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Rebaudioside F, a diterpene glycoside from *Stevia rebaudiana*

Alvin N. Starratt[a,*], Christopher W. Kirby[b], Robert Pocs[a], James E. Brandle[a]

[a]*Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, 1391 Sandford Street, London, ON, N5V 4T3 Canada*
[b]*Department of Chemistry, University of Western Ontario, London, ON, N6A 5B7 Canada*

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Abstract

The sweet diterpenoid glycoside, rebaudioside F, was isolated from leaves of a high rebaudioside C producing line of *Stevia rebaudiana,* and its structure was established by chemical and spectral studies.

Keywords: *Stevia rebaudiana*; Compositae; Diterpenoid glycoside; Rebaudioside F

1. Introduction

Stevia rebaudiana (Bertoni) Bertoni produces in remarkably high yield several high-potency low-calorie sweeteners in its leaf tissues (Brandle et al., 1998). These substances, the major ones being stevioside, rebaudiosides A and C, and dulcoside A, glycosides of the diterpene steviol (*ent*-13-hydroxykaur-16-en-19-oic acid), exhibit characteristic organoleptic properties (Phillips, 1989). Since flavour of the individual glycosides has a significant effect on their economic value, the discovery of new glycosides which have the potential for novel sensory characteristics is of considerable interest. During plant breeding studies, we noticed an unidentified peak in chromatograms of extracts of a *S. rebaudiana* line high in rebaudioside C (Brandle, 1999). This paper describes the isolation and structural elucidation of the compound responsible for this peak, a new sweet steviol glycoside which has been named rebaudioside F (**1**).

2. Results and discussion

Analysis of methanolic extracts of a high rebaudioside C-producing line of *S. rebaudiana* (Brandle, 1999) indicated the following composition expressed on a dry weight basis: stevioside (0.3%), rebaudioside A (0.6%), rebaudioside C (14.4%), dulcoside A (0.9%) and a new compound, rebaudioside F (2.7%, based on the HPLC response factor for stevioside). A mixture of rebaudiosides C and F, obtained by column chromatography, could be separated by HPLC to give a small quantity of non-crystalline rebaudioside F for characterization.

Rebaudioside F (**1**) showed a molecular ion peak at m/z 959.4103 $[M+Na]^+$ in the ESI mass spectrum corresponding to a molecular formula of $C_{43}H_{68}O_{22}$. The ^{13}C NMR spectrum of **1** (Tables 1 and 2) showed typical signals of a steviol glycoside with a pattern very similar to that of rebaudioside C (Sakamoto et al., 1977). As in the case of rebaudioside C, peaks attributable to the aglycone and three glucose units were observed. The remaining peaks in the spectrum indicated that rebaudioside F contained a pentose instead of the hexose rhamnose present in rebaudioside C. The ^1H NMR spectrum showed signals for the anomeric protons at δ 6.10 ($J=8.1$ Hz) for a β-glucosyl ester and δ 5.02 ($J=7.4$ Hz), 5.26 ($J=7.7$ Hz), and 5.42 ($J=7.3$ Hz) for β-glycosidic linkages. Acid hydrolysis yielded isosteviol (*ent*-16-oxobeyeran-19-oic acid) and a mixture of glucose and xylose in a 3:1 ratio.

Alkaline hydrolysis of rebaudioside F afforded a non-crystalline product, **2**, with a molecular ion peak in the ESI-MS at m/z 797.3540 $[M+Na]^+$ indicating the loss of a glucose unit. This was confirmed when this product was hydrolyzed with acid. Isosteviol was produced together with glucose and xylose in a 2:1 ratio. Based on this evidence, we have concluded that rebaudioside F is a steviol derivative with a glucose ester moiety at C-19 and a trisaccharide residue consisting of two glucose and a xylose unit linked through oxygen to C-13.

* Corresponding author. Tel.: +1-519-457-1470; fax: +1-519-457-3997.

E-mail address: starratta@em.agr.ca (A.N. Starratt).

