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Isolation and Structure Elucidation of a New Diterpene Glycoside from *Stevia rebaudiana* Bertoni

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Abstract

A new minor diterpene glycoside has been isolated from a commercial extract of the leaves of *Stevia rebaudiana* and its structure was identified as 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-[2-*O*-(2-*O*-β-D-glucopyranosyl)-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl] ester (**1**) based on extensive NMR (1D and 2D) and mass spectroscopic data, and hydrolysis studies.

Keywords: *Stevia rebaudiana*, Compositae, Asteraceae, diterpene glycosides, structural characterization, spectral data, acid and enzymatic hydrolysis

1. Introduction

Recent interest of food and beverage industry towards reduction in calories by introducing natural non-caloric sweeteners into their systems together with the increasing awareness of obesity problem and the health impacts associated with certain artificial sweeteners has driven research focus towards natural high-potency sweeteners. *Stevia rebaudiana* (Bertoni) Bertoni, a perennial shrub often referred to as "the sweet Herb of Paraguay" (Mosettig et al., 1963; Mosettig & Nes, 1955) belong to the Asteraceae (Compositae) have been used for decades to sweeten food and beverages in Japan, South America and China. *S. rebaudiana* is well known to produce a number of potently sweet diterpene glycosides stevioside, rebaudiosides A, D, M and dulcoside A (Brandle et al., 1998). Due to increase in demand for pure steviol glycosides, it is grown commercially in several countries, particularly in Japan, Taiwan, Korea, Thailand and Indonesia.

As a part of our continuing research to discover natural sweeteners from the leaves of *S. rebaudiana*, we have obtained commercial extracts from various suppliers around the World and isolated various new steviol glycosides (Chaturvedula & Zamora, 2014; Chaturvedula 2014; Chaturvedula & Meneni 2016, 2017).

2. Materials and Methods

2.1 General Experimental Procedures

HPLC separation and analysis has been performed using an Agilent (Wilmington, DE) 1100 HPLC System, including a quaternary pump, a temperature controlled column compartment with an additional 6 port switching valve, an auto sampler and VWD absorbance detector. Data acquisition was done using a Chemstation A 10.02 software using an UV detector set-up at 210 nm. A reversed-phase C18 (2) Phenomenex (Torrance CA) (250 × 4.6 mm, 5 μm) column used for HPLC purification whereas sugar analysis was performed with a Phenomenex Luna C18 column [150 x 4.6 mm (5 u)]; Branson Ultrasonic Cleaner Model 2510 (Maplewood, NJ) was used for degassing HPLC solvents. NMR spectra were acquired on Bruker Avance DRX 500 MHz or Varian INOVA 600 MHz instrument using standard pulse sequences and high Resolution Mass Spectral (HRMS) data were generated with a LTQ Orbitrap Discovery instrument with its resolution set to 30k. Injection sample was prepared with 2:2:1 CH_3CN:MeOH:water (same as infusion eluent) and injected 50 microliters. Sugar identification was partially performed by TLC which was performed on Baker Si-C_{18}F plates. Identification of spots on TLC plate was carried out by spraying 10% H_2SO_4 in EtOH and heating plate at about 80^o C.

2.2 Plant Material

The commercial sample of stevia extract from the leaves of *S. rebaudiana* was obtained from Sinochem Qingdao Co Ltd, China with Lot No: 20170512. The authenticity of the commercial extract was confirmed by performing

its retention time (t_R) comparison with the internal standard compounds of known steviol glycosides isolated from *S. rebaudiana* using the HPLC method reported earlier (Chaturvedula & Zamora 2014). A voucher specimen is deposited at Wisdom Natural Brands.

2.3 Extraction and Isolation

Compound 1 was purified using an Agilent 1100 HPLC system with Phenomenex column (250 × 4.6 mm, 5 μm) by RP-HPLC in 3 stages. The first method utilized an isocratic elution using the mobile phase acetonitrile/phosphate buffer (20:80) with a flow rate of 2 mL/min (injection volume: 50 μL) with an UV detection at 210 nm. The eluent collected between *t*R 6.5 and 8 min has been combined over several runs; dried the corresponding solution under nitrogen yielded a mixture (10.8 mg), which on second round of purification with an isocratic mobile phase acetonitrile/phosphate buffer (25:75); flow rate: 1 mL/min; injection volume: 10 μL; detection: 210 nm. The eluent collected between *t*R 12 and 13 min has been combined over several runs; dried the corresponding solution under nitrogen yielded a mixture (6.8 mg), which has been purified with an isocratic mobile phase acetonitrile/phosphate buffer (32:68); flow rate: 0.5 mL/min; injection volume: 10 μL; detection: 210 nm. The peak eluting at *t*R 12.35 min has been collected over multiple runs and dried the corresponding solution under vacuum yielded 1 (3.2 mg).

