Antitumor Agents. 261. 20(*S*)-Protopanaxadiol and 20(*S*)-Protopanaxatriol as Antiangiogenic Agents and Total Assignment of ^{1}H NMR Spectra[#]

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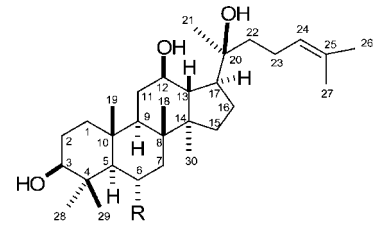
Angiogenesis is a critical step in tumor progression and involves several steps including endothelial cell (EC) proliferation, migration, and matrix remodeling. We investigated the antiangiogenic effects of 20(*S*)-protopanaxadiol (**1**) and 20(*S*)-protopanaxatriol (**2**), the sapogenins of two major ginseng saponins, in an angiogenesis model of human umbilical vein endothelial cells (HUVECs). These compounds inhibited the proliferative activity of HUVECs in a dose-dependent manner and have potential as anticancer drug candidates. In addition, we report the complete and unambiguous assignment of ^{1}H NMR spectra of **1** and **2**, based on analyses of 2D NMR spectra including COSY, NOESY, HSQC, and HMBC. This report is the first to completely assign the ^{1}H NMR signals of **2**, together with correction of data for **1** from prior reports.

The roots of *Panax* species (e.g., *P. ginseng* C. A. Meyer, *P. quinquefolium* L.) have long been prized as a panacea in Chinese medicine. In 500 A.D., ginseng was ranked as an upper grade medicine, meaning it is nontoxic and can be used as a tonic to maintain physical vitality, in *Shennong Bencao Jing*, a Chinese compilation of medicinal herbs.[1,2] People in the Far East, especially in China, Hong Kong, Taiwan, and Korea, use ginseng as a dietary supplement to improve health and vitality. Numerous studies have investigated ginseng's immunomodulatory effects, cancer chemopreventive action, and anticancer activity,[1–7] but various biological activities of ginseng remain unclear.[8,9]

The pharmacologically active constituents of *Panax* species are a group of dammarane-type triterpene saponins known as ginsenosides. Shibata et al. performed the first isolation and structural characterization studies on ginsenosides and their corresponding aglycons (sapogenins).[10–14] The most abundant ginsenosides can be subdivided into two classes on the basis of the aglycons, 20(*S*)-protopanaxadiol (**1**) and 20(*S*)-protopanaxatriol (**2**).[15–17] The structural difference between **1**-ginsenosides (e.g., Rb1, Rb2, and Rg3) and **2**-ginsenosides (e.g., Re and Rg1)[18] is the presence of an additional OH group at C-6 in the latter compound class. Recent studies have demonstrated that most ginsenosides are metabolized in the gastrointestinal tract through a series of deglycosylation steps into active aglycon derivatives, including **1** and **2**.[19,20]

Tanaka and co-workers first assigned the ^{13}C NMR spectra of **1** and **2** in 1977.[21] However, only an incomplete ^{1}H NMR assignment of **1** has been reported,[22,23] and a ^{1}H NMR assignment of **2** is not found in the literature. Because complete and confident assignment of spectroscopic data is crucial for confirming chemical structures and evaluating purity of natural products, as well as synthetic derivatives, we undertook a thorough spectroscopic analysis of authentic aglycons **1** and **2**, which were obtained by hydrolysis of an extract of American ginseng. Herein, we present a complete assignment of ^{1}H NMR signals of **2**, together with reassignment of those of **1**, on the basis of extensive analysis of DEPT and 2D-NMR spectra, including COSY, NOESY, HSQC, and HMBC.



1, 20(*S*)-Protopanaxadiol: R = H
2, 20(*S*)-Protopanaxatriol: R = OH

In addition, our laboratories are quite interested in new drug development, including antiangiogenic agents. Angiogenesis, the growth of new vascular capillary channels from pre-existing vessels, is responsible for the progression of solid tumors, diabetic retinopathy, psoriasis, and rheumatoid arthritis and is also of fundamental importance in numerous physiological processes such as embryonic development and wound healing. In tumor progression, angiogenesis involves several steps including endothelial cell (EC) proliferation, migration, and matrix remodeling.[24,25] Studies have shown that various **1**-ginsenosides (ginsenosides Rb2 and Rg3) can inhibit tumor-induced angiogenesis.[26–28] However, it was also demonstrated that a ginseng extract with predominance of Rg1 (a **2**-ginsenoside) promoted angiogenesis, whereas a predominance of Rb1 (a **1**-ginsenoside) exerted an opposing effect (antiangiogenesis).[29] Therefore, in the current study, we investigated the antiangiogenic effects of aglycons **1** and **2**, using human umbilical vein endothelial cells (HUVECs) as a model, and demonstrated that **1** and **2** exerted antiangiogenic activity at low concentrations by inhibiting the proliferation of HUVECs.

