Bioorganic & Medicinal Chemistry Letters 21 (2011) 2840–2844

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

New monoterpene glycosides and phenolic compounds from *Distylium racemosum* and their inhibitory activity against ribonuclease H

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ARTICLE INFO

Article history:
Received 31 January 2011
Revised 18 March 2011
Accepted 23 March 2011
Available online 30 March 2011

Keywords:
Distylium racemosum
Monoterpene glycosides
Megastigmane glucoside
Ribonuclease H

ABSTRACT

Two new monoterpene glycosides, distyloside A–B (**1**–**2**), and a new megastigmane glucoside, *iso*-dihydrodendranthemoside A (**3**) were isolated from twigs and leaves of *Distylium racemosum*, along with five known phenolic compounds (**4**–**8**). The structures were established via spectroscopic techniques and chemical transformations, and the absolute stereochemistry of **3** was determined by Mosher's esterification. A homogeneous fluorescence resonance energy transfer (FRET) quenching assay was used to determine the inhibitory activity of isolates (**1**–**8**) on the ribonuclease H enzymes from HIV-1, 2, human, and *Escherichia coli*. Among them, 6″-*O*-galloylsalidroside (**6**) showed potent inhibitory effects with an IC_{50} value of 3.5 μM on HIV-2, and 1.7 μM on human RNase H, respectively.

Reverse transcription is the most vital process in the retroviral life cycle as a result of which reverse transcriptase (RT) has three different activities including RNA dependent DNA polymerase, DNA dependent DNA polymerase, and ribonuclease H (RNase H) activities.[1] Retroviral RNase H is necessary for the proliferation of retroviruses such as human immunodeficiency virus (HIV) and murine leukemia virus.[2] Inhibition of this enzyme has therefore been an important pharmaceutical target against diseases like AIDS. HIV, the causative agent of AIDS, is a dynamic virus that undergoes rapid genetic variation upon infecting a host, resulting in strains resistant to treatment.[3] The rapid emergence of drug-resistant variants of HIV demands the discovery of potent and selective anti-AIDS agents. However, the development of inhibitors of HIV RNase function has been slow and still has not resulted in clinical candidates.

Thus, we have sought potent antiviral components from natural sources since inhibitors of HIV remain an urgent priority. The methanol extract of *Distylium racemosum* Siebold & Zucc. (Hamamelidaceae), a small evergreen tree found on Jeju Island in South Korea,[4] was found to completely inhibit RNase H enzymatic activity at the concentration of 50 μg/mL in a fluorescence resonance energy transfer (FRET) quenching assay.[5] Herein, we report the phytochemical isolation and RNase H inhibition of components from the methanol extract of *D. racemosum*, and elucidated the structures of three new compounds **1**–**3** via spectroscopic techniques and chemical transformations.

The plant material was collected at Halla Mountain, Jeju Island, in December 2003 and taxonomically identified by one of the authors (Professor Young Ho Kim). A voucher specimen (CNU03152) was deposited at the Herbarium of the College of Pharmacy, Chungnam National University, Korea.

Preliminary examination of the crude methanol extract found that activity could be eluted from a polyamide column, indicating that tannins were not solely responsible for the observed activity.[7]

To obtain a bulk extract, twigs and leaves of *D. racemosum* (1.1 kg) were macerated three times with MeOH (10 L) at room temperature for 7 days. The solvent was concentrated in vacuo to give a greenish extract (70.0 g), which was suspended in H_2O and partitioned with CH_2Cl_2 and EtOAc to give a CH_2Cl_2-soluble fraction (14.0 g), an EtOAc-soluble fraction (12.0 g), and a H_2O-soluble fraction (44.0 g). In the enzymatic assay, the H_2O-fraction showed very strong inhibition of RNase H activity with an IC_{50} value of 0.2 μg/mL. It was applied to a Diaion HP-20 column and eluted stepwise with mixtures of H_2O/MeOH (0, 25, 50, 75, 100%, v/v). Subsequent separations of the 25% MeOH fraction (F2, 8.0 g) were performed on reverse phase C-18, Sephadex LH-20, and silica gel columns[8] to ultimately yield two new monoterpene glycosides, distyloside A–B (**1**–**2**), a new megastigmane glucoside, *iso*-dihydrodendranthemoside A (**3**), and five known phenolic compounds,

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doi:10.1016/j.bmcl.2011.03.091

(−)-epigallocatechin (**4**),[9] salidroside (**5**),[10] 6″-*O*-galloylsalidroside (**6**),[11] chlorogenic acid methyl ester (**7**),[12] and syringin (**8**)[13] (Fig. 1).

