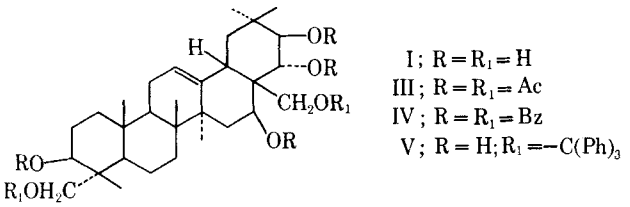
Constituents from *Gymnema sylvestre* Leaves VIII: Isolation, Chemistry, and Derivatives of Gymnemagenin and Gymnestrogenin

G. SUBBA RAO* and JOSEPH E. SINSHEIMER

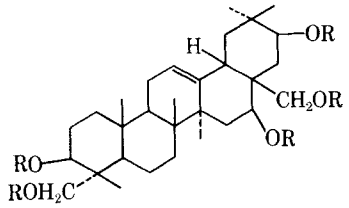
Abstract □ Isolation of crystalline gymnemagenin and gymnestrogenin directly from the leaves of *Gymnema sylvestre*, together with preparation of various derivatives of the two aglycones, is described.

Keyphrases □ *Gymnema sylvestre* leaves—constituents □ Gymnestrogenin, gymnemagenin—isolation, identification □ IR spectrophotometry—structure □ UV spectrophotometry—structure

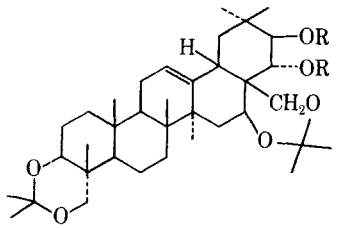
In an earlier paper (1) on the constituents of gymnemic acid, the antisweet principle of *Gymnema sylvestre* R. Br. (Asclepiadaceae) leaves, the authors showed gymnemagenin (I) and gymnestrogenin (II) to be the aglycones of gymnemic acids A and B and C and D, respectively. The antiviral activity of acids A and B was also noted (2). To elucidate the structure of gymnemagenin and to obtain material for antiviral structure–activity studies, it became necessary to develop an isolation procedure for securing substantial quantities of this aglycone directly from the leaves of *G. sylvestre*. The present paper describes: (*a*) procedures for the isolation of crystalline gymnemagenin and gymnestrogenin; (*b*) the chemistry of gymnemagenin to support Structure I, deduced recently on the basis of spectral data (3–5); and (*c*) the synthesis of derivatives of the aglycones and of gymnemic acid A for antiviral activity studies.



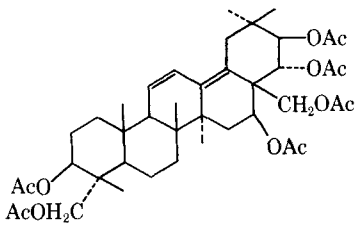
I; R = R₁ = H
III; R = R₁ = Ac
IV; R = R₁ = Bz
V; R = H; R₁ = —C(Ph)₃



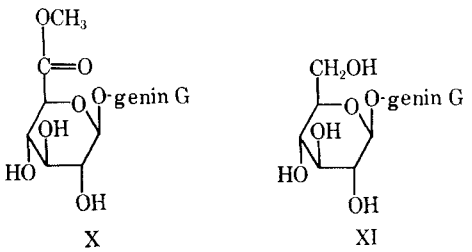
II; R = H
VI; R = Ac



VII; R = H
VIII; R = Ac



IX



X XI

Acidic and basic hydrolyses of the crude gymnemic acid mixture, obtained by mineral acid precipitation (6, 7) from the aqueous extracts of *G. sylvestre* leaves, gave gymnemagenin containing a trace amount of gymnestrogenin. Resolution of this genin mixture was carried out by silicic acid chromatography. Recrystallization from chloroform–methanol gave gymnemagenin (I) as stout, white needles, m.p. 329.5–331° [lit. (8), m.p. 328–335°], and gymnestrogenin (II) as fine needles, m.p. 286–288° [lit. (9), m.p. 288–289°]. Identity of gymnemagenin and gymnestrogenin with reference samples (8, 9) was established through undepressed mixed melting point, superimposable IR spectra, and identical TLC behavior. Similarly, the hexaacetate of gymnemagenin (III) and the pentaacetate of gymnestrogenin (VI) were found to be identical to the corresponding reference compounds.

