Two New Flavonol Glycosides from *Otostegia limbata* Benth.

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A BuOH-soluble part of the methanolic extract from the roots of *Otostegia limbata* yielded two new flavonol glycosides; kaempferol 3-*O*-[β-D-glucopyranosyl-(1→2)-{β-D-glucopyranosyl-(1→3)}-{β-D-glucopyranosyl-(1→4)}-α-L-rhamnopyranoside]-7-*O*-[α-L-rhamnopyranoside] (1) and kaempferol 3-*O*-[β-D-glucopyranosyl-(1→4)-β-D-6‴″[4-hydroxy (*E*)-cinnamoyl]glucopyranosyl-(1→3)-{β-D-glucopyranosyl-(1→2)}-α-L-rhamnopyranoside]-7-*O*-[α-L-rhamnopyranoside] (2). The structures of these compounds were elucidated by spectroscopic and chemical means. To the best of our knowledge, these are the largest flavonoids derivatives described so far from the genus *Otostegia*.

Key words *Otostegia limbata*; Lamiaceae; flavonol glycoside; chemotaxonomy

The genus *Otostegia* (Lamiaceae) consists of about 33 species, mainly occurring in the Mediterranean region.[1] In Pakistan, only three species have been found namely *Otostegia aucheri*, *O. persica* and *O. limbata* (Benth.). The species *O. limbata* is vernacularly called "spin aghzai" or "chiti booti."[2] It is used in folk medicine for the treatment of wound healing in beast and man, children gum diseases and for ophthalmia in man.[3] Moreover, the constituents of different species of the genus *Otostegia* have shown antiulcer, antispasmodic, antidepressant, anxiolytic, sedative and antioxidant activities.[4,5] Previously, we have isolated clerodane diterpenoids from the plant *Otostegia limbata*.[6,7] Here we report the isolation and structure elucidation of two new flavonol glycosides **1** and **2** (Fig. 1) from the same plant.

Results and Discussion

Compound **1** was isolated as a pale yellow, gummy solid. The high resolution-electrospray ionization-quadrupole-time of flight-mass spectrum (HR-ESI-Q-TOF-MS) (+ve) showed quasi-molecular ion peaks at *m/z* 1087.3814 $[M+Na]^+$ and 1065.3960 $[M+H]^+$ indicating a molecular formula of $C_{45}H_{60}O_{29}$. This formula was further augmented by UV, IR, and NMR data. The characteristic absorption bands in IR spectrum of compound **1** were found at 3421 (O–H), 2925 (C–H), 1654 (α,β-unsaturated C=O), 1602 (C=C), 1493 (C=O, aromatic), and 1108—1018 cm^{-1} (glycosidic nature). When recorded in methanol, the UV spectrum of **1** showed the characteristic maxima at 265 and 355 nm of kaempferol

