Current Topics

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Regular Article

Isolation of Three New Diterpenes from *Dodonaea viscosa*

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An investigation into the methanol extracts obtained from the stems of *Dodonaea viscosa* led to the isolation of one nor-clerodane diterpene (1) and two labdane diterpenes (2, 3), as well as 17 known compounds (4–20). The structures of these compounds were elucidated based on chemical and spectral evidence. The stereochemical structure of the nor-clerodane diterpene was confirmed *via* its circular dichroism spectrum and calculated electronic circular dichroism spectrum. Isolated compounds were evaluated for their inhibitory effects on collagenase and tyrosinase. Since 5,7,4′-trihydroxy-3′-(4-hydroxy-3-methylbutyl)-5′-(3-methylbut-2-enyl)-3,6-dimethoxyflavone (9) showed collagenase inhibitory activity and scopoletin (12) had significant tyrosinase inhibitory activity, they were considered to be good candidates for cosmetic agents.

Key words *Dodonaea viscosa*; Sapindaceae; diterpene; tyrosinase inhibitor; collagenase inhibitor; circular dichroism

Introduction

Dodonaea viscosa Jacquin (family: Sapindaceae) is a small evergreen tree (around 3–5 m in height) that is naturally distributed in Japan (Nansei Islands and Ogasawara Islands), Australia, New Zealand, and other tropical to subtropical regions of the world. It branches from the lower section of the aerial part to form an oval-shaped tree; its leaves are glossy green and alternately oblong at all edges. From March to April, it forms short panicles to produce inconspicuous yellow-green flowers.

D. viscosa and its parts have several reported properties and uses. For example, a compound isolated from this plant, dodoviscin A, has been reported to have an inhibitory effect on melanin production.[1] Several parts of *D. viscosa* have been used in traditional medicine to treat several diseases in East Africa, and some studies have reported the various pharmacological activities of *D. viscosa* extracts, which include antiherpes,[2] gastroprotective,[3] antibacterial and antiviral,[4,5] and anti-inflammatory activities.[6] In addition, previous chemical studies of *D. viscosa* have led to the isolation of *ent*-labdane- and clerodane-type diterpenes,[7,8] as well as flavonoids,[9] which have characteristically been prenylated.[10] In the present study, as part of a continuous study of Okinawan plants, we describe the isolation of three new diterpenes (**1**–**3**) (Fig. 1) and 17 known compounds (**4**–**20**) (Fig. 2) from the leaves of *D. viscosa*. Furthermore, we elucidated the structures of these compounds based on extensive chemical and spectral evidence. We also describe the inhibitory effects of the isolated constituents of *D. viscosa* toward collagenase activity and mushroom tyrosinase activity, which could help to identify extracted compounds that would be useful as cosmetic agents.

Results and Discussion

The stems of *D. viscosa* were air-dried and an extract was produced using three MeOH extractions, each conducted at room temperature. The resultant MeOH extract was partitioned into an EtOAc–H_2O (1 : 1, v/v) mixture to create an EtOAc-soluble fraction and an aqueous phase. The EtOAc-soluble fraction was subjected to normal and reversed-phase silica gel column chromatography followed by repeated HPLC separation. This process gave three new compounds (**1**–**3**) as well four diterpenes [labd-13(*E*)-en-8,15-diol (**4**),[11,12] *ent*-15,16-epoxy-9αH-labda-13(16),14-diene-3β,8α-diol (**5**),[13] hautriwaic acid (**6**),[14,15] and 6-hydroxyhardwickjic acid (**7**),[16]], four flavonoids [viscosine (**8**),[17] 5,7,4′-trihydroxy-3′-(4-hydroxy-3-methylbutyl)-5′-(3-methylbut-2-enyl)-3,6-dimethoxyflavone (**9**),[10,18] alizarin (**10**),[18,19] and dodovisone C (**11**)[20]], three coumarins [scopoletin (**12**),[21] isofraxidin (**13**),[22,23] and fraxetin (**14**)[24]], one lignane [(+)-pinoresinol (**15**)[25]] and five other compounds [protocatechuic acid (**16**),[26] (*E*)-cinnamic acid (**17**),[27] methyl hydrogen azelate (**18**),[28] β-hydroxylpropiovanillone (**19**),[29] and 4-hydroxyacetophenone (**20**)[30]]. These known compounds were identified by comparing their observed spectroscopic data with previously reported data. However, the structures of the new compounds were elucidated by high-resolution electrospray-ionization (HR-ESI)-

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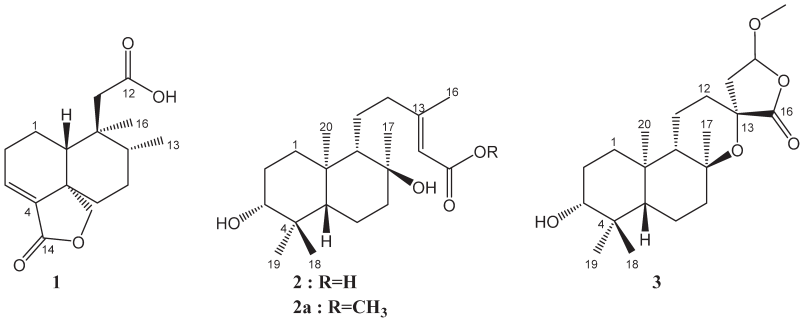


