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Research Article

STRUCTURAL CHARACTERIZATION OF THE HYDROLYSIS PRODUCTS OF THE SWEET PRINCIPLE REBAUDIOSIDE-F

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ABSTRACT

Hydrolysis of the diterpene glycoside rebaudioside F isolated from *Stevia rebaudiana* was performed using enzymatic and acid conditions. Acid hydrolysis was carried out using H_2SO_4 and HCl whereas enzymatic hydrolysis was performed using pectinase. Utilizing these methods, aglycone moieties of rebaudioside F and its sugar residues were identified. The structures of the acid and enzymatic hydrolysis products were achieved on the basis of extensive 1D and 2D NMR spectral data. Further, the configuration of sugar moieties in glycosides were confirmed by preparing their corresponding thiocarbamoyl-thiazolidine carboxylate derivative with L-cysteine methyl ester and *O*-tolyl isothiocyanate in comparison of their retention times with standard sugars.

Keywords*: Stevia rebaudiana*, rebaudioside A, diterpene glycoside, acid hydrolysis, enzymatic hydrolysis, structure characterization.

INTRODUCTION

Rebaudioside F, an *ent*-kaurane diterpene glycoside was 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" [1-2]. The major constituents from *S. rebaudiana* are the potently sweet diterpenoid glycosides also known as stevia sweeteners [3]. Rebaudioside F is about 200 times sweeter compared to sucrose. These compounds are all glycosides of the diterpene 13-hydroxy *ent*-kaur-16-en-19-oic acid known as steviol [4].

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 *S. grosvenorii* [5-10]. Apart from isolating novel compounds from various plant sources and utilizing them as possible natural sweeteners or sweetness enhancers, we are also engaged in understanding the physicochemical profiles of di and triterpene glycosides in various systems of interest, stability under various conditions and characterization of various degradation products [11-12]. In this article, we are describing the enzymatic and acid hydrolysis of rebaudioside F (**1**), one of the major sweet constituents of *S. rebaudiana*, and characterization of the various hydrolysis products obtained during the course of reaction. The structure of rebaudioside F which consists of a 13-hydroxy *ent*-kaur-16-en-19-oic acid (steviol) skeleton with a (2-*O*-β-D-xylopyranosyl-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).

EXPERIMENTAL

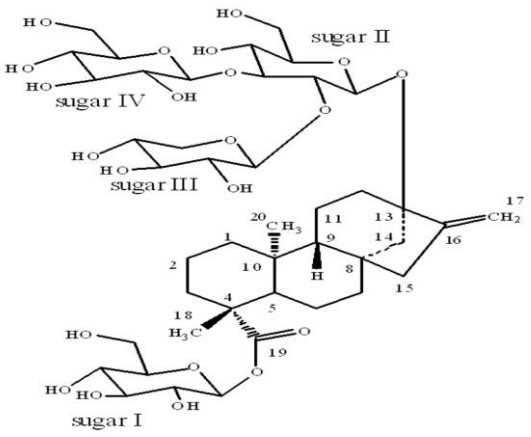
MATERIAL AND METHODS

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).

Instrumentation and Conditions

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). The column was maintained at a temperature of 55°C and the flow rate was 1.0 ml/minute. The injection volume of each sample was 100 µl, which were kept at ambient temperature while in the autosampler. Charged Aerosol Detector (CAD) was used for the analysis of all steviol glycosides with a total run time of 43 min (Table 1).



Rebaudioside F (**1**)

Figure 1: Structure of rebaudioside F

Analytical HPLC was carried out with a Waters 600E multisolvent delivery system using a Phenomenex Luna C_{18} (150 x 4.6 mm, 5 µ**m)** column. NMR spectra were acquired on Bruker Avance DRX 500 MHz and Varian Unity Plus 600 MHz instruments using standard pulse sequences. The spectra were recorded in $CDCl_3$; chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. HRMS data were generated with a Thermo Fisher Discovery OrbiTrap in the electrospray positive mode. Samples were diluted with MeOH/pyridine and introduced via infusion using the onboard syringe pump.