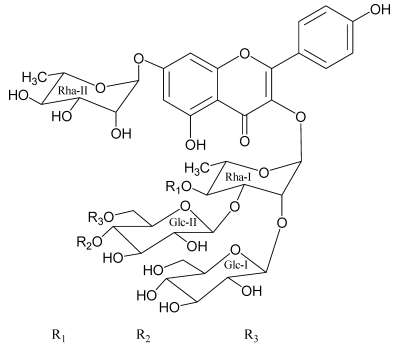

Fig. 1. Structures of Compounds **1** and **2**

3-*O*-glycosides or 3,7-*O*-glycosides.[8] By the addition of standard shift reagents in methanol spectrum it was found that the hydroxyl groups at C-3 and C-7 of kaempferol were substituted while the C-5 and C-4′ hydroxyl groups were free.[9,10] The collision-induced dissociation (CID)-MS/MS of $[M+Na]^+$ peak at *m/z* 1087.3 yielded characteristic product ions at *m/z* 941 (loss of rhamnosyl) and 655 (loss of flavonol+rhamnosyl). The first fragmentation of the $[M+Na]^+$ ion was due to the breakdown of the *O*-glycosidic bond at C-7 position of flavonoid showing the presence of one rhamnosyl moiety at this position.[11] The ^{1}H- and ^{13}C-NMR spectra revealed that compound **1** was a pentaglycoside with five anomeric protons' signals at δ_H 5.61 (br d, J=1.9 Hz), 5.55 (br d, J=2.1 Hz), 4.65 (d, J=8.2 Hz), 4.62 (d, J=8.0 Hz), 4.54 (d, J=7.8 Hz) and the anomeric carbon resonances at δ_C 102.6, 99.8, 104.2, 105.3, and 106.2, respectively. In proton NMR spectrum, two protons at δ_H 6.46 and 6.72 (each d, J=1.9 Hz) respectively correlated to δ_C 100.7 and 95.6 *via* heteronuclear single quantum coherence (HSQC) experiment which are characteristic for 6- and 8-position in ring A of the flavonoids.[12] The aglycone was identified as kaempferol from the chemical shift and coupling constant data of the aromatic protons together with their corresponding ^{13}C-NMR chemical shifts (Table 1).[13] Compound **1** contained three glucopyranosyl (Glc) and two rhamnopyranosyl (Rha) moieties indicated by coupling constants of the anomeric protons and the corresponding carbon resonances together with two secondary methyl signals at δ_H 1.04 and 1.24 (each d, J=6.1 Hz). The identification of the two rhamnopyranosyl and three glucopyranosyl units was realized from the combined analysis of ^{1}H–^{1}H-correlation spectroscopy (COSY), HMBC and 1D-total correlation spectroscopy (TOCSY) spectra. However, the selective 1D-TOCSY on the anomeric proton of Glc I could not give information for its isolated spin cage because Glc I H-1 and Rha I H-2 had the same chemical shift, therefore, the Glc I H-3 and Glc I H-6 were selected for the identification of spin system of Glc I. The anomeric configurations were assigned as β for the glucopyranosyl and α for the rhamnopyranosyl groups from their coupling constants. The ^{13}C-NMR spectrum revealed that the C-3 and C-7 position of kaempferol were glycosylated as indicated by the upfield shift of these positions to δ_C 136.6 and 163.6, respectively.[14] The sugar se-

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Table 1. ^{1}H- and ^{13}C-NMR Data for Compounds **1** and **2** in CD_3OD[a)]

