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Research Article

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Controlled hydrolysis studies of the diterpene glycosides rebaudioside D, and rebaudioside E of *Stevia rebaudiana* Bertoni

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ABSTRACT

*Controlled hydrolysis studies of the two sweet diterpene glycosides of Stevia rebaudiana Bertoni namely 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oic acid-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) ester (rebaudioside D, **1**) resulted in the formation of rebaudioside A, whereas 13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] ent-kaur-16-en-19-oic acid-(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl) ester (rebaudioside E, **2**) yielded stevioside and steviolbioside. The structures of the isolated compounds were characterized on the basis of spectroscopic data as well as comparison with standard compounds.*

Keywords: *Stevia rebaudiana*; Asteraceae; rebaudioside A; rebaudioside E; hydrolysis studies; structural characterization

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INTRODUCTION

Stevia rebaudiana Bertoni, a perennial shrub belong to the family of Asteraceae (Compositae) native to Paraguay and Brazil. Extracts of the leaves of *S. rebaudiana* have been used for decades to sweeten food and beverages in Japan, South America and China [1-2]. The major constituents in the leaves of *S. rebaudiana* are the potently sweet glycosides namely stevioside, and rebaudiosides A; which are glycosides of *ent*-13-hydroxykaur-16-en-19-oic acid (steviol) [3-4]. These compounds are also known as Stevia sweeteners. Rebaudioside A has a 2,3-disubstituted-β-D-glucotriosyl unit at *C*-13 position in the form of an ether and a 2-β-D-glucobiosyl unit at *C*-19 position in the form of an ester of the aglyconesteviol; whereas rebaudioside Eis having two 2-β-D-glucobiosyl units at *C*-13 and *C*-19 positions in the form of an ether and ester of the aglyconesteviol(Figure 1). Rebaudioside A (**1**) tastes about 200-300 times sweeter than sucrose; and rebaudioside E(**2**) tastes about 150-200 times sweeter than sucrose; both are non-caloric.

As a part of our research to discover natural sweeteners, we have collected commercial extracts of *S. rebaudiana* from various suppliers all over the world and in the process of isolating minor novel diterpene glycosides [5-6]. Apart from isolating novel compounds from *S. rebaudiana* and utilizing them as possible natural sweeteners or sweetness enhancers, we are also engaged in developing analytical methods for separation of steviol glycosides [7-8], stability studies of steviol glycosides, and synthetic methodology for important steviol glycosides of commercial significance and present in trace quantities in the original *S. rebaudiana* extract. In this paper, we are describing the synthesis of the two major steviol glycosides rebaudioside A (**3**) and stevioside (**4**) as well as the minor compound steviolbioside (**5**) (Figure 2) from the controlled hydrolysis studies of rebaudioside D (**1**), and rebaudioside E (**2**).

