New Non-Glycosidic Diterpenes from the Leaves of *Stevia rebaudiana*

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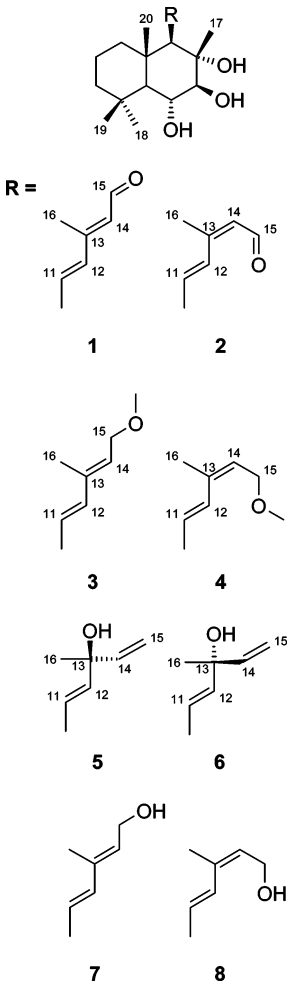
Six new labdane-type, non-glycosidic diterpenes, sterebins I−N (**1**−**6**), were isolated from the leaves of *Stevia rebaudiana*. Their structures, analogous to those of the previously described sterebins A−H, were elucidated on the basis of spectroscopic and chemical studies.

Stevia rebaudiana (Bertoni) Bertoni is a plant of the Compositae family native to Paraguay,[1] where its leaves have long been known to be sweet tasting. Recently, it has been introduced as a crop in a number of countries, including Canada, and has become a popular natural-source high-potency sweetener and dietary supplement. This plant produces, in its leaves, several ent-kaurene glycosides of the diterpene steviol that are up to 450 times as sweet as sucrose.[2] Other previously identified constituents from the leaves include a variety of volatile oil constituents, sterols, triterpenoids, flavonoids, coumarins, and caffeic and chlorogenic acids, as well as 13 non-glycosidic diterpenes.[3] Included among these are eight labdane-type diterpenes, designated as sterebins A−H.[4,5] With a view to developing *S. rebaudiana* lines in which levels of sweet-tasting steviol glycosides are maximized and non-glycosidic diterpenoids are minimized, the present work was undertaken to characterize some previously unknown low-polarity *S. rebaudiana* leaf components. We report here the isolation and characterization of six new non-glycosidic labdane diterpenes, designated sterebins I−N (**1**−**6**), which are structurally similar to the previously identified sterebins A−H.[4,5]

Extraction of freeze-dried *S. rebaudiana* leaves, followed by a series of chromatographic separations, yielded a mixture of sterebins I (**1**) and J (**2**), whose UV spectra were identical to those previously reported for the conjugated diene aldehydes **9** and **10**,[6] indicating that they are *trans* and *cis* isomers, respectively, of a doubly unsaturated aldehyde. Reanalysis of HPLC-isolated samples of sterebin I (**1**) and sterebin J (**2**) by HPLC again showed the presence of *both* compounds, suggesting that the two stereoisomers were readily interconverted, a behavior consistent with *cis*/*trans* isomerization of α,*β*,*γ*,*δ*-unsaturated aldehydes. This conclusion was supported by the ^{1}H and ^{13}C NMR spectra of sterebin J (**2**) (Table 1), which exhibited aldehyde resonances at 10.1 and 191.4 ppm, respectively. The mixture of sterebins I (**1**) and J (**2**) yielded, upon reduction with sodium borohydride, a mixture of sterebins E (**7**) and F (**8**)[5] (identified by HPLC, UV, ^{1}H and ^{13}C NMR, and MS), confirming the structures of sterebins I and J as shown. Comparison of ^{1}H and ^{13}C NMR spectra of **2** (Table 1) with spectral data for compounds **3**, **4**, **5**, and **6** (Tables 1 and 2) allowed assignment of ring and methyl signals for sterebin J. Assignment of ^{1}H and ^{13}C NMR chemical shifts for the side chain of **2** (Table 1) was achieved by comparison of our data with ^{1}H and ^{13}C NMR data reported for 8α-hydroxy-11*E*,13*Z*-labdadien-15-al.[7] Sterebin I (**1**) was not isolated in sufficient purity to allow comparable NMR spectra to be obtained. The accurate mass of sodiated sterebin J (**1**) as determined by HRESIMS (*m*/*z* 359.2192) established the molecular formula as $C_{20}H_{32}O_4$ (calculated for $C_{20}H_{32}O_4Na$, 359.2198).



