New Hydrolyzable Tannins, Shephagenins A and B, from *Shepherdia argentea* as HIV-1 Reverse Transcriptase Inhibitors[1)]

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Two new hydrolyzable tannins, shephagenins A and B, were isolated along with hippophaenin A and strictinin from the leaf extract of *Shepherdia argentea*, which showed a remarkable inhibitory activity against human immunodeficiency virus (HIV)-1 reverse transcriptase. Their structures, having a gluconic acid core, have been elucidated on the basis of spectroscopic and chemical methods. The inhibitory effect of the leaf extract on HIV-1 reverse transcriptase was found to be due to tannins, and their activities were stronger than that of (−)-epigallocatechin gallate as a positive control.

Key words *Shepherdia argentea*; Elaeagnaceae; shephagenin A; shephagenin B; HIV-1 reverse transcriptase

In a survey of bioactive compounds from plants grown in western North America,[2)] we have found that a leaf extract of *Shepherdia argentea* NUTT. (Elaeagnaceae) exhibits a potent inhibitory effect on human immunodeficiency virus (HIV) reverse transcriptase. We previously reported that some hydrolyzable tannins show inhibitory effects against retroviral reverse transcriptase,[3)] and also against HIV replication.[4)] These previous findings, together with the fact that the elaeagnaceous plants are often rich in tannins,[5)] prompted us to investigate the tannin constituents of *S. argentea*. The fractionation, guided by inhibitory effect against HIV-1 reverse transcriptase, led to the isolation of two new tannins named shephagenins A (**3**) and B (**4**) as effective inhibitors. This paper describes in detail the isolation and structure elucidation of the active tannins.

The 70% ethanol extract of the dried leaves of *S. argentea* was partitioned between water and *n*-BuOH. The aqueous extract showed a stronger inhibitory effect (93.8%) than the *n*-BuOH extract (54.3%) at a concentration of 2 μg/ml. Repeated column chromatography of the active aqueous extract over polystyrene and/or polyvinyl gels afforded four polyphenols. Among these compounds, two were identified as strictinin (**1**)[6)] and hippophaenin A (**2**),[5)] by direct comparison of their spectral and HPLC data with those of authentic specimens.

The new tannins, shephagenins A (**3**) and B (**4**), were characterized as ellagitannins by their positive coloration with the ferric chloride and acetic acid-nitrous acid[7)] reagents on a TLC plate. The ^{1}H-NMR spectrum of **3** showed a two-proton singlet at δ 7.09 attributable to a galloyl group and five one-proton singlets at δ 6.22, 6.52, 6.65, 6.72 and 7.10, which suggested the presence of a hexahydroxydiphenoyl (HHDP) and a valoneoyl group in the molecule. The aliphatic proton signals constitute a sequentially coupled six-spin system which was assigned with the aid of the ^{1}H–^{1}H shift correlation spectrum (COSY), and their coupling pattern and chemical shifts were closely similar to those of hippophaenin A (**2**). The presence of a gluconic acid core in **3** was also suggested by its ^{13}C-NMR spectrum, which exhibited only five sp^3 carbon signals in the region of δ 64—110 ppm and a carboxyl carbon resonance at δ 168.8 (broad). These component units were confirmed by acid hydrolysis of **3**, yielding gallic acid, ellagic acid and valoneic acid dilactone. As the similarity of the chemical shifts of the sugar proton signals between **2** and **3** indicated that all the hydroxyl groups of gluconic acid are acylated, shephagenin A was regarded as a congener of **2**, in which one of the two HHDP groups in **2** is replaced by a valoneoyl group. This was substantiated by the following observations. The FAB-MS data which showed the $[M+H]^+$ and $[M+Na]^+$ ion peaks at *m/z* 1121 and *m/z* 1143, respectively, were consistent with the molecular formula $C_{48}H_{32}O_{32}$. Methylation of **3** with dimethyl sulfate and potassium carbonate in acetone afforded a nonadecamethyl derivative (**3a**), *m/z* 1387 $[M+H]^+$, which upon methanolysis gave methyl tri-*O*-methylgallate, dimethyl hexamethoxydiphenate and trimethyl octa-*O*-methylvaloneate. Upon heating of the aqueous solution of **3**, gallic acid and a hydrolyzate were produced, and the latter was identified as hippophaenin A (**2**). The formation of **2** from **3** can be interpreted on the basis of ether cleavage of the valoneoyl group followed by disproportionation reaction.[8)]

