Phytochemical Study on American Plants I. Two New Phenol Glucosides, together with Known Biflavones and Diterpene, from Leaves of *Juniperus occidentalis* Hook.

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Two new phenol glucosides termed juniperosides I (1) and II (2) were isolated, together with known two biflavones, cupressuflavone and amentoflavone and a diterpene, 3β-hydroxy sandaracopimaric acid, from leaves of *Juniperus occidentalis* Hook. (Cupressaceae) collected in Oregon, U.S.A., and their structures were established as (1*S*)- and (1*R*)-1-(2′-hydroxy-6′-methylphenyl)ethanol 2′-*O*-β-D-glucopyranosides (1, 2), respectively, on the basis of spectral, chemical, and synthetic evidence. The glycosides 1 and 2, as well as the corresponding aglycones 1a and 2a, are apparently novel types of naturally occurring compounds; to our knowledge, isolation of these types of natural phenol derivatives has only rarely been reported from the vegetable kingdom.

Key words *Juniperus occidentalis*; Cupressaceae; phenol glucoside; juniperoside; biflavone; diterpene

In a screening of anti-human immunodeficiency virus (HIV-1) replication activity of North American plants,[1] the possible presence of active components in the aerial part (that is, the leaves, stems, and/or fruits) of *Juniperus occidentalis* Hook. (Cupressaceae) was mentioned. This prompted us to investigate the constituents in the ethanol extract of the leaves of the plant and to isolate two new phenol glucosides called juniperosides I (**1**) and II (**2**), together with two known biflavones and a diterpene. Unfortunately, none of the five substances isolated here showed the anti-HIV-1 activity on our *in vitro* assay, but both **1** and **2** proved to be novel and rare naturally occurring compounds. In this paper we fully describe their isolation and structural elucidation based on spectral, chemical, and synthetic evidence. Identification of the three known compounds is also briefly referred to.

The ethanol extract of the dried leaves of *J. occidentalis* was separated by silica gel column chromatography, followed by octadecyl silica gel (ODS) column chromatography and/or repeated HPLC (ODS) separation to afford the new glucosides (**1**, **2**), along with known cupressuflavone, amentoflavone, and 3β-hydroxy sandaracopimaric acid.

Juniperoside I (**1**), colorless needles of mp 89—91 °C, $[\alpha]_D$ −26.3° (c=1.0, pyridine) and juniperoside II (**2**), colorless needles, mp 97—99 °C, $[\alpha]_D$ −8.4° (c=1.0, pyridine) gave the same mass number of quasimolecular ions ($[M-H]^-$) at m/z 313 in the FAB-MS (negative mode). High resolution (HR) studies in the same mode revealed that the molecular formula common to **1** and **2** is consistent with $C_{15}H_{22}O_7$, and hence **1** and **2** are isomeric. In the FAB-MS (negative mode) **1** and **2** also afforded a common significant fragment at m/z 151, arising from the loss of a hexosyl unit from the $[M-H]^-$ ion. These MS spectral data indicated that both **1** and **2** are monoglycosides and carry a hexosyl residue as the sugar part. The D-glucosyl moiety was identified in each one as the corresponding thiazolidine derivative by gas liquid chromatography (GLC) (see Experimental).

In further structural studies for **1** and **2**, the ^{1}H- and ^{13}C-NMR assignments were performed with the aid of two dimensional (2D)-NMR [^{1}H–^{1}H and ^{13}C–^{1}H shift-correlation spectroscopy (COSY), nuclear Overhauser enhancement spectroscopy (NOESY), and heteronuclear multiple bond correlation spectroscopy (HMBC)] experiments, and all protons and carbons were assigned as shown in Tables 1 and 2. The assignments (chemical shifts, multiplicities, and coupling constants) for all protons (Table 1) and carbons (Table 2) due to each glucosyl part in **1** and **2** substantiated the presence of a β-D-glucopyranosyl ($^4C_1$ form) moiety as the common monosaccharide in **1** and **2**.