Fig. 1. Chemical Structures of Compounds **1**–**3**

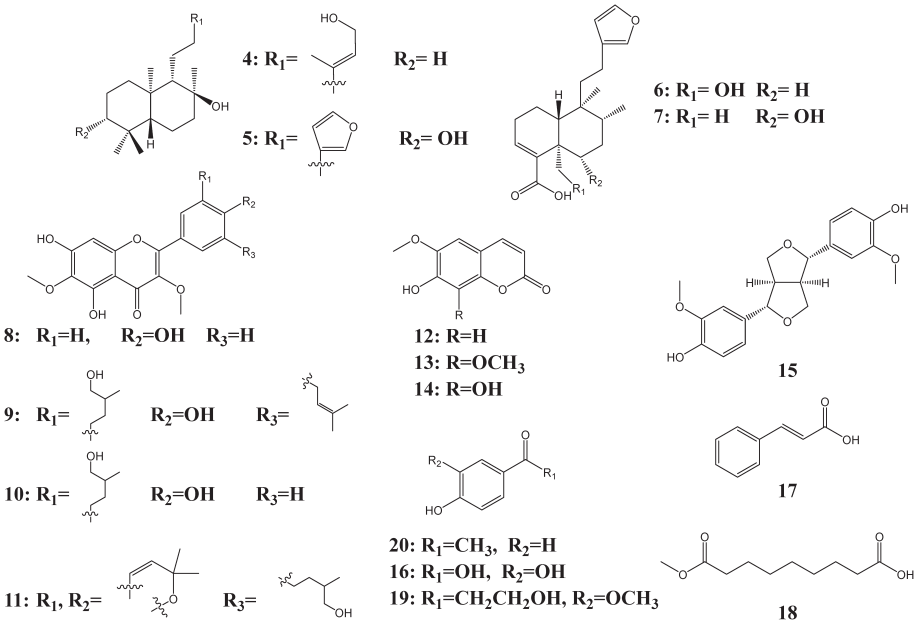


Fig. 2. Chemical Structures of Known Compounds

MS, one dimensional (1D)- and 2D-NMR data, and chemical methods.

Compound 1 (**1**), $[\alpha]_D$ −130.5, was isolated as an amorphous powder and its elemental composition was determined to be $C_{16}H_{22}O_4Na$ by HR-ESI-MS. The IR spectrum exhibited absorption bands ascribable to hydroxy groups (3431 cm^{-1}) a γ-lactone group (1762 cm^{-1}) and a carboxy group (1727 cm^{-1}). Compound **1** was identified as a clerodane-type diterpene derivative by its characteristic ^{1}H- and ^{13}C-NMR (Table 1) and its heteronuclear single quantum coherence (HSQC) spectra, in which a tertiary methyl group appeared as a singlet at δ_H 0.67, δ_C 17.4 was assigned to CH_3-16, a secondary methyl group was a doublet at δ_H 0.95 (d, J = 6.9 Hz), and δ_C 16.4 was assigned to CH_3-13.[31] The presence of an α,β-unsaturated γ-lactone moiety was evident from the ^{1}H-NMR signals of δ_H 6.75 (dd, J = 7.6, 2.1 Hz) for an olefinic β-proton at C-3 (δ_C 138.1). In addition, the ^{1}H-signals at δ 4.02 (1H, d, J = 8.2-Hz) and δ 4.41 (1H, d, J = 8.2 Hz) were for oxymethylene at C-15 (δ_C 73.4). Based on heteronuclear multiple-bond correlation (HMBC) spectra (Fig. 3), a correlation was observed between H_2-11 (δ_H 2.417 and 2.424) and C-8 (δ_C 39.3), C-9 (δ_C 41.4), C-10 (δ_C 50.1), and C-12 (δ_C 175.3), thereby indicating the location of the carboxylic acid group at C-11. The olefinic proton at C-3 showed a HMBC with C-5 (δ_C 47.1) and 14 (δ_C 172.0). In a nuclear Overhauser enhancement spectroscopy (NOESY) experiment, the nuclear Overhauser effect (NOE) cross-peak between H-10 and H-6β, and between H-8 and H-11, indicated that H-8, H-10, and C-11 were in β-orientation. Furthermore, the NOE cross-peak between H-15α (δ_H 4.02) and H-1α, H-16, and between H-15β (δ_H 4.41) and H-7, H-16, suggested that C-15 was in α-orientation (Fig. 4). The circular dichroism spectrum of compound **1** displayed a negative Cotton effect at 244 nm ($\Delta\varepsilon$ −4.08) (Fig. 5), which was consistent with the Cotton effect from the calculated electronic circular dichroism spectrum with the configurations 5*S*, 8*R*, 9*S*, and 10*R*. Therefore, the absolute structure of compound **1** was determined to be that shown in Fig. 1.

Compound 2 (**2**) was isolated as an amorphous powder with a negative specific rotation ($[\alpha]_D$ −21.4) and its elemental composition was determined to be $C_{20}H_{34}O_4$. The ^{13}C-NMR spectrum showed five methyl carbons (δ_C 28.8, 24.0, 19.3, 16.3, and 16.2), six methylenes (δ_C 45.6, 45.2, 39.5, 27.9, 25.2 and 21.4), three methines including oxygenated ones (δ_C 79.6, 62.5 and 56.6), an oxymethine (δ_C 79.6), an acetate (δ_C 104.4), and a carbonyl carbon (δ_C 180.5). The ^{1}H- and ^{13}C-NMR spectra were similar to those of (+)-(3*S**,5*R**,8*R**,9*R**,10*S**)-lab-13-en-3β,8α-diol-15-oic acid.[32] Moreover, the HMBC and correlation spectroscopy (COSY) correlations supported

Table 1. ^{13}C-NMR and ^{1}H-NMR Spectroscopic Data for Compounds **1**–**3** (150 MHz and 600 MHz, Respectively, Methanol-d_4)

