Journal of Medicinal Plants Research Vol. 5(19), pp. 4838-4842, 23 September, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Diterpene glycosides from *Stevia rebaudiana*

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Accepted 22 July, 2011

A new diterpenoid glycoside, 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-(4-*O*-(2-*O*-α-D-glucopyranosyl)-α-D-glucopyranosyl-β-D-glucopyranosyl) ester (1) was isolated from the commercial extract of the leaves of *Stevia rebaudiana* along with the known steviol glycosides including stevioside, rebaudiosides A-G, rubusoside and dulcoside A. The complete ^{1}H and ^{13}C NMR assignments of the new compound and rebaudioside G (2) were achieved by the extensive NMR (^{1}H and ^{13}C, COSY, 1D TOCSY, HSQC, HMBC) and MS spectral data as well as chemical studies.

Key words: ^{1}H NMR, ^{13}C NMR, 2D NMR, MS, diterpene glycosides, *Stevia rebaudiana* Bertoni, chemical studies.

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”. 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 known as steviol, *ent*-13-hydroxykaur-16-en-19-oic acid (Brandle et al., 1998). The biological properties of *S. rebaudiana* have been reported by Madan et al. (2010) exclusively in their review indicating that steviol glycosides possesses activities like antioxidant, mutagenic and bactericidal, antiviral, gastro protective, and their effectiveness on renal function, blood pressure and blood glucose.

MATERIALS AND METHODS

General experimental procedures

NMR spectra were acquired on Bruker Avance DRX 500 MHz and Varian Unity Plus 600 MHz instruments using 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 are given in δ (ppm), and coupling constants are reported in Hz. 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 ThermoFisher Discovery OrbiTrap in the positive mode electrospray. Samples were diluted with water: acetonitrile (1:1) containing 0.1% formic acid and introduced via infusion using the onboard syringe pump. Preparative HPLC was performed on an Agilent 1100 system using a Phenomenex Prodigy ODS (3) column (250 × 21.2 mm, 5 µm). Analytical HPLC was carried out with a Waters 600 E multisolvent delivery system using a Phenomenex Synergi Hydro RP column (250 × 4.6 mm, 5 µm) or Waters Atlantis dC_{18} (250 × 4.6 mm, 5 µm) column.

Plant material

Stevia extract SG95, the commercial sample consisting of a mixture of diterpenoid glycosides from the leaves of *S. rebaudiana* was obtained from Pure Circle (Kuala Lumpur, Malaysia). A voucher specimen is deposited at The Coca-Cola Company, No. VSPC-3166-002.

Isolation

Preliminary separation of the crude stevioside extract was carried out using a preparative HPLC method employing a 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 of the baseline material eluting between 12.58 and 13.33 min was collected over several injections and dried in a rotary evaporator under reduced pressure, and named as fraction (1). Further purification of the fraction (1) over several runs employing the aforementioned HPLC method at a flow rate of 5 ml/min and collected the peaks eluting at 12.10 and 22.8 min which on concentration under reduced pressure in a rotary evaporator

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furnished 1 (1.3 mg) and 2 (1.7 mg) respectively. All the known compounds were identified in comparison of their retention times with authentic standards using the HPLC-MS method as described previously (Clos et al., 2008) and the spectral data that were 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, b).

13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oic acid-(4-O-(2-O-α-D-glucopyranosyl)-α-D-glucopyranosyl-β-D-glucopyranosyl) ester (1)

Colorless film; ^{1}H NMR (CD_3OD, δ ppm) and ^{13}C NMR (CD_3OD, δ ppm) spectroscopic data +ESI TOFMS *m/z* 1129.4951 (calcd for $C_{50}H_{81}O_{28}$: 1129.4914) Table 1.