On the other hand, each of these new glucosides gave the following proton signals ascribed to its aglycone: an aromatic methyl [δ 2.58 (s) in **1**; δ 2.33 (s) in **2**], three kinds of aromatic protons [δ 6.89 (d, J=7.8 Hz), 7.10 (dd, J=7.8, 7.8 Hz), and 7.54 (d, J=7.8 Hz) in **1**; δ 6.87 (d, J=7.8 Hz), 7.10 (dd, J=7.8, 7.8 Hz), and 7.65 (d, J=7.8 Hz) in **2**], and a 1-hydroxyethyl substituent [–CH(OH)–CH_3] on an aromatic ring [δ 1.81 (3H, d, J=6.6 Hz), 5.50 (1H, br s, OH), and 5.96 (1H, q, J=6.6 Hz) in **1**; δ 1.86 (3H, d, J=6.6 Hz), 6.60 (1H, d, J=8.4 Hz, OH), and 5.52 (1H, dq, J=8.4, 6.6 Hz) in **2**] (Table 1). This ^{1}H-NMR evidence, coupled with the FAB-MS (*vide ante*) and ^{13}C-NMR data, suggested that the aglycones in **1** and **2** are both phenol derivatives, have 1,2,3-trisubstituted benzene ring, and are stereoisomeric.

Precise structures for these aglycones were decided as follows: on enzymatic hydrolysis with almond emulsin, **1** and **2** afforded the corresponding aglycones having the same molecular formula, $C_9H_{12}O_2$, that is, **1a**, colorless needles of mp 78—79 °C, $[\alpha]_D$ −42.5° (c=0.80, MeOH) and **2a**, colorless needles, mp 80—81 °C, $[\alpha]_D$ +43.8° (c=0.22, MeOH), respectively. Both **1a** and **2a** gave the same spectra in the electron impact (EI)-MS, and ^{1}H- and ^{13}C-NMR measurements, suggesting that the two compounds have the same gross structure and appear to be structurally different only in the absolute configuration of the chiral carbon (C-1) on the 1-hydroxyethyl (=*sec* alcoholic) substituent. The multiplicities and J-values of the three kinds of aromatic protons in **1**, **2**,

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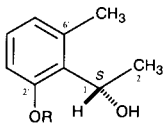
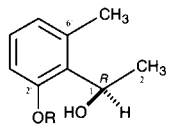
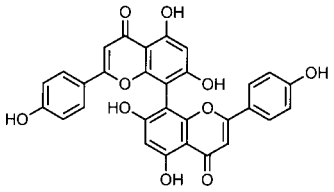


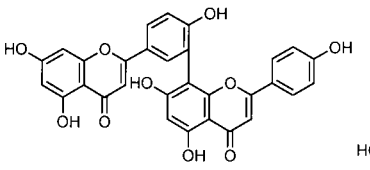
1 : R=β-D-glucopyranosyl
1a : R=H



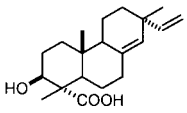
2 : R=β-D-glucopyranosyl
2a : R=H



cupressuflavone



amentoflavone



3β-hydroxy sandaracopimaric acid

Fig. 1

Table 1. ^{1}H-NMR Data of **1**, **2**, **1a** and **2a** in C_5D_5N[a)]

Proton No.	**1**[b)]	**2**[b)]	**1a**, **2a**[c)]
1	5.96 (q, 6.6)	5.52 (dq, 8.4, 6.6)	5.57 (q, 6.6)
1-OH	5.50 (br s)	6.60 (br d, 8.4)	8.34 (s)
2	1.81 (3H, d, 6.6)	1.86 (3H, d, 6.6)	1.66 (3H, d, 6.6)
2′-OH			10.67 (s)
3′	7.54 (d, 7.8)	7.65 (d, 7.8)	7.05 (d, 7.8)
4′	7.10 (dd, 7.8, 7.8)	7.10 (dd, 7.8, 7.8)	7.14 (dd, 7.8, 7.8)
5′	6.89 (d, 7.8)	6.87 (d, 7.8)	6.73 (d, 7.8)
6′-CH_3	2.58 (3H, s)	2.33 (3H, s)	2.22 (3H, s)
Glc 1″	5.50 (d, 7.8)	5.37 (d, 7.8)	
2″	4.28[d)]	4.31 (dd, 7.8, 9.0)	
3″	4.28[d)]	4.26 (dd, 9.0, 9.0)	
4″	4.28[d)]	4.28 (dd, 9.0, 9.0)	
5″	4.02 (ddd, 9.0, 5.4, 2.4)	4.07 (ddd, 9.0, 5.4, 2.4)	
6″	4.51 (dd, 12.0, 2.4)	4.58 (dd, 12.0, 2.4)	
	4.37 (dd, 12.0, 5.4)	4.40 (dd, 12.0, 5.4)	

