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Phenylethanoid oligoglycosides and acylated oligosugars with vasorelaxant activity from *Cistanche tubulosa*☆

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Abstract—The methanolic extract from the dried stems of *Cistanche tubulosa* (Schrenk) R. Wight was found to show an inhibitory effect on contractions induced by noradrenaline in isolated rat aortic strips. From the extract, new phenylethanoid oligoglycoside constituents, kankanosides F and G, and an acylated oligosugar, kankanose, were isolated together with 14 known compounds. The structures of these new compounds were determined on the basis of their chemical and physicochemical evidence. In addition, principal constituents, kankanoside F, kankanose, echinacoside, acteoside, and cistanoside F, showed vasorelaxant activity, and several structural requirements for the activity were clarified.

1. Introduction

The Orobanchaceae parasitic plant, *Cistanche tubulosa* (Schrenk) R. Wight, is widely distributed in North Africa, Arabia, and Asian countries, and the stems of *C. tubulosa* as well as *C. salsa* and *C. deserticola* have been traditionally used as a promoting agent of blood circulation and treatment of impotence, sterility, lumbago, body weakness, and tonic.[2,3] During the course of our characterization studies on bioactive constituents from Chinese natural medicines,[1,4–14] we have reported the isolation and structure elucidation of five iridoids, kankanosides A–D and kankanol, and a monoterpene glycoside, kankanoside E, together with 16 known compounds from the stems of *C. tubulosa*.[1] As a continuation of the study on this herbal medicine, the methanolic extract was found to show an inhibitory effect on contractions induced by noradrenaline in isolated rat thoracic aortic strips. From this methanolic extract, we additionally isolated phenylethanoid oligoglycosides, kankanosides F (**1**) and G (**2**), and an acylated oligosugar, kankanose (**3**), and 14 known compounds, echinacoside (**4**), acteoside (**5**), isoacteoside (**6**), 2′-acetylacteoside (**7**), tubulosides A (**8**) and B (**9**), cistanoside F (**10**), salidroside (**11**), (2*R*,3*R*)-butane-2,3-diol 2-*O*-β-D-glucopyranoside, *meso*-butane-2,3-diol 2-*O*-β-D-glucopyranoside, ethyl β-D-glucopyranoside, 3-methylbutanol β-D-glucopyranoside, (+)-pinoresinol 4′-*O*-β-D-glucopyranoside, and (+)-syringaresinol 4′-*O*-β-D-glucopyranoside. This paper deals with the structure elucidation of new compounds (**1**–**3**) as well as the vasorelaxant effects of the principal constituents (**1**, **3**–**6**,**10**, and **11**) on contractions induced by noradrenaline in isolated rat aortic strips based on the traditional application for blood circulation (Fig. 1).

2. Results and discussion

2.1. Vasorelaxant effect of methanolic extract from the stems of *C. tubulosa* on the contraction induced by noradrenaline in isolated rat thoracic aorta

As shown in Table 1, the methanolic extract from the stems of *C. tubulosa* (purchased in Urumuqi, Xinjiang Province, China) was found to show vasorelaxant activity on contractions induced by noradrenaline in isolated rat aortic strips, time- and concentration-dependently (30–300 μg/mL). The methanolic extract was further

Keywords: *Cistanche tubulosa*; Phenylethanoid oligoglycoside; Kankanoside F; Kankanoside G; Kankanose; Vasorelaxant activity.
☆ See Ref. 1.
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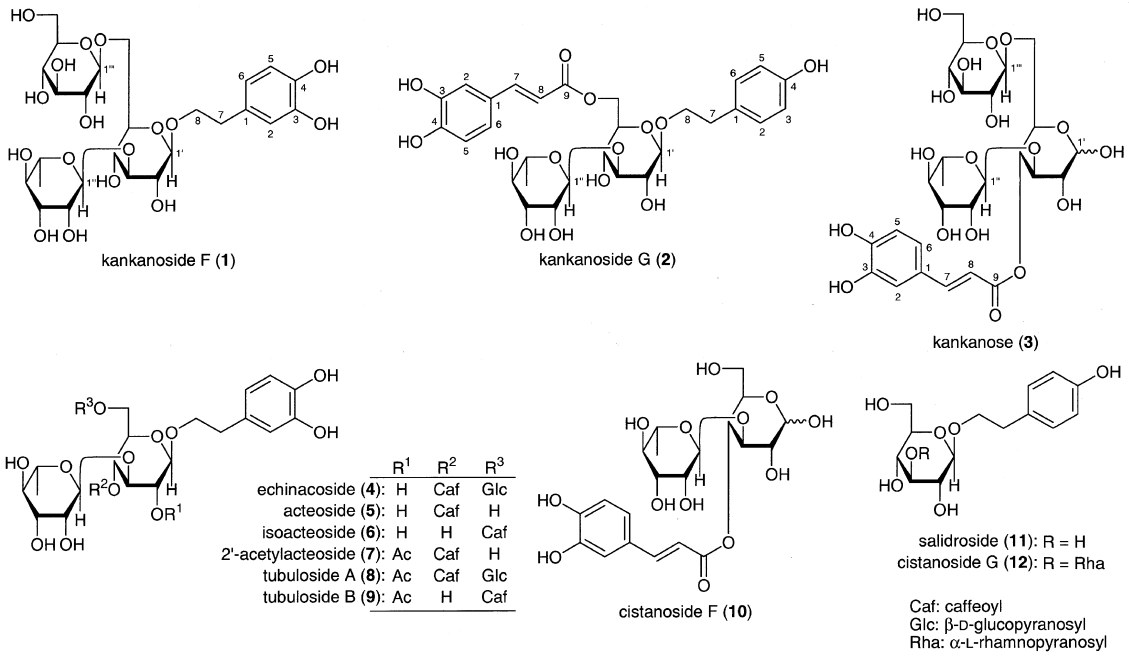

Figure 1. Chemical structures of compounds **1**–**11** from the stems of *C. tubulosa*.

