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Studies on the Chinese Crude Drug "Forsythiae Fructus." VI.[1] The Structure and Antibacterial Activity of Suspensaside isolated from *Forsythia suspensa*

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A new caffeoyl glycoside of β,3,4-trihydroxyphenethyl alcohol, designated as suspensaside (**1**), was isolated from the fruits of *Forsythia suspensa* Vahl (Oleaceae). The structure of **1** was established as dl-β,3,4-trihydroxyphenethyl-*O*-α-l-rhamnopyranosyl-(1→6)-4-*O*-caffeoyl-β-d-glucopyranoside on the basis of analysis of the carbon-13 nuclear magnetic resonance spectrum and chemical evidence.

Compound **1** exhibited antibacterial activity against *Staphylococcus aureus* Terashima, with MIC 4.1 mM (2.6 mg/ml).

Keywords——*Forsythia suspensa*; Oleaceae; new caffeoyl glycoside of β,3,4-trihydroxyphenethyl alcohol; suspensaside; dl-β,3,4-trihydroxyphenethyl-*O*-α-l-rhamnopyranosyl-(1→6)-4-*O*-caffeoyl-β-d-glucopyranoside; ^{13}C-NMR spectra; antibacterial activity

In a previous paper,[1] we reported the isolation of a new caffeoyl glycoside of 3,4-dihydroxyphenethyl alcohol, designated forsythiaside, from the fruits of *Forsythia suspensa* Vahl (Oleaceae). Hikino *et al.*[2] independently reported the isolation of the same glycoside, designated forsythoside A, from the leaves of *F. suspensa* and described its antibacterial activity.

As a continuation of our investigation on the Chinese crude drug "Forsythiae Fructus," we examined the antimicrobial activity of two kinds of the crude drug of different origin; one was from the fruits of *F. suspensa* and the other from the fruits of *F. viridissima* (Table I). Higher antibacterial activity against *Staphylococcus aureus* was observed in the fruits of *F. suspensa*.

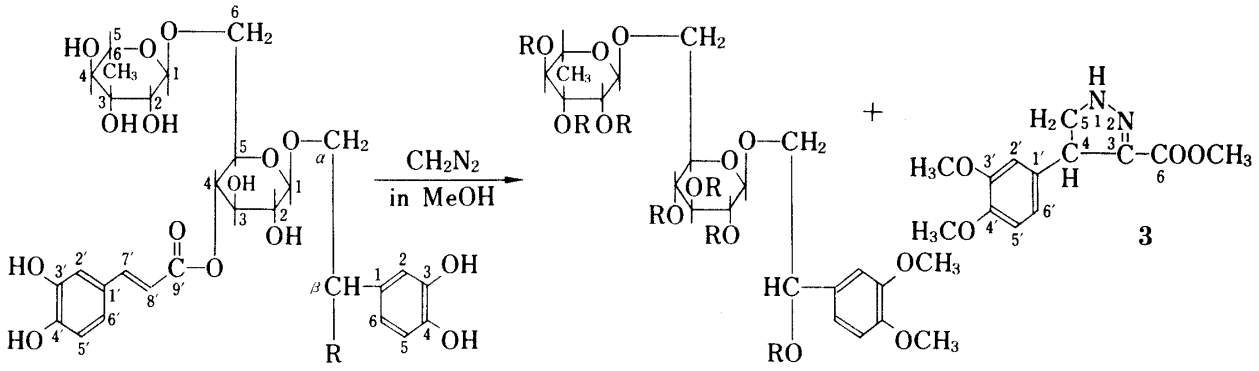
Table I. Antimicrobial Activity of Forsythiae Fructus

Strains	Crude drug[a]	
	F. suspensa	*F. viridissima*
Staphylococcus aureus Terashima	4	10
Staphylococcus aureus 209 P	6	10
Streptococcus mutans BHT	18	>20
Streptococcus faecalis ATCC 6057	>20	>20
Streptococcus salivarius ATCC 9759	<10	18
Lactobacillus casei	50	60
Candida albicans ATCC 1012	>50	>50
Bacillus subtilis	>50	>50
Escherichia coli C 600	14	>16

a) w/v%: Based on dry weight of crude drug.