The location of the valoneoyl group in **3** was established by the ^{1}H–^{13}C long-range shift correlation spectroscopy (see Table 1). The valoneoyl H_B signal was assigned by its correlation through two- or three-bond couplings with the lowest field aromatic carbon signal (δ 147 ppm), assignable to C-4′ of the B-ring, and also with the C-1′ signal (δ 117.1 ppm).[9)] The H_B signal also correlated with a carbonyl carbon signal at δ 168.5 ppm, which in turn was associated with the sugar H-6 (δ 4.93) by three-bond coupling, indicating that the valoneoyl group is at O-6/O-4 with the orientation shown in the formula (**3**). The other

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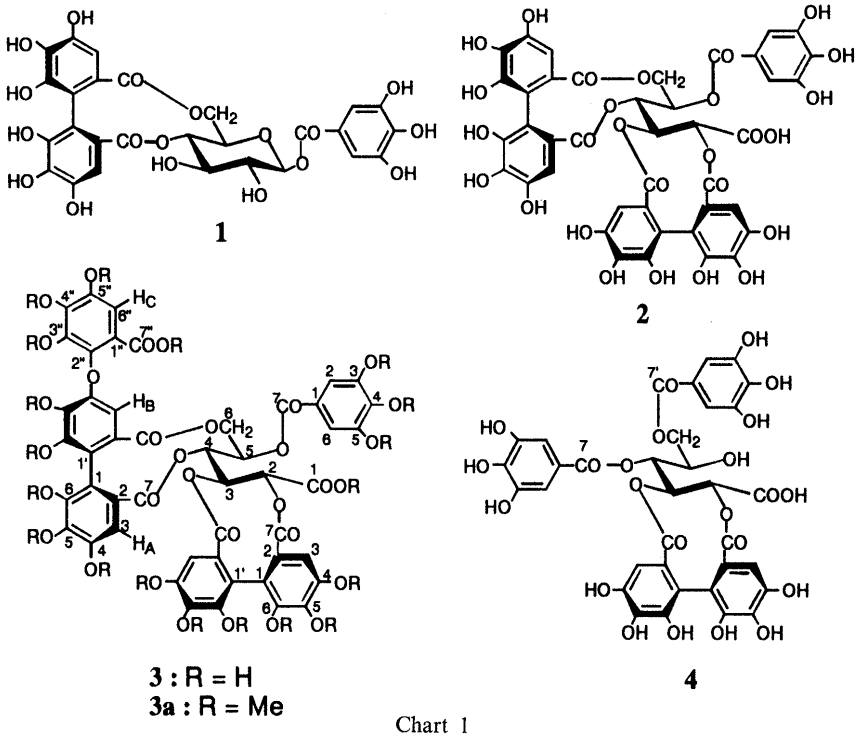

Chart 1

Table 1. One-Bond and Long-Range ^{1}H–^{13}C Correlation Data[a] for Shephagenin A (3)