a) Chemical shifts are in δ-values from internal TMS and are followed by multiplicities and *J*-values (in Hz). *b*) Measured at 600 MHz. *c*) Measured at 400 MHz. *d*) Overlapping with other signal(s), hence, its multiplicity and *J*-value are both obscure.

Table 2. ^{13}C-NMR Data for **1**, **2**, **1a** and **2a** in C_5D_5N[a)]

Carbon No.	**1**[b)]	**2**[b)]	**1a**, **2a**[c)]
1	65.2	66.0	67.7
2	23.5	23.4	23.0
1′	134.4	134.0	129.0
2′	156.6	158.4	157.7
3′	115.2	115.9	115.6
4′	127.8	128.2	128.2
5′	126.0	125.5	122.0
6′	137.3	136.2	135.1
6′-CH_3	20.4	20.0	19.2
Glc 1″	103.7	104.6	
2″	74.9	75.4	
3″	78.7	78.4	
4″	71.3	71.3	
5″	78.8	79.1	
6″	62.4	62.5	

a) Assignments were determined based on ^{1}H–^{13}C COSY and HMBC experiments. *b*) Measured at 150 MHz. *c*) Measured at 100.5 MHz.

1a, and **2a** (Table 1) suggested both of the aglycones (**1a**, **2a**) have a 1,2,3-trisubstituted benzene ring. In addition, the significant cross peak observed between aromatic methyl (=the

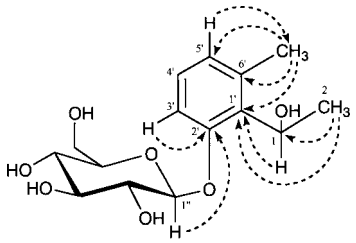


Fig. 2. Key HMBC Correlations Observed in Both **1** and **2**

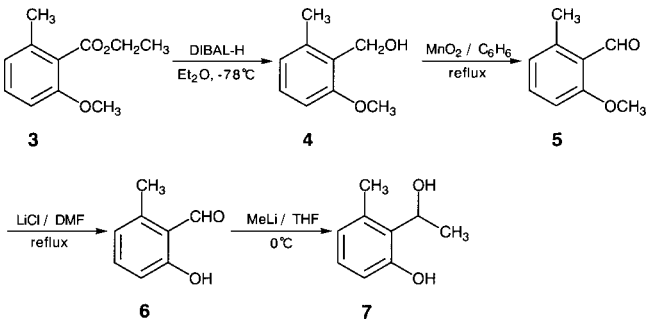


Chart 1

singlet methyl) and the ethanolic methyl (=the doublet methyl) in the NOESY spectra of **1** and **2** indicated that methyl and 1-hydroxyethyl substituents locate at the vicinal carbons on the benzene ring. This ^{1}H-NMR and NOESY evidence, coupled with the detailed analysis of HMBC networks observed in **1** and **2** (Fig. 2), demonstrated 1-(2′-hydroxy-6′-methylphenyl) ethanol (**7**) to be consistent with the gross structure for both aglycones (**1a**, **2a**), *i.e.*, the racemic form of both **1a** and **2a**.

The validity of this gross structure established for the aglycones was further corroborated by their direct comparison with a synthetic sample, *i.e.*, a new racemate (**7**) which had first been prepared by us from commercially available 2-methoxy-6-methylbenzoic acid ethyl ester (**3**) according to the following route (also see Chart 1). The ethoxycarbonyl substituent in the starting compound (**3**) was converted to the formyl substituent in **5** *via* the hydroxymethyl group in **4**. Next, the methoxy group in **5** was demethylated to afford the phenol **6** and **6** was subsequently, reacted with methyllithium

to yield the desired 1-phenylethanol derivative (**7**) in a racemic form, which was identified with each of the aglycones **1a** and **2a** by comparison of ^{1}H- and ^{13}C-NMR (400, 100.5 MHz, respectively; in pyridine-d_5) and EI-MS spectra.