Table 1. Vasorelaxant effect of methanolic extract from the stems of *C. tubulosa* on the contractions induced by noradrenaline in isolated rat thoracic aorta

	Concn (μg/mL)	Time						
		5 min	10 min	20 min	30 min	40 min	50 min	60 min
Contraction (%) dl-noradrenaline (1 μM)								
Control	—	99.5 ± 0.5	100.4 ± 0.8	100.1 ± 0.7	100.0 ± 0.3	100.2 ± 0.2	99.4 ± 0.4	99.7 ± 0.7
Methanolic extract	30	99.3 ± 0.6	99.3 ± 0.9	99.3 ± 1.7	97.7 ± 1.9	95.4 ± 2.1	90.3 ± 3.7	78.8 ± 9.0[a]
	100	100.1 ± 0.5	100.0 ± 0.9	98.1 ± 1.7	89.6 ± 7.4	75.2 ± 15.3	52.3 ± 14.4[a]	19.3 ± 9.2[a]
	300	101.8 ± 0.7	99.8 ± 0.8	88.4 ± 5.2	55.9 ± 13.6[a]	23.0 ± 11.0[a]	6.7 ± 4.1[a]	1.9 ± 1.2[a]

Each value represents the mean ± SEM ($N = 4$–5).
Significantly different from the control: [a] $p < 0.01$.

purified by normal- and reverse-phase column chromatography and finally HPLC to give **1** (0.016%), **2** (0.025%), **3** (0.020%), **4** (3.09%),[3] **5** (0.68%),[3] **6** (0.096%),[3] **7** (0.017%),[3] **8** (0.052%),[3] **9** (0.0036%),[3] **10** (0.025%),[15] **11** (0.044%),[16] (2*R*,3*R*)-butane-2,3-diol 2-*O*-β-D-glucopyranoside (0.0087%),[17] *meso*-butane-2,3-diol 2-*O*-β-D-glucopyranoside (0.0065%),[17] ethyl β-D-glucopyranoside (0.055%),[17] 3-methylbutanol β-D-glucopyranoside (0.0030%),[17] (+)-pinoresinol 4′-*O*-β-D-glucopyranoside (0.016%),[18] and (+)-syringaresinol 4′-*O*-β-D-glucopyranoside (0.022%).[15]

2.2. Structures of kankanosides F (1) and G (2)

Kankanoside F (**1**) was obtained as a white powder with negative optical rotation ($[\alpha]_D^{28}$ −44.8° in MeOH). The IR spectrum of **1** showed absorption bands at 1560, 1508, and 1458 cm^{-1} ascribable to an aromatic ring and strong absorption bands at 3432, 1075, and 1044 cm^{-1}, suggestive of an oligoglycoside structure. In the positive- and negative-ion FAB-MS of **1**, quasi-molecular ion peaks were observed at *m/z* 647 $(M+Na)^+$ and 623 $(M-H)^-$, and high-resolution FAB-MS analysis revealed the molecular formula of **1** to be $C_{26}H_{40}O_{17}$. The ^1H and ^{13}C NMR (CD_3OD, Table 2) spectra of **1**, which were assigned by various NMR experiments,[19] showed signals assignable to two methylenes [δ 2.78 (2H, m, H_2-7), 3.70, 4.00 (1H each, both m, H_2-8)] and *ortho*- and *meta*-coupled ABC-type aromatic protons [δ 6.56 (1H, dd, $J = 1.9$, 7.9 Hz, H-6), 6.67 (1H, d, $J = 7.9$ Hz, H-5), 6.69 (1H, d, $J = 1.9$ Hz, H-2)] together with three glycopyranosyl moieties [δ 4.30 (1H, d, $J = 8.0$ Hz, H-1′), 4.37 (1H, d, $J = 8.0$ Hz, H-1‴), 5.16 (1H, br s, H-1″)]. The oligoglycoside structure in **1** was characterized by heteronuclear multiple bond connectivity (HMBC) experiment, which showed long-range correlations between the following proton and carbon pairs (H-1′ and C-8; H-1″ and C-3′; H-1‴ and C-6′) as shown in Figure 2. Enzymatic deacylation of echinacoside (**4**), whose absolute stereostructure was reported previously,[3] with tannase[13,14] gave **1**. On the basis of the above-mentioned evidence, the structure of kankanoside F was determined to be 2-(3,4-dihydroxyphenyl)ethyl

Table 2. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectral data for compounds **1** and **2** in CD_3OD (ppm, *J* in Hz)