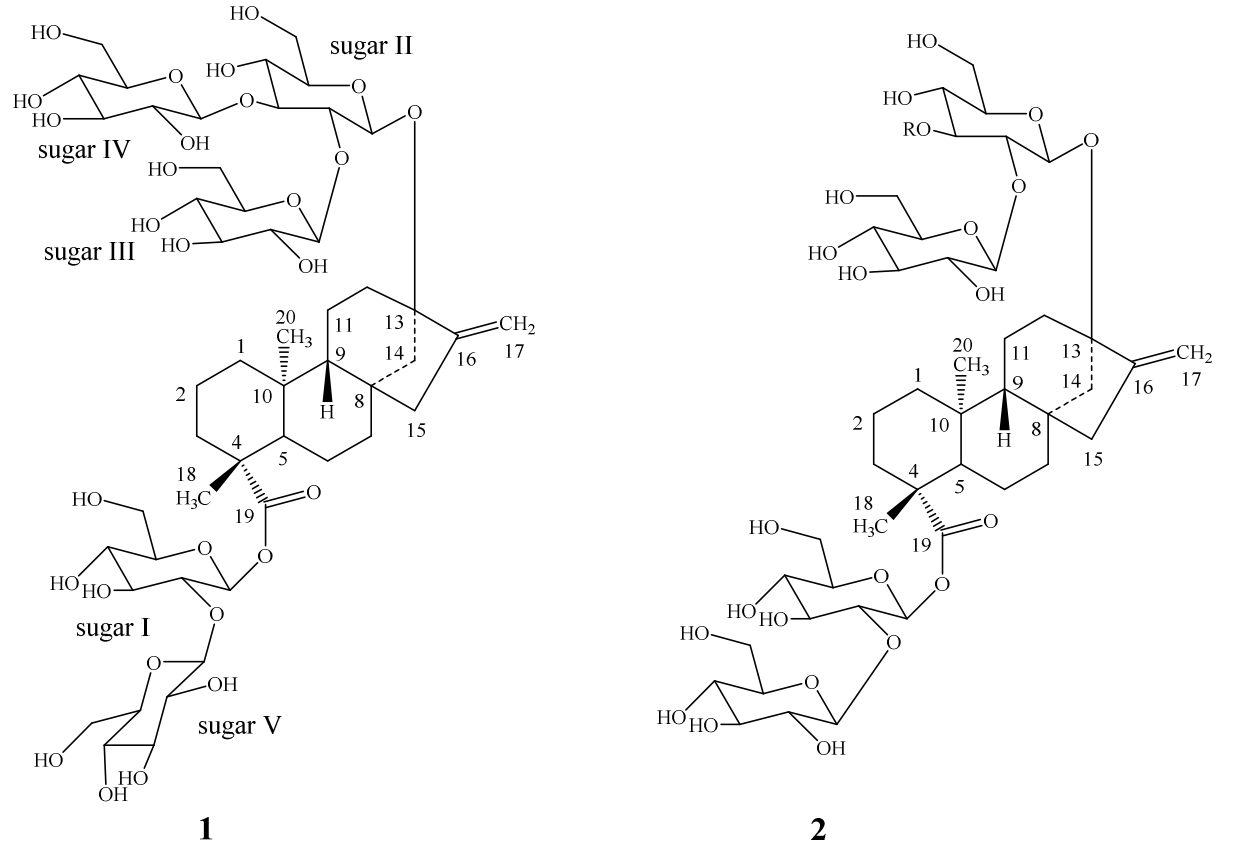


Figure 1. Structures of rebaudioside D (1), and rebaudioside E (2)