This paper describes the isolation and structure determination of the antibacterial constituents from the fruits of *F. suspensa*. The extraction and separation were carried out as described in "Experimental."

Two glycosides were isolated as the antibacterial constituents, and one of them was identified as forsythiaside. The other glycoside, designated suspensaside (**1**), was obtained as a colorless powder, $C_{29}H_{36}O_{16}$, mp 177—182°C, $[\alpha]_D^{18}$ −18.7° (methanol), whose molecular weight was confirmed by the observation of *m/z* 640 (M^+) on field desorption mass spectrometry (FD-MS).



1 : R=OH
forsythiaside : R=H

2 : R=H
4 : R=CH_3CO

Chart 1

The molecula formula of **1** differs in composition by an increment of one unit of oxygen atom relative to that of forsythiaside. The ultraviolet (UV) spectrum of **1** showed absorption maxima at 219, 245, 290, 302 and 332 nm. The bathochromic shift of the absorption maximum with base was very similar to that of forsythiaside. The infrared (IR) spectrum of **1** suggested the presence of a conjugated ester (1690 cm^{-1}) and an aromatic ring (1600 cm^{-1}), while the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **1** resembled that of forsythiaside except for disappearance of the signal (δ 2.80, 2H, t, J=7 Hz, for forsythiaside) assigned to two benzyl protons of the phenethyl moiety. These data suggest that **1** bears a marked structural resemblance to forsythiaside.

The reaction of **1** in methanol with excess diazomethane gave two compounds, **2** as an amorphous powder, $C_{22}H_{34}O_{13}$, $[\alpha]_D^{20}$ −36.0° (methanol) and **3** as colorless needles, $C_{13}H_{16}O_4N_2$, mp 119—120°C, $[\alpha]_D^{20}$ +62.4° (chloroform).

The UV spectrum of **2** gave no bathochromic shift on addition of base. No IR absorption assignable to a conjugated ester was observed in the IR spectrum of **2**. The ^{1}H-NMR spectrum of **2** exhibited signals at δ 1.23 (3H, d, J=6 Hz) due to methyl protons of the sugar moiety, at δ 3.76 and 3.79 (6H, each s) due to aromatic methoxy protons, at δ 4.30 (1H, d, J=8 Hz) and 4.83 (1H, br s) due to anomeric protons of the sugar moiety, and at δ 6.80—7.00 (3H, m) due to aromatic protons. Acetylation of **2** with acetic anhydride–pyridine gave **4** as an amorphous powder, $C_{36}H_{48}O_{20}$, $[\alpha]_D^{20}$ −40.9° (chloroform). The ^{1}H-NMR spectrum of **4** showed the presence of seven alcoholic acetoxys (δ 1.96, 2.00 and 2.06), two aromatic methoxys (δ 3.80 and 3.83) and a proton (δ 5.77, t, J=6 Hz) at the benzyl position bearing an acetoxy group, whose chemical shift was confirmed by comparison with that of 5-acetoxymatairesinol dimethyl ether (δ 5.83, d, J=6 Hz).[3] It was clearly suggested that **2** consists of a β-hydroxy-dimethoxyphenethyl moiety and a sugar moiety containing no acyl group.

The acid hydrolysis of **2** gave **5** as a yellow oil, $C_{10}H_{12}O_3$, which was identical with authentic 3,4-dimethoxyphenylacetaldehyde. The genuine aglycone of **2**, β-hydroxy-3,4-dimethoxyphenethyl alcohol, could not be obtained from the hydrolyzate, because β-hydroxy-3,4-dimethoxyphenethyl alcohol is very sensitive to acid and is readily converted to 3,4-dimethoxyphenylacetaldehyde (**5**), as shown in Chart 2. The presence of D-glucose and L-rhamnose in

the hydrolyzate was detected in a ratio of 1:1 by gas chromatography (GC). Therefore, the structure of **2** is proposed to be rhamno-glucoside of β-hydroxy-3,4-dimethoxyphenethyl alcohol, *i.e.*, deacylsuspensaside dimethyl ether.

