Constituents from *Gymnema sylvestre* Leaves VI: Acylated Genins of the Gymnemic Acids—Isolation and Preliminary Characterization

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Abstract □ With the aid of a selective enzyme system, genins G, K, N, and gymnestrogenin were isolated and shown to be the aglycones of gymnemic acids A–D, respectively. Genin G was found to be an acylated derivative of gymnemagenin containing formic, acetic, isovaleric, and tiglic acids, while genin K differed from G by the absence of the acetic acid residue. Genin N was observed to be gymnestrogenin tiglate. Genin J, probably an artifact originating from genin G, was also isolated and indicated to be gymnemagenin esterified with acetic, isovaleric, and tiglic acids. The sugar moieties of acids A and B are not acylated, while those of acids C and D are indicated to be esterified with ferulic acid.

Keyphrases □ *Gymnema sylvestre* leaves—acylated genins, isolation □ Genins, acylated—*G. sylvestre* gymnemic acids □ Paper chromatography—identity □ TLC—identity □ IR spectrophotometry—structure □ GLC—identity

In the preceding paper (1), the authors described the isolation and preliminary characterization of the major constituents, acids A–D, of gymnemic acid, the anti-sweet principle of *Gymnema sylvestre* R. Br. (*Asclepiadaceae*) leaves. Gymnemic acids A–D were shown to be acidic glycosides containing glucuronic acid as the sugar component, while acids C and D were also observed to possess glucose. This paper deals with further developments in the structural elucidation of these gymnemic acids.

Acidic or enzymatic hydrolysis of individual gymnemic acids yielded genin mixtures consistent with the presence of acylated genins in the parent glycosides. Characterization of neutral components, obtained after acidic and basic hydrolyses of individual acids A–D, was carried out to establish their genin structure after complete deacylation. Identification of carboxylic acids in the basic hydrolysates of gymnemic acids A–D was then performed to characterize further each gymnemic acid. A selective enzyme procedure, recently developed by Kapadia (2), was utilized to secure the intact acylated genins of the gymnemic acids which were then available for structural investigation. A comparative study of the acylated genins obtained in this investigation with those isolated by Stöcklin *et al.* (3) was also conducted.

EXPERIMENTAL[1]

Reagents—Firebrick (Gas-Chrom RA, 80/100 mesh) and neopentylglycolsuccinate, regular (HI-EFF) (both from Applied Science Labs.); β-glucuronidase aryl sulfatase from *Helix pomatia*, B grade, 160,000 Fishman units/ml. (Calbiochem); and a commercial insecticide ("Real-Kill," Real-Kill Products, Division of Cook Chemical Co., Kansas City, Mo.).

Paper Chromatography—Hydroxamic derivatives (4) (from 10 mcg. of reference carboxylic acids and 100–500 mcg. of sample fractions) were spotted on Whatman No. 1 paper (57 × 19.5 cm.) and saturated for 16 hr. with the aqueous phase of solvent systems employed for development. Chromatograms were developed by descending technique to a distance of 48 cm. For analysis of hydroxamic derivatives of carboxylic acids, Solvent System A, *n*-butanol–dimethyl formamide–water (9:1:1), and Solvent System B, *n*-butanol–acetic acid–water (4:1:5) (4), were employed. Ferric chloride spray reagent was used to visualize hydroxamic acids on chromatograms (4).

Thin-Layer Chromatography—Silica gel G TLC of the genins was performed as in the case of the gymnemic acids (1). Solvent Systems I–IV are described in Table I. Ceric sulfate–sulfuric acid reagent (6) was used as the chromogen.

Identification of ferulic acid by silica gel G TLC was carried out in the following solvent systems: V, ethanol–water–ammonium hydroxide (90:25:4); VI, benzene–dioxane–acetic acid (90:25:4); and VII, *n*-butyl ether (water-saturated)–acetic acid (10:1) (7). Detection of ferulic acid on chromatograms was by spraying with diazotized sulfanilic acid reagent (8).

