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Isolation and Structural Characterization of a New Minor Steviol Glycoside of *Stevia rebaudiana*

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Abstract: A new minor *ent*-kaurane diterpene glycoside having five β-D-glucopyranosyl units with a rare attachment of β-D-glucopyranosyl units at C-13 position has been isolated from the commercial extract of the leaves of *Stevia rebaudiana* Bertoni. The chemical structure of the new compound was characterized as 13-[(3-*O*-{6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl}-β-D-glucopyranosyl) oxy] *ent*-kaur-16-en-19-oic acid-(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester (**1**) on the basis of extensive 1D (^{1}H & ^{13}C) and 2D NMR (TOCSY, HMQC, and HMBC), and High Resolution (HR) mass spectroscopic data as well as enzymatic and acid hydrolysis studies.

Keywords*: Stevia rebaudiana; Diterpene glycoside; Isolation; Structure elucidation; Spectral data; Hydrolysis studies.*

I. INTRODUCTION

Stevia rebaudiana (Bertoni) Bertoni often referred to as "the sweet herb of Paraguay" is a perennial shrub of the Asteraceae (Compositae) family native to Paraguay and Brazil [1]. Extracts of the leaves of *S. rebaudiana* have been used for decades to sweeten food and beverages in Japan, South America and China [2]. The major constituents responsible for the potential sweetness of the leaves of *S. rebaudiana* are the diterpene glycosides which are known as Stevia sweeteners. All the isolated diterpene glycosides from *S. rebaudiana* are having the aglycone moiety as *ent*-13-hydroxykaur-16-en-19-oic acid also known as steviol [3-4].

As a part of our research to discover natural sweeteners and their potential usage into food and beverage industry; we have collected commercial extracts of *S. rebaudiana* from various suppliers all over the world and in the process of isolating minor novel diterpene glycosides [5-6]. In this paper, we are describing the isolation and structure elucidation of a minor new diterpenoid glycoside, 13-[(3-*O*-{6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl}-β-D-glucopyranosyl) oxy] *ent*-kaur-16-en-19-oic acid-(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester (**1**) based on extensive spectroscopic (NMR and MS) and hydrolysis studies (Figure 1), and in comparison with the spectral data of the known steviol glycoside rebaudioside D (**2**) and rebaudioside G (**3**).

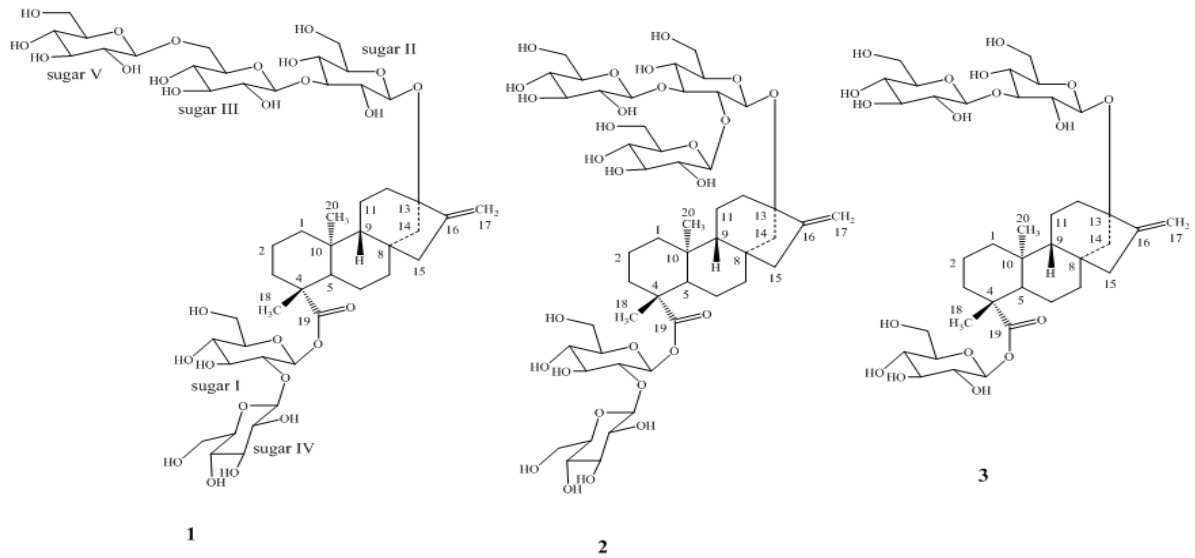

Figure 1: Structures of new compound (**1**), rebaudioside D (**2**) and rebaudioside G (**3**)