The absolute structures for the two aglycones were determined as follows. Aglycones **1a** ($[\alpha]_D$ −42.5°, MeOH) and **2a** ($[\alpha]_D$ +43.8°, MeOH) showed optical rotations of the same magnitude in an opposite sense, indicating that they are an enantiomeric pair. The signs in the optical rotations of **1a** and **2a** were compared with those of a model enantiomeric pair, *i.e.*, commercially available authentic (1*R*)- and (1*S*)-1-phenylethanols [the (1*R*)-isomer: $[\alpha]_D$ +44.65° (c=0.98, MeOH) and the (1*S*)-isomer: $[\alpha]_D$ −44.12° (c=0.98, MeOH)] and it was concluded that **1a** corresponds to the (1*S*)-isomer and hence, **2a** is the (1*R*)-isomer, the antipode of **1a**.

As the final step in the structural elucidation of both glucosides, the location of the β-D-glucopyranosyl residue on each aglycone is decided as follows. Each glucoside showed the NOESY cross peak between the anomeric H and the aromatic 3′-H, as well as the HMBC correlation between the anomeric H and 2′-C bearing the phenolic O-function (Fig. 2), proving that the β-D-glucopyranosyl moiety is linked at the phenolic (2′-) OH of the respective aglycones in **1** and **2**. In conclusion, the entire structures of **1** and **2** are defined as (1*S*)- and (1*R*)-1-(2′-hydroxy-6′-methylphenyl)ethanol 2′-*O*-β-D-glucopyranosides, respectively.

To our knowledge, the aglycones in **1** and **2** must belong to structurally novel and unique phenolic compounds which have not yet been found in nature. Furthermore, glucosides **1** and **2** must also be novel and rare types of naturally occurring components.

In this study, two known biflavones, cupressuflavone and amentoflavone and a known diterpene, 3β-hydroxy sandaracopimaric acid were also isolated from the same plant material for the first time. Identities of cupressuflavone[2,3] and amentoflavone[3,4] were established by comparison of ^{1}H- and ^{13}C-NMR data with those reported for the respective authentic specimens. The physicochemical and spectral data {mp, $[\alpha]_D$, ^{1}H- and ^{13}C-NMR (600, 150 MHz, respectively), EI-MS} of the isolated diterpene were in agreement with the reported values for authentic 3β-hydroxy sandaracopimaric acid.[5]

Although none of juniperosides I and II, cupressuflavone, amentoflavone, and 3β-hydroxy sandaracopimaric acid showed significant anti-HIV-1 activity, further component analysis of the aerial part of the plant is continuing with the intention of isolating the anti-HIV-1 active compound.

Experimental

General Remarks All melting points were recorded on a Yanagimoto melting point apparatus without correction. ^{1}H- and ^{13}C-NMR spectra were measured on a GE-Omega 600 (^{1}H at 600 and ^{13}C at 150 MHz) and a JEOL JNM-GX 400 (^{1}H at 400 and ^{13}C at 100.5 MHz) spectrometers with pyridine-d_5 as a solvent. Chemical shifts are given in δ (ppm) values relative to internal tetramethylsilane (TMS). FAB- and HR-FAB-MS spectra in a negative mode (matrix, triethanolamine), along with EI- and HR-EI-MS spectra, were obtained with a JEOL JMS-700T spectrometer. Optical rotations were determined on a JASCO DIP-140 polarimeter. GLC was carried out on a Shimadzu GC-7AG instrument. For column chromatography, Kieselgel 60 (Merck) and Chromatorex ODS DM1020T (Fuji Silysia) were used. Preparative HPLC was performed on a JAI LC-908 instrument and unless otherwise noted, a JAIGEL-ODS column was used. The almond emulsin used in this work was commercially obtained from Sigma-Aldrich Japan Co. and the starting material in the synthetic work, 2-methoxy-6-methylbenzoic acid ethyl ester, from Tokyo Kasei Kogyo Co., Ltd., respectively. The authentic samples of (*R*)- and (*S*)-1-phenylethanols were also available as (*R*)-(+)- and (*S*)-(−)-1-phenylethyl alcohols from Tokyo Kasei Kogyo Co., Ltd.