	δ_C	δ_H	
		Proton coupled *via* one bond	Proton coupled *via* two or three bonds
Gluconic acid			
C-1	168.8		5.37 (gluconic acid H-2)
C-2	72.8	5.37	
C-3	73.6	5.57	
C-4	70.1	5.72	
C-5	69.9	5.57	
C-6	64.7		
HHDP			
C-1	113.8		6.52 (HHDP-H_A)
C-2	126.3[b]		
C-3	107.3	6.52	
C-4	145.1[c]		
C-5	136.2		6.52 (HHDP-H_A)
C-6	144.3[d]		
C-7	167.9		6.52 (HHDP-H_A) 5.37 (gluconic acid H-2)[h]
C-1′	113.6		6.72 (HHDP-H_B)
C-2′	126.5[b]		
C-3′	107.3	6.72	
C-4′	145.2[c]		
C-5′	136.1		6.72 (HHDP-H_B)
C-6′	144.4[d]		
C-7′	168.3		6.72 (HHDP-H_B) 5.57 (gluconic acid H-3)
Valoneoyl			
C-1	116.0		6.65 (valoneoyl-H_A)
C-2	124.5[e]		
C-3	107.5	6.65	
C-4	145.3[f]		
C-5	136.9		6.65 (valoneoyl-H_A)
C-6	145.1[f]		
C-7	168.7		6.65 (valoneoyl-H_A)
C-1′	117.1		6.22 (valoneoyl-H_B)
C-2′	125.7[e]		
C-3′	104.8	6.22	
C-4′	147.0		6.22 (valoneoyl-H_B)
C-5′	136.7		6.22 (valoneoyl-H_B)
C-6′	145.1[f]		
C-7′	168.5		6.22 (valoneoyl-H_B) 4.93 (gluconic acid H-6)
C-1″	115.4		
C-2″	137.3		7.10 (valoneoyl-H_C)
C-3″	140.1		
C-4″	139.7		7.10 (valoneoyl-H_C)
C-5″	143.1		
C-6″	109.8	7.10	
C-7″	167.3		7.10 (valoneoyl-H_C)
Galloyl			
C-1	120.4		
C-2, 6	110.1	7.09	
C-3, 5	145.9		7.09 (galloyl-H)
C-4	139.3		
C-7	165.9		7.09 (galloyl-H) 5.57 (gluconic acid H-5)[g]

a) J_{CH} = 10 Hz. *b*—*f*) Each interchangeable. *g*) J_{CH} = 7 Hz. *h*) J_{CH} = 8 Hz.

sets (aromatic proton–carbonyl carbon–sugar proton) of three-bond long-range correlations (Table 1) were consistent with the proposed structure (**3**) for shephagenin A, although the connectivity between sugar H-4 and valoneoyl H_A through ester carbonyl carbon could not be observed upon measurements of the spectra set at J_{CH} 7,

8 and 10 Hz.

The (*S*)-configuration of the HHDP and valoneoyl moieties was indicated by the strong positive Cotton effect at 229 nm ($[\theta]+14\times10^4$) in the circular dichroism (CD) spectrum of **3**.[10]

Shephagenin B (**4**) was obtained as an off-white amorphous powder. Its ^{1}H-NMR spectrum showed two 2H-singlets (δ 7.14 and 7.15) and two 1H-singlets (δ 6.65 and 6.73) in the aromatic region, indicating the presence of two galloyl groups and an HHDP group in the molecule. These constituent units were also substantiated by the ^{13}C-NMR spectrum (see Experimental). Evidence for the gluconic acid core in **4** was provided by the well-defined six sugar proton signals, and also by the five aliphatic carbon resonances, and a carboxyl carbon signal (δ 171.3), besides signals [δ 166.6, 167.5 and 169.2 (2C)] due to four ester carbonyl carbons in the ^{13}C-NMR spectrum. A remarkable upfield shift ($\Delta\delta$ 1.38 ppm) of the sugar H-5 signal compared with that (δ 5.64)[5] of **2** indicated that the C-5 hydroxyl group in **4** is unacylated. Another distinct feature of the sugar protons between **4** and **2** was the chemical shifts of the methylene proton signals on the gluconic acid core. Namely, a large chemical shift difference between each methylene proton, which is characteristic of ellagitannins[8] having bridged ester linkages at O-4/O-6, was found in the latter (δ 5.02 and 4.07), but not in the former (δ 4.44 and 4.23), suggesting the absence of an HHDP group at O-4/O-6 in **4**. The positions of the acyl groups in **4** were unequivocally determined from the heteronuclear multiple bond correlation spectrum (HMBC; $J_{CH}=7$ Hz) as follows. Two ^{1}H-singlets at δ 6.65 and 6.73 due to the HHDP group showed three-bond coupling with the ester carbonyl carbons at δ 169.2 (2C), which in turn showed cross peaks with the H-2 and H-3 signals of the gluconic acid core, establishing the position of the HHDP group at O-2/O-3. Two-bond coupling between the H-2 and C-1 carboxyl carbon signals was also demonstrated. The ester carbonyl carbon signal (δ 167.5), which was assigned to C-7 of one of the galloyl groups by correlation with the 2H-singlet at δ 7.14, gave a cross peak with the H-6 signal. The remaining galloyl group should be at O-4 of the sugar portion, although this was not confirmed by HMBC. The (*S*)-configuration of the chiral HHDP group was determined from the CD spectrum of **4**, which showed a positive Cotton effect at 234 nm ($[\theta]+7.1\times10^4$) with an amplitude of a half of that of **3**. Based on these findings, the structure of shephagenin B was concluded to be represented by **4**. It should be noted that shephagenins A and B (in addition to hippophaenin A) are quite unusual in having a gluconic acid core among the hundreds of hydrolyzable tannins characterized so far.[11]

