Cowaniin, a *C*-Glucosidic Ellagitannin Dimer Linked through Catechin from *Cowania mexicana*

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A new complex tannin, cowaniin (1) was isolated from the leaves and stems of *Cowania mexicana* (Rosaceae), and its structure was characterized as novel *C*-glucosidic tannin dimer linked through (+)-catechin on the basis of spectral and chemical evidence. The inhibitory effect on activation of the Epstein-Barr virus early antigen was assessed for cowaniin. Six known polyphenols and related compounds, including a nitrile glucoside, purshianin, were also characterized.

Key words *Cowania mexicana*; Rosaceae; cowaniin; complex tannin; *C*-glucosidic ellagitannin dimer; Epstein-Barr virus

Cowania mexicana D. Don (Rosaceae) is distributed in western North America, and its aerial parts have been used as a cough suppressant and a remedy for respiratory disease by America Indians. As chemical constituents of this plant, the presence of triterpenoids, such as ursolic acid[1] and cucurbitan-type triterpenes[2] as inhibitors of Epstein-Barr virus early antigen (EBV-EA) activation were reported. We reported the isolation and characterization of nine tannins and related polyphenols [casuarinin (**2**), stachyurin (**3**), pterocarinin A, stenophyllanin A (**4**), alienanin B, casuglaunin A, (−)-epicatechin, procyanidin B-4 and ellagic acid] along with two nitrile glucosides (menisdaurin and lithospermoside) from the leaves and stems of *C. mexicana*. Their inhibitory effects on *in vitro* EBV-EA activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and on *in vivo* two-stage carcinogenesis on mouse back-skin were also reported.[3] Further chemical investigation on a polar fraction of the plant extract has resulted in the isolation of a new complex tannin dimer named cowaniin (**1**) and a known nitrile glucoside. This paper describes the isolation and structure elucidation of the new tannin, along with five known polyphenols from a non-polar fraction.

A concentrated 70% aqueous acetone homogenate of the dried aerial parts of *C. mexicana* was extracted with Et_2O, EtOAc and water-saturated *n*-BuOH to give the respective extracts and the water-soluble portion. The Et_2O extract was chromatographed over polyvinyl gels to afford five known compounds, quercetin 3-*O*-α-L-arabinofuranoside,[4] and *p*-coumaric, *p*-hydroxybenzoic, protocatechuic and gallic acids. The separation and purification of the *n*-BuOH and water-soluble portion by polystylene and/or polyvinyl gels gave cowaniin (**1**) and a known nitrile glucoside, purshianin.[5] The known compounds were identified by direct comparison with authentic samples and/or by their spectral comparisons with data reported in the literature.

Cowaniin (**1**) was obtained as an off-white amorphous powder and exhibited an $[M+NH_4]^+$ ion peak at *m*/*z* 2162 in electrospray ionization (ESI)-MS. The molecular formula was determined to be $C_{97}H_{68}O_{57}$ by elemental analysis and NMR spectra along with MS data. The ^{1}H-NMR spectrum of **1** exhibited two 2H singlets (δ 7.09, 7.06) and seven 1H singlets (δ 6.83, 6.72, 6.62, 6.55, 6.53, 6.51, 6.25), which were assignable to two galloyl and four hexahydroxydiphenoyl (HHDP) groups. These acyl groups were consistent with the appearance of ten ester carbonyl carbon signals in ^{13}C-NMR (see text) and chemically confirmed by the production of methyl tri-*O*-methylgallate (**7**) and dimethyl hexamethoxydiphenate (**8**) upon methanolysis of the methylated derivative of **1**. The odd number of aromatic 1H singlets in the ^{1}H-NMR spectrum of **1** suggested that one of the HHDP groups may participate in C–C bond formation with an anomeric carbon of open-chain glucose residue as characterized by *C*-glucosidic ellagitannins such as casuarinin (**2**) and stachyurin (**3**). The presence of two open-chain glucose residues was indicated by the coupling patterns and chemical shifts of aliphatic proton signals which were assigned by ^{1}H–^{1}H correlated spectroscopy (COSY) (see Experimental). Two anomeric proton signals were observed at δ 4.64 (d, J=1.5 Hz) and 5.85 (d, J=2.5 Hz), the former of which was characteristic of that of complex tannin, a condensate of *C*-glucosidic tannin with catechin (or epicatechin) such as **4**. In fact, the occurrence of a catechin unit in the molecule was implied by two methine signals [δ 4.73 (d, J=4 Hz) and 3.98 (dt, J=5, 4 Hz)] and methylene signals [δ 2.61 (dd, J=4, 16 Hz) and 2.55 (dd, J=5, 16 Hz)] as well as ABX-type signals in the aromatic region of the ^{1}H-NMR spectrum. ^{13}C-NMR resonances of glucose and catechin residues of **1**, which were assigned by the heteronuclear multiple quantum connectivity (HMQC) spectrum, indicated a close resemblance to those of stachyuranin A (**6**)[6] and stenophyllanin B (**5**)[7] rather than stenophyllanin A (**4**) as shown in Table 1. These NMR spectral features and MS data, taking the absence of A-ring protons of catechin into consideration, suggested the dimeric structure of cowaniin, in which the 6- and 8-positions of the catechin unit bind respectively to each anomeric carbon of ellagitannin monomers with an open-chain glucose core. The binding mode of the ellagitannin monomers to catechin as shown in **1** was determined by the heteronuclear multiple bond connectivity (HMBC) spectrum. Among three oxygen-bearing carbon signals (δ 150.1, 153.5,

