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Full Length Research Paper

Structure elucidation of three new diterpene glycosides from *Stevia rebaudiana*

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Three new minor diterpene glycosides were isolated from the commercial extract of the leaves of *Stevia rebaudiana* and their structures were identified as 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-15-en-19-oic acid β-D-glucopyranosyl ester (1),13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-15-en-19-oic acid β-D-glucopyranosyl ester (2) and 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl]- β-D-glucopyranosyl)oxy] 17-hydroxy-*ent*-kaur-15-en-19-oic acid β-D-glucopyranosyl ester based on extensive spectroscopic data and chemical studies.

Key words: *Stevia rebaudiana*, compositae, asteraceae, diterpene glycosides, structural characterization, spectral data, acid and enzymatic hydrolysis.

INTRODUCTION

Stevia rebaudiana (Bertoni) Bertoni is a perennial shrub of the Asteraceae (Compositae) family native to certain regions of South America (Paraguay and Brazil). It is often referred to as “the sweet Herb of Paraguay” (Mosettig et al., 1963; Mosettig et al., 1955) but now is grown commercially in a number of countries, particularly in Japan, Taiwan, Korea, Thailand and Indonesia. The major constituents in the leaves of *S. rebaudiana* are the potently sweet diterpenoid glycosides stevioside, rebaudiosides A and D and dulcoside A. These compounds are all glycosides of the diterpene *ent*-13-hydroxykaur-16-en-19-oic acid known as steviol (Brandle et al., 1998). As a part of our continuing research to discover natural sweeteners, we have obtained commercial extracts of the leaves of *S. rebaudiana* from various suppliers around the world. Recently, we have reported several novel diterpene glycosides from the commercial extract of *S. rebaudiana* obtained from pure circle, Malaysia (Chaturvedula et al., 2011a-e).

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Abbreviations: IR, Infrared; **FT-IR,** fourier transform infrared; **UATR,** universal attenuated total reflectance; **NMR,** nuclear magnetic resonance; **MS,** mass spectrometer; **Q-Tof,** quadrupole time-of-flight; **HPLC,** high performance liquid chromatography; **TFA,** trifluoroacetic acid; **UV,** ultraviolet; **HPLC,** high performance liquid chromatography mass spectrometer; **HRMS,** high resolution mass spectral data.

EXPERIMENTAL

General experimental procedures

Melting points were reported using SRS Optimelt, MPA 100 instrument is uncorrected. Optical rotation was performed using Rudolph Autopol V at 25°C and Infrared (IR) spectral data was acquired using a Perkin Elmer 400 Fourier Transform Infrared (FT-IR) spectrometer with Universal attenuated total reflectance (UATR) polarization accessory. Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker Advance DRX 500 MHz with a 5 mm inverse detection probe and Varian unity plus 600 MHz instruments was used as standard pulse sequences. The spectra were referenced to the residual solvent signal (δ_H 3.30, δ_C 49.0 for CD_3OD), chemical shifts were given in δ (ppm) and coupling constants are reported in Hz. mass spectrometer (MS) and MS/MS data were generated with a Waters Premier Quadrupole Time-of-Flight (Q-Tof) mass spectrometer equipped with an electrospray ionization source operated in the positive-ion mode and Thermo Fisher Discovery OrbiTrap in the positive mode electrospray. Samples were diluted with water: acetonitrile (1:1) containing 0.1% formic acid was introduced via infusion using the onboard syringe

pump. Preparative high performance liquid chromatography (HPLC) was performed on an Agilent 1100 system using a Phenomenex Prodigy ODS (3) column (250 × 21.2 mm and 5 µm). Semi-preparative HPLC was carried out with waters 600 E multisolvent delivery system using a Phenomenex Synergi Hydro RP column (250 × 10 mm and 4 µm) and analytical HPLC was performed using Phenomenex Luna C_{18} (150 × 4.6 mm and 5 µm) column.

Plant material

A commercial sample of Stevia extract (KD 260112) consisting of a mixture of diterpenoid glycosides (73% Rebaudioside A) from the leaves of *S. rebaudiana* was obtained from Daepyung Company Limited, Korea. In the absence of the plant material to identify botanically, the authenticity of the crude extract was confirmed by performing its retention time (t_R) comparison with the standard compounds of known steviol glycosides namely rebaudioside A to D, stevioside and dulcoside A using the preparative HPLC method as reported earlier (Clos et al., 2008). A voucher specimen is deposited at The Coca-Cola Company, No. VSPC-3166-152.