Plant Material Leaves of *J. occidentalis* were collected in Harney county, Oregon, U.S.A. in 1992. A voucher specimen (Murata J. *et al.*, No. 363) was deposited in the Herbarium of the Botanical Gardens, Faculty of Science, University of Tokyo.

Extraction and Isolation The dried and cut leaves (584.3 g) were soaked in acetone (2 l), and the washed plant materials were extracted twice with EtOH (each 3 l) at room temperature for 1 week. The resulting EtOH extract (16.0 g) was chromatographed on silica gel, and eluted successively with $CHCl_3$–MeOH (50 : 1→20 : 1), the lower phase of $CHCl_3$–MeOH–H_2O (9 : 3 : 1), and a mixture solvent of $CHCl_3$–MeOH–H_2O (6 : 4 : 1) to give 13 fractions (Nos. 1 to 13). Fraction No. 7 (830 mg) was rechromatographed over silica gel [eluting with $CHCl_3$–MeOH (10 : 1)], followed by preparative HPLC [70% aqueous (=aq.) MeOH] to afford 3β-hydroxy sandaracopimaric acid (15.4 mg). Fraction No. 8 (530 mg) was subjected to reversed-phase (ODS) column chromatography (eluting with 70% aq. MeOH), followed by preparative HPLC separation (JAIGEL-GS column; eluting with MeOH) to yield cupressuflavone (39.0 mg) and amentoflavone (37.8 mg) in that eluting order. Fraction No. 9 (350 mg) was further column-chromatographed over silica gel [eluting with $CHCl_3$–MeOH (7 : 1)] and ODS (eluting with 50% aq. MeOH), successively, followed by preparative HPLC separation (eluting with 50% aq. MeOH) to give **1** (73.9 mg) and **2** (21.9 mg).

Juniperoside I (**1**): Colorless needles from MeOH, mp 89—91 °C, $[\alpha]_D$ −26.3° (c=1.0, pyridine). FAB- and HR-FAB-MS (negative mode) *m/z*: 313.1281 (Calcd for $C_{15}H_{21}O_7$, $[M-H]^-$: 313.1288), 151 $[M-H-Glc]^-$. ^{1}H- and ^{13}C-NMR: Given in Tables 1 and 2, respectively.

Juniperoside II (**2**): Colorless needles from MeOH, mp 97—99 °C, $[\alpha]_D$ −8.4° (c=1.0, pyridine). FAB- and HR-FAB-MS (negative mode) *m/z*: 313.1281 (Calcd for $C_{15}H_{21}O_7$, $[M-H]^-$: 313.1288), 151 $[M-H-Glc]^-$. ^{1}H- and ^{13}C-NMR: Given in Tables 1 and 2, respectively.

Enzymatic Hydrolysis of 1 A solution of **1** (20.0 mg) in acetate buffer (pH 5.0; 2 ml) was incubated with almond emulsin (20.0 mg) at 37 °C for 2 h. The reaction mixture was extracted with ether and the solvent was evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography (*n*-hexane : ether=3 : 1 as the eluent) to yield **1a** (4.5 mg), colorless needles of mp 78—79 °C, $[\alpha]_D$ −42.5° (c=0.80, MeOH). EI- and HR-EI-MS *m/z* (%): 152.0841 (Calcd for $C_9H_{12}O_2$, M^+: 152.0837, 30), 134 ($[M-H_2O]^+$, 100). ^{1}H- and ^{13}C-NMR: Given in Tables 1 and 2.

Enzymatic Hydrolysis of 2 A solution of **2** (10.0 mg) in acetate buffer (pH 5.0; 2 ml) was incubated with almond emulsin (10.0 mg) at 37 °C for 2 h. Work-up and purification procedure of the reaction mixture as described in the hydrolysis of **1** furnished **2a** (2.2 mg), colorless needles, mp 80—81 °C, $[\alpha]_D$ +43.8° (c=0.22, MeOH). EI- and HR-EI-MS *m/z* (%): 152.0840 (Calcd for $C_9H_{12}O_2$, M^+: 152.0837, 30), 134 ($[M-H_2O]^+$, 100). ^{1}H- and ^{13}C-NMR: Given in Tables 1 and 2.