The UV spectra of sterebins K (**3**) and L (**4**), λ_{max} (MeOH/H_2O) 236 and 238 nm, respectively, indicated the presence

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Table 1. ^{1}H and ^{13}C NMR Data (δ in ppm, J in Hz) for Sterebins J−L (**2**−**4**)

	sterebin J (**2**)		sterebin K (**3**)			sterebin L (**4**)		
position	δ_C[a]	δ_H[b]	δ_C[c]	δ_H[d]	HMBC[e]	δ_C[f]	δ_H[g]	HMBC[e]
1	40.9 (CH_2)		41.0	0.81 (t, 12.3)	20	41.3	0.84 (t, 12.4)	
				1.34 (m)			1.35 (m)	
2	18.1 (CH_2)		18.4	1.36 (m)		18.3	1.35 (m)	
				1.53 (qt, 13.1, 3.2)			1.54 (m)	
3	43.3 (CH_2)		43.6	1.22 (m)		44.0	1.18	
				1.34 (m)	5		1.35 (m)	
4	33.9 (C)		34.4			34.2		
5	57.2 (CH)	1.14 (d, 11.0)	57.6	1.12 (d, 10.9)	6, 7, 9, 20	57.7	1.14 (d, 10.9)	6, 7, 9
6	71.6 (CH)	3.71 (dd, 10.8, 9.6)	71.8	3.72 (t, 9.5)	5, 7	72.1	3.72 (dd, 11.0, 9.5)	5, 7, 10
7	84.3 (CH)	3.43 (d, 9.5)	84.5	3.44 (d, 9.5)	5, 6, 8, 17	84.7	3.45 (d, 9.2)	5, 6, 8, 17
8	75.2 (C)		75.1			75.4		
9	64.0 (CH)	2.00 (d, 9.5)	64.6	1.86 (d, 10.1)	1, 5, 7, 8, 10, 11, 12, 17, 20	64.8	1.90 (d, 10.4)	1, 5, 8, 10, 11, 12, 17, 20
10	38.2 (C)		38.4			38.6		
11	133.7 (CH)	6.19−6.31 m	123.9	5.62 (dd, 15.5, 10.1)	9, 13	126.9	5.71 (dd, 15.1, 10.4)	9, 10, 13
12	138.8 (CH)	6.19−6.31 m	140.4	6.18 (d, 15.4)	9, 13, 14, 16	133.0	6.48 (d, 15.4)	9, 13, 14, 16
13	153.3 (C)		136.8			135.8		
14	129.6 (CH)	5.92 (d, 8.0)	128.1	5.59 (t, 6.7)		126.3	5.50 (t, 6.3)	12
15	191.4 (CH)	10.09 (d, 8.0)	69.1	4.03 (d, 6.7)	13, 14, OCH_3	68.5	4.05 (m)	13, 14, OCH_3
16	13.3 (CH_3)	2.02 (s)	13.3	1.80 (s)	12, 13, 14	21.1	1.88 (s)	12, 13, 14
17	19.9 (CH_3)	1.23 (s)	20.5	1.19 (s)	7, 8, 9	20.7	1.20 (s)	7, 8, 9
18	36.2 (CH_3)	1.16 (s)	36.5	1.15 (s)	3, 4, 5, 19	36.5	1.17 (s)	3, 4, 5, 19
19	22.1 (CH_3)	1.02 (s)	22.4	1.00 (s)	3, 4, 5, 18	22.8	1.02 (s)	3, 4, 18
20	17.3 (CH_3)	1.01 (s)	17.5	0.98 (s)	1, 5, 9, 10	18.0	1.00 (s)	1, 5, 10
OCH_3			58.6	3.34 (s)	15	58.7	3.34 (s)	15

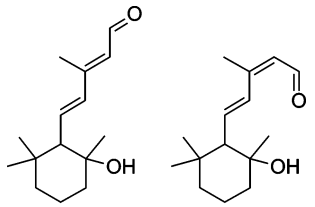
[a] Carbon type in parentheses as determined by DEPT spectrum (recorded in $CDCl_3$, 100 MHz). [b] Recorded in $CDCl_3$, 500 MHz. [c] Assignments were based on HMQC (or HSQC) and HMBC experiments (recorded in $CDCl_3$, 500 MHz). [d] Assignments were based on ^{1}H, HMQC (or HSQC), and HMBC experiments (recorded in $CDCl_3$, 500 MHz). [e] Carbons correlating with proton resonance. [f] Assignments were based on gHSQC and gHMBC experiments (recorded in $CDCl_3$, 600 MHz). [g] Assignments were based on ^{1}H, gHSQC, and gHMBC experiments (recorded in $CDCl_3$, 600 MHz).