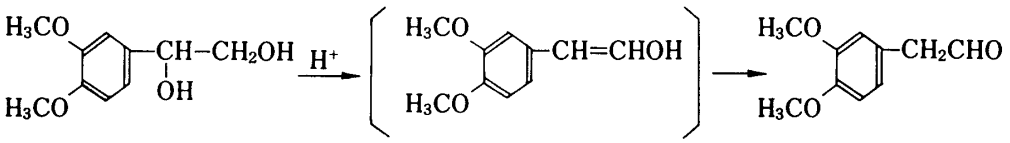


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Chart 2

On the other hand, IR, UV, ^{1}H-NMR and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectral data suggested **3** to be a pyrazoline derivative of caffeic acid, and **3** was found to be identical with 4-[3′,4′-dimethoxyphenyl]-2-pyrazoline-3-carboxylic acid methyl ester obtained by the reaction of 3,4-dimethoxycinnamic acid in methanol with excess diazomethane.

The ^{13}C-NMR spectra of **1** and **2** were correlated with those of known compounds, *i.e.*, forsythiaside, β,3,4-trihydroxyphenethyl alcohol,[4] β-hydroxy-3,4-dimethoxyphenethyl alcohol and rutin bearing the rutinose moiety, respectively. Tables II and III present the ^{13}C-NMR data and their assignments. The ^{13}C-NMR of **1** supported the attachment of the caffeate moiety at the C-4 carbon of glucose (Glc-4), and the chemical shift of the C-α carbon of **1** at 76.8 ppm suggested the linkage of the β,3,4-trihydroxyphenethyl moiety to the C-1 carbon of glucose (Glc-1). With regard to the problem of the absolute configuration at the C-β position in **1**, a comparison of the molecular optical rotation differences between forsythiaside derivatives and suspensaside derivatives suggested that the β,3,4-trihydroxyphenethyl moiety is a racemate.

Consequently, the structure of **1** has been established as DL-β,3,4-trihydroxyphenethyl-*O*-α-L-rhamnopyranosyl-(1→6)-4-*O*-caffeoyl-β-D-glucopyranoside.

The antibacterial activity of **1** and related compounds against *Staphylococcus aureus* TERASHIMA is summarized in Table V. The data indicate that the antibacterial activity of **1** might be attributed to the phenolic moieties, *i.e.*, caffeate and β,3,4-trihydroxyphenethyl

TABLE II. ^{13}C-NMR Chemical Shifts[a]

	β,3,4-Trihydroxyphenethyl moiety				Rutinose moiety			Caffeate moiety	
	1	Forsythiaside	β,3,4-Trihydroxyphenethyl alcohol		**1**	Forsythiaside		**1**	Forsythiaside
C-1	133.6	131.3	134.4	Glc-1	104.4	104.4	C-1′	127.8	127.6
C-2	114.8	116.3	114.5	Glc-2	75.4	75.1	C-2′	115.5	115.2
C-3	146.2	146.0	145.7	Glc-3	75.6	75.8	C-3′	146.8	146.7
C-4	145.9	144.6	145.4	Glc-4	75.1	75.1	C-4′	149.6	149.7
C-5	116.7	117.1	116.0	Glc-5	74.8	74.7	C-5′	116.4	116.5
C-6	119.3	121.3	118.9	Glc-6	67.7	67.6	C-6′	123.2	123.0
C-α	76.8	72.2	68.3	Rham-1	102.2	102.2	C-7′	147.8	147.5
C-β	73.6	36.7	75.4	Rham-2	72.0	72.0	C-8′	114.8	114.7
				Rham-3	72.4	72.2	C-9′	168.4	168.2
				Rham-4	74.0	73.9			
				Rham-5	69.9	69.8			
				Rham-6	18.1	18.0			

a) The spectra were taken with a JNM-FX 60 spectrometer (15.00 MHz) in CD_3OD with TMS as an internal reference, using micro cells.

