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Spectral Analysis and Chemical Studies of the Sweet Constituent, Rebaudioside A

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Research Article

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ABSTRACT

Aim: To study NMR spectral analysis and hydrolysis products of Rebaudioside A (**1**) that has been isolated from *Stevia rebaudiana.*
Study Design: Acquiring NMR and high resolution mass spectral (HRMS) data of Rebaudioside A for structural characterization, and identification of hydrolysis products.
Place of Study: Organic Chemistry Department of The Coca-Cola Company, Atlanta, GA 30313, USA.
Methodology: The 1D (^{1}H & ^{13}C) and 2D (COSY, HSQC, and HMBC) NMR spectral data of was acquired using Varian Unity Plus 600 MHz instrument using standard pulse sequences. Hydrolysis studies were performed on Rebaudioside A using acid, base and enzymatic studies to identify partial degradation products, aglycone and sugar residues.
Results: The proton and carbon NMR values of Rebaudioside A were assigned based on NMR and HRMS data as well as chemical studies. The aglycone was identified as steviol on the basis of enzymatic hydrolysis, sugar was identified as D-glucose from acid hydrolysis and the partial hydrolyzed product was identified as Rebaudioside B from alkaline hydrolysis.

Keywords: Stevia rebaudiana; compositae; asteraceae; Rebaudioside A; spectral data; TLC comparison; hydrolysis studies.

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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 countries, particularly in Japan, Taiwan, Korea, Thailand and Indonesia [1-2] Please write the reference not numbers (Mosettig et al., 1963; Mosettig and Nes, 1955). Extracts of the leaves of *S. rebaudiana* have been used for decades to sweeten food and beverages in Japan, South America and China. The major constituents in the leaves of *S. rebaudiana* are the potently sweet glycosides namely Steviolbioside, Stevioside, Rebaudiosides A and E, dulcoside A and Rubusoside; which are glycosides of the diterpene steviol, *ent*-13-hydroxykaur-16-en-19-oic acid [3]. These compounds are also known as Stevia sweeteners. Rebaudioside A tastes about 200-300 times sweeter than sucrose and is non-caloric. The biological properties of *S. rebaudiana* have been reported by Madan et al [4] 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.

As a part of our continuing research to discover natural sweeteners, we have isolated several diterpene and triterpene glycosides from the commercial extracts of the leaves of *S. rebaudiana* and *Siraitia grosvenorii* [5-10]. Apart from isolating novel compounds from *S. rebaudiana* and utilizing them as possible natural sweeteners or sweetness enhancers, we are also engaged in understanding the stability of the steviol glycosides in various systems of interest and identification of degradation products using various spectroscopic analysis [11-12] as well as synthesis using naturally occurring starting materials [13]. In this article, we are describing the isolation, characterization and complete ^{1}H and ^{13}C NMR spectral assignments for the diterpene glycoside 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy] *ent*-kaur-16-en-19-oic acid-β-D-glucopyranosyl ester (**1**) which is also known as Rebaudioside A (Figure 1). The complete NMR assignments were achieved on the basis of 1D (^{1}H and ^{13}C) and 2D (COSY, HSQC and HMBC) NMR as well as high resolution mass spectroscopic data. Acid, alkaline and enzymatic hydrolysis studies on compound **1** were carried out to identify partial hydrolyzed products, aglycone and sugar residues.

2. MATERIALS AND METHODS

2.1 General Instrumentation Procedures

Melting points were measured using a SRS Optimelt MPA 100 instrument and are uncorrected. Optical rotation was performed using Rudolph Autopol V at 25° C and IR spectral data was acquired using a Perkin Elmer 400 Fourier Transform Infrared (FT-IR) Spectrometer with Universal attenuated total reflectance (UATR) polarization accessory. HPLC analysis was performed using an Agilent (Wilmington, DE) 1200 system, including a quaternary pump, a temperature controlled column compartment with additional 6-port switching valve, an autosampler and a UV absorbance detector. The reversed phase (RP) HPLC was employed using a Phenomenex (Torrance, CA) Synergi-Hydro column (250 mm x 4.6 mm, 4 µm) with a Phenomenex Security guard C_{18} cartridge and a tertiary solvent mobile phase (A: 0.040% NH_4OAc/AcOH buffer, B: MeCN and C: 0.040% AcOH). The column was maintained at a temperature of 55°C. Charged Aerosol Detector (CAD) was used for the purification of the steviol glycoside **1** with a total run time of 43 min (Table 1).