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157.5) of the A-ring of catechin, the signal at δ 150.1 was assigned to C-8a by its three-bond correlation with H-2 (δ 4.73), while the signal at δ 157.5 was assigned to C-7 based on the correlations with both anomeric proton signals at δ 4.64 (H-1) and 5.85 (H-1′). The H-1 signal (δ 4.64) also showed the correlation with another oxygen-bearing carbon resonance (C-5) at δ 153.5 through long-range coupling. These HMBC data along with the correlation between H-1′ and C-8 of the A-ring indicated the structure of cowaniin as **1**. The (*S*)-configurations at all biphenyl moieties in **1** were evidenced by a strong positive Cotton effect at 234 nm[8] in the CD spectrum.

Table 1. ^{13}C-NMR Spectral Data for the Sugar and Catechin Moieties of **1** and **4**—**6**

Carbon	**1**	**6**	**5**[a]	**4**[a]
Glucose				
C-1	38.5		38.1	38.1
C-2	81.2		81.3[b]	81.8[c]
C-3	75.0		74.9[b]	74.0[c]
C-4	73.4		73.2[b]	73.0[c]
C-5	71.4		71.2[b]	69.4[c]
C-6	64.1		64.4	64.8
C-1′	68.3	68.3		
C-2′	78.8	78.4		
C-3′	76.2	76.1		
C-4′	70.1	70.0		
C-5′	71.8	71.7		
C-6′	64.1	64.2		
Catechin				
C-2	80.5	81.0	82.0	82.4
C-3	66.8	67.0	68.2	67.7
C-4	25.8	25.7	31.6	31.6
C-4a	99.2	99.5	101.0	101.0
C-5	153.5	157.3	155.0	153.8
C-6	108.3	96.4	107.5	96.5
C-7	157.5	156.7	155.2	155.3
C-8	103.2	103.0	96.5	107.6
C-8a	150.1	151.7	155.9	156.3

a) Data from ref. 7. *b*, *c*) Interchangeable.

Following only two known *C*-glucosidic tannin dimers linked through catechin, anogeissinin from *Anogeissus acuminata* (Combretaceae)[9] and casuglaunin B (**9**) from *Casuarina glauca* (Casuarinaceae)[10] and *Elaeagnus umbellate* (Elaeaganaceae),[11] cowaniin (**1**) is the third example of this type of tannins in nature, and is considered a plausible biogenetical precursor of **9**. Therefore, biomimetic chemical conversion of **1** into **9** was attempted in a way similar to the transformation of stachyuranin A (**6**) into stenophyllanin A (**4**) by polyphosphoric acid treatment in hot dioxane.[6] Reversed-phase HPLC of the reaction mixture showed a sole major peak with a retention time identical to that of casuglaunin B (**9**), thus confirming the gross structure **1** for cowaniin including the absolute configuration in catechin moiety.