Enzymatic hydrolysis of (1): A solution of (1) (500 µg) was dissolved in 5 ml of 0.1 M sodium acetate buffer, pH 4.5 and crude pectinase from *Aspergillus niger* (100 ul, Sigma-Aldrich, P2736) was added. The mixture was stirred at 50°C for 48 h. The product precipitated out during the reaction was filtered and then crystallized from methanol (MeOH). The resulting steviol (4, 65 µg) was identical to an authentic sample by TLC and ^{1}H NMR (Ohtani et al., 1992).

Determination of the configuration of sugars in (1): Compound (1) (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% TFA water, 1 ml/min; UV detection at 250 nm. The sugars were identified as D-glucose (*t*R, 12.26 min) [authentic samples, D-glucose (*t*R, 12.35) and L-glucose (*t*R, 11.12 min) (Tanaka et al., 2007).

13-[(3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oic acid β-D-glucopyranosyl ester (2)

Colorless film; ^{1}H NMR (CD_3OD, δ ppm) and ^{13}C NMR (CD_3OD, δ ppm) spectroscopic data +ESI TOFMS *m/z* 805.3882 (calcd for $C_{38}H_{61}O_{18}$: 805.3858) Table 1.

Enzymatic hydrolysis of (2): Hydrolysis of (2) (500 µg) as described previously furnished (4) (76 µg).

Acid hydrolysis of 3: Hydrolysis of 2 (250 µg) as described above furnished D-glucose (*t*R, 12.18 min) [authentic samples, D-glucose (*t*R, 12.35) and L-glucose (*t*R, 11.12 min).

RESULTS

As a part of our continuing research to discover natural sweeteners (Chaturvedula et al., 2011a, b), we have obtained several extracts of the leaves of *S. rebaudiana* from various commercial suppliers around the world.

Purification of the commercial extract from the leaves of *S. rebaudiana* obtained from Pure Circle (Kuala Lumpur, Malaysia) resulted in the isolation of an additional new diterpenoid glycosides (1), and the known steviol glycosides, rebaudiosides A-F, rebaudioside G (2), rubusoside (**3**), 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 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, b). This paper describes the isolation and structure elucidation of the new diterpene glycoside 1 as well as the complete NMR spectral assignments of 2 based on the 2D NMR and mass spectral data, spectroscopic data as well as chemical studies.

DISCUSSION

Compound (1) was isolated as a colorless oil and its molecular formula has been deduced as $C_{50}H_{81}O_{28}$ on the basis of its positive ESI TOF (time of flight) mass spectrum which showed an $[M+H]^+$ ion at *m/z* 1129.4951 together with $[M+NH_4]^+$ and $[M+Na]^+$ adducts at *m/z* 1146.5215 and 1151.4792, and this composition was supported by the ^{13}C NMR spectral data. The ^{1}H NMR spectrum of (1) showed the presence of two methyl singlets at δ 0.98 and 1.21, two olefinic protons as singlets at δ 4.85 and 5.19 of an exocyclic double bond, nine methylene and two methine protons between δ 0.86 to 2.27, characteristic for the *ent*-kaurane diterpenoids isolated earlier from the genus *Stevia* (Avent et al., 1990; Ohta et al., 2010; Starratt et al., 2002). The basic skeleton of kaurane diterpenoids was supported by COSY (H-1/H-2; H-2/H-3; 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, C-14, C-15; H-14/C-8, C-9, C-13, C-15, C-16 and H-17/C-13, C-15, C-16) correlations. The fragment ions observed at *m/z* 967, 805, 643, 481, and 319 in the positive ESI mode MS/MS spectrum of 1 indicating the presence of five hexose moieties in its structure. This was further supported by the ^{1}H NMR spectrum of 1 which showed the presence of five anomeric protons at δ 4.58, 4.59, 5.13, 5.21 and 5.38. Enzymatic hydrolysis of 1 furnished an aglycone which was identified as steviol (4) by comparison of ^{1}H NMR spectral data (Ohtani et al., 1992). Acid hydrolysis of (1) afforded a sugar unit which was identified as D-glucose 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 (Tanaka et al., 2007). The ^{13}C NMR values for all the carbons were assigned on the basis of COSY, HSQC and HMBC correlations. A close comparison of the ^{1}H and ^{13}C NMR spectrum of (1) (Table 1) with rubusoside (3) (Ohtani et al., 1992) suggested that it is also a steviol glycoside having one glucose moiety

