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NEW SWEET DITERPENE GLUCOSIDES FROM *STEVIA REBAUDIANA*

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Abstract—From the leaves of *Stevia rebaudiana*, two new sweet glucosides, rebaudiosides A and B, were isolated besides the known glucosides, stevioside and steviolbioside. On the basis of IR, MS, ^1H and ^{13}C NMR as well as chemical evidences, the structure of rebaudioside B was assigned as 13-*O*-[β-glucosyl(1-2)-β-glucosyl(1-3)]-β-glucosyl-steviol and rebaudioside A was formulated as its β-glucosyl ester.

INTRODUCTION

Stevioside (**1**), a principal constituent of the leaves of *Stevia rebaudiana* Bertoni, a wild herb of Paraguay is known to be one of the sweetest natural products [1]. In an attempt to produce **1** as a substitute for synthetic sweeteners, this plant has been cultivated extensively in Japan. The present authors have previously investigated chemical constituents of several *Stevia* species [2,3]; this paper reports the isolation and structural determination of two new sweet steviol glucosides of *S. rebaudiana*.

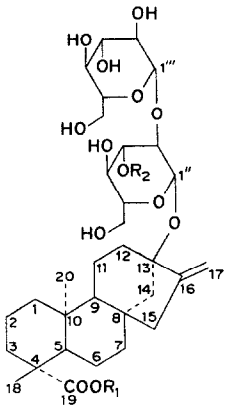
RESULTS AND DISCUSSION

An aqueous suspension of MeOH extract of the dried leaves was washed with Et_2O and then extracted with *n*-BuOH. Recrystallization of the BuOH-soluble fraction from MeOH gave **1**. The mother liquor which was shown to contain several glycosides by TLC was chromatographed affording **1**, steviolbioside (**2**) [1], and two new glycosides, named rebaudiosides A (**3**) and B (**4**).

Rebaudioside A (**3**) (yield 1.4%), $C_{44}H_{70}O_{23}\cdot 3H_2O$ exhibited an IR band due to ester-glycoside linkage at 1728 cm^{-1} (KBr). Rebaudioside B (**4**) (yield 0.04%), $C_{38}H_{60}O_{18}\cdot 2H_2O$ showed an IR band attributable to COOH at 1700 cm^{-1} (Nujol). On methylation by Hakomori's procedure [4], **4** yielded a methyl ester of decamethyl ether (**5**), $C_{49}H_{82}O_{18}$, IR ν_{max} 1730 cm^{-1} ($COOCH_3$), no OH absorption (CCl_4), PMR; see Experimental. Alkaline saponification of **3** yielded **4** and 1,6-anhydroglucopyranose [1], which was identified by PMR [5] and its conversion into glucose by acid treatment.

Acid hydrolysis of **3** or **4** gave glucose and isosteviol (**6**), which is a rearranged aglycone previously obtained from **1** by acid hydrolysis [1]. During the course of the structural elucidation of **1**, the acid unstable genuine aglycone, steviol (**7**) could not be prepared from **1** by acid hydrolysis but obtained only by enzymatic hydrolysis [1,6]. It has been reported that hydrolysis of **1** with snail enzyme [6] and pectinase [7] gives (**7**) and recently, a glucosidase in the crude preparation of hesperidinase [8] was found to give **7** from **1** almost quantitatively [9]. Treatment of **3** with this crude enzyme however did not yield **7** but yielded glucose and a partially hydrolyzed product which was proved to be identical with **4**. Identification of the genuine aglycone of **3** and **4** as well as respective correlation of **3** and **4** with **1** and **2** were achieved effectively by CMR. All of the signals due to the aglycone moiety of **1** and **2** in the CMR of kaurene type diterpenes and their glucosides have been assigned [10]. The spectra of **3** and **4** exhibited signals due to carbons of the aglycone moiety at almost the same position as those of **1** and **2**, respectively, except for slight differences (0.6–0.7 ppm) of the signals of C-12 and -13 carbons (Table 1). This indicates that **3** and **4** have the same aglycone, steviol (**7**) as that of **1** and **2**. Further, it has been noted that in the spectra of **1** and **2**, anomeric carbon signals linked with the tertiary OH at C-13 or with 19-COOH appears at somewhat higher region than that of a β-glucoside of a usual secondary OH [10] (Table 1). Comparison of the anomeric carbon signals of **3** and **4** with those of **1** and **2** coupled with the evidence of the alkaline saponification of **3** to **4** led to structures for **4** and **3** as 13-*O*-(tri-β-glucosyl)-steviol and its β-glucosyl ester, respectively. A fragment ion at *m/e* 627 (deca-*O*-methyl-triglucosyl ion (**8**)) of MS of **5** supported this formulation and β-glucosyl linkage in **3** and **4** were substantiated also by coupling constant (J = 6–8 Hz) of PMR signals of anomeric protons of **3**, **4**, and **5** (see Experimental).

