3 μg/ml.

Keywords: NMR; ^{1}H NMR; ^{13}C NMR; 2D NMR; selaginellin G; selaginellin H; *Selaginella pulvinata*; Selaginellaceae

Introduction

Selaginella pulvinata (Hook. et Grev.) Maxim. (Selaginellaceae) is a perennial herb widely distributed in China. As one of the two qualified species listed in Chinese Pharmacopoeia (2005 edition),[1] it has been widely used in traditional Chinese medicine for the treatment of cancer, cardiovascular problems and skin diseases.[2] Our previous phytochemical research on the whole plant of *S. pulvinata* revealed the presence of a series of interesting selaginellin derivatives,[3] whose structural novelty and biological promise stimulated our interest in searching for their potential biogenetic precursor. Continued investigation of the title plant led to the isolation of two new selaginellin derivatives, selaginellins G (**1**) and H (**2**), together with five known biflavonoids and a flavone. In addition, all isolated compounds were tested for antimicrobial activities against *Staphylococcus aureus* and *Candida albicans*. Herein, we report the isolation and structural elucidation of these new compounds, as well as the evaluation of their bioactivity.

Results and Discussion

Selaginellin G (**1**) was obtained as pale yellow crystals from methanol. Its high-resolution electrospray ionization mass spectrometry (HR-ESIMS) exhibited an $[M + Na]^{+}$ ion at *m/z* 523.1525 (calcd *m/z* 523.1521), corresponding to a molecular formula of $C_{33}H_{24}O_5$, with 22 degrees of unsaturation. The UV spectrum (λ_{max} 242, 333, 340 nm) suggested the presence of a conjugated system. The IR spectrum showed absorption bands for hydroxy (3422 cm^{-1}), α,β-unsaturated carbonyl (1630 cm^{-1}) and aromatic (1598, 1513 cm^{-1}) functionalities.

The ^{1}H and ^{13}C NMR spectra (Table 1) of compound **1** showed similarities to those of selaginellin.[3,4] In particular, the presence of an ABC spin system at δ_H 7.20 (2H, d, J = 7.6 Hz) and δ_H 7.38 (1H, t, J = 7.6 Hz), originating from *ortho*-trisubstituted A-ring, and four AA′XX′ systems at δ_H 6.42 (4H, d, J = 8.5 Hz), 6.98 (4H, d, J = 8.5 Hz), 6.55 (2H, d, J = 8.6 Hz), 6.97(2H, d, J = 8.6 Hz), 6.66 (2H, d, J = 8.6 Hz) and 7.34 (2H, d, J = 8.6 Hz), representing the protons of the respective 1,4-disubstituted B-, C-, D- and E-ring systems, were indicative of the skeleton of a selaginellin derivative. However, further comparison of its NMR data with those of selaginellin revealed that the two carbons of the triple bond in the latter were converted into a ketonic carbon (δ_C 192.1, C27) and an olefinic carbon (δ_C 123.5, C26), connected to a deshielded olefinic proton at δ_H 7.03. The downfield shifts for the two aromatic protons (H28/32) indicated that the carbonyl moiety was attached to C33 (B-ring). This was further supported by HMBC correlations between H28/32 (B-ring) and C27. Similarly, an exocyclic alkene was positioned at C14 (A-ring), as evident from the HMBC experiment. For instance, H26 exhibited ^{2}J and ^{3}J correlations to C14 (δ_C 142.6), C15 (δ_C 129.8), C19 (δ_C 139.2), C27 and C33 (δ_C 131.8), whereas H16 showed ^{3}J coupling to C14. The C- and D-rings were connected via C7, as deduced from HMBC correlations of H3/5 and H8/12 to C7. The E-ring was found to reside at C18, based on correlations from H20/24 to C18, and H17 to C25; thus, C7 could only be located next to C19 in the A-ring. The *E*-configuration of the double bond between C14 and C26 was established by the correlation between H26 and H5/12 in the ROESY spectrum. Chemical equivalence of the 1,4-disubstituted phenolic rings C and D as follows from the NMR spectral data in Table 1 is not normally expected, due to free rotation around C4–C7 and C7–C13 bonds.

