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A PHYTOCHEMICAL SCREENING PROCEDURE FOR SWEET *ENT*-KAURENE GLYCOSIDES IN THE GENUS *STEVIA* [1,2]

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ABSTRACT.—Altogether, 110 species of the genus *Stevia*, comprising both herbarium and fresh leaf samples, were screened for the presence of sweet *ent*-kaurene glycosides, using a combination of tlc and hplc, followed by gc/ms. Stevioside and rebaudiosides A and C were detected in a *Stevia rebaudiana* herbarium specimen collected in Paraguay in 1919, and stevioside was observed as a constituent of a *Stevia phlebophylla* herbarium specimen collected in Mexico in 1889. Steviol glycosides were not detected in any of the other 108 *Stevia* species studied. The phytochemical results obtained in this study are correlated with those of preliminary organoleptic tests on the sweetness of these *Stevia* samples, and the chemotaxonomic implications of the present findings are discussed.

In preliminary studies designed to investigate the distribution of sweet *ent*-kaurene glycosides in the genus *Stevia*, we have studied organoleptically 184 *Stevia* leaf samples taken from an herbarium (1), as well as the leaves of 31 species of this genus collected in the field (2). Although a number of stored and fresh *Stevia* leaves exhibited taste sensations that were perceived as being slightly sweet, none approached the intense sensations of sweetness produced by leaves of *Stevia rebaudiana* (Bertoni) Bertoni, as demonstrated by both a specimen preserved for more than 60 years as well as a live specimen collected in its native habitat in northeastern Paraguay (1,2).

Despite the current commercial use of *S. rebaudiana* products in Japan and Brazil for sweetening purposes (1), and the fact that many *Stevia* species are used medicinally (3), only about 20 members of this large genus have been studied phytochemically to date. Thus far, none of the eight, sweet *ent*-kaurene glycoside constituents of *S. rebaudiana*—namely, dulcoside A, rebaudiosides A-E, steviolbioside, and stevioside—have been reported as constituents of any other *Stevia* species. However, a group of nonsweet *ent*-kaurene glycosides, paniculosides I-V, have been reported to occur in the leaves of *S. paniculata* Lag. (4) and *S. ovata* Lag. (5). Other phytochemical papers have reported in *Stevia* species bisabolane (6-9), germacrane (6-8,10-12), guaiane (8,10,13,14), humulane (7,10), α-longipinane (6,7,10,15), and pseudoguaiane (16) sesquiterpene derivatives, clerodane (8,17), kaurene (8,10,18), and labdane (6,8,18-20) diterpene derivatives, as well as sterols (10,18,20,21), triterpenes (10,20), flavonoids (6,19,21,22), chromenes (6,18,23), euparin derivatives (10), and miscellaneous volatile constituents (7,8,21,24,25). Phytochemical screening studies to determine the infrageneric distribution of either *ent*-kaurene glycosides or any of the other known *Stevia* constituents are so far lacking; thus, the chemotaxonomic significance of all of the secondary metabolites so far isolated from plants in this genus is presently unknown.

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[1]Part IV in the series "Potential Sweetening Agents of Plant Origin." For the previous article in this series, see reference 1.

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In the present paper, we describe a phytochemical screening method to detect sweet steviol (13-hydroxy-*ent*-kaurenoic acid) glycoside constituents in 184 *Stevia* herbarium samples (110 species, 121 taxa) and in seven of these species that were collected in the field in Peru and Mexico. Such a study was initiated to locate new sources of sweet *ent*-kaurene glycosides within the genus *Stevia*.

RESULTS AND DISCUSSION

The sweet compound stevioside was detected by analytical tlc and hplc in leaf herbarium samples of *S. rebaudiana*, collected in Paraguay in 1919 (*Gosling s.n.*), and *S. phlebophylla* A. Gray, collected in Mexico in 1889 (*Pringle 2291*). The additional sweet compounds, rebaudiosides A and C, were detected in the *S. rebaudiana* sample. These observations confirm our previous assertion of the sweet nature of these same samples after organoleptic evaluation (1). None of the above-mentioned compounds, nor any of the other known sweet *ent*-kaurene constituents of *S. rebaudiana*, was identified in leaf samples of the remaining 108 *Stevia* species investigated.