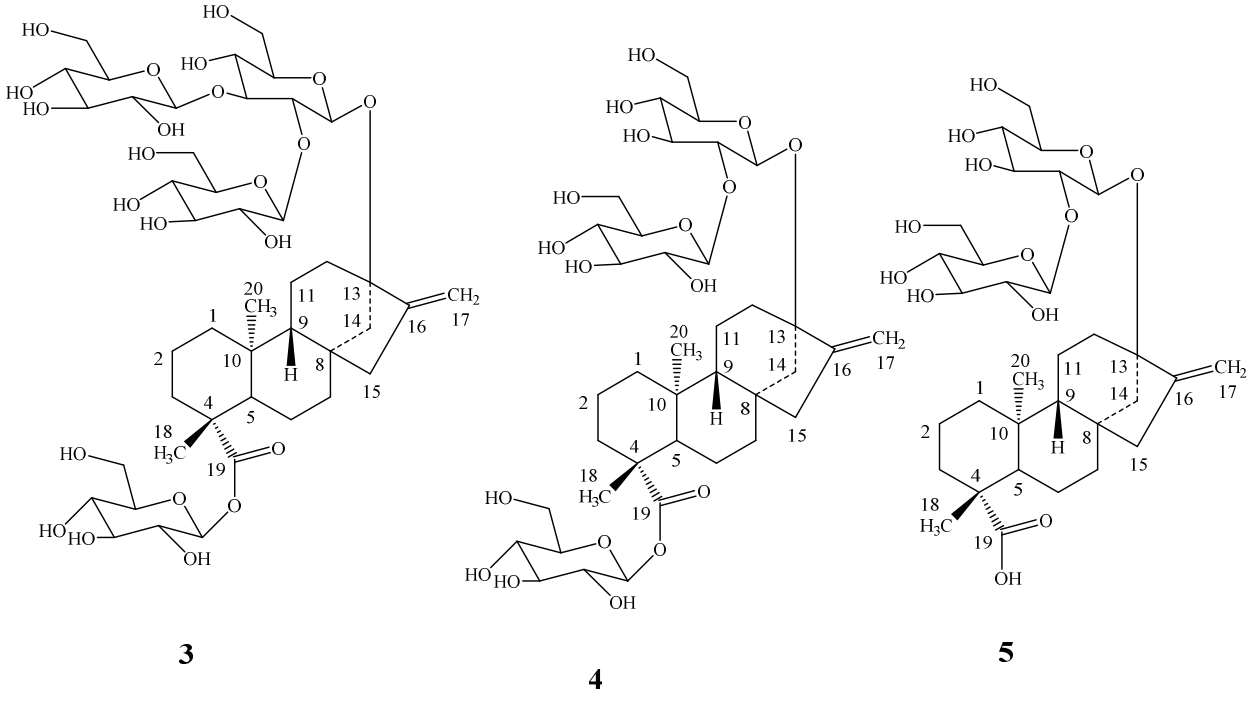


Figure 2. Structures of rebaudioside A (3), stevioside (4), and steviolbioside (5)

EXPERIMENTAL SECTION

General Experimental Procedures

Melting points were measured using a SRS Optimelt MPA 100 instrument and are uncorrected. 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. The data acquisition was done using a Chemstation A 10.02 software. The column used for HPLC analysis was a reversed-phase C18 (2) 100 A Phenomenex (Torrance CA) (Length: 250 mm, inner diameter 4.6 mm, particle size: 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 ^{o}C, and tube lens voltage = 75. Sample was diluted with 2:2:1 CH_3CN:MeOH:water (same as infusion eluent) and injected 50 microliters. TLC was performed on Baker Si-C_{18}F plates with mobile phase H_2O-MeOH (80:20). Identification of the spots on the TLC plate was carried out by spraying 10% H_2SO_4 in EtOH and heating the plate at about 80^{o} C.

Synthesis of rebaudioside A (3) from rebaudioside D (1)

Compound **1** (10 mg) was dissolved in 20 ml of MeOH at room temperature and added 20 ml of 5% aqueous NaOH. The solution was heated to reflux and the reaction mixture was continuously monitored by TLC against the standard compounds rebaudioside D(**1**) and rebaudioside A (**3**).After 2 h, a compound having retention factor (R_f) corresponding to rebaudioside A (**3**)has been appeared in TLC and continued refluxing. After an additional three hours reflux, the yield of the compound corresponding tothe R_f of rebaudioside A (**3**) is increased, and in addition a compound corresponding to the R_f of stevioside (**4**) was observed on TLC. Further reflux of the reaction mixture for 3 h resulted in the absence of the compound corresponding to the **3** and formation of compounds corresponding to the R_f of stevioside (**4**) and steviolbioside (**5**). Since the goal is to synthesize rebaudioside A (**3**), performed the same reaction once again with **1**and continued reflux for 5 h only. The mixture was cooled to room temperature and neutralized to pH 4.0 with 1 N HCl at 5-10^{o} C. The solvent was concentrated under vacuum and the product was extracted with *n*-BuOH (3 x 25 ml). The *n*-BuOH layer was washed with water (2 x 15 ml) and concentrated under vacuum at low temperature afforded a crude solid which was purified on reversed-phase preparative TLC using water: MeOH (80:20) yielded a pure compound (3.8 mg), which was identified to be rebaudioside A (**3**) in comparison of the co-TLC and co-HPLC [7, 8] with standard compound, and was also further confirmed on the basis of NMR and HRMS data [9].