	Compound **1**		Compound **2**		Compound **3**	
	^{13}C	^{1}H	^{13}C	^{1}H		
1	21.4	*α* 1.16 (1H, *dq*, *J* = 3.7, 11.7 Hz)	39.5	ax. 1.15 (1H, *m*)	38.8	ax. 1.06 (1H, *dt*-like, *J* = 5.0, 13.2 Hz)
		β 2.01 (1H, *m*)		eq. 1.70 (1H, *m*)		eq. 1.67 (1H, *m*)
2	28.8	*α* 2.38 (1H, *dddd*, *J* = 1.6, 3.4, 7.6, 17.9 Hz)	27.9	1.66 (2H, *m*)	28.1	ax. 1.67 (1H, *m*)
		β 2.20 (1H, *m*)				eq. 1.62 (1H, *m*)
3	138.1	6.75 (1H, *dd*, *J* = 2.1, 7.6 Hz)	79.6	3.17 (1H, *m*)	79.6	3.17 (1H, *dd*, *J* = 5.6, 10.7 Hz)
4	139.9		40.1		40.1	
5	47.1		56.6	0.93 (1H, *m*)	56.7	1.00 (1H, m)
6	35.6	*α* 1.85 (1H, *td*-like, *J* = 3.2, 12.8 Hz)	21.4	ax. 1.39 (1H, *m*)	20.7	ax. 1.40 (1H, *m*)
		β 1.24 (1H, *m*)		eq. 1.68 (1H, *m*)		eq. 1.70 (1H, *m*)
7	28.9	*α* 1.64 (1H, *m*)	45.2	ax. 1.41 (1H, *m*)	43.1	ax. 1.54 (1H, *dt*, *J* = 4.0, 13.2 Hz)
		β 1.59 (1H, *m*)		eq. 1.81 (1H, *m*)		eq. 1.74 (1H, *m*)
8	39.3	1.92 (1H, *m*)	74.9		78.3	
9	41.4		62.5	1.10 (1H, *t*, *J* = 3.4 Hz)	58.6	1.35 (1H, *dd*, *J* = 2.6, 11.9 Hz)
10	50.1	2.10 (1H, *d*, *J* = 11.9 Hz)	40.2		37.9	
11	43.1	2.417 (1H, *s*-like)	25.2	1.63 (1H, *m*)	16.09	ax. 1.59 (1H, *m*)
		2.424 (1H, *s*-like)		1.66 (1H, *m*)		eq. 1.41 (1H, *dq*-like, *J* = 3.7, 13.3 Hz)
12	175.3		45.6	2.19 (1H, *dt*, *J* = 3.7, 12.6 Hz)	36.1	ax. 1.73 (1H, *m*)
				2.38 (1H, *dt*, *J* = 3.7, 12.6 Hz)		eq. 2.10 (1H, *td*, *J* = 3.5, 12.9 Hz)
13	16.4	0.95 (3H, *d*, *J* = 6.9 Hz)	162.7		77.1	
14	172.0		116.4	5.67 (1H, *m*)	43.7	2.51 (1H, *s*-like)
						2.52 (1H, *s*-like)
15	73.4	4.02 (1H, *d*, *J* = 8.2 Hz)	170.6		104.4	5.45 (1H, *d*, *J* = 2.1, 4.8 Hz)
		4.41 (1H, *d*, *J* = 8.2 Hz)				
16	17.4	0.67 (3H, *s*)	19.3	2.15 (3H, *d*, *J* = 1.1 Hz)	180.5	
17			24.0	1.14 (3H, *s*)	23.5	1.27 (3H, *s*)
18			28.8	0.98 (3H, *s*)	28.6	0.98 (3H, *s*)
19			16.2	0.76 (3H, *s*)	16.07	0.76 (3H, *s*)
20			16.3	0.85 (3H, *s*)	16.5	0.82 (3H, *s*)
15-OMe					57.4	3.46 (3H, *s*)