Before commenting on the implications of these results, it is pertinent to discuss briefly the methodology described here for the purpose of phytochemical screening of *Stevia* species for the presence of sweet *ent*-kaurene glycosides. While it was not possible to isolate the sweet constituents in the limited amounts of plant material available, the positive glycoside identifications proposed here for the *S. rebaudiana* and *S. phlebophylla* samples were based on a combination of tlc and hplc data, backed up by the unequivocal generation of isosteviol methyl ester from both plants by acid hydrolysis and methylation, as demonstrated by gc/ms. Isosteviol, a known product of stevioside on acid hydrolysis (26,27), was also shown here to be produced after reflux of rebaudiosides A-E, delcoside A, and steviolbioside, a treatment undergone by all of the *Stevia* samples examined in this study. The failure to detect isosteviol on acid hydrolysis of the other 108 *Stevia* species studied not only confirms the negative results obtained after analytical tlc, but also strongly suggests that steviol glycosides, whether sweet or nonsweet, were absent from the samples studied of these species. It also may be pointed out that it was found empirically that the passage of *Stevia* *n*-BuOH-soluble fractions through alumina columns enabled the removal of many colored polar constituents while allowing the elution of the *ent*-kaurene glycosides. This stage greatly facilitated effective sweet-compound detection by tlc and hplc. The persistence of detectable amounts of stevioside in the *S. phlebophylla* sample, which was one of the oldest specimens available to us (1), not only attests to the stability of this compound on protracted herbarium storage but also to the validity of this type of approach to the study of this distribution of sweet *ent*-kaurene glycosides in the genus *Stevia*.

The apparent absence of sweet *ent*-kaurene glycosides in *Stevia* leaves previously recorded by us as exhibiting slight, sweet-taste sensations after organoleptic evaluation (1,2) may be explained by either the presence of sweet constituents other than *ent*-kaurene glycosides in the specimens or as a result of errors in the subjective judgment of the sweetness of these samples. Both the *n*-BuOH- and aqueous-soluble fractions of these slightly sweet species were analyzed by tlc, in an attempt to rule out the presence of low concentrations of rebaudiosides D and E, which are preferentially soluble in H_2O (28). Also, our failure to detect isosteviol on the acid hydrolysis of the *n*-BuOH fractions of these slightly sweet species appears to preclude the existence of novel steviol glycosides, because such compounds presumably would also hydrolyze to isosteviol under these conditions. In view of the detection here of stevioside in a *S. phlebophylla* sample that was perceived as being no more sweet than the other slightly sweet *Stevia* leaf specimens tasted (1,2), it would seem unlikely that undetected trace amounts of steviol glycosides were present in the slightly sweet species other than *S. phlebophylla*.

From a chemotaxonomic standpoint, the occurrence of stevioside in both *S. rebaudiana* and *S. phlebophylla* is somewhat unexpected. These species are distantly separated, taxonomically as well as geographically. *S. rebaudiana* is restricted in its natural distribution to Paraguay, in the Cordillera of Amambay in the northeastern part of the country, whereas *S. phlebophylla* is restricted to Mexico, in the mountains surrounding Guadalajara in the state of Jalisco. The major taxonomic differences between these two species lie in the habit of the plants, the arrangement of capitula, and the type of achenes. *S. rebaudiana* is a rhizomatous perennial herb, with paniculately arranged capitula and multiaristate achenes, while *S. phlebophylla* is a large shrub with corymbously arranged capitula and non-aristate achenes (29,30).

Grashoff (30) recognized three subgeneric groupings of the North American *Stevia* (Colombo-Panamanian border to southern United States), with taxonomic status of Series, on the basis of habit and arrangement of capitula. These three Series are Podocephalae, Corymbosae, and Fruticosae. Members of the Series Fruticosae consist of shrubs, within which *S. phlebophylla* belongs. According to Robinson (31), three subgeneric groupings with the taxonomic status of Sections may be recognized for the Peruvian and other South American *Stevia*—namely Eustevia, Multiaristatae, and Breviaristatae—all of which are herbaceous in habit. Members of the Section Multiaristatae, within which *S. rebaudiana* belongs, are characterized by the possession of achenes with a multiaristate pappus (20-30 awns per achene). It is interesting to note that achenes with a multiaristate pappus are completely absent in North American *Stevia* species (30). Grashoff (30) has speculated that the multiaristate members of the genus represent the more primitive and ancestral forms, whereas the North American species represent a derived condition, through the total or partial loss of awns. Hard and substantial evidence, however, is needed to support Grashoff's assertions.

