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Diterpene-Glycosides of *Stevia Paniculata* Lag.: Structures of Aglycones

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The leaves of *Stevia paniculata* (*Compositae*) which are not sweet unlike the leaves of *S. rebaudiana*, were shown to contain several glycosides. The enzymatic hydrolysis of the crude glycosides fraction of the leaves gave four aglycones A (II), B (XVI), C (XV) and D (IV). It was proved that II and IV were identical with the known diterpene acids, *ent*-15α-hydroxykaur-16-en-19-oic acid and *ent*-16α,17-dihydroxykauran-19-oic acid, respectively. On the basis of the chemical and physical investigation as well as the comparison of the known kaurene-type diterpenes, XV and XVI could be formulated as *ent*-11α,15α-dihydroxykaur-16-en-19-oic acid and its 15-oxo derivative, respectively.

From the ether-soluble fraction, taraxasterol and its acetate were also isolated and identified.

The diterpene-glucoside, stevioside (I) which is the principal constituent of *Stevia rebaudiana* Bertoni[2] (*Compositae*), a wild herb of Paraguay, has recently attracted much attention as a substitute of synthetic sweeteners. In this respect, *S.* rebaudiana and its related species have been cultivated and investigated from pharmaceutical and agricultural point of view by our research group. The present paper deals with the isolation and the structural elucidation of the aglycones of the diterpene glycosides of *S. paniculata* Lag.

The suspension of the methanolic extract of the dried leaves (25 g) in water was extracted with ether and then with *n*-butanol, successively. The column chromatography of the ethereal fraction afforded taraxasterol and its acetate.

The butanolic fraction which was not sweet, was revealed to contain several glycosides by thin-layer chromatography (TLC). The enzymatic hydrolysis of this fraction with the crude hesperidinase[3] and the subsequent column chromatography of the resulted crude hydrolysate on silica gel gave three substances designated as aglycones A, BC, and D. The column chromatography of aglycone BC on silica gel impregnated with $AgNO_3$ furnished the separation into aglycones B and C.

On the basis of IR, NMR, and mass spectral studies, aglycone A, mp 214.5—217° (yield 93 mg) (II), (methyl ester, mp 166—169° (III)) and aglycone D, mp 257—259° (yield 20 mg) (IV) (methyl ester, mp 149—150° (V)) were suggested to be *ent*-15α-hydroxykaur-16-en-19-oic acid[4] and *ent*-16β,17-dihydroxykauran-19-oic acid,[5,6] respectively. The confirmation of the structure of aglycone A was conducted by the comparison of its methyl ester (III) with an authentic sample derived from methyl grandiflorolinate (VI)[7] *via* the 15-ketone (VII). The identification of aglycone D was completed by the direct comparison with an authentic sample. Although both aglycones A and D have already been found in nature as a free form, this is the first example of their occurrence as glycosides.

1) Location: *a) Kasumi, 1-2-3, Hiroshima-shi; b) Kasukabe, Kasukabe-shi, Saitama.*
2) E. Mosettig, U. Beglinger, F. Dolder, H. Lichiti, P. Quitt, and J.A. Waters, *J. Am. Chem. Soc.*, **85**, 2305 (1963) and references cited therein.
3) H. Kohda and O. Tanaka, *Yakugaku Zasshi*, **95**, 246 (1975).
4) J.R. Cannon, P.W. Chow, P.R. Jefferies, and G.V. Meehan, *Aust. J. Chem.*, **19**, 861 (1966).
5) P.R. Jefferies and T.G. Payne, *Aust. J. Chem.*, **18**, 1441 (1965).
6) S. Mihashi, I. Yanagisawa, O. Tanaka, and S. Shibata, *Tetrahedron Letters*, **1969**, 1683.
7) F. Piozzi, V. Sprio, S. Passannanti, and R. Mondelli, *Gass. Chim. Ital.*, **98**, 907 (1968); S. Yahara, M. Ishida, K. Yamasaki, O. Tanaka, and S. Mihashi, *Chem. Pharm. Bull.* (Tokyo), **22**, 1629 (1974).