Gymnemagenin formed the hexaacetate (III), m.p. 290–291° [lit. (8), m.p. 290–291°], as well as an hexabenzoate (IV), m.p. 186–188°, under mild conditions. This indicated the presence of six sterically unhindered primary and/or equatorially oriented secondary hydroxyl groups (10). Further confirmation for these six hydroxyl groups was attained by spectrophotometric determination (11). Of these six hydroxyl groups, two were found to be primary since gymnemagenin gave rise to a dityrityl derivative (V), m.p. 268–269°, upon treatment with triphenyl chloromethane (10, 12). While reactions of gymnemagenin with lead tetraacetate (13) and sodium periodate (14) were suggestive of a 1,2-glycol system, as shown by Stöcklin (4), its *trans*-configuration was inferred in the present investigation by the reversible reaction of gymnemagenin with monopotassium triacetylosmate (15).

Copper pyrolysis (12) of gymnemagenin at 270–290° generated formaldehyde, which was characterized as its dimedone derivative. Treatment of gymnemagenin with acetone–sulfuric acid at 0° for 48 hr. gave a di-*O*-isopropylidene derivative (VII),[1] m.p. 301–303°. Acet-

[1] Structure elucidation of the isopropylidene derivatives, VII and VIII, is reported separately (16).

ylation of VII yielded a diacetate (VIII),[1] m.p. 275–276°. These results were indicative of two 1,3-glycol systems in gymnemagenin (17, 18).

The presence of the β-amyrin skeleton in gymnemagenin was substantiated by refluxing its hexaacetate (III) with selenium dioxide in acetic acid for 17 hr. (12). Dehydrogenation occurred to yield the heteroannular 11,13(18)-diene (IX). The diene (IX) was characterized by triple maxima at 241, 250, and 259 nm. (log ε 4.16, 4.21, and 4.00, respectively). This facile dehydrogenation has been considered characteristic of the β-amyrin derivatives (10, 17, 19).

Thus, the chemical evidence obtained in the present investigation confirms gymnemagenin (I) to be a derivative of β-amyrin, containing one 1,2-*trans*-glycol and two 1,3-glycol systems with a total of six sterically unhindered primary and/or equatorially oriented secondary hydroxyl groups. This is consistent with the structure, I, proposed for gymnemagenin on the basis of spectral data (3, 5).

The derivatives were also of interest as part of an antiviral structure–activity study of triterpenoid compounds. In addition, two derivatives of gymnemic acid A (6), its methyl ester and its diborane reduction product, were prepared for this study. Thus, the free carboxyl function of the glucuronic acid group of gymnemic acid A was reacted with diazomethane to yield the methyl ester (X); treatment of acid A with diborane (20, 21) resulted in the reduction of the glucuronic acid moiety to yield a glucoside (XI). Formation of the glucoside (XI) was confirmed by hydrolysis with ethanolic hydrochloric acid and identification of β-D-glucose in the hydrolysate by paper chromatography and the glucostat test (6).

EXPERIMENTAL[2]

Dry leaves of *G. sylvestre* were employed in this investigation.[3] Melting points were taken on the Kofler micro hot stage and are uncorrected. Spectra were recorded on Perkin-Elmer 137B and 337 IR and Beckman DK-2A UV spectrophotometers. A Zeiss model PMQ II spectrophotometer was employed for a quantitative determination of hydroxyl groups in gymnemagenin (I) based upon the method of Umbreit and Houtman (11).

Silica gel G TLC analysis was carried out as described previously (6). Solvent systems employed were as follows: benzene–methanol (7:3), chloroform–methanol (9:1) (8), benzene–chloroform–methanol (5:8:3), and benzene–methanol–acetic acid (45:8:4) (22) for I and II; and benzene–ethyl ether (1:1) (8) and chloroform–ethyl acetate–methanol (97:2:1) (8) for acetate and isopropylidene derivatives. Visualization of the compounds on chromatograms was carried out by spraying with either modified Liebermann–Burchard (23) or ceric sulfate–sulfuric acid (24) reagents.

Isolation of Gymnemagenin (I)—A crude gymnemic acid mixture was obtained from the aqueous extracts of *G. sylvestre* leaves by precipitation with 10% hydrochloric acid as described earlier (6). A 115-g. quantity of crude gymnemic acid mixture was extracted with 3.5 l. of hot acetone (in seven batches of about 15 g. each, with 500 ml. of acetone per batch) in a large beaker and filtered; the acetone extract was concentrated to a volume of about 200 ml. Addition of 200 ml. of chloroform to this concentrated acetone extract caused copious precipitation which settled as a gummy mass. The clear supernatant liquid was decanted; the gummy precipitate, upon drying, yielded 40.17 g. of a light-brown residue.

