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Structural characterization of the hydrolysis products of Rebaudioside M, a minor steviol glycoside of *Stevia rebaudiana* Bertoni

Venkata Sai Prakash Chaturvedula[a*], Oliver Yu[b] and Guohong Mao[a]

[a] *Natural Ingredient Development, Blue California, 30111 Tomas, Rancho Santa Margarita, CA 92688, USA*
[b] *Wuxi NewWay Biotechnology, 401 North Xingyuan Road, Wuxi 210043, P.R. China*

ABSTRACT

Hydrolysis of the diterpene glycoside, rebaudioside M isolated from Stevia rebaudiana Bertoni was performed using acid and base conditions. Acid hydrolysis was carried out using H_2SO_4 and HCl, whereas base hydrolysis was performed using NaOH. This is the first report of the synthesis of isosteviol and rebaudioside B from the diterpene steviol glycoside rebaudiosides M. The structures of the acidic and basic hydrolysis products were achieved on the basis of extensive spectral data and literature comparison. Further, configuration of sugar moieties in the steviol glycoside rebaudioside M obtained during the course of acid hydrolysis studies and its base hydrolyzed product rebaudioside B were confirmed by preparing their corresponding thiocarbamoyl-thiazolidine carboxylate derivatives with L-cysteine methyl ester and O-tolyl isothiocyanate and in comparison of their retention times with standard sugars.

Keywords: Rebaudioside M, Diterpenoid glycoside, 1D and 2D NMR spectral data, Acid hydrolysis, Base hydrolysis, Structure characterization

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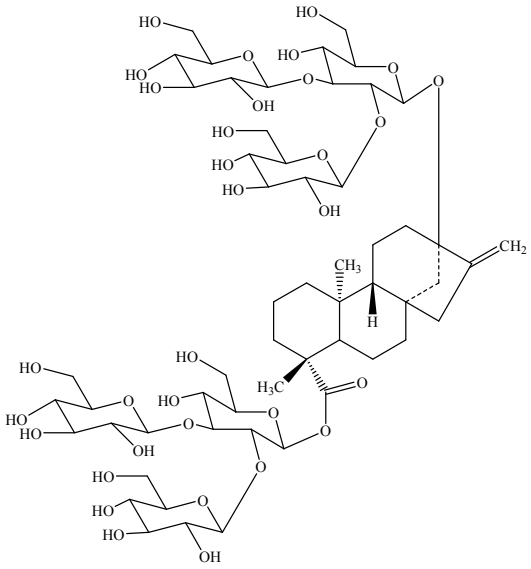
INTRODUCTION

Rebaudioside M, also known as rebaudioside X is the hexa pyranosyl *ent*-kaurane diterpene glycoside 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]. Recently, several minor steviol glycosides have been reported including rebaudioside M from *S. rebaudiana* Morita, which was developed as a cultivar by selective breeding of *S. rebaudiana* Bertoni [2]. *S. rebaudiana* Bertoni is native to Brazil and Paraguay which has been used for decades to sweeten food and beverages in Japan, South America and China, but now is grown commercially in a number of countries, particularly in Japan, China, Taiwan, Korea, Thailand and Indonesia [3-4]. The major constituents in the leaves of *S. rebaudiana* are the diterpenoid glycosides namely stevioside, and rebaudioside A, which are potently sweet glycosides of the diterpene steviol, *ent*-13-hydroxykaur-16-en-19-oic acid. These are also known as Stevia sweeteners [5].

In our continuing research to discover natural sweeteners, we have isolated several minor diterpene glycosides from the commercial extracts of *S. rebaudiana* Bertoni [6-7]. In this article, we are describing the acidic and basic hydrolysis studies of the steviol diterpene glycoside rebaudiosides M, the minor constituent isolated from *S. rebaudiana* Bertoni, and characterization of the various hydrolyzed products obtained during the course of reaction. Rebaudioside M is a hexapyranosyl steviol glycoside having two 2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl units in its structure, one at C-13 position as an ether and another at C-19 position as an ester (**1**) (Figure 1). The structural characterization of the acid and base hydrolysis products namely isosteviol (**2**) and rebaudioside B (**3**) respectively were achieved on the basis of 1D (^{1}H and ^{13}C) and 2D (COSY, HMQC and HMBC) NMR and high resolution mass spectroscopic (MS) data, as well as in comparison of the physical and spectral data of their respective standard compounds reported from the literature. Further, configuration

of sugar moieties in the diterpene glycoside **1** and rebaudioside B (**3**), its base hydrolyzed product were confirmed by preparing their corresponding thiocarbamoyl-thiazolidine carboxylate derivative with L-cysteine methyl ester and *O*-tolyl isothiocyanate, and in comparison of their retention times with standard sugars.