Position	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
Aglycone				
2	159.9	—	158.8	—
3	136.6	—	135.4	—
4	179.7	—	179.2	—
5	162.9	—	162.9	—
6	100.7	6.46 d (1.9)	100.6	6.36 d (1.8)
7	163.6	—	163.5	—
8	95.6	6.72 d (1.9)	95.3	6.52 br s
9	158.1	—	157.7	—
10	107.5	—	107.5	—
1′	122.2	—	122.3	—
2′/6′	132.0	7.79 d (8.7)	132.0	7.75 d (8.6)
3′/5′	117.3	6.94 d (8.7)	117.0	6.91 d (8.7)
4′	161.8	—	162.6	—
Rhamnose-I				
1″	102.6	5.61 br d (1.9)	101.2	5.74 br s
2	80.7	4.55 br d (3.8)	80.6	4.43 br d (3.6)
3	81.5	4.15 dd (3.1, 9.7)	80.9	4.05 dd (3.0, 9.9)
4	78.3	3.75 t (9.7)	72.7	3.26 t (8.6)
5	70.83	3.58 pseudo dq/m (6.1, 9.7)	70.87	3.22 m
6	18.0	1.04 d (6.1)	17.95	0.95 d (6.2)
Rhamnose-II				
1‴	99.8	5.55 br d (2.1)	99.9	5.54 br d (2.3)
2	71.6	4.01 br d (4.1)	71.7	4.02 dd (1.7, 3.3)
3	72.0	3.83 br d (10.2)	72.1	3.82 dd (3.4, 9.4)
4	73.5	3.47 t (9.4)	73.6	3.48 t (9.5)
5	71.10	3.58 pseudo dq/m (6.1, 9.7)	71.1	3.64 m
6	18.1	1.24 d (6.1)	18.1	1.27 d (6.1)
Glucose-I				
1⁗	106.2	4.54 d (7.8)	105.9	4.54 d (7.9)
2	75.3	3.18 t (8.4)	75.4	3.20 dd (7.9, 9.1)
3	78.5	3.30 t (9.0)	77.9	3.37 t (9.9)
4	71.13	3.37 t (9.0)	71.8	3.29
5	77.88	3.20 m	77.9	3.27 m
6	62.4	3.64 dd (5.3, 11.7)	62.8	3.68 dd (4.8, 12.1)
		3.67 dd (2.3, 12.2)		3.78 br d (11.8)
Glucose-II				
1⁗′	105.3	4.62 d (8.0)	105.4	4.71 d (7.8)
2	75.4	3.28 t (8.0)	75.3	3.30 dd (7.87, 7.96)
3	77.79	3.20 m	78.2	3.41 t (9.0)
4	71.3	3.32 t (8.7)	78.4	3.73 t (9.3)
5	78.0	3.41 m	76.1	3.72 ddd (2.7, 6.9, 9.8)
6	62.3	3.78 br d (12.1)	65.2	4.38 br d (10.8)
		3.91 br d (11.6)		4.60 dd (8.2, 11.5)
Glucose-III				
1⁗″	104.2	4.65 d (8.2)	104.0	4.66 d (8.0)
2	75.6	3.11 t (8.27)	75.8	3.04 t (8.6)
3	77.75	3.22 m	78.7	3.24 t (9.0)
4	71.7	3.26 dd (8.0, 9.4)	71.4	3.27 t (9.0)
5	77.78	3.20 m	77.6	3.20 m
6	62.8	3.62 dd (5.6, 12.24)	62.9	3.59 dd (4.0, 12.0)
		3.81 br d (12.44)		3.79 br d (11.4)
4-Hydroxy (*E*)-cinnamoyl moiety				
1⁗‴	—	—	168.9	—
2	—	—	114.9	6.18 d (15.9)
3	—	—	146.6	7.48 d (15.9)
4			126.5	—
5/9	—	—	130.4	7.00 d (8.5)
6/8	—	—	116.4	6.29 d (8.5)
7	—	—	160.9	—

a) Chemical shifts (δ) in ppm relative to respective residual non-deuterated solvent peaks (δ_H 3.31 and δ_C 49.0 from CD_3OD); coupling constants (J in Hz) are given in parantheses.