In conclusion, it seems worthwhile to mention that the discovery of stevioside in *S. phlebophylla* will probably not have significant economic consequences since this constituent was found to occur only in trace quantities, and there is a possibility that this species may now be extinct (30). However, the present study has only embraced about 50% of the species in the genus *Stevia*, and the sweet *ent*-kaurene glycosides may be better represented in the other half of this group. In light of the recent reports of stevioside as a constituent of *Rubus suavissimus* S. Lee (32) and of the sweet steviol glycoside rubusoside, in *R. chingii* Hu (33), the phytochemical screening of further members of the genus *Rubus* (family Rosaceae) also may elucidate additional sweet *ent*-kaurene glycosides.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were measured with a Kofler apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer 241 polarimeter. A Beckman DB-G grating spectrophotometer was used to obtain the uv spectrum. The ir spectrum was obtained with a Nicolet MX-1 FT-IR spectrophotometer, and the pmr spectrum was recorded in $CDCl_3$, using TMS as internal standard, on a Varian T-60A instrument (60 MHz), with a Nicolet TT-7 Fourier Transform attachment. The low-resolution solid probe mass spectrum was obtained on a Varian MAT 112S instrument, operating at 70 eV. Analytical hplc was conducted using a Beckman Model 324 Gradient Liquid Chromatograph, equipped with a Model 421 Microprocessor Solvent System Controller and a Model 210 sample injection valve, and a Perkin-Elmer LC-85 Spectrophotometric Detector and LC Autocontrol. Combined gc/ms (70 eV) was performed on the Varian MAT 112S mass spectrometer, linked to a Varian 1440 model gas chromatograph and a Varian 166 data system.

PLANT MATERIAL.—Altogether, 184 *Stevia* leaf herbarium samples, embracing 110 species and 121 taxa, were obtained from the John G. Searle Herbarium, Field Museum of Natural History, Chicago, IL. The Latin binomial, collector, collection number, country, and date of collection of each specimen studied have been noted previously (1). In addition, seven of the 31 species collected from their native habitat between December 1980, and June 1981, were also studied as freshly dried specimens: *S. mandonii* Sch.-Bip. (*Soejarto et al. 5250*) (collected in Peru) and *S. caracasana* DC. (*Compadre & Compadre 16*), *S. monar-*

difolia HBK. (*Soejarto & Compadre 5307*), *S. origanoides* HBK. (*Compadre & Compadre 9*), *S. salicifolia* Cav. var. *salicifolia* (*Compadre & Compadre 6*), *S. serrata* Cav. var. *serrata* (*Soejarto & Compadre 5367*) and *S. subpubescens* Lag. var. *subpubescens* (*Compadre & Compadre 3*) (collected in Mexico). Voucher specimens were deposited in the Herbarium of the Field Museum of Natural History.

REFERENCE COMPOUNDS.—Authentic samples of stevioside, steviolbioside, rebaudiosides A-C, and dulcoside A were isolated from *S. rebaudiana* leaves or procured as described previously (28).

Rebaudiosides D and E were isolated from an aqueous-soluble extract of *S. rebaudiana* leaves,[6] utilizing repeated gradient elution column chromatography over silica gel,[7] with mixtures of $CHCl_3$-MeOH-H_2O in the proportions of 45:15:2 to 9:9:2. Isolated rebaudiosides D and E were recrystallized from H_2O-MeOH and exhibited mp and optical rotation data consistent with literature values (34).

MICROEXTRACTION PROCEDURE.—Powdered *Stevia* leaves (*ca.* 50-100 mg per sample) were macerated in three 7-ml portions of MeOH-H_2O (4:1), with each maceration stage lasting 48 h. Macerates were combined and filtered, with solvent being removed under reduced pressure at 55°. Each residue was dissolved in H_2O (10 ml) and defatted with three 3-ml portions of EtOAc. Aqueous layers were partitioned with *n*-BuOH (three times with 5 ml), with the combined organic phases being washed with H_2O and evaporated to dryness. The EtOAc and aqueous layers of each sample analyzed were also dried and stored.

n-BuOH extracts were passed through Pasteur pipettes plugged with cotton and packed with aluminum oxide[8] (2 g, neutral activity I), eluted with 20 ml MeOH-H_2O (1:1). Samples were prepared for preliminary tlc by removing solvent under reduced pressure.