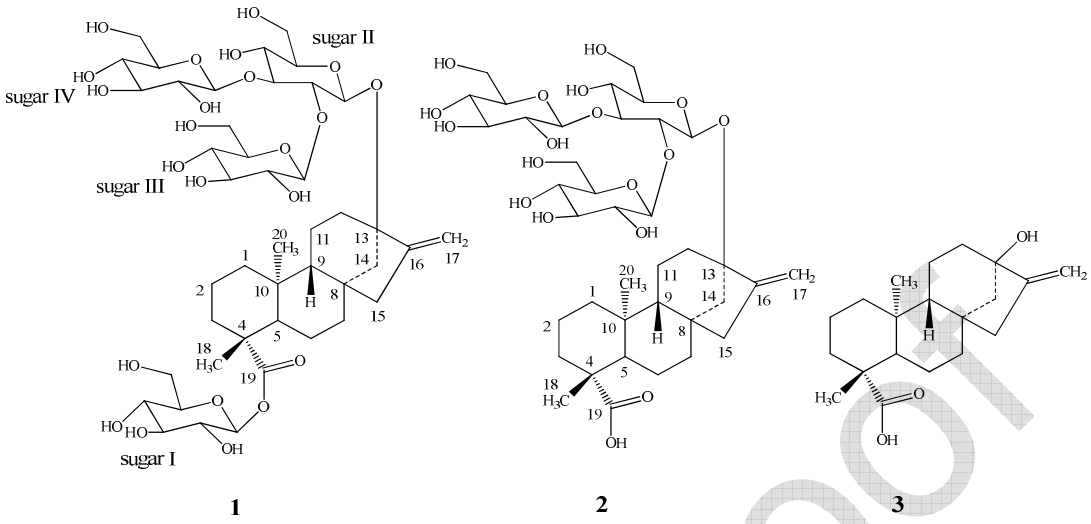

Figure 1: Structure of rebaudioside A (**1**) and other compounds

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Table 1. RP-HPLC method utilized for the purification of Rebaudioside A (1)

Time (min)	% of Mobile Phase A	% of Mobile Phase B	% of Mobile Phase C
0.0	75	25	0
8.5	75	25	0
10.0	71	29	0
16.5	70	30	0
18.5	0	34	66
24.5	0	34	66
26.5	0	52	48
29.0	0	52	48
31.0	0	70	30
37.0	0	70	30
37.1	0	90	10
40.0	0	90	10
40.1	75	25	0
43.0	75	25	0

Analytical HPLC was carried out with a Waters 600E multisolvent delivery system using a Phenomenex Luna C_{18} (150 x 4.6 mm, 5 µm) column. NMR spectra were acquired on Varian Unity plus 600 MHz instrument using standard pulse sequences. The NMR spectra was performed in CD_3OD and D_2O mixture (9:1) mixture; chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. HRMS 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 electrospray positive mode respectively. Samples were diluted with water: acetonitrile (1:1) containing 0.1% formic acid and introduced via infusion using the onboard syringe pump.

2.2 Plant Material

SG95, the commercial aqueous extract consisting of a mixture of diterpenoid glycosides of the leaves of *S. rebaudiana* was obtained from the Pure Circle (Kuala Lumpur, Malaysia). The authenticity of the crude 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 preparative HPLC method as reported earlier [14]. A voucher specimen is deposited at The Coca-Cola Company, No. VSPC-3166-002.