Methanolysis of **5** yielded methyl 2,3,4,6-tetra-*O*-methyl-glucopyranoside and a methyl di-*O*-methylglucopyranoside (**9**). Recently, Matsubara and Hayashi reported that MS of partially methylated methyl glucosides and their derivatives (TMSi) are quite effective for their identification and mutual differentiation [11]. MS of **9** and it TMSi derivative showed the same fragment ions with similar relative intensities as those of methyl 4,6-di-*O*-methylglucopyranoside and its TMSi derivative, respectively, being clearly different from those of other partially

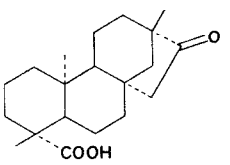


(**1**) R_1 = Glc, R_2 = H

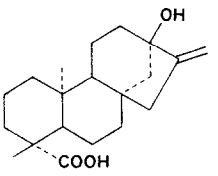
(**2**) R_1 = R_2 = H

(**3**) R_1 = R_2 = Glc

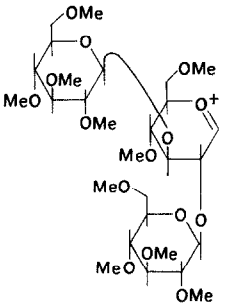
(**4**) R_1 = H, R_2 = Glc



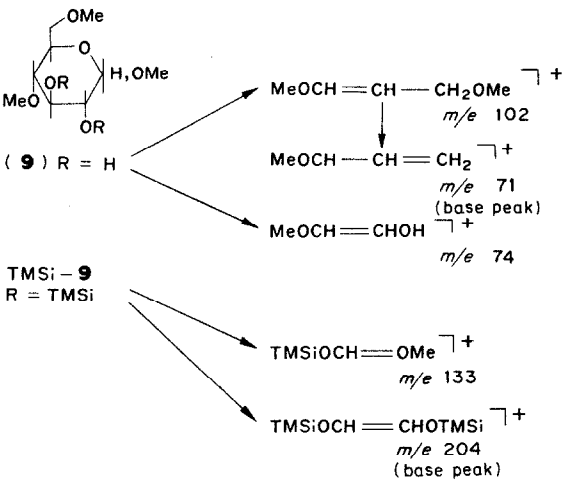
(**6**)



(**7**)



(**8**) *m/e* 627



methylated methyl glucosides and their TMSi derivatives. It follows that **4** should be represented by 13-*O*-[β-glucopyranosyl(1–2)-β-glucopyranosyl(1–3)]-β-glucopyranosyl-steviol and consequently **3** must be its β-glucosyl ester.

EXPERIMENTAL

Mp's are uncorrected; MS were determined at 75 eV. NMR were determined on JEOL PS-100 FT spectrometer at 25° unless otherwise stated; PMR at 100 MHz and CMR at 25 MHz.

Table 1. CMR chemical shift for stevioside (**1**), steviolbioside (**2**), rebaudiosides A (**3**) and B (**4**) and steviol (**7**). (δ ppm from TMS) in C_5D_5N

	Compound				
	1	**2**	**3**	**4**	**7**
C-1	40.7	40.9	40.9	41.0	41.1
2	19.2	19.7	19.4	19.8	19.8
3	38.1	38.5	38.3	38.7	38.6
4	43.9	43.8	44.0	43.8	43.9
5	57.3	56.9	57.4	57.0	57.1
6	22.0	22.4	22.2	22.5	22.6
7	41.5	41.5	41.8	41.8	41.8
8	42.5	42.4	42.4	42.2	41.8
9	53.8	54.1	54.0	54.1	54.3
10	39.7	39.6	39.8	39.8	39.8
11	20.6	20.5	20.7	20.6	20.8
12	36.6	37.2	37.3	37.8	40.7
13	85.9	86.1	86.6	86.8	79.8
14	44.3	44.7	44.5	44.5	47.4
15	47.5	47.9	47.9	47.9	48.1
16	154.3	154.0	153.9	153.7	157.6
17	104.5	104.9	104.5	104.6	102.9
18	28.2	29.2	28.3	29.3	29.3
19	177.0	180.1	177.0	180.2	180.0
20	15.4	15.9	15.5	16.1	15.9
1′	95.6	—	95.6	—	—
1″	97.7	97.6	97.9	97.8	—
1‴	106.5	106.2	104.5	104.4	—
1⁗	—	—	104.5	104.6	—

Plant material. S. rebaudiana was cultivated in Kasukabe Experimental Station of Medicinal Plants, Kasukabe-shi, Saitama-ken and harvested in September, 1974.