Compound **1**[14] was obtained as an amorphous powder with the molecular formula $C_{16}H_{30}O_8$, based on the positive HRESIMS (experimental *m*/*z* [M+Li]$^+$ 357.2131, calculated 357.2101). Acid hydrolysis liberated D-glucose, which was identified by GC.[15] The ^{1}H and ^{13}C NMR spectral data (Table 1) showed the presence of three methyl singlets, three methylenes, two methines (one oxygenated), two oxygenated quaternary carbons, and a β-D-glucosyl unit. This was almost identical with (1*R*,2*R*,4*S*)-*p*-menthane-1,2,8-triol 8-*O*-β-D-glucopyranoside, except for a down-field shift difference for C-1 [δ 78.6 (+7.9)] and an up-field shift for C-8 [δ 71.7 (−8.0)].[17] The HMBC spectrum of **1** confirmed that the position of the glucosyl unit attachment was at C-1 by the observed cross-peak between the anomeric H-1′ and C-1 (Fig. 2). Furthermore, (1*S*,2*S*,4*R*)-*p*-menthane-1,2,8-triol 2-*O*-β-D-glucopyranoside showed a positive optical rotation [$[\alpha]_D^{21}$ +9.0 (MeOH)][18] contrary to those of (1*R*,2*R*,4*S*)-*p*-menthane-1,2,8-triol 8-*O*-β-D-glucopyranoside [$[\alpha]_D^{23}$ −31.0 (MeOH)][17] and **1** [$[\alpha]_D^{27}$ −31.0 (MeOH)].[13] On the basis of this evidences, the structure of compound **1** was determined to be (1*R*,2*R*,4*S*)-*p*-menthane-1,2,8-triol 1-*O*-β-D-glucopyranoside, which was named distyloside A (**1**).

Compound **2**[14] was obtained as an amorphous powder with the molecular formula $C_{21}H_{36}O_{11}$, based on HRESIMS (experimental *m*/*z* [M+Li]$^+$ 471.2437, calculated 471.2418). Acid hydrolysis liberated D-glucose and D-xylose, which were identified by GC.[15] A comparison of the ^{1}H- and ^{13}C NMR spectroscopic data for **2** with those of (4*R*)-*p*-menth-1-ene-7,8-diol 7-*O*-β-D-glucopyranoside[19] revealed the additional β-D-xylopyranosyl unit [δ 105.1 (C-1″), 72.3 (C-2″), 74.1 (C-3″), 69.4 (C-4″), 66.6 (C-5″) and δ 4.32 (1H, d, *J* = 6.5 Hz, H-1″), 3.60 (1H, m, H-2″), 3.52 (1H, m, H-3″), 3.80 (1H, m, H-4″), 3.53 (1H, m, H-5″), 3.86 (1H, dd, *J* = 3.0, 12.5 Hz, H-5″)] in the structure of **2**. From analysis of their HMBC correlations (Fig. 2), the linkage of xylose and glucose was confirmed to be β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl and the location of the glucosyl group was indicated to be at C-7. Enzymatic hydrolysis of **2** gave an aglycone [**2a**, $C_{10}H_{18}O_2$, an amorphous powder, $[\alpha]_D^{27}$ +43.1° (*c* 0.1, MeOH)], which matched literature values for (4*R*)-*p*-menth-1-ene-7,8-diol[19] and accordingly implied that **2** has the same absolute configuration. Therefore, compound **2** was determined to be (4*R*)-*p*-menth-1-ene-7,8-diol 7-*O*-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl] ester, which was named distyloside B (**2**).