ANALYTICAL THIN-LAYER CHROMATOGRAPHY.—Portions (*ca.* 10-20 μl) of *ca.* 1% w/v solutions in MeOH of the *n*-BuOH extracts of each *Stevia* leaf sample (stored and fresh) were subjected to analytical tlc on silica gel GHLF (250 μm, 20×20 cm) in solvents S_1 (EtOAc-EtOH-H_2O, 130:27:20), S_2 ($CHCl_3$-MeOH-H_2O, 6:3:1, lower layer), and S_3 (EtOAc-HOAc-H_2O, 8:3:2). Plates were visualized after development using the spray reagents R_1, naphthoresorcinol-H_2SO_4,[9] R_2, Ac_2O-H_2SO_4,[9] and R_3, 60% w/v H_2SO_4. Tlc plates were heated at 110° for 10 min after spraying, and were examined in both daylight (D) and long-wave ultraviolet light (UVL).

Preliminary identifications of sweet *ent*-kaurene glycosides present in the *Stevia* leaf *n*-BuOH extracts were made by comparison with the following analytical tlc data obtained for the sweet *S. rebaudiana ent*-kaurene glycosides of known structure: dulcoside A, Rf: S_1 0.28, S_2 0.24, S_2 0.46; colors with acid sprays: R_1 red-purple (D), purple (UVL), R_2 yellow-gray (D), pink (UVL), R_3 green-brown (D), pink (UVL). Rebaudioside A, Rf: S_1 0.13, S_2 0.13, S_3 0.27; colors with acid sprays: R_1 pink (D), purple (UVL), R_2 gray (D), pink (UVL), R_3 gray (D), purple (UVL). Rebaudioside B, Rf: S_1 0.22, S_3 0.22, S_3 0.47; colors with acid sprays: R_1 pink (D), purple (UVL), R_2 gray (D), pink (UVL), R_3 gray (D), purple (UVL). Rebaudioside C, Rf: S_1 0.17, S_2 0.14, S_3 0.38; colors with acid sprays: R_1 red-purple (D), purple (UVL), R_2 yellow-gray (D), pink (UVL), R_3 green-brown (D), pink (UVL). Rebaudioside D, Rf: S_1 0.05, S_2 0.02, S_3 0.13; colors with acid sprays, R_1 pink (D), purple (UVL), R_2 gray (D), purple (UVL), R_3 gray (D), purple (UVL). Rebaudioside E, Rf: S_1 0.06, S_2 0.03, S_3 0.16; colors with acid sprays, R_1 pink (D), purple (UVL), R_2 gray (D), yellow (UVL), R_3 gray (D), purple (UVL). Steviolbioside, Rf: S_1 0.32, S_2 0.31, S_3 0.58; colors with acid sprays: R_1 pink (D), purple (UVL), R_2 gray (D), pink (UVL), R_3 gray (D), purple (UVL). Stevioside, Rf: S_1 0.20, S_2 0.18, S_3 0.38; colors with acid sprays: R_1 pink (D), purple (UVL), R_2 gray (D), pink (UVL), R_3 gray (D), purple (UVL). The aqueous leaf extracts of *Stevia* specimens perceived as possessing a slight degree of sweetness in preliminary organoleptic studies (1,2) were also subjected to an initial tlc examination in this manner.

ANALYTICAL HIGH-PRESSURE LIQUID CHROMATOGRAPHY.—The identities of *ent*-kaurene glycosides in *Stevia* leaf extracts were further checked by hplc under the following conditions: column, Dupont Zorbax NH_2 (25 cm×4.6 mm internal diameter); detection, 210 nm; eluant, acetonitrile-H_2O (21:4, adjusted to pH 5 with H_3PO_4); flow rate, 2 ml/min; pressure, 176 bar; temperature, ambient; chart speed, 10 mm/min. Samples were applied to the column in 20 μl aliquots (*ca.* 0.1 μg/μl) and retention times (R_T min) were compared with the following reference solutes: steviolbioside, 4.7; dulcoside A, 7.9; rebaudioside B, 8.9; stevioside, 12.9; rebaudioside C, 16.6; rebaudioside A, 25.3. Using this isocratic elution procedure, rebaudiosides E and D were eluted, in turn, as flat peaks with retention times in excess of 1 h.

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[6]Kindly provided by Stevia Inc., Arlington Heights, IL.

[7]Merck, Darmstadt, W. Germany.

[8]Woelm, Eschwenge, W. Germany.

[9]Constituted according to Stähl (35).