* *Correspondence to: Qiang Wang, Department of Chinese Materia Medica Analysis, China Pharmaceutical University, No. 24, Tongjia Lane, Nanjing 210009, People's Republic of China. E-mail: qwang1949@126.com*

a *Department of Chinese Materia Medica Analysis, China Pharmaceutical University, Nanjing 210009, People's Republic of China*

b *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, People's Republic of China*

c *Jiangsu Provincial Institute of Materia Medica, Nanjing 210009, People's Republic of China*

Table 1. NMR spectral data of compounds **1** and **2** (δ in ppm, J in Hz)

Position	**1** (CD_3OD)			**2** (CD_3OD)		
	δ_C (DEPT)	δ_H	HMBC H → C	δ_C (DEPT)	δ_H	HMBC H → C
1	157.2 (C)	–	–	158.8 (C)	–	–
2/6	115.2 (CH)	6.42 (d, 8.5)	1, 4	115.5 (CH)	6.61 (d, 8.7)	1, 4
3/5	131.2 (CH)	6.98 (d, 8.5)	1, 4, 7	131.2 (CH)	6.81 (d, 8.7)	1, 4, 7
4	134.6 (C)	–	–	131.4 (C)	–	–
7	155.6 (C)	–	–	95.0 (C)	–	–
8/12	131.2 (CH)	6.98 (d, 8.5)	7, 10, 13	131.2 (CH)	6.81 (d, 8.7)	7, 10, 13
9/11	115.2 (CH)	6.42 (d, 8.5)	10, 13	115.5 (CH)	6.61 (d, 8.7)	10, 13
10	157.2 (C)	–	–	158.8 (C)	–	–
13	134.6 (C)	–	–	131.4 (C)	–	–
14	142.6 (C)	–	–	123.4 (C)	–	–
15	129.8 (CH)	7.20 (d, 7.6)	14, 16, 17, 19, 26	143.0 (C)	–	–
16	128.2 (CH)	7.38 (t, 7.6)	14, 15, 17, 18	128.7 (C)	7.75 (d, 7.7)	14, 18, 19, 26, 27
17	129.8 (CH)	7.20 (d, 7.6)	15, 16, 18, 19, 25	138.9 (C)	7.45 (d, 7.7)	15, 16, 19, 25
18	142.6 (C)	–	–	139.5 (C)	–	–
19	139.2 (C)	–	–	152.4 (C)	–	–
20/24	130.5 (CH)	6.97 (d, 8.6)	18, 22, 25	131.9 (CH)	6.48 (d, 8.7)	18, 22, 25
21/23	115.8 (CH)	6.55 (d, 8.6)	22, 25	115.3 (CH)	6.45 (d, 8.7)	22, 25
22	159.7 (C)	–	–	158.0 (C)	–	–
25	133.8 (C)	–	–	130.8 (C)	–	–
26	123.5 (CH)	7.03 (s)	14, 15, 19, 27, 33	60.7 (CH_2)	5.18 (s)	14, 15, 16
27	192.1 (C)	–	–	172.0 (C)		
28/32	131.5 (CH)	7.34 (d, 8.6)	27, 30, 33		–	–
29/31	115.9 (CH)	6.66 (d, 8.6)				
30	163.3 (C)	–	–			
33	131.8 (C)	–	–			

On the basis of the above spectral data, the structure of selaginellin G (**1**) was unambiguously established as (*E*)-2-(6-(bis(4-hydroxyphenyl)methylene)-5-(4-hydroxyphenyl) cyclohexa-2,4-dienylidene)-1-(4-hydroxyphenyl)ethanone (Fig. 1). The full assignments of the ^1H and ^{13}C NMR data of compound **1** (Table 1) were achieved in combination with HMBC, HSQC, COSY and ROESY experiments.

Selaginellin H (**2**) was obtained as off-white crystals from methanol. Its molecular formula was determined as $C_{27}H_{20}O_6$ according to HR-ESIMS. UV spectroscopy (λ_{max} 230 nm) indicated the presence of the double bond. NMR data for compound **2** resembled those for **1**. The key differences included the absence of one 1,4-disubstituted phenolic ring, the olefinic CH group and the ketone group, whereas one additional CH_2OH group, one additional quaternary carbon at δ_C 95.0 (C7) and one ester carbonyl carbon at δ_C 172.1 (C27) were observed, suggesting the presence of a lactone functionality. This new lactone moiety added another degree of unsaturation satisfying the unsaturation degree of 18. However, there are two possible modes of formation of the lactone ring. One mode involves the linkage between C7 and C14, the other involves that between C14 and C15. A free OH was observed at δ_H 5.37 (1H, brs) and located at C26 based on its correlation to H_2-26 in the COSY spectrum, thus excluding the possibility of a C14–C15 linkage. The presence of the oxygenated quaternary carbon C7 also confirmed that the γ-lactone adopted the C7–O–C27–C14 linkage. Consequently, the structure of selaginellin H (**2**) was assigned as 7-(hydroxymethyl)-3,3,4-tris(4-hydroxylphenyl)isobenzofuran-1(3H)-one (Fig. 1). The full assignments of the ^1H and ^{13}C NMR data of compound **2** (Table 1) were achieved in combination with HMBC, HSQC, COSY and ROESY experiments.

