Anti-ischemia Steroidal Saponins from the Seeds of *Allium fistulosum*

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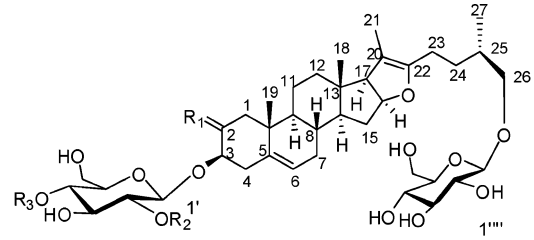
Six new furostanol saponins (**1**−**6**), named fistulosaponins A−F, three known furostanol saponins (**7**−**9**), and seven known aromatic compounds were isolated from seeds of Welsh onion (*Allium fistulosum*). The structures of these compounds were characterized by spectroscopic analyses including 2D NMR spectroscopy, mass spectrometry, and acid hydrolysis. The protective effect of the saponins on hypoxia-induced human umbilical vein endothelial cell injury was evaluated.

Allium fistulosum L. (Alliaceae), or Welsh onion, is widely used in cooking as an ingredient in Asian cuisine, especially in East and Southeast Asia. In Traditional Chinese Medicine (TCM), Welsh onion seeds are used for the treatment of renal deficiency, dizziness, and colds.[1,2] Water extracts of Welsh onion seeds were shown to have protective effects against acute myocardial ischemia in dogs and myocardial ischemia-reperfusion injury in rats.[3] Steroidal saponins were reported to be responsible for the cardiovascular activity in members of the Liliaceae family.[4] These findings prompted us to carry out a systematic study on steroidal saponins of Welsh onion seeds. Six new furostanol saponins (**1**−**6**), three known furostanol saponins (**7**−**9**), and seven known aromatic compounds were isolated, the structures of these compounds were determined, and the protective effect of the saponins on hypoxia/reoxygenation (H/R)-induced human umbilical vein endothelial cell (HUVEC) injury was evaluated.

Results and Discussion

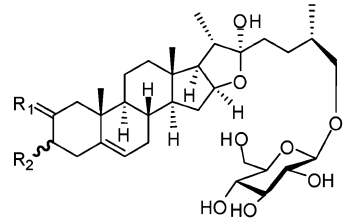
Air-dried seeds of *A. fistulosum* were refluxed with water. The concentrated extract was chromatographed on HP-20 polystyrene resin, silica gel, octadecylsilanized (ODS) silica gel, and semi-preparative RP-HPLC to afford 16 compounds.

Compound **1**, an amorphous powder, exhibited positive reactions to the Liebermann-Burchard and Ehrlich reagents, indicating the presence of a furostanol skeleton.[5] The molecular formula was determined to be $C_{51}H_{80}O_{22}$ by positive-ion HRESIMS (*m*/*z* 1067.5001 $[M + Na]^+$, calcd for 1067.5033) and confirmed by ^{13}C and DEPT NMR data. The ^1H NMR spectrum of **1** showed three methyl singlets (δ 0.67, 1.04, 1.61), three methyl doublets [δ 0.99 ($J = 6.6$ Hz), 1.59 ($J = 6.0$ Hz), 1.77 ($J = 6.0$ Hz)], four anomeric proton signals [δ 4.78 (d, $J = 7.2$ Hz), 4.98 (d, $J = 7.2$ Hz), 5.81 (brs), 6.35 (brs)], and an olefinic proton signal at δ 5.42 (brs) (Table 1). Among the 51 carbon signals in the ^{13}C NMR spectrum, 27 signals were assigned to the aglycone and the remaining 24 signals attributed to four hexose units. The ^{13}C NMR spectrum of **1** had signals at δ 103.5, 123.0, 137.5, 152.4, and 205.0, indicating the presence of a carbonyl carbon and two olefinic bonds. Comparison of the aglycone signals of **1** in the ^{13}C NMR spectrum with those of (25*R*)-26-*O*-β-D-glucopyranosyl-5,20-dienefurostan-3β,26-diol-3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→3)]-β-D-glucopyranoside (taccaoside A)[6] showed that they had the same B, C, D, and E rings, but differed in the A ring (Table 2). All the proton resonances were assigned to the relevant carbon atoms by using the HMQC spectrum. In the HMBC spectrum, the methyl proton signal at δ 1.04 (H-19) showed long-range correlations with the carbon signals at δ 49.7 (C-9), 42.2 (C-10), 52.7 (C-1), and 137.5 (C-5), and the proton signal at δ 5.42 (H-6) showed long-



1 $R_1 = O$ $R_2^{II} = R_3^{III} =$ Rha

5 $R_1 = \beta$-H, α-OH $R_2 =$ Glc--^{2}--Glc $R_3 =$ H



2 $R_1 = O$ $R_2 =$ β-O-Glc--^{2}--Rha (|^4 Rha)