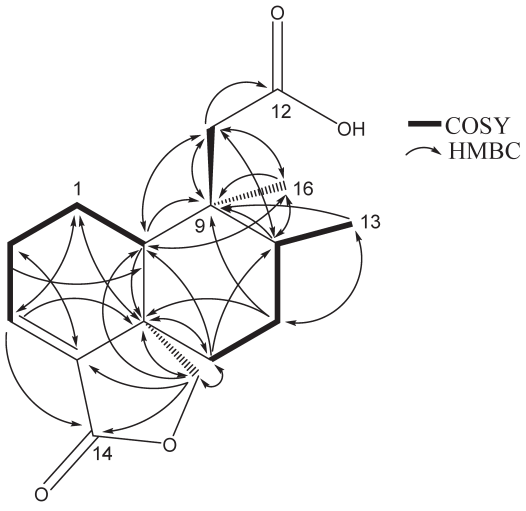


Fig. 3. 2D-NMR Correlations in Compound **1**

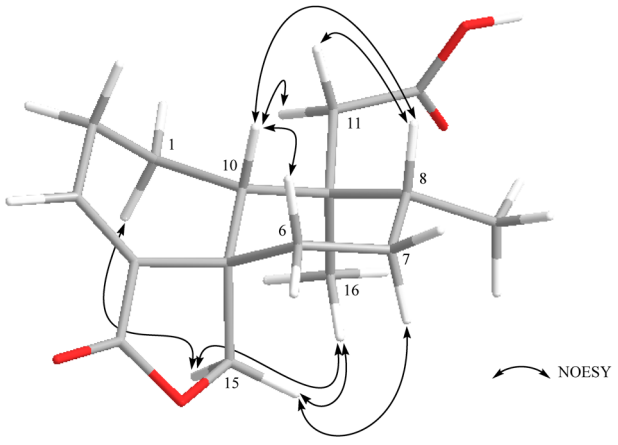
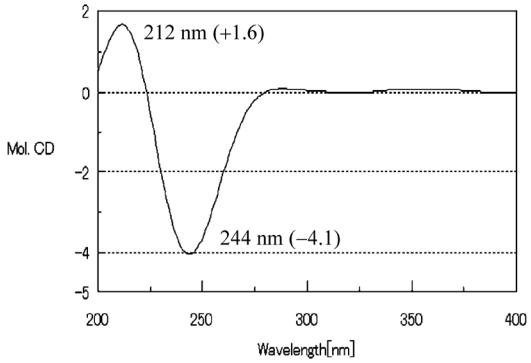


Fig. 4. Key NOEs in Compound **1**

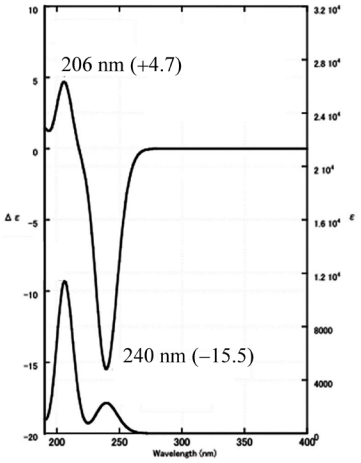
(Color figure can be accessed in the online version.)

their planar structure. (Fig. 6) The NOESY correlations of H_3-20 and H-6 (ax), H_3-17; and H-6 (ax) and H_3-19 suggested that H_3-17, H_3-19, and H_3-20 were in *α*-orientation. Furthermore, the NOE cross-peaks between H-5 and H-3, H-7, H-9; and H-5 and H_3-18 indicated that H-3, H-5, and H-9 were in *β*-orientation (Fig. 7). From the above results, compound **2** was an enantiomer of reported compound.[32] Optical rotation was used to compare a methyl ester derivative (**2a**) of compound **2** with reported data (**2a**: $[\alpha]_D$ −25.0; reported data: $[\alpha]_D$ +6.8).[33] Therefore, the absolute structure of compound **2** was determined as 3*R*, 5*S*, 8*S*, 9*S*, 10*R*.

Compound 3 (**3**), $[\alpha]_D$ +22.6, was isolated as an amorphous powder and its elemental composition was determined to be $C_{21}H_{34}O_5Na$ by HR-ESI-MS. The IR spectrum exhibited absorption bands ascribable to hydroxy groups (3435 cm^{-1}) and



Experimental CD spectrum of compound 1



Calculated CD and UV spectra of Compound 1

Fig. 5. CD Spectra of Compound **1**

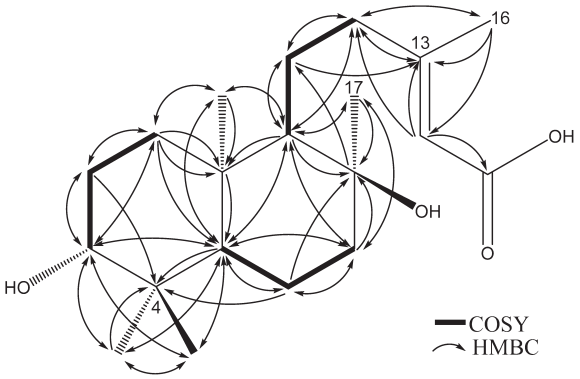


Fig. 6. 2D-NMR Correlations in Compound **2**

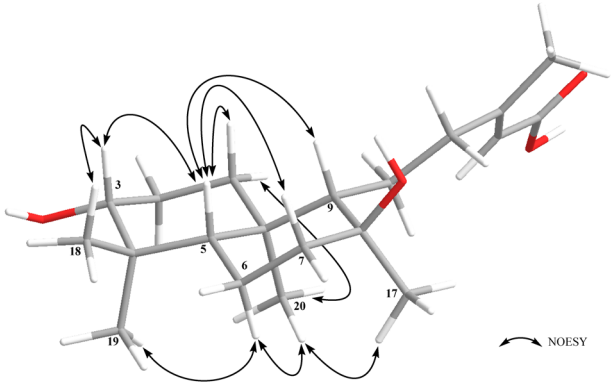


Fig. 7. Key NOEs in Compound **2**
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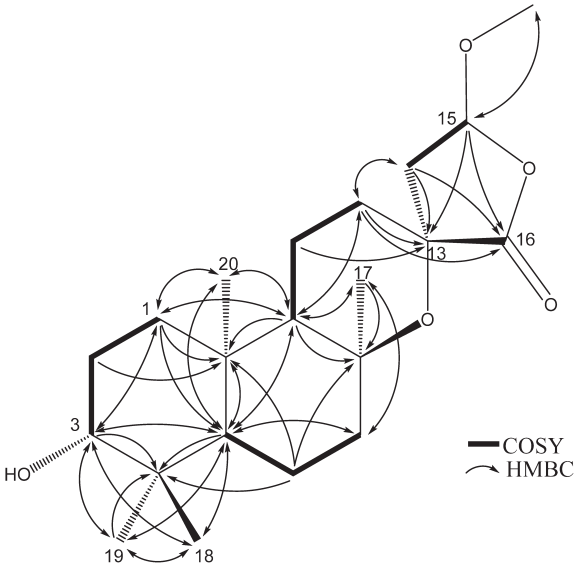