II. EXPERIMENTAL

General Instrumentation Methods

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 was used for analysis. The detector was set-up at UV 210 nm and the data acquisition was done using a Chemastation A 10.02 software. The column used for HPLC analysis was a reversed-phase C18 (2) 100 A Phenomenex (Torrance CA) (250 × 4.6 mm, 5 µm); pH was measured using meter Metler Toledo seven compact pH/ion S220 (Switzerland); Branson Ultrasonic Cleaner Model 2510 (Maplewood, NJ) was used for degassing HPLC solvents. Identification of the spots on the TLC plate was carried out by spraying 10% H_2SO_4 in EtOH and heating the plate at about 80-100^o C. 1D and 2D NMR spectra were acquired on Bruker Avance DRX 500 MHz or Varian INOVA 600 MHz instrument instruments using standard pulse sequences. High Resolution Mass Spectral (HRMS) data were generated with a LTQ Orbitrap Discovery instrument with its resolution set to 30k. The needle voltage was set to 4 kV; the other source conditions were sheath gas = 25, aux gas = 0, sweep gas = 5 (all gas flows in arbitrary units), capillary voltage = 30V, capillary temperature = 300 ^{o}C, and tube lens voltage = 75. Sample was diluted with 2:2:1 CH_3CN: MeOH: water (same as infusion eluent) and injected 50 microliters. TLC was performed on Baker Si-C_{18}F plates with mobile phase H_2O-MeOH (80:20).

Plant Material

The commercial sample of stevia extract from the leaves of *S. rebaudiana* which is a mixture of diterpene glycosides was obtained from Sinochem Qingdao Co Ltd, China with Lot No: 20140611. The authenticity of the commercial extract was confirmed by performing its retention time (t_R) comparison with the internal standard compounds of known JECFA steviol glycosides isolated from *S. rebaudiana* using the HPLC method as reported earlier [7]. A voucher specimen is deposited at Wisdom Natural Brands.

Isolation and purification of 13-[(3-*O*-{6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl}-β-D-glucopyranosyl) oxy] *ent*-kaur-16-en-19-oic acid-(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester (1)

Compound **1** was purified from the commercial stevia extract obtained from Sinochem Qingdao Co Ltd, China using an Agilent 1100 HPLC system with Phenomenex column (250 × 4.6 mm, 5 µm) by RP-HPLC in 2 stages. The first stage utilized an isocratic elution method using the mobile phase acetonitrile/phosphate buffer (35:65); flow rate: 2 mL/min; injection volume: 50 µL; detection: 210 nm. The eluent collected between *t*R 4.8 and 5.6 min has been combined over several runs; dried the corresponding solution under nitrogen yielded a mixture (16.2 mg), which on second stage of purification with an isocratic mobile phase acetonitrile/phosphate buffer (25:75); flow rate: 1 mL/min; injection volume: 25 µL; detection: 210 nm. The peak eluting at *t*R 6.45 min has been collected over multiple runs; dried the corresponding solution under nitrogen yielded **1** (4.1 mg).

Identification and spectroscopic data of Compound 1. White powder; ^{1}H NMR (600 MHz, C_5D_5N, δ ppm) and ^{13}C NMR (150 MHz, C_5D_5N, δ ppm) spectroscopic data see Table 1; HRMS $(M+Na)^+$ *m/z* 1151.4716 (calcd. for $C_{50}H_{80}O_{28}Na$: 1151.4734).

Acid Hydrolysis of 1. Compound **1** (250 µg) is dissolved in MeOH (2 ml) and added 5% H_2SO_4 (5 mL). The mixture was heated to reflux and stirred for 16 hours. The reaction mixture was cooled to temperature and neutralized with saturated aqueous sodium carbonate. The aqueous phase was extracted with ethyl acetate (EtOAc, 2 x 15 ml) to separate an EtOAc fraction containing the aglycone part. 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) [8-10]; the sugars were identified as glucose.

Enzymatic hydrolysis of 1. Compound **1** (500 µg) was dissolved in 5.0 mL of 0.1 M sodium acetate buffer (pH 4.5) and crude pectinase from *Aspergillus niger* (250 µL, Sigma-Aldrich, P2736) was added. The reaction mixture was stirred at 50^o C for 96 hr and precipitated product was extracted with ethyl acetate (EtOAc, 2 x 15 ml) and the organic layer was dried under vacuum. The crude product is purified using reversed-phase preparative TLC using water: MeOH (70:30) yielded a pure compound, which was identified as steviol by comparison with co-TLC and ^{1}H-NMR of an authentic sample as well as from the spectral data from the literature [11].