3 $R_1 = O$ $R_2 =$ α-O-Glc--^{2}--Rha--^{4}--Rha

4 $R_1 = O$ $R_2 =$ β-O-Glc--^{2}--Glc--^{2}--Glc

6 $R_1 = \beta$-H, α-OH $R_2 = \beta$-O-Glc--^{2}--Glc--^{2}--Glc

7 $R_1 =$ H, H $R_2 = \beta$-O-Glc--^{2}--Rha (|^3 Glc)

8 $R_1 =$ H, H $R_2 = \beta$-O-Glc--^{2}--Rha (|^4 Rha)

9 $R_1 =$ H, H $R_2 = \beta$-O-Glc--^{4}--Rha--^{4}--Rha (|^2 Rha)

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Table 1. ^{1}H NMR Spectroscopic Data (600 MHz) for the Aglycone Portions of Compounds **1**−**6**[a] (δ in ppm, C_5D_5N, J in Hz)

position	1	2	3	4	5	6
1	2.51, d (12.6)	2.48, d (12.6)	3.30, d (12.6)	2.46, d (12.6)	2.30, dd (12.6, 3.6)	2.30, dd (12.6, 3.6)
	2.03, d (12.6)	2.01, o	2.53, d (12.6)	2.06, d (12.6)	1.27, dd (12.6, 12.6)	1.27, dd (12.6, 12.6)
2					4.11, dd (12.6, 9.0)	4.13, d (12.6)
3	4.73, dd (11.4, 7.2)	4.71, dd (11.4, 7.8)	4.48, o	4.83, dd (10.4, 8.4)	3.83, dd (11.4, 9.0)	3.80, dd (8.4, 8.4)
4	3.12, dd (13.2, 7.2)	3.11, dd (13.2, 7.8)	2.86, dd (15.0, 3.6)	3.06, dd (13.2, 6.6)	2.67, dd (12.6, 4.8)	2.68, dd (13.2, 4.8)
	3.08, m	3.03, dd (11.4, 11.4)	2.63, d (15.0)	2.71, dd (12.0, 12.0)	2.55, dd (12.6, 11.4)	2.55, dd (13.2, 8.4)
6	5.42, brs	5.40, brs	5.52, brs	5.41, brs	5.29, brs	5.26, brs
7	1.90, dd (13.2, 6.6)	1.80, o	1.96, m	1.81, d (15.0)	1.81, m	1.96, m
	1.87, m	1.41, m	1.43, o	1.36, o	1.79, m	1.80, d (14.2)
8	1.40, d (6.6)	1.45, m	1.59, o	1.38, m	1.44, m	1.47, m
9	1.02, o	0.97, o	2.07, o	1.01, o	1.04, o	0.89, o
11	1.43, m	1.28, o	1.41, dd (12.0, 4.2)	1.31, o	1.46, m	1.44, m
	1.31, o	1.25, o	1.07, d (7.2)	1.27, o	1.37, o	1.37, o
12	1.67, d (12.0)	1.64, m	1.70, o	1.64, m	1.68, d (12.6)	1.70, m
	1.09, o	1.02, o	1.34, o	1.10, m	1.11, dd (12.6, 12.6)	1.06, d (12.6)
14	0.85, m	0.98, o	1.40, dd (12.0, 3.6)	1.00, m	0.82, m	0.99, d (10.2)
15	2.10, ddd (13.2, 6.6, 6.6)	1.98, o	2.05, o	1.98, m	2.06, ddd (12.0, 6.0, 6.0)	2.02, o
	1.48, dd (12.6, 6.6)	1.47, m	1.39, o	1.47, dd (10.2, 10.2)	1.41, o	1.39, m
16	4.76, o	4.89, dd (14.4, 14.4)	4.78, m	4.90, d (7.2)	4.75, o	4.89, m
17	2.42, d (10.2)	1.87, m	1.74, dd (7.2, 7.2)	1.91, o	2.41, d (10.2)	1.98, m
18	0.67, s	0.82, s	0.89, s	0.82, s	0.69, s	0.84, s
19	1.04, s	1.01, s	1.00, s	0.83, s	0.94, s	0.92, s
20		2.17, dd (6.6, 6.6)	2.17, dd (6.6, 6.6)	2.17, dd (6.0, 6.0)		2.19, dd (6.6, 6.6)
21	1.61, s	1.28, d (6.6)	1.23, d (6.6)	1.29, d (6.6)	1.61, s	1.30, d (7.8)
23	1.79, o	2.00, o	2.01, o	1.98, m	1.78, m	2.00, o
	1.45, dd (14.4, 7.8)	1.99, o	2.00, o	1.95, o	1.43, o	1.90, m
24	2.21, m	2.01, o	1.97, o	2.03, o	2.20, m	2.01, m
	2.19, dd (14.4, 8.4)	1.60, m	1.64, o	1.61, m	2.19, m	1.67, m
25	1.97, o	1.86, m	1.90, m	1.87, m	1.92, dd (12.0, 6.6)	1.88, m
26	3.99, dd (8.4, 8.4)	3.96, m	3.91, dd (7.8, 7.8)	3.97, dd (9.0, 9.0)	3.98, m	3.99, dd (7.8, 7.8)
	3.60, dd (9.0, 6.0)	3.57, dd (9.0, 6.0)	3.59, dd (8.4, 6.0)	3.59, dd (8.4, 6.0)	3.60, dd (9.0, 6.0)	3.59, m
27	0.99, d (6.6)	0.95, d (6.0)	0.95, d (6.6)	0.95, d (6.6)	1.00, d (6.0)	0.96, d (7.2)

[a] Assignments are based on DEPT, ^{1}H−^{1}H COSY, HSQC, and HMBC experiments.