The 1D (^{1}H and ^{13}C NMR, DEPT) and 2D (COSY, NOESY, HSQC, and HMBC) spectra of purified **1** and **2** were measured in pyridine-d_5 (Table 1) and $CDCl_3$ (Table 2). Complete assignments of ^{1}H NMR signals are presented in Tables 1 and 2, together with ^{13}C NMR signals.[21] NOESY and HMBC cross-peaks are also shown in the tables.

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Table 1. NMR Data of **1** and **2** in Pyridine-d_5[d]

	1				**2**			
position	δH (*J* in Hz)	δC	HMBC	NOESY	δH (*J* in Hz)	δC	HMBC	NOESY
1	1.65[a]	39.4, t	9		1.69[b], m	39.4[a], t	3, 5	6
	0.96[a]				1.05[c], m		3	
2	1.83[a]	28.3, t	1		1.93[a]	28.2, t	1, 4	
	1.83[a]				1.88[a]		1, 4	
3	3.45, dd; (11.0,5.0)	78.0, d	4, 28, 29		3.55, ddd (11.4,5.0,4.6)	78.4, d	28, 29	5, 28
4		39.6, s				40.4, s		
5	0.85, dd (11.7,1.8)	56.4, d	1, 6, 7, 9, 10, 28, 29	9, 28	1.22, d (9.5)	61.8, d	3, 4, 6, 7, 9, 19, 28, 29	1[c], 3, 9, 28
6	1.59[a]	18.8, t	8	18	4.40, dddd (10.5,9.5,6.6,3.9)	67.7, d	4, 5	18, 19, 29
	1.48[a]		8, 10					
7	1.55[a]	35.2, t	5, 8, 9, 14, 18		1.98[a]	47.6, t	5, 6, 14, 18	
	1.28[a]		5, 6, 8, 9		1.91[a]		5, 6, 9, 18	
8		40.1, s				41.2, s		
9	1.50[a]	50.5, d	8, 10, 11, 18	5, 30	1.60[a]	50.1, d	8, 12	5
10		37.4, s				39.4[a], s		
11	2.12[a]	32.1, t	8, 9, 13		2.17[b], br dd (9.6,5.0)	32.1, t	8, 12, 13	
	1.57[a]		8		1.59[a,c]		8	
12	3.94, br td (10.8,7.1)	71.1, d	13		3.95, m	71.1, d	17, 30	
13	2.08[a]	48.6, d	8, 11, 12, 16, 17, 20		2.06[a]	48.3, d	8, 11, 12, 14, 16, 17	18
14		51.7, s				51.7, s		
15	1.62[a]	31.4, t	14, 30		1.62[a]	31.4, t	30	
	1.08[a]		14		1.06[a]		13, 30	
16	1.91[a]	26.9, t	13, 15, 17		1.89[a]	26.9, t	13, 17	
	1.41[a]				1.40[a]		14, 20	
17	2.37, td (10.8,7.1)	54.8, d	13, 16, 22	30	2.36, td (10.8,7.3)	54.8, d	12, 13, 20, 21, 22	30
18	1.02	15.9, q	7, 8, 9, 14	6	1.13	17.5, q	8, 9, 14	13, 29
19	0.90	16.5, q	1, 5, 9, 10	29	1.02	17.6, q	1, 5, 9	18, 29
20		72.9, s				73.0, s		
21	1.45	27.1, q	17, 20, 22		1.43, s	27.1, q	20	
22	2.07[a]	35.9, t	23		2.05[a]	35.9, t	20, 23	
	1.73, td (13.0,5.5)		23		1.71, m		20	
23	2.63, m	23.0, t	21, 24, 25	21, 27	2.63, m	23.0, t	22, 24, 25	27
	2.30. m		21, 22, 24, 25	21, 27	2.30, m		22, 24, 25	27
24	5.34, br t (7.1)	126.3, d	23, 26, 27	23, 23′, 26	5.32, br t (7.1)	126.3, d	25, 26, 27	26
25		130.8, s				130.8, s		
26	1.67, s	25.8, q	24, 25, 27	24	1.67, s	25.8, q	24, 25, 27	
27	1.64, s	17.7, q	24, 25, 26	23, 23′	1.64, s	17.7, q	23, 24, 25, 26	23, 23′
28	1.25, s	28.7, q	3, 4, 5, 29		2.01, s	32.0, q	3, 4, 5, 29	24
29	1.05, s	16.3, q	3, 4, 5, 28	19	1.47, s	16.5, q	3, 4, 5, 28	19
30	0.95, s	17.1, q	8, 14, 15	17	0.98, s	17.1, q	8, 13, 14, 15	12, 17
3-OH	5.78, br s				5.74, br d (4.6)		3, 4	
6-OH					5.26, d (6.6)		5, 6	
12-OH	7.26, br s			12	7.27, br d (1.6)		11, 13	
20-OH	7.00, br s				6.99, br s		17, 20, 21, 22	

[a] Overlapping signals, [b] Equatorial, [c] Axial. [d] br = broad, d = doublet, m = multiplet, q = quartet, s = singlet, t = triplet.