Extraction and isolation

Preliminary separation of the crude extract (2.5 g) from the leaves of *S. rebaudiana* consisting of a mixture of diterpenoid glycosides including 73% Rebaudioside A was carried out using a preparative HPLC method employing a mobile phase of water/acetonitrile (B) gradient (25% B for 8.5 min, 25 to 29% B over 1.5 min, 29 to 30% B over 5.5 min, 30 to 34% B over 2.0 min, 34% B for 6 min, 34 to 52% B over 2.0 min, 52% B for 3.0 min, 52 to 70% B over 1.0 min, 70% B for 5.5 min) at a flow rate of 20 ml/min. All the baseline materials eluting between 7.0 to 8.0 min, 19.0 to 20.0 min and 20.0 to 21.0 min were collected over several injections and dried in a rotary evaporator under reduced pressure as crude fractions 1, 2 and 3, respectively. Final purification of crude fractions 1 to 3 were then performed by injecting each of them over several runs using semi-preparative HPLC by employing the above gradient method at a flow rate of 5 ml/min furnished 2 (1.8 mg, *t*R 7.56 min), 1 (2.3 mg, *t*R 19.70 min) and 3 (1.8 mg, *t*R 20.46 min), respectively.

13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]ent-kaur-15-en-19-oic acid β-D-glucopyranosyl ester (1)

White powder. $[\alpha]_D^{25}$ +2.02 (*c* 0.1, H_2O); IR (KBr) ν_{max}: 3348, 1725, 1020, 965 cm^{-1}; high resolution mass spectral data (HRMS) *m/z* 827.3661 (Calcd for $C_{38}H_{60}O_{18}Na$: 827.3677); ^1H NMR (CD_3OD, δ ppm) and ^{13}C NMR (CD_3OD, δ ppm) spectroscopic data Tables 1 and 2.

13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-15-en-19-oic acid β-D-glucopyranosyl ester (2)

White powder. $[\alpha]_D^{25}$ +4.86 (*c* 0.1, H_2O); IR (KBr) ν_{max}: 3345, 1723, 1025, 958 cm^{-1}; HRMS *m/z* 989.4188 (Calcd for $C_{44}H_{70}O_{23}Na$: 989.4206); ^1H NMR (CD_3OD, δ ppm) and ^{13}C NMR (CD_3OD, δ ppm) spectroscopic data Tables 1 and 2.

13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl]-β-D-glucopyranosyl)oxy]-17-hydroxy-ent-kaur-15-en-19-oic acid β-D-glucopyranosyl ester (3)

White powder. $[\alpha]_D^{25}$ +2.46 (*c* 0.1, H_2O); IR (KBr) ν_{max}: 3340, 1727, 1028, 956 cm^{-1}; HRMS *m/z* 1005.4132 (Calcd for $C_{44}H_{70}O_{24}Na$: 1005.4155); ^1H NMR (CD_3OD, δ ppm) and ^{13}C NMR (CD_3OD, δ ppm) spectroscopic data Tables 1 and 2.

General procedure for acid hydrolysis and determination of sugar configuration in 1 to 3

Each compound (500 µg) was hydrolyzed with 0.5 M HCl (0.5 ml) for 1.5 h. After cooling, the mixture was passed through an Amberlite IRA400 column and the eluate was lyophilized. The residue was dissolved in pyridine (0.25 ml) and heated with L-cysteine methyl ester HCl (2.5 mg) at 60°C for 1.5 h, and then *O*-tolyl isothiocyanate (12.5 ul) was added to the mixture and heated at 60°C for an additional 1.5 h. The reaction mixture was analyzed by HPLC column Phenomenex Luna C18, 150 × 4.6 mm (5 u); 25% acetonitrile-0.2% Trifluoroacetic Acid (TFA) water, 1 ml/min; ultraviolet (UV) detection at 250 nm. The sugar was identified as D-glucose in each experiment (*t*R, 12.15 to 12.28 min) [authentic samples, D-glucose (*t*R, 12.35) and L-glucose (*t*R, 11.12 min)] (Tanaka et al., 2007).

Enzymatic hydrolysis of 1 to 3

Each compound (250 µg) was dissolved in 2.5 ml of 0.1 M sodium acetate buffer, pH 4.5 and crude pectinase from *Aspergillus niger* (50 ul, Sigma-Aldrich, P2736) was added. The mixture was stirred at 50°C for 48 h. The product precipitated out during the reaction and was filtered and then crystallized. The resulting product obtained from the hydrolysis of 1 and 2 was identified as *ent*-13-hydroxykaur-15-en-19-oic acid (4) whereas from 3 was identified as *ent*-13, 17-dihydroxykaur-15-en-19-oic acid by comparison of their ^1H NMR spectral data (Ohtani et al., 1992). This is the first report of the isolation of the three diterpene glycosides 1 to 3 in nature from *S. rebaudiana* whereas these were reported earlier as degradation products (Clos et al., 2008; DuBois et al., 2009; Lee, 2009). Also, their complete spectral data were not characterized previously and this is the first report of their complete ^1H and ^{13}C NMR spectral assignments that were made on the basis of spectral (COSY, HSQC, HMBC, HRMS and MS/MS) and chemical studies. The discovery of these compounds is an important addition in expanding our understanding of the diversity of the diterpenoid glycosides of *ent*-13-hydroxykaur-15-en-19-oic acid and *ent*-13, 17-dihydroxykaur-15-en-19-oic acid skeletons from *S. rebaudiana* in nature.