Table 2. ^{13}C NMR Spectroscopic Data (150 MHz) for the Aglycone Portions of Compounds **1**−**6**[a] (δ in ppm, C_5D_5N)

position	taccaoside A	1	2	3	4	5	6
1	37.7, CH_2	52.7, CH_2	52.5, CH_2	49.7, CH_2	52.4, CH_2	45.8, CH_2	45.7, CH_2
2	30.6, CH_2	205.0, qC	205.1, qC	210.5, qC	206.5, qC	70.1, CH	70.0, CH
3	78.5, CH	80.0, CH	80.0, CH	78.9, CH	79.5, CH	84.8, CH	84.6, CH
4	39.8, CH_2	39.3, CH_2	39.5, CH_2	39.4, CH_2	39.7, CH_2	37.7, CH_2	37.7, CH_2
5	140.9, qC	137.5, qC	137.5, qC	135.8, qC	137.4, qC	140.1, qC	140.0, qC
6	121.9, CH	123.0, CH[b]	123.0, CH[b]	123.7, CH[b]	123.7, CH	121.9, CH	121.9, CH
7	32.6, CH_2	32.2, CH_2	32.2, CH_2	32.0, CH_2	32.0, CH_2	32.3, CH_2	32.3, CH_2
8	31.6, CH	31.1, CH	31.2, CH	31.5, CH	31.2, CH	30.8, CH	31.0, CH
9	50.4, CH	49.7, CH	49.6, CH	47.6, CH	49.6, CH	50.2, CH	50.1, CH
10	37.2, qC	42.2, qC	42.1, qC	42.8, qC	42.2, qC	37.9, qC	37.8, qC
11	21.4, CH_2	21.2, CH_2	21.0, CH_2	20.9, CH_2	21.0, CH_2	21.3, CH_2	21.1, CH_2
12	38.8, CH_2	39.6, CH_2	39.5, CH_2	39.3, CH_2	39.5, CH_2	39.6, CH_2	39.8, CH_2
13	43.6, qC	43.3, qC	40.6, qC	40.5, qC	40.6, qC	43.4, qC	40.7, qC
14	55.1, CH	54.6, CH	56.2, CH	55.9, CH	56.2, CH	54.8, CH	56.4, CH
15	34.6, CH_2	34.4, CH_2	32.0, CH_2	32.4, CH_2	32.2, CH_2	34.4, CH_2	32.1, CH_2
16	84.6, CH	84.4, CH	80.9, CH	81.0, CH	81.1, CH	84.4, CH	81.0, CH
17	64.6, CH	64.4, CH	63.6, CH	63.5, CH	63.7, CH	64.5, CH	63.7, CH
18	14.3, CH_3	14.0, CH_3	16.2, CH_3	16.3, CH_3	16.3, CH_3	14.1, CH_3	16.4, CH_3
19	19.5, CH_3	20.1, CH_3	20.0, CH_3	19.8, CH_3	20.0, CH_3	20.4, CH_3	20.3, CH_3
20	103.6, qC	103.5, qC	40.5, CH	40.6, CH	40.5, CH	103.5, qC	40.6, CH
21	11.9, CH_3	11.7, CH_3	16.2, CH_3	16.3, CH_3	16.3, CH_3	11.7, CH_3	16.3, CH_3
22	152.5, qC	152.4, qC	110.6, qC	110.4, qC	110.6, qC	152.4, qC	110.6, qC
23	33.8, CH_2	31.4, CH_2	36.9, CH_2	37.1, CH_2	37.1, CH_2	31.4, CH_2	37.1, CH_2
24	23.8, CH_2	23.6, CH_2	28.2, CH_2	28.2, CH_2	28.3, CH_2	23.7, CH_2	28.3, CH_2
25	31.6, CH	33.5, CH	34.1, CH	34.1, CH	34.2, CH	33.5, CH	34.2, CH
26	75.3, CH_2	74.9, CH_2	75.1, CH_2	75.2, CH_2	75.2, CH_2	74.9, CH_2	75.2, CH_2
27	17.3, CH_3	17.3, CH_3	17.3, CH_3	17.3, CH_3	17.3, CH_3	17.3, CH_3	17.4, CH_3

[a] Assignments are based on DEPT, ^{1}H−^{1}H COSY, HSQC, and HMBC experiments. [b] Signal overlapped by solvent peaks.