Position	**1**		Position	**2**	
	δ	δ_C		δ	δ_C
Aglycon					
1		131.5 (s)	1		130.7 (s)
2	6.69 (d, 1.9)	117.2 (d)	2,6	7.03 (2H, d, 8.6)	131.0 (d)
3		146.1 (s)	3,5	6.65 (2H, d, 8.6)	116.2 (d)
4		144.7 (s)	4		156.8 (s)
5	6.67 (d, 7.9)	116.4 (d)			
6	6.56 (dd, 1.9, 7.9)	121.3 (d)			
7	2.78 (2H, m)	36.6 (t)	7	2.82 (2H, m)	36.5 (t)
8	3.70, 4.00 (both m)	72.4 (t)	8	3.70, 3.96 (both m)	72.5 (t)
β-D-Glucopyranosyl (inner)					
1′	4.30 (d, 8.0)	104.3 (d)	1′	4.33 (d, 7.9)	104.4 (d)
2′	3.28 (m)	75.7 (d)	2′	3.33 (m)	75.7 (d)
3′	3.49 (dd, 8.9, 8.9)	84.1 (d)	3′	3.54 (m)	84.0 (d)
4′	3.45 (m)	70.1 (d)	4′	3.40 (m)	70.5 (d)
5′	3.45 (m)	77.0 (d)	5′	3.55 (m)	75.4 (d)
6′	3.80 (dd, 4.9, 11.6)	69.8 (t)	6′	4.36 (dd, 6.1, 11.6)	64.7 (t)
	4.14 (dd, 1.8, 11.6)			4.50 (dd, 2.8, 11.6)	
α-L-Rhamnopyranosyl					
1″	5.16 (br s)	102.8 (d)	1″	5.19 (br s)	102.8 (d)
2″	3.94 (m)	72.4 (d)	2″	3.95 (m)	72.4 (d)
3″	3.70 (m)	72.3 (d)	3″	3.71 (m)	72.3 (d)
4″	3.39 (m)	74.0 (d)	4″	3.40 (m)	74.0 (d)
5″	4.01 (m)	70.1 (d)	5″	4.01 (m)	70.1 (d)
6″	1.24 (3H, d, 6.1)	17.9 (q)	6″	1.25 (3H, d, 6.1)	17.9 (q)
β-D-Glucopyranosyl (terminal)			Caffeoyl		
1‴	4.37 (d, 8.0)	104.9 (d)	1		127.7 (s)
2‴	3.19 (m)	74.8 (d)	2	7.04 (d, 1.9)	115.1 (d)
3‴	3.37 (m)	78.0 (d)	3		146.9 (s)
4‴	3.27 (m)	71.8 (d)	4		149.7 (s)
5‴	3.28 (m)	78.1 (d)	5	6.78 (d, 8.3)	116.6 (d)
6‴	3.64 (dd, 6.4, 11.9)	62.8 (t)	6	6.89 (dd, 1.9, 8.3)	123.2 (d)
	3.86 (dd, 2.2, 11.9)		7	7.56 (d, 15.9)	147.3 (d)
			8	6.29 (d, 15.9)	114.9 (d)
			9		169.1 (s)

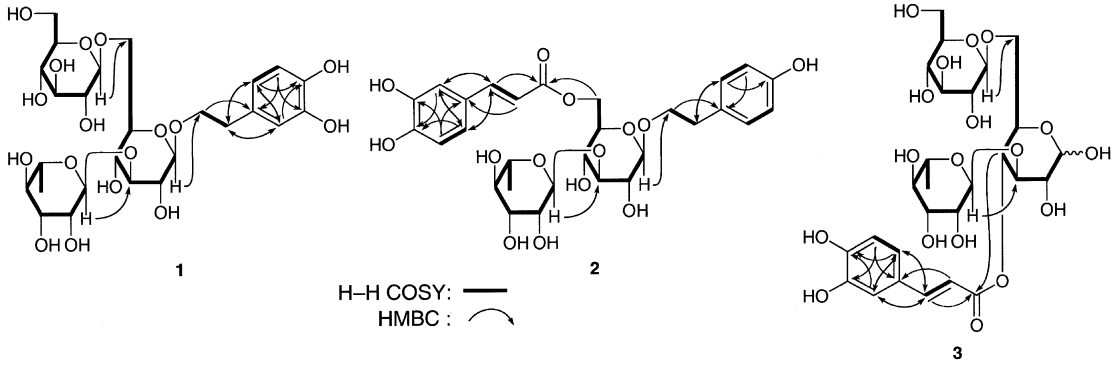

Figure 2. H–H COSY and HMBC correlations of **1**–**3**.

O-α-L-rhamnopyranosyl(1 → 3)[β-D-glucopyranosyl(1 → 6)]-β- D-glucopyranoside (**1**).

Kankanoside G (**2**) was also obtained as a white powder with negative optical rotation ($[\alpha]_D^{26}$ −35.0° in MeOH). The IR spectrum of **2** showed absorption bands at 3432, 1709, 1638, 1518, 1447, and 1040 cm^{-1}, ascribable to hydroxyl, conjugated ester carbonyl, and ether functions and aromatic ring. The molecular formula, $C_{29}H_{36}O_{14}$, of **2** was determined from the positive- and negative-ion FAB-MS [*m*/*z* 631 $(M+Na)^+$ and 607 $(M-H)^-$] and by high-resolution FAB-MS. Enzymatic deacylation of **2** with tannase yielded cistanoside G (**12**), whose absolute stereostructure was reported previously.[16] On alkaline hydrolysis of **2** with 10% aqueous potassium hydroxide (KOH)—50% aqueous