Inhibitory effect on EBV-EA activation induced by TPA according to the reported method[3] was tested for cowaniin in order to compare with the previously reported effects[3] of the co-existing anti-tumor promoting polyphenols, alienanin B and stenophyllanin A (**4**); however the potency (44.9% inhibition) of **1** was much weaker than those of alienanin B (81.5% inhibition) and **4** (79.1% inhibition) at a concentration of 500 mol ratio per TPA.

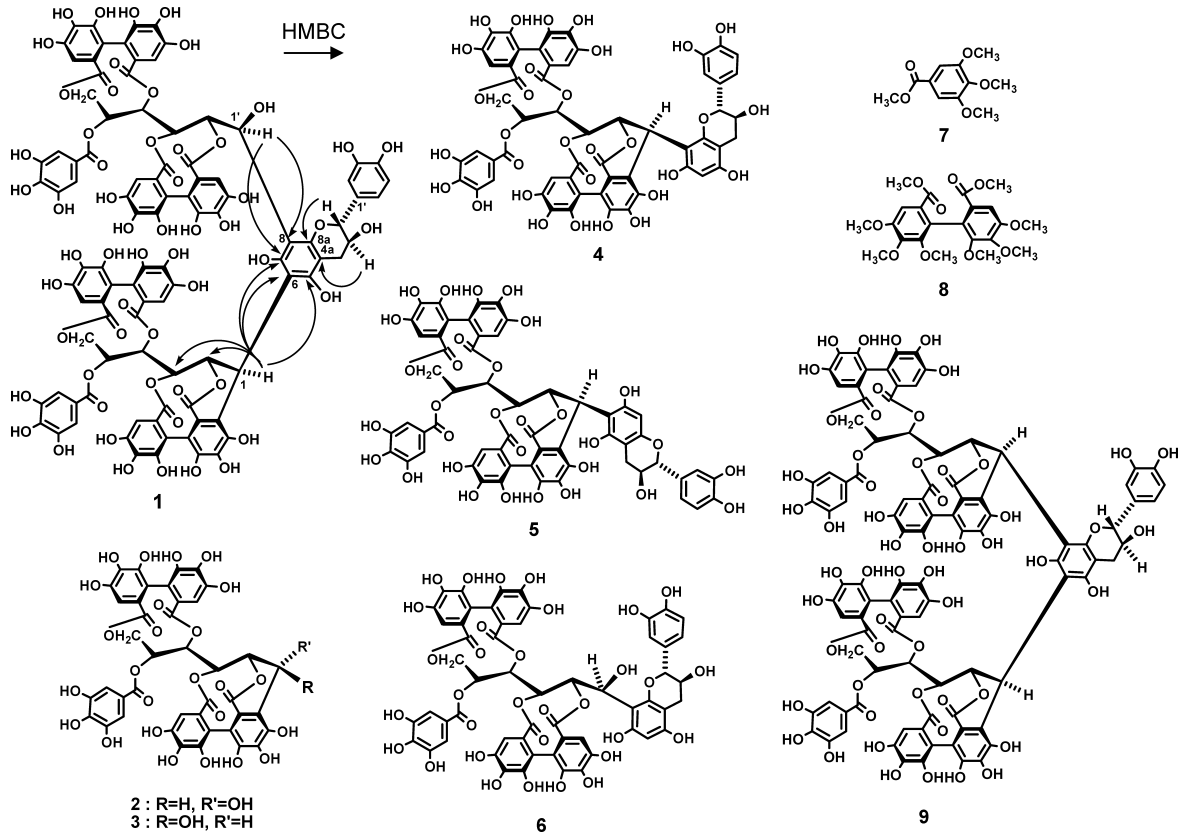

Fig. 1. Structures of Tannins from *C. mexicana* and Related Tannins

Experimental

Optical rotations were recorded on a JASCO DIP-1000 polarimeter and elemental analyses on a Yanaco CHN recorder MT-5. UV spectra were measured on a HITACHI U-2001 spectrophotometer and CD spectra on a JASCO J-720W spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian VXR-500 instrument (500 MHz for ^{1}H and 126 MHz for ^{13}C) and chemical shifts are given in δ (ppm) values relative to those of the solvent [acetone-d_6 (δ_H 2.04; δ_C 29.8)] on a tetramethylsilane scale. The standard pulse sequences programmed into the instrument (VXR-500) were used for each two-dimensional measurement. The J_{CH} value was set at 6 Hz in the HMBC spectra. ESI-MS spectra were recorded on a Micromass Auto Spec OA-Tof mass spectrometer (solvent: 50% aqueous MeOH+0.1% NH_4OAc). Normal phase HPLC was conducted on a YMC-Pack SIL A-003 (YMC Co., Ltd.) column (4.6 i.d.×250 mm) developed with *n*-hexane–MeOH–tetrahydrofuran–formic acid (60 : 45 : 15 : 1) containing oxalic acid (500 mg/1.2 l) (flow rate: 1.5 ml/min; 280 nm UV detection) at room temperature. Reversed-phase HPLC was performed on a YMC-Pack ODS-A A-302 (YMC Co., Ltd.) column (4.6 i.d.×150 mm) developed with 10 mM H_3PO_4–10 mM KH_2PO_4–EtOH–EtOAc (42.5 : 42.5 : 10 : 5) (solvent A), 60% aqueous MeOH (solvent B) or H_2O–CH_3CN–HCOOH (90 : 6 : 4) (solvent C) [each flow rate: 1.0 ml/min; detection, 280 nm UV or diode array detector (DAD) (HITACHI L-7445) 200—600 nm] at 40 °C. Column chromatography was carried out on Diaion HP-20, MCI GEL CHP-20P (Mitsubishi Kasei Co.), and Toyopearl HW-40 (coarse grade; Tosoh Co.).