range correlations with the carbon signals at δ 32.2 (C-7) and 42.2 (C-10). The carbon signal at δ 137.5 (C-5) showed long-range correlations with the proton signals at δ 4.73 (H-3), 3.08 (H-4b), 2.51 (H-1a), and 1.04 (H-19). Long-range correlations between the carbonyl carbon signal (δ 205.0) and the proton signals at δ 2.51 (H-1a), 2.03 (H-1b), 4.73 (H-3), 3.12 (H-4a), and 3.08 (H-4b) suggested that the carbonyl was at C-2. The 25*R* configuration of the 27-methyl group was deduced by the resonances of protons and carbons at C-25, C-26, and C-27[7,8] and by the J values between H-25 and H-26 (J = 8.4 Hz), in comparison with literature data.[9,10] The differences observed in ^{1}H NMR chemical shifts of the geminal protons H-26a (δ 3.99) and H-26b (δ 3.60) (Δ_{ab} = 0.39 < 0.48) also supported a 25*R* configuration.[9,11] NOESY correlations between H-8 and H-19, H-11b and H-19/H-18, H-9 and H-14, H-14 and H-16/H-17, H-16 and H-17/H-26, and H-17 and H-21 allowed assignment of relative configurations in the B/C *trans*, C/D *trans*,

and D/E *cis* rings of **1**.[10] On the basis of these data, the structure of the aglycone was determined as depicted in the formula.

Compound **1** was subjected to acid hydrolysis with 2 M HCl, followed by trimethylsilylation and GC analysis on a chirasil column, and the sugar residues were found to be D-glucose and L-rhamnose in a 1:1 ratio. This procedure was then applied to all of the new compounds (**1**−**6**). The sequence of sugars in the oligosaccharide chain and its linkage to the aglycone were determined by the analysis of a combination of ^{1}H−^{1}H COSY, TOCSY-HSQC, ROESY, and HMBC spectra (Tables 4 and 5 in the Supporting Information). In the HMBC experiment, long-range correlations between the anomeric proton H-1′ and C-26 of the aglycone, and between the anomeric proton H-1″ and C-3 of the aglycone, allowed us to identify C-26 and C-3 as the glucosidic linkage sites. Long-range correlations between H-1‴ and C-2″ and between H-1⁗ and C-4″ suggested that the rhamnopyranose units were linked to C-2 and C-4 of the glucopyranose at C-3. The β-anomeric conformations of both glucose units were judged from their large $^3J_{H1,\ H2}$ coupling constants (J = 7.2 Hz).[12] For the rhamnosyl moieties, the chemical shift at C-5 (δ 69.7, 70.4) and three-bond-coupled strong HMBC correlations from the anomeric proton to the C-3 and C-5 carbons indicated that each anomeric proton was equatorial, thus possessing an α-pyranoid anomeric form.[12,13] On the basis of these findings, **1** was identified as (25*R*)-26-*O*-β-D-glucopyranosyl-5,20-dienefurostan-2-one-3β,26-diol-3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside, and it was named fistulosaponin A.

Compound **2** was isolated as an amorphous powder. The molecular formula was determined to be $C_{51}H_{82}O_{23}$ by HRESIMS, ^{13}C NMR, and DEPT NMR data, differing from that of **1** by H_2O. The ^{1}H NMR spectrum contained signals for two singlet methyl groups (δ 0.82 and 1.01), four secondary methyl groups (δ 0.95, 1.28, 1.54, and 1.73), an olefinic proton (δ 5.40), and four anomeric protons (δ 4.75, 4.95, 5.74, and 6.28). The ^{13}C NMR spectrum of **2** showed two olefinic carbon signals and a carbonyl signal. Acid hydrolysis of **2** with 2 M HCl gave D-glucose and L-rhamnose in a 1:1 ratio. The triglycoside rhamnosyl-(1→2)-[rhamnosyl-(1→4)]-glucosyl was linked to C-3 of the aglycone, as for **1**, and this was confirmed by HMBC correlations between H-1″ and C-3 of the aglycone, between H-1‴ and C-2″ of the glucosyl unit at δ 77.5, and between H-1⁗ and C-4″ of the glucosyl unit at δ 78.7. Correlation between H-1′ and C-26 was also observed. A feature differing from the aglycone skeleton of **1** was the substitution of the double bond on C-22 by an OH. This was verified by the HMBC correlations between C-22 (δ 110.6) and H-20 (δ 2.17, dd, J = 6.6, 6.6 Hz), H-21 (δ 1.28, d, J = 6.6 Hz), and H-23a (δ 2.00, o) and between C-20 (δ 40.5) and H-17 (δ 1.87, m) and H-21 (δ 1.28, d, J = 6.6 Hz). Configuration of the OH group at C-22 was assigned as α by analysis of NMR data and comparison with other saponins as previously described.[10,14] Thus, **2** was characterized as (25*R*)-26-*O*-β-D-glucopyranosyl-5-enefurostan-2-one-3β,22α,26-triol-3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside, and it was named fistulosaponin B.