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 namely rebaudioside A-D, and dulcoside A isolated from *S. rebaudiana* using the preparative HPLC method as reported earlier [12]. A voucher specimen is deposited at The Coca-Cola Company, No. VSPC-3166-002.

Isolation

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, collected the peaks eluting at t_R 21.06 min; and dried the corresponding solution under nitrogen yielded **1**. The structure of **1** was characterized on the basis of spectral data and in comparison with the reported literature values [13] as well as retention time comparison with that of its standard compound using HPLC method as described earlier by Clos et al [14].

Identification and spectroscopic data for the hydrolysis products

*Enzymatic hydrolysis of **1**:* Compound **1** (250 µg) was dissolved in 25 ml of 0.1 M sodium acetate buffer, pH 4.5 and crude pectinase from *Aspergillus niger* (500 uL, Sigma-Aldrich, P2736) was added. The mixture was stirred at 50° C for 48 hr. The product precipitated out during the reaction and was filtered and then crystallized. The resulting product obtained from the hydrolysis was identified as *ent*-13-hydroxykaur-16-en-19-oic acid (steviol, **2**), which was identified by comparison of their NMR spectral data [15] and co-TLC with standard compound.

*Steviol (13-hydroxy ent-kaur-16-en-19-oic acid, **2**)*: White powder; mp 206-209 °C; $[\alpha]_D^{25}$ -161.77.56 (*c* 0.01 EtOH/0.1N NaOH); ^{1}H-NMR (500 MHz, CD_3OD, δ ppm) and ^{13}C-NMR (125 MHz, CD_3OD, δ ppm) spectroscopic data see Table 1; HRMS $(M+H)^+$ *m/z* 319.2269 (calcd. for $C_{20}H_{31}O_3$: 319.2273); $(2M+H)^+$ *m/z* 637.4465 (calcd. for $C_{40}H_{61}O_6$: 637.4468).

*Acid hydrolysis of **1**:* To a solution of **1** (250 µg) in MeOH (1 mL) was added 1 mL of 10% 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 EtOAc layer was washed with brine solution (2 x 5 mL) and dried over anhydrous $MgSO_4$. Concentration of the EtOAc layer furnished a product which on crystallization with aqueous EtOH yielded a white solid that was identified as isosteviol (**3**) on the basis of NMR and mass spectral data as well comparison with the spectral data reported in the literature [16-17]. 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) [18-20]; the two sugars were identified as xylose and glucose.

*Isosteviol (13-methyl-16-oxo-17-nor-ent-kauran-19-oic acid, **3**)*: White powder; mp 232-235 °C; $[\alpha]_D^{25}$ -75.77.56 (*c* 0.01 MeOH); ^{1}H-NMR (500 MHz, CD_3OD, δ ppm) and ^{13}C-NMR (125 MHz, CD_3OD, δ ppm) spectroscopic data see Table 1; HRMS $(M+H)^+$ *m/z* 319.2268 (calcd. for $C_{20}H_{31}O_3$: 319.2273); $(M+NH_4)^+$ *m/z* 336.2533 (calcd. for $C_{20}H_{34}O_3N$: 336.2539); $(M+Na)^+$ *m/z* 341.2088 (calcd. for $C_{20}H_{34}O_3Na$: 341.2093).

*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 uL) was added to the mixture and heated at 60ºC for an additional 1.5 h. The reaction mixture was analysed by HPLC: column Phenomenex Luna C18, 150 x 4.6 mm (5 u); 25% acetonitrile-0.2% TFA water, 1 mL/min; UV detection at 250 nm. The sugars were identified as D-glucose (*t*R, 12.26 min) and D-xylose (*t*R, 14.12 min) [authentic samples, D-glucose (*t*R, 12.35) and L-glucose (*t*R, 11.12 min); D-xylose (*t*R, 14.23) and L-xylose (*t*R, 13.06 min) [21].