RESULTS AND DISCUSSION

Purification of the commercial extract from the leaves of *S. rebaudiana* obtained from Daepyung Company Limited, Korea resulted in the isolation of three additional new diterpenoid glycosides 1 to 3 and the known steviol glycosides, rebaudiosides A to F, rebaudioside G, rubusoside, stevioside and dulcoside A (Figure 1). The structures of all the known compounds were identified by comparison of their retention times with authentic standards using the high performance liquid chromatography- mass spectrometer (HPLC-MS) method reported earlier (Clos et al., 2008) and the spectral data reported in the literature (Avent et al., 1990; Kobayashi et al., 1977; Kohda et al., 1976; Ohta et al., 2010; Starratt et al., 2002; Sakamoto et al., 1977a, 1977b). In this article, we

Table 1. ^{1}H NMR spectral data for 1 to 3 isolated from *Stevia rebaudiana* in CD_3OD [a-b].

Position	**1** (δ_H)	**2** (δ_H)	**3** (δ_H)
1	0.86 and 1.85 m	0.85 and 1.85 m	0.86 and 1.86 m
2	1.40 and 1.94 m	1.42 and 1.96 m	1.43 and 1.98 m
3	1.06 and 2.14 d (12.2)	1.03 m and 2.12 d (12.2)	1.06 and 2.14 d (12.6)
5	1.10 m	1.10 d and 12.0	1.12 d and 12.0
6	1.81 and 1.99 m	1.76 and 1.96 m	1.83 and 2.00 m
7	1.48 and 1.61 m	1.46 and 1.60 m	1.55 and 1.65 m
9	0.85 m	0.86 m	0.91 m
11	1.51 and 1.69 m	1.50 and 1.67 m	1.63 and 1.72 m
12	1.64 and 1.69 m	1.64 and1.69 m	1.69 and 1.81 m
14	1.67 m and 2.25 d (10.2)	1.67 m and 2.27 d (10.0)	1.73 m and 2.32 d (10.4)
15	5.10 s	5.12 s	5.36 s
17	1.71 s	1.71 s	4.13 dd (1.5, 14.2) 4.31 dd (1.5, 14.0)
18	1.20 s	1.21 s	1.23 s
20	1.00 s	0.98 s	0.97 s
1′	5.37 d (8.2)	5.39 d (8.7)	5.39 d (8.4)
2′	3.35 m	3.36 m	3.36 m
3′	3.43 m	3.46 m	3.46 m
4′	3.36 m	3.32 m	3.34 m
5′	3.36 m	3.38 m	3.39 m
6′	3.68 and 3.82 m	3.68 and 3.82 m	3.68 and 3.82 m
1″	4.63 d (8.5)	4.62 d (8.4)	4.67 d (7.6)
2″	3.68 m	3.65 m	3.68 m
3″	3.54 m	3.75 m	3.78 m
4″	3.26 m	3.34 m	3.38 m
5″	3.24 m	3.32 m	3.29 m
6″	3.68 and 3.82 m	3.65 and 3.83 m	3.65 and 3.83 m
1‴	4.55 d (7.8)	4.63 d (7.6)	4.62 d (7.6)
2‴	3.29 m	3.28 m	3.26 m
3‴	3.36 m	3.30 m	3.32 m
4‴	3.29 m	3.33 m	3.36 m
5‴	3.25 m	3.44 m	3.44 m
6‴	3.68 and 3.82 m	3.78 and 4.10 m	3.78 and 4.10 m
1⁗		4.78 d (7.4)	4.80 d (7.8)
2⁗		3.26 m	3.24 m
3⁗		3.44 m	3.49 m
4⁗		3.29 m	3.28 m
5⁗		3.32 m	3.34 m
6⁗		3.58 and 3.84 m	3.61 and 3.86 m

[a] assignments made on the basis of COSY, HSQC and HMBC correlations; [b] Coupling constants given in parentheses are in Hz.