Plant Material Leaves and stems of *C. mexicana* were collected at Beaver Dam Mts, Utah, U.S.A. in May 1992, and the plant was identified by Dr. R. J. Kass, Brigham Young University, Utah. A voucher specimen is deposited at the Herbal Garden of Kyoto Pharmaceutical University.

Extraction and Isolation The dried leaves and stems (1 kg) of *C. mexicana* were homogenized in 70% aqueous acetone (3 l×3), and the concentrated solution (1 l) was extracted with Et_2O (1 l×3), EtOAc (1 l×6) and water-saturated *n*-BuOH (1 l×6), successively, to give Et_2O (51.0 g), EtOAc (29.8 g), *n*-BuOH (85.8 g) and water (95.4 g) extracts. The Et_2O extract (12.6 g) was chromatographed over Toyopearl HW-40 (2.2 i.d.×64 cm) with aqueous MeOH (50, 60, 70%)→70% aqueous acetone. The 50% MeOH eluate was divided into two portions (110, 691 mg), and each portion was separately subjected to column chromatography over MCI-GEL CHP-20P (1.1 i.d.×23 cm) with aqueous MeOH to yield *p*-coumaric acid (12 mg), *p*-hydroxybenzoic acid (36 mg), protocatechuic acid (5 mg) and gallic acid (30 mg). The 60% MeOH eluate (260 mg) was purified by column chromatography over MCI-GEL CHP-20P (1.1 i.d.×38 cm) with aqueous MeOH to afford quercetin 3-*O*-α-L-arabinofuranoside (23 mg). A part (4.4 g) of *n*-BuOH was fractionated by column chromatography over Diaion HP-20 (12 i.d.×40 cm) with aqueous MeOH (H_2O→20%→40%→60%→100% MeOH). The 40% MeOH eluate (1.0 g) was subjected to repeated column chromatography over Toyopearl HW-40 and MCI GEL CHP-20P using aqueous MeOH to furnish purshianin (5 mg). The water extract (94.0 g) was subjected to column chromatography over Diaion HP-20 (6.8 i.d.×40 cm) using aqueous MeOH (H_2O→20%→40%→60%→100% MeOH). The water eluate (6.0 g) was further separated and purified by column chromatography over Toyopearl HW-40 and MCI GEL CHP-20P with aqueous MeOH to give cowaniin (**1**) (70 mg).