quence was determined on the basis of both 1D- and 2D-NMR, particularly HMBC and rotating frame nuclear Overhauser effect spectroscopy (ROESY) experiments. The positions of attachment of glycoside moieties with the aglycone were confirmed by the ^{1}H–^{13}C long-range HMBC correlations of δ_H 5.55 (Rha II H-1) to δ_C 163.6 (aglycone-C-7) and δ_H 5.61 (Rha I H-1) to δ_C 136.6 (aglycone-C-3). The point of attachment of Rha II was further confirmed through ROEs observed between H-1‴ (δ_H 5.55) and H-6/8 (δ_H 6.46/6.72) indicating a 7-*O*-linkage in conjunction with COSY correlations. The chemical shift values for the carbon resonances assigned to the Rha II unit indicated that it was a terminal rhamnose attached directly to the aglycone.[15] However, C-2 (δ_C 80.7), C-3 (δ_C 81.5), and C-4 (δ_C 78.3) resonances of the Rha I moiety were shifted downfield due to glycosylation and provided the sites of attachment of Rha I to Glc I, Glc II, and Glc III at these positions, respectively. Additional long-range correlations were noted between Glc I H-1 (δ_H 4.54) and Rha I C-2 (δ_C 80.7), between Glc II H-1 (δ_H 4.62) and Rha I C-3 (δ_C 81.5), and between Glc III H-1 (δ_H 4.65) and Rha I C-4 (δ_C 78.3). There were also noted the inverse correlations between Rha I H-2 (δ_H 4.55) and Glc I C-1 (δ_C 106.2), between Rha I H-3 (δ_H 4.15) and Glc II C-1 (δ_C 105.3), and between Rha I H-4 (δ_H 3.75) and Glc III C-1 (δ_C 104.2). These observations supported a tetraglycosidic oligosaccharide structure at C-3 of kaempferol having an inner rhamnose and three terminal glucose moieties. The interglycosidic linkage was confirmed in the ROESY spectrum by the observation of ROEs between the anomeric protons and the glycosidic protons of glycosylated positions. Thus ROEs were observed between Glc II H-1 and Rha I H-3, and between Glc III H-1 and Rha I H-4. However, ROEs could not be observed between Glc I H-1 and Rha I H-2 because they had almost the same chemical shift in ^{1}H-NMR (Table 1). The ^{1}H- and ^{13}C-NMR spectra of **1** were in full agreement with the proposed structure. Thus, on the basis of above spectroscopic techniques the structure of **1** was established as kaempferol 3-*O*-[β-D-glucopyranosyl-(1→2)-{β-D-glucopyranosyl-(1→3)}-{β-D-glucopyranosyl-(1→4)}-α-L-rhamnopyranoside]-7-*O*-[α-L-rhamnopyranoside] which is unprecedented.

Compound **2** was isolated as a pale yellow, gummy solid. The HR-ESI-Q-TOF-MS (+ve) showed quasi-molecular ion peaks at *m/z* 1233.3929 $[M+Na]^+$ and 1211.3979 $[M+H]^+$ indicating a molecular formula of $C_{54}H_{66}O_{31}$. By the MS/MS of $[M+Na]^+$ peak at *m/z* 1233.3 characteristic product ion peaks were obtained at *m/z* 1087 (loss of rhamnosyl), 801 (loss of flavone+rhamnosyl), 639 (loss of flavone+rhamnosyl+glucosyl), 287 $[kaempferol+H]^+$, and 147.0405 (4-hydroxy (*E*)-cinnamoyl). The UV spectral data of **2** when recorded in methanol showed similarity to the characteristic maxima of kaempferol 3-*O*-glycosides or 3,7-*O*-glycosides at 268 and 316 nm. The maximum at 316 nm corresponded to the 4-hydroxy (*E*)-cinnamoyl residue.[16] The ^{1}H- and ^{13}C-NMR spectra of **2** were very similar to those of **1** (Table 1), except for the difference in interglycosidic linkages at C-3 of the two compounds as well as the additional signals due to 4-hydroxy (*E*)-cinnamoyl moiety in compound **2**. The third glucopyranosyl unit (Glc III), in compound **2**, was linked to the C-4⁗ of Glc II unit instead of Rha I C-4″ as in compound **1**. In compound **2**, the C-4⁗ signal of Glc II unit shifted downfield at δ_C 78.4 due to glycosylation. The point of attachment of Glc III unit with C-4⁗ of Glc II was also confirmed by HMBC interactions of H-4⁗ (δ_H 3.73) of Glc II with C-1 (δ_C 104.0) of Glc III and the inverse correlations of H-1 (δ_H 4.66) of Glc III with C-4 of Glc II (δ_C 78.4) (Fig. 2). These observations were supported by ROESY spectra. Thus the ROEs were observed between H-1⁗ (δ_H 4.66) of Glc III and the H-4⁗ (δ_H 3.73) of Glc II. The signals due to 4-hydroxy (*E*)-cinnamoyl moiety were observed at δ_H 6.18 and 7.48 (each d, J=15.9 Hz), and δ_H 7.00 and 6.29 (each d, J=8.5 Hz)[17] with corresponding carbon resonances at δ_C 114.9, 146.6, 130.4 and 116.4, plus a carbonyl signal at δ_C 168.9 and two quaternary signals at δ_C 126.5 and 160.9. As the H-6⁗ signals of Glc II were deshielded at δ_H 4.38 (br d, J=10.8 Hz) and 4.60 (dd, J=8.2, 11.5 Hz) compared with those of **1**, C-6⁗ had to be the point of attachment of 4-hydroxy (*E*)-cinnamoyl moiety. Accordingly, the C-6⁗ resonance was shifted downfield at δ_C 65.2 while the C-5⁗ resonance was shifted upfield at δ_C 76.1 due to β-effect.[18] An HMBC experiment confirmed the site of attachment of 4-hydroxy (*E*)-cinnamoyl moiety, showing a ^{1}H–^{13}C long-range correlation between the carbonyl resonance (δ_C 168.9, C-1⁗⁗) of 4-hydroxy (*E*)-cinnamoyl moiety and the H-6⁗ (δ_H 4.38, and 4.60) of Glc II unit. The ^{1}H- and ^{13}C-NMR spectra of **2** were in full agreement with the proposed structure. On the basis of these results, the structure of compound **2** was established as kaempferol 3-*O*-[β-D-glucopyranosyl-(1→4)-β-D-6⁗[4-hydroxy (*E*)-cinnamoyl]glucopyranosyl-(1→3)-{β-D-glucopyranosyl-(1→2)}-α-L-rhamnopyranoside]-7-*O*-[α-L-rhamnopyranoside] which is unprecedented.