Aglycone B, mp 259—260°, $C_{20}H_{28}O_4$ (yield 67 mg) exhibited IR absorption due to hydroxyl, carbonyl, and carboxyl groups and NMR signals assignable to two *tert*-methyls at δ 1.17 and 1.32 ppm (in pyridine-d_5). The secondary-axial nature of its hydroxyl group was disclosed by the NMR signal at δ 4.24 ppm (1H broad s, 1/2W=7.0 Hz) (in pyridine-d_5). The presence of the α,β-unsaturated five membered ring ketone system such as VIII was demonstrated by its UV absorption, λ_{max}^{EtOH} 237.5 nm (log ε 3.79), IR band at 1720 cm^{-1} (in $CHCl_3$) and NMR signals at δ 5.21, 5.98 (1H each, broad s) and 2.98 ppm (1H m) (in pyridine-d_5) in comparison with those of VII, λ_{max}^{EtOH} 233.5, $\nu_{max}^{CHCl_3}$ 1720 cm^{-1}, NMR δ 5.18, 6.00 (1H, each, broad s, >C=CH_2) and 2.91 ppm (1H m, C-13 methine) (in pyridine-d_5).

Aglycone C, mp 244.5—245.5°, $C_{20}H_{30}O_4$ (yield 85 mg) afforded a methyl ester, mp 161—163° (IX) being proved to be a dihydroxy-acid by the IR and NMR spectra. Reduction of aglycone B with $NaBH_4$ yielded aglycone C leading to the presence of the allyl alcohol system such as X on the basis of the stereochemistry of the metal hydride reduction of the α,β-unsaturated ketone VIII.[8,9] As shown in Table I, the chemical shift difference ($\Delta\delta$) of the corresponding *tert*-methyl NMR signals in pyridine-d_5 between aglycone C and its methyl

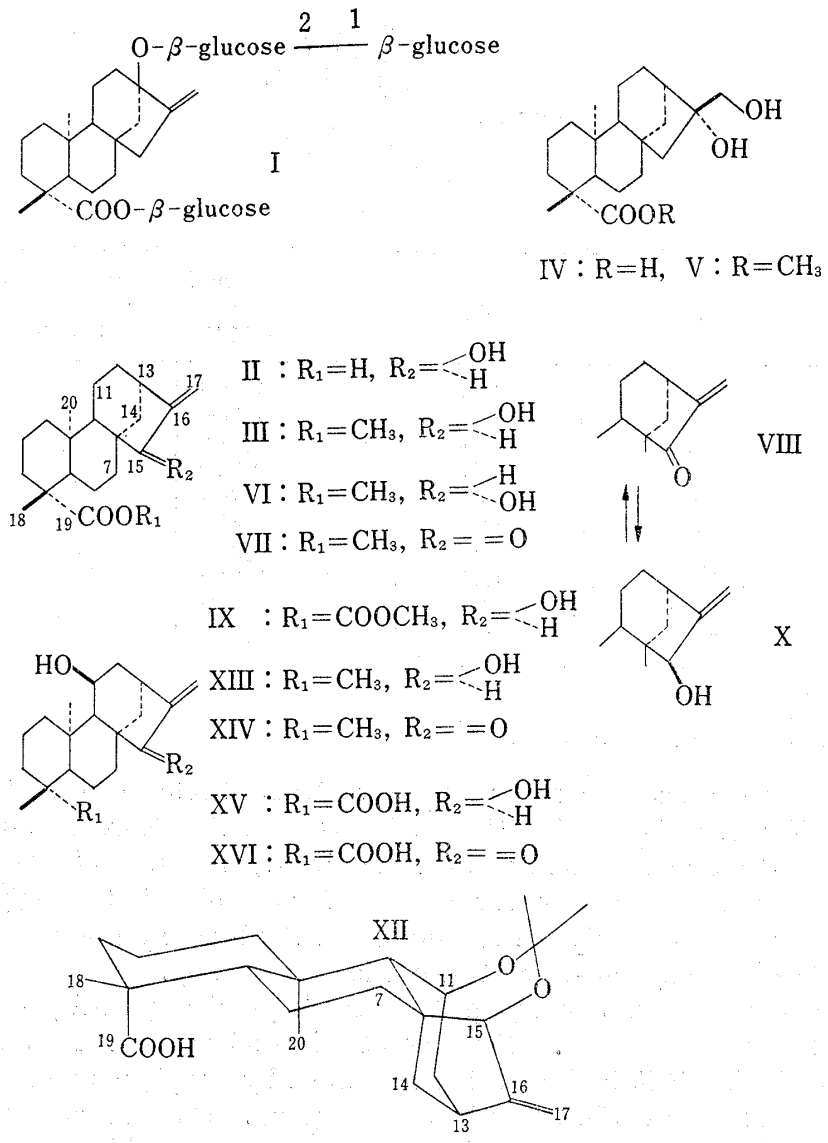


Chart 1

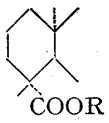
8) M.F. Barns and J. MacMillan, *J. Chem. Soc.* (*C*), **1967**, 361.
9) J.D. Connolly and I.M.S. Thornton, *J. Chem. Soc.*, Perkin I, **1973**, 736.