13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]*ent*-kaur-16-en-19-oicacid-[2-*O*-(2-*O*-β-D-glucopyranosyl)-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl] ester (**1**): White powder. HRMS *m/z* 1291.5456 (Calcd for $C_{56}H_{91}O_{33}$: 1291.5443); ^{1}H NMR and ^{13}C NMR spectroscopic data (C_5D_5N, δ ppm): Table 1.

Enzymatic hydrolysis of 1: Compound **1** (250 μg) was dissolved in 2.5 ml of 0.1 M sodium acetate buffer by maintaining pH at 4.5 and 50 uL of crude pectinase from *Aspergillus niger* (Sigma-Aldrich, P2736) was added. The mixture was stirred at 50^o C for 48 hr and the product precipitated out during the reaction was filtered and then crystallized. The resulting product obtained was identified as steviol by comparison of their ^{1}H NMR spectral data (Ohtani et al., 1992).

Acid Hydrolysis of 1. Compound **1** (500 μg) is dissolved in MeOH (3 ml) and added 5% H_2SO_4 (10 mL). The mixture was refluxed for 16 hours, cooled to room temperature and then neutralized with saturated sodium carbonate. The aqueous phase was extracted with ethyl acetate (EtOAc, 2 x 15 ml) and the aqueous layer was concentrated and compared with standard sugars using the TLC system EtOAc/*n*-butanol/water (2:7:1) and CH_2Cl_2/MeOH/water (10:6:1) (Bedir et al., 2001; Chaturvedula et al., 2003; Huan et al., 1998); the sugars in **1** were identified as D-glucose.

Determination of sugar configuration 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 ℃ for 1.5 h, and then *O*-tolyl isothiocyanate (12.5 uL) was added to the mixture and heated at 60 ℃ for an additional 1.5 h. HPLC analysis of the reaction mixture was performed by a Phenomenex Luna column [C18, 150 x 4.6 mm (5 u)] using the mobile phase 25% acetonitrile-0.2% TFA water, 1 mL/min under UV detection at 250 nm. The sugar was identified as D-glucose (*t*R, 12.38) [authentic samples, D-glucose (*t*R, 12.28) and L-glucose (*t*R, 11.24 min)] (Tanaka et al., 2007).

3. Results and Discussion

Purification of the commercial extract from the leaves of *S. rebaudiana* obtained from SinoChem Qingdao Co Ltd, China resulted in the isolation of an additional new diterpenoid glycoside **1** and its structure elucidation has been deduced based on the 1D (^{1}H and ^{13}C NMR), and 2D NMR spectral assignments (TOCSY, HSQC, HMBC) as well as enzymatic and acid hydrolysis studies. The structure elucidation of the novel compound was further supported by high resolution mass spectral data (HRMS), and MS/MS data and hydrolysis studies.

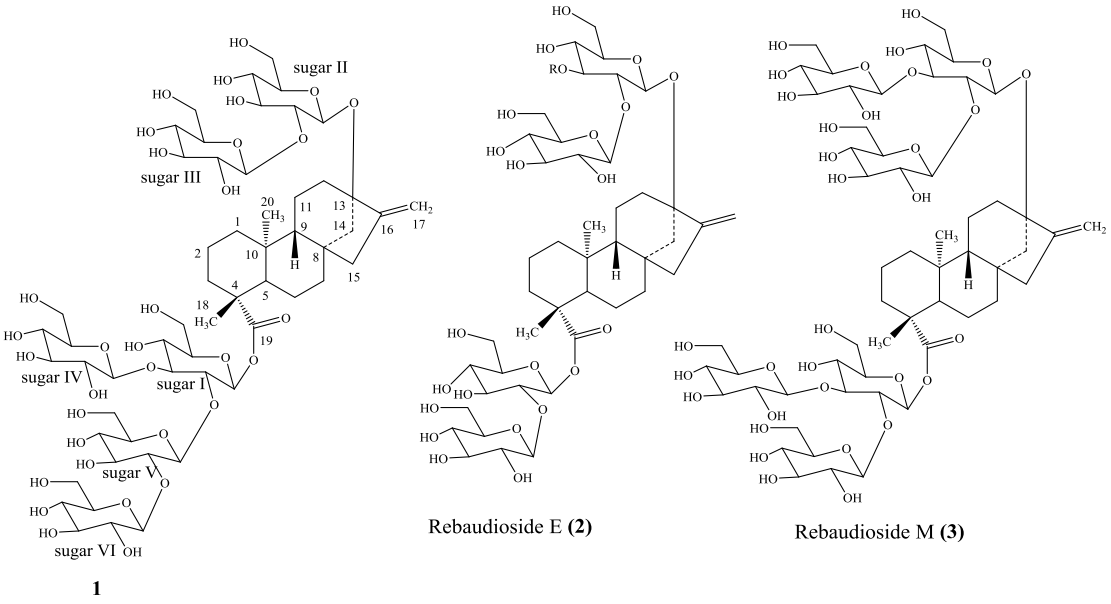

Figure 1. Structures of new compound **1**, rebaudioside E (**2**), and rebaudioside M (**3**)