we describe the isolation and structure elucidation of three new minor diterpene glycosides 1 to 3 based on the basis of 1D and 2D NMR spectral assignments (^{1}H and ^{13}C NMR, COSY, HSQC and HMBC) similar to the methods adopted earlier in elucidation of the structures of clerodane diterpenoids (Yamale et al., 2009) as well as enzymatic and acid hydrolysis studies. The structure elucidation of the novel compounds was further supported by HRMS and MS/MS data. Compound 1 was obtained as white powder and its molecular formula was assigned as $C_{38}H_{60}O_{18}$ from its HRMS data which showed $(M+Na)^+$ ion at *m/z* 827.3661; this was supported by the ^{13}C NMR spectral data. The ^{1}H NMR spectrum of 1 showed the presence of three methyl singlets at δ 1.00, 1.20 and 1.71, eight methylene and two methine protons between δ 0.86 to 2.25 and a trisubstituted olefinic proton as a singlet at δ 5.10, similar to *ent*-13-hydroxykaur-15-en-19-oic acid (4) (Ohtani et al., 1992; Ohta et al., 2010; Starratt et al., 2002). The basic skeleton of *ent*-kaurane diterpenoids was supported by COSY (H-1/H-2; H-2/H-3;

Table 2. ^{13}C NMR spectral data for 1 to 3 isolated from *S. rebaudiana* in CD_3OD [a].

Position	1 (δ_C)	2 (δ_C)	3 (δ_C)
1	41.7	41.4	41.8
2	19.8	19.6	19.8
3	38.8	38.9	38.8
4	44.6	44.3	44.7
5	58.0	58.2	58.0
6	21.5	21.5	21.2
7	40.5	40.4	40.1
8	49.1	49.1	49.3
9	48.1	47.9	47.7
10	40.1	40.3	40.1
11	21.7	21.5	21.4
12	29.9	30.2	30.4
13	90.9	90.7	90.5
14	48.5	48.6	49.6
15	136.6	136.7	136.7
16	143.8	143.6	146.9
17	12.1	12.0	59.0
18	28.5	28.4	28.4
19	178.1	178.3	178.1
20	15.8	15.9	15.8
1′	96.6	96.5	96.3
2′	73.7	73.8	73.8
3′	78.3	78.5	78.5
4′	70.7	70.9	70.9
5′	78.4	78.2	78.2
6′	62.1	62.2	62.2
1″	95.7	95.6	95.3
2″	82.6	80.1	79.6
3″	77.9	87.5	87.6
4″	71.6	70.2	69.9
5″	77.5	77.3	77.2
6″	62.8	62.4	62.3
1‴	104.8	103.4	103.2
2‴	75.8	74.8	74.6
3‴	77.6	77.3	77.4
4‴	71.3	70.3	70.2
5‴	78.1	77.1	77.2
6‴	61.6	61.4	61.3
1⁗		103.8	103.8
2⁗		74.8	74.6
3⁗		77.1	77.0
4⁗		70.3	70.4
5⁗		77.4	77.2
6⁗		61.5	61.3

[a] assignments made on the basis of COSY, HSQC and HMBC correlations.

H-5/H-6; H-6/H-7; H-9/H-11; H-11/H-12) and HMBC (H-1/C-2, C-10; H-3/C-1, C-2, C-4, C-5, C-18, C-19; H-5/C-4, C-6, C-7, C-9, C-10, C-18, C-19, C-20; H-9/C-8, C-10, C-11, C-12; H-14/C-8, C-9, C-13, C-15, C-16 and H-17/C-13, C-15, C-16) correlations.

In addition, the ^1H NMR spectrum of 1 also showed three anomeric protons as doublets at δ 4.55, 4.63 and 5.37 suggesting the presence of three sugar units in its