2.3 Isolation and Characterization

Compound **1** was purified by using an Agilent HPLC 1200 system equipped with a Phenomenex Synergi-Hydro column (250 mm x 4.6 mm, 4 µm) with a Phenomenex Security guard C_{18} cartridge. Using the above mentioned HPLC method shown in Table 1, collected the peak eluting at t_R 18.453 min; and dried the corresponding solution under nitrogen yielded **1**.

2.3.1 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oic acid β-D-glucopyranosyl ester (Rebaudioside A, 1)

White powder; mp 242-244 ℃; $[\alpha]_D^{25}$ -31.0 (*c* 0.5, H_2O); IR ν_{max}: 3350, 2916, 1728, 1035, 886 cm^{-1}; ^{1}H-NMR (600 MHz, CD_3OD + D_2O, δ ppm) and ^{13}C-NMR (150 MHz, CD_3OD + D_2O, δ ppm) spectroscopic data see Table 2; HRMS $(M+NH_4)^+$ *m/z* 984.4653 (calcd. for $C_{44}H_{74}O_{24}N$: 984.4652); $(M+Na)^+$ *m/z* 989.4203 (calcd. for $C_{44}H_{70}O_{24}Na$: 989.4206).

2.3.2 Acid hydrolysis of 1

To a solution of compound of **1** (250 µg) in MeOH (1 ml) was added 1 ml of 5% H_2SO_4 and the mixture was refluxed for 8 hours. The reaction mixture was then neutralized with saturated sodium carbonate and extracted with ethyl acetate (EtOAc) (2 x 5 ml) to give an aqueous fraction containing sugars and an EtOAc fraction containing the aglycone part. The aqueous phase was concentrated and compared with standard sugars using the TLC systems EtOAc/*n*-butanol/water (2:7:1) and CH_2Cl_2/MeOH/water (10:6:1) [15-17]; the sugar was identified as glucose [9].

2.3.3 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 60ºC for 1.5 h, and then *O*-tolyl isothiocyanate (12.5 µL) was added to the mixture and heated at 60ºC for an additional 1.5 h. The reaction mixture was analyzed by HPLC: column Phenomenex Luna C18, 150 x 4.6 mm (5 µm); 25% acetonitrile-0.2% TFA water, 1 mL/min; UV detection at 250 nm. The sugar was identified as D-glucose (*t*R, 12.24 min) [authentic samples, D-glucose (*t*R, 12.35) and L-glucose (*t*R, 11.12 min)] [18].

2.3.4 Alkaline hydrolysis of 1

To a solution of NaOH 800 mg dissolved in 90 ml of MeOH at room temperature, was added Rebaudioside A (**1**, 400 mg) and the solution was heated to reflux. The mixture was refluxed for 14 hrs under continuous stirring. The mixture was cooled to room temperature and then neutralized to pH 4.0 with 1 N HCl at 10^{o} C. The solvent was concentrated under vacuum and the product was extracted with *n*-BuOH. The *n*-BuOH layer was washed with water and concentrated under vacuum at low temperature afforded a crude solid which was crystallized with methanol-acetone (1:1) mixture yielded a compound [9], which was identical to Rebaudioside B (**2**) by comparison of the TLC with standard compound and NMR as well as mass spectral data [19].

2.4.5 Enzymatic hydrolysis of 1

Compound **1** (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^{o} C for 48 hr. The product precipitated out during the reaction and was filtered and then crystallized. The resulting product obtained from the hydrolysis of **1** was identified as steviol (**3**) by comparison of its co-TLC with standard compound and ^{1}H NMR spectral data [20].