Table I

Compound	18 or 19 CH_3 signal δ ppm	20-CH_3 signal δ ppm
Aglycone C (XV)	1.32	1.10
Methyl ester (IX)	1.16	0.81
$\Delta\delta$ (acid-ester)	0.16	0.29
$\Delta\delta$ (acid-ester)[10] of type XIax	0.17—0.20	0.27
$\Delta\delta$ (acid-ester)[10] of type XIeq	0.17—0.20	0.08

in pyridine-d_5

XIax



XIeq

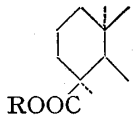
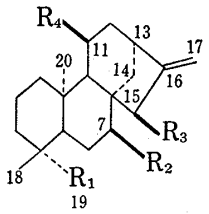


Table II.



Compound	R_1	R_2	R_3	R_4	20-CH_3 signal δ ppm	20-CH_3 signal $\Delta\delta$ ppm	other CH_3 signal δ ppm
Aglycone C (XV)	COOH	H	OH	OH	1.10[a]	0.10	1.32
Aglycone A (II)	COOH	H	OH	H	1.20[a]		1.32
IX	$COOCH_3$	H	OH	OH	0.81[a]	0.11	1.16
III	$COOCH_3$	H	OH	H	0.92[a]		1.15
IX	$COOCH_3$	H	OH	OH	0.75[b]	0.10	1.19
III	$COOCH_3$	H	OH	H	0.85[b]		1.17
XIII[9]	CH_3	H	OH	OH	0.94[b]	0.10	0.81, 0.88
XVII[8]	CH_3	H	OH	H	1.04[b]		—[c]
XVIII[11]	$COOCH_3$	OH	H	H	0.85[b]	0.00	1.16
XIX[8]	$COOCH_3$	H	H	H	0.85[b]		1.16

a) in pyridine-d_5
b) in $CDCl_3$
c) Data were not given in the literature[9].

ester (IX) demonstrated that the partial structure XIax should be present in aglycone C according to the Narayanan's rule.[10] These evidences and the comparison of the CD curve (n-π^*) of aglycone B (negative max. at 343 nm in MeOH) with that of VII (negative max. at 350 nm in MeOH) as well as the co-occurrence of *ent*-kaurenoic acid type aglycones A and D led to assign the structures of aglycones B and C as *ent*-x-hydroxy-15-oxo-kaur-16-en-19-oic acid and *ent*-x,15α-dihydroxykaur-16-en-19-oic acid, respectively.

The position of the second hydroxyl group (secondary-axial by NMR) of aglycone C was revealed by the following evidences. The concentration-independent IR band at 3440 cm^{-1} in the spectrum of IX in $CHCl_3$ indicated that the second hydroxyl group must be intramolecularly hydrogen bonded with the other functional group. On treatment with anhydrous acetone and active silica gel, aglycone C yielded an unstable acetonide (XII), mp 204—207°. These results unequivocally place the second hydroxyl group at either C-7 or C-11.

10) C.R. Narayanan and N.K. Venkatasubramanian, *Tetrahedron Letters*, **1965**, 3639.

In 1973, Connolly, *et al.* reported the isolation and the structural elucidation of *ent*-11α,15α-dihydroxykaur-16-ene (XIII) and its 15-oxo derivative (XIV) (UV λ_{max} 238 nm).[9] As shown in Table II, the introduction of an axial hydroxyl group at C-11 of the kaurene-skeleton causes the up-field shift of the 20-methyl NMR signal by *ca.* 0.10 ppm. On the contrary, no remarkable effect of the C-7 axial hydroxyl group to the 20-methyl signal is observed.[11] The 20-methyl signals of aglycone C and its methyl ester (IX) appeared at *ca.* 0.10 ppm higher position than those of the corresponding 11-deoxy diterpene acid (aglycone A (II)) and its methyl ester (III), respectively. It follows that aglycone C can be formulated as *ent*-11α,15α-dihydroxykaur-16-en-19-oic acid (XV) and accordingly aglycone B must be represented by its 15-oxo derivative (XVI).