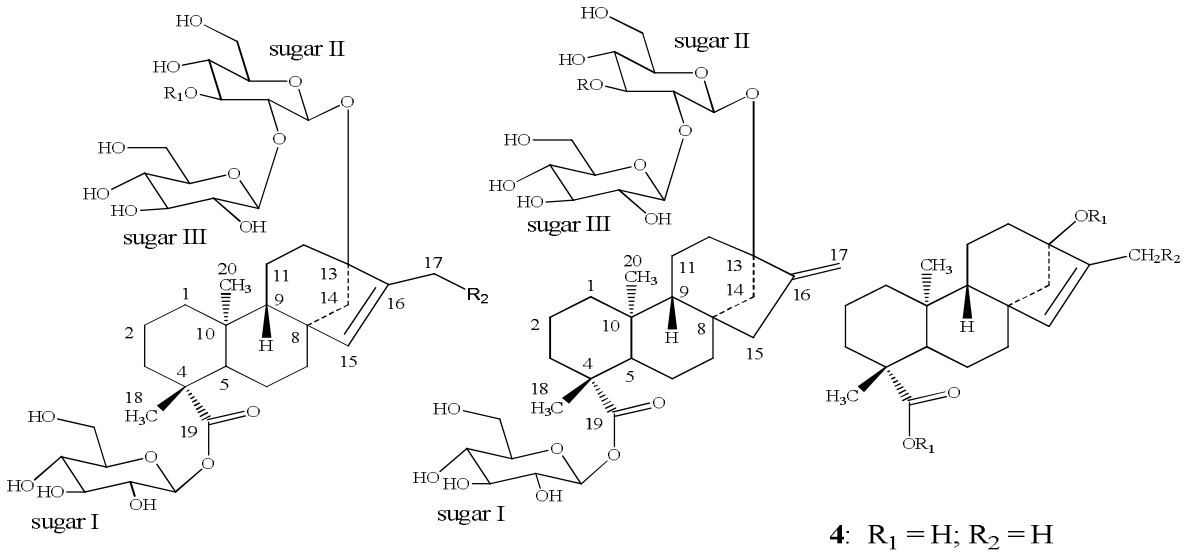

Figure 1. Structures of 1 to 3 and other compounds.

structure which was further supported by the fragment ions observed at *m/z* 643, 481 and 319 corresponding to the successive loss of three hexose moieties from its $[M+H]^+$ ion in the ESI MS/MS spectrum. Enzymatic hydrolysis of 1 furnished a compound which was found identical to 4 on the basis of NMR spectral data (Ohtani et al., 1992). Acid hydrolysis of 1 afforded D-glucose that was identified by preparing its corresponding thiocarbamoyl-thiazolidine carboxylate derivative with L-cysteine methyl ester and *O*-tolyl isothiocyanate and in comparison of its retention time with the standard sugars as described in the literature comparison (Tanaka et al., 2007). The ^1H and ^{13}C NMR values for all the protons and carbons were assigned on the basis of COSY, HSQC and HMBC correlations and were given in Tables 1 and 2. A close comparison of the ^1H and ^{13}C NMR values of 1 with steviol glycosides obtained from *S. rebaudiana* (Avent et al., 1990; Ohta et al., 2010; Starratt et al., 2002) suggested a 2-substituted β-D-glucobiosyl unit at C-13 and a glucosyl unit at C-19 as in stevioside (5) and migration of the exocyclic double bond from C-16/C-17 to C-15/C-16. This was supported by the ^{13}C NMR values for the trisubstituted double bond between C-15 and C-16 which were observed at δ 136.6 and 143.8, respectively and was further confirmed by the HMBC correlations: H-12/C-9, C-11, C-13, C-14, C-16; H-15/C-8, C-9, C-14, C-16, C-17 and H-17/C-13, C-15, C-16. The large coupling constants observed for the three D-glucosyl anomeric protons suggested their β-orientation as reported for steviol glycosides. The key COSY and HMBC correlations for 1 were shown in Figure 2, and it supported the structure completely. Thus, structure of 1 was established unambiguously as 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-15-en-19-oic acid β-D-glucopyranosyl ester.

Compound 2 was also obtained as white powder and its molecular formula was assigned as $C_{44}H_{70}O_{23}$ from the positive ESI spectrum which showed $[M+H]^+$ ion at *m/z* 967 together with an $[M+Na]^+$ adduct at *m/z* 989; this was supported by the high resolution mass spectral data. The ^1H NMR spectrum of 2 showed the presence of three methyl singlets at δ 0.98, 1.21 and 1.71, eight methylene and two methine protons between δ 0.85 to 2.27, a trisubstituted olefinic proton as a singlet at δ 5.12; similar to 1. The ^1H NMR of 2 showed the presence of four anomeric protons as doublets at δ 4.62 (*J*=8.4 Hz, 1H), 4.63 (*J*=7.6 Hz, 1H), 4.78 (*J*=7.4 Hz, 1H) and 5.39 (*J*=8.7 Hz, 1H), suggesting the presence four sugar units in its structure. Enzymatic and acid hydrolysis of 2 furnished 4 and D-glucose, respectively, suggesting the identical aglycone and sugar moieties in its structure similar to 1. The large coupling constants observed for the four anomeric protons suggested their β-orientation similar to 1 as well as for the reported steviol glycosides. The NMR values for all the protons and carbons were assigned on the basis of COSY, HSQC and HMBC correlations and were given in Tables 1 and 2. From the above spectral (NMR and MS) and hydrolysis data, it was incurred that 2