1,4-dioxane (1:1, v/v), caffeic acid was identified by HPLC analysis. The ^{1}H and ^{13}C NMR (CD_3OD, Table 2) spectra[19] of **2** indicated the presence of the following functions: 4-hydroxyphenylethyl alcohol part {two methylenes [δ 2.82 (2H, m, H_2-7), 3.70, 3.96 (1H each, both m, H_2-8)] and *ortho*-coupled A_2B_2-type aromatic protons [δ 6.65, 7.03 (2H each, both d, J = 8.6 Hz, H-3,5 and H-2,6)]} and two glycopyranosyl moieties [δ 4.33 (1H, d, J = 7.9 Hz, H-1′), 5.19 (1H, br s, H-1″)] together with a caffeoyl moiety {an *trans*-olefin [δ 6.29, 7.56 (1H each, both d, J = 15.9 Hz, H-8 and H-7)] and pyrocatechol-type dihydroxybenzene [δ 6.78 (1H, d, J = 8.3 Hz, H-5), 6.89 (1H, dd, J = 1.9, 8.3 Hz, H-6), 7.04 (1H, d, J = 1.9 Hz, H-2)]}. In the HMBC experiment on **2**, a long-range correlation was observed between the 6′-proton [δ 4.36 (1H, dd, J = 6.1, 11.6 Hz), 4.50 (dd, J = 2.8, 11.6 Hz)] and the caffeoyl carbonyl carbon (δ_C 169.1). Consequently, the structure of kankanoside G was determined to be 4-hydroxyphenethyl-*O*-α-L-rhamnopyranosyl(1 → 3)-(6-*O*-caffeoyl)-β-D-glucopyranoside (**2**).

2.3. Structure of kankanose (3)

Kankanose (**3**), $[\alpha]_D^{27}$ −35.3° (MeOH), was also obtained as a white powder. The IR spectrum of **3** showed absorption bands at 3430, 1702, 1639, 1611, 1458, and 1071 cm^{-1}, ascribable to hydroxyl, conjugated ester carbonyl, and ether functions and aromatic ring. The positive- and negative-ion FAB-MS of **3** showed quasimolecular ion peaks at *m/z* 673 $(M+Na)^+$ and *m/z* 649 $(M-H)^-$, respectively. The high-resolution FAB-MS of **3** revealed the molecular formula to be $C_{27}H_{38}O_{18}$. Alkaline hydrolysis of **3** with 10% KOH–50% aqueous 1,4-dioxane liberated caffeic acid, which was identified by HPLC analysis, together with an oligosugar. The acid hydrolysis of this oligosugar with 1.0 M hydrochloric acid (HCl) liberated L-rhamnose and D-glucose, which were identified by HPLC analysis using an optical rotation detector.[1,13,14] The ^{1}H and ^{13}C NMR (CD_3OD, Table 3) spectra[19] of **3** showed signals assignable to the trisaccharide moiety {δ [5.11 (d, J = 3.4 Hz), 4.57 (d, J = 7.9 Hz), α- and β-form mixture of H-1′ (ca. 1:1)], 4.25, 4.27 (both d, J = 7.7 Hz, H-1‴), 5.13, 5.18 (both br s, H-1″)} together with a cafferoyl group [δ 6.28, 6.29 (both d, J = 15.9 Hz, H-9), 6.78 (d, J = 8.0 Hz, H-5), 6.96 (dd, J = 2.0, 8.0 Hz, H-6), 7.06 (d, J = 2.0 Hz, H-2), 7.60 (d, J = 15.9 Hz, H-7)]. In the HMBC experiments on **3**, long-range correlations were observed between the 4′-proton [δ 5.04, 4.99 (both dd, J = 9.5, 9.8 Hz)] and the caffeoyl carbonyl carbon (δ_C 168.53, 168.65). On the basis of above-mentioned evidence, the structure of kankanose was elucidated to be α-L-rhamnopyranosyl(1 → 3)[β-D-glucopyranosyl(1 → 6)]-*O*-(4-*O*-caffeoyl)-D-glucopyranose (**3**).

2.4. Vasorelaxant effect of principal constituents (1, 3–6, 10, 11) from *C. tubulosa* on the contraction induced by noradrenaline and high K^+ in isolated rat thoracic aorta

As shown in Table 4 and Figure 3, inhibitory effects of the principal constituents, kankanoside F (**1**) and kankanose (**3**), echinacoside (**4**), acteoside (**5**), isoacteoside (**6**), cistanoside F (**10**), and salidroside (**11**) on noradrenaline-induced contractions in isolated rat thoracic aorta were examined. As a result, compounds **3**–**5**, and **10** having a caffeoyl group in the 4′-position significantly inhibited the contractions, time- and/or concentration-dependently (10–100 μM), while **6** having the 6′-*O*-caffeoyl moiety showed weak activity than **4** and **5**. On the other hand, compound **1** with a catechol group showed significant inhibition, but **11** with a phenol group did not show such effect. These results led us to presume that the 4′-*O*-caffeoyl moiety and the 1′-*O*-dihydroxyphenethyl moiety are essential for the strong vasorelaxant activity. On the other hand, compounds **1**, **3**–**6**, and **10** (100 μM) did not show any effects on contractions induced by high concentration of potassium cation (high K^+) different from nifedipine, a voltage-dependent calcium channel blocker, in the same tissues (data not shown). This finding suggested that these active constituents inhibited the contractions via receptor-operated calcium channel, but not via voltage-dependent calcium channel. Recently, Iizuka et al. reported that a phenylethanoid, forsythiaside, from the fruit of *Forsythia suspensa* inhibited the noradrenaline-induced contractions in rat aortic ring preparations and suggested that the inhibition by forsythiaside of