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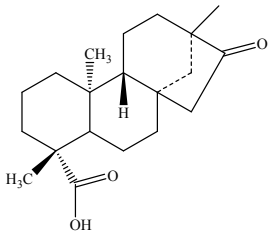

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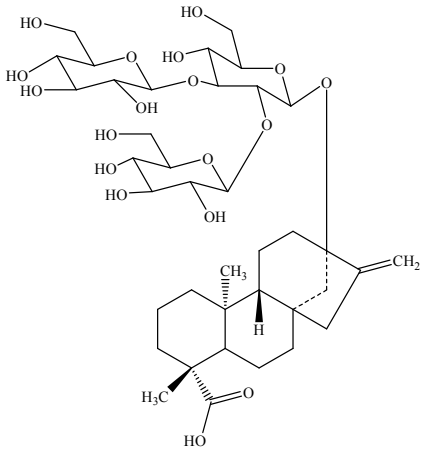

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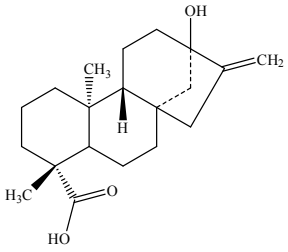

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Figure 1: Structure of rebaudioside M (1) and other compounds

EXPERIMENTAL SECTION

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^{o} C. HPLC analysis was performed using a Dionex UPLC ultimate 3000 system (Sunnyvale, CA), including a quaternary pump, a temperature controlled column compartment, an auto sampler and a UV absorbance detector. Phenomenex Luna NH_2 with guard column, 150x3.0 mm, 3µm (100A) were used for the characterization of rebaudioside M as well as hydrolysis products. 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 Bruker Avance DRX 500 MHz or Varian INOVA 600 MHz instrument instruments using standard pulse sequences. The NMR spectra were performed in C_5D_5N or $CDCl_3$; chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. MS and MS/MS data were generated with a Thermo LTQ-FTMS mass spectrometer (100,000 resolutions) equipped with a Nano spray ionization source. Samples were diluted with methanol and introduced via infusion using the onboard syringe pump.

Isolation of 1

Compound **1** was purified by the repeated isocratic elution (72% acetonitrile in water) of the commercial extract of *S. rebaudiana* Bertoni using Dionex Ultra Performance Liquid Chromatography (UPLC) ultimate 3000 system with Phenomenex Luna NH_2 guard column. Collected the peak eluting at t_R 5.70min and dried the aqueous organic layer under nitrogen yielded pure **1**.

Identification and spectroscopic data for the acid and base hydrolysis products of 1

*Acid hydrolysis of **1**:* Compound **1** (2.5 mg) has been dissolved in MeOH (10 ml) and added 10% H_2SO_4 (10 ml); the mixture was refluxed for 8 hours. The reaction mixture was neutralized with aqueous solution of saturated sodium carbonate and extracted with ethyl acetate (EtOAc) (2 x 50 ml). The organic (EtOAc) layer was washed with brine solution (2 x 50 ml) and dried over anhydrous $MgSO_4$, and after concentration furnished a product which on crystallization with aqueous EtOH (1:1) yielded a white solid that was identified as isosteviol (**2**) on the basis of NMR and mass spectral data as well comparison with the spectral data reported in the literature. The aqueous phase containing sugars was concentrated and compared with standard sugars suggesting the presence of the two sugars as glucose using the TLC systems EtOAc/*n*-butanol/water (2:7:1) and CH_2Cl_2/MeOH/water (10:6:1) [8-10].

*Isosteviol (13-methyl-16-oxo-17-nor-ent-kauran-19-oic acid, **2**)*: White powder; mp 230-233 °C; $[\alpha]_D^{25}$ -75.56 (*c* 0.01 MeOH); ^{1}H-NMR (600 MHz, $CDCl_3$, δ ppm) and ^{13}C-NMR (150 MHz, $CDCl_3$, δ ppm) spectroscopic data see Table 1; HRMS $(M+H)^+$ *m/z* 319.2274 (calcd. for $C_{20}H_{31}O_3$: 319.2273); $(M+Na)^+$ *m/z* 341.2096 (calcd. for $C_{20}H_{30}O_3Na$: 341.2093).