Synthesis of the Racemic Aglycone (7) The preparation of the synthetic aglycone (**7**) from the commercially available starting compound (**3**) *via* 4 reaction processes (Chart 1) is briefly described below.

2-Methoxy-6-methylbenzyl Alcohol (4) A 1 M solution of DIBAL-H in *n*-hexane (10 ml, 1 mmol) was added to a stirred solution of **3** (970.0 mg, 0.5 mmol) in dry ether (10 ml) at −78 °C under nitrogen. The mixture was stirred at −78 °C for a further 10 min under nitrogen, acidified with 5% hydrochloric acid, and extracted into ether. The organic layer was washed with brine until neutral, dried (anhydrous Na_2SO_4), and evaporated *in vacuo* to afford **4** (734.0 mg; in a 97% yield) as a pale yellow oil.

2-Methoxy-6-methylbenzaldehyde (5) A mixture of **4** (734.0 mg, 0.48 mmol) and active MnO_2 (3.67 g) in benzene (10 ml) was refluxed with stirring for 5 h. The mixture was filtered through a short bed of Celite and the filtrate was evaporated *in vacuo* to give **5** (619.0 mg; in a 86% yield) as a pale yellow oil.

2-Hydroxy-6-methylbenzaldehyde (6) A mixture of **5** (300.0 mg, 0.2 mmol) and LiCl (254 mg, 0.6 mmol) in *N*,*N*-dimethylformamide (DMF) (5 ml) was refluxed with stirring for 15 h. The cooled reaction mixture was diluted with 10% aq. NaOH and washed with ether. The aq. solution was acidified with 10% hydrochloric acid and extracted with ether. The ether layer was washed with brine and dried (anhydrous Na_2SO_4). After evaporation of the solvent, the residue was purified by silica gel column chromatography (elution with *n*-hexane : ether=3 : 1) to give **6** (111.3 mg; in a 41% yield) as colorless needles.

1-(2′-Hydroxy-6′-methylphenyl)ethanol (7) A 1.09 M solution of MeLi

in ether (1.96 ml, 0.16 mmol) was added to a stirred solution of **6** (111.3 mg, 0.082 mmol) in dry THF (2.0 ml) at 0 °C under nitrogen. The mixture was stirred at 0 °C under nitrogen for a further 10 min, poured into a mixture of ice and water, and extracted with ether. The ether layer was washed with brine, dried (anhydrous Na_2SO_4), and evaporated. The residual product was purified by silica gel column chromatography (eluted with *n*-hexane : ether = 3 : 1) to give the desired synthetic aglycone **7** (114.3 mg; in a 92% yield) as colorless needles. The ^{1}H- and ^{13}C-NMR (400, 100.5 MHz, respectively) and EI-MS spectra of **7** were superimposable on those of **1a** and **2a**.

Identification of D-Configuration of the Glucosyl Moiety in 1 and 2[6] A solution of each of **1** and **2** (each 1.0 mg) in 2 M HCl–EtOH (1 : 1; 0.5 ml) was refluxed for 2 h. The mixture solution was extracted with Et_2O and the residual aq. layer was neutralized with Ag_2CO_3. After centrifugation of the inorganic precipitate, the supernatant was concentrated *in vacuo* to afford a glucosyl residue. The residue was subjected to the preparation of the corresponding thiazolidine derivative, followed by trimethylsilylation and GLC analysis, according to the reported procedure.[6] The D-configuration for glucose obtained from each of **1** and **2** was decided, based on direct comparison with the D- and L-standards of glucose (t_R: D-Glc, 14 min 25 s; L-Glc, 15 min 10 s). GC conditions: capillary column, TC-1 (0.32 mm i.d.×30 m, GL Sciences Inc.); detector, hydrogen flame ionization detector; column temperature, 230 °C; injection temperature, 250 °C; carrier N_2 gas.

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