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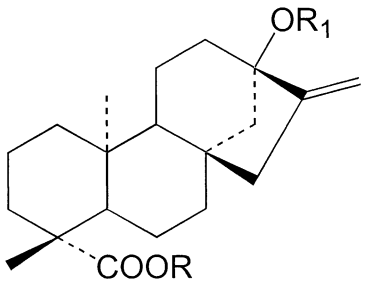

	R	R_1
1	β-Glc	β-Glc^{2}-β-Xyl $\vert^{3}$ β-Glc
2	H	β-Glc^{2}-β-Xyl $\vert^{3}$ β-Glc

Table 1
^{1}H NMR and ^{13}C NMR spectroscopic data for the aglycone moieties of compounds **1** and **2** in pyridine–d_5

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1	0.77, 1.78	40.8	0.79, 1.82	41.1
2	1.41, 2.20	19.4	1.46, 2.20	19.8
3	1.00, 2.32	38.4	1.05, 2.45	38.7
4	–	44.0	–	43.9
5	1.02	57.3	1.03	57.0
6	1.89, 2.44	22.1	1.92, 2.21	22.6
7	1.28	41.7	1.38	41.9
8	–	42.5	–	42.7
9	0.89	54.0	0.95	54.2
10	–	39.8	–	39.8
11	1.66	20.6	1.68	20.6
12	1.85, 2.28	37.0	1.87, 2.22	37.9
13	–	86.4	–	86.7
14	1.79, 2.65	44.3	1.77, 2.59	44.4
15	2.03, 2.14	47.7	2.06, 2.14	48.0
16	–	154.2	–	153.8
17	4.99, 5.63	104.6	5.04, 5.71	105.0
18	1.20	28.3	1.31	30.0
19	–	177.0	−180.2	
20	1.31	15.5	1.26	16.0

Consideration of the ^{1}H NMR and ^{13}C NMR spectral data for **1** and **2** and detailed 2-D NMR studies using gHSQC, gHSQC-TOCSY, gHMBC, ROESY and selective TOCSY experiments, established the structure of rebaudioside F as 19-*O*-β-glucopyranosyl-13-*O*-[β-xylopyranosyl-(1→2)-β-glucopyranosyl-(1→3)-β-glucopyranosyl]-steviol. ^{1}H NMR and ^{13}C NMR chemical shifts and important gHMBC interactions for the sugar moieties are presented in Table 2.

3. Experimental

3.1. General

NMR spectra of samples in pyridine-d_5 were recorded using a Varian Inova 600 NMR spectrometer operating at 599.7 MHz (^{1}H NMR) and 150.8 MHz (^{13}C NMR). ESI-MS were obtained with a Micromass LCT mass spectrometer with introduction of samples via a syringe pump. Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter.

3.2. Extraction and analysis of diterpenoid glycosides

Ground leaves (300 mg) of line 41-412 *S. rebaudiana* (1996 crop), grown as described by Brandle and Rosa (1992), were extracted twice for 15 min periods with 10 ml CH_3CN-H_2O (1:1) and ultrasonification at room temperature. After centrifugation, the supernatants were separated and the solvent removed in vacuo to yield the crude extract (83 mg). For HPLC analysis of the diterpenoid glycosides, aliquots were filtered (0.45 μm) and applied to a Waters carbohydrate cartridge column (4.6×250 mm, 4 μm) using linear gradients of 13–17.5% H_2O in CH_3CN for 12 min and then 17.5–24% H_2O in CH_3CN for 14 min at 1.5 ml/min and UV detection at 205 nm. Typical retention times observed for the diterpenoid glycosides were: dulcoside A (14.1 min), stevioside (16.4 min), rebaudioside C (17.3 min), rebaudioside F (17.7 min), and rebaudioside A (19.3 min). The response factor for a stevioside standard was used to determine the amount of the other glycosides after correcting for differences in molecular weight.

3.3. Isolation of rebaudioside F (*1*)

S. rebaudiana leaf extract (1.5 g), obtained as described above, was chromatographed over a column (23×380 mm) packed with 3-aminopropyl-functionalized silica gel (Aldrich) using CH_3CN-H_2O (84:16) at 0.5 ml/min. Residue in fractions containing rebaudioside C which were enriched in rebaudioside F was further separated by HPLC with a Waters carbohydrate column (7.8×250 mm, 4 μm packing) using a flow rate of 1.4 ml/min and linear gradients of 6–16% H_2O in CH_3CN over 12 min followed by 16–23% H_2O in CH_3CN over the next 15 min. At this time, the flow rate was reduced linearly to 0.7 ml/min over 14.5 min. This method permitted the separation of rebaudioside C (retention time, 41.1 min) and rebaudioside F (retention time, 43.4 min).