Gas-Liquid Chromatography—Preparation of a stationary phase of firebrick (Gas-Chrom RA, 80/100 mesh) treated with 10% neopentylglycolsuccinate and 2% phosphoric acid, and packing of columns [1.52 m. × 0.32 cm. (5 ft. × 0.125 in.) copper] were carried out as described in the literature (9–11). Columns were conditioned for 12 hr. as follows: oven temperature, 195°; detector temperature, 200°; injection port temperature, 200°, and carrier gas, helium, at 40 p.s.i. Carboxylic acids (sample size: reference acids, 15 mcg.; sample fraction, ~200 mcg.) were chromatographed under the following conditions: oven temperature, 125°; detector temperature, 210°; injection port temperature, 200°; carrier gas, helium, 60 ml./min. (80 p.s.i.); hydrogen, 50 ml./min. (16 p.s.i.); air, 380 ml./min. (16 p.s.i.); attenuation, 1; and range, 50.

Acidic Hydrolysis—Thirty-milligram quantities of the gymnemic acids were refluxed with 4 ml. of 3 *N* ethanolic hydrochloric acid for 72 hr. The warm hydrolysates were treated with 5 mg. of activated charcoal[2] and concentrated to 3 ml. The concentrates were diluted with water to precipitate genins which were collected by centrifugation after 1 hr. of refrigeration. After washing with water twice, genin precipitates were dried to yield white residues weighing 12–15 mg. TLC Systems I–III (Table I) were employed to analyze these genin residues.

Basic Hydrolysis and Identification of Carboxylic Acids—Basic hydrolysis of gymnemic acids was performed by refluxing 20-mg. samples in 5 ml. of 5% methanolic potassium hydroxide solution for 2 hr. Cooled reaction mixtures were poured into 10-ml. portions of water and precipitated genins were filtered off. After removal of methanol from alkaline filtrates *in vacuo*, they were acidified with 2 *N* sulfuric acid and extracted with ether (6 × 10 ml.). Ether extracts were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness *in vacuo*. Yellow oily residues weighing 4–7 mg. were obtained in each case. These residues were dissolved in minimal amount (~0.1 ml.) of either ether or methanol, and 3–6 μl. of these fractions were analyzed for the presence of free carboxylic acids by GLC. Table II summarizes GLC results. Identification of ferulic acid was carried out by TLC in Solvent Systems V–VII.

Methyl esters of carboxylic acids present in these described fractions were prepared by treatment with diazomethane (12) and

[1] Melting points were recorded on a Kofler hot stage and are uncorrected. IR spectra were taken on Perkin-Elmer spectrophotometer models 137B and 337. F&M model 700 gas chromatograph was employed for GLC of carboxylic acids. Enzymatic hydrolyses were carried out in either a Precision constant-temperature water bath or a Precision incubator maintained at the desired temperature. Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich.

[2] Norit, American Norit Co.

Table I—Thin-Layer Chromatography of the Genins

Genin	R_f Value in Solvent System[a] I	II	III	IV
G	0.64	0.63	0.70	0.73
J	0.55	0.49	0.62	0.67
K	0.47	0.38	0.54	0.63
N	0.39	0.35	0.50	0.61
Gymnestrogenin	0.32	0.16	0.44	0.56
Gymnemagenin	0.23	0.10	0.27	0.40

[a] Solvent Systems: I, benzene–methanol–acetic acid (45:8:4) (5); II, chloroform–methanol (9:1) (3); III, benzene–chloroform–methanol (5:8:3); and IV, benzene–methanol (7:3).

Table II—Gas-Liquid Chromatography of the Carboxylic Acids from the Gymnemic Acids

Acid	R_t, min.[a]
Formic, acetic[b]	1.57
Propionic	2.51
Isobutyric	3.06
n-Butyric	4.15
Isovaleric	4.93
Tiglic	10.81
Unidentified	20.05
Unidentified	21.54

[a] Relative to solvent front. Ferulic acid could not be chromatographed under the conditions employed. [b] Identification of these two acids was accomplished by paper chromatography of their hydroxamic acid derivatives (3, 4).

then converted to hydroxamic acids by reacting with hydroxylamine according to the procedure of Bayer and Reuther (4). These hydroxamic acids were analyzed by PC in Solvent Systems A and B for the presence of formic and acetic acid derivatives.