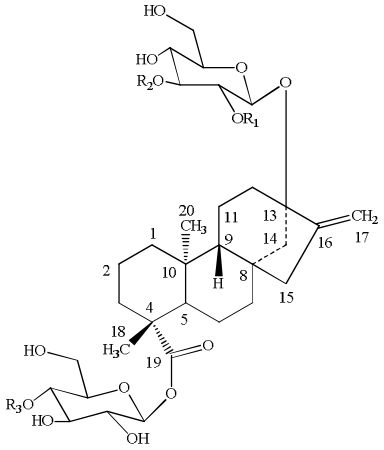
Table 1. ^{1}H and ^{13}C NMR spectral data (chemical shifts and coupling constants) for compounds (1–2) in CD_3OD[a]

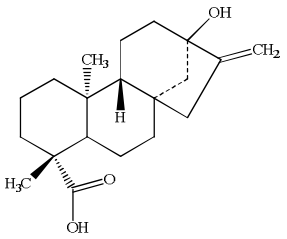
Position	1		2	
	δ_H	δ_C	δ_H	δ_C
1	0.86 m, 1.87 m	41.5	0.85 m, 1.87 m	41.6
2	1.41 m, 1.93 m	20.1	1.44 m, 1.93 m	19.8
3	1.06 m, 2.15 m	38.6	1.06 m, 2.17 m	38.7
4		44.6		44.3
5	1.12 d (11.5)	58.2	1.12 d (12.2)	58.4
6	1.84 m, 2.04 m	22.6	1.83 m, 2.08 m	22.8
7	1.41 m, 1.55 m	42.3	1.43 m, 1.55 m	42.5
8		43.1		43.0
9	0.97 m	54.9	0.98 m	54.7
10		40.3		39.7
11	1.65 m, 1.79 m	21.2	1.66 m, 1.82 m	21.0
12	1.53 m, 1.97 m	38.2	1.43 m, 2.01 m	37.8
13		88.3		87.9
14	1.54 m, 2.27 m	44.9	1.63 m, 2.18 m	45.0
15	2.04 m, 2.14 m	48.4	2.03 m, 2.10 m	48.3
16		153.6		154.4
17	4.85 s, 5.19 s	105.5	4.82 s, 5.11 s	104.9
18	1.21 s	28.8	1.21 s	28.8
19		178.3		178.2
20	0.98 s	16.3	1.00 s	16.0
1′	5.38 d (8.2)	95.4	5.37 d (8.5)	95.5
2′	3.39 m	73.8	3.36 m	73.8
3′	3.72 m	78.0	3.46 m	78.3
4′	3.62 m	79.7	3.36 m	71.1
5′	3.45 m	77.8	3.36 m	77.6
6′	3.60 m, 3.82 m	62.3	3.64 m, 3.80 m	62.2
1″	4.59 d (7.8)	97.3	4.54 d (7.4)	98.8
2″	3.44 m	82.3	3.38 m	74.6
3″	3.53 m	78.2	3.64 m	87.8
4″	3.25 m	71.8	3.34 m	71.1
5″	3.27 m	74.7	3.28 m	78.3
6″	3.64 m, 3.80 m	62.6	3.56 m, 3.74 m	62.7
1‴	4.58 d (7.8)	104.9	4.56 d (7.4)	105.0
2‴	3.27 m	74.4	3.26 m	75.3
3‴	3.34 m	77.3	3.42 m	78.6
4‴	3.13 m	71.2	3.27 m	71.3
5‴	3.44 m	77.6	3.34 m	77.8
6‴	3.56 m, 3.81 m	63.4	3.48 m, 3.72 m	62.4
1⁗	5.21 d (3.7)	102.1		
2⁗	3.49 m	81.3		
3⁗	3.75 m	74.7		
4⁗	3.29 m	73.1		
5⁗	3.76 m	74.3		
6⁗	3.56 m, 3.81 m	62.1		
1⁗′	5.13 d (3.7)	102.6		
2⁗′	3.43 m	73.8		
3⁗′	3.68 m	75.0		
4⁗′	3.25 m	71.2		
5⁗′	3.77 m	74.3		

Table 1. Contd.