Further, the NMR signals of IX at δ 5.08, 5.00 (each 1H broad s), 3.96 (1H, d-like),[12] 3.75 (1H broad s, 1/2W=7 Hz) and 2.60 ppm (1H m) (in $CDCl_3$) are very similar to the corresponding signals of XIII, δ 5.08, 4.98 (>C=CH_2), 3.98, 3.72 (carbinol protons), and 2.60 ppm (C-13 bridge head proton) even in the coupling features.[9] Although the precise comparison of the NMR spectra of the aglycones B and C with those of corresponding diterpenes XIII and XIV in the same solvent ($CDCl_3$) could not be made owing to the insufficient solubility of these aglycones in $CDCl_3$, the anomalous down-field shift of the C-14 axial proton signal (δ 2.40 ppm (1H d, J=12 Hz[13] in $CDCl_3$))[9] in the NMR of XIV was also observed in the spectrum of aglycone B (XVI) (δ 2.40 ppm (1H d, J=12 Hz in pyridine-d_5)), supporting the present assignment of its structure.

It should be noted that the genuine aglycones A(II), C(XV), and D(IV) would not be obtained by the mineral acid hydrolysis of the glycosides because of the acid catalyzed garryfoline-cuauchichicine rearrengement[8] (in case of II and XV) or dehydration (in case of IV). The isolation and the structural determination of the glycosides are in progress.

Experimental

All melting points were taken on a micro hot-stage and uncorrected. NMR spectra were taken on JEOL PS-100 (100 MHz). Mass spectra were determined on JEOL 01-SG-2 (double focus, high resolution).

Extraction——The dried and powdered leaves (25 g) of *S. paniculata* which was cultivated at the Experimental Station of Medicinal Plants[1b] were extracted with hot MeOH and the MeOH solution was concentrated to dryness *in vacuo*. The suspension of the residue in H_2O was extracted with $(C_2H_5)_2O$ and then with *n*-BuOH (saturated with H_2O), successively.

The $(C_2H_5)_2O$ layer was evaporated and the residue was chromatographed on silica gel. The elution with $CHCl_3$: MeOH (1000: 1) afforded taraxasteryl acetate, mp 238—241° (from $CHCl_3$–MeOH) which was identified with an authentic sample by mixed melting point and the comparison of TLC, NMR, IR and mass spectra.

The further elution with $CHCl_3$: MeOH (200: 1) gave taraxasterol, mp 218—220° (from $CHCl_3$–MeOH) which was proved to be identical with the authentic sample by mixed melting point and the comparison of TLC, IR, NMR and mass spectra.

Enzymatic Hydrolysis of the Crude Glycosides Fraction——The *n*-BuOH layer of the above separation was concentrated to dryness affording the crude glycosides fraction in a yield of 6.2%, the TLC of which indicated the presence of several glycosides; solvent $CHCl_3$: MeOH: H_2O (65: 35: 10) on silica gel. No stevioside (I) was detected in this fraction. To a solution of this fraction (1.2 g) in phosphate buffer (0.2M pH 4.0, 200 ml) were added the crude hesperidinase (produced by Tanabe Pharm. Ind. Co. Ltd.; crude preparation before the addition of the diluent was used[3]) (1.0 g) and three drops of toluene. The mixture was incubated at 37° for 48 hr and then extracted with $(C_2H_5)_2O$. The $(C_2H_5)_2O$ layer was concentrated to dryness and the residue (470 mg) was chromatographed on silica gel by the gradient elution with $CHCl_3$: MeOH.

From the eluate with $CHCl_3$: MeOH (100: 2), aglycone A (II) was obtained as colourless prisms, mp 214.5—217° (from C_6H_6), $[\alpha]_D$−83.5° ($CHCl_3$) (93 mg). On methylation with CH_2N_2 in $(C_2H_5)_2O$, II yielded the methyl ester (III), colourless prisms, mp 166—169° (from $(C_2H_5)_2O$–petroleum ether) which was proved

11) J.R. Hanson and A.F. White, *Tetrahedron*, **25**, 2743 (1969) and the references cited therein.
12) C-15 carbinol proton, long-range coupling with C-13 proton.
13) The torsion angle between C-13 proton and C-14 axial proton is *ca.* 80—90° irrespective of ring C being in a boat or a chair form. This requires coupling constant of *ca.* 0 Hz between these protons.[9]

to be identical with an authentic specimen of methyl *ent*-15α-hydroxykaur-16-en-19-oate by mixed melting point and comparison of TLC, optical rotation and IR. The authentic sample of III was prepared from VI[7] according to the procedure reported by MacMillan, *et al.*[8]: Oxidation of VI with CrO_3 in pyridine at room temperature for 14 hr gave the 15-ketone (VII), mp 149—151° (from n-C_6H_{14}) as colourless prisms. On reduction with $NaBH_4$ in MeOH at room temperature overnight, VII yielded III, colourless prisms, mp 166—169° (from $(C_2H_5)_2O$-petroleum ether).