Results of these chromatographic identifications of carboxylic acids in the hydrolysates of various gymnemic acids are given in Table III.

Isolation of Genins—*Gymnemagenin*—Gymnemic acid A (30 mg.) was refluxed with 5 ml. of 8% sulfuric acid in 50% aqueous ethanol for 6 hr. The reaction mixture was then concentrated to about 1 ml. *in vacuo* and poured into 20 ml. of ice water. The precipitated material was collected by centrifugation, dried, and refluxed with 5 ml. of 5% methanolic potassium hydroxide for 6 hr. Concentration of the reaction mixture to about 1 ml. and pouring into 20 ml. of ice water yielded 18.1 mg. of a white residue. Recrystallization of this residue from chloroform–methanol gave gymnemagenin as white needles, m.p. 329.5–331° [lit. (3) m.p. 328–335°]; $\nu_{max.}^{KBr}$ 3450 (O—H), 2900 (C—H), 1635 (trisubstituted C=C), 1250 (H-bonded O—H), 1070 and 1040 cm.$^{-1}$ (C—O or C—OH).

Anal.—Calcd. for $C_{30}H_{50}O_6$: C, 71.11; H, 9.95. Found: C, 70.95; H, 9.91.

Identity of gymnemagenin with that isolated by Reichstein's group (3) was established by undepressed mixed melting point, superimposable IR spectra, and TLC in Solvent Systems I–IV.

Gymnestrogenin—To a suspension of 10 mg. of gymnemic acid D in 10 ml. of water, 0.1 ml. of β-glucuronidase (*Helix pomatia*) preparation and 2 drops of insecticide (Real-Kill) were added and kept at 40 ± 1° for 5 days. The enzyme digest was then boiled for 1 min., cooled, diluted with 50 ml. of ethanol, and filtered through a bed of diatomaceous earth.[3] The filtrate was evaporated *in vacuo* and the residue obtained was extracted with chloroform (4 × 10 ml.). Pooled chloroform extracts, upon removal of solvent, gave 2.9 mg. of a white residue which was recrystallized from chloroform–methanol to yield fine needles of gymnestrogenin, m.p. 286–288° [lit. (13) m.p. 288–289°]; $\nu_{max.}^{KBr}$ 3470 (O—H), 2945 (C—H), 1635 (trisubstituted C=C), 1070 and 1040 cm.$^{-1}$ (C—OH).

Gymnestrogenin was observed to be homogeneous by TLC in Solvent Systems I–IV and identical to a reference sample (13) by TLC, undepressed mixed melting point, and superimposable IR spectra.

Genin G—To a suspension of 100 mg. of gymnemic acid A in 50 ml. of water, 0.2 ml. of β-glucuronidase (*Helix pomatia*) and 5 drops of insecticide were added and kept at 40 ± 1° for 5 days. Isolation of 34.6 mg. (34.6%) of hydrolysis product was carried out as in the case of gymnestrogenin. This product was observed to be homogeneous by TLC in Solvent Systems I–IV. Recrystallization of the hydrolysis product from petroleum ether–chloroform gave genin G as shiny white crystals, m.p. 164–166°; $\nu_{max.}^{KBr}$ 3450 (O—H), 2960 (C—H), 1740 (C=O), 1460 and 1385 (side-chain methylene and methyl of $-CH_2-O-COCH_3$, respectively), 1260 (H-bonded O—H), 1080 and 1040 cm.$^{-1}$ (C—OH).

Anal.—Calcd. for $C_{43}H_{66}O_{11}$: C, 69.51; H, 8.95. Found: C, 69.52; H, 9.11.

Genin J—A 450-mg. sample of the parent gymnemic acid mixture isolated by the ethyl acetate procedure (1) was dissolved in 150 ml. of 0.01 *N* potassium bicarbonate, 1 ml. of β-glucuronidase (*Helix pomatia*) preparation added, and kept at 37 ± 0.5° for 5 days (3). The enzyme digest was then diluted with 750 ml. of ethanol, filtered through a bed of diatomaceous earth and the filtrate evaporated *in vacuo* to yield 398.7 mg. of a pale-yellow residue. This residue was found by TLC (Table I) to be a mixture composed of genins G, J, K, gymnestrogenin, and gymnemagenin.