Structurally, compounds **1** and **2** might be regarded as the biogenetic precursors of other known selaginellins.[3,4]

A comparison of the spectroscopic and physical data with those published allowed us to establish the structures of the known compounds as ginkgetin, isoginkgetin,[5] isocryptomerin, hinokiflavone, amentoflavone[6] and apigenin.[7] All known compounds except amentoflavone and apigenin were isolated from *S. pulvinata* for the first time.

All isolated compounds were evaluated for antimicrobial activity against *S. aureus* and *C. albicans*. Only compound **1** displayed antifungal activity against *C. albicans* with an IC_{50} value of 5.3 μg/ml.

Experimental

General procedures

Melting points were determined on an XT-4 micromelting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1020 polarimeter. UV spectra were recorded on a Shimadzu 2401-PC spectrophotometer. IR spectra were acquired on a Bruker Tensor 27 FT-IR spectrometer. 1D and 2D NMR spectra were recorded on Bruker AV-400 and AV-500 spectrometers. MS were measured on a VG Auto Spec-3000 mass spectrometer. Column chromatography was performed over silica gel H (200–300 mesh and 10–40 μm) and Sephadex LH-20 (40–70 μm; Amersham Pharmacia Biotech AB, Uppsala, Sweden). TLC was conducted on precoated silica gel plates GF_{254} (Qingdao Marine Chemical Ltd., Qingdao, People's Republic of China).

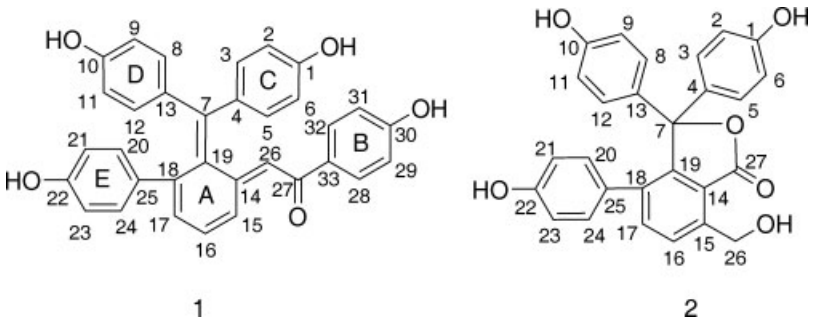

Figure 1. Structures of selaginellins G (**1**) and H (**2**).

Plant material

S. pulvinata was collected from Yunnan Province, P.R. China in October 2007. The taxonomic identification was established by Dr Prof. Qiang Wang, Department of Pharmacognosy, China Pharmaceutical University, where a voucher specimen (2007-10-018) was preserved.

NMR spectroscopy

All NMR experiments were recorded on a Bruker AV-500 spectrometer operating at 500 and 125 MHz for ^{1}H and ^{13}C, respectively, using standard pulse sequences supplied by the manufacturer. The spectrometer was equipped with a QNP 5-mm probe (^{1}H 90° pulse width = 10.0 μs, ^{13}C 90° pulse width = 9.4 μs), and detections were carried out at 298 K using approximately 6 mg of compounds **1** and **2**. Chemical shifts are given in ppm, referenced to residual solvent signals. Coupling constants (*J*) are expressed in Hertz.

1D spectra were acquired using 32 K data points and spectral widths of 8993 and 23 585 Hz for ^{1}H and ^{13}C, respectively; 32 K data points were used for the processing with an exponential function (LB = 0.4 for ^{1}H NMR, LB = 1.0 for ^{13}C NMR).

The 2D spectra used 1024 × 128 (COSY), 1024 × 128 (ROESY), 1024 × 128 (HSQC) and 1024 × 512 (HMBC) data point matrices, which were zero filled to 1024 × 512, 1024 × 1024, 1024 × 1024 and 2048 × 1024, respectively. A relaxation delay of 2.0 s was used in all experiments. The HSQC data were acquired with two transients per *t*1 increment. Spectral widths of 12 575 and 3005 Hz were used for ^{13}C and ^{1}H, respectively. The HMBC experiment was performed with 40 transients per *t*1 increment. Spectral widths of 22 637 and 4496 Hz were used for ^{13}C and ^{1}H, respectively. For COSY and ROESY, a spectral width of 3205 Hz was used in both dimensions. The number of transients collected for each time increment in the indirect dimension was 2 and 8 for ^{1}H–^{1}H COSY and ROESY, respectively. The mixing time for ROESY was 800 ms.

