

A New Penta β -D-Glucopyranosyl Diterpene from *Stevia rebaudiana*

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Abstract

A new steviol diterpene glycoside, 13-[(2-*O*- β -D-glucopyranosyl)- β -D-glucopyranosyl]oxy] *ent*-kaur-16-en-19-oic acid-{2-*O*-[6-*O*- β -D-glucopyranosyl]- β -D-glucopyranosyl]- β -D-glucopyranosyl} ester (**1**) has been isolated from the commercial extract of the leaves of *Stevia rebaudiana* Bertoni. Structure of the new compound has been established on the basis of extensive NMR spectroscopy (^1H & ^{13}C , TOCSY, HMQC, and HMBC) and High Resolution (HR) mass spectroscopic data as well as enzymatic and acid hydrolysis studies.

Keywords

Stevia rebaudiana, Compositae, Asteraceae, Diterpenoid Glycoside, Structure Characterization, Spectral Data, Hydrolysis Studies

1. Introduction

Stevia rebaudiana (Bertoni) is a perennial shrub belonging to the family of Asteraceae (Compositae) native to Brazil and Paraguay, but now grown commercially in a number of regions, particularly in China, Japan, Taiwan, Korea, India, Thailand and Indonesia. Extracts of the leaves of *S. rebaudiana* have been used for decades to sweeten food and beverages in Japan, South America and China [1]. The major constituents in the leaves of *S. rebaudiana* are the potentially sweet glycosides namely stevioside, and rebaudiosides A; which are glycosides of the diterpene steviol, *ent*-13-hydroxykaur-16-en-19-oic acid [2] [3]. As a part of our ongoing research in discovering new natural sweeteners and their potential usage into food and beverage industry [4] [5] [6]; we have isolated a new diterpene glycoside from the commercial extract of the leaves of *S. rebaudiana* from China. The structure of new compound has been characterized as 13-[(2-*O*- β -D-glucopyranosyl)- β -D-glucopyranosyl]oxy] *ent*-kaur-16-en-19-oic acid-{2-*O*-[6-*O*- β -D-glucopyranosyl]- 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 E (**2**) reported earlier from *S. rebaudiana*.

2. Experimental

2.1. General Instrumentation

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. 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°C, and tube lens voltage = 75. Sample was diluted with 2:2:1 CH₃CN:MeOH:water (same as infusion eluent) and injected 50 microliters. TLC was performed on Baker Si-C₁₈F plates with mobile phase H₂O-MeOH (80:20). Identification of the spots on the TLC plate was carried out by spraying 10% H₂SO₄ in EtOH and heating the plate at about 80°C.

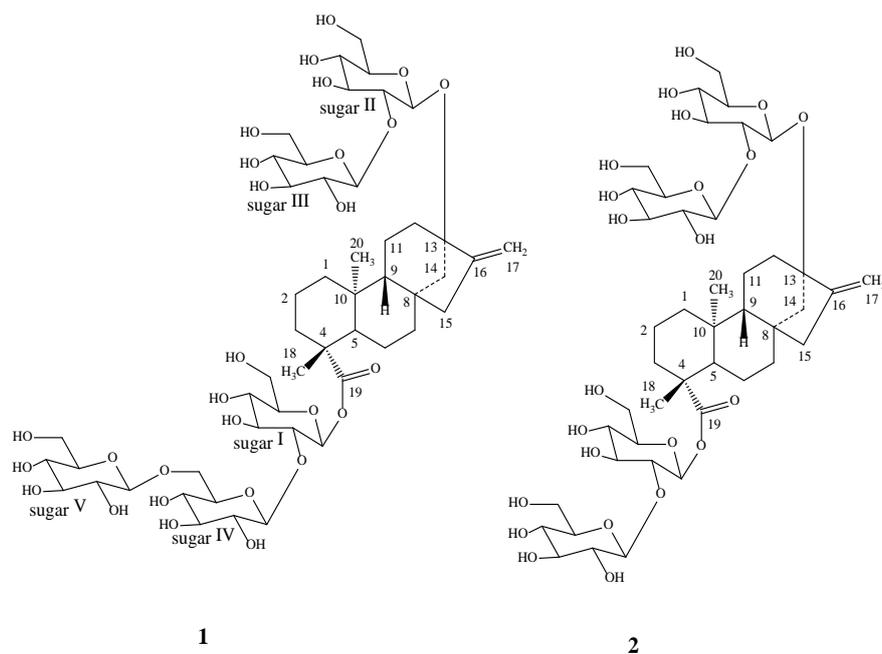


Figure 1. Structure of new compound (**1**), and rebaudioside E (**2**).

2.2. 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.: 20161201. 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 preparative HPLC method as reported earlier [7]. A voucher specimen is deposited at Wisdom Natural Brands.

