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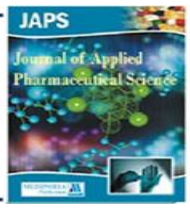

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Acid and Alkaline Hydrolysis Studies of Stevioside and Rebaudioside A

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ABSTRACT

Hydrolysis studies of the two diterpene glycosides stevioside and rebaudioside A that were isolated from *Stevia rebaudiana* were performed using acid and alkaline conditions. Acid hydrolysis was carried out using H_2SO_4 whereas alkaline hydrolysis was performed using NaOH. Using these methods, partial hydrolyzed glycosides of stevioside and rebaudioside A and their sugar residues were identified from the alkaline and acid hydrolysis studies respectively. The structures of the acid and alkaline hydrolysis products were achieved on the basis of extensive NMR and high resolution mass spectral data, and in comparison with the data reported in the literature as well as TLC comparison with their corresponding standard compounds.

Key words: Stevioside, Rebaudioside A, Diterpene glycosides, Acid and Alkaline hydrolysis, Spectral data, TLC comparison.

INTRODUCTION

Stevioside and Rebaudioside A are the *ent*-kaurane diterpene glycosides isolated from *Stevia rebaudiana* (Bertoni); a perennial shrub of the Asteraceae (Compositae) family native to certain regions of South America (Paraguay and Brazil) which is often referred to as "the sweet herb of Paraguay" (Mosettig, *et al*., 1955, 1963). 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 diterpenoid glycosides namely stevioside, and rebaudioside A; which are glycosides of the diterpene steviol, *ent*-13-hydroxykaur-16-en-19-oic acid (Brandle, *et al*., 1998). Stevioside tastes about 150-250 times sweeter than sucrose whereas rebaudioside A tastes about 200-300 times sweeter than sucrose; both are non-caloric. These compounds are also known as Stevia sweeteners. In our continuing research to discover natural sweeteners, we have collected commercial extracts of *S. rebaudiana* from various suppliers all over the World and isolated several novel diterpene glycosides (Chaturvedula, *et al.*, 2011a-g). 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 (Chaturvedula, *et al*., 2011h-i) as well as synthesis using naturally occurring starting materials (Chaturvedula, *et al*., 2011j). In this article, we are describing the acid and alkaline hydrolysis of stevioside and rebaudioside A, the major sweet constituents of *S. rebaudiana*, and characterization of the various hydrolysis products obtained

during the course of reaction. The structure of stevioside (**3**) consists of an aglycone 13-hydroxy *ent*-kaur-16-en-19-oic acid (steviol) skeleton with a 2-*O*-β-D-glucopyranosyl-(β-D-glucopyranosyl)oxy moiety at its C-13 position in the form of an ether and an additional β-D-glucopyranosyl at C-19 position in the form of an ester whereas that of rebaudioside A (**4**) contains 2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-(β-D-glucopyranosyl) oxy moiety at its C-13 position in the form of an ether and an additional β-D-glucopyranosyl at C-19 position in the form of an ester (Figure 1).

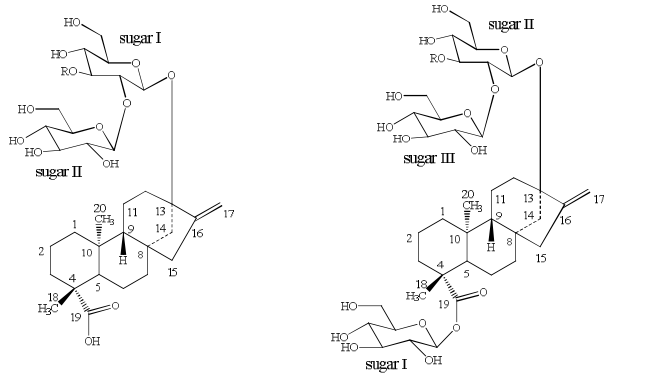

Figure 1: Structures of **1-2** and other compounds

MATERIAL AND METHODS

General Instrumentation Procedures

Melting points were measured using a SRS Optimelt MPA 100 instrument and are uncorrected. 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).