Compound **4** was assigned the molecular formula $C_{51}H_{82}O_{25}$ as determined by HRESIMS. ESIMS showed *m*/*z* 1117.5 $[M + Na]^+$, 955.6 $[M + Na - 162]^+$, 793.5 $[M + Na - 2 \times 162]^+$, and 631.3 $[M + Na - 3 \times 162]^+$, attributable to sequential loss of four aldohexose residues. The spectroscopic data of **4** showed that it possessed the same aglycone as that of **2** (Tables 1 and 2), but differed in the oligosaccharide chain. The ^{1}H and ^{13}C NMR spectra of **4** contained resonances for four anomeric protons and resonances for four steroidal methyl groups and an olefinic proton. Acid hydrolysis of **4** yielded only D-glucose. In the HMBC spectrum, long-range correlations were observed between the anomeric proton signal at δ 4.74 (H-1′) and the carbon signal at δ 75.2 (C-26), between the anomeric proton signal at δ 4.91 (H-1″) and the carbon signal at δ 79.5 (C-3), between the anomeric proton signal at δ 5.02 (H-1‴) and the carbon signal at δ 80.9 (C-2″), and between the anomeric proton signal at δ 5.17 (H-1⁗) and the carbon signal at δ 86.0 (C-2‴). The anomeric centers of the sugar moieties were β-oriented, as indicated by their large $^3J_{H1,\ H2}$ coupling constants (J = 7.2−7.8 Hz). Accordingly, **4** was determined to be (25*R*)-26-*O*-β-D-glucopyranosyl-5-enefurostan-2-one-3β,22α,26-triol-3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside, and it was named fistulosaponin D.

Compound **3** had the molecular formula $C_{51}H_{82}O_{23}$. The ^{1}H NMR spectrum of **3** showed signals for two singlet methyl groups, four secondary methyl groups, an olefinic proton, and four anomeric protons. Acid hydrolysis of **3** gave D-glucose and L-rhamnose in a ratio of 1:1. Comparison of the ^{1}H and ^{13}C NMR spectra of **3** and **2** indicated that both compounds had the same aglycone moiety, but differed in configuration and sequence of the oligosaccharide chains (Tables 1 and 2). The diaxial coupling between H-3 (δ 4.48, o) and H-19 (δ 1.00, s) detected in the ROESY spectrum indicated the α-orientation of the OH at C-3. Linkage of the triglycoside to C-3 of the aglycone was confirmed by HMBC. The HMBC spectrum showed long-range correlations between H-1″ (δ 4.72, d, J = 8.4 Hz) and C-3 (δ 78.9) of the aglycone, between H-1‴ (δ 6.52, brs) and C-2″ (δ 76.2), between H-1⁗ (δ 5.79, brs) and C-4‴ (δ 78.2), and between H-1′ (δ 4.78, d, J = 7.8 Hz) and C-26 (δ 75.2). These findings confirmed the structure of **3** as (25*R*)-26-*O*-β-D-glucopyranosyl-5-enefurostan-2-one-3α,22α,26-triol-3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, and it was named fistulosaponin C.

The molecular formula of **5** was $C_{51}H_{82}O_{24}$, as determined by HRESIMS and ^{13}C NMR data. The ^{1}H NMR spectrum showed signals for four typical steroid methyl groups, four anomeric protons, and an olefinic proton. The ^{13}C NMR spectrum of **5** showed four olefinic carbon signals, which were suggestive of a 5(6),20(22)-diene furostanol skeleton. Comparison of the ^{1}H and ^{13}C NMR spectra of **5** with those of **1** indicated that they differed in the chemical shifts of C/H atoms around C-2. In particular, C-2 was found to be an oxygenated methine (δ 70.1), and in addition, α-orientation of OH-2 was defined by the coupling constants of H-2 (dd, J = 12.6, 9.0 Hz). This was verified by the HMBC correlations between C-2 and H-1b (δ 1.27, dd, J = 12.6, 12.6 Hz) and H-4a (δ 2.67, dd, J = 12.6, 4.8 Hz) and by the NOESY cross-peak of H-2 with H-19. Acid hydrolysis of **5** afforded only D-glucose. In the HMBC experiments, correlations between H-1″ and C-3 of the aglycone, H-1‴ and C-2″, H-1⁗ and C-2‴, and H-1′ and C-26 were observed. Thus, compound **5** was (25*R*)-26-*O*-β-D-glucopyranosyl-5,20-dienefurostan-2α,3β,26-triol-3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside, and it was named fistulosaponin E.

Compound **6** had the molecular formula $C_{51}H_{84}O_{25}$, and the ^{1}H and ^{13}C NMR spectra were similar to those of **5**, except for signals for ring E and the side chain. The higher field chemical shifts observed for C-20 and C-22 corroborated the presence of an OH group at C-22. The structure of **6**, named fistulosaponin F, was characterized as (25*R*)-26-*O*-β-D-glucopyranosyl-5-enefurostan-2α,3β,22α,26-tetraol-3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside.