Table 2
^{1}H NMR and ^{13}C NMR spectroscopic data for the sugar moieties of **1** and **2** in pyridine-d_5[a]

Position	**1**			**2**		
	δ_H	δ_C	gHMBC[b]	δ_H	δ_C	gHMBC[b]
Glc						
1	5.02 (*d*, 7.4)	97.9 (*d*)	C-13, Glc-5	5.05 (*d*, 7.7)	97.7 (*d*)	C-13, Glc-5
2	4.22 (*dd*, 9.3, 7.4)	80.7 (*d*)	Xyl-1, Glc-1, Glc-3, Glc-4	4.24 (*dd*, 9.5, 7.7)	80.7 (*d*)	Xyl-1, Glc-1, Glc-3, Glc-4
3	4.06 (*dd*, 9.3, 8.8)	88.3 (*d*)	Glc′-1, Glc-1, Glc-2, Glc-4	4.18 (*dd*, 9.5, 8.1)	88.6 (*d*)	Glc′-1, Glc-2, Glc-4
4	3.83 (*dd*, 9.7, 8.8)	70.6 (*d*)	Glc-2, Glc-3, Glc-5, Glc-6	4.08 (*dd*, 9.9, 8.1)	70.0 (*d*)	Glc-2, Glc-3, Glc-5, Glc-6
5	3.72 (*ddd*, 9.7, 6.3, 1.4)	77.3 (*d*)	Glc-3, Glc-4, Glc-6	3.60 (*ddd*, 9.9, 9.7, 1.8)	77.6 (*d*)	Glc-1, Glc-4, Glc-6
6	4.42 (*dd*, 9.7, 1.4) 4.02 (*dd*, 9.7, 6.3)	62.6 (*t*)	Glc-4, Glc-5	4.23 (*dd*, 11.9, 9.7) 4.12 (*dd*, 11.9, 1.8)	62.3 (*t*)	Glc-4, Glc-5
Glc′						
1	5.26 (*d*, 7.7)	104.8 (*d*)	Glc-3, Glc′-5	5.31 (*d*, 7.7)	104.9 (*d*)	Glc-3, Glc′-5
2	4.01 (*dd*, 9.0, 7.7)	75.2 (*d*)	Glc′-1, Glc′-3, Glc′-4	4.04 (*dd*, 9.9, 7.7)	75.3 (*d*)	Glc′-1, Glc′-3, Glc′-4
3	4.17 (*dd*, 9.0, 8.3)	79.0 (*d*)	Glc′-1, Glc′-2, Glc′-4	4.20 (*dd*, 9.9, 8.1)	78.6 (*d*)	Glc′-1, Glc′-2, Glc′-4, Glc′-5
4	4.13 (*dd*, 9.4, 8.3)	71.5 (*d*)	Glc′-3, Glc′-5, Glc′-6	4.13 (*dd*, 9.2, 8.1)	71.6 (*d*)	Glc′-3, Glc′-5, Glc′-6
5	4.02 (*ddd*, 9.4, 8.7, 1.8)	78.6 (*d*)	Glc′-1, Glc′-3, Glc′-4, Glc′-6	4.02 (*ddd*, 9.6, 9.2, 1.7)	78.6 (*d*)	Glc′-1, Glc′-6
6	4.51 (*dd*, 10.4, 1.8) 4.26 (*dd*, 10.4, 8.7)	62.3 (*t*)	Glc′-4, Glc′-5	4.52 (*dd*, 11.3, 1.7) 4.25 (*dd*, 11.3, 9.6)	62.4 (*t*)	Glc′-4, Glc′-5
Xyl						
1	5.42 (*d*, 7.3)	105.4 (*d*)	Glc-2, Xyl-3, Xyl-5	5.43 (*d*, 7.7)	105.4 (*d*)	Glc-2, Xyl-5
2	4.10 (*dd*, 9.9, 7.3)	75.9 (*d*)	Xyl-1, Xyl-3, Xyl-4	4.06 (*dd*, 9.5, 7.7)	76.2 (*d*)	Xyl-1, Xyl-3
3	4.12 (*dd*, 9.9, 7.8)	78.6 (*d*)	Xyl-2, Xyl-4, Xyl-5	4.13 (*dd*, 9.5, 8.1)	78.6 (*d*)	Xyl-2, Xyl-4
4	4.23 (*ddd*, 10.0, 7.8, 5.5)	71.2 (*d*)	Xyl-3, Xyl-5	4.20 (*ddd*, 10.9, 8.1, 5.1)	71.3 (*d*)	Xyl-3, Xyl-5
5	4.35 (*dd*, 11.6, 5.5) 3.65 (*dd*, 11.6, 10.0)	67.5 (*t*)	Xyl-1, Xyl-3, Xyl-4	4.32 (*dd*, 11.0, 5.1) 3.62 (*dd*, 11.0, 10.9)	67.6 (*t*)	Xyl-1, Xyl-3, Xyl-4
Glc″						
1	6.10 (*d*, 8.1)	95.8 (*d*)	C-19			
2	4.19 (*dd*, 9.9, 8.1)	73.9 (*d*)	Glc″-1, Glc″-3			
3	4.10 (*dd*, 9.9, 8.0)	79.2 (*d*)	Glc″-1, Glc″-4			
4	4.24 (*dd*, 9.0, 8.0)	71.0 (*d*)	Glc″-3, Glc″-5			
5	3.93 (*ddd*, 9.0, 2.7, 1.7)	78.5 (*d*)	Glc″-1, Glc″-4			
6	4.39 (*dd*, 11.9, 1.7) 4.30 (*dd*, 11.9, 2.7)	62.1 (*t*)	Glc″-4, Glc″-5			