RESULTS AND DISCUSSION

Compound **2** was isolated as white powder and its molecular formula has been deduced as $C_{20}H_{30}O_3$ on the basis of its HRMS data which showed the presence of an $[M+H]^+$ ion at *m/z* 319.2268 together with $[2M+H]^+$ ion at *m/z* 637.4465, and this composition was supported by the ^{13}C NMR spectral data. The compound gave a positive Liebermann-Burchard test for terpenoids. The ^{1}H NMR spectrum of **2** showed the presence of two methyl singlets at δ 0.96 and 1.19, two olefinic protons as singlets at δ 4.82 and 4.99 of an exocyclic double bond, methylene and methine protons between δ 0.83-2.22. The ^{13}C NMR data of **2** coupled with HSQC and HMBC spectra showed the presence of nine sp3 methylenes, two sp3 methines, four sp3 quaternary carbons, one sp2 methylene, one sp2 quaternary carbon and two methyl groups (Table 1). The above ^{1}H and ^{13}C NMR data was characteristic for the diterpenes belongs to the class of *ent*-kaurenes isolated earlier from the genus *Stevia* [3-10]. The basic skeleton of *ent*-kaurene diterpenoids was supported by the 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. In addition, the ^{13}C NMR data of **2** also showed the presence of a carbonyl group resonating at δ 183.3, which did not show correlation to any proton suggesting its presence in the form of a free acid functional group. The ^{13}C NMR values for all the carbons were assigned on the basis of COSY, HSQC and HMBC correlations and are given in Table 1. On the basis of above spectral data as well as the key COSY and HMBC correlations shown in Figure 2, the structure of **2** was suggested as an *ent*-kaurane diterpenoid having an exocyclic double bond between C-15 and C-16 with hydroxyl and carboxylic groups at C-13 and C-19 positions respectively. A close comparison of the physical and spectral data of **2** with the data reported in the literature [15] suggested its structure as steviol (13-hydroxy *ent*-kaur-16-en-19-oic acid).

The molecular formula of compound **3** was also deduced as $C_{20}H_{30}O_3$ from the $[M+H]^+$ ion observed at *m/z* 319.2268, together with an $[M+H]^+$ and $[M+Na]^+$ adduct ions at *m/z* 336.2533 and 341.2088 respectively. The ^{1}H-NMR spectrum of **3** (Table 1) showed the presence of three methyl singlets at δ 0.79, 0.98 and 1.25; nine methylene and two methine protons between δ 0.96 and 2.64. The ^{13}C NMR data of **3** showed the presence of nine sp3 methylenes, two sp3 methines, four sp3 quaternary carbons, and three methyl groups (Table 1). The ^{13}C NMR data of **3** also showed the presence of a saturated carbonyl group at δ 223.0 and another carbonyl group δ 184.1. From the proton and carbon NMR data of **3**, it was found that this compound also belongs to an *ent*-kaurane diterpenoid similar to **2** with the absence of an exocyclic double bond and the presence of additional methyl and carbonyl groups. The ^{13}C NMR values for all the carbons in **3** were assigned on the basis of COSY, HSQC and HMBC correlations and are given in Table 1. The singlet group resonating at δ 1.25 corresponding to C-18 methyl group showed a correlation with the carbonyl group corresponding at δ 184.1 suggested the presence of a free acid group at C-19 position as in **2**. In the absence of unsaturated carbons together with the appearance of a methyl group at δ 0.98 together with a carbonyl group resonating at δ 223.0 suggested the presence of an 13-methyl-16-oxo-17-nor-*ent*-kauran-19-oic acid (isosteviol) skeleton to compound **3**. The structure was further supported by the key COSY and HMBC correlations as shown in Figure 3 and in comparison of the ^{1}H NMR and ^{13}C NMR spectral data with isosteviol reported from the literature [16-17].