The inhibitory effect of tannins isolated in the present study on HIV reverse transcriptase was evaluated in a reaction mixture containing $(rA)_n\cdot(dT)_{12-18}$ as the template-primer and [^{3}H]thymidine triphosphate (TTP) as the triphosphate substrate, under the optimized reaction conditions for HIV-1 reverse transcriptase.[2a,12] Shephagenins A (**3**) and B (**4**), strictinin (**1**) and hippophaenin A (**2**) exhibited potent inhibitory effects with IC_{50} values of 4.9×10^{-8} M, 7.4×10^{-8} M, 8.7×10^{-8} M and 5.2×10^{-8} M, respectively, all of which are stronger than that (IC_{50} 2.5×10^{-7} M) of (−)-epigallocatechin gallate as a positive control.[12] These compounds thus seem to be candidates for further investigation at the cell level.

Experimental

Optical rotations were recorded on a JASCO DIP-4 polarimeter. ^{1}H- and ^{13}C-NMR spectra were measured in acetone-d_6-D_2O and methanol-d_4 on Varian VXR-500 (500 MHz for ^{1}H-NMR and 125.7 MHz for ^{13}C-NMR) and GE NMR OMEGA (600 MHz for ^{1}H and 150 MHz for ^{13}C) instruments. Chemical shifts are given in δ (ppm) values relative to that of the solvent [acetone-d_6 (δ_H 2.04; δ_C 29.8), methanol-d_4 (δ_H 3.35; δ_C 49.8)] on a tetramethylsilane scale. FAB-MS were taken on a VG 70-SE mass spectrometer using 3-nitrobenzyl alcohol as the matrix agent. CD spectra were measured on a JASCO J-500A spectrometer equipped with a DP-501N data processor. Normal-phase HPLC was conducted on a Superspher Si 60 (Merck) column (4 × 125 mm) developed with *n*-hexane–MeOH–THF–formic acid (55:33:11:1) containing oxalic acid (450 mg/l) (flow rate, 1.5 ml/min; detection 280 nm) at room temperature. Reversed-phase HPLC was performed on a LiChrospher RP-18 column (5 μm; 4 × 250 mm) developed with 10 mM H_3PO_4–10 mM KH_2PO_4–MeCN (9:9:2) (flow rate, 1 ml/min; detection 280 nm) at 40 °C. Analytical and preparative TLC were carried out on Kieselgel PF_{60} with benzene–acetone (4:1). Solvents were evaporated under reduced pressure below 40 °C.

Plant Materials Leaves and stems of *S. argentea* NUTT. were collected in Harney County, Oregon, U.S.A. in 1992. A voucher specimen (Murata *et al.*, No. 426) was deposited in the Herbarium, University of Tokyo (TI).

Bioassay The purified HIV-1 reverse transcriptase, which was a generous gift from Dr. S. H. Wilson, NIH, U.S.A., was obtained from *E. coli* harboring an expression plasmid for the coding sequence of the enzyme. The HIV-1 reverse transcriptase-inhibitory activity of the extracts and tannins was measured using [^{3}H]TTP and $(rA)_n\cdot(dT)_{12-18}$ (2:1), under the reaction conditions previously described.[2a]