Table III. ^{13}C-NMR Chemical Shifts[a)]

	β-Hydroxy-3,4-dimethoxyphenethyl moiety			Rutinose moiety				
	2	β-Hydroxy-3,4-dimethoxyphenethyl alcohol		2	Rutin		2	Rutin
C-1	135.1	136.2	Glc-1	104.6	104.5	Rham-1	102.2	102.1
C-2	111.5	111.5	Glc-2	75.2	75.5	Rham-2	72.2	71.8
C-3	150.4	150.3	Glc-3	77.9	77.9	Rham-3	72.4	72.0
C-4	150.0	149.8	Glc-4	71.7	71.7	Rham-4	74.1	73.7
C-5	113.0	112.9	Glc-5	76.9	76.9	Rham-5	69.8	69.4
C-6	120.1	119.9	Glc-6	68.4	68.3	Rham-6	18.1	17.6
C-α	76.7	68.6						
C-β	73.6	75.5						
OCH_3	56.6	56.5						

a) The spectra were taken with a JNM-FX 60 spectrometer (15.00 MHz) in CD_3OD with TMS as an internal reference, using micro cells.

Table IV. Molecular Optical Rotation Differences

	$[\alpha]_D$	$[M]_D$	$\Delta[M]_D$
Suspensaside (1)	−18.7°	−119.7°	−3.6°
Forsythiaside	−18.6°	−116.1°	
Deacylsuspensaside dimethyl ether (2)	−36.0°	−182.2°	+3.0°
Deacylforsythiaside dimethyl ether	−37.8°	−185.2°	

Table V. Antibacterial Activity against *Staphylococcus aureus* Terashima

	MIC
Suspensaside (1)	4.1 mM (2.6 mg/ml)
β,3,4-Trihydroxyphenethyl alcohol	3.5 mM (0.6 mg/ml)
Caffeic acid	4.5 mM (0.8 mg/ml)
Forsythiaside	3.2 mM (2.0 mg/ml)

moiety. Compound **1** also shows high inhibitory activity against cyclic adenosine monophosphate(cAMP)-phosphodiesterase *in vitro* (IC_{50} 18.3×10^{-5} mol/l).[5)]

Experimental

All melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. The following instruments were used: optical rotation, Yanagimoto OR-10; UV spectra, Shimadzu UV-210; IR spectra, Shimadzu IR-400; ^{1}H-NMR, Hitachi R-40 with tetramethylsilane ($\delta=0$) as an internal reference; ^{13}C-NMR spectra, JEOL JNM-FX 60, equipped with a JEC-980 computer; FD-MS, JEOL JMS-DX 300; MS, Hitachi RMU-7L; GC, Shimadzu GC-6AM. The abbreviations used are as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br s, broad singlet.

The conditions for GC were as follows: glass column (3 mm × 1 m), 1.5% OV-1 on shimalite-W (80—100 mesh); column temp., 150—200°C (3°/min); injection and detector temp., 230°C; carrier gas, N_2 (20 ml/min).

Pre-coated high-performance thin-layer chromatogaphy (HPTLC) plates silica gel $60F_{254}$ (Merck) were used for thin-layer chromatography (TLC). Pre-coated TLC plates silica gel $60F_{254}$ (Merck) were used for preparative TLC.

Isolation——"Forsythiae Fructus" (500 g, fruits of *Forsythia suspensa* Vahl) were crushed and extracted with hot water (2.5 l). The extract was cooled and the precipitate was filtered off. The filtrate was lyophilized to give a powder (39.6 g). The powder (23.8 g) was subjected to column chromatography on Sephadex

LH-20, eluting with H_2O. The fractions (20 ml each) were monitored by TLC using the upper layer of $CH_3COC_2H_5$–AcOEt–HCOOH–H_2O–C_6H_6 (4: 3: 1: 1: 2) as a developer, and those showing a TLC spot at *Rf* 0.35, which gave a greenish-blue color with dil. $FeCl_3$ soln., were concentrated to afford crude forsythiaside. This was purified by repeated re-chromatography on Sephadex LH-20, giving 215.3 mg of forsythiaside.