Extraction and isolation

The air-dried whole plant of *S. pulvinata* (6 kg) was cut into small pieces and extracted with 95% EtOH. The ethanolic extract was combined and concentrated under reduced pressure to yield a brown residue (380 g) which was defatted with petroleum ether (PE, 60–90 °C; 6 l, 3×) and then successively extracted with EtOAc (6 l, 3×) and *n*-BuOH (6 l, 3×). The EtOAc extract (48 g) was fractionated using silica gel column chromatography with gradient elution ($CHCl_3$–MeOH, 100 : 0 to 20 : 80) to yield eight fractions. Fraction 5 (3.8 g) was further separated using silica gel column chromatography and produced seven subfractions (5.1–5.7). Subfraction 5.2 (36 mg) was repeatedly chromatographed over silica gel to afford **2** (6.8 mg). Compound **1** (18 mg) was obtained from subfraction 5.5 (61 mg) after repeated purification with Sephadex LH-20 (MeOH and MeOH–H_2O, 7 : 3).

*Selaginellin G (**1**)*

Pale yellow crystals (MeOH); mp 194–195 °C; $[\alpha]_D^{23}$ −3.2 (*c* 0.14, MeOH); UV (MeOH) λ_{max} (log ε) 242 (1.72), 333 (1.41), 340 (1.41) nm; IR (KBr) ν_{max} 3422, 1630, 1598, 1513, 1218, 835 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HR-ESIMS *m/z* 523.1525 $[M + Na]^+$ (calcd 523.1521 for $C_{33}H_{24}O_5Na$).

*Selaginellin H (**2**)*

Off-white crystals (MeOH); mp 170–171 °C; $[\alpha]_D^{23}$ −1.6 (*c* 0.25, MeOH); UV (MeOH) λ_{max} (log ε) 230 (1.89) nm; IR (KBr) ν_{max} 3414, 2924, 2854, 1728, 1612, 1597, 1514, 1230, 1174, 833 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HR-ESIMS *m/z* 463.1171 $[M + Na]^+$ (calcd 463.1157 for $C_{27}H_{20}O_6Na$).

In vitro antimicrobial bioassay

The antimicrobial activity assay was performed against *S. aureus* and *C. albicans* by turbidimetric method with ampicillin (Keygen Biotech Co. Ltd, Nanjing, China) and miconazole nitrate salt (Sigma, Cat. No. M-3512) as positive control, respectively. All organisms were obtained from China General Microbiological Culture Collection Center and included *S. aureus* CGMCC 1.2465 and *C. albicans* CGMCC 2.2086. Inocula were prepared by correcting the OD_{620} of microbe suspensions in incubation broth [Mueller Hinton Broth (Oxiod, CM 0405, Hampshire, England) for *S. aureus*, and PDA broth (potato extract/2% dextrose/2% agar) for *C. albicans*] to McFarland standard 0.5 and diluted with medium to 1×10^6 CFU/ml. Aliquots of 90 μl were filled in 96-well U-bottomed microplate. Ten microliters of sample, dissolved in DMSO and diluted with the medium, was dispensed in the wells with a final concentration of 10 μg/ml. After cultivation at 37 °C for 24 h, absorbance was measured at 620 nm with the microplate reader (Molecular Devices, SPECTRA MAX 340, Silicon Valley, CA, United States). The level of inhibition was calculated as the percentage of maximum absorbance (0.04% DMSO control) after adjusting for minimum absorbance (medium control).

Acknowlegements

We thank Dr Lukas Oberer (Novartis Pharma AG) for technical assistant, and the members of the analytical group (State Key

Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese academy of Sciences) for measurements of the spectra. We are also indebted to Mr M. X. Lai (Guangxi Academy of Chinese Medicine and Pharmaceutical Science) for collecting plant material.

References

[1] Chinese Pharmacopoeia Commission, in *Pharmacopoeia of the People's Republic of China*, vol. 1, People's Medical Publishing House: Beijing, **2005**, p 154.

[2] Jiangsu New Medical College, in *Zhong Yao Da Ci Dian*, Shanghai Science and Technology Press: Shanghai, **2004**, pp 1472.

[3] Y. Cao, J. J. Chen, N. H. Tan, L. Oberer, T. Wagner, Y. P. Wu, G. Z. Zeng, H. Yan, Q. Wang, *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2456.

[4] L. P. Zhang, Y. M. Liang, X. C. Wei, D. L. Cheng, *J. Org. Chem.* **2007**, *72*, 3921.

[5] P. Y. Sun, Y. Xu, Y. Wen, Y. P. Pei, Y. J. Chen, *Chin. J. Med. Chem.* **1998**, *8*, 122.

[6] G. L. Silva, H. Chai, M. P. Gupta, N. R. Fransworth, G. A. Cordell, J. M. Pezzuto, C. W. W. Beecher, A. D. Kinghorn, *Phytochemistry* **1995**, *40*, 129.

[7] X. Zheng, D. F. Liao, B. Y. Zhu, Q. H. Tuo, Y. L. Xu, *Zhongcaoyao* **2001**, *32*, 17 (C.A. **2002**, *135*, 43484).