Compound **3**[14] was isolated as an amorphous powder and its molecular formula was determined to be $C_{21}H_{36}O_{11}$, based on HRESIMS (experimental *m*/*z* [M+Li]$^+$ 399.2586, calculated 399.2570). Acid hydrolysis liberated D-glucose, which was identified by GC.[15] The ^{1}H and ^{13}C NMR spectral data (Table 1) indicated the presence of a β-D-glucopyranose unit and a megastigmane skeleton including two oxymethines (δ_C 69.7 and 75.4) and one oxygenated quaternary carbon (δ_C 77.7). Close inspection of COSY and HMBC spectrum (Fig. 2) established its structure to be megastigman-3,6,9-triol 3-*O*-β-D-glucopyranoside. Compound **3** showed similar chemical shift values in the ^{1}H and ^{13}C NMR spectra with dihydrodendrant-

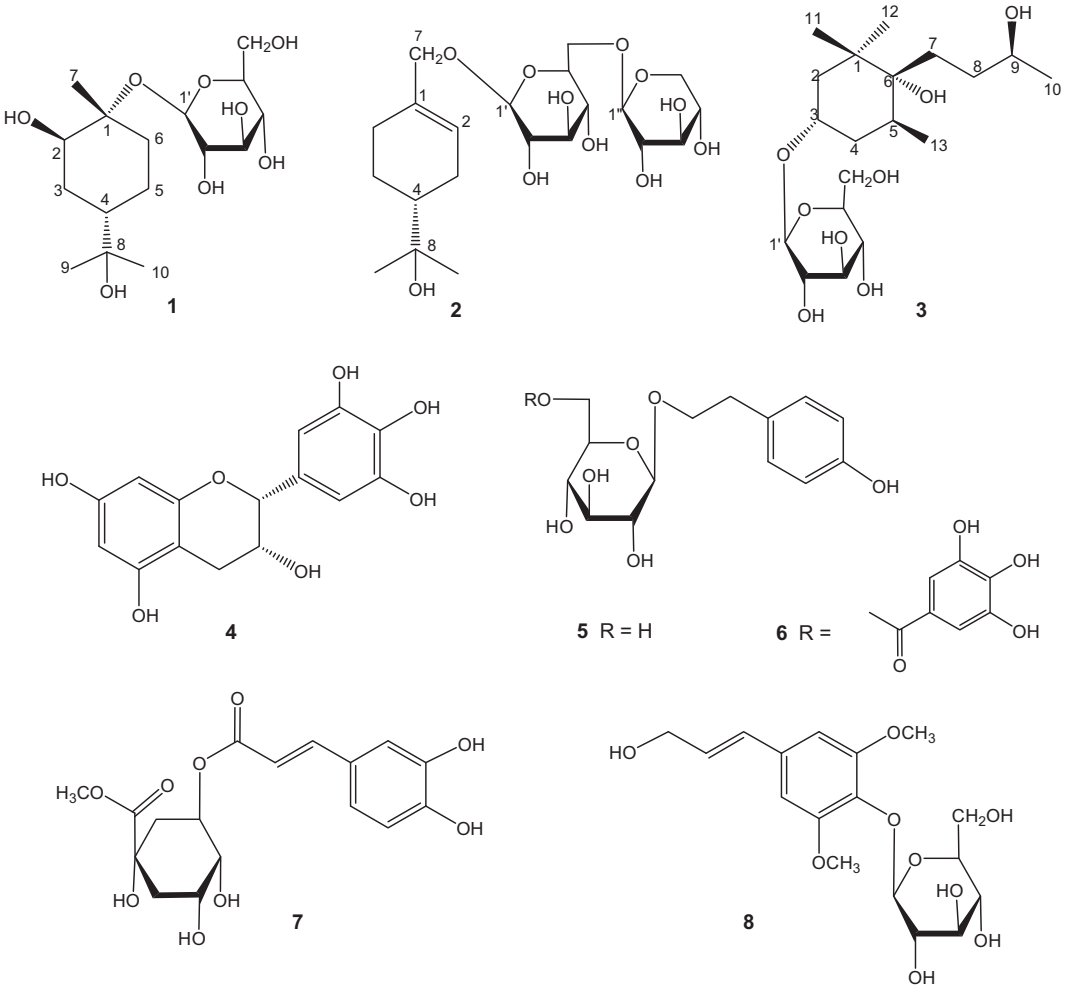

Figure 1. Structures of compounds **1**–**8**.