Determination of sugar configuration in 1. Compound **1** (1 mg) has been hydrolyzed with 0.5 M HCl (2.0 mL) for 1.5 hours at reflux temperature. The mixture was cooled to room temperature and passed through an Amberlite IRA400 column and the eluate was lyophilized to get a crude residue which was dissolved in pyridine (0.25 mL) and heated with L-cysteine methyl ester HCl (3.0 mg) at 60 °C for 1.5 h, and then *O*-tolyl isothiocyanate (15 µL) 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 µ); 25% acetonitrile-0.2%

TFA water, 1 mL/min; UV detection at 250 nm. The sugar was identified as D-glucose (*t*R, 11.16 min) [authentic samples, D-glucose (*t*R, 11.25) and L-glucose (*t*R, 9.85 min)] [12].

III. RESULTS AND DISCUSSION

The molecular formula of compound **1** has been deduced as $C_{50}H_{80}O_{28}$ on the basis of its positive high resolution (HR) mass spectrum which showed adduct ion corresponding to $[M+ Na]^+$ at *m/z* 1151.4716; this composition was supported by the ^{13}C NMR spectral data. The ^1H NMR spectral data of **1** showed the presence of two methyl singlets at δ 1.08 and 1.45, two olefinic protons as singlets at δ 5.11 and 5.70 of an exocyclic double bond, nine sp3 methylene and two sp3 methine protons between δ 0.72-2.82, characteristic for the *ent*-kaurane diterpenoids isolated earlier from the genus *Stevia* [5-6]. Enzymatic hydrolysis of **1** furnished an aglycone which was identified as steviol by comparison of ^1H-NMR and co-TLC with standard compound [11] supported the presence of steviol backbone in its structure. The ^1H NMR spectrum of **1** also showed the presence of five anomeric protons resonating at δ 5.03, 5.07, 5.29, 5.46, and 6.32; suggesting five sugar units in its structure. Acid hydrolysis of **1** with 5% H_2SO_4 afforded glucose which was identified by direct comparison with authentic sample by TLC [8-10]. Further, the configuration of the glucosyl moieties present in **1** was achieved by preparing its corresponding thiocarbamoyl thiazolidine carboxylate derivative with L-cysteine methyl ester and *O*-tolyl isothiocyanate, and in comparison of retention times with the standard sugars as described in the literature [12]. The large coupling constants observed for the five anomeric protons of the glucosyl moieties at δ 5.03 (d, *J*=7.2 Hz), 5.07 (d, *J*=7.2 Hz), 5.29 (d, *J*=8.4 Hz), 5.46 (d, *J*=7.8 Hz), and 6.32 (d, *J*=7.8 Hz), suggested their β-orientation as reported for steviol glycosides isolated from *S. rebaudiana* [5-6]. The ^1H and ^{13}C NMR values for compound **1** were assigned on the basis of TOCSY, HMQC and HMBC data and are given in Table 1.

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

Position	^1H NMR	^{13}C NMR
1	0.72 t (12.6), 1.68 m	41.2
2	1.46 m, 2.10 m	20.6
3	1.06 m, 2.82 d (11.4)	38.4
4	---	44.9
5	0.96 d (11.8)	58.1
6	1.85 m, 2.14 m	22.6
7	1.24 m, 1.58 m	42.2
8	---	43.3
9	0.89 d (7.5)	54.5
10	---	40.3
11	1.72 m	21.2
12	1.86 m, 2.18 m	38.2
13	---	86.8
14	1.65 d (10.8), 2.44 d (11.2)	44.9
15	1.98 m, 2.13 m	48.6
16	---	154.98
17	5.11 s, 5.70 s	105.4
18	1.45 s	29.9
19	---	176.3
20	1.08 s	17.3
1′	6.32 d (7.8)	93.9
2′	4.36 m	81.5
3′	4.25 m	78.5
4′	4.22 m	72.0
5′	3.96 m	79.6
6′	4.28 m, 4.38 m	62.7
1″	5.07 d (7.2)	99.9
2″	3.94 m	74.5
3″	4.04 m	89.5
4″	4.23 m	71.0
5″	3.98 m	78.1
6″	4.32 m, 4.58 dd (2.6, 8.4)	63.1
1‴	5.29 d (8.4)	106.5

2‴	4.18 m	76.0
3‴	4.35 m	79.3
4‴	4.26 m	72.2
5‴	3.79 m	78.5
6‴	4.32 m, 4.56 m	69.8
1⁗	5.46 d (7.8)	106.4
2⁗	4.04 m	77.1
3⁗	4.24 m	78.7
4⁗	4.38 m	71.4
5⁗	3.98 m	79.4
6⁗	4.38 m, 4.56 m	63.5
1⁗′	5.03 d (7.2)	105.7
2⁗′	4.04 m	77.4
3⁗′	4.23 m	78.8
4⁗′	4.27 m	72.6
5⁗′	3.96 m	78.9
6⁗′	4.26 m, 4.42 m	63.2