Isolation of Tannins The dried leaves (83 g) of *S. argentea* were washed with acetone (1 l), and soaked twice in EtOH (each 1.2 l × 1 week), and then in 70% aqueous EtOH (1.2 l × 1 week) at room temperature to yield the EtOH extract (7 g) and 70% aqueous EtOH extract (19.5 g). The inhibitory effect on HIV-1 reverse transcriptase was 31.7% at a concentration of 2 μg/ml for the EtOH extract and 95.3% for the 70% aqueous EtOH extract. The active latter extract was partitioned between water and *n*-BuOH. A part (5 g) of the highly active aqueous extract (16.2 g) was subjected to column chromatography over Diaion HP-20 with H_2O containing increasing amounts of MeOH in a stepwise gradient mode. The 10% MeOH eluate (439 mg) (98.9% inhibition at 1.0 μg/ml) was rechromatographed over Toyopearl HW-40 (coarse grade) with H_2O–MeOH (8:2→7:3→6:4→4:6) to give shephagenins A (**3**) (181 mg) and B (**4**) (8 mg) from the 30% MeOH eluate, and hippophaenin A (**2**) (44 mg) from the 40% MeOH eluate. The 20% MeOH eluate (370 mg) (97.2% inhibition at 1.0 μg/ml) from Diaion HP-20 column chromatography was purified by chromatography on Sephadex LH-20 (EtOH–MeOH) to yield strictinin (**1**) (17 mg).

Shephagenin A (3) An off-white amorphous powder, $[\alpha]_D$ +116° ($c=1.0$, MeOH). UV λ_{max}^{MeOH} nm (log ε): 229 (4.93), 277 (4.59). *Anal.* Calcd for $C_{48}H_{32}O_{32}\cdot6H_2O$: C, 46.91; H, 3.58. Found: C, 46.83; H, 4.02. FAB-MS *m/z*: 1143 $(M+Na)^+$. CD (MeOH) [θ] (nm): $+1.4\times10^5$ (229), -2.3×10^4 (278), $+3.7\times10^4$ (280). ^{1}H-NMR (acetone-d_6+D_2O) δ: 7.10, 6.65, 6.22 [each 1H, s, valoneoyl (Val)], 7.09 [2H, s, galloyl (Gal)], 6.72, 6.52 (each 1H, s, HHDP), 5.37 [1H, d, $J=9.5$ Hz, gluconic acid (Gluc) H-2], 5.57 (1H, d, $J=9.5$ Hz, Gluc H-3), 5.72 (1H, d, $J=9.5$ Hz, Gluc H-4), 5.57 (1H, dd, $J=3.5$, 9.5 Hz, Gluc H-5), 4.93 (1H, dd, $J=3.5$, 13 Hz, Gluc H-6), 4.01 (1H, d, $J=13$ Hz, Gluc H-6). ^{13}C-NMR (acetone-d_6+D_2O): see Table 1.

Acid Hydrolysis of Shephagenin A (3) A solution of **3** (5 mg) in 3% H_2SO_4 was heated in a boiling water-bath for 8 h. After cooling, the reaction mixture was extracted with EtOAc. The EtOAc-soluble portion was analyzed by reversed-phase HPLC to detect gallic acid (t_R 3.2 min), ellagic acid (t_R 10.7 min) and valoneic acid dilactone (t_R 8.4 min). The aqueous layer was neutralized with Amberlite IR-120 (OH form), and evaporated to dryness. The GC analysis of the syrupy residue after trimethylsilylation showed a peak identical with that of glucono-δ-lactone

(t_R 7.1 min).

Methylation of 3 A mixture of **3** (50 mg), anhydrous K_2CO_3 (100 mg) and Me_2SO_4 (250 μl) in dry acetone (10 ml) was stirred overnight at room temperature, and then refluxed for 4 h. After removal of the inorganic materials by filtration, the filtrate was concentrated *in vacuo*, and subjected to preparative TLC (Kieselgel PF_{254}, light petroleum–$CHCl_3$–acetone, 2 : 1 : 2) to give the permethylated derivative (**3a**) (20 mg) of shephagenin A as a white powder. $[\alpha]_D$ +100.4° (c=0.5, MeOH). FAB-MS m/z 1387 $(M+H)^+$. ^{1}H-NMR (acetone-d_6) δ: 7.37 (2H, s, Gal), 7.23, 7.16, 6.89, 6.79, 6.44 (each 1H, s, HHDP, Val), 5.60 (1H, dd, J=3, 9.5 Hz, Gluc H-5), 5.56 (1H, br d, J=10 Hz, Gluc H-3), 5.54 (1H, d, J=8.5 Hz, Gluc H-2), 5.52 (1H, br d, J=9.5 Hz, Gluc H-4), 4.89 (1H, dd, J=3, 13.5 Hz, Gluc H-6), 4.14 (1H, d, J=13.5, Gluc H-6), 4.03, 3.90, 3.88, 3.84, 3.84, 3.80, 3.80, 3.77, 3.76, 3.74, 3.70, 3.64, 3.59 (each 3H, s, OMe × 13), 3.94, 3.86, 3.82 (each 6H, s, OMe × 6).