Fig. 8. 2D-NMR Correlations in Compound **3**

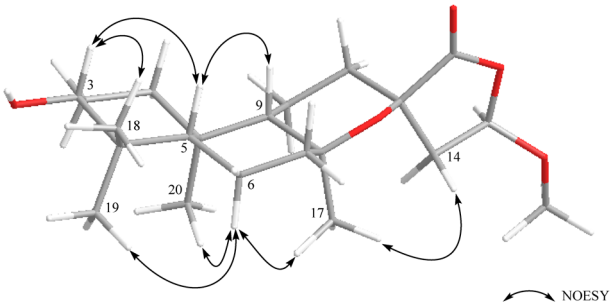

Fig. 9. Key NOEs in Compound **3**
(Color figure can be accessed in the online version.)

a carbonyl functional group (1777 cm^{-1}). The ^{13}C-NMR spectrum displayed five methyl carbons (δ_C 57.4, 28.6, 23.5, 16.5, and 16.07), seven methylenes (δ_C 43.7, 43.1, 38.8, 36.1, 28.1, 20.7, and 16.09), two methines (δ_C 58.6 and 56.7), an oxymethine (δ_C 79.6), an acetate (δ_C 104.4), four quaternary carbons (δ_C78.3, 77.1, 40.1, and 37.9), and a carbonyl carbon (δ_C 180.5). A ^{1}H-^{1}H COSY experiment and a HSQC experiment indicated that compound **3** had four structural fragments as shown by the bold lines in Fig. 8. The HMBC cross-peaks between H-15 and C-13, C-16; and H-14 and C-13, C-16 demonstrated the presence of a lactone ring. Moreover, the cross-peak between H-21 and C-15 suggested that a methoxy group combined at C-15. Furthermore, additional HMBC cross-peaks indicated that compound **3** was a labdane-type diterpene derivative. The relative configuration of compound **3** was determined by a NOESY experiment (Fig. 9). The NOESY correlations of H-6 (ax) and H_3-17, H_3-19, H_3-20 suggested that H-17, H-19, and

Table 2. Collagenase Inhibitory Activities (IC_{50} Values, μM)

Compounds	$IC_{50} \pm$ S.E. (μM)	compounds	$IC_{50} \pm$ S.E. (μM)
1	>200	11	94.5 ± 17.7
2	>200	12	139 ± 1.28
3	>200	13	163 ± 16.1
4	>200	14	>200
5	>200	15	>200
6	>200	18	>200
7	>200	19	>200
8	129 ± 6.00	20	>200
9	42.9 ± 5.97	Positive control	
10	111 ± 3.89	Caffeic acid	89.7 ± 4.80

H-20 were in α-orientation, and other correlation of H-17 and H-14 meant that H-14 was in α-orientation. Furthermore, the NOE cross-peaks between H-3 and H-5, H_3-18; H-5 and H-9; and indicated that H-3, H-5, and H-9 were in β-orientation and that H-14 was also in β-orientation. Since, compound **2** was defined as a member of the *ent*-labdane type and some *ent*-labdane diterpenes were reported from same plant so compound **3** was expected as same type.[2,7] Thus, the relative structure of compound **3** was determined to be that shown in Fig. 1.

Dodoviscin A, a compound isolated from *D. viscosa*, has an inhibitory effect on melanin production.[1] However, a detailed cosmetic investigation of this plant species has yet to be conducted. Collagen is a major component of the dermis that keeps the skin elastic and firm, whereas collagenase is an enzyme that breaks down the collagen contained in the dermis of the skin and causes skin aging (*e.g.* as wrinkles). Although collagenase is also an important enzyme for degrading old collagen and maintaining skin firmness, suppressing collagenase activity would be effective for keeping skin firm. Therefore, plant extracts with anti-collagenase activity would be useful in the cosmetics industry. Collagenase is also known to be a member of the matrix metalloproteinases, which play crucial roles in normal physiological processes such as embryogenesis and wound healing.[34] In the present study, we evaluated the collagenase inhibitory activity, as well as the tyrosinase (an enzyme that catalyzes the production of melanin and other pigments) inhibitory activity, of all the isolated compounds.[35,36] As shown in Table 2, compound **9** showed the most potent collagenase inhibitory activity, while compound **11** showed almost the same activity as the positive control (caffeic acid). Compounds **8** and **12** showed moderate tyrosinase inhibitory activities with inhibition percent values of 34.6 and 48.8%, recpectively, the inhibitory activity of a positive control, arbutin, being 32.1%. Similar to dodoviscin A, compounds **9**–**11** were prenylated flavonoids. Taken together, these results suggest that compounds **9** and **12** would be the best candidates for use as cosmetic agents.

Conclusion

Three new diterpenoids (**1**–**3**) were isolated from the stems of *D. viscosa*, and their structures were determined on the basis of chemical and physicochemical evidence, and cotton effect. However, compound **3** has an acetal and a methoxy group, thus, this compound may be an artifact. Other study has reported that an isolated compound from this plant has an inhibitory effect on melanin production. The results of the current study suggest that *D. viscosa* plant was beneficial for cosmetic utilization.