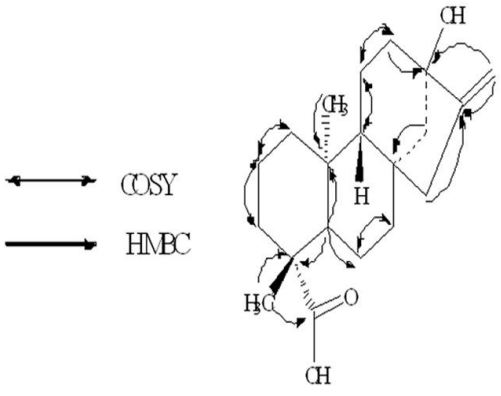


Fig 2 : Key COSY and HMBC correlation of 2

Table 1: ^{1}H and ^{13}C NMR chemical shift values for compounds 1 and 2 in $CDCl_3$ [a-c]

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
1	0.83 (m, 1H), 1.88 (m, 1H)	41.4	0.96 (m, 1H), 1.72 (m, 1H)	39.9
2	1.43 (m, 1H),1.89 (m, 1H)	19.2	1.44 (m, 1H), 1.84 (m, 1H)	19.1
3	1.08 (m, 1H), 2.10 (d, 12.4, 1H)	38.6	1.05 (m, 1H), 2.17 (d, 13.2, 1H)	37.9
4		43.8		43.9
5	1.12 (m, 1H)	57.0	1.16 (d, 12.0, 1H)	57.2
6	1.78 (m, 1H), 1.94 (m, 1H)	22.0	1.76 (m, 1H), 1.92 (m, 1H)	21.8
7	1.44 (m, 1H), 1.56 (m, 1H)	41.4	1.48 (m, 1H), 1.68 (m, 1H)	41.6
8		42.0		48.6
9	0.98 (m, 1H)	54.0	1.20 (m, 1H)	54.9
10		39.7		38.4
11	1.57 (m, 1H), 1.78 (m, 1H)	20.6	1.23 (m, 1H), 1.72 (m, 1H)	20.5
12	1.46 (m, 1H), 1.77 (m, 1H)	40.7	1.38 (m, 1H), 1.62 (m, 1H)	37.5
13		80.5		39.7
14	1.28 (m, 1H), 1.57 (m, 1H)	47.0	1.43 (m, 1H), 1.58 (m, 1H)	54.4
15	1.88 (m, 1H), 2.22 (m, 1H)	47.6	1.80 (m, 1H), 2.64 (dd, 3.6, 18.0, 1H)	48.9
16		156.0		223.0
17	4.82 (s, 1H), 4.99 (s, 1H)	103.2	0.98 (s, 3H)	20.0
18	1.24 (s, 3H)	29.0	1.25 (s, 3H)	29.2
19		183.3		184.1
20	0.96 (s, 3H)	15.6	0.79 (s, 3H)	13.5

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

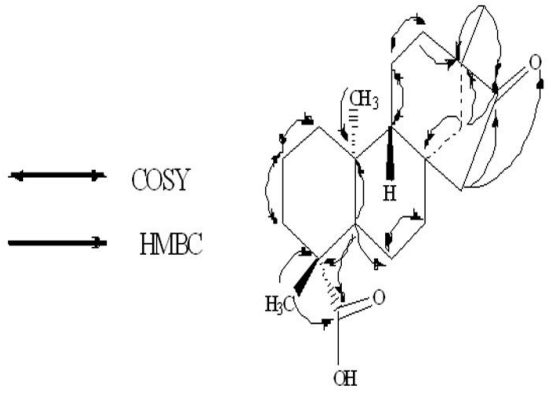


Fig 3 : Key COSY and HMBC correlations of 3.

CONCLUSION

Based on the enzymatic and acid hydrolysis experiments utilized in this study, the aglycone moieties present in rebaudioside F were identified. Enzymatic hydrolysis of rebaudioside F furnished steviol whereas its acid hydrolysis using 10% H_2SO_4 furnished isosteviol. The structures of the two aglycone moieties steviol and isosteviol obtained by enzymatic and acid hydrolysis studies respectively were achieved on the basis of NMR and HRMS spectral data. Further, identification of the sugars present in rebaudioside F as well as their configurations were achieved by the acid hydrolysis experiment using HCl.