Table 1. RP-HPLC method for the identification of steviolbioside (**1**) and rebaudioside B (**2**).

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

The column was maintained at a temperature of 55°C and charged Aerosol Detector (CAD) was used for the purification of the steviol glycosides **1** and **2** with a total run time of 43 min (Table 1).

IR spectral data was acquired using a Perkin Elmer 400 Fourier Transform Infrared (FT-IR) Spectrometer with Universal attenuated total reflectance (UATR) polarization accessory and NMR spectra were acquired on Bruker Avance DRX 500 MHz and Varian Unity Plus 600 MHz instruments using standard pulse sequences. The NMR spectra were acquired in CD_3OD and D_2O (9:1) mixture; chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. HRMS 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. Samples were diluted with water: acetonitrile (1:1) containing 0.1% formic acid and introduced via infusion using the onboard syringe pump.

Reference Standards and Other Compounds

All reference standards were isolated by AMRI (Bothell, WA) or prepared by The Coca-Cola Company and were certified by Chromadex (Irvine, CA).

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 (Clos, *et al*., 2008). A voucher specimen is deposited at The Coca-Cola Company, No. VSPC-3166-002.

Isolation and Characterization

Compounds **1** and **2** were 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 HPLC method shown in Table 1, collected the peaks eluting at t_R 26.872 and 26.879 min; and dried the corresponding solutions under nitrogen yielded **1** and **2** respectively. The structures of **1** and **2** were characterized on the basis of spectral data and in comparison with the reported literature values as well as retention time comparison with that of its standard compound using HPLC method as described earlier (Clos, *et al.*, 2008).

Identification and spectroscopic data for the alkaline hydrolysis (1-2) and reference compounds (3-4)

*13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-ent-kaur-16-en-19-oic acid (steviolbioside, **1**)*

White powder; mp 195-200 °C; IR ν_{max}: 3307, 2937, 1605, 1053, 970 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 1.00 (s, 3H,

C_{20}-CH_3), 1.19 (s, 3H, C_{18}-CH_3), 4.86 (s, 1H, C_{17}-H), 5.21 (s, 1H, C_{17}-H), 4.61 (d, *J*=7.9 Hz, 1H), 4.64 (d, *J*=7.9 Hz, 1H); HRMS $(M+Na)^+$ *m/z* 665.3140 (calcd. for $C_{32}H_{50}O_{13}Na$: 665.3149) (Chaturvedula, *et al*., 2011g).

*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**)*

White powder; mp 240-250 °C; IR ν_{max}: 3305, 2932, 1615, 1055, 976 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 0.95 (s, 3H, C_{20}-CH_3), 1.23 (s, 3H, C_{18}-CH_3), 4.88 (s, 1H, C_{17}-H), 5.22 (s, 1H, C_{17}-H), 4.66 (d, *J*=7.8 Hz, 1H), 4.75 (d, *J*=7.8 Hz, 1H), 4.82 (d, *J*=7.8 Hz, 1H); HRMS $(M+Na)^+$ *m/z* 827.3661 (calcd. for $C_{38}H_{60}O_{18}Na$: 827.3677) (Kohda, *et al*., 1976).

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

White powder; mp 196-198 °C; ^1H NMR (500 MHz, CD_3OD) δ 0.98 (s, 3H, C_{20}-CH_3), 1.21 (s, 3H, C_{18}-CH_3), 4.86 (s, 1H, C_{17}-H), 5.21 (s, 1H, C_{17}-H), 4.59 (d, *J*=7.9 Hz, 1H), 4.62 (d, *J*=7.9 Hz, 1H), 5.38 (d, *J*=7.9 Hz, 1H); HRMS $(M+Na)^+$ *m/z* 827.3661 (calcd. for $C_{38}H_{60}O_{18}Na$: 827.3677) (Chaturvedula, *et al*., 2011g).