Table 3. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectral data for compound **3** in CD_3OD (ppm, J in Hz)

Position	3	
	δ	δ_C
α-D-Glucopyranosyl/β-D-glucopyranosyl		
1′	5.11 (d, 3.4)/4.57 (d, 7.9)	94.19/98.24 (d)
2′	3.58 (m)/3.20 (m)	74.69/75.20 (d)
3′	4.05 (dd, 9.2, 9.5)/3.82 (dd, 9.2, 9.5)	79.27/81.75 (d)
4′	5.04 (dd, 9.5, 9.8)/4.99 (dd, 9.5, 9.8)	70.82/70.88 (d)
5′	4.21 (m)/3.58 (m)	75.17/74.73 (d)
6′	3.94 (2H, m)	69.68/69.93 (t)
α-L-Rhamnopyranosyl		
1″	5.13 (br s)/5.18 (br s)	103.10/103.19 (d)
2″	3.93 (m)	72.37 (d)
3″	3.58 (m)	72.13 (d)
4″	3.29 (m)	73.82/73.88 (d)
5″	3.56 (m)	70.45/70.49 (d)
6″	1.09 (d, 6.1)	18.51 (q)
β-D-Glucopyranosyl		
1‴	4.25 (d, 7.7)/4.27 (d, 7.7)	104.52/104.56 (d)
2‴	3.20 (m)	75.20 (d)
3‴	3.35 (m)	77.82 (d)
4‴	3.25 (m)	71.48/71.51 (d)
5‴	3.35 (m)	77.89/77.91 (d)
6‴	3.64 (br d, ca. 12)	62.67 (t)
	3.82 (m)	
Caffeoyl		
1		127.70/127.73 (s)
2	7.06 (d, 2.0)	115.32 (d)
3		146.88 (s)
4		149.85/149.87 (s)
5	6.78 (d, 8.0)	116.57 (d)
6	6.96 (dd, 2.0, 8.0)	123.31 (d)
7	7.60 (d, 15.9)	148.21/148.24 (d)
8	6.28 (d, 15.9)/6.29 (d, 15.9)	114.78/114.90 (d)
9		168.53/168.65 (s)

Table 4. Vasorelaxant effects of **1**, **3**–**6**, **10**, and **11** on the contractions induced by noradrenaline in isolated rat thoracic aorta

	Concn (μM)	Time						
		5 min	10 min	20 min	30 min	40 min	50 min	60 min
Contraction (%) dl-noradrenaline (1 μM)								
Control	—	99.7 ± 0.2	99.6 ± 0.4	100.3 ± 1.0	100.5 ± 1.5	100.4 ± 1.4	100.9 ± 1.8	100.6 ± 1.9
Echinacoside (**4**)	10	100.0 ± 0.0	99.6 ± 0.6	92.6 ± 2.5	74.0 ± 7.9	32.0 ± 6.7[b]	5.5 ± 1.3[b]	0.4 ± 0.4[b]
	30	99.5 ± 0.7	99.9 ± 1.7	88.5 ± 6.7	56.7 ± 16.3	24.5 ± 11.7[b]	7.4 ± 3.9[b]	3.0 ± 2.1[b]
	100	100.0 ± 0.0	99.1 ± 0.9	82.4 ± 7.8	35.4 ± 17.5[b]	14.9 ± 12.9[b]	5.9 ± 5.9[b]	2.8 ± 2.8[b]
Acteoside (**5**)	10	103.1 ± 3.9	102.2 ± 5.7	91.6 ± 10.9	67.8 ± 21.0	55.1 ± 21.3[a]	41.5 ± 20.2[b]	29.6 ± 16.4[b]
	30	96.0 ± 1.8	91.9 ± 3.1	73.2 ± 8.6[a]	45.6 ± 13.4[a]	20.3 ± 9.4[b]	5.4 ± 4.0[b]	1.6 ± 1.6[b]
	100	96.2 ± 1.9	91.6 ± 4.5	83.0 ± 9.7	53.3 ± 16.0[a]	23.3 ± 11.4[b]	8.1 ± 5.2[b]	2.8 ± 2.8[b]
Isoacteoside (**6**)	10	100.6 ± 0.3	101.1 ± 0.4	101.3 ± 0.4	100.7 ± 0.9	98.8 ± 1.6	96.4 ± 2.3	89.1 ± 6.2
	30	99.6 ± 0.3	99.5 ± 0.5	98.5 ± 0.4	96.1 ± 0.5	90.9 ± 1.9	87.5 ± 5.1	72.0 ± 8.2
	100	99.9 ± 1.0	101.1 ± 0.7	100.4 ± 1.0	97.6 ± 1.8	90.6 ± 3.8	76.9 ± 6.8	59.6 ± 9.9[b]
Control	—	101.1 ± 0.1	99.9± 0.5	100.3 ± 0.3	100.6 ± 0.3	100.9 ± 0.3	100.1 ± 0.7	100.5 ± 0.9
Kankanoside F (**1**)	100	98.8 ± 0.4	97.1 ± 1.7	31.3 ± 14.7[b]	2.5 ± 1.8[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
Kankanose (**3**)	100	97.7 ± 0.7	96.2 ± 2.7	65.8 ± 14.4	9.6 ± 4.4[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
Cistanoside F (**10**)	100	98.8 ± 0.5	97.0 ± 1.3	30.1 ± 12.4[b]	3.5 ± 1.3[b]	0.4 ± 0.4[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]
Salidroside (**11**)	100	99.7 ± 0.2	99.9 ± 0.3	99.9 ± 0.3	98.6 ± 0.6	98.3 ± 0.8	97.4 ± 1.3	96.3 ± 1.9
Prazosin	0.01	83.0 ± 6.8[b]	64.4 ± 9.3[b]	33.6 ± 4.8[b]	27.7 ± 3.8[b]	25.0 ± 3.7[b]	24.4 ± 3.0[b]	22.6 ± 3.0[b]
	0.1	7.2 ± 0.3[b]	0.3 ± 0.2[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]