A 2.6-mg. quantity of genin J was isolated from the above genin mixture by preparative TLC[4] using Solvent System II for development. Recrystallization from petroleum ether–chloroform yielded genin J as white crystals, m.p. 195–196° [lit. (3) m.p. 193–196°]; $\nu_{max.}^{KBr}$ 3450 (O—H), 2960 (C—H), 1740 (C=O), 1460 (side-chain methylene), 1390 (side-chain methyl), 1260 (H-bonded O—H), 1080 and 1040 cm.$^{-1}$ (C—OH). Genin J was found to be homogeneous by TLC in Solvent Systems I–IV.

Genin K—To a suspension of 20 mg. of gymnemic acid B in 10 ml. of water, 0.1 ml. of β-glucuronidase (*Helix pomatia*) and 2 drops of insecticide were added and kept at 40 ± 1° for 5 days. Isolation of genin K from the hydrolysate was carried out as described for gymnestrogenin. A yield of 6.1 mg. of genin K was obtained. Recrystallization from petroleum ether–chloroform gave genin K as white crystals, m.p. 146–148°; $\nu_{max.}^{KBr}$ 3450 (O—H), 2950 (C—H), 1710 (C=O), 1455 and 1390 (side-chain methylene and methyl, respectively), 1265 (H-bonded O—H), 1080 and 1045 cm.$^{-1}$ (C—OH). Genin K was observed to be homogeneous by TLC in Solvent Systems I–IV.

Genin N—A 10-mg. quantity of gymnemic acid C was subjected to enzymatic hydrolysis with the selective β-glucuronidase (*Helix pomatia*) preparation as described in the isolation of genin K. The hydrolysis product (3.6 mg.) was found to consist of genin N with trace amounts of genins G and K, based upon TLC in Solvent Systems I–IV.

RESULTS AND DISCUSSION

Detection of the Acylated Genins—Stöcklin *et al.* (3) observed that hydrolysis of their gymnemic acid A mixture[5] by the snail (*Helix pomatia*) β-glucuronidase yielded a genin mixture composed of genins G, J, K, and gymnemagenin. A direct TLC comparison of this genin mixture with that obtained during similar enzymatic hydrolysis of gymnemic acid A from these studies established the two genin mixtures to consist of identical components. Also, TLC analysis of nonsugar fractions secured by acidic hydrolysis of gymnemic acids A–D from these studies revealed them to be mixtures of closely related genins.

The production of genin mixtures during acidic hydrolysis of the gymnemic acids is consistent with the reported (3) presence of acyl groups which would undergo partial hydrolysis in acid medium (14). Indeed, when the genin mixture resulting from gymnemic acid A was refluxed with 5% methanolic potassium hydroxide solution, it yielded a single compound, gymnemagenin,[6] in a completion of

[3] Celite 535, Johns-Manville Co.

[4] E. Merck silica gel GF 254 preparative plates (thickness 2 mm.).

[5] The Reichstein group described this mixture to be composed of acids A_1–A_4, of which acids A_1 and A_2 were found to be identical to the authors' acids A and B (1).

[6] Gymnemagenin was found to be identical to a reference sample isolated by Reichstein's group (3) by TLC, undepressed mixed melting point, and superimposable IR spectra. Structure I has been recently assigned for gymnemagenin (15, 16).

Table III—Constituents of Gymnemic Acids A–D

Gymnemic Acid	Sugar	Acylated Aglycone	Parent Genin	Sugar Residue	Carboxylic Acids[a] in Aglycone: Formic	Acetic	Isovaleric	Tiglic
A	Glucuronic acid	G	Gymnemagenin	None	+	+	+	+
		J	Gymnemagenin	—		+	+	+
B	Glucuronic acid	K	Gymnemagenin	None	+		+	+
C	Glucuronic acid Glucose	N	Gymnestrogenin	Ferulic[b]				+[b]
D	Glucuronic acid Glucose	—	Gymnestrogenin	Ferulic				