From the eluate with $CHCl_3$: MeOH (100: 4), a mixture of aglycones B and C (211 mg) was obtained which was subjected to the second chromatography on silica gel impregnated with 8% $AgNO_3$. Elution with $CHCl_3$: MeOH (100: 2) afforded aglycone B (XVI) (67 mg) and further elution with $CHCl_3$: MeOH (100: 3) gave aglycone C (XV) (85 mg).

From the eluate of the first chromatography on silica gel with $CHCl_3$: MeOH (100: 6), there was obtained aglycone D (IV) as colourless prisms, mp 257—259° (from $CHCl_3$–MeOH), $[\alpha]_D$ −85.0° (pyridine) (20 mg), which was proved to be identical with *ent*-16β,17-dihydroxykauran-19-oic acid by mixed melting point and the comparison of TLC, optical rotation IR and NMR with an authentic sample.[6]

Aglycone B (XVI)——Colourless needles, mp 259—260° from C_6H_6 or $CHCl_3$, $[\alpha]_D$ −159.0° (MeOH). M^+ Calcd. for $C_{20}H_{28}O_3$, 332.19876. Found 332.19889. IR $\nu_{max}^{CHCl_3}$ 3620, 3520 (OH), 3200—2400, 1690 (COOH), and 1720 cm^{-1} (C=O).

Aglycone C (XV)——Colourless prisms, mp 244.5—245.5° from $CHCl_3$, $[\alpha]_D$ −77.9° (MeOH). The molecular formula of XV, $C_{20}H_{30}O_4$ was determined by the high resolution mass spectrometry of its methyl ester (IX) (see below). IR ν_{max}^{KBr} 3350 (OH) and 3200—2400, 1695 cm^{-1} (COOH), NMR (in pyridine-d_5) (methyl signals; see Table I and II) δ 2.60 (1H, m, C-13H), 4.02 (1H broad s, 1/2W=7 Hz, C-11H), 4.16 (1H d-like,[12] C-15H), 5.12 and 5.33 ppm (each 1H broad s, C-17 vinyl protons). To a suspension of XV in $(C_2H_5)_2O$ was added a solution of CH_2N_2 in $(C_2H_5)_2O$ and the mixture was allowed to stand untill all of the crystals (XV) were dissolved. After working up in the usual way, the product was recrystallized from MeOH to give the methyl ester (IX), colourless prisms, mp 161—163°. M^+ Calcd. for $C_{31}H_{52}O_4$, 348.2294. Found: 348.2300. IR $\nu_{max}^{CHCl_3}$ 3600 (free OH), 3440 (concentration-independent, intramolecularly hydrogen bonded OH), and 1720 cm^{-1} ($COOCH_3$), NMR (in $CDCl_3$) δ 3.67 ppm (3H s, $COOCH_3$).

Reduction of XVI to XV——Aglycone B (XVI) (10 mg) was dissolved in dioxane–H_2O (1: 1) and to this solution was added a solution of $NaBH_4$ in dioxane. The reaction mixture was allowed to stand at room temperature overnight. After worked up in the usual way, the crude products were purified by column chromatography on silica gel impregnated with 8% $AgNO_3$. Elution with C_6H_6–AcOEt (2: 3) afforded XV (6 mg) after recrystallization from $CHCl_3$. The identification was achieved by mixed melting point and the comparison of TLC (on 8% $AgNO_3$–silica gel, solvent C_6H_6: AcOEt 1: 2), IR and NMR with the natural aglycone C.

Formation of the Acetonide (XII) from XV——To a solution of XV (20 mg) in anhydrous acetone (20 ml) was added activated silica gel (for column chromatography, activated at 120° for 3 hr, 50 mg) and the mixture was refluxed for 5 hr. The silica gel was removed by filtration and the filtrate was concentrated to dryness. The residue was recrystallized from C_6H_6 to give XII as colourless prisms, mp 204—207°, which was readily regenerated XV on treatment with MeOH. This acetonide (XII) exhibited no OH band in its IR and showed NMR signals (in pyridine-d_5) at δ 1.12 (3H s), 1.33 (3H s), 1.46 (6H s, methyls of acetonide), 2.60 (1H m, C-13H), 4.08, 4.20 (each 1H broad s, carbinol protons at C-11 and -15), 5.16, and 5.38 ppm (each 1H broad s, C-17 vinyl protons). The instability of the sterically hindered acetonide was reported by Y. Tsuda, T. Sano and K. Isobe (*Chem. Pharm. Bull.* (Tokyo), 22, 2396 (1974)).

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