Compound **1** was obtained as white powder and its molecular formula was assigned as $C_{56}H_{91}O_{33}$ from its HRMS data which showed $(M+H)^+$ ion at *m/z* 1291.5456; this was supported by the ^{13}C NMR spectral data. The ^1H NMR spectrum of **1** showed the presence of two methyl singlets at δ 1.43, and 1.17, nine methylene and two methine protons between δ 0.74-2.71, and two singlets at δ 5.02 and 5.69 corresponding to an exocyclic double bond, similar to the *ent*-kaurane diterpenoids isolated earlier from *S. rebaudiana* (Ohtani et al., 1992; Ohta et al., 2010; Starratt et al., 2002). The basic skeleton of *ent*-kaurane diterpenoids was supported by the key TOCSY (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. Further, the ^1H NMR spectrum of **1** showed six anomeric protons as doublets at δ 5.10, 5.12, 5.42, 5.49, 5.59, and 6.34 suggesting the presence of six sugar units in its structure which was supported by the ESI MS/MS fragment ions observed at *m/z* 1129, 967, 805, 643, 481, and 319 corresponding to the successive loss of three hexose moieties from its $[M+H]^+$ ion. Acid hydrolysis of **1** with 5% H_2SO_4 afforded D-glucose which was identified by direct comparison with authentic sample by TLC suggested the presence of six glucopyranosyl moieties in its molecular structure (Bedir et al., 2001; Chaturvedula et al., 2003; and Huan et al., 1998). Enzymatic hydrolysis of **1** furnished a compound which was found identical to steviol based on NMR spectral data (Ohtani et al, 1992). The configuration of D-glucose 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 based on TOCSY, HSQC and HMBC correlations and were given in Table 1.

Table 1. ^{1}H and ^{13}C NMR spectral data (chemical shifts and coupling constants) of **1** in d5-pyridine (C_5D_5N) [a-c].

Position	^{1}H NMR	^{13}C NMR
1	0.74 t (12.8), 1.69 m	41.3
2	1.45 m, 2.21 m	20.1
3	1.06 m, 2.36 d (12.4)	38.7
4	---	45.1
5	0.98 d (11.7)	57.9
6	1.85 m, 2.12 m	22.4
7	1.24 m, 1.36 m	42.1
8	---	43.2
9	0.91 d (7.5)	54.5
10	---	39.9
11	1.68 m	21.2
12	1.92 m, 2.18 m	37.7
13	---	86.9
14	1.98 m, 2.71 m)	44.8
15	1.86 m, 2.08 m	48.5
16	---	154.9
17	5.02 s, 5.69 s	105.1
18	1.43 s	28.7
19	---	176.5
20	1.17 s	17.2
1′	6.34 d (7.4)	94.0
2′	4.46 m	81.4
3′	5.08 m	88.7
4′	4.23 m	72.0
5′	4.12 m	78.9
6′	4.20 m, 4.38 m	63.5
1″	5.12 d (7.8)	98.2
2″	4.16 m	84.8
3″	4.34 m	78.4
4″	4.20 m	71.6
5″	3.82 m	78.6
6″	4.18 m, 4.40 m	62.8
1‴	5.42 d (7.5)	106.4
2‴	4.12 m	77.2
3‴	4.26 m	78.4
4‴	4.32 m	72.4
5‴	3.90 m	79.3
6‴	4.28 m, 4.43 m	63.4
1⁗	5.49 d (7.5)	106.0
2⁗	4.02 m	77.2
3⁗	4.36 m	78.6
4⁗	4.18 m	72.1
5⁗	3.96 m	79.1
6⁗	4.18 m, 4.36 m	63.1
1′′′′′	5.10 d (7.8)	105.3
2′′′′′	4.19 m	84.2
3′′′′′	4.29 m	76.6
4′′′′′	4.16 m	70.9
5′′′′′	4.05 m	78.6
6′′′′′	4.28 m, 4.41 m	62.6
1′′′′′	5.59 d (7.5)	104.3
2′′′′′′	3.99 m	75.2
3′′′′′′	4.18 m	78.6
4′′′′′′	4.24 m	71.4
5′′′′′′	4.01 m	78.3
6′′′′′′	4.23 m, 4.43 m	62.2