Table 1
NMR data for compounds **1**–**3**

No.	**1**[a]		**2**[b]		**3**[b]	
	δ_C[c]	δ_H[d] (J in Hz)	δ_C[c]	δ_H[d] (J in Hz)	δ_C[c]	δ_H[d] (J in Hz)
1	78.6	—	135.5	—	39.9	—
2	72.9	4.37 m	126.6	5.76 m	43.4	2 ax 1.82 dd (14.0, 3.5) 2 eq 1.47 br d (14.0)
3	31.1	3 ax 2.30 m 3 eq 2.56 ddd (13.0, 13.0, 2.5)	27.8	3 ax 1.85 m 3 eq 2.13 ddd (13.0, 3.0, 3.0)	75.4	3.97 m
4	42.1	2.38 dddd (2.5, 2.5, 13.0, 13.0)	46.3	1.52 dddd (3.0, 3.0, 13.0, 13.0)	34.9	1.61 m
5	22.7	5 ax 1.86 ddd (2.5, 2.5, 13.0) 5 eq 2.32 m	24.8	5 ax 1.23 ddd (4.0, 13.0, 13.0) 5 eq 1.94 d (13.0)	31.3	2.22 m
6	31.2	6 ax 2.11 m, 6 eq 2.12 m	27.1	6 ax 2.03 dd (13.0, 16.0) 6 eq 2.24 dd (4.0, 16.0)	77.7	—
7	25.0	1.78 s	73.9	4.04 d (11.5), 4.16 d (11.5)	33.0	1.55 m, 1.80 m
8	71.7	—	73.1	—	35.9	1.58 m
9	28.0	1.40 s	27.8	1.15 s	69.7	3.64 m
10	27.6	1.39 s	26.3	1.14 s	23.4	0.93 d (8.5)
11					26.6	1.15 s
12					27.4	1.13 s
13					16.6	0.92 d (6.5)
1′	98.6	5.18 d (8.0)	102.7	4.23 d (8.0)	102.6	4.28 d (8.0)
2′	75.6	4.04 m	75.0	3.19 m	75.3	3.14 m
3′	79.1	4.25 m	77.9	3.33 m	78.4	3.34 m
4′	72.0	4.21 m	71.6	3.33 m	71.7	3.27 m
5′	78.1	3.94 m	76.8	3.39 m	77.7	3.23 m
6′	63.0	4.33 m, 4.49 m	69.4	3.72 dd (5.5, 11.5) 4.08 dd (2.0, 11.5)	62.8	3.65 dd (5.5, 12.0) 3.84 dd (2.0, 12.0)
1″			105.1	4.32 d (6.5)		
2″			72.3	3.60 dd (7.0, 17.5)		
3″			74.1	3.52 m		
4″			69.4	3.80 m		
5″			66.6	3.53 m 3.86 dd (3.0, 12.5)		

[a] C_5D_5N was used for NMR solvent.
[b] CD_3OD was used for NMR solvent.
[c] Recorded at 125 MHz.
[d] Recorded at 500 MHz.

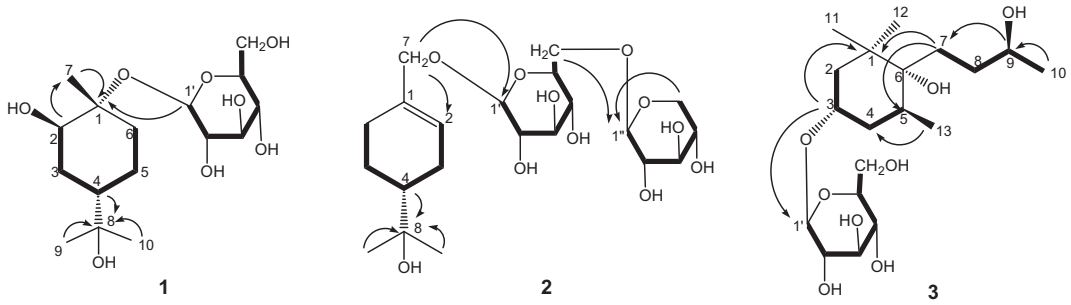

Figure 2. Selected ^{1}H–^{1}H COSY (—) and selected HMBC correlations (H→C) of **1**–**3**.