*Base Hydrolysis of rebaudioside M (**1**)*

Compound **1** (500 µg) has been dissolved in 10 ml of MeOH at room temperature and added solid NaOH (500 mg). The mixture was stirred at room temperature for 30 min and heated to reflux for 24 hrs under continuous stirring. The reaction mixture was allowed to cool to room temperature and neutralized to pH 4.0 with 1 N HCl at 0-5^o C. The reaction solvent (MeOH) was concentrated under vacuum yielded a residue which was extracted with *n*-BuOH (3 x 25 ml). The *n*-BuOH layer was washed with water and concentrated under vacuum at low temperature to afford a crude solid, which was crystallized with methanol-acetone (1:1) yielded a pure compound which was identified as rebaudioside B (**3**) by ^{1}H and ^{13}C NMR and mass spectral data as well as in comparison of the co-TLC and spectral data with standard compound [11].

*13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oic acid (Rebaudioside B, **3**)*

White powder; mp 239-250 °C; $[\alpha]_D^{25}$ +26.24 (*c* 0.1, MeOH); ^{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 $(M+H)^+$ *m/z* 805.3848 (calcd. for $C_{38}H_{61}O_{18}$: 805.3858); $(M+Na)^+$ *m/z* 827.3672 (calcd. for $C_{38}H_{60}O_{18}Na$: 827.3677).

General procedure for acid hydrolysis and determination of sugar configuration in 1, and 3

Each compound **1** and **3** (500 µg) were hydrolyzed using 0.5 M HCl (0.5 ml) by refluxing the mixture for 1.5 h. The reaction mixture was cooled to room temperature. The reaction mixture is passed through an Amberlite IRA400 column and the eluate after chromatography has been lyophilized. The residue obtained after lyophilization 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 subsequently added *O*-tolyl isothiocyanate (12.5 ul) to the mixture and heated at 60ºC for an additional 1.5 h. The obtained reaction mixture was analyzed by HPLC [column and conditions: Phenomenex Luna C18, 150 x 4.6 mm (5 u); 25% acetonitrile-0.2% TFA water, 1 ml/min; UV detection at 250 nm]. Based on the preparation of the corresponding thiocarbamoyl-thiazolidine carboxylate derivatives, the sugars present in **1** and **3** were identified as D-glucose (*t*R, 13.12 min for **1**; and *t*R, 13.08 min for **3**) [authentic samples, D-glucose (*t*R, 13.15) and L-glucose (*t*R, 11.82 min) [12].

RESULTS AND DISCUSSION

The molecular formula of compound **2** which was isolated as a white powder was deduced as $C_{20}H_{30}O_3$ from the $[M+H]^+$ ion observed at *m/z* 319.2274, together with an adduct ion corresponding to $[M+Na]^+$ observed at *m/z* 341.2096. The ^{1}H-NMR spectrum of **2** showed the presence of three methyl singlets resonated at δ 0.78, 0.98 and 1.25; peaks between δ 0.96 and 2.64 corresponding to the nine methylene and two methine protons (Table 1). The ^{13}C NMR data obtained from HMQC and HMBC spectral data of **2** showed the presence of nine sp3 methylenes, two sp3 methines, four sp3 quaternary carbons, and three methyl groups. The ^{13}C NMR data of **2** also showed the presence of two carbonyl groups; a saturated carbonyl group at δ 222.8 and another carbonyl group δ 183.9. From the above NMR spectral data of **2**, it was found that the compound belongs to an *ent*-kaurane diterpenoid with the absence of an exocyclic double bond between C-16/C-17, and the presence of additional methyl and carbonyl

groups. The ^{13}C NMR values for all the carbons in **2** were assigned on the basis of COSY, HMQC and HMBC correlations and are given in Table 1.