Each value represents the mean ± SEM ($N = 4$–8).
Significantly different from the control: [a]$p < 0.05$, [b]$p < 0.01$.

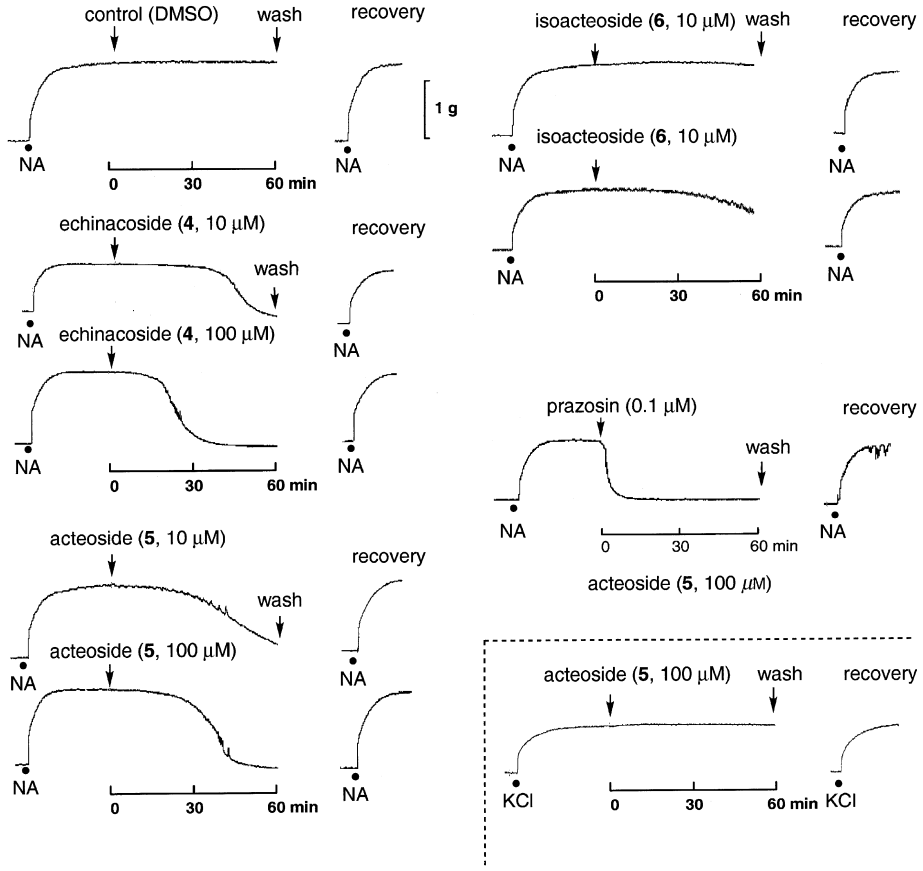

Figure 3. Effects of **4**–**6** on noradrenaline (NA, 1 μM)- and/or 54 mM KCl-induced contractions in isolated rat thoracic aorta.

noradrenaline-induced contraction is partly due to a decrease in noradrenaline-induced calcium influx.[20] However, in the present study, the relaxant effects of the active constituents were observed ca. 30 min after addition of noradrenaline in a different way from that of prazosin, an adrenaline α_1-receptor antagonist. The mechanisms of action of active constituents need to be studied further.

3. Experimental

3.1. General experimental procedure

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter ($l = 5$ cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; ^{1}H NMR spectra, JEOL JNM-LA500 (500 MHz) spectrometer; ^{13}C NMR spectra, JEOL JNM-LA500 (125 MHz) spectrometer with tetramethylsilane as an internal standard; FABMS and HR-FAB-MS, JEOL JMS-SX 102A mass spectrometer; HPLC detector, Shimadzu RID-6A refractive index and SPD-10A UV–vis detectors. HPLC column, YMC-Pack ODS-A (250×4.6 mm id) and (250×20 mm id) columns were used for analytical and preparative purposes, respectively.

The following experimental conditions were used for chromatography: normal-phase silica gel column chromatography, silica gel BW-200 (Fuji Silysia Chemical, Ltd, 150–350 mesh); reversed-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd, 100–200 mesh); TLC, pre-coated TLC plates with silica gel 60F_{254} (Merck, 0.25 mm) (normal-phase) and silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed-phase); reversed-phase HPTLC, pre-coated TLC plates with silica gel RP-18 WF_{254S} (Merck, 0.25 mm); detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4, followed by heating.