Synthesis of stevioside (4) from rebaudioside E (2)

Compound **2** (5 mg) was dissolved in 10 ml of MeOH at room temperature and added 10 ml of 5% aqueous NaOH. The solution was heated to reflux and the reaction mixture was continuously monitored by TLC against the standard compounds rebaudioside E(**2**) and stevioside (**4**). After 6 h, a compound having retention factor (R_f) corresponding to stevioside (**4**) has been appeared in TLC and continued refluxing. After an additional three hours reflux, the yield of the compound corresponding to the R_f of stevioside (**4**) is increased, and in addition a compound corresponding to the R_f of steviolbioside (**5**) has been appeared on TLC. Further reflux of the reaction mixture for 3 h more resulted in the absence of the compound corresponding to**4**and formation of a compound corresponding to the R_f of steviolbioside (**5**). Since the goal is to synthesize stevioside (**4**), performed the same above reaction once again with **1** and continued reflux for 9 h. The mixture was cooled to room temperature and neutralized to pH 4.0 with 1 N HCl at 5-10^{o} C. The solvent was concentrated under vacuum and the product was extracted with *n*-BuOH (3 x 25 ml). Work-up of the reaction as in *2.2.*, and purification of the crude extract of the *n*-BuOH layer on reversed-phase preparative TLC yielded a pure compound (2.6 mg), identified to stevioside (**4**) on the basis of the co-TLC and co-HPLC [7, 8] with standard compound, and was also further confirmed on the basis of NMR and HRMS data [10].

Synthesis of steviolbioside (5) from rebaudioside E (2)

To a solution of compound **2** (10 mg) in 20 ml of MeOH at room temperature was added 20 ml10% aqueous NaOH and the mixture was heated to refluxfor 16 h under continuous stirring. The reaction mixture was cooled to room temperature and then neutralized to pH 4.0 with 1 N HCl at 5-10^{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) yielded a pure compound (3.2 mg), which was identified assteviolbioside (**5**) based on the co-TLC and co-HPLC [7, 8] with standard compound, and was also further confirmed on the basis of NMR and HRMS data [10].

Physical and Spectroscopic Data of Compounds 3-5

*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, **3**):*

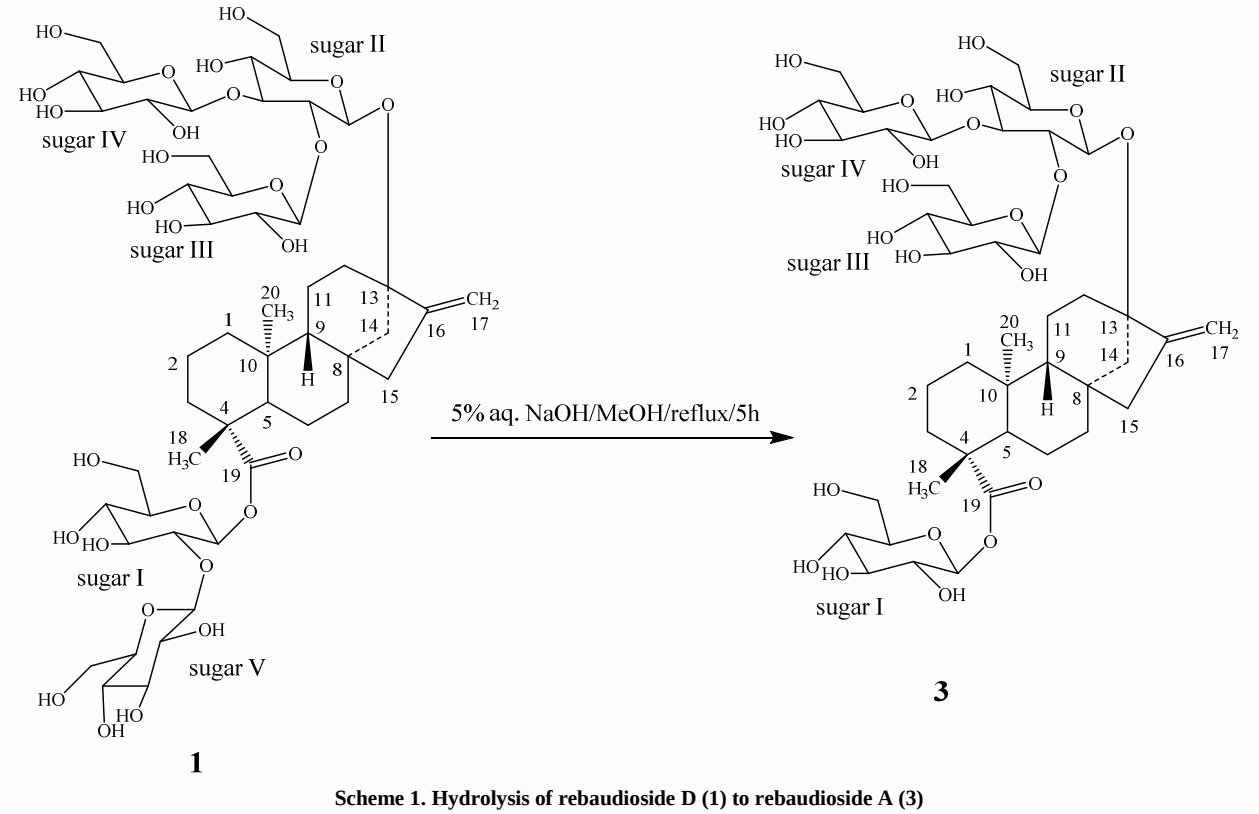
White powder; mp 240-243 °C; ^{1}H-NMR (600 MHz, CD_3OD + D_2O, δ ppm) and ^{13}C-NMR (150 MHz, CD_3OD + D_2O, δ ppm) spectroscopic data see Table 1; HRMS $(M+Na)^+$ m/z 989.4194 (calcd. for $C_{44}H_{70}O_{24}Na$: 989.4206).