hemoside A, obtained by catalytically reducing dendranthemoside A isolated from the leaves of *Alangium premnifolium*.[21] But the main difference in ^{13}C NMR spectral data was an up-field shift of C-4 [δ 35.0 (−4.0)] and C-5 [δ 31.4 (−5.6)], which indicated that **3** has an *S* orientation on C-5, not *R*. To confirm the stereochemistry of **3**, the glucose unit was removed by enzymatic hydrolysis.[20] The aglycone **3a** was separated into two equal portions and treated with (*S*)-(+)-*R*- and (*R*)-(−)-*R*-methoxy-*R*-(trifluoromethyl)phenylacetyl chloride in anhydrous pyridine at room temperature overnight, to afford the corresponding MTPA esters, respectively.[22] As shown in Fig. 3, the $\Delta\delta$ values of the MTPA esters were consistent with 3*S* and 9*S* configurations, and NOESY correlations from H-3 to H-2 eq and H-13 supported the configuration of C-3 as well as C-5 to be 3*S* and 5*S*, respectively. In addition, H-2 ax was unambiguously correlated with H-4 but not with H-7, which gives supports a 6*R* assignment. On the basis of the evidence, the structure of **3** was determined to be (3*S*,5*S*,6*R*,9*S*)-megastigman-3,6,9-triol 3-*O*-β-D-glucopyranoside, which was named as *iso*-dihydrodendranthemoside A (**3**).

To evaluate these compounds for antiviral activity, the isolates (**1**-**8**) were tested for inhibition of RNase H enzymatic activity from HIV-1, HIV-2, human, and *Escherichia coli* sources, using the RNase H FRET quenching assay as described previously.[5] Among the eight compounds evaluated, 6″-*O*-galloylsalidroside (**6**) exhibited the most potent inhibitory activity with IC_{50} values of 3.5 against HIV-2 and 1.7 μM against human RNase H, while (−)-epigallocatechin (**4**) showed moderate inhibition with IC_{50} value of 16.4 μM against the human enzyme. HIV standard inhibitor, *N*-(3,4,5-trihydroxybenzoyl)-1-naphthaldehyde hydrazone (KMMP) was used as a positive control in this FRET assay. The IC_{50} value of KMMP was 5.0 μM. Compound **6** (NSC# 741644) exhibited moderate effects with an IC_{50} value of 17.4 μM against HIV-1 and the

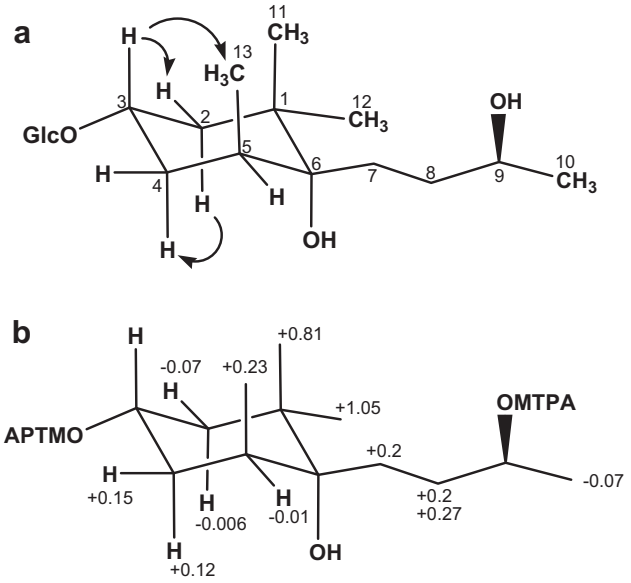

Figure 3. Diagnostic NOESY correlation of **3** (a) and results with the modified Mosher's methods ($\Delta\delta_S - \delta_R$) of **3a** (b).