Methanolysis of 3a An MeOH solution (1 ml) of **3a** (4 mg) containing 1% NaOMe (50 μl) was left standing at room temperature for 10 h to give methyl tri-*O*-methylgallate (t_R 1.4 min), dimethyl hexamethoxyldiphenate (t_R 2.6 min) and trimethyl octa-*O*-methylvaloneate (t_R 7.5 min), which were shown to be identical with authentic samples by co-chromatography using HPLC and TLC (Kieselgel PF_{254}, light petroleum–$CHCl_3$–acetone, 2 : 1 : 1). HPLC conditions: Superspher Si 60 (4 mm i.d. × 125 mm; Merck); solvent system, *n*-hexane–EtOAc (2 : 1), at room temperature.

Chemical Conversion of 3 into 2 A solution of **3** (10 mg) in H_2O (10 ml) was heated in a water bath at 90 °C, and the reaction process was monitored by HPLC, which showed the formation of gallic acid (t_R 1.8 min) and hippophaenin A (**2**) (t_R 3.2 min). The residue obtained after removal of the solvent was subjected to chromatography over MCI-gel CHP-20P to give **2** (2 mg). The identities of **2** and **4** were proved by co-chromatography with the authentic samples on HPLC. Compound **2** was further confirmed by direct comparison of the ^{1}H-NMR spectrum with that of an authentic specimen.

Shephagenin B (4) An off-white amorphous powder, $[\alpha]_D$ +142.5° (c=0.4, MeOH). UV λ_{max}^{MeOH} nm (log ε): 218 (4.77), 268 (4.38). FAB-MS m/z: 803 $(M+H)^+$, 825 $(M+Na)^+$. CD (MeOH) [θ] (nm): $+7.1\times10^4$ (234), -1.7×10^4 (260), $+2.0\times10^4$ (282). ^{1}H-NMR (methanol-d_4) δ: 7.15, 7.14 (each 2H, s, Gal), 6.73, 6.65 (each 1H, s, HHDP), 5.60 (1H, d, J=9.5 Hz, Gluc H-2), 5.90 (1H, br d, J=9.5 Hz, Gluc H-3), 5.82 (1H, br d, J=9.5 Hz, Gluc H-4), 4.26 (1H, m, Gluc H-5), 4.44 (1H, br d, J=9.5 Hz, Gluc H-6), 4.23 (1H, m, Gluc H-6). ^{13}C-NMR δ: 171.3 (Gluc C-1), 169.2 (2C) (HHDP C-7, 7′), 167.5 (Gal C-7′), 166.6 (Gal C-7), 145.5, 145.4 (Gal C-3, 5, 3′, 5′), 144.9 (2C) (HHDP C-4, 4′), 143.6 (2C) (HHDP C-6, 6′), 139.3, 138.8 (Gal C-4, 4′), 136.2, 136.1 (HHDP C-5, 5′), 126.2, 125.8 (HHDP C-2, 2′), 120.4, 119.6 (Gal C-1, 1′), 109.5 (2C), 109.4 (2C) (Gal C-2, 6, 2′, 6′), 107.0 (2C) (HHDP C-3, 3′), 75.8 (Gluc C-2), 75.2 (Gluc C-3), 71.6 (Gluc C-4), 68.4 (Gluc C-5), 66.1 (Gluc C-6).

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References and Notes

1) Part 2 in the series of "Tannins and Related Polyphenols from Elaeagnaceous Plants." For Part 1, see ref. 5. This paper also constitutes Part 3 in the series of "Western North American Plants with Antiviral and Antitumor Activities," and ref. 2*a* and 2*b* correspond to Parts 1 and 2 of the series, respectively.
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