*13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-ent-kaur-16-en-19-oic acid β-D-glucopyranosyl ester (stevioside, **4**):*
White powder; mp 194-196 °C; ^{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 $(M+Na)^+$*m/z*827.3658 (calcd. for $C_{38}H_{60}O_{18}Na$: 827.3677).

*13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-ent-kaur-16-en-19-oic acid (steviolbioside, **5**):*
White powder; mp 192-195 °C; ^{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 1; HRMS (M + Na)$^+$*m/z* 665.3132 (calcd. for $C_{32}H_{50}O_{13}Na$: 665.3149).

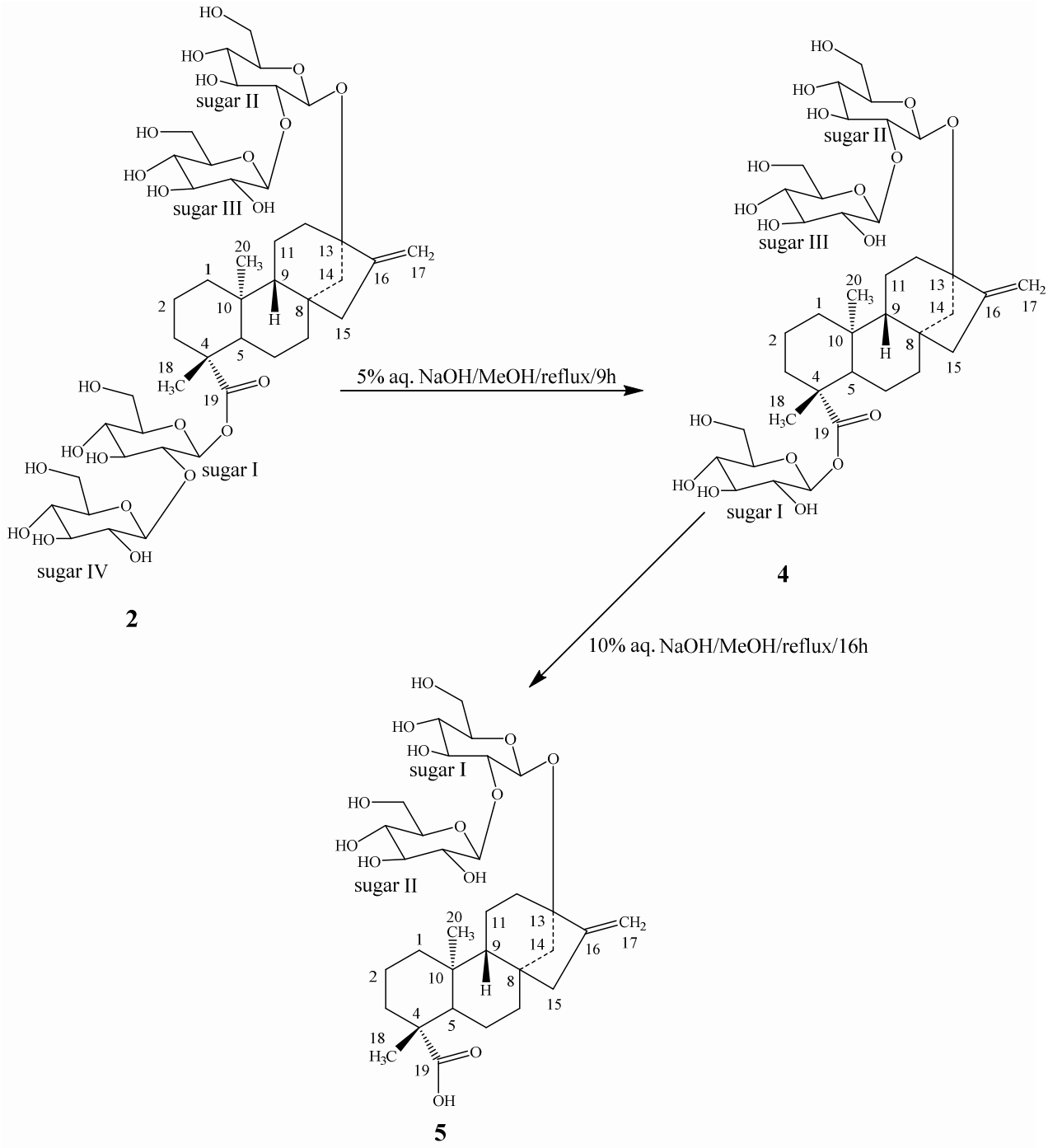
RESULTS AND DISCUSSION

Hydrolysis of the two *ent*-kaurane diterpene glycosides isolated from *S. rebaudiana* were performed using aqueous NaOH solution; rebaudioside D (**1**)furnished rebaudioside A (**3**) as shown in Scheme 1, whereas and rebaudioside E (**2**) yielded stevioside (**4**), and steviolbioside (**5**) as shown in Scheme 2.



Scheme 1. Hydrolysis of rebaudioside D (1) to rebaudioside A (3)

Spectroscopy and Structural Characterization of Hydrolyzed Compounds 3-5
Structural characterization of the hydrolyzed compounds **3**–**5** obtained by the aqueous NaOH of the two *ent*-kaurane diterpene glycosides isolated from *S. rebaudiana*; rebaudioside D (**1**),and rebaudioside E (**2**) has been