Extraction and separation of glucosides. Dried leaves (224 g) were extracted with hot MeOH and the MeOH-soln was concentrated to dryness. A suspension of the residue (70 g) in H_2O was washed with Et_2O and then extracted with *n*-BuOH. The BuOH-layer was evaporated to dryness and the residue (54 g) was recrystallized from MeOH affording **1** as colourless needles (4.9 g). The mother liquor was evaporated to dryness and the residue was chromatographed on Si gel. Elution with $CHCl_3$–MeOH–H_2O (45:9:1) gave **2** (90 mg) which was identified with an authentic sample prepared from **1** by alkaline hydrolysis. Further elution with $CHCl_3$–MeOH–H_2O (45:15:2) afforded **4** (93 mg), **1** (750 mg), and finally **3** (3.2 g). TLC: On Si gel, solvent $CHCl_3$–MeOH–H_2O (30:20:4 homogeneous), detection H_2SO_4. R_f values **3**: 0.38, **1**: 0.49, **4**: 0.59, and **2**: 0.77.

Rebaudioside A (**3**). Colourless needles (from MeOH), mp 242–244°, $[\alpha]_D^{24}$ −20.8° (*c* = 0.84, MeOH). PMR (C_5D_5N at 80°): δ 1.24(6H, *s*), 4.91(1H, *d*, *J* 8 Hz, anomeric H), 5.10(1H, *d*, *J* 6 Hz, anomeric H), 5.34(1H, *d*, *J* 6 Hz, anomeric H), 5.94(1H, *d*, *J* 7 Hz, anomeric H), 4.95 and 5.52 (each 1H, *br s*, C-17 vinylic protons). Found: C, 52.06; H, 7.77. $C_{44}H_{70}O_{23}\cdot 3H_2O$ requires C, 51.76; H, 7.50%.

Rebaudioside B (**4**) Colourless needles (from MeOH), mp 193–195°, $[\alpha]_D^{24}$ −45.4° (*c* = 0.96, MeOH). PMR (C_5D_5N): δ 1.20(3H, *s*), 1.31(3H, *s*), 5.30(1H, *d*, *J* 7 Hz, anomeric H), 5.50(1H, *d*, *J* 7 Hz, anomeric H), 5.64(1H, *br s*, C-17 vinylic proton), one of the anomeic proton signals was overlapped on one of the C-17 vinylic proton signals at δ 5.03. Found: C, 53.94, H, 7.45. $C_{38}H_{60}O_{18}\cdot 2H_2O$ requires C, 54.27; H, 7.67%.

Methylation of **4**. A mixture of NaH (300 mg) and DMSO (10 ml) was heated at 65° for 1 hr under N_2 and to this mixture

was added a solution of **4** (300 mg) in DMSO (10 ml). After heating at 65° for 15 min, MeI (5 ml) was added to this mixture and the soln was stirred at room temp for 3 hr. After dilution with excess of H_2O, the reaction mixture was extracted with $CHCl_3$ and the $CHCl_3$-layer was washed, dried, and evaporated to dryness. The resulted residue was crystallized from *n*-C_6H_{14} to yield **5** (247 mg), colourless needles, mp 147–148.5°, $[\alpha]_D^{24}$ -10.0° (*c* = 0.90, $CHCl_3$), PMR ($CDCl_3$): δ 0.84(3H, *s*), 1.16(3H, *s*), 3.30(3H × 2, *s*, 2 × OMe), 3.37(3H, *s*, OMe), 3.44(3H × 2, *s*, 2 × OMe), 3.48(3H, *s*, OMe), 3.50(3H × 2, *s*, 2 × OMe), 3.53(3H, *s*, OMe), 3.55(3H, *s*, OMe), 3.62(3H, *s*, OMe), 4.38(1H, *d*, *J* 7 Hz, anomeric H), 4.80(1H, *d*, *J* 8 Hz, anomeric H), 4.88(1H, *d*, *J* 7 Hz, anomeric H), 4.76 and 5.16 (each 1H, *br s*, C-17 vinylic protons). Found: C, 61.66; H, 8.47. $C_{49}H_{82}O_{18}$ requires C, 61.36; H, 8.62%.