The nine isolated saponins were tested for antihypoxic activity against H/R-induced HUVEC injury. The MTT assay showed that H/R significantly decreased the cell viability to (54.3 ± 4.4)% compared to the control [(100.3 ± 2.2)%, $P < 0.01$], and treatment with compounds **1**−**9** significantly improved the survival of these H/R-treated HUVEC ($P < 0.05$). All nine saponins exhibited good antihypoxic effect in a dose-dependent manner (Table 3). Fistulosaponin A (**1**) was more effectively resistant to H/R-induced HUVEC injury, with a cell viability of (59.5 ± 3.0)%, (76.3 ± 3.3)%, (80.1 ± 3.6)%, (82.7 ± 4.1)%, (86.3 ± 4.6)%, and (78.2 ± 2.8)% for the six dose groups (0.5, 1, 5, 10, 50, and 100 μM), respectively.

Table 3. Protective Effects of Different Concentrations of Compounds **1**−**9** on HUVEC Injury Induced by Hypoxia (8 h) and Reoxygenation (12 h)[a]

drugs	0.5 μM	1 μM	5 μM	10 μM	50 μM	100 μM
positive	65.8 ± 3.3[b]	69.6 ± 2.7[c]	75.8 ± 1.8[c]	79.9 ± 2.9[c]	81.0 ± 3.4[c]	77.3 ± 4.5[c]
fistulosaponin A	59.5 ± 3.0	76.3 ± 3.3[c]	80.1 ± 3.6[c]	82.7 ± 4.1[c]	86.3 ± 4.6[c]	78.2 ± 2.8[c]
fistulosaponin B	65.7 ± 2.2[b]	71.8 ± 3.8[c]	77.2 ± 3.9[c]	79.4 ± 3.9[c]	82.1 ± 2.6[c]	74.8 ± 3.2[c]
fistulosaponin C	71.7 ± 2.1[c]	74.8 ± 3.2[c]	79.7 ± 3.9[c]	81.2 ± 3.2[c]	84.6 ± 3.6[c]	69.5 ± 3.1[b]
fistulosaponin D	68.9 ± 2.8[b]	72.6 ± 3.3[c]	78.2 ± 3.8[c]	79.8 ± 3.5[c]	82.4 ± 4.0[c]	75.6 ± 3.3[c]
fistulosaponin E	60.9 ± 3.5	64.3 ± 3.6	70.2 ± 4.4[b]	77.9 ± 2.9[c]	75.4 ± 4.6[c]	68.0 ± 3.2[b]
fistulosaponin F	62.3 ± 2.8[b]	68.3 ± 3.7[b]	74.1 ± 3.1[c]	75.5 ± 2.6[c]	77.4 ± 4.2[c]	71.2 ± 3.2[c]
protogracillin	60.9 ± 3.3	69.5 ± 3.4[b]	75.2 ± 4.0[c]	77.9 ± 2.3[c]	78.2 ± 3.9[c]	68.0 ± 2.8[b]
compound **8**	60.7 ± 2.2[b]	63.5 ± 4.1[b]	69.4 ± 4.7[b]	72.1 ± 2.5[c]	74.3 ± 3.4[c]	68.6 ± 2.4[b]
compound **9**	64.0 ± 2.2[b]	65.2 ± 4.3[b]	69.5 ± 3.3[b]	73.3 ± 2.5[c]	73.6 ± 4.7[c]	62.8 ± 2.4[b]

[a] Cell viability was measured by the MTT assay. Data are expressed as mean ± SEM of three independent experiments. [b] $P < 0.05$ vs H/R. [c] $P < 0.01$ vs H/R.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. IR analyses were performed with a Bruker Vector-22 infrared spectrometer (Bruker Ltd. Co, Germany). ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra and all 2D NMR spectra were recorded on a Bruker Avance 600 NMR spectrometer (Bruker Co., Germany) in C_5H_5N. ESIMS were recorded on a Mat 211 mass spectrometer (Varian Technologies, USA). HRESIMS were obtained from either an Agilent 6220 TOF LC/MS instrument (Agilent Technologies, MA, USA) or a Q-Tof micro (Waters Technologies, Manchester, UK). Materials for CC were Diaion HP-20 (Mitsubishi Chemical, Japan), YMC-GRL ODS-A (50 μm; YMC, MA, USA), and silica gel (200−300 mesh). Semipreparative RP-HPLC isolation was achieved with an Agilent 1200 instrument using a YMC 5 μm C8 column (250 mm × 10 mm) eluting with MeCN−H_2O at 1.5 mL/min. Peak detection was made with a refractive index detector (RID). GC-MS was conducted on a Thermo Finnigan Trace GC apparatus using an L-Chirasil-Val column (25 m × 0.32 mm i.d.).

Plant Material. The plant material was collected during September 2004 in Zigong, Sichuan Province, China, and identified by Professor Hanming Zhang (School of Pharmacy, Second Military Medical University). A voucher specimen (No. 20040947) was deposited at the Department of Pharmacognosy of the Second Military Medical University School of Pharmacy.