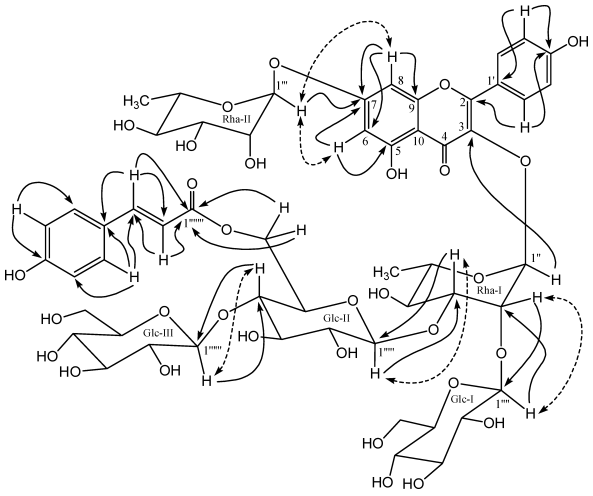

Fig. 2. Important HMBC (⟶) and ROESY (◀⋯▶) Correlations of **2**

The absolute configuration of the sugar units was assigned after hydrolysis of crude fraction (no. 12) with HCl. The hydrolyzed sugars were converted into acetylated thiazolidine derivatives, and the GC retention time of each sugar was compared with those of authentic sugar samples prepared in the same manner.

Chemotaxonomic Significance Flavonoid 4-hydroxy (*E*)-cinnamoyl glucosides are commonly found in some genera of the Lamiaceae and they are generally considered as valuable markers in this family for a chemotaxonomic point of view.[19] The isolation of compounds **1** and **2** is of chemotaxonomic interest as kaempferol pentaglycosides are re-

ported here for the first time from the genus *Otostegia*. These compounds may be useful for the authentication and standardization of extracts of medicinal interest.