3.2. Plant material

Dried stems of *C. tubulosa* were purchased at Urumuqi, Xinjiang Province, China, in January 2005 via Eishin Trading Co., Ltd, Osaka, Japan, as described previously.[1]

3.3. Extraction and isolation

Fractions 1-2 (590 mg), 2-1 (466 mg), 2-3 (3931 mg), 3-3 (3610 mg), 3-4 (190 mg), 4-5 [=echinacoside (**4**, 2595 mg, 0.44%)], 4-6 (1635 mg), 5-2 (1114 mg), 5-3 (306 mg), 5-4 (347 mg), 5-5 (=**4**, 1620 mg, 2.03%), and 5-6 (1453 mg) were obtained from the methanolic extract of the stems of *C. tubulosa* as reported previously.[1] Fraction 1–2 (590 mg) was purified by HPLC [MeOH/H_2O (35:65, v/v)] to give 3-methylbutanol β-D-glucopyranoside (18 mg, 0.0030%), (+)-pinoresinol 4′-*O*-β-D-glucopyranoside (96 mg, 0.016%), and (+)-syringaresinol 4′-*O*-β-D-glucopyranoside (129 mg, 0.022%) together with cistanochlorin (21 mg, 0.0035%). Fraction 2-1 (466 mg) was separated by HPLC [MeOH/H_2O (5:95, v/v)] to give (2*R*,3*R*)-butane-2,3-diol 2-*O*-β-D-glucopyranoside (12 mg, 0.0087%), (2*R*,3*S*)-butane-2,3-diol 2-*O*-β-D-glucopyranoside (9 mg, 0.0065%), and ethyl β-D-glucopyranoside (76 mg, 0.055%) together with uridine (0.0069%). Fraction 2-2 (535 mg) was purified by HPLC [MeOH/H_2O (5:95, v/v)] to afford salidroside (**11**, 41 mg, 0.022%) together with antirrhide (15 mg, 0.0079%) and 6-deoxycatalpol (214 mg, 0.11%). Fraction 2-3 (535 mg) was separated by HPLC [MeOH/H_2O (5:95, v/v)] to furnish **11** (17 mg, 0.022%) together with gluroside (110 mg, 0.14%) and bartsioside (164 mg, 0.21%). Fraction 3-3 (510 mg) was subjected to HPLC [MeOH/H_2O (40:60, v/v)] to give acteoside (**5**, 257 mg, 0.31%), isoacteoside (**6**, 79 mg, 0.096%), and 2′-acetylacteoside (**7**, 14 mg, 0.017%). Fraction 3-4 (190 mg) was further purified by HPLC [MeOH/H_2O (40:60, v/v)] to give kankanoside G (**2**, 17 mg, 0.0029%) and tubuloside B (**9**, 21 mg, 0.0036%). Fraction 4-6 (75 mg) was purified by HPLC [MeOH/H_2O (35:65, v/v)] to afford **5** (18 mg, 0.065%). Fraction 5-2 (1114 mg) was subjected to HPLC [MeOH/H_2O (10:90, v/v)] to give **2** (24 mg, 0.030%). Fraction 5-3 (306 mg) was purified by HPLC [MeOH/H_2O (15:85, v/v)] to give kankanoside F (**1**, 13 mg, 0.016%). Fraction 5-4 (347 mg) was subjected to HPLC [MeOH/H_2O (10:90, v/v)] to give kankanose (**3**, 30 mg, 0.038%) and cistanoside F (**10**, 20 mg, 0.025%). Fraction 5-6 (530 mg) was purified by HPLC [MeOH/H_2O (35:65, v/v)] to afford **4** (185 mg, 0.62%), **5** (91 mg, 0.30%), and tubuloside A (**8**, 16 mg, 0.052%).

3.3.1. Kankanoside F (1). A white powder, $[\alpha]_D^{28}$ −44.8° (*c* 0.50, MeOH). High-resolution positive-ion FAB-MS *m*/*z*: Calcd for $C_{26}H_{40}O_{17}Na$ $(M+Na)^+$, 647.2163. Found: 647.2160. UV [MeOH, nm (log ε)]: 224 (3.83), 283 (3.37). IR (KBr): 3432, 2940, 1560, 1508, 1458, 1075, 1044 cm^{-1}. ^{1}H NMR (500 MHz, CD_3OD) δ: given in Table 2. ^{13}C NMR data (125 MHz, CD_3OD) δ_C: given in Table 2. Positive-ion FAB-MS *m*/*z*: 647 $(M+Na)^+$. Negative-ion FAB-MS *m*/*z*: 623 $(M-H)^-$, 477 $(M-C_6H_{11}O_4)^-$, 623 $(M-C_6H_{11}O_5)^-$, 623 $(M-C_{12}H_{21}O_9)^-$.

3.3.2. Kankanoside G (2). A white powder, $[\alpha]_D^{26}$ −35.0° (*c* 0.80, MeOH). High-resolution positive-ion FAB-MS *m*/*z*: Calcd for $C_{29}H_{36}O_{14}Na$ $(M+Na)^+$, 631.2003. Found: 631.1996. UV [MeOH, nm (log ε)]: 329 (4.10). IR (KBr): 3432, 2924, 1709, 1638, 1518, 1447, 1264, 1040 cm^{-1}. ^{1}H NMR (500 MHz, CD_3OD) δ: given in Table 2. ^{13}C NMR data (125 MHz, CD_3OD) δ_C: given in Table 2. Positive-ion FAB-MS *m*/*z*: 631 $(M+Na)^+$. Negative-ion FAB-MS *m*/*z*: 607 $(M-H)^-$, 461 $(M-C_6H_{11}O_4)^-$.