Table 2. ^{1}H and ^{13}C NMR Data (δ in ppm, J in Hz) for Sterebins M (**5**) and N (**6**)

	sterebin M (**5**)			sterebin N (**6**)		
position	δ_C[a]	δ_H[b]	HMBC[c]	δ_C[a]	δ_H[b]	HMBC[c]
1	40.5	0.81	20	40.5	0.81	20
		1.33			1.33	
2	18.0	1.33		18.0	1.33	
		1.41			1.41	
3	43.3	1.17[d]		43.5	1.13[d]	
		1.36			1.32	
4	33.7			33.7		
5	57.1	1.10 (d, 11.3)	4, 6, 7, 9, 10, 18, 19, 20	57.1	1.10 (d, 11.3)	4, 6, 7, 9, 10, 18, 19, 20
6	71.3	3.68 (dd, 11.3, 9.4)	5, 7	71.3	3.69 (dd, 11.3, 9.4)	5, 7
7	84.2	3.40 (d, 9.4)	5, 6, 8, 17	84.2	3.40 (d, 9.4)	5, 6, 8, 17
8	74.9			74.9		
9	63.5	1.79 (d, 10.0)	8, 10, 11, 12, 17, 20	63.3	1.79 (d, 10.0)	8, 10, 11, 12, 17, 20
10	37.3			37.3		
11	123.1	5.65	12, 13	123.9	5.62 (dd, 15.6, 10.0)	12, 13
12	141.7	5.67	9, 11, 13	142.1	5.70 (d, 15.6)	9, 11, 13, 14, 16
13	73.2			73.2		
14	144.1	5.93 (dd, 10.6, 17.4)	12, 13	143.5	5.97 (dd, 10.8,17.2)	13
15	112.2	5.22 (dd, 17.4, 1.0)	13, 14	112.7	5.25 (dd, 17.2, 1.0)	13, 14
		5.04 (dd, 10.6, 1.0)	13		5.10 (dd, 10.8, 1.0)	13
16	27.4	1.39 (s)	12, 13, 14	28.4	1.37 (s)	12, 13, 14
17	19.7	1.21[e] (s)	7, 8, 9	19.7	1.14[e] (s)	7, 8, 9
18	36.1	1.15 (s)	3, 4, 5, 9, 19	36.1	1.15 (s)	3, 4, 5, 9, 19
19	22.0	1.00 (s)	3, 4, 5, 18	22.0	0.96 (s)	3, 4, 5, 18
20	17.1	0.93 (s)	1, 5, 9, 10	17.1	0.93 (s)	1, 5, 9, 10

[a] Assignments were based on HMQC (or HSQC) and HMBC experiments (recorded in $CDCl_3$, 500 MHz). [b] Assignments were based on ^{1}H, HMQC (or HSQC), and HMBC experiments (recorded in $CDCl_3$, 500 MHz). [c] Carbons correlating with proton resonance. [d,e] Assignments may be reversed between the two compounds.

of conjugated double bonds. The ^{1}H NMR spectra of sterebins K (**3**) and L (**4**) (Table 1) were almost identical to those of sterebins E (**7**) and F (**8**),[5] with the addition of a methoxy resonance at 3.34 ppm for both compounds. HMBC and HSQC experiments allowed assignment of all proton and carbon resonances for both compounds (Table 1) and showed that the methoxy group was attached to C-15 in each case. NOESY experiments indicated significant NOEs between H-16 and H-15 and between H-12 and H-14 for sterebin K (**3**) (*trans* stereochemistry for the double bond between C-13 and C-14), between H-16 and H-14 and between H-12 and H-15 for sterebin L (**4**) (*cis* stereochemistry for the double bond between C-13 and C-14), and between H-11 and H-16 and between H-9 and H-12 for both compounds (*trans* stereochemistry for the double bond between C-11 and C-12). The coupling constant between H-11 and H-12 for sterebins K (**3**) and L (**4**) (each 15.4 Hz) also indicated *trans* stereochemistry. The accurate



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mass of sodiated sterebin K established by ESIMS was *m*/*z* 375.2479 (calculated for $C_{21}H_{36}O_4Na$, 375.2511). It should be noted, however, that sterebins K and L could not be found by HPLC-ESIMS analysis of a hexane extract of *S. rebaudiana* leaves which had had no contact with methanol, suggesting that these two methyl ethers may have been formed by methanolysis of sterebins E (**7**) and F (**8**), respectively, perhaps during column chromatography with methanol used as an eluting solvent.