Table 2
Inhibitory activity of isolated compounds (**1**–**8**) on ribonuclease H

Compounds	RNase H IC_{50}[a] (μM)			
	HIV-1	HIV-2	Human	*E. coli*
1	>20.0	>20.0	>20.0	>20.0
2	>20.0	>20.0	>20.0	>20.0
3	>20.0	>20.0	>20.0	>20.0
4	>20.0	>20.0	16.4	>20.0
5	>20.0	>20.0	>20.0	>20.0
6	17.4	3.5	1.7	>20.0
7	>20.0	>20.0	>20.0	>20.0
8	>20.0	>20.0	>20.0	>20.0
KMMP[b]	5.0			

[a] All data are derived from triplicate tests with the variation of the mean averaging 10%.
[b] *N*-(3,4,5-Trihydroxybenzoyl)-1-naphthaldehyde hydrazone, positive control ($n = 8$).

remaining compounds yielded negligible effects with IC_{50} values more than 20.0 μM against all four RNase H RTs. In addition, none of the compounds inhibited the activity of *E coli* RNase H in our assay system (Table 2).

This study indicated the structures (**4** and **6**) bearing a pyrogalloyl (1,2,3-trihydroxybenzyl) group might play an important role in inhibition of viral activity and is consistent with previous reports that polyphenolic tannins formed by polymerization of phenol, catechol, and pyrogallol precursors possess a range of biological activities, such as antioxidant, anti-carcinogenic, antiviral, and anti-inflammatory properties.[23]

Thus, the present study suggests a potent possibility of contribution on antiviral properties by compound **6** isolated from *D. racemosum*.

Acknowledgments

This work was supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093815). This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract no. HHSN26120080001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government. This Research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

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8. *Isolation*: The 25% MeOH fraction (F2, 8.0 g) was separated on an ODS C-18 column successively eluting with mixtures of H_2O/MeOH (1:0, 1:3, 1:1, 3:1, v/v) to yield three fractions (F21–F23). Fraction F21 (5.9 g) was partitioned on a Sephadex LH-20 column using pure MeOH as eluting solvent to give **4** (6.0 mg) and five fractions (F211–F215). The fraction F211 (130.1 mg) was further separated on an silica gel column using CH_2Cl_2/MeOH (10:1, v/v) as an eluent to yield five fractions (F2111–F2115). The fraction F2112 (5.0 mg) was purified by preparative ODS HPLC, using CH_3CN/H_2O (17:83, 3.0 mL/min) as solvent system to afford **8** (t_R 7.4 min, 1.2 mg). The fractions F2113 (22.7 mg) and F2115 (8.6 mg) were purified by ODS gel column chromatography with H_2O/MeOH (8:1, 9:1, v/v) as an eluent to give new compounds **3** (6.8 mg) and **1** (2.7 mg), respectively. The fraction F212 (1.2 g) was chromatographed on a silica gel column using a CH_2Cl_2/MeOH (1:1, 9:1, 8:2, v/v) as an eluent to yield seven fractions, F2121–F2127. The fraction F2125 (20.0 mg) was purified by preparative ODS HPLC, using CH_3CN/H_2O (20:80, 3.0 mL/min) as solvent system to afford **7** (t_R 9.8 min, 10.7 mg). The fraction of F2127 (68.0 mg) was chromatographed on a silica gel column using a CH_2Cl_2/MeOH (9:1, v/v) as an eluent to yield compound **5** (46.5 mg). Fraction F22 (1.3 g) was chromatographed over a Sephadex LH-20 column using pure MeOH as eluting solvent to afford six fractions (F221–F226). Further purification of the fractions F221 (484.0 mg) on a silica gel column using a CH_2Cl_2/MeOH (8:1, v/v) as an eluent resulted in the isolation of new compound **2** (7.2 mg). The fraction F223 (106.0 mg) was purified by preparative ODS HPLC, using CH_3CN/H_2O (18:82, 2.0 mL/min) as solvent system to afford **6** (t_R 7.8 min, 3.5 mg).
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14. Distyloside A (**1**), NSC# 742190: an amorphous powder; $[\alpha]_D^{27}$ −31.0 (*c* 0.1, MeOH); ^{1}H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz), see Table 1; ESIMS *m/z* 357.2 $[M+Li]^+$; HRESIMS *m/z* 357.2131 $[M+Li]^+$ (calcd for $C_{16}H_{30}O_8Li$, 357.2101). Distyloside B (**2**), NSC# 742191: an amorphous powder; $[\alpha]_D^{27}$ −18.2 (*c* 0.5, MeOH); ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz), see Table 1; ESIMS *m/z* 471.2 $[M+Li]^+$; HRESIMS *m/z* 471.2437 $[M+Li]^+$ (calcd for $C_{21}H_{36}O_{11}Li$, 471.2418). *iso*-Dihydrodendrantheroside (**3**), NSC# 742189: an amorphous powder; $[\alpha]_D^{27}$ −37.0 (*c* 0.1, MeOH); ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz), see Table 1; HRESIMS *m/z* 399.2586 $[M+Li]^+$ (calcd for $C_{19}H_{36}O_8Li$, 399.2570).
15. *Acid hydrolysis and sugar determination of compounds* **1**–**3**: Each compound (**1**–**3**, 0.1 mg) was dissolved in 6 N HCl (dioxane–H_2O, 1:1, v/v, 200 μL) and then heated to 110 °C in a sand bath for 4 h. The reaction mixture was then concentrated to dryness under N_2 gas overnight. The residue was dissolved in 100 μL of trimethylsilylimidazole and 100 μL of pyridine. Heating at 60 °C was