3.3.3. Kankanose (3). A white powder, $[\alpha]_D^{27}$ −35.3° (*c* 0.50, MeOH). High-resolution positive-ion FAB-MS *m*/*z*: Calcd for $C_{27}H_{38}O_{18}Na$ $(M+Na)^+$, 673.1956. Found: 673.1959. UV [MeOH, nm (log ε)]: 219 (4.10), 247 (3.96), 333 (4.21). IR (KBr): 3430, 2962, 1702, 1639, 1611, 1458, 1264, 1071 cm^{-1}. ^{1}H NMR (500 MHz, CD_3OD) δ: given in Table 3. ^{13}C NMR data (125 MHz, CD_3OD) δ_C: given in Table 3. Positive-ion

FAB-MS *m/z*: 673 $(M+Na)^+$. Negative-ion FABM-S *m/z*: 649 $(M-H)^-$, 487 $(M-C_9H_8O_3)^-$, 325 $(M-C_{15}H_{18}O_8)^-$.

3.4. Enzymatic deacylation of 2 and 4 with tannase

A solution of **2** (4.8 mg, 0.008 mmol) in H_2O (1.0 mL) was treated with tannase (3.7 mg, from *Aspergillus oryzae*, Wako Pure Chemical Ind., Ltd, Osaka, Japan) and the solution was stirred at 37 °C for 24 h. After EtOH was added to the reaction mixture, the solvent was removed under reduced pressure and the residue was purified by HPLC [MeOH/H_2O (35:65, v/v)] to furnish cistanoside G (**12**, 2.8 mg, 80%).[16] Through a similar procedure, a solution of **4** (9.2 mg, 0.012 mmol) in H_2O (2.0 mL) was treated with tannase (7.5 mg) and the solution was stirred at 37 °C for 24 h. Work-up of the reaction mixture as described above gave a residue, which was purified by HPLC [MeOH/H_2O (15:85, v/v)] to furnish **1** (4.7 mg, 62%).

3.5. Alkaline and acid hydrolysis of 2 and 3

A solution of **2** and **3** (each 3.5 mg) in 50% aqueous 1,4-dioxane (1.0 mL) was treated with 10% aqueous KOH (1.0 mL) and the whole was stirred at 37 °C for 1 h, respectively. Removal of the solvent under reduced pressure gave a reaction mixture, which was subjected to HPLC analysis [column: YMC-Pack ODS-A, 250 × 4.6 mm id; mobile phase: MeOH/H_2O (30:70, v/v); detection: UV (254 nm); flow rate: 1.0 mL/min] to identify caffeic acid (t_R: 13.1 min). Then the reaction mixture from **3** was dissolved in 1 M HCl (2.0 mL) and heated under reflux for 3 h. After cooling, the reaction mixture was extracted with EtOAc. The aqueous layer was subjected to HPLC analysis under the following conditions, respectively: HPLC column, Kaseisorb LC NH_2-60-5, 250 × 4.6 mm id (Tokyo Kasei Co., Ltd, Tokyo, Japan); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd, Tokyo, Japan); mobile phase, CH_3CN/H_2O (85:15, v/v); flow rate 0.8 mL/min]. Identification of L-rhamnose and D-glucose present in the aqueous layer was carried out by comparison of their retention time and optical rotation with those of authentic samples, t_R: 9.9 min (L-rhamnose, negative optical rotation) and 17.9 min (D-glucose, positive optical rotation).

3.6. Vasorelaxant activity

3.6.1. Tissue preparation. Male Sprague–Dawley rats weighing 250–350 g were sacrificed by severing both carotid arteries under anesthesia, and the thoracic aorta was isolated and cut into helical strips (2–3 mm × 15–20 mm). Physiological salt solution contained NaCl (118.0 mM), KCl (4.7 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), $CaCl_2$ (2.5 mM), $NaHCO_3$ (25.0 mM), and D-glucose (10.0 mM). The solution was aerated with a 95% O_2–5% CO_2 gas mixture and kept at 37 °C. To investigate the mechanical response, each preparation was suspended in an organ bath (6 ml) and subjected to an initial load of about 1 g. One hour equilibration period was allowed before initiation of the experiments. Contractions were measured isometrically via a force-displacement transducer (AD Instruments) and recorded on a polygraph.

3.6.2. Inhibitory effect on the contraction induced by dl-noradrenaline or high K^+. After equilibration, dl-noradrenaline (final concentration: 1 μM) or 3 M KCl (0.1 mL, final concentration of K^+: 54 mM) was added to the bath. The tissues were washed three times and re-equilibrated after the contraction had reached the maximum level. Sustained contraction was induced again by the addition of dl-noradrenaline or KCl, and then test compound was applied at 10–100 μM (noradrenaline) or 100 μM (high K^+). The contractile response prior to the application of test sample was taken to be 100%. Prazosin hydrochloride and nifedipine were used as reference compounds.

3.6.3. Statistical analysis. Values are expressed as means ± SEM. For statistical analysis, one-way analysis of variance followed by Dunnett's test for multiple comparison analysis was used. Probability (p) values less than 0.05 were considered significant.

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