Extraction and Isolation. Air-dried and powdered seeds of *A. fistulosum* (20 kg) were refluxed with water (20 L × 3) for 2 h. The extract was filtered and evaporated to 15 L under reduced pressure and then subjected to a Diaion HP-20 (5 kg) column, eluting with H_2O and 10% (15 L), 50% (20 L), and 95% EtOH (10 L). After evaporation under reduced pressure, 50% of the EtOH-eluted residue (174 g) was suspended in water (500 mL) and partitioned with *n*-BuOH (500 mL × 3), yielding the corresponding *n*-BuOH fraction (42 g) after evaporation of the solvents. Repeated separation of the target fraction was performed on an ODS (200 g) column, using a linear gradient solvent system from H_2O to MeOH. After evaporation of the solvents, five fractions were obtained: F1 (H_2O), F2 (MeOH−H_2O, 3:7), F3 (MeOH−H_2O, 5:5), F4 (MeOH−H_2O, 7:3), and F5 (MeOH−H_2O, 9:1). F2 (3.2 g) was then separated by HPLC on a semipreparative C_{18} column with the mobile phase MeCN−H_2O (23:77) (total time: 165 min) to afford **4** (t_R = 136.0 min; 36 mg), **5** (t_R = 145.4 min; 29 mg), **6** (t_R = 154.4 min; 21 mg), and **9** [t_R = 157.5 min; 8 mg, (25*R*)-26-*O*-β-D-glucopyranosyl-5-enefurostan-3β,22α,26-triol-3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside].[15] F3 (7.4 g) was purified by HPLC using MeCN−H_2O (25:75) as eluent (total time: 145 min) to afford **1** (t_R = 112.4 min; 25 mg), **3** (t_R = 125.1 min; 16 mg), and **7** (t_R = 138.2 min; 25 mg, protogracillin).[16] F4 (4.6 g) was subjected to HPLC using MeCN−H_2O (27:73) as eluent (total time: 140 min). Two fractions were obtained, which yielded compounds **2** (t_R = 107.5 min; 38 mg) and **8** [t_R = 118.9 min; 27 mg, (25*R*)-26-*O*-β-D-glucopyranosyl-5-enefurostan-3β,22α,26-triol-3-*O*-(2,4-di-*O*-α-L-rhamnopyranosyl)-β-D-glucopyranoside].[17]

Fistulosaponin A (1): colorless, amorphous powder; $[\alpha]^{25}_D$ −69.6 (*c* 0.24, H_2O); IR (KBr) ν_{max} 3425, 2932, 1725, 1382, 1042, 629 cm^{-1}; ^{1}H NMR (C_5D_5N, 600 MHz) (Tables 1 and 4) and ^{13}C NMR (C_5D_5N, 150 MHz) (Tables 2 and 5); ESIMS *m*/*z* 1043.5 [M − H]$^-$, 897.4 [M − H − 146]$^-$, 879.2 [M − H − 146 − H_2O] $^-$, 589.3 [M − H − 2 × 146 − 162]$^-$; HRESIMS *m*/*z* 1067.5001 [M + Na]$^+$ (calcd for $C_{51}H_{80}NaO_{22}$, 1067.5033).

Fistulosaponin B (2): colorless, amorphous powder; $[\alpha]^{25}_D$ −65.0 (*c* 0.25, H_2O); IR (KBr) ν_{max} 3420, 2906, 1731, 1457, 1141 cm^{-1}; ^{1}H NMR (C_5D_5N, 600 MHz) (Tables 1 and 4) and ^{13}C NMR (C_5D_5N, 150 MHz) (Tables 2 and 5); ESIMS *m*/*z* 1061.5 [M − H]$^-$, 915.5 [M − H − 146]$^-$, 897.4 [M − H − 146 − H_2O]$^-$, 769.4 [M − H − 2 × 146]$^-$, 607.3 [M − H − 2 × 146 − 162]$^-$, 445.3 [M − H − 2 × 146 − 2 × 162]$^-$; HRESIMS *m*/*z* 1085.5128 [M + Na]$^+$ (calcd for $C_{51}H_{82}NaO_{23}$, 1085.5139).

Fistulosaponin C (3): colorless, amorphous powder; $[\alpha]^{25}_D$ −5.0 (*c* 0.22, H_2O); IR (KBr) ν_{max} 3291, 2933, 1733, 1071 cm^{-1}; ^{1}H NMR (C_5D_5N, 600 MHz) (Tables 1 and 4), and ^{13}C NMR (C_5D_5N, 150 MHz) (Tables 2 and 5); ESIMS *m*/*z* 1085.5 [M + Na]$^+$, 939.4 [M + Na − 146]$^+$, 793.5 [M + Na − 2 × 146]$^+$, 631.6 [M + Na − 2 × 146 − 162]$^+$; HRESIMS *m*/*z* 1085.5151 [M + Na]$^+$ (calcd for $C_{51}H_{82}NaO_{23}$, 1085.5145).

Fistulosaponin D (4): colorless, amorphous powder; $[\alpha]^{25}_D$ −40.5 (*c* 0.22, H_2O); IR (KBr) ν_{max} 3309, 2900, 1699, 1456, 1074 cm^{-1}; ^{1}H NMR (C_5D_5N, 600 MHz) (Tables 1 and 4), and ^{13}C NMR (C_5D_5N, 150 MHz) (Tables 2 and 5); ESIMS *m*/*z* 1117.5 [M + Na]$^+$, 955.6 [M + Na − 162]$^+$, 793.5 [M + Na − 2 × 162]$^+$, 631.3 [M + Na − 3 × 162]$^+$; HRESIMS *m*/*z* 1117.5003 [M + Na]$^+$ (calcd for $C_{51}H_{82}NaO_{25}$, 1117.5037).