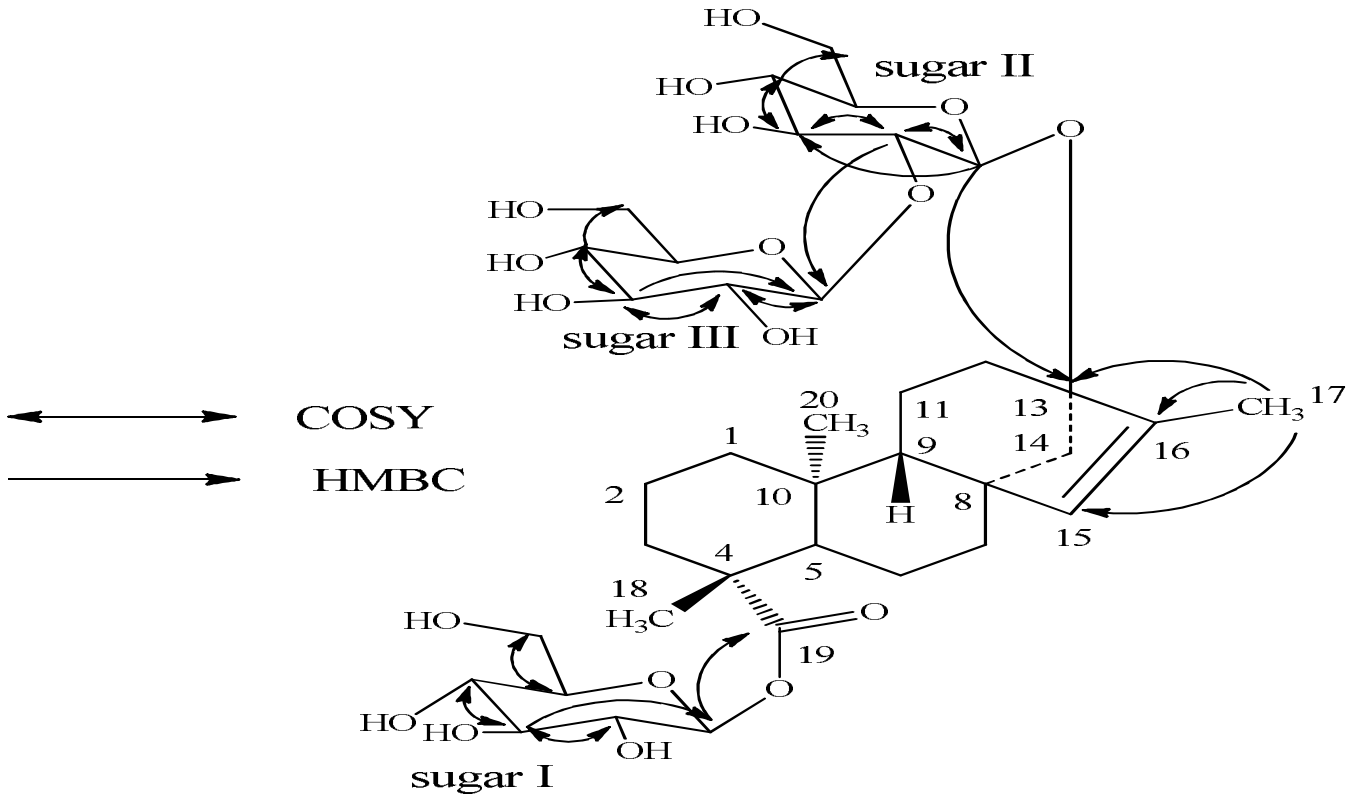

Figure 2. Key COSY and HMBC correlations of 1.

was identical to 1 except for the presence of an additional β-D-glucosyl moiety. A close comparison of the ^{1}H and ^{13}C NMR values of 2 with 1 and rebaudioside A (6) suggested the presence of the additional glucose moiety at C-3″ position of sugar II as a 2, 3-branched β-D-glucotriosyl substituent at C-13 and another glucosyl unit at C-19 with a trisubstituted double bond between C-15 and C-16 as shown in Figure 3, which was supported by the key COSY and HMBC correlations. Thus, the structure of 2 was deduced as 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-15-en-19-oic acid β-D-glucopyranosyl ester.