The 26-Me and 27-Me protons of **2** were confirmed from the presence of NOESY cross-peaks between H-26/H-24 and H-27/H-23_A, H-23_B protons. Likewise, 28-Me and 29-Me protons were confirmed on the basis of NOESY cross-peaks between H-3/H-28 and H-19/H-29 protons. Other important NOESY correlations in **2** are illustrated in Tables 1 and 2 and are consistent with the dammarane skeleton.

In the same manner, the proton signals of **1** in pyridine-d_5 were reassigned, and the revised data are listed in Table 1. NOESY and HMBC correlations are also shown in Table 1. The 29-Me and 19-Me assignments in the prior study[22] were corrected, and the revised assignments agreed with those in another partial assignment reported in the literature.[23]

The assignments of **1** and **2** in $CDCl_3$ are also presented in Table 2, together with important NOESY and HMBC correlations. These standard data should be significantly useful, because $CDCl_3$ is a more widely used deuterated NMR solvent. Generally, the differences between the two solvents were not excessive. However, the chemical shifts of H-28 in **2** were quite different, δ 2.01 in pyridine-d_5 and δ 1.32 in $CDCl_3$.

In summary, we achieved the first complete assignment of the ^{1}H NMR spectroscopic data of **2**, together with reassignment of the ^{1}H NMR of **1** and ^{13}C NMR of both compounds on the basis of modern NMR techniques.

Because ginsenosides are known to be metabolized in the gastrointestinal system into aglycon derivatives, the antiangiogenic activity of ginsenosides could be attributed to their aglycons. Consequently, we evaluated the effects of agyclons **1** and **2** on HUVEC proliferation. Both compounds significantly inhibited proliferation in a dose-dependent manner after 72 h, with EC_{50} values of 2.16 and 6.64 μg/mL, respectively. At the highest dose tested (20 μg/mL), the number of viable cells was reduced to 16.5–27.5% of control. The results show that both aglycons possess antiangiogenic activity and that **1** is more potent than **2**.

Most solid tumors are dependent on neovascularization to supply nutrients to the growing tumor; therefore, angiogenesis inhibition is a novel target for anticancer therapy. Recently, it was shown that **1** can induce different forms of programmed cell death, including both typical apoptosis and autophagy through both caspase-dependent and-independent mechanisms.[30] These results and those from our present study suggest that **1** and **2** represent potential lead compounds for further structural modification targeting anticancer drug development. In conclusion, these studies provide a basis for further development of novel antiangiogenesis agents as anticancer drugs. We are currently conducting the evaluation of various dammarane-type triterpenoids, including derivatives of **1** and **2**.