Experimental

General Experimental Procedures Optical rotations were measured on a JASCO DIP-360 digital polarimeter using a 5 cm cell tube. UV spectra were obtained on a Hitachi U-3200 spectrophotometer in MeOH solutions and presented as λ_{max} nm ($\log \varepsilon$). IR (KBr) spectra were recorded using a Bruker Vector 22 FTIR spectrometer. 1D- and 2D-NMR spectra were run on a Bruker AV (Avance) 600 MHz instrument. Chemical shifts values were reported in δ (ppm) with reference to the residual nondeuterated solvent peaks (δ_H 3.31 and δ_C 49.0 from CD_3OD) and coupling constants (J) were measured in Hz. ESI-MS were recorded on QStar XL Hybrid LC/MS/MS spectrometer (Applied Biosystems). For the collision induced dissociation (CID) mass spectra, the collision energy was kept at 60 or 70 eV with nitrogen as collision gas. Gas Chromatography was carried out on GC-17A (Shimadzu) instrument, equipped with Optima-5-Accent column (0.25 μm, 30 m×0.25 mm i.d., Macherey-Nagel) and FID detector, under the following conditions: N_2: carrier gas; column initial temp.: 50 °C; temp. increased: 5 °C/min; column final temp.: 240 °C; inj. port temp.: 240 °C. Recycling Preparative HPLC was carried out on LC-908W (Japan Analytical Industries Co., Ltd.) equipped with J'sphere ODS-M80 column (S-4 μm, 8 nm, 20×250 mm i.d., YMC) or Hibar LiChrosorb RP-18 column (7 μm, 25×250 mm i.d., Merck) using a JAI RI-5 refractive index detector and a JAI UV-310 detector. MPLC was run on Eyela VSP-3050 attached with a column (25×200 mm i.d., Eyela) filled with polygroprep C-18 (25—40 μm, 10 nm, Macherey-Nagel). Reverse phase column chromatography was carried out on Sephadex LH-20 (25—100 μm, Sigma-Aldrich). TLC was performed on pre-coated RP-18 F_{254s} plates (Merck) and the spots were observed first under UV (254/366 nm), and then stained with cerium(IV) sulphate spray reagent in 10% H_2SO_4 solution and heated until coloration developed.

Plant Material The root parts of *Otostegia limbata* (Lamiaceae) were collected in July 2002 from Abbottabad, Pakistan, and identified by Dr. Manzoor Ahmad (taxonomist) at the Department of Botany, Post-Graduate College, Abbottabad, Pakistan. A voucher specimen (No. 6872) has been deposited to the herbarium of the same department.

Extraction and Purification The air-dried roots of *Otostegia limbata* (30 kg) were extracted repeatedly with methanol (50 l×3), at room temperature. The solvent was evaporated under reduced pressure to give a dark residue (450 g), which was partitioned between *n*-hexane (50 g), $CHCl_3$ (80 g), EtOAc (120 g), *n*-BuOH (170 g) and water (30 g). The *n*-BuOH extract was subjected to LH-20 column and eluted with H_2O (2 l), followed by increasing concentrations of MeOH in H_2O (0→100 MeOH/H_2O in steps of 25% of MeOH, each 2 l) as eluent, yielded five fractions. The fraction eluted with MeOH : H_2O (1 : 1) was then applied to repeated Sephadex LH-20 column with increasing concentrations of MeOH in H_2O (0→100% MeOH) to remove the saponin part. The flavonoid part was then subjected to MPLC (Polygroprep C-18 column) and eluted with H_2O followed by increasing concentrations of MeOH in H_2O (0→100% MeOH) to give fourteen fractions. Fraction no. 12 was then subjected to further purification on preparative recycling HPLC (ODS-M80 column, 1 : 1 MeOH/H_2O). As a result two sub-fractions 12.1 and 12.2 were obtained. Sub-fraction 12.1 was subjected to repeated HPLC (Hibar LiChrosorb RP-18 column, 1 : 1 MeOH/H_2O) and yielded compound **1** (10 mg, t_R: 38 min, flow rate: 3 ml/min) while sub-fraction 12.2 when subjected to repeated HPLC (Hibar LiChrosorb RP-18 column, 1 : 1 MeOH/H_2O) afforded compound **2** (6 mg, t_R: 17 min, flow rate: 9 ml/min). The elution of these compounds was monitored through UV and RI detectors, simultaneously.