Compound 3 was also obtained as white powder and its molecular formula was assigned as $C_{44}H_{70}O_{24}$ from the high resolution mass spectral data which showed $(M+Na)^{+}$ ion at *m/z* 1005.4132. The ^{1}H NMR spectrum of 3 showed the presence of two methyl singlets at δ 0.97 and 1.23, eight methylene and two methine protons between δ 0.86 to 2.32, an oxymethylene group as a doublet of doublets at δ 4.13 (*J* = 1.5, 14.2 Hz) and 4.31 (*J* = 1.5, 14.0 Hz) and a trisubstituted olefinic proton as a singlet at δ 5.36; similar to *ent*-13,17-dihydroxykaur-15-en-19-oic acid (7) (Ohtani et al., 1992). In addition, the ^{1}H NMR spectrum of 3 showed the presence of four anomeric protons that were observed as doublets at δ 4.62 (*J*=7.6, 1H), 4.67 (*J*=7.6, 1H), 4.80 (*J*=7.8, 1H), and 5.39 (*J*=8.4, 1H). Enzymatic and acid hydrolysis of 3 furnished an aglycone which was identified as *ent*-13, 17-dihydroxykaur-15-en-19-oic acid (**7**) and D-glucose, respectively. The large coupling constants observed for the four anomeric protons suggested their β-orientation similar to 1 and 2. A close comparison of the ^{1}H and ^{13}C NMR values of 3 with 2 and suavioside J (8) isolated from *Rubus suavissimus* (Ohtani et al., 1992) suggested a trisubstituted double bond between C-15 and C-16 having an additional hydroxyl group present at the C-17 position, with a 2, 3-branched β-D-glucotriosyl substituent at C-13 and another β-D-glucosyl unit at C-19 in its structure. This was supported by the ^{13}C NMR values for the oxymethylene group at C-17 which appeared at δ 59.0 and a trisubstituted double bond between C-15 and C-16 which were observed at δ 136.7 and 146.9, respectively as well as the HMBC correlations: H-12/C-9, C-11, C-13, C-14, C-16; H-15/C-8, C-9, C-14, C-16, C-17 and H-17/C-13, C-15, C-16. The structure was further supported from the key COSY and HMBC correlations as shown in Figure 4. The ^{1}H and ^{13}C NMR values for all the protons and carbons were assigned on the basis of COSY, HSQC and HMBC correlations and were given in Tables 1 and 2. Thus, structure of 3 was established as 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl]- β-D-glucopyranosyl)oxy]-17-hydroxy-*ent*-kaur-15-en-19-oic acid β-D-glucopyranosyl ester.