3. RESULTS AND DISCUSSION

Compound **1** was isolated as a white powder and its molecular formula has been deduced as $C_{44}H_{70}O_{23}$ on the basis of its positive HR mass spectrum which showed an $[M+NH_4]^+$ ion at *m/z* 984.4653 together with $[M+Na]^+$ adducts at *m/z* 989.4203; this composition was supported by ^{13}C NMR spectral data. The ^{1}H NMR spectrum of **1** showed the presence of two methyl singlets at δ 0.86 and 1.13, two olefinic protons as singlets at δ 4.80 and 5.13 of an exocyclic double bond, nine methylene and two methine protons between δ 0.75-2.13 characteristic for the *ent*-kaurane diterpenoids isolated earlier from the genus *Stevia* [3-10]. The basic skeleton of *ent*-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 positive mode of ESI MS/MS spectrum of **1** showed the fragment ions observed at *m/z* 805, 643, 481, and 319 suggesting the presence of four hexose moieties in its structure. The presence of four sugar units in its structure was supported by the ^{1}H NMR spectrum of **1** which showed the anomeric protons at δ 4.53, 4.59, 4.75, and 5.30. Enzymatic hydrolysis of **1** furnished an aglycone which was identified as steviol (**3**) by comparison of ^{1}H NMR [20] and co-TLC with standard compound. Acid hydrolysis of **1** with 5% H_2SO_4 afforded glucose which was identified by direct comparison with authentic samples by TLC [15-17]. The stereochemistry of the sugar was identified as D-glucose by preparing its corresponding thiocarbamoyl-thiazolidine carboxylate derivatives with L-cysteine methyl ester and *O*-tolyl isothiocyanate, and in comparison of their retention times with the standard sugars as described in the literature [18]. Alkaline hydrolysis of **1** furnished a compound which was identified as 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy] *ent*-kaur-16-en-19-oic acid (Rebaudioside B, **2**) on the basis of comparison of the spectral data reported from the literature and co-TLC with standard compound [19-21]. The ^{1}H and ^{13}C NMR values for all the carbons in **1** were assigned on the basis of COSY, HSQC and HMBC correlations and are given in Table 2.

Table 2. ^{1}H and ^{13}C NMR spectral data (chemical shifts and coupling constants) for Rebaudioside A (1) in CD_3OD+D_2O (9:1) [a-c]

Position	^{1}H NMR	^{13}C NMR
1	0.75 (m, 1H), 1.88 (m, 1H)	41.4
2	1.38 (m, 1H), 1.90 (m, 1H)	20.1
3	0.98 (m, 1H), 2.08 (m, 1H)	37.8
4		43.8
5	1.02 (m, 1H)	57.3
6	1.82 (m, 1H), 1.94 (m, 1H)	21.8
7	1.36 (m, 1H), 1.54 (m, 1H)	41.9
8		40.4
9	0.95 (m, 1H)	53.9
10		40.5
11	1.65 (m, 1H), 1.80 (m, 1H)	21.8
12	1.51 (m, 1H), 1.93 (m, 1H)	39.4
13		87.6
14	1.53 (m, 1H), 2.13 (d, *J*=12.2, 1H)	44.2
15	2.05 (m, 1H), 2.06 (d, *J*=17.4, 1H)	48.3
16		152.3
17	4.80 (s, 1H), 5.13 (s, 1H)	104.7
18	1.13 (s, 3H)	27.8
19		177.9
20	0.86 (s, 3H)	15.2
1′	5.30 (d, *J*=7.8, 1H)	94.6
2′	3.57 (m, 1H)	74.0
3′	3.40 (m, 1H)	77.2
4′	3.34 (m, 1H)	70.2
5′	3.36 (m, 1H)	77.4
6′	3.60 (m, 1H), 3.82 (m, 1H)	61.4
1″	4.59 (d, *J*=7.6, 1H)	96.2
2″	3.60 (m, 1H)	78.7
3″	3.68 (m, 1H)	86.2
4″	3.38 (m, 1H)	69.7
5″	3.30 (m, 1H)	77.2
6″	3.60 (m, 1H), 3.82 (m, 1H)	62.2
1‴	4.53 (d, *J*=7.6, 1H)	102.5
2‴	3.18 (m, 1H)	74.6
3‴	3.32 (m, 1H)	76.8
4‴	3.13 (m, 1H)	70.3
5‴	3.44 (m, 1H)	76.9
6‴	3.56 (m, 1H), 3.81 (m, 1H)	61.3
1⁗	4.75 (d, *J*=7.8, 1H)	102.9
2⁗	3.26 (m, 1H)	74.6
3⁗	3.42 (m, 1H)	76.8
4⁗	3.32 (m, 1H)	70.9
5⁗	3.36 (m, 1H)	76.9
6⁗	3.62 (m, 1H), 3.82 (m, 1H)	61.2