REFERENCES

1. Mosettig E, Beglinger U, Dolder F, Lichiti H, Quitt P, Waters JA. The absolute configuration of steviol and isosteviol. J. Am. Chem. Soc. 1963; 85: 2305-2309.
2. Mosettig E, Nes WR. Stevioside. II. The structure of the aglucon. J. Org. Chem. 1955; 20: 884-899.
3. Wayne ES, Lin L. NMR studies of the conformation of the natural sweetener rebaudioside A. Carbohydr. Res. 2009; 344: 2533-2538.
4. Brandle JE, Starrratt AN, Gijen M. *Stevia rebaudiana*: its agricultural, biological and chemical properties. Can. J. Plant Sci. 1998; 78: 527-536.
5. Chaturvedula VSP, Mani U, Prakash I. Diterpene glycosides from *Stevia rebaudiana*. Molecules. 2011; 16: 3552-3562.
6. Chaturvedula VSP, Prakash I. A new diterpenoid glycoside from *Stevia rebaudiana*. Molecules. 2011; 16: 2937-2943.
7. Chaturvedula VSP, Prakash I. Structures of the novel diterpene glycosides from *Stevia rebaudiana*. Carbohydr. Res., 2011; 346: 1057-1060.
8. Chaturvedula VSP, Rhea J, Milanowski D, Mocek U, Prakash I. Two minor diterpene glycosides from the leaves of *Stevia rebaudiana*. Nat. Prod. Commun. 2011; 6: 175-178.
9. Chaturvedula VSP, Prakash I. Additional minor diterpene glycosides from *Stevia rebaudiana*. Nat. Prod. Commun. 2011; 6: 1059-1062.
10. Chaturvedula VSP, Prakash I. Cucurbitane glycosides from *Sitaitia grosvenorii*. J. Carb. Chem. 2011; 30: 16-26.
11. Chaturvedula VSP, Clos JF, Prakash I. Stability study of steviol glycosides in mock beverages using fluorescent light exposure under ICH guidelines, Int. J. Pharm. Pharm. Sci. 2011; 3: 316-323.
12. Chaturvedula VSP, Clos JF, Prakash I. Stability of steviol glycosides in mock beverages under acidic conditions, Int. J. Pharm. Pharm. Sci. 2011; 3 (suppl 5): 421-425.
13. Starratt AN, Kirby CW, Pocs R, Brandle JE. Rebaudioside F, a diterpene glycoside from *Stevia rebaudiana*. Phytochemistry 2002; 59: 367-370.
14. Clos JF, DuBois GE, Prakash I. Photostability of Rebaudioside A and Stevioside in Beverages. J. Agric. Food Chem. 2008; 56, 8507-8513.
15. Ohtani K, Aikawa Y, Kasai R, Chou W, Yamasaki K, Tanaka O. Minor diterpene glycosides from sweet leaves of *Rubus suavissimus*. Phytochemistry 1992; 31: 1553-1559.
16. Kohda H, Kasai R, Yamsaki K, Murakami K, Tanaka O. New sweet diterpene glucosides from *Stevia rebaudiana*. Phytochemistry 1976; 15: 981-983.
17. Avent AG, Hanson JR, DeOliviera BH. Hydrolysis of the diterpenoid glycoside, Stevioside. Phytochemistry 1990; 29: 2712-2715.
18. Bedir E, Toyang NJ, Khan IA, Walker LA, Clark AM. A new dammarane type triterpene glycoside from *Polyscias fulva*. J. Nat. Prod. 2001; 64: 95-97.
19. Chaturvedula VSP, Schilling JK, Miller JS, Andriantsiferana R, Rasamison VE, Kingston DGI. New cytotoxic oleanane saponins from the infructescences of *Polyscias amplifolia* from the Madagascar rainforest. Planta Med. 2003; 69: 440-444.
20. Huan VD, Yamamura S, Ohtani K, Kasai R, Yamasaki K, Nham NT. Oleanane saponins from *Polyscias fructicosa*. Phytochemistry 1998; 47: 451-457.
21. Tanaka T, Nakashima T, Ueda T, Tomii K, Kouno I. Facile discrimination of aldose enantiomers by reversed-phase HPLC. Chem. Pharm. Bull. 2007; 55: 899-901.