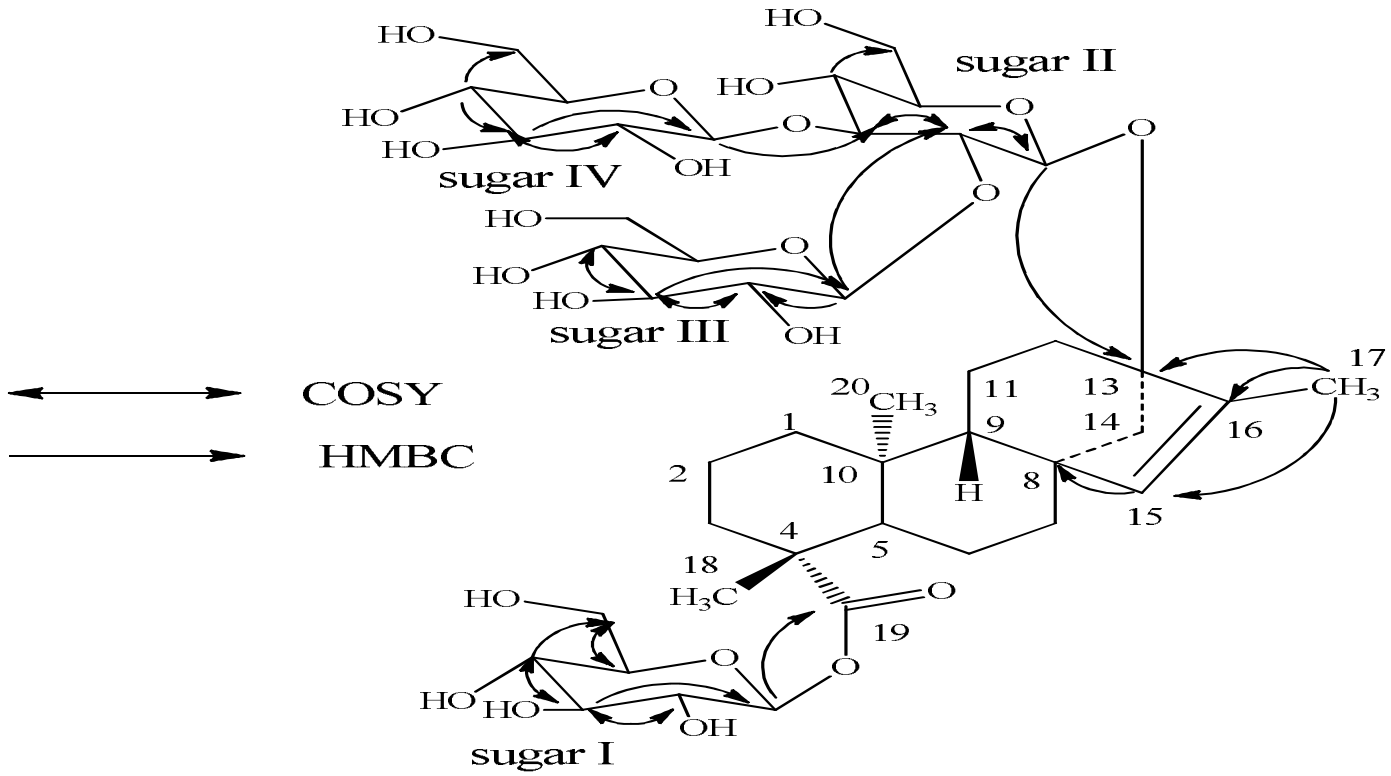

Figure 3. Key COSY and HMBC correlations of 2.

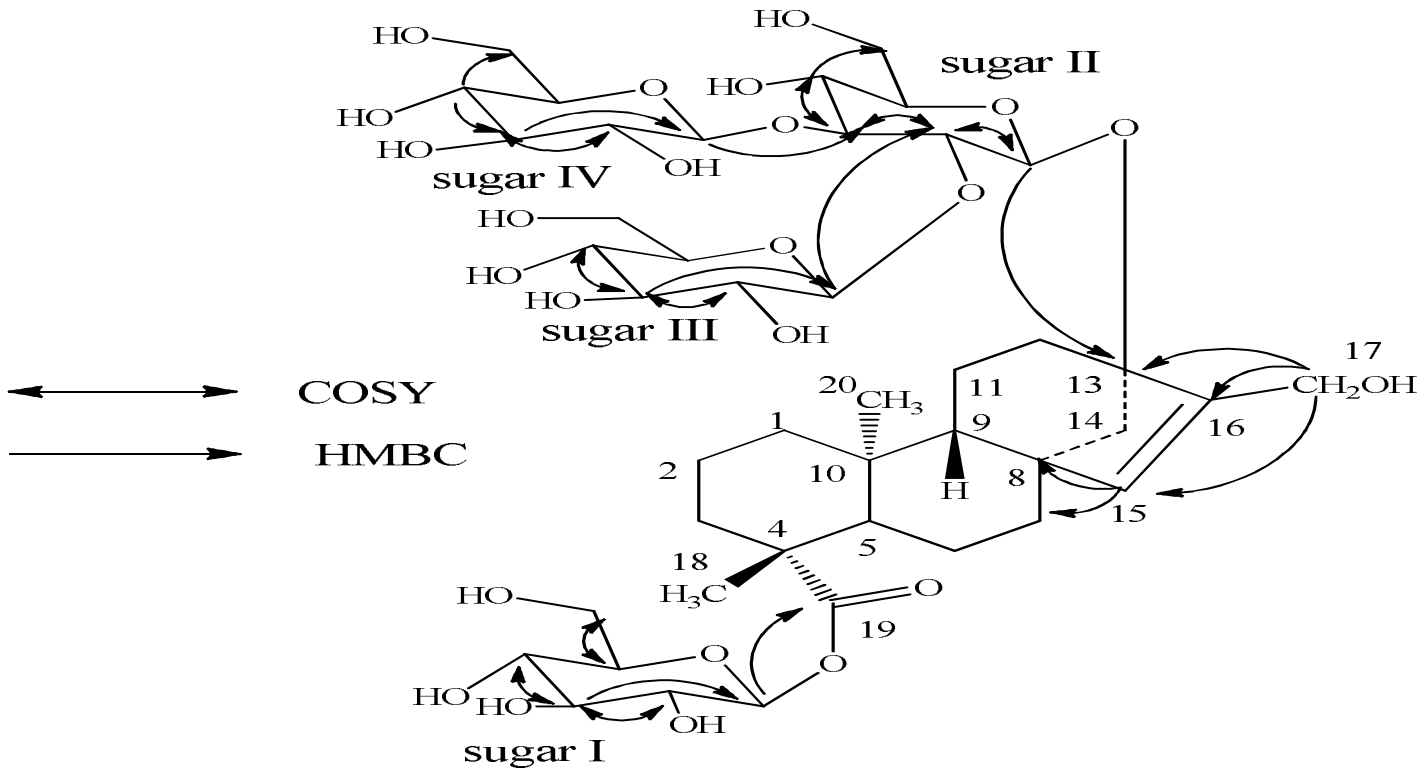

Figure 4. Key COSY and HMBC correlations of 3.

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