A 30-g. quantity of this residue was dissolved in 500 ml. of methanol and refluxed with 80 ml. of concentrated hydrochloric acid for 20 hr. The reaction mixture was concentrated *in vacuo* to about 100 ml. and then poured into 2 l. of ice water with vigorous stirring to cause precipitation. After allowing the precipitate to settle for 1 hr., it was filtered, washed with water until washings were neutral (pHydrion paper), and dried in a vacuum desiccator for 18 hr. to yield 16.69 g. of a light-yellow residue.

The acid-hydrolyzed fraction (16.37 g.) was dissolved in 500 ml. of methanol, with 20 g. of potassium hydroxide pellets added, and refluxed for 20 hr. The reaction mixture was then concentrated *in vacuo* to about 100 ml. and poured into 2 l. of ice water with stirring. The resulting fine precipitate was centrifuged, washed repeatedly with water until washings were neutral, and then dried to yield 9.77 g. of a pale-yellow residue.

Since the mother liquor decanted off during centrifugation was not clear, it was extracted with 1 l. of ethyl acetate for 48 hr. continuously in a liquid–liquid extractor. The ethyl acetate extract, after removal of the solvent, gave 0.75 g. of a light-yellow residue. This residue was combined with the main precipitate obtained during centrifugation to give a total yield of 10.5 g. (9.1% of crude gymnemic acid mixture).

The acid- and base-hydrolyzed fraction, obtained as described, was dissolved in 300 ml. of methanol and decolorized twice with activated charcoal.[4] The residue obtained after removal of the solvent was recrystallized from chloroform–methanol to yield 5.68 g. of a white crystalline product, sintering at 298° and melting at 314–316°. This crystalline material was observed to be comprised of gymnemagenin (I) and a trace amount of gymnestrogenin (II) by TLC.

A 2-g. quantity of this genin mixture was subjected to column (1.2 × 95 cm.) chromatography on 50 g. of silicic acid. Elution was carried out with chloroform–methanol (9:1). The solvent flow rate was maintained at 10 ml./hr., and 150 fractions (5 ml. each) were collected. Fractions 50–80 were pooled based upon TLC results, and evaporation of the solvent gave 1.12 g. of a white, homogeneous residue. Three recrystallizations of this residue from chloroform–methanol gave 0.87 g. of gymnemagenin (I) as stout, white needles, m.p. 329.5–331° [lit. (8), m.p. 328–335°]; $\nu_{max.}^{KBr}$ 3450 (O—H), 2900 (C—H), 1635 (trisubstituted C=C), 1250 (O—H), 1070 and 1040 cm.$^{-1}$ (C—OH).

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

Isolation of Gymnestrogenin (II)—A 17.8-mg. quantity of gymnestrogenin (II) was isolated from Fractions 28–31 obtained during silicic acid column chromatographic purification of gymnemagenin (I). Upon recrystallization from chloroform–methanol, II was obtained as fine needles, m.p. 286–288° [lit. (9) m.p. 288–289°]; $\nu_{max.}^{KBr}$ 3470 (O—H), 2945 (C—H), 1635 (trisubstituted C=C), 1070 and 1040 cm.$^{-1}$ (C—OH).

3β,16β,21β,23,28-Penta-*O*-acetylolean-12-ene (VI)—A 10-mg. quantity of gymnestrogenin (II) was treated with 1 ml. of dry pyridine and 0.8 ml. of acetic anhydride and left at 37° for 18 hr. The reaction mixture was evaporated *in vacuo*, and the residue obtained was extracted with chloroform (5 × 10 ml.). The chloroform extract, after washing with 20-ml. portions of 2 *N* hydrochloric acid, 10% potassium bicarbonate, and water, was dried over anhydrous sodium sulfate. Removal of the solvent from the chloroform extract yielded 11.4 mg. of a white residue. Recrystallization of this residue from petroleum ether–chloroform gave the pentaacetate (VI) as thin flakes, m.p. 121–123°; $\nu_{max.}^{KBr}$ 1740 (C=O, acetoxyl), 1460 and 1375 cm.$^{-1}$ (side-chain methylene and methyl of —CH_2—O—CO—CH_3, respectively). This pentaacetate was found to be identical by IR and TLC in two systems to a reference sample of noncrystalline material previously reported by Stöcklin (9).