Alkaline saponification of **3**. A soln of **3** (300 mg) in 10% KOH-EtOH (18 ml) was refluxed for 2 hr. The solution was diluted with H_2O, acidified with AcOH, and extracted with *n*-BuOH. The BuOH-layer was washed with H_2O and concentrated at low temperature *in vacuo*. The residue was crystallized from MeOH to give **4** (100 mg), identical with natural rebaudioside B by comparison of TLC and PMR and mmp. The H_2O-layer was neutralized by passing through a column of ion exchange resin (Amberlite MB-3) and evaporated to dryness. The crystalline residue which was homogeneous by TLC (solvent: $CHCl_3$–MeOH–H_2O 15:10:2, Si gel) was proved to be 1,6-anhydro-β-glucopyranose by comparison of PMR(D_2O) with that reported by Heyns and Weyer [5]. This was further confirmed by conversion of this residue to glucose by refluxing with 5% H_2SO_4 (5 ml) for 2 hr. Detection of glucose: GLC of TMSi, detector–dual FID, N_2 at 1.2 kg cm^2, isothermal 170°, column–3 mm × 2 m packed with 1.5% OV-1.

Acid hydrolysis of **3** *and* **4**. A soln of **3** (50 mg) in 10% H_2SO_4 (15 ml) was refluxed for 2 hr and the reaction mixture was extracted with Et_2O. Et_2O-layer was washed and concentrated to dryness. On recrystallization from aqueous EtOH, the residue gave colourless needles, mp 223°, $[\alpha]_D^{24}$ −69.3° (*c* = 0.075, 95% EtOH), identical with an authentic sample of **6** by mmp, and TLC comparison, GLC of the methyl ester (dual FID detector, N_2 1 kg/cm^2, isothermal 200°, column–3 mm × 2 m packed with 1.5% OV-1, retention time of the methyl ester of **6** – 18.9 min) and optical rotation. The aq layer was deionized and concentrated affording glucose which was identified by GLC of TMSi and PC. Rebaudioside B (**4**) was hydrolyzed in the same way as above also yielding **6** and glucose.

Enzymatic hydrolysis of **3**. A soln of **3** (5 mg) and crude hesperidinase (5 mg, produced by Tanabe Pharm. Ind. Co. Ltd.) in 0.2 M Pi buffer (pH 4.0, 7 ml) with 2 drops of $C_6H_5CH_3$ was incubated at 40° for 48 hr. The product was recrystallized from MeOH to give **4** identical with an authentic sample by mmp and comparison of PMR (C_5D_5N) and TLC. The aqueous layer was dialyzed against H_2O and the dialyzed fraction was deionized and concentrated; glucose was detected.

Methanolysis of **5**. A soln of **5** (100 mg) in 8% HCl–MeOH (3 ml) was refluxed for 2 hr. To the reaction mixture was added Ag_2CO_3 and the ppt. was removed by filtration. The filtrate was concentrated to dryness and GLC of the residue indicated the presence of methyl 2,3,4,6-tetra-*O*-methyl-α- and β-glucopyranosides (retention time: 3.8 and 5.1 min) and methyl 4,6-di-*O*-methyl-α- and β-glucopyranosides (**9**) (retention time: 22.1 and 27.5). Condition of GLC: Dual FID detector, N_2 1 kg/cm^2, isothermal 170°, column 3 mm × 2 m packed with 5% NPGS. The residue was chromatographed on Si gel and elution with C_6H_6–Me_2CO (4:1) afforded **9**: MS of **9** (75 eV) *m/e* (relative intensity) 159(2), 133(5), 102(24), 101(15), 88(13), 87(71), 75(27), 74(78), 71(100), 45(7). MS of TMSi **9** *m/e* (relative intensity) 217(10), 204(100), 159(60), 147(69), 146(29), 133 (69), 101(14), 88(15), 87(21), 75(48), 74(28), 71(79), 45(43). It should be noted that the fragment ion of TMSi-**9** at *m/e* 204 (base peak) is characteristic of methyl 2,3-di-*O*-TMSi-4,6-di-*O*-methylglucopyranoside and is detected in MS of none of other methyl di-*O*-TMSi-di-*O*-methylglucosides [11] (Chart 2).

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