[a] assignments made on the basis of COSY, HSQC 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 four β-D-glucosyl units in its structure. The COSY and HMBC correlations shown in Figure 2 suggested that compound **1** is a steviol glycoside which has three glucose residues that are attached at the C-13 hydroxyl as a 2,3-branched β-D-glucotriosyl substituent and another β-D-glucosyl moiety in the form of an ester at C-19. The large coupling constants observed for the four anomeric protons of the glucose moieties at δ 4.73 (d, *J*=7.6 Hz), 4.59 (d, *J*=7.6 Hz), 4.75 (d, *J*=7.8 Hz), and 5.30 (d, *J*=7.8 Hz), suggested their β-orientation as reported for steviol glycosides [5-10, 22]. Based on the results from chemical and spectral studies, **1** was assigned as 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy] *ent*-kaur-16-en-19-oic acid β-D-glucopyranosyl ester and its spectral studies are consistent with the literature data of reabudioside A [19].

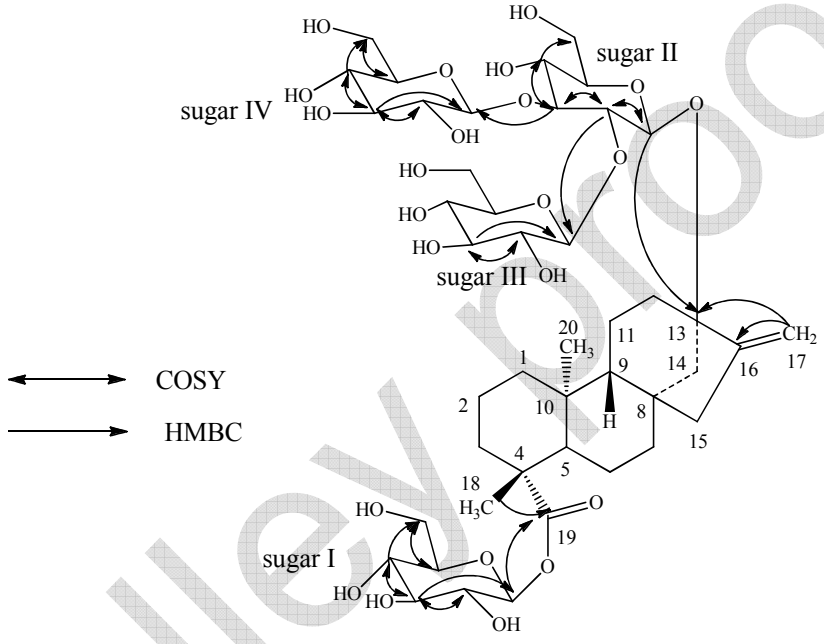

Figure 2: Key COSY and HMBC correlations of **1**

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4. CONCLUSIONS

We are herewith reporting the complete ^{1}H and ^{13}C NMR spectral assignments for 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy] *ent*-kaur-16-en-19-oic acid-β-D-glucopyranosyl ester (Rebaudioside A, **1**) that were made on the basis of extensive 1D and 2D NMR as well as high resolution mass spectral data. Further, alkaline hydrolysis of **1** yielded Rebaudioside B (**2**) by the cleavage of its β-D-glucobiosyl unit present at C-19 position and enzymatic hydrolysis furnished steviol whereas acid hydrolysis furnished glucose whose configuration was established by preparing its corresponding thiocarbamoyl-thiazolidine carboxylate derivatives with L-cysteine methyl ester and *O*-tolyl isothiocyanate.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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