The fractions showing a TLC spot at *Rf* 0.18, which gave a greenish-blue color with dil. $FeCl_3$ soln., were concentrated to afford crude suspensaside (**1**). Repeated re-chromatography on Sephadex LH-20 gave 430.2 mg of **1**.

Suspensaside (**1**)——Colorless powder, mp 177—182°C, $[\alpha]_D^{18}$ −18.7° (c=1.7 in MeOH). UV λ_{max}^{EtOH} nm (log ε): 219 (4.21), 245 (3.92), 290 (3.99), 302 (3.99), 332 (4.12). UV $\lambda_{max}^{EtOH+NaOH}$ nm: 292, 363. IR ν_{max}^{KBr} cm^{-1}: 3600—3100 (OH), 1690 (conjugated CO), 1600 (C=C). FD-MS m/z: 640 (M^+, $C_{29}H_{36}O_{16}$). ^{1}H-NMR (in CD_3OD) δ: 1.20 (3H, d, J=6 Hz, rhamnose-CH_3), 4.43 (1H, d, J=8 Hz, glucose-anomeric H), 4.65 (1H, br s, rhamnose-anomeric H), 6.30 (1H, d, J=15 Hz, Ar–C<u>H</u>=CH–), 6.60—7.20 (6H, m, arom.H), 7.56 (1H, d, J=15 Hz, Ar–CH=C<u>H</u>–).

Reaction of Suspensaside (**1**) **in Methanol with Excess Diazomethane**——A solution of 99.1 mg of **1** in methanol was treated with excess diazomethane, and the mixture was left to stand overnight in a refrigerator. Then the reaction mixture was evaporated to dryness. The residue was extracted with $CHCl_3$. The insoluble portion was purified by preparative TLC using $CHCl_3$–MeOH–H_2O (65: 35: 10) as developer to give 55.4 mg of **2**, and the soluble portion was purified by preparative TLC using $CHCl_3$–AcOEt (1: 1) as developer to give 38.7 mg of **3**.

Deacylsuspensaside Dimethyl Ether (**2**)——Amorphous powder, $[\alpha]_D^{20}$ −36.0° (c=0.9 in MeOH). UV λ_{max}^{EtOH} nm (log ε): 230 (3.94), 278 (3.51). IR ν_{max}^{KBr} cm^{-1}: 3600—3100 (OH), 1600, 1590, 1510 (arom. C=C). MS: Calcd for $C_{22}H_{34}O_{13}$, 506.1984. Obsd., 506.1983. ^{1}H-NMR (in CD_3OD) δ: 1.23 (3H, d, J=6 Hz, rhamnose-CH_3), 3.76, 3.79 (6H, each s, 2×CH_3O), 4.30 (1H, d, J=8 Hz, glucose-anomeric H), 4.83 (1H, br s, rhamnose-anomeric H), 6.80—7.00 (3H, m, arom.H).

4-[3′,4′-Dimethoxyphenyl]-2-pyrazoline-3-carboxylic Acid Methyl Ester (**3**)——Colorless needles from MeOH, mp 119—120°C, $[\alpha]_D^{20}$ +62.4° (c=0.7 in $CHCl_3$). UV λ_{max}^{EtOH} nm (log ε): 226 (3.98), 288 (3.96). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400 (NH), 1700 (CO), 1600, 1590, 1510 (arom. C=C), 1560 (C=N). MS: Calcd for $C_{13}H_{16}O_4N_2$, 264.1087. Obsd., 264.1085. ^{1}H-NMR (in $CDCl_3$) δ: 3.70 (3H, s, CH_3O), 3.80 (6H, s, 2×CH_3O), 3.50—4.45 (3H, m, $C_{4,5}$–H), 6.23 (1H, br s, NH, quenched by addition of D_2O), 6.60—6.90 (3H, m, arom.H). ^{13}C-NMR (in CD_3OD) δ: 145.0 (C-3), 49.3 (C-4), 58.7 (C-5), 164.5 (C-6), 52.0 (OCH_3), 135.6 (C-1′), 112.3 (C-2′), 150.6 (C-3′), 149.6 (C-4′), 113.3 (C-5′), 120.5 (C-6′), 56.4 (OCH_3).