2.3. Isolation and Purification of 13-[(2-O- β -D-Glucopyranosyl)- β -D-Glucopyranosyl]oxy] *ent*-kaur-16-en-19-oic Acid-{2-O-[6-O- β -D-Glucopyranosyl)- β -D-Glucopyranosyl]- β -D-Glucopyranosyl} Ester (1)

Compound **1** was purified using an Agilent 1100 HPLC system with Phenomenex column (250 \times 4.6 mm, 5 μ m) by RP-HPLC in 3 stages. The first method utilized an isocratic elution using the mobile phase acetonitrile/phosphate buffer (20:80); flow rate: 2 mL/min; injection volume: 50 μ L; detection: 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 μ L; detection: 210 nm. The eluent collected between t_R 10.5 and 12 min has been combined over several runs; dried the corresponding solution under nitrogen yielded a mixture (6.8 mg), which on final round of purification with an isocratic mobile phase acetonitrile/phosphate buffer (32:68); flow rate: 0.5 mL/min; injection volume: 10 μ L; detection: 210 nm. The peak eluting at t_R 14.85 min has been collected over multiple runs; dried the corresponding solution under nitrogen yielded **1** (3.2 mg).

2.4. Identification and Spectroscopic Data of 13-[(2-O- β -D-Glucopyranosyl)- β -D-Glucopyranosyl]oxy] *ent*-Kaur-16-en-19-oic acid-{2-O-[6-O- β -D-Glucopyranosyl)- β -D-Glucopyranosyl]- β -D-Glucopyranosyl} Ester (1)

White powder; ^1H NMR (600 MHz, $\text{C}_5\text{D}_5\text{N}$, δ ppm) and ^{13}C NMR (150 MHz, $\text{C}_5\text{D}_5\text{N}$, δ ppm) spectroscopic data see **Table 1**; HRMS ($\text{M}+\text{Na}$) $^+$ m/z 1151.4713 (calcd. for $\text{C}_{50}\text{H}_{80}\text{O}_{28}\text{Na}$: 1151.4734).

2.5. Acid Hydrolysis of 1

Compound **1** (500 μ g) is dissolved in MeOH (3 ml) and added 5% H_2SO_4 (10 mL). The mixture was refluxed for 16 hours and then neutralized with saturated sodium carbonate after cooling to room temperature. The aqueous phase was extracted with ethyl acetate (EtOAc, 2 \times 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] [9] [10]; the sugars were identified as D-glucose.

Table 1. ^1H and ^{13}C NMR spectral data (chemical shifts and coupling constants) of **1** in $\text{d}_5\text{-pyridine (C}_5\text{D}_5\text{N)}^{\text{a-c}}$.

Position	^1H NMR	^{13}C NMR
1	0.76 t (12.4), 1.69 m	41.3
2	1.45 m, 2.23 m	20.3
3	1.03 m, 2.42 d (12.6)	38.7
4	---	45.1
5	1.01 d (10.5)	57.9
6	1.85 m, 2.10 m	22.4
7	1.28 m, 1.36 m	42.1
8	---	43.2
9	0.89 d (7.2)	54.5
10	---	39.9
11	1.70 m	21.2
12	1.94 m, 2.21 m	37.7
13	---	86.9
14	1.76 d (10.5), 2.65 d (11.6)	44.8
15	1.33 m, 2.08 m	48.5
16	---	154.9
17	5.04 s, 5.68 s	105.1
18	1.39 s	28.7
19	---	176.5
20	1.15 s	17.2
1'	6.31 d (7.4)	94.0
2'	4.38 m	81.4
3'	4.28 m	78.7
4'	4.23 m	72.0
5'	3.92 m	78.9
6'	4.24 m, 4.42 m	63.5
1''	5.14 d (7.8)	98.4
2''	4.18 m	84.6
3''	4.32 m	78.8
4''	4.24 m	71.8
5''	3.80 m	78.9
6''	4.33 m, 4.43 m	63.0
1'''	5.23 d (7.8)	107.0
2'''	4.15 m	77.6
3'''	4.25 m	78.7
4'''	4.32 m	72.4

Continued

5'''	3.94 m	79.1
6'''	4.25 m, 4.43 m	63.3
1''''	5.44 d (7.5)	106.0
2''''	4.05 m	77.2
3''''	4.16 m	78.6
4''''	4.28 m	72.1
5''''	3.96 m	79.1
6''''	4.38 m, 4.62 m	70.2
1'''''	5.04 d (7.6)	106.3
2'''''	3.99 m	77.2
3'''''	4.21 m	78.6
4'''''	4.30 m	71.9
5'''''	4.05 m	79.1
6'''''	4.28 m, 4.41 m	63.2

^aassignments made on the basis of TOCSY, HMQC and HMBC correlations; ^bChemical shift values are in δ (ppm); ^cCoupling constants are in Hz.

2.6. Enzymatic Hydrolysis of 1

Compound **1** (500 μ g) was dissolved in 5.0 mL of 0.1 M sodium acetate buffer (pH 4.5) and crude pectinase from *Aspergillus niger* (250 μ L, Sigma-Aldrich, P2736) was added. The mixture was stirred at 50°C for 96 hr. The product precipitated out during the reaction was filtered and then purified using reversed-phase preparative TLC using water: MeOH (70:30) yielded a pure compound, which was identified as steviol (**3**) by comparison with co-TLC and ¹H-NMR of an authentic sample as well as from the spectral data from the literature [11].