Fistulosaponin E (5): colorless, amorphous powder; $[\alpha]^{25}_D$ −15.0 (*c* 0.25, H_2O); IR (KBr) ν_{max} 3309, 2933, 1436, 1075 cm^{-1}; ^{1}H NMR (C_5D_5N, 600 MHz) (Tables 1 and 4) and ^{13}C NMR (C_5D_5N, 150 MHz) (Tables 2 and 5); ESIMS *m*/*z* 1101.5 [M + Na]$^+$, 917.5 [M + H − 162]$^+$, 755.5 [M + H − 2 × 162]$^+$, 593.4 [M + H − 3 × 162]$^+$, 431.4 [M + H − 4 × 162]$^+$; HRESIMS *m*/*z* 1101.5059 [M + Na]$^+$ (calcd for $C_{51}H_{82}NaO_{24}$, 1101.5064).

Fistulosaponin F (6): colorless, amorphous powder; $[\alpha]^{25}_D$ −5.1 (*c* 0.455, H_2O); IR (KBr) ν_{max} 3290, 2902, 1472, 1074 cm^{-1}; ^{1}H NMR (C_5D_5N, 600 MHz) (Tables 1 and 4) and ^{13}C NMR (C_5D_5N, 150 MHz) (Tables 2 and 5); ESIMS *m*/*z* 1119.5 [M + Na]$^+$, 957.5 [M + Na − 162]$^+$, 795.5 [M + Na − 2 × 162]$^+$, 633.4 [M + Na − 3 × 162]$^+$; HRESIMS *m*/*z* 1119.5220 [M + Na]$^+$ (calcd for $C_{51}H_{82}NaO_{25}$, 1119.5199).

Acid Hydrolysis of Compounds 1−6. Each saponin (2 mg) was heated in 2 M HCl (1 mL) at 120 °C for 1 h. The mixture was concentrated, the residue was dissolved in 1-(trimethylsilyl)imidazole (Trisil-Z) and pyridine (0.2 mL), and the solution was stirred at 60 °C for 5 min. After drying the solution, the residue was partitioned between CH_2Cl_2 and H_2O (1 mL, 1:1 v/v). The organic layer was analyzed by GC-MS using an L-Chirasil-Val column (0.32 mm × 25 m). Temperature of the injector and detector was 200 °C for both. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. Retention times for authentic samples after simultaneous treatment with Trisil-Z were detected at 9.68 and 10.71 min (L-rhamnose), 9.74 and 10.80 min (D-rhamnose), 14.71 min (D-glucose), and 14.80 min (L-glucose). Peaks of the derivatives of L-rhamnose (9.67 and 10.70 min) and D-glucose (14.72 min) were detected in **1**, **2**, and **3**, with a molecular ratio of 1:1. Only D-glucose (14.70 min) was detected in **4**, **5**, and **6**.

Biological Assays. Ischemic injuries are mainly due to reduction in oxygen supply. H/R are principal components of ischemia/repefusion and have distinctive effects on the tissue. In vitro cell culture of H/R

injury can simulate in vivo ischemic injury. HUVEC obtained from the Global Bioresource Center (ATCC, USA) were cultured in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal calf serum (Invitrogen, USA) at 37 °C and 5% CO_2. Cells were digested with 0.25% trypsin (Invitrogen, USA) during the exponential phase, seeded on 96-well plates (Corning, USA), and then submitted to hypoxia for 8 h by transferring the culture to a hypoxic incubator (95% N_2 + 5% CO_2) (MCO-18 M O_2/CO_2 incubator, SANYO, Japan) at 37 °C. Reoxygenation was performed by placing the cells in an incubator under normoxic conditions for 12 h (MCO-15AC CO_2 incubator, SANYO, Japan). The control culture was maintained in normal conditions. Dioscin, the major component (about 40%) of Diao-xin-xue-kang (an extract of the root of *Dioscorea anthaica* Prain et Burkill), which is generally accepted as a clinical drug for preventing myocardial ischemia in China, was used as the positive control. The isolated compounds **1**−**9** and positive control (0.5, 1, 5, 10, 50, and 100 μM) were added to the culture during H/R. Cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, 20 μL of MTT (dissolved in PBS, 5 mg/mL) was added to each well. After 4 h of incubation, the supernatant was removed and 100 μL of DMSO was added to solubilize the crystals. The plate was shaken for 5 min, and the absorbance values at 570 nm were read by a microplate reader. Data were expressed as a percentage of control cell viability.

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Supporting Information Available: HRESIMS and 1D and 2D NMR spectra and tables of ^{1}H and ^{13}C NMR spectroscopic data for the sugar moieties of fistulosaponins A−F (Tables 4 and 5) (**1**−**6**). This material is available free of charge via the Internet at http://pubs.acs.org.

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