CONVERSION OF SWEET STEVIOL GLYCOSIDES TO ISOSTEVIOL METHYL ESTER.—Crude stevioside (23 g) was prepared from *S. rebaudiana* leaves (250 g), by MeOH precipitation of a *n*-BuOH extract (28). Hydrolysis of this material was conducted with 20% w/v H_2SO_4 (27) (750 ml) on a steam bath for 5 h. The reaction mixture was cooled and extracted with $CHCl_3$ (five times with 300 ml), and the pooled $CHCl_3$ layers were washed with H_2O and evaporated *in vacuo* to yield a residue. This residue, when chromatographed over 200 g of silica gel[7] by elution with $CHCl_3$, afforded isosteviol, after removal of solvent and crystallization from MeOH, mp 221-223°, $[\alpha]^{25}D$ −78.1° (c 0.28, $CHCl_3$) [lit. mp 223°, $[\alpha]^{24}D$ −69.3° (c 0.075, 95% EtOH)] (36); uv, λ max 202 nm (log ε) 3.17; ir, ν max (KBr) 3400-2850 br, 1735, 1688, 1450, 1255, 1181, 1110, 1088, 980, 845 and 805 cm^{-1}; pmr, ($CDCl_3$, 60 MHz) δ 0.79, 0.98 (6H, two s, 10-, 13-CH_3), 1.25 (3H, s, 18-CH_3), 1.50-2.84 (18H, m, methylene-CH_2) and 11.3 ppm (1H, br s, CO_2H); ms *m/z* 318 (M^+, 49%), 300 (35), 275 (27), 274 (26), 273 (21), 272 (21), 259 (20), 203 (22), 165 (35), 152 (49), 121 (57), 109 (67), 107 (56), 95 (52), 93 (55), 91 (47), 81 (77), 79 (55), 67 (51), 55 (80), and 41 (100); elemental formula determination,[10] found C 75.37%, H 9.37%, O 15.11%, $C_{20}H_{30}O_3$ requires C 75.47%, H 9.43%, O 15.09%; Rf, toluene-$MeCO_2$-HOAc (200:55:0.7) (S_4) 0.55; isopropyl ether-Me_2CO-HOAc (160:45:0.5) (S_5) 0.69; color with acid spray, R_3 gray-brown (D), purple (UVL).

Isosteviol was methylated by treatment overnight with CH_2N_2[11] and subjected to gc/ms under the following conditions: column, glass (2 m×3 mm); stationary phase, 0.3% OV-101 on Gas Chrom Q (80-100 mesh); column temperature, 200-360°, programmed 4°/min; He flow rate, 18 ml/min; interface temperature, 290°; spectra were recorded with 6 sec between scans. Isosteviol methyl ester exhibited the following data: R_T 6.8 min; ms *m/z* 332 (M^+, 70%), 310 (15), 300 (66), 289 (17), 273 (100), 272 (42), 203 (19), 152 (24), 123 (31), 121 (67), 109 (58), 107 (52), 101 (35), 95 (38), 81 (51), 79 (49), 55 (61), and 41 (64).

Samples (*ca.* 5 mg) of dulcoside A, rebaudiosides A-E and steviolbioside were refluxed at 100° with 10 ml 20% w/v H_2SO_4 for 2 h. On cooling, the reaction mixtures were extracted with $CHCl_3$ (three times with 15 ml). The combined $CHCl_3$ layers were washed with 20 ml H_2O. On removal of solvent and analytical tlc of 10-20 μg portions in S_4 and S_5, isosteviol was found to be a major product for each starting compound. Identity was confirmed in each case by the detection of isosteviol methyl ester by gc/ms after methylation with CH_2N_2.[11]

The *n*-BuOH fraction (*ca.* 2-10 mg) of each *Stevia* leaf herbarium specimen studied was also subjected to the above hydrolysis, work-up, and analytical tlc procedure in order to detect the presence of isosteviol as a hydrolytic product from any steviol (13-hydroxy-*ent*-kaurenoic acid) glycosides originally present. Isosteviol methyl ester, produced in each case by methylation of the acid-hydrolyzed *S. rebaudiana* and *S. phlebophylla* leaf herbarium samples, was indistinguishable by gc/ms from the authentic sample described above.

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[10]The elemental analysis was determined by Micro-Tech Laboratories, Inc., Skokie, IL.

[11]An ethereal solution of diazomethane was prepared using a Diazald Kit, Aldrich Chemical Company, Inc., Milwaukee, WI.

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