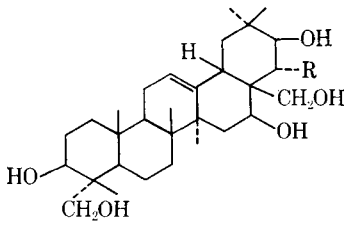
[a] Results obtained from alkaline hydrolysis of genins G, J, and K have been supported by preliminary NMR and mass spectrometric studies (26). [b] This distribution of ferulic and tiglic acid is deduced by comparison to gymnemic acid C and not by direct identification of the acids in the hydrolysate of genin N.

the hydrolysis. Alkaline hydrolysis of the genin mixture obtained by the Swiss investigators also gave them gymnemagenin. Similarly, acidic and basic hydrolyses of gymnemic acid B afforded gymnemagenin, while acids C and D upon identical treatment produced gymnestrogenin.[7] Chromatographic examination of alkaline hydrolysates of the gymnemic acids confirmed the presence of carboxylic acids, thus establishing these glycosides to be acylated derivatives.

Identification of the Carboxylic Acids—From the hydrolysate obtained after enzymatic and alkaline hydrolyses of their gymnemic acid A_1–A_4 mixture, Reichstein's group (3) identified formic, acetic, *n*-butyric, isovaleric, and tiglic acids by GLC and PC. Gymnemic acid A (the present authors' designation), when subjected to a similar detection procedure, revealed the presence of formic, acetic, isovaleric, and tiglic acids in the basic hydrolysate, while gymnemic acid B yielded all these acids except acetic acid. Ferulic and tiglic acids were found in the alkaline hydrolysate of gymnemic acid C while acid D yielded only ferulic acid. The basic hydrolysate of the ethyl acetate parent mixture (1) of gymnemic acids A–D was also examined by GLC and PC. In addition to those compounds given above as arising from acids A–D, trace amounts of *n*-butyric (as noted by the Swiss investigators), isobutyric, propionic, and two unidentified acids were detected (Table II)

Isolation of the Acylated Genins—During enzymatic hydrolysis of their gymnemic acid A mixture, Stöcklin *et al.* (3) found that cleavage of acyl groups present in these glycosides had occurred along with liberation of glucuronic acid. These investigators indicated that this cleavage of acyl groups could be due to either saponification under the alkaline condition (0.01 *N* potassium bicarbonate) employed or to the action of esterase enzymes known to be present in snail β-glucuronidase preparations (3, 17–19). In the present investigation, hydrolysis of individual gymnemic acids by similar enzyme preparation[8] in neutral solutions also cleaved acyl groups along with glycosidic linkages. This suggested esterase activity of the β-glucuronidase preparation to be responsible for the hydrolysis of acyl groups.

During a structural elucidation of the cardenolide glycoside, acospectoside A, Kapadia (2) recently developed a procedure for a selective enzymatic hydrolysis of the glycosidic linkage in the presence of an acyl group. This was accomplished by inhibiting esterase activity of the enzyme preparation through addition of a few drops of the insecticide which contains an esterase inhibitor, *O,O*-dimethyl-*O*-(2,2-dichlorovinyl) phosphate (DDVP) (25). In the present investigation, a similar selective enzyme system made from the commercial β-glucuronidase (*Helix pomatia*) preparation was also found to cleave selectively glycosidic linkages in the gymnemic acids.



I, R = OH
II, R = H

By use of this selective enzyme preparation, gymnemic acids A, B, and D were observed to produce single genins G, K, and gymnestrogenin, respectively, in neutral solutions at 40 ± 1°. However, if hydrolysis was allowed to proceed beyond 6 days, degradation products of the initially formed genins also appeared. Accordingly, crystalline genins G, K, and gymnestrogenin were isolated from the corresponding enzyme digests by allowing hydrolysis to proceed for 5 days. Similar hydrolysis of gymnemic acid C yielded a new genin, N,[9] as a major product with trace amounts of genins G and K. The latter two genins can be assigned to traces of acids A and B originally present in the gymnemic acid C sample employed. These enzymatic hydrolyses were followed by TLC in Solvent System II.