The ^{1}H NMR spectrum of an isolated fraction initially thought to contain a single compound indicated the presence of two compounds, sterebins M (**5**) and N (**6**). These two compounds, only partially resolved by reversed-phase HPLC, had identical UV spectra (λ_{max} [MeOH/H_2O] 198 nm) that indicated the absence of conjugated double bonds. COSY, HMQC, and HMBC experiments allowed assignment of all proton and carbon resonances (Table 2) for both compounds. The accurate mass by EIMS (mixture of two compounds) was *m*/*z* 320.2352 [M − H_2O]$^+$; calculated for $C_{20}H_{32}O_3$, 320.2351. The coupling constant between H-11 and H-12 in sterebin N (**6**) was 15.6 Hz, indicating *trans* stereochemistry. The ^{1}H chemical shifts of H-11 and H-12 in sterebin M (**5**) were too close together for coupling to be observed. If sterebins M (**5**) and N (**6**) were *cis*/*trans* isomers, the ^{13}C NMR resonances of C-9 and C-13 would be expected to be 4−6 ppm lower for the *cis* isomer than for the *trans*,[8] but that was not the case in the present investigation. Furthermore, with the exception of resonances for positions C-6 and C-7, the ^{13}C and ^{1}H NMR chemical shifts of sterebins M (**5**) and N (**6**) closely matched those of (11*E*,13*S*)-11,14-labdadiene-8,13-diol and (11*E*,-13*R*)-11,14-labdadiene-8,13-diol, respectively.[9] These observations led to the conclusion that both compounds have *trans* stereochemistry at C-11 and that sterebin M has the 13*S* configuration (**5**) and sterebin N the 13*R* configuration (**6**).

The identification of the new sterebin compounds described in this paper demonstrates the complexity of the metabolome of *S. rebaudiana*. While the concentration of individual labdane diterpenes is low,[10] taken together the labdane concentration is significant and suggests the possible importance of inhibiting biosynthetic pathways of the labdane-type diterpenes to enhance levels of sweet-tasting diterpene glycosides. Efforts to reduce levels of these less polar substances may also simplify the purification of the sweet steviol glycosides.

Experimental Section

General Experimental Procedures. Optical rotations were measured in MeOH using a Perkin-Elmer model 241 polarimeter. UV spectra were obtained using the diode-array UV detector on a Hewlett-Packard 1050 HPLC system. ^{1}H NMR spectra were obtained on Varian Inova (400 and 600 MHz) and Unity (500 MHz) spectrometers. ^{13}C and DEPT NMR spectra were obtained on a Varian VXR (400 MHz) spectrometer. Accurate mass measurements were obtained by electrospray ionization mass spectrometry (ESIMS) using an Autospec high-resolution mass spectrometer (Micromass) and by electron impact mass spectrometry (EIMS) using a Finnigan MAT 8200 mass spectrometer. Analytical HPLC was performed on a Waters system consisting of a U6K injector and two M510 pumps controlled by Millennium 2010 Chromatography Manager with a Shimadzu model SPD-M6A photodiode-array detector using a Waters Nova-Pak C_{18} column (150 × 3.9 mm, 4 μm). Semipreparative HPLC was performed on the above system using a Waters μBondapak C_{18} column (150 × 19 mm, 10 μm) or on a Hewlett-Packard 1050 HPLC system equipped with a diode-array UV detector using a Supelcosil C_{18} column (250 × 10 mm, 5 μm). TLC was carried out on silica gel precoated plastic sheets (Polygram Sil G/UV_{254} from Machery-Nagel or Baker-flex IB-F from J.T. Baker) or plates (Kieselgel 60; EM Science), and spots were viewed under UV light or visualized by exposure to iodine vapor, by spraying with sulfuric acid and heating, or by immersing in anisaldehyde stain[11] and heating. Column and flash chromatography were performed on silica gel (SiliTech 32-63, 60 Å; ICN), aluminum oxide (Grade 5; BDH), and octadecyl-functionalized silica gel [Aldrich or Waters Prep C_{18} (55−105 μm, 125 Å)].

Plant Material. Leaves of cultivated *Stevia rebaudiana* from a landrace cultivar imported from the People's Republic of China[12] were collected at the Delhi, Ontario, site of the Southern Crop Protection and Food Research Centre in August 1991 and freeze-dried. A voucher specimen (accession number 791791) was deposited in the Vascular Plant Herbarium, Agriculture and Agri-Food Canada, Ottawa.