continued for 5 min, and the reaction mixture was evaporated to give a dried product, which was then partitioned between CH_2Cl_2 and H_2O (each 100 μL). The CH_2Cl_2 layer was analyzed by GC procedure.[16] The peaks of hydrolysates were detected at 14.11 min for D-glucose and 8.21 min D-xylose, respectively. The retention times of the authentic samples (Sigma–Aldrich), after being treated in a similar manner, were 14.11 (D-glucose), 14.24 (L-glucose), 8.21 (D-xylose), and 8.66 min (L-xylose).

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20. *Enzymatic hydrolysis of* **2** and **3**: A mixture of each compound (**2** and **3**, 4.0 mg) and β-glucosidase (5 mg, TOYOBO Co., Ltd) in water (5.0 ml) was shaken in a water bath at 37 °C for 7 d. The mixture was concentrated in vacuo to dryness and the residue was chromatographed over silica gel eluting with $CHCl_3$–MeOH (9:1), to afford aglycones **2a** (1.0 mg) and **3a** (2.0 mg), respectively.
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22. *Preparation of the (R)- and (S)-MTPA ester derivatives of compound* **3a**: Two portions (each 0.4 mg) of **3a** were treated with either (*S*)-(+)-*R*- or (*R*)- (−)-*R*-methoxy-*R*(trifluoromethyl)phenylacetyl chloride (5.0 μL) in anhydrous pyridine (50.0 μL) at room temperature overnight. The reaction mixtures were purified over small silica gel columns with *n*-hexane–EtOAc (8:1) as the elution solvent, to afford the (*R*)- and (*S*)-MTPA ester derivatives of **3a**, respectively. *R-MTPA ester of* **3a**: δ 1.89 (1H, d, J = 14.0 Hz, H-2 ax), 1.26 (1H, d, J = 14.0 Hz, H-2 eq), 5.22 (1H, m, H-3), 1.83 (1H, m, H-4 ax), 1.53 (1H, m, H-4 eq), 1.29 (1H, m, H-5), 1.84 (1H, m, H-7 ax), 1.53 (1H, m, H-7 eq), 1.71 (1H, m, H-8 ax), 1.64 (1H, m, H-8 eq), 5.01 (1H, m, H-9), 1.25 (3H, J = 9.0 Hz, H-10), 1.84 (3H, s, H-11), 1.53 (3H, s, H-12), 0.85 (3H, J = 6.5 Hz, H-13). *S-MTPA ester of* **3a**: δ 1.90 (1H, J = 14.0 Hz, H-2 ax), 1.33 (1H, d, J = 14.0 Hz, H-2 eq), 5.17 (1H, m, H-3), 1.67 (1H, m, H-4 ax), 1.41 (1H, m, H-4 eq), 1.30 (1H, m, H-5), 1.84 (1H, m, H-7 ax), 1.33 (1H, m, H-7 eq), 1.44 (1H, m, H-8 ax), 1.44 (1H, m, H-8 eq), 4.97 (1H, m, H-9), 1.31 (3H, J = 9.0 Hz, H-10), 0.79 (3H, s, H-11), 0.72 (3H, s, H-12), 0.62 (3H, J = 6.5 Hz, H-13).
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