achieved on the basis of one dimensional (^{1}H, ^{13}C), two-dimensional (^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC, ^{1}H-^{13}C HMBC) NMR and HR mass spectral data as well as in comparison with the co-TLC of standard compounds. The selected proton and carbon NMR spectral data of the hydrolyzed compounds **3**-**5**for the sp3 methyl groups, exocyclic doubles bonds, carboxylic acid group, and anomeric carbons are given in in Table 1.



Scheme 2. Hydrolysis of rebaudioside E (2) to stevioside (4) and steviolbioside (5)

Table 1.[1]H and [13]C NMR spectral data (chemical shifts and coupling constants) for rebaudioside A (3), stevioside (4), and steviolbioside (5) [a–c]

Position	3		4		5	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
16		152.3		153.5		154.0
17	4.81 (s, 1H), 5.15 (s, 1H)	104.7	4.85 (s, 1H), 5.20 (s, 1H)	105.4	4.86 (s, 1H), 5.24 (s, 1H)	105.7
18	1.1 (s, 3H)	28.0	1.30 (s, 3H)	28.5	1.18 (s, 3H)	28.7
19		178.1		178.8		180.6
20	0.87 (s, 3H)	15.3	1.24 (s, 3H)	16.4	1.00 (s, 3H)	16.5
1′	5.29 (d, *J*=8.4, 1H)	94.7	5.40 (d, 8.2, 1H)	95.7	4.61 (d, 7.8, 1H)	97.7
1″	4.60 (d, *J*=7.8, 1H)	96.4	4.61 (d, 7.8, 1H)	97.5	4.60 (d, 8.1, 1H)	105.4
1‴	4.58 (d, *J*=7.6, 1H)	102.7	4.58 (d, 7.8, 1H)	105.0		
1⁗	4.75 (d, *J*=7.4, 1H)	103.0				

[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.