Table 2. NMR Data of **1** and **2** in $CDCl_3$

	1				**2**			
position	δH (*J* in Hz)	δC	HMBC	NOESY	δH (*J* in Hz)	δC	HMBC	NOESY
1	1.72[a]	38.9[a], t	2, 5		1.72, m	38.7, t	2, 3, 5	
	1.01[a]		2		1.04, m		3	
2	1.63[a]	27.4, t			1.65[a]	27.0, t		
	1.60[a]				1.60[a]			
3	3.20, dd (10.4,7.4)	78.9, d	1, 4, 28, 29	5, 28	3.19, dd (11.9,5.0)	78.6, d	2, 4, 28, 29	5, 28
4		38.9[a], s	28, 29			39.2, s		
5	0.73, d (11.4)	55.8, d	6, 7, 9, 10, 19, 28, 29	3, 9, 28	0.98, d (10.5)	61.1, d	3, 4, 6, 9, 7, 19, 28, 29	3, 28
6	1.55[a]	18.3, t	5, 10		4.12, td (10.3,3.9)	68.7, d	5, 4, or 10	18, 19, 29
	1.46[a]		5					
7	1.49[a]	34.8, t	5, 6		1.60[a]	47.0, t		
	1.30[a]		5, 6, 8, 9		1.56[a]			
8		39.7, s				41.0, s		
9	1.42[a]	50.0, d	7, 8, 10, 11	5, 12	1.44, dd (13.0,2.0)	49.5, d		
10		37.1, s				39.3, s		
11	1.84[a]	31.0, t	8		1.88[a]	30.93[b], t	12, 13	
	1.26[a]		12		1.23[a]			
12	3.60, td (10.3,5.0)	70.9, d	9, 17	9, 17, 30	3.59, td (10.3,5.3)	70.7, d	13, 17	9, 17, 30
13	1.74[a]	47.8, d	11, 14, 15, 17		1.72[a]	47.5, d	8, 12, 13, 14, 15, 16, 17, 20	
14		51.6, s				51.4, s		
15	1.51[a]	31.0, t	14, 16		1.52[a]	31.0, t		
	1.04[a]		13, 14, 17		1.05[a]		13, 17, 30	
16	1.85[a]	26.5, t	13, 17		1.87[a]	26.4, t	12, 13, 14	
	1.26[a]		13, 14, 17		1.27[a]			
17	2.03, td (10.8,7.3)	53.4, d	13, 16	12	2.04, td (10.7,7.4)	53.3, d	12, 13, 16, 20, 22	
18	0.99	15.7, q	7, 8, 9, 14	19	1.07, d (0.6)	17.3, q	7, 8, 9, 14	6, 19
19	0.88	16.1, q	5, 7, 9	18, 29	0.94, s	17.2, q	1, 5, 9, 10	6, 18, 29
20		74.6, s				74.7, s		
21	1.20	27.1, q	16, 17, 20	23, 23′	1.20, s	27.1, q		
22	1.66[a]	34.4, t	20		1.68[a]	34.2, t		
	1.42[a]		20		1.41, ddd (13.8,10.3,5.5)			
23	2.17	22.4, t	20, 22, 24, 25	21, 27	2.17, m	22.4, t	22, 24, 25	27
	2.03[a]		20, 22, 24, 25	21, 27	2.05, m		22, 24, 25	27
24	5.17, br t (7.1)	124.9, d	22, 23, 26, 27	26	5.16, br t (6.7)	124.7, d	22, 23, 26, 27	22, 23, 26
25		132.0, s				132.1, s		
26	1.70, s	25.8, q	24, 25	24	1.69, s	25.8, q	24, 25	24
27	1.64, s	17.6, q	24, 25	23, 23′	1.64, s	17.8, q	24, 25	23, 23′
28	0.98, s	28.0, q	3, 4, 5, 29	3, 29	1.32, s	30.87[b], q	3, 4, 5, 29	3, 5
29	0.78, s	15.3, q	3, 4, 5, 28	19, 29, 30	0.99, s	15.5, q	3, 4, 5, 28	6, 19
30	0.89, s	16.8, q	8, 13, 14, 15	12	0.91, s	16.8, q	8, 13, 14, 15	12

[a] Overlapping signals. [b] Shifts recorded to 0.01 ppm to distinguish C-11 and C-28.

Experimental Section

General Experimental Procedures. The aglycons (**1** and **2**) were prepared by alkaline hydrolysis of an extract of American ginseng (*P. quinquefolium* L.) according to literature procedures.[23,31] The hydrolyzed saponin mixture was separated using preparative TLC (CH_2Cl_2/MeOH, 95:5) followed by reversed-phase HPLC [Shimadzu LC-10 with UV detection at 210 nm, column Alltech C18 (22 × 250 mm), solvent acetonitrile/H_2O, 70:30] to give **1** and **2**, comparable with prior literature methodology.[32,33] Their identity and purity were confirmed by examination of ^{1}H NMR spectra, and the compounds were dissolved in dimethylsulfoxide and added to the experimental media. ^{1}H and ^{13}C NMR spectra were obtained on a Varian INOVA-500 spectrometer with 63K data points and spectral width of 8996.7 and 31421.8 Hz, respectively. The 90° pulse widths for ^{1}H and ^{13}C spectra were 9.5 and 17.0 μs, respectively. Data point matrices for COSY and NOESY experiments were 2048 × 258. HSQC and HMBC spectra were measured by a pulse field gradient method with data point matrix of 2048 × 512.

Cell Culture. Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex Bio Science. HUVECs were grown in EBM-2 supplemented with 5% FBS and growth factors at 37 °C in a 5%/95% air atmosphere.[34]

Growth Inhibition Assay. This assay was conducted according to the method of the NCI Angiogenesis Resource Center.[35] HUVECs (5×10^4/mL) were seeded in a 96-well plate in 100 μL of EGM-2 (Clonetic #CC3162). After 24 h (day 0), compounds (100 μL) were added to each well at the desired concentration in EGM-2 medium. On day 0, one plate was stained with 0.5% crystal violet in 20% MeOH for 10 min, rinsed with water, and air-dried. The remaining plates were incubated for 72 h at 37 °C. After 72 h, plates were stained with 0.5% crystal violet in 20% MeOH, rinsed with water, and air-dried. The stain was eluted with 0.05 M sodium citrate in 50% EtOH (including day 0 plate), and absorbance was measured at 540 nm with an ELISA reader.

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