*3-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-ent-kaur-16-en-19-oic acid β-D-glucopyrano syl ester (Rebaudioside A, **4**)*

White powder; mp 240-245 °C; ^1H NMR (500 MHz, CD_3OD) δ 0.95 (s, 3H, C_{20}-CH_3), 1.23 (s, 3H, C_{18}-CH_3), 4.88 (s, 1H, C_{17}-H), 5.22 (s, 1H, C_{17}-H), 4.61 (d, *J*=7.9 Hz, 1H), 4.68 (d, *J*=7.9 Hz, 1H), 4.84 (d, *J*=7.9 Hz, 1H), 5.38 (d, *J*=8.2 Hz, 1H); HRMS $(M+Na)^+$ *m/z* 989.4203 (calcd. for $C_{44}H_{70}O_{23}Na$: 989.4206) (Kohda, *et al*., 1976).

Acid hydrolysis of Stevioside (3)

To a solution of stevioside (**3**, 2.50 mg) in MeOH (10 ml) was added 10 ml of 5% H_2SO_4 and the mixture was refluxed for 8 hrs. 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) (Bedir, *et al*., 2001; Chaturvedula, *et al*., 2003; Huan, *et al*., 1998); the sugar was identified as D-glucose.

Alkaline hydrolysis of Stevioside (3)

To a solution of NaOH 800 g (20 mol) dissolved in 9 l of MeOH at room temperature, was added stevioside (**3**, 418.60, 0.52 mol) 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 pure **1**, which was identical to steviolbioside (**1**) by comparison of the TLC with standard compound and NMR as well as mass spectral data (Chaturvedula, *et al.*, 2011g).

Acid hydrolysis of rebaudioside A (4)

Acid hydrolysis of **4** (2.5 mg) was performed using the method described above for **1** furnished D-glucose.

Alkaline hydrolysis of rebaudioside A (4)

Alkaline hydrolysis of rebaudioside A (**4**, 502.84 g, 0.52 mol) was performed as described above yielded rebaudioside B (**2**) which was identified by comparison of the TLC with standard compound and NMR as well as mass spectral data (Kohda, *et al*., 1976).

RESULTS AND DISCUSSION

Chemistry and Spectroscopy

The two compounds stevioside (**3**) and rebaudioside A (**4**) were hydrolyzed under acidic and alkaline conditions using H_2SO_4 and NaOH respectively. Alkaline hydrolysis of stevioside (**3**) and rebaudioside A (**4**) furnished their partially hydrolyzed compounds **1** and **2** whose structural characterization has been given below. Alkaline hydrolysis of stevioside (**3**) furnished a compound (**1**) which was isolated as white powder and its molecular formula has been deduced as $C_{32}H_{50}O_{13}$ on the basis of its HRMS data which showed the presence of an $[M+NH_4]^+$ ion at *m/z* 660.3589 together with $[M+Na]^+$ adduct at *m/z* 665.3140, this composition was supported by the ^{13}C NMR spectral data. The ^1H NMR spectrum of **1** showed the presence of two methyl singlets at δ 1.00 and 1.19, two olefinic protons as singlets at δ 4.86 and 5.21 of an exocyclic double bond, nine methylene and two methine protons characteristic for the diterpenes belongs to the class of *ent*-kaurenes isolated earlier from the genus *Stevia*. The ^1H NMR spectrum of **1** also indicated the presence of two anomeric protons at δ 4.61 (d, *J* = 7.9 Hz), and 4.64 (d, *J* = 7.9 Hz) indicating the presence of two sugar units in its structure and their large coupling constants suggesting their β-orientation as in stevioside. Acid hydrolysis of **1** with 5% H_2SO_4 afforded D-glucose which was identified by direct comparison with authentic samples (Bedir, *et al*., 2001; Chaturvedula, *et al*., 2003; Huan, *et al*., 1998); confirming the presence of a β-glucopyranosyl moiety in its structure. Based on the above spectral and hydrolysis studies, it was confirmed that one of the three β-D-glucopyranosyl units present in stevioside (**3**) has been hydrolyzed. Further from the HMBC correlations of **1** (Figure 2), it was observed that the carbonyl group did not show correlation to either of the two anomeric protons in its HMBC spectrum suggested the presence of an acid group at C-19 position. Based on the above results, the structure of **1** was assigned unambiguously as 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*- kaur -16- en- 19- oic acid (steviolbioside) and its spectral studies are consistent with the literature data (Chaturvedula, *et al*., 2011g).