6‴″	3.53 m, 3.78 m	62.6

[a] Assignments made on the basis of COSY, HSQC and HMBC correlations; [b] Coupling constants are in Hz; [c] Chemical shift values are in δ (ppm).





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Compound	R_1	R_2	R_3
1	βglc	H	$\alpha^{2}glc\text{-}\alpha^{1}glc$
2	H	βglc	H
3	H	H	H

βglc = β–D-glucopyranosyl

Figure 1. Structures of **1-2** and other compounds.

attached to the C-13 hydroxyl and another glucose moiety in the form of an ester at C-19 leaving the assignments of the other three glucosyl units. From the COSY (H-2″/H-1″, H-3″) and HMBC (H-2″/C-1″, C-3″, C-4″, C-1‴) correlations, it was observed that the anomeric proton of sugar II (H-1″) appeared at δ 4.59 showed a correlation to the H-2″ proton resonating at δ 3.44 and its corresponding carbon was observed in the downfield at δ 82.3 indicating the presence of a 2-substituted D-glucobiosyl unit at C-1 in (1); suggesting the presence of additional two glucosyl units attached to the sugar I at C-19. From the combination of COSY, 1D TOCSY, HSQC and HMBC experiments of (1), the complete proton and carbon values for sugar I were identified and found that the δ_H and δ_C values of its C-4 position were appeared in the downfield region at δ 3.89 and δ 79.7 respectively; suggesting the presence of a D-glucobiosyl unit at this position. The presence of 2-substituted D-glucobiosyl unit at C-4′ was inferred by the key HMBC correlations: H-4′/C-2′, C-3′, C-5′, C-6′, C-1‴; and H-2‴/C-1‴, C-3‴, C-1⁗ correlations (Figure 2). The anomeric protons corresponding to the two sugars IV and V were observed at δ 5.21 and 5.13 respectively showed coupling constant of 3.7 Hz, suggesting their α-orientation similar to the two saponins vina-ginsenosides R5 and R6 isolated from Vietnamese ginseng, *Panax vietnamensis* having α-glycosyl linkage (Duc et al., 1994). The large coupling constants observed for the remaining three anomeric protons at δ 4.58 (d, *J* = 7.8 Hz), 4.59 (d, *J* = 7.8 Hz), and5.38 (d, *J* = 8.2 Hz) suggested their β-orientation as reported for steviol glycosides (Avent et al., 1990;Kobayashi et al., 1977; Kohda et al., 1976; Ohta et al., 2010; Starratt et al., 2002; Sakamoto et al., 1977a, b). Based on the results from chemical and spectral studies, (1) was assigned as 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-(4-*O*-(2-*O*-α-D-glucopyranosyl)-α-D-glucopyranosyl-β-D glucopyranosyl) ester.

The molecular formula of compound (2) was established as $C_{38}H_{61}O_{18}$ from the positive ESI TOF mass spectrum and ^{13}C NMR spectral data. The ^{1}H NMR spectrum of (2) showed the presence of two methyl singlets at δ 1.00 and1.21, nine methylene and two methine protons between δ 0.85 to 2.18, two protons as singlets at δ 4.82