This product was identical with 4-[3′,4′-dimethoxyphenyl]-2-pyrazoline-3-carboxylic acid methyl ester obtained by the reaction of 3,4-dimethoxycinnamic acid in methanol with excess diazomethane.

Deacylsuspensaside Dimethyl Ether Heptaacetate (**4**)——**2** was acetylated with acetic anhydride–pyridine in the usual way. The crude acetate was purified by preparative TLC using $CHCl_3$–AcOEt (1: 1) as a developer to give **4**. Amorphous powder, $[\alpha]_D^{20}$ −40.9° (c=0.6 in $CHCl_3$). UV λ_{max}^{EtOH} nm (log ε): 231 (4.01), 278 (3.57). IR ν_{max}^{KBr} cm^{-1}: 1740 (CO), 1600, 1590, 1510 (arom. C=C). MS: Calcd for $C_{36}H_{48}O_{20}$, 800.2783. Obsd., 800.2788. ^{1}H-NMR (in $CDCl_3$) δ: 1.26 (3H, d, J=6 Hz, rhamnose-CH_3), 1.96, 2.00, 2.06 (21H, each s, 7× alcoholic $OCOCH_3$), 3.80, 3.83 (6H, each s, 2×CH_3O), 5.77 (1H, t, J=6 Hz, Ar–C<u>H</u>–$OCOCH_3$), 6.60—6.90 (3H, m, arom.H). MS m/z: 800 (M^+, $C_{36}H_{48}O_{20}$), 577 ($C_{24}H_{33}O_{16}^+$), 561 ($C_{24}H_{33}O_{15}^+$), 511 ($C_{24}H_{31}O_{12}^+$), 273 $C_{12}H_{17}O_7^+$), 223 ($C_{12}H_{15}O_4^+$).

Acid Hydrolysis of Deacylsuspensaside Dimethyl Ether (**2**)——**2** in 1% H_2SO_4 soln. was heated on a water bath for 1 h, then cooled. The mixture was extracted with Et_2O. The Et_2O layer was washed and evaporated to dryness. The residue was purified by preparative TLC using $CHCl_3$–AcOEt (1: 1) as a developer to give **5**. The aq.layer was neutralized with $BaCO_3$ and the precipitate was filtered off. The filtrate was evaporated to dryness, and the residue was examined by GC to identify L-rhamnose (t_R: 4.14, 5.23 for TMS ether) and D-glucose (t_R: 10.53, 13.97 for TMS ether) in a ratio of 1: 1 in comparison with the products obtained from rutin by the same procedure.

3,4-Dimethoxyphenylacetaldehyde (**5**)——Yellow oil. UV λ_{max}^{EtOH} nm (log ε): 230 (4.18), 275 (3.98), 308 (3.85). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1690 (CO), 1605, 1600, 1520 (arom. C=C). MS: Calcd for $C_{10}H_{12}O_3$, 180.0747. Obsd., 180.0743. ^{1}H-NMR (in $CDCl_3$) δ: 3.56 (2H, d, J=2 Hz, –C<u>H</u>$_2$–), 3.83 (6H, s, 2×CH_3O), 6.60—7.00 (3H, m, arom.H), 9.63 (1H, t, J=2 Hz, –C<u>H</u>O).

This product was identical with authentic 3,4-dimethoxyphenylacetaldehyde.

Antimicrobial Activity Test——The minimum inhibitory concentration (MIC) was determined by the broth dilution method.[6] Each strain listed in Table I was grown in penassay broth overnight at 37°C. The overnight culture was diluted 100-fold with physiological saline solution and inoculated into Müller Hinton medium or Trypticase soy broth containing aqueous extract of Forsythiae Fructus.

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References and Notes

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