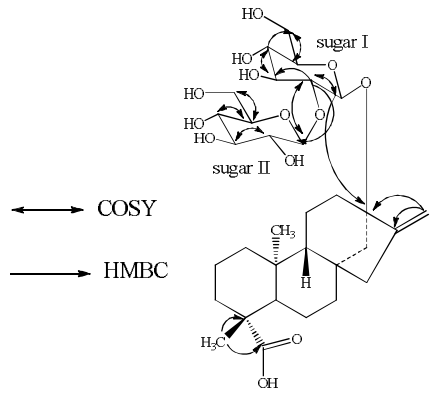

Figure 2: Key COSY and HMBC correlations of **1**

Compound **2** which was obtained from the alkaline hydrolysis of rebaudioside A (**4**) was also obtained as a white powder and its molecular formula was assigned as $C_{38}H_{60}O_{18}$ from its HRESI mass spectrum, which showed a $(M+Na)^+$ ion at *m/z* 827.3661. The ^{1}H NMR spectrum of **2** also showed the presence of two methyl singlets at δ 0.95, and 1.23, two olefinic protons of an exocyclic double bond as singlets at δ 4.88 and 5.22, nine methylene and two methine protons, similar to **1**. The ^{1}H-NMR spectrum of **2** also showed three anomeric protons as doublets at δ 4.66 (*J* = 7.8 Hz), 4.75 (*J* = 7.8 Hz), and 4.82 (*J* = 7.8 Hz) suggesting the presence in its structure of three sugar units. Acid hydrolysis of **2** afforded sugar unit which was identified as D-glucose as described in **1**. A close comparison of the NMR values of **2** with those of rebaudioside A (**4**) (Kohda, *et al.*, 1976) suggested a 2,3-branched β-D-glucotriosyl substituent at C-13 as well as the absence of a glucosyl unit at C-19. This was supported by the COSY and HMBC correlations as shown in Figure 3. The large coupling constants observed for the three D-glucose anomeric protons suggested their β-orientation as reported for steviol glycosides **1** and **3**-**4**. Thus, on the basis of above chemical and spectral studies, the structure of **2** was confirmed to be 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid (rebaudioside B) (Kohda, *et al.*, 1976).

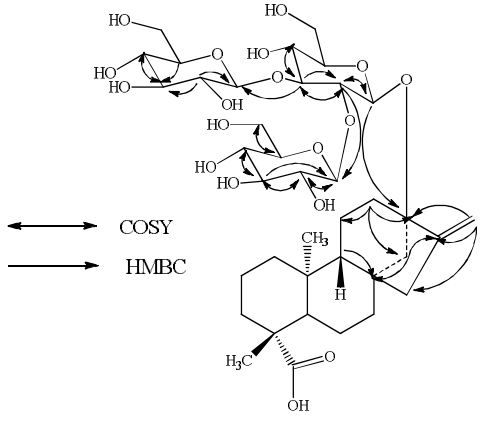

Figure 3: Key COSY and HMBC correlations of **2**

CONCLUSION

Based on the acid and alkaline hydrolysis experiments utilized in this study, the partial degraded products of the two major steviol glycosides, stevioside (**3**) and rebaudioside A (**4**) obtained from *S. rebaudiana* were identified and characterized. Alkaline hydrolysis of **3** and **4** yielded steviolbioside (**1**) and rebaudioside B (**2**) respectively by the cleavage of the β-D-glucopyranosyl unit present at their C-19 position whereas acid hydrolysis furnished D-glucose for both the compounds. The structures of the two compounds **1** and **2** were achieved on the basis of NMR and HRMS spectral data as well as comparative spectral data reported from literature.

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