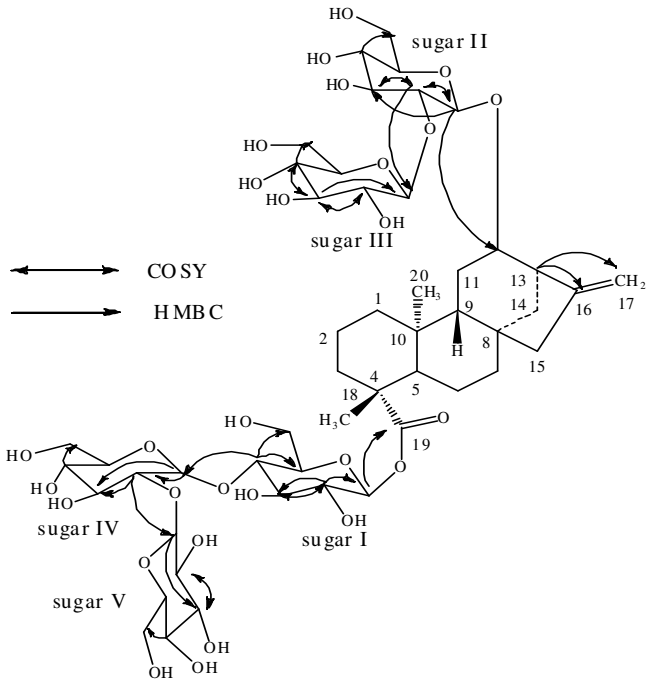

Figure 2. Key COSY and HMBC correlations of (1).

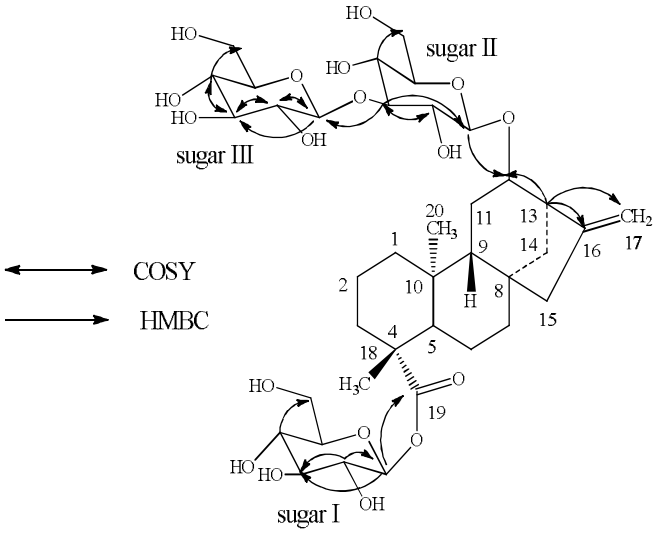

Figure 3. Key COSY and HMBC correlations of (2).

and 5.11 of an exocyclic double bond; similar to (1). The ^{1}H NMR data of (2) showed also the presence of three anomeric protons at δ 4.54, 4.56 and 5.37 suggesting three sugar moieties in its structure and acid hydrolysis identified the sugar units as D-glucose. COSY and HMBC correlations (Figure 3) suggested the placement of a 3-substituted β-D glucobiosyl unit at C-13 and another β-D glucosyl unit at C-19 position on the aglycon moiety of (4). The complete ^{1}H and ^{13}C NMR assignment of (2) was made on the basis of COSY, HSQC and HMBC correlations and were given in Table 1.

The large coupling constants observed for the three anomeric glucose protons suggested the β-orientation similar to (1). These data indicated that (2) was identical to a previously described compound, rebaudioside G (Ohta et al., 2010). Partial NMR data was given earlier in C_5D_5N, therefore a complete ^{1}H and ^{13}C NMR spectral data for the entire proton and carbons were assigned out using 2D NMR (COSY, HSQC and HMBC) correlations in CD_3OD along with other isolated compounds are reported herewith. The structure of (2) was then confirmed as 13-[(3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid□ β-D-glucopyranosyl ester.

ACKNOWLEDGEMENTS

We wish to thank Pure Circle (Kuala Lumpur, Malaysia) for providing the stevia extract SG-95.

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