Stöcklin *et al.* (3) attempted to isolate the acylated genins from the snail enzyme hydrolysate of their gymnemic acid A mixture by silicic-acid chromatography. This resulted in the isolation of genin J in crystalline form, m.p. 193–196°, while genins G and K were obtained as amorphous solids. However, the latter two genins yielded crystalline acetate derivatives.

Due to its apparent structural similarity and potential utility in the structure proof of the acylated genins, genin J was also isolated in this investigation. This was accomplished by preparative TLC from the genin mixture obtained by nonselective enzymatic hydrolysis of the parent mixture of acids obtained by the ethyl acetate procedure (1).

Characterization of Acyl Functions—Reichstein's group (3), based upon elemental analysis and the identification of carboxylic acids in their gymnemic acid mixture, postulated gymnemic acid A_1

[7] Gymnestrogenin was observed to be identical to a reference sample isolated by Stöcklin (13) directly from the leaves of *G. sylvestre* and he has also designated Structure II to this compound.

[8] The β-glucuronidase preparation from the snail, *Helix pomatia*, has been demonstrated to effect the cleavage of β-linked D-glucose (20) as well as D-glucuronic acid (21) present in several steroid and triterpenoid glycosides. In addition, this snail enzyme preparation has been found to cleave D-glucose residues from various glycosides that were resistant to similar hydrolysis by other enzymes known to cleave β-linked D-glucose, *e.g.*, strophanthobiase (20, 22, 23). It may be mentioned here that gymnemic acids A–D could not be hydrolyzed by either β-glucuronidase (bovine liver) or β-glucosidase (almond emulsion) preparations (24).

[9] Naming of the new aglycone, genin N, is a continuation of Stöcklin's nomenclature (13).

(the present authors' acid A), $C_{49}H_{74}O_{16}$, to be the glucuronide of gymnemagenin containing 1 mole of formic, acetic, isovaleric, and tiglic acids through ester linkages. Similarly, genin J, $C_{40}H_{64}O_8$, was hypothesized to be mono-*o*-isovaleryl-mono-*o*-tiglyl-gymnemagenin. Except for the IR spectra, no further work on genins G, J, K, and their acetates has been reported by Reichstein's group.

The present authors' experiments utilizing the selective enzyme preparation clearly established genins G, K, N, and gymnestrogenin to be the aglycones of gymnemic acids A, B, C, and D, respectively. Basic hydrolysis of genins G, J, and K showed them to be acylated derivatives of gymnemagenin, while similar hydrolysis of genin N proved it to be an acylated derivative of gymnestrogenin. These relationships are summarized in Table III. It may be noted that genin J differs from genin G only by absence of a formyl group. It is suggested that genin J arises primarily as an artifact of the further hydrolysis of genin G rather than from a minor gymnemic acid. This is based upon the isolation of genin G in significant yields after nonspecific enzymatic hydrolysis of the ethyl acetate-extracted acids, a fraction relatively free of minor gymnemic acids.

A distribution of carboxylic acids between sugar and aglycone portions of gymnemic acids A–D is also indicated in Table III. Basic hydrolysis of both gymnemic acid A and genin G yielded identical mixtures of formic, acetic, isovaleric, and tiglic acids. Similarly, alkaline hydrolysis of gymnemic acid B and genin K gave identical carboxylic acid mixtures (formic, isovaleric, and tiglic acids). This deleted the possibility of hydroxyl groups in the sugar moieties of gymnemic acids A and B being esterified. However, since gymnemic acid D yields gymnestrogenin upon selective enzyme treatment and liberates ferulic acid only after its basic hydrolysis, it is indicated that ferulic acid is present in the sugar part of this glycoside. Based upon the similarity between gymnemic acids C and D, both in their genin and sugar portions, it is postulated that the ferulic acid found in the basic hydrolysate of acid C is also present in the sugar residue of this acid. However, an identification of carboxylic acids in a basic hydrolysate of genin N, the aglycone of gymnemic acid C, has not been carried out due to a lack of pure sample.

Investigations directed toward the position assignment of acyl and sugar residues in the basic genin structure of gymnemic acids A–D are currently in progress.

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