Experimental Procedure

Optical rotations were measured on a JASCO P-1030 spectropolarimeter. IR and UV spectra were measured on Horiba FT-710 and JASCO V-520 UV/Vis spectrophotometers, respectively. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance III spectrometer at 600 MHz and 150 MHz, respectively, with TMS or a residual solvent as an internal standard. Positive-ion HR-ESIMS was acquired on a Thermo Fisher Scientific Orbitrap. Silica gel column chromatography (CC) and reversed-phase (ODS) open CC were performed on silica gel 60 (Merck, Darmstadt, Germany) and Cosmosil 75C18-OPN (Nacalai Tesque, Kyoto, Japan). HPLC was performed on an Inertsil ODS-3 column (GL Science, Tokyo, Japan; $\Phi = 10$ mm, $L = 25$ cm) and a Cosmosil πNAP column (Nakalai Tesque; $\Phi = 10$ mm, $L = 25$ cm), and the eluate was monitored with a refractive index monitor. Precoated silica gel 60 F254 plates (E. Merck; 0.25 mm in thickness) were used for thin layer chromatography analyses, which were visualized by spraying with a 10% solution of H_2SO_4 in ethanol and heating to around 150 °C on a hotplate. Fluorescence was measured by EnSpire (PerkinElmer, Inc., U.S.A.) and Absorbance was measured by Multiskan Go (Thermo Fisher Scientific, U.S.A.).

Plant Material The stems of *D. viscosa* were collected in Nakagami-gun, Okinawa in June 2004, A voucher specimen was deposited in the Herbarium of the Department of Pharmacognosy, Graduate School of Biomedical and Health Sciences, Hiroshima University (04-DV-Okinawa-0630). The plant was identified by Dr. Takakazu Shinzato of Faculty of Agriculture, University of the Ryukyus whom the authors acknowledge.

Extraction and Isolation Air-dried stems of *D. viscosa* (900 g, Okinawa) were extracted three times with MeOH (15 L × 3) at room temperature for one week. Evaporation of the solvent under reduced pressure provided a MeOH extract (58.9 g). The MeOH extract (57.0 g) was divided into an EtOAc–H_2O (1 : 1, v/v) mixture to obtain an EtOAc-soluble fraction (24.2 g) and H_2O-soluble fraction (32.6 g). The EtOAc-soluble fraction (24.2 g) was subjected to normal-phase silica gel column chromatography [600 g, Hexane : EtOAc (10 : 1→5 : 1→2 : 1→1 : 1→2 : 3)→$CHCl_3$: MeOH (20 : 1→10 : 1)→MeOH] to obtain twelve fractions [fr. 1 (1.8 g), fr. 2 (0.7 g), fr. 3 (1.9 g), fr. 4 (0.5 g), fr. 5 (1.0 g), fr. 6 (2.7 g), fr. 7 (0.8 g), fr. 8 (0.9 g), fr. 9 (1.2 g), fr. 10 (1.2 g), fr. 11 (7.9 g) and fr. 12 (3.2 g)]. Fraction 5 (1.0 g) was separated by reversed-phase silica gel column chromatography (CC) [30 g, MeOH : H_2O (1 : 1→3 : 2→7 : 3→4 : 1→9 : 1)→MeOH→Acetone] to yield eleven fractions [fr. 5-1 (9.4 mg), fr. 5-2 (19.6 mg), fr. 5-3 (31.1 mg), fr. 5-4 (47.6 mg), fr. 5-5 (10.8 mg), fr. 5-6 (58.0 mg), fr. 5-7 (56.7 mg), fr. 5-8 (86.0 mg), fr. 5-9 (83.5 mg), fr. 5-10 (235 mg) and fr. 5-11 (164 mg)]. Fraction 5-2 (19.6 mg) was a mixture of compounds **17** and **18**. Fraction 6 (2.8 g) was separated by reversed-phase silica gel column chromatography (CC) [90 g, MeOH : H_2O (1 : 1→3 : 2→7 : 3→4 : 1→9 : 1)→MeOH→Acetone] to yield eleven fractions [fr. 6-1 (25.6 mg), fr. 6-2 (68.0 mg), fr. 6-3 (970 mg, **5**), fr. 6-4 (523 mg), fr. 6-5 (89.2 mg), fr. 6-6 (72.3 mg), fr. 6-7 (155 mg), fr. 6-8 (34.8 mg), fr. 6-9 (39.2 mg), fr. 6-10 (33.3 mg) and fr. 6-11 (71.3 mg)]. Fraction 6-2 (68.0 mg) was purified by HPLC [MeOH : H_2O (38 : 62, v/v)] to obtain **20** (2.7 mg). 6-Hydroxyhardwickjic acid (**7**, 4.1 mg) was recrystallized from fr. 6-4. Fraction 7 (0.8 g) was separated by reversed-phase silica