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

A close comparison of the ^{1}H and ^{13}C NMR values of **1** with known steviol glycosides rebaudioside E (**2**) (Chaturvedula & Prakash 2013) and rebaudioside M (**3**) (Prakash et al., 2013) isolated earlier from *S. rebaudiana* suggested a 2-substituted β-D-glucobiosyl unit at C-13 and a 2,3-substituted β-D-glucotriosyl unit at C-19 leaving the assignment of the additional β-D-glucosyl unit. The downfield shift for both the ^{1}H and ^{13}C chemical

shifts at 2′-position of sugar V of the β-D-glucosyl moiety suggested that the additional β-D-glucosyl unit has been attached at this position, which was supported by the key HMBC correlations: H-1‴″/C-2′, C-2‴″, C-3‴″; H-1⁗/C-2⁗, C-2⁗ and C-3⁗. The large coupling constants observed for the five D-glucosyl anomeric protons suggested their β-orientation as reported for steviol glycosides. The key TOCSY and HMBC correlations for **1** were shown in Figure 2, supported the structure completely. Thus, structure of **1** was established unambiguously as 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-[2-*O*-(2-*O*-β-D-glucopyranosyl)-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl] ester.

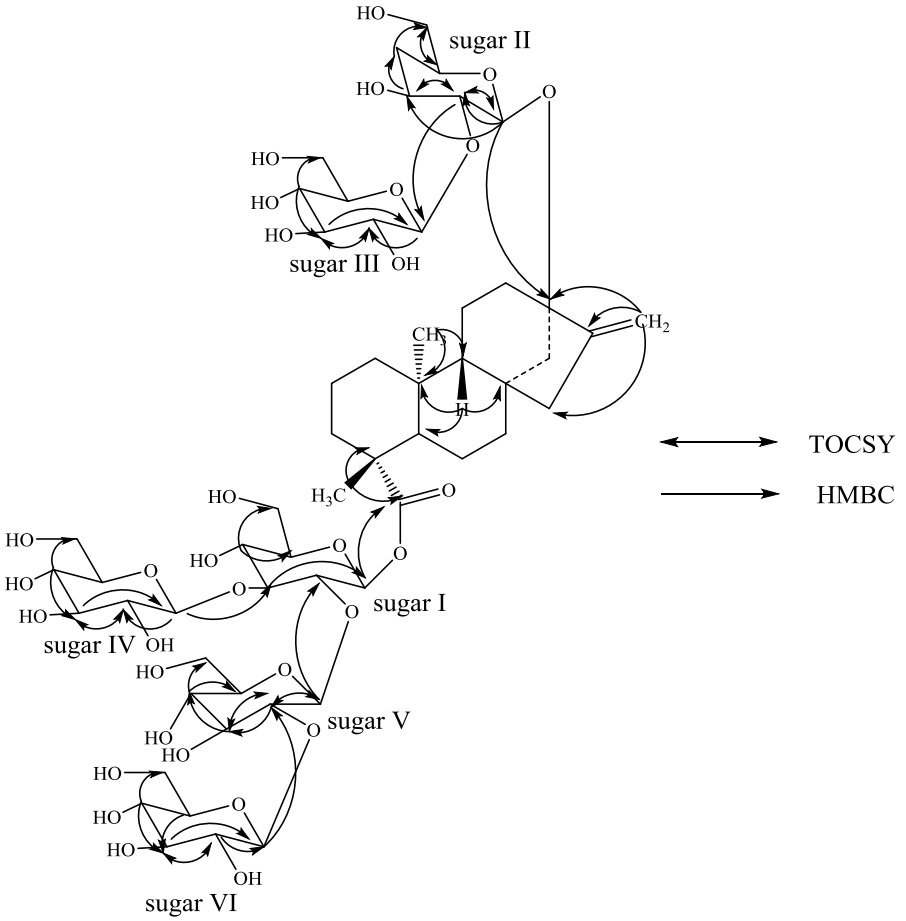

Figure 2. Key TOCSY, and HMBC correlations of 1

4. Conclusion

This is the first report of the isolation of the new diterpene glycoside 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]*ent*-kaur-16-en-19-oic acid-[2-*O*-(2-*O*-β-D-glucopyranosyl)-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl] ester (**1**) in nature and its complete ^{1}H and ^{13}C NMR spectral assignments were made based on spectral (TOCSY, HSQC, HMBC, HRMS, and MS/MS) and chemical studies. The discovery of this new compound is an important addition in expanding our understanding of the diversity of the diterpenoid glycosides of *ent*-13-hydroxykaur-16-en-19-oic acid from *S. rebaudiana* in nature.

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