Compound **3** 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 HRMS, which showed an $[M+Na]^+$ adduct ion at *m/z*989.4194. The ^{1}H NMR spectrum of **3** showed the presence of two methyl singlets at δ 0.88 and 1.15, two olefinic protons as singlets at δ 4.81 and 5.15 of an exocyclic double bond, nine methylene and two methine protons between. The ^{1}H NMR spectrum of **3** also showed the presence four sugar units in its structure which were supported by the anomeric protons at δ 4.58, 4.60, 4.77, and 5.31. Acid hydrolysis of **3** with 5% H_2SO_4 afforded glucose which was identified by direct comparison with authentic samples by TLC. Further, the large coupling constants observed for the four anomeric protons of the glucose moieties suggested their β-orientation as reported for steviol glycosides [11-15]. The COSY and HMBC correlations suggested that compound **3** is having three glucose residues that are attached at the *C*-13 hydroxyl as a 2,3-branched β-D-glucotriosyl substituent in the form of an ether and another β-D-glucosyl unit in the form of an ester at *C*-19. Based on the results from chemical and spectral studies, **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 β-D-glucopyranosyl ester and its spectral studies are consistent with the literature data of reabudioside A [9].

The molecular formula of compound **4** was deduced as $C_{38}H_{60}O_{18}$from its HRMS data that showed an $[M+Na]^+$ adduct ion at *m/z* 827.3658. The ^{1}H NMR spectrum of **4** showed the presence of two methyl singlets, two protons as singlets for an exocyclic double bond, nine methylene and two methine protons. The ^{1}H NMR of **4** showed the presence of three anomeric protons as doublets suggesting the presence three sugar residues in its structure which was confirmed as D-glucose on the basis of acid hydrolysis, together with the coupling constants of the anomeric protons confirming the presence of three β-D-glucosyl moieties in **4**.The attachment of the threeβ-D-glucosyl units were assigned on the basis of COSY, HMQC and HMBC correlations and were identified as a 2-β-D-glucobiosyl substituent in the form of an ether at *C*-13 position and another β-D-glucosyl unit at *C*-19 in the form of an ester. Based on the above spectral and chemical results, **4** was assigned as 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-*ent*-kaur-16-en-19-oic acid β-D-glucopyranosyl ester (stevioside) [10].

Compound **5** was also 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 $[M+Na]^+$ adduct at *m/z* 665.3132. The ^{1}H NMR spectrum of **5** showed the presence of two methyl singlets, two olefinic protons as singlets of an exocyclic double bond, nine methylenes and two methines, as in **3** and **4**. The ^{1}H NMR spectrum of **5** also indicated the presence of two anomeric protons suggesting the presence of two sugar units in its structure, and acid hydrolysis confirmed the sugars as D-glucose. The large coupling constants observed for the two anomeric protons of the glucose moieties suggested the β-orientation as reported for steviol glycosides [11-15]. The COSY, HMQC and HMBC correlations suggested the presence of a 2-β-D-glucobiosyl substituent at *C*-13 position with a free carboxylic acid at *C*-19 position confirming the structure as 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid (steviolbioside) which was further supported by the comparison with literature data [10].

CONCLUSION

Hydrolysis studies of rebaudioside D (**1**), and rebaudioside E (**2**), using aqueous sodium hydroxide solutions resulted in the formation of rebaudioside A, stevioside and steviolbioside. The structures of the isolated compounds were characterized on the basis of spectroscopic data (NMR and HRMS) as well as comparison with standard compounds. To the best of our knowledge this is the first report of the synthesis of rebaudioside A, stevioside and steviolbioside from the known steviol glycosides rebaudioside D, and rebaudioside E.

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