Cowaniin (**1**) An off-white amorphous powder. $[\alpha]_D^{20}$ +55.4° (c=1.0, MeOH). ESI-MS *m/z* 2162 $(M+NH_4)^+$. *Anal*. Calcd for $C_{97}H_{68}O_{57}\cdot 16H_2O$: C, 47.9; H, 4.11. Found: C, 47.8; H, 3.94. UV λ_{max} (MeOH) nm (log ε): 213 (5.26), 268 (4.81). CD (MeOH) [θ] (nm): $+2.95\times10^5$ (234), -7.1×10^4 (261), $+5.1\times10^4$ (283). ^{1}H-NMR (acetone-d_6+D_2O) δ: 7.09, 7.06 (each 2H, s, galloyl-H), 6.83, 6.72, 6.62, 6.55, 6.53, 6.51, 6.25 (each 1H, s, HHDP-H), 6.53 [1H, d, J=2 Hz, catechin unit (Cat) H-2′], 6.50 (1H, d, J=8 Hz, Cat H-5′), 6.38 (1H, dd, J=2, 8 Hz, Cat H-6′), 5.85 (1H, d, J=2.5 Hz, H-1′), 5.70 (1H, br d, J=7 Hz, H-4′), 5.64 (1H, dd, J=2, 8.5 Hz, H-3′), 5.59 (1H, dd, J=2, 8 Hz, H-4), 5.46 (1H, dd, J=2.5, 8.5 Hz, H-2′), 5.40 (1H, br d, J=7 Hz, H-5′), 5.33 (1H, dd, J=3, 8 Hz, H-5), 5.10 (1H, t, J=2 Hz, H-3), 4.83 (1H, dd, J=3, 13 Hz, H-6), 4.73 (1H, br t, J=1.5 Hz, H-2), 4.73 (1H, d, J=4 Hz, Cat H-2), 4.64 (1H, d, J=1.5 Hz, H-1), 4.54 (1H, dd, J=3, 13 Hz, H-6′), 4.22 (1H, br d, J=13 Hz, H-6′), 4.04 (1H, d, J=13 Hz, H-6), 3.98 (1H, dt, J=5, 4 Hz, Cat H-3), 2.61 (1H, dd, J=4, 16 Hz, Cat H-4), 2.55 (1H, dd, J=5, 16 Hz, Cat H-4). ^{13}C-NMR (acetone-d_6+D_2O) δ: 169.7, 169.15, 169.06, 168.9, 168.5, 168.4, 168.3, 167.1, 166.1, 165.9 (each ester carbonyl-C), 146.0 (2C), 145.8 (2C), 145.7, 145.1 (4C), 145.0, 144.9 (2C), 144.8, 144.6, 144.4, 144.3, 144.2, 144.1, 143.8, 143.1, 139.3 (3C), 137.5, 136.8, 136.7, 136.1 (3C), 135.9 (2C), 135.4, 131.6 (Cat C-1′), 127.6, 127.0 (2C), 126.9, 126.1, 124.9, 124.8, 124.2, 122.7, 120.8, 120.6 (2C), 118.5 (Cat C-6′), 116.8, 115.9 (Cat C-5′), 115.7, 115.5, 115.3, 115.0, 114.7, 114.5, 114.1, 113.7 (Cat C-2′), 110.1 (2C), 110.0 (2C), 109.0, 108.8, 107.6, 107.3 (2C), 105.8, carbons for sugars and a part of the catechin unit, see Table 1.

Methylation of 1 followed by Methanolysis A solution of **1** (1 mg) in EtOH (0.1 ml) was treated with ethereal diazomethane for 6 h at room temperature. After removal of the solvent, the residue was directly methanolyzed with 1% NaOMe (0.1 ml) in absolute MeOH (1 ml) at room temperature for 12 h. The reaction mixture was acidified with a few drops of AcOH and evaporated *in vacuo*. The residue was re-dissolved in MeOH and analyzed by reversed-phase HPLC (solvent B), which exhibited the production of methyl tri-*O*-methylgallate (**7**) (t_R 6.98 min) and dimethyl hexamethoxydiphenate (**8**) (t_R 9.63 min).

Chemical Conversion of Cowaniin (1) into Casuglaunin B (9) A dioxane solution (0.3 ml) of cowaniin (**1**) (0.5 mg) containing polyphosphoric acid (3 mg) was heated in a boiling water bath for 1 h. The reaction mixture was analyzed by reversed-phase HPLC (solvent C) equipped with a diode-array detector. A major product peak (14.6 min), besides the peak of **1** (13.2 min), was identified as that of casuglaunin B (**9**) by co-chromatography and UV comparison with an authentic specimen.

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