[a] Multiplicities and coupling constants (*J*) in Hz are given in parentheses. Carbon multiplicity and directly attached protons were determined from gHSQC experiments. Proton multiplicity and *J*-values were determined using selective TOCSY experiments.
[b] Correlations from H to the indicated carbons.

3.4. Rebaudioside F (*1*)

Non-crystalline solid; $[\alpha]_D$ −25.5 °C (MeOH; *c* 1.0); ^{1}H NMR and ^{13}C NMR spectral data (Tables 1 and 2); ESI–MS *m/z*: 959.4103 $[M+Na]^+$ (calc. for $C_{43}H_{68}O_{22}Na$: 959.4100).

3.5. Alkaline hydrolysis of *1*

A solution of **1** (10 mg) in 10% KOH-EtOH (0.75 ml) was heated at 100 °C for 2 h. After acidification with HOAc, the solvent was removed and the product was partitioned between *n*-BuOH and H_2O. The BuOH layer was concentrated in vacuo to yield non-crystalline **2**; $[\alpha]_D$ −31.2 °C (MeOH; *c* 1.0); ^{1}H NMR and ^{13}C NMR spectral data (Tables 1 and 2); ESI–MS *m/z* 797.3540 $[M+Na]^+$ (calc. for $C_{37}H_{58}O_{17}Na$: 797.3571). HPLC on a Waters Resolve C_{18} column (3.9×150 mm, 5 μm) using 30% CH_3CN in 10 mM K-Pi buffer (pH 5.8) gave a peak at 47.2 min while the corresponding compound from alkaline hydrolysis of rebaudioside C eluted at 44.2 min.

3.6. Acid hydrolysis of *1* and *2*

Rebaudioside F (1 mg), **1**, in 0.5 ml 2 M TFA was heated for 3 h at 100 °C. After drying under N_2, the residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was shown to contain isosteviol (Mosettig and Nes, 1955) by comparison of HPLC retention times with that for an authentic specimen (UV detection at 193 nm) on (1) a Waters Symmetry C_{18} column (3.9×150 mm, 5 μm) using CH_3CN-H_2O (3:7) and (2) the Waters analytical carbohydrate column and solvent system described above for analysis of the stevia glycosides. The aqueous layer was shown to contain glucose and xylose in a ratio of 3:1 by GC–MS of the TMSi derivatives (Sweeley et al., 1963) on a J & W Scientific DB 1701 capillary column (30 m×0.25 mm i.d.) as described by Court and Hendel (1986). Compound **2** was hydrolyzed and the products identified similarly as isosteviol and a mixture of glucose and xylose in a 2:1 ratio.

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