Table 1. ^{1}H and ^{13}C NMR spectral data (chemical shifts and coupling constants) for isoteviol (2) and rebaudioside B (3) [a-c]

Position	2		3	
	δ_H	δ_C	δ_H	δ_C
1	0.93 (m, 1H), 1.70 (m, 1H)	39.7	0.80 (m, 1H), 1.80 (m, 1H)	40.3
2	1.41 (m, 1H), 1.85 (m, 1H)	20.3	1.42 (m, 1H), 1.924(m, 1H)	20.3
3	1.04 (m, 1H), 2.16 (br d, 12.4, 1H)	37.6	1.07 (m, 1H), 2.49 (m, 1H)	38.2
4		43.7		45.0
5	1.17 (m, 1H)	57.0	1.06 (m, 1H)	57.4
6	1.74 (m, 1H), 1.94 (m, 1H)	21.6	1.81 (m, 1H), 1.95 (m, 1H)	23.1
7	1.46 (m, 1H), 1.64 (m, 1H)	41.4	1.38 (m, 1H), 1.51 (m, 1H)	42.3
8		48.4		41.5
9	1.20 (m, 1H)	54.7	0.95 (m, 1H)	54.6
10		38.2		40.3
11	1.24 (m, 1H), 1.66 (m, 1H)	19.8	1.67 (m, 1H), 1.75 (m, 1H)	21.1
12	1.34 (m, 1H), 1.70 (m, 1H)	37.3	1.73 (m, 1H), 2.22 (m, 1H)	38.3
13		39.7		88.8
14	1.45 (m, 1H), 1.74 (m, 1H)	54.3	1.74 (m, 1H), 2.56 (d, *J*=11.6, 1H)	45.0
15	1.77 (m, 1H), 2.63 (dd, 3.3, 17.6, 1H)	48.7	2.06 (m, 1H), 2.20 (m, 1H)	48.5
16		222.8		154.5
17	0.98 (s, 3H)	18.8	5.06 (br s, 1H), 5.71 (br s, 1H)	105.3
18	1.25 (s, 3H)	28.9	1.34 (s, 3H)	29.8
19		183.9		180.6
20	0.78 (s, 3H)	13.3	1.25 (s, 3H)	16.6
1′			5.38 d (8.1)	98.5
2′			4.57 m	81.3
3′			4.32 m	88.8
4′			4.21 m	70.4
5′			3.91 m	78.0
6′			4.12 m, 4.47 m	62.8
1″			5.59 d (8.4)	105.0
2″			4.12 m	76.8
3″			4.36 m	78.5
4″			4.28 m	72.1
5″			3.86 m	79.0
6″			4.38 m, 4.56 m	63.7
1‴			5.08 d (8.1)	105.1
2‴			4.06 m	75.8
3‴			4.38 m	79.2
4‴			4.24 m	72.8
5‴			4.08 m	78.8
6‴			4.22 m, 4.50 m	62.9

[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

The presence of a free acid group in **2** at C-19 position was suggested by the key HMBC correlation, which showed that the singlet resonating at δ 1.25 corresponding to C-18 methyl group showed a correlation with the carbonyl group corresponding at δ 183.9. In the absence of any unsaturated carbons together with the presence of a saturated a carbonyl group resonating at δ 222.8 in its ^{13}C NMR spectral data with an additional methyl group at δ 0.98 in its ^{1}H NMR spectral data suggested the presence of a 13-methyl-16-oxo-17-nor-*ent*-kauran-19-oic acid (isosteviol) skeleton in compound **2**. The key COSY and HMBC correlations shown in Figure 2 and comparison of the ^{1}H NMR and ^{13}C NMR spectral data with isosteviol reported from the literature supported the structure completely [11,13].