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

Based on the results from NMR spectral data and hydrolysis experiments of **1**, it was concluded that there are five β-D-glucosyl units in its structure connected to the aglycone steviol. A close comparison of the ^{1}H and ^{13}C NMR values of **1** with rebaudioside D (**2**) and rebaudioside G (**3**) reported literature data [13] suggested the presence of a steviol aglycone moiety with a 3-O-β-D-glucobiosyl unit at C-13 in the form of ether linkage and another 2-O-β-D-glucobiosyl unit at C-19 position in the form of an ester linkage, leaving the assignment of the additional β-D-glucosyl unit. The downfield shift for both the ^{1}H and ^{13}C chemical shifts at 6-position of sugar III of the β-D-glucosyl moiety suggested that the additional β-D-glucosyl unit has been attached at this position. The structure was further supported by the key TOCSY and HMBC correlations as shown in Figure 2.

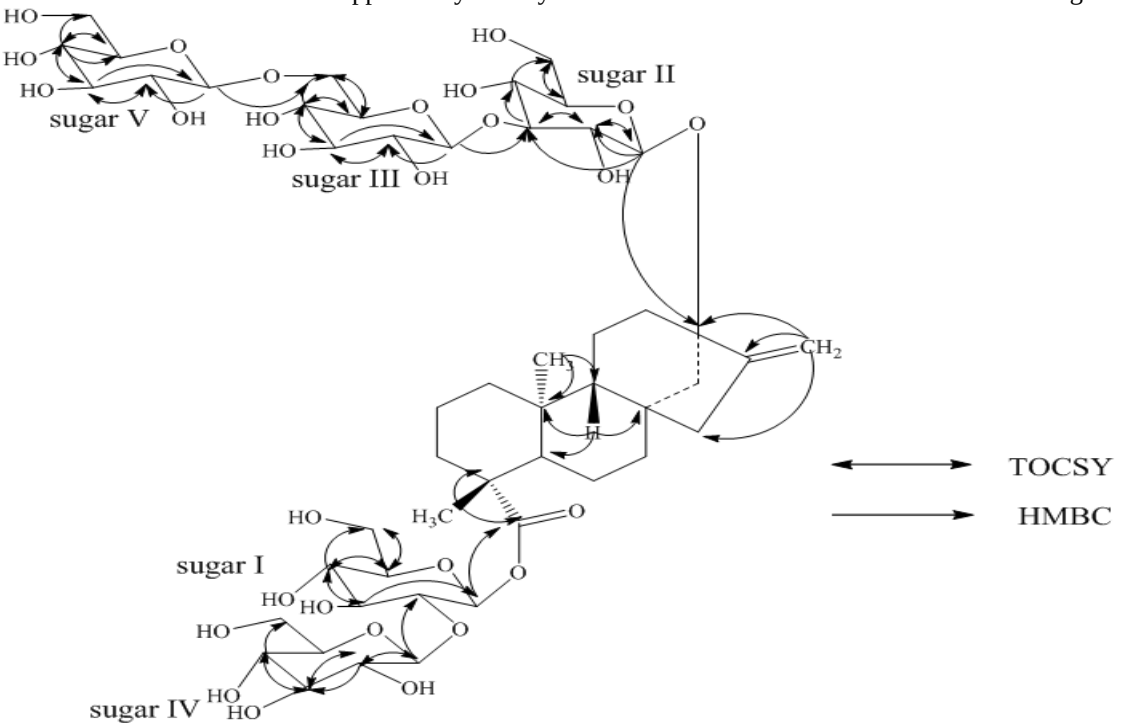

Figure 2: Key TOCSY and HMBC correlations of new compound **1**

Based on the results of NMR and mass spectral data as well as hydrolysis studies, the structure of **1** was deduced as 13-[(3-*O*-{6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl}-β-D-glucopyranosyl) oxy] *ent*-kaur-16-en-19-oic acid-(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester.

IV. CONCLUSIONS

We are herewith reporting the isolation and structural characterization for the new diterpenoid glycoside, 13-[(3-*O*-{6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl}-β-D-glucopyranosyl) oxy] *ent*-kaur-16-en-19-oic acid-(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester (**1**) that has been isolated from the commercial extract of the leaves of *S. rebaudiana*. The new compound was identified and characterized based on the basis of NMR and HR mass spectral data as well as enzymatic and acid hydrolysis studies. This is the first report of the isolation of this new diterpene glycoside in nature, which is an important addition in expanding our understanding of the diversity of the diterpenoid glycosides present in the leaves of *S. rebaudiana* and their structure-activity relationship.

V. ACKNOWLEDGMENTS

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