3. Results and Discussion

The molecular formula of compound **1** has been deduced as C₅₀H₈₀O₂₈ on the basis of its positive high resolution (HR) mass spectrum which showed an ion corresponding to [M+ Na]⁺ at m/z 1151.4713 and this composition was supported by the ¹³C NMR spectral data. The ¹H NMR spectral data of **1** showed the presence of two methyl singlets at δ 1.15 and 1.39, two olefinic protons as singlets at δ 5.04 and 5.68 of an exocyclic double bond, nine sp³ methylene and two sp³ methine protons between δ 0.76-2.65, very similar to the *ent*-kaurane diterpenoids isolated earlier from *S. rebaudiana* [12] [13] [14]. The ¹H NMR spectrum of **1** also showed the presence of five anomeric protons resonating at δ 5.04, 5.14, 5.23, 5.44, and 6.31 suggesting five sugar units in its structure. Acid hydrolysis of **1** with 5% H₂SO₄ afforded D-glucose which was identified by direct comparison with authentic sample by TLC suggested the presence of five glucopyranosyl moieties in its molecular structure [8] [9] [10]. The positive mode of ESI MS/MS spectrum of **1** showed the fragment ions observed at m/z 967, 805,

643, 481, and 319 supported the presence of five hexose moieties in its structure. Further, the large coupling constants observed for the five anomeric protons of the glucose moieties at δ 5.04 (d, $J = 7.6$ Hz), 5.14 (d, $J = 7.8$ Hz), 5.23 (d, $J = 7.8$ Hz), 5.44 (d, $J = 7.5$ Hz), and 6.32 (d, $J = 7.4$ Hz), suggested their β -orientation as reported for steviol glycosides [11] [12] [13] [14]. Enzymatic hydrolysis of **1** furnished an aglycone which was identified as steviol by comparison of $^1\text{H-NMR}$ and co-TLC with standard compound [11]. 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. 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.

Based on the results from NMR spectral data and hydrolysis experiments, the basic skeleton of **1** has been deduced as a steviol aglycone with five β -D-glucosyl units in its structure. A close comparison of the ^1H and ^{13}C NMR values of **1** with rebaudioside E (**2**) [15] suggested the presence of a steviol aglycone moiety with a 2-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 ^1H and ^{13}C chemical shifts at 6-position of sugar IV of the β -D-glucosyl moiety suggested that the additional β -D-glucosyl unit has been attached at this position, supported by the key HMBC correlations: H-6^{''''}/C-1^{''''}, H-6^{''''}/C-5^{''''} and C-4^{''''}.

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

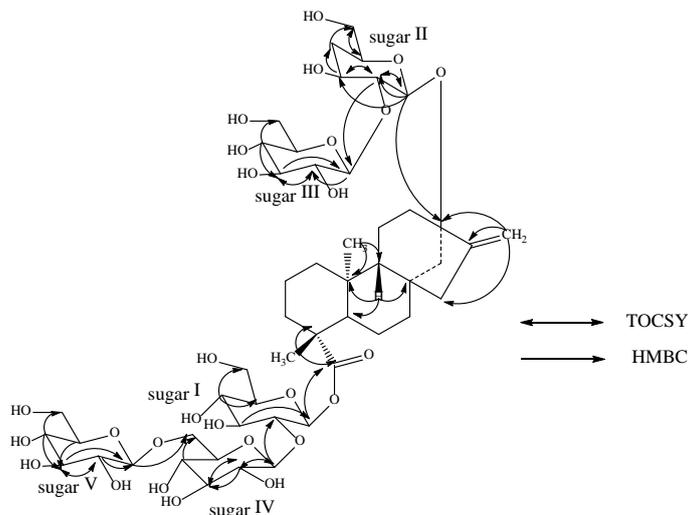


Figure 2. Key TOCSY, and HMBC correlations of **1**.

4. Conclusion

A new diterpenoid glycoside, 13-[(2-*O*- β -D-glucopyranosyl)- β -D-glucopyranosyl] *ent*-kaur-16-en-19-oic acid-{2-*O*-[6-*O*- β -D-glucopyranosyl]- β -D-glucopyranosyl} ester (1) has been isolated from the commercial extract of the leaves of *S. rebaudiana* obtained from Sinochem Qingdao Co. Ltd. (China). The new compound was identified and characterized based on the basis of NMR and HR mass spectral data as well as hydrolysis studies. This is the first report of the isolation of this new diterpene glycoside from *S. rebaudiana* in nature, which is an important addition in expanding our understanding of the diversity of the β -D-glucopyranosyl units attached at multiple positions on diterpenoid glycosides present in the *S. rebaudiana* and their structure-activity relationship.

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