gel CC [24 g, MeOH : H_2O (2 : 3→1 : 1→3 : 2→7 : 3→4 : 1→9 : 1)→MeOH→Acetone] to yield eleven fractions [fr. 7-1 (27.6 mg), fr. 7-2 (9.2 mg), fr. 7-3 (62.6 mg), fr. 7-4 (105.5 mg), fr. 7-5 (92.0 mg), fr. 7-6 (35.7 mg), fr. 7-7 (54.5 mg), fr. 7-8 (49.3 mg), fr. 7-9 (95.5 mg), fr. 7-10 (21.6 mg) and fr. 7-11 (25.6 mg)]. Fraction 7-3 (62.6 mg) was purified by HPLC [MeOH : H_2O (57.5 : 42.5, v/v)] to obtain **1** (4.4 mg). Fraction 7-4 (105.5 mg) was purified by HPLC [MeOH : H_2O (54 : 46, v/v)] to give **3** (7.5 mg). Compound **6** (26.9 mg) was recrystallized from fr. 7-5. Fraction 8 (0.9 g) was separated by reversed-phase silica gel CC [27 g, MeOH : H_2O (2 : 3→1 : 1→3 : 2→7 : 3→4 : 1→9 : 1)→MeOH→Acetone] to yield eleven fractions [fr. 8-1 (46.9 mg), fr. 8-2 (31.1 mg), fr. 8-3 (53.3 mg, **8**), fr. 8-4 (74.2 mg), fr. 8-5 (71.2 mg), fr. 8-6 (48.4 mg, **9**), fr. 8-7 (57.0 mg), fr. 8-8 (75.4 mg), fr. 8-9 (58.9 mg), fr. 8-10 (79.9 mg) and fr. 8-11 (37.1 mg)]. Fraction 8-7 (57.0 mg) was purified by HPLC [MeOH : H_2O (81 : 19, v/v)] to obtain **11** (6.0 mg) and **4** (12.3 mg). Fraction 9 (1.2 g) was separated by reversed-phase silica gel CC [36 g, MeOH : H_2O (1 : 4→3 : 7 →2 : 3→1 : 1→3 : 2→7 : 3→4 : 1→9 : 1)→MeOH→Acetone] to yield twelve fractions [fr. 9-1 (27.5 mg, **16**), fr. 9-2 (37.5 mg), fr. 9-3 (25.0 mg, **12**), fr. 9-4 (8.6 mg), fr. 9-5 (22.5 mg), fr. 9-6 (8.0 mg, **15**), fr. 9-7 (41.5 mg), fr. 9-8 (166 mg), fr. 9-9 (57.6 mg), fr. 9-10 (90.4 mg), fr. 9-11 (95.3 mg) and fr. 9-12 (309 mg)]. Fraction 10 (1.2 g) was separated by reversed-phase silica gel CC [36 g, MeOH : H_2O (1 : 4→3 : 7 →2 : 3→1 : 1→3 : 2→7 : 3→4 : 1→9 : 1)→MeOH→Acetone] to yield twelve fractions {[fr. 10-1 (28.5 mg), fr. 10-2 (24.7 mg), fr. 10-3 (10.0 mg), fr. 10-4 (63.8 mg), fr. 10-5 (116 mg), fr. 10-6 (138 mg, **2**), fr. 10-7 (31.8 mg, **10**), fr. 10-8 (49.7 mg), fr. 10-9 (58.4 mg), fr. 10-10 (102.6 mg), fr. 10-11 (131 mg) and fr. 10-12 (137 mg)}. Fraction 10-2 (24.7 mg) was purified by HPLC [MeOH : H_2O (43 : 57, v/v)] to afford **13** (4.3 mg). Fraction 11 (7.9 g) was separated by reversed-phase silica gel CC [240 g, MeOH : H_2O (2 : 3→1 : 1→3 : 2→7 : 3→4 : 1→9 : 1)→MeOH→Acetone] to yield ten fractions [fr. 11-1 (377 mg), fr. 11-2 (1.2 g), fr. 11-3 (1.3 g), fr. 11-4 (763 mg), fr. 11-5 (612 mg), fr. 11-6 (1.2 g), fr. 11-7 (1.1 g), fr. 11-8 (588 mg), fr. 11-9 (160 mg) and fr. 11-10 (335 mg)]. Fraction 11-1 (377 mg) was purified by HPLC [MeOH : H_2O (40 : 60, v/v)] to afford **19** (11.1 mg) and **14** (15.6 mg).

Compound 1 (1) Amorphous powder; $[\alpha]_D^{24}$ −130.5 ($c = 0.21$, MeOH); IR ν max (film) cm^{-1}: 3431, 2928, 1762, 1727, 1656, 1450, 1201, 1009; UV λ max (MeOH) nm ($\log\varepsilon$): 247 (2.80), 204 (3.77); CD ($c = 1.46 \times 10^{-5}$ M, MeOH) $\Delta\varepsilon$ (nm): -4.08 (244), +1.64 (212); HR-ESI-MS (positive) m/z : 301.1412 $[M + Na]^+$ (Calcd for $C_{16}H_{22}O_4Na$: 301.1410)

Compound 2 (2) Amorphous powder; $[\alpha]_D^{22}$ −32.1 ($c = 0.13$, MeOH), $[\alpha]_D^{24}$ −21.4 ($c = 2.20$, $CHCl_3$); IR ν max (film) cm^{-1}: 3407, 2943, 1678, 1644, 1231, 1126, 1022, 930; UV λ max (MeOH) nm ($\log\varepsilon$): 215 (4.09); HR-ESI-MS (positive) m/z : 361.2351 $[M + Na]^+$ (Calcd for $C_{20}H_{34}O_4Na$: 361.2349)

Compound 3 (3) Amorphous powder; $[\alpha]_D^{24}$ +22.6 ($c = 0.50$, MeOH); IR ν max (film) cm^{-1}: 3435, 2946, 2863, 1777, 1645, 1058, 1017; HR-ESI-MS (positive) m/z : 389.2301 $[M + Na]^+$ (Calcd for $C_{21}H_{34}O_5Na$: 389.2298)

Methyl Ester (2a) of Compound 2[37] A solution of compound **2** (5.4 mg) was treated with a solution of $TMSCHN_2$ (10% in hexane, 0.3 mL), and stirred for 5 min at room temperature. The solvent was vacuum distilled. The product was chromatographed over a preparative thin-layer chromatography with elution by hexane : EtOAc (9 : 1) to afford compound **2a** (2.8 mg).