Compound **1**: Pale yellow gummy solid. ^{1}H-NMR (CD_3OD, 600 MHz), see Table 1. ^{13}C-NMR (CD_3OD, 150 MHz), see Table 1. IR ν_{max} (KBr) cm^{-1}: 3421, 2925, 1654, 1602, 1493, 1455, 1373, 1283, 1210, 1175, 1108—1018, 962, 894, 838, 811. UV λ_{max} (MeOH) nm ($\log \varepsilon$): 230.7 (4.25), 265.5 (4.42), 289.8 (4.11), 339.8 (4.24), 355.8 (4.22); λ_{max} ($AlCl_3$) nm: 236, 275, 351.5; λ_{max} ($AlCl_3$–HCl) nm: 234.6, 274, 340, 389; λ_{max} (NaOAc) nm: 266, 339, 346. ESI-Q-TOF-MS/MS (positive mode) *m/z* (%): 1087 $[M+Na]^+$ (7), 941 (14), 655 (100), 475 (72), 313 (92), 295 (37), 203 (70), 85 (27), 51 (48). HR-ESI-Q-TOF-MS (+ve) *m/z*: 1087.3814 $[M+Na]^+$ (Calcd for $C_{45}H_{60}O_{29}$+Na: 1087.3090), 1065.3960 $[M+H]^+$ (Calcd for $C_{45}H_{60}O_{29}$+H: 1065.3279). $[\alpha]_D^{29}$ −125.2° (c=0.212, MeOH).

Compound **2**: Pale yellow gummy solid. ^{1}H-NMR (CD_3OD, 600 MHz), see Table 1. ^{13}C-NMR (CD_3OD, 150 MHz), see Table 1. IR ν_{max} (KBr) cm^{-1}: 3408, 2924, 2856, 1691, 1656, 1605, 1514, 1493, 1450, 1353, 1264, 1207, 1170, 1110—1027, 966, 829. UV λ_{max} (MeOH) nm ($\log \varepsilon$): 226.7 (4.25), 268 (4.31), 316 (4.34); λ_{max} ($AlCl_3$) nm: 232.7, 277, 304, 389.6; λ_{max} ($AlCl_3$–HCl) nm: 234, 276, 302, 319.8, 389.4; λ_{max} (NaOAc) nm: 266, 311, 390. ESI-Q-TOF-MS/MS (positive mode) *m/z* (%): 1233 $[M+Na]^+$ (92), 1087 (42), 801 (100), 639 (11), 625 (6), 475 (42), 349 (58), 313 (29), 295 (23), 287 (11), 147 (58), 119 (11). HR-ESI-Q-TOF-MS (+ve) *m/z*: 1233.3929 $[M+Na]^+$ (Calcd for $C_{54}H_{66}O_{31}$+Na: 1233.3456), 1211.3979 $[M+H]^+$ (Calcd for $C_{54}H_{66}O_{31}$+H: 1211.3645). $[\alpha]_D^{29}$ +18.3° (c=0.048, MeOH).

Acid Hydrolysis of Crude Extract A solution of semi-purified fraction no. 12 (10 mg) was dissolved in 10% aqueous HCl and refluxed for 3 h. On cooling, the reaction mixture was extracted with EtOAc. After separating the organic layer, the aqueous phase was neutralized with Na_2CO_3 and concentrated. The sugars were identified as glucose and rhamnose by co-TLC with authentic samples by using the standard method.[20]

Determination of Absolute Configuration The concentrated residue of the hydrolyzed sugars in pyridine (0.04 mol/l) and L-cysteine ethyl ester hydrochloride (0.06 mol/l) were mixed and the solution was warmed at 60 °C for 1 h. Acetic anhydride (150 μl) was then added and the mixture was warmed at 90 °C for another 1 h. After evaporation of pyridine and acetic anhydride *in vacuo*, each residue was dissolved in acetone (350 μl) and the solution (1 μl) was subjected to GC.[21] The peaks for peracetylated thiazolidine derivatives with retention time of 31.68 and 44.57 min were observed for the samples which were, respectively, identical to the derivatives of authentic D-glucose and L-rhamnose prepared in the same manner.

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