Extraction and Isolation. A total of 400 g of finely ground leaves was extracted in eight portions, each with 350 mL of hexane in a Soxhlet apparatus. Removal of the solvent afforded a residue (17.47 g), which was subjected to column chromatography on alumina (Grade 5, BDH). Elution with petroleum ether−EtOAc (9:1), petroleum ether−EtOAc (4:1), petroleum ether−EtOAc (1:1), EtOAc, EtOAc−MeOH (9:1), EtOAc−MeOH (3:1), and MeOH indicated a complex mixture of compounds which, in general, increased in polarity with the fraction number (TLC evidence). A group of EtOAc−MeOH (3:1) fractions that showed TLC similarity were combined (yielding a total of 955 mg) and further fractionated on a column prepared with Waters Prep C_{18} bulk packing. The column was eluted with a methanol−water gradient consisting of 30−60% MeOH with collection of 30-min fractions (approximately 7 mL each). A group of fractions that were eluted with 50−60% MeOH were combined on the basis of similarity of their HPLC profiles and further purified by silica gel TLC using $CHCl_3$−MeOH (9:1) and semipreparative HPLC on a Supelcosil C_{18} column (250 × 10 mm) using MeOH−water (70:30) as mobile phase at a flow rate of 2.5 mL/min. Under these conditions, sterebin I (**1**) was eluted at 29 min, sterebin J (**2**) was eluted at 35 min, and sterebins M (**5**) and N (**6**) were eluted as unresolved peaks at 37−39 min. Several isolated fractions were found to contain two additional peaks, which were eluted at 15 min [sterebin K (**3**)] and 17 min [sterebin L (**4**)] by semipreparative HPLC using a mobile phase of MeOH−water (80:10). HPLC analysis of fractions from the Waters Prep C_{18} column indicated that the extract contained 52 mg (0.013%) of sterebin I (**1**), 112 mg (0.028%) of sterebin J (**2**), and a total of 212 mg (0.053%) of sterebins M (**5**) and N (**6**).

Sterebin I (1): UV (MeOH−water) λ_{max} 236, 286 nm.

Sterebin J (2): colorless amorphous solid; $[\alpha]^{23}_D$ +34.0° (*c* 0.42, MeOH); UV (MeOH−water) λ_{max} 289 nm; ^{1}H ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; EIMS *m*/*z* 318 [M − H_2O]$^+$ (4), 300 (3), 275 (11), 262 (8), 247 (8), 177 (11), 166 (14), 153 (21), 135 (27), 123 (27), 109 (70), 95 (100), 81 (54), 69 (79), 55 (85); HRESIMS *m*/*z* 359.2192 [M + Na]$^+$ (calcd for $C_{20}H_{32}O_4Na$, 359.2198).

Sterebin K (3): colorless amorphous solid; $[\alpha]^{23}_D$ +53.7° (*c* 0.14, MeOH); UV (MeOH−water) λ_{max} 236 nm; ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR (derived from HMQC, HSQC, and HMBC in $CDCl_3$, 500 MHz), see Table 1; HRESIMS *m*/*z* 375.2479 [M + Na]$^+$ (calcd for $C_{21}H_{36}O_4Na$, 375.2511).

Sterebin L (4): colorless amorphous solid; UV (MeOH−water) λ_{max} 238 nm; ^{1}H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR

(derived from gHSQC and gHMBC in $CDCl_3$, 600 MHz), see Table 1; ESIMS *m*/*z* 375 $[M + Na]^+$.

Sterebins M (5) and N (6): colorless crystals from EtOAc, mp 178−181 °C (mixture of sterebins M and N); UV (MeOH−water) λ_{max} 198 nm; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR (derived from HMQC, HSQC, and HMBC in $CDCl_3$, 500 MHz), see Table 2; EIMS (mixture) *m*/*z* 320 $[M - H_2O]^+$ (4), 302 (3), 287 (4), 277 (8), 259 (16), 247 (25), 246 (29), 231 (22) 161 (41), 121 (62), 109 (67), 107 (56), 95 (77), 93 (72), 81 (82), 69 (100), 55 (90); ESIMS (mixture) *m*/*z* 361 $[M + Na]^+$; HREIMS (mixture) *m*/*z* 320.2352 $[M - H_2O]^+$ (calcd for $C_{20}H_{32}O_3$, 320.2351).

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