Compound 2a (2a) Amorphous powder; $[\alpha]_D^{24}$ −25.0 ($c = 0.20$, MeOH), $[\alpha]_D^{24}$ −22.9 ($c = 1.5$, $CHCl_3$); ^{1}H-NMR (600 MHz, CD_3OD) δ: 5.69 (1H, br d, $J = 1.1$ Hz, H-14), 3.66 (3H, s, H-OMe), 3.17 (1H, dd, $J = 5.0$, 11.8 Hz, H-3), 2.34, 2.19 (both 1H, each dt-like, $J = 5.0$, 13.0 Hz, H_2-12), 2.16 (3H, d, $J = 1.1$ Hz, H-16), 1.13 (3H, s, H-17), 1.08 (1H, t-like, $J = 3.9$ Hz, H-9), 0.98 (3H, s, H-18), 0.93 (1H, dd-like, $J = 2.3$, 11.9 Hz, H-5), 0.85 (3H, s, H-20), 0.76 (3H, s, H-19); ^{13}C-NMR (150 MHz, CD_3OD) δ: 169.2 (C-15), 163.1 (C-13), 115.7 (C-14), 79.6 (C-3), 74.9 (C-8), 62.5 (C-9), 56.6 (C-5), 51.4 (C-OMe), 45.5 (C-12), 45.1 (C-7), 40.2 (C-10), 40.1 (C-4), 39.5 (C-1), 28.8 (C-18), 27.9 (C-2), 25.2 (C-11), 23.9 (C-17), 21.4 (C-6), 19.3 (C-16), 16.3 (C-20), 16.2 (C-19); HR-ESI-MS (positive-ion mode) m/z: 375.2507 $[M + Na]^+$ (Calcd for $C_{21}H_{36}O_4Na$: 375.2506).

Electronic Circular Dichroism (ECD) Caluculaion Conformational search was performed with CONFLEX 8 (Ver. 8.A.0901 by CONFLEX, Tokyo)[38–40] using a commercially available PC (operating system: Windows 10 Pro for Workstations, CPU: Intel Xeon E5-1650 processor 3.60 GHz, RAM 32 GB) and DFT calculations were conducted with Gaussian 09 (Revivion E.01 by Gaussian, Wallingford, CT)[41] with a PC (Operating System: CentOS 7 a Linux, CPU: Intel Xeon E5-1660 processor 3.20 GHz x16, RAM 32 GB). Theoretical CD spectrum was obtained from a typical calculation procedure[42] as described in the followings. The initial structure was constructed on a graphical user interface considering the absolute configurations of interest and subjected to a conformational search with CONFLEX8 using MMFF94S (2010-12-04HG) as the force field, where initial stable conformers were generated for up to 50 kcal/mol. Both two stable conformers obtained were further optimized by the density functional theory method supposing methanol as the solvent (polarizable continuum models: PCM) with the hybrid B3LYP functional and the double-zeta 6-31G(d) basis set. The obtained conformers were analyzed their populations with their Boltzmann distribution. Based on their energies that were obtained from the internal energies and vibrational corrections, one of the two stable conformers showed 100% of abundance. This most stable conformer was subjected to time-dependent simulations at the level of double-zeta approximation using cc-pVDZ and aug-cc-pVDZ basis sets and hybrid functional B3LYP, cam-B3LYP, BHandHLYP, and PBE1PBE in all possible combinations in MeOH (PCM method). The resultant rotational strengths were converted into Gaussian curves (bandwidth sigma = 3000 cm^{-1}) and summed to give the theoretical CD spectrum. The best match between the experimental and the theoretical ECD curves was obtained when cam-B3LYP/aug-cc-pVDZ was applied.

Collagenase Inhibitory Activity Collagenase Type V (EC3.4.24.3; Sigma-Aldrich, Tokyo, Japan) and the substrate MOCAc-PRO-Leu-Gly-Leu-A_2pr(Dnp)-Ala-Arg-NH_2 (Peptide Institute Inc., Osaka, Japan) were used to test for collagenase inhibitory activity. To the sample solution (50 μL) and 100 μL of 10 μg/mL of enzyme solution in 50 mM tricine buffer (pH 7.50) were added to a 96-well plate. Then it was preincubated at 37 °C for 10 min. After preincubation, 50 μL of substrate solution (10 μg/mL) in the same buffer was added to the mixture to begin the reaction. The fluorescence values were measured at an excitation of 320 nm and an emission of 405 nm during incubation at 37 °C for 30 min using a fluorescent plate reader.

Caffeic acid was used as a positive control. The inhibition of collagenase was calculated as:

$$\text{Inhibition ratio (\%)} = [1-(A_{\text{sample 30min}} - A_{\text{sample 0min}})/A_{\text{blank 30min}} - A_{\text{blank 0min}})]\times 100$$

A_{sample}: test sample. A_{blank}: without test sample.

Tyrosinase Inhibitory Activity Tyrosinase from mushroom lyophilized powder, purchased from Sigma-Aldrich (Tokyo, Japan), and the substrate L-(−)-tyrosine, from Tokyo Chemical Ind. (Tokyo, Japan), were used. First, 100 μL of mushroom tyrosinase (100 U/mL) in 0.1 M phosphate buffer (pH 6.80) was added to a solution containing the sample (20 μL) and 2.5 mM L-dihydroxyphenylalanine (80 μL) in the same buffer. This was then incubated at 25 °C for 5 min. The amounts of dopachrome formed were photometrically determined by measuring the optical density at 405 nm using a microplate reader. Kojic acid was used as a positive control. The inhibition of tyrosinase was calculated as follows:

$$\text{Inhibition ratio (\%)} = [1-(A_{\text{sample}} - A_{\text{blank}})/A_{\text{control}} - A_{\text{blank}})]\times 100$$

A_{control}: without test sample;

A_{blank}: without test sample and tyrosinase.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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