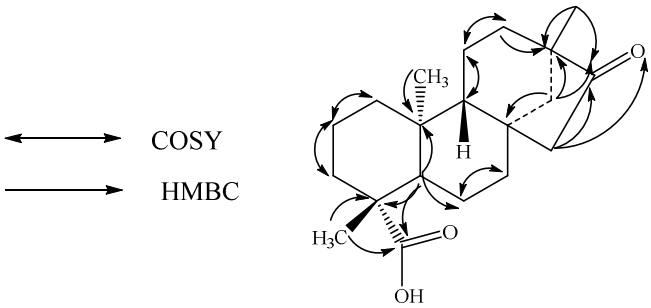

Figure 2: Key COSY and HMBC correlations of 2

Compound **3** was also isolated as a white powder; its molecular formula has been deduced as $C_{38}H_{60}O_{18}$ on the basis of its positive HR mass spectrum which showed an $[M+H]^+$ ion at *m/z* 805.3848, together with an $[M+Na]^+$ adduct ion observed at m/z 827.3672. This molecular composition was supported by ^{13}C NMR chemical shift vaues obtained based on HMQC and HMBC spectral data. The ^1H NMR spectrum of **3** showed the presence of methyl singlets at δ 1.25 and 1.34, two olefinic protons of an exocyclic double bond as broad singlets resonating at δ 5.06 and 5.71, nine methylene and two methine protons between δ 0.80-2.56 characteristic for the *ent*-kaurane diterpenoids isolated earlier from the genus *Stevia* [14-18]. The fragment ions observed at *m/z* 643, 481, and 319 in the positive mode of ESI MS/MS spectrum of **3** suggesting the presence of three hexose moieties in its structure, which was supported bytThe ^1H NMR spectrum of **3** also showed anomeric protons appeared at δ 5.08, 5.38, and 5.59. Enzymatic hydrolysis of **3** furnished an aglycone which was identified as steviol (**4**) by comparison of ^1H NMR and co-TLC with standard compound [11]. The ^1H and ^{13}C NMR values for all the carbons in **3** were assigned on the basis of COSY, HMQC and HMBC correlations and are given in Table 1. From the literature it was found that the basic skeleton of *ent*-kaurane diterpenoids was supported in the structure of **3** by the key 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. Acid hydrolysis of **3** with 5% H_2SO_4 afforded glucose which was identified by direct comparison with authentic samples by TLC [8-10]. 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 [12].

From the NMR spectral data and hydrolysis experiments of **3**, it was concluded that there are three β-D-glucosyl units in its structure. Further, the presence of two β-D-glucosyl units has been confirmed at C-2′ and C-3′ of sugar I in **3** by the downfield shift for both the ^1H and ^{13}C chemical shifts supporting the presence of a 2,3-branched β-D-glucotriosyl unit in its structure. Comparison of the ^1H and ^{13}C NMR spectral data of **3** with rebaudiosides M (**1**) isolated from *S. rebaudiana* Bertoni suggested that it is also a steviol glycoside having three glucose residues that are attached at the C-13 hydroxyl as a 2,3-branched β-D-glucotriosyl substituent with a free carboxylic acid group at C-19 position, which was supported by the HMBC spectral data in which none of the three anomeric protons showed correlation to the carbonyl group appeared at δ 180.6, whereas the C-18 methyl group resonating at δ 1.34 showed a correlation. The large coupling constants for the three anomeric protons observed at δ 5.08 (d, *J*=8.1 Hz), 5.38 (d, *J*=8.1 Hz), and 5.59 (d, *J*=8.4 Hz) suggested their β-orientation as reported for steviol glycosides [14-18] isolated earlier. Thus, the structure of **3** 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 which was confirmed by the key COSY and HMBC correlations as shown in Figure 3; that are consistent with the literature data of reabudioside B [11].

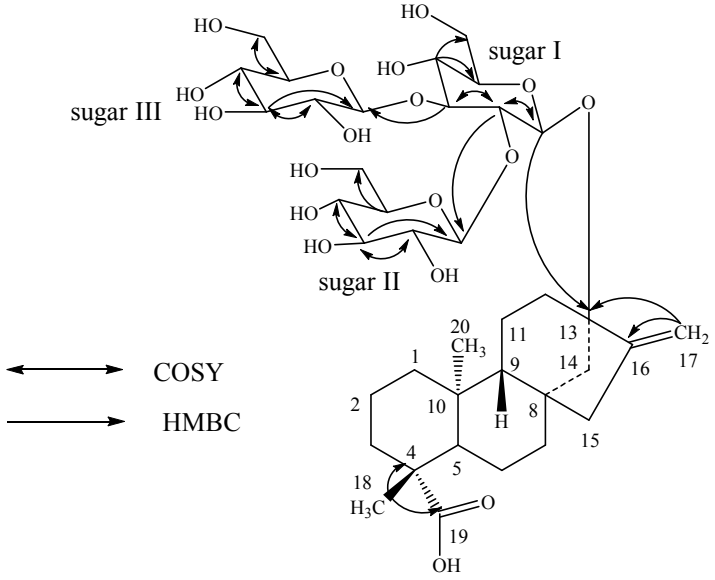

Figure 3: Key COSY and HMBC correlations of 3

CONCLUSION

We are herewith reporting the synthesis of isosteviol and rebaudioside respectively from the acid and base hydrolysis of rebaudioside M (**1**) for the first time. The complete ^1H and ^{13}C NMR spectral assignments for isosteviol (**2**) and 13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-

19-oic acid (rebaudioside B, **3**) were assigned on the basis of extensive 1D and 2D NMR as well as mass spectral data.

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