3β,16β,21β,22α,23,28-Hexa-*O*-acetylolean-12-ene (III)—A 500-mg. quantity of gymnemagenin (I) was acetylated with 10 ml. of acetic anhydride and 16 ml. of dry pyridine at 0° for 18 hr. The reaction product (785.3 mg.), processed as in the case of VI, was found to be a mixture by TLC. It was chromatographed on a column (1.2 × 28 cm.) of 15 g. of neutral alumina using benzene–ether (2:1) for elution to yield 314.4 mg. of hexaacetate (III). Recrystallization from petroleum ether–chloroform afforded III as fine needles, m.p. 290–291° [lit. (4) m.p. 290–291°]; $\nu_{max.}^{KBr}$ 1740

[2] Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich.

[3] Prachi Gobeson Co., Calcutta, India, and the Himalaya Drug Co., Bombay, India.

[4] Norit, American Norit Co., Jacksonville, Fla.

(C=O, acetoxyl), 1250 (asymmetric stretching of acetate —C—O—C), 1460 and 1375 cm.$^{-1}$, the intensity of the 1375 band being much stronger than the 1460 band (side-chain methylene and methyl, respectively, of —CH_2—O—CO—CH_3).

Anal.—Calcd. for $C_{42}H_{60}O_{12}$: C, 66.46; H, 8.23. Found: C, 66.33; H, 8.57.

3β,16β,21β,22α,23,28-Hexa-*O*-benzoyloolean-12-ene (IV)—A 100-mg. quantity of gymnemagenin (I) in 5 ml. of dry pyridine was heated with 2 ml. of benzoyl chloride on a water bath (70–74°) for 1 hr. The cooled reaction mixture was poured into 25 ml. of water, and the precipitate formed was filtered. This precipitate was stirred with 5 ml. of 5% sodium carbonate, filtered, washed with water, and dried to yield 148.2 mg. of a white residue. Recrystallization of this residue from chloroform–methanol gave hexabenzoate (IV) as white prisms, m.p. 186–188°; $\nu_{max.}^{KBr}$ 1700, 1730 (C=O stretching of benzoyloxy group), 1600 (phenyl nucleus), 1135 and 1295 cm.$^{-1}$ (C—O stretching of benzoates).

Anal.—Calcd. for $C_{72}H_{74}O_{12}$: C, 76.44; H, 6.59. Found: C, 76.72; H, 6.81.

3β,16β,21β,22α-Tetrahydroxy-23,28-ditrityloolean-12-ene (V)—A mixture of 100 mg. of gymnemagenin (I) and 300 mg. of triphenyl chloromethane in 5 ml. of dioxane–pyridine (1:1) was heated on a steam bath for 16 hr. The reaction mixture was then evaporated *in vacuo*, and the residue obtained was extracted with ether (3 × 10 ml.). The ether extract, after removal of the solvent, was chromatographed on a column (1.2 × 36 cm.) of neutral alumina (20 g.). Elution with benzene (100 ml.) gave triphenyl carbinol (108.6 mg.), while benzene–methanol (7:1) (300 ml.) eluted the dityrityl derivative (V). Recrystallization from benzene–methanol gave 38.8 mg. of V as colorless needles, m.p. 268–269°; $\nu_{max.}^{KBr}$ 3540 (O—H), 1600, 1585, 1495 (phenyl nucleus), 750, 705 cm.$^{-1}$ (5 adjacent H on phenyl nucleus).

Anal.—Calcd. for $C_{68}H_{78}O_6$: C, 82.39; H, 7.93. Found: C, 82.53; H, 8.19.

3β,16β,21β,22α,23,28-Hexa-*O*-acetyloolean-11,13(18)-diene (IX)—A 50-mg. sample of hexaacetate (III) in 5 ml. of glacial acetic acid was refluxed with 30 mg. of selenium dioxide for 18 hr.; the red metallic selenium that formed was filtered over a bed of diatomaceous earth[5] using methanol for transfer and washing. The filtrate was evaporated *in vacuo* to yield 29.8 mg. of a white residue. Recrystallization of this residue from petroleum ether–methanol afforded the diene (IX) as white crystals, m.p. 286–288°; $\lambda_{max.}^{EtOH}$ 241, 250, and 259 nm. (log ε 4.16, 4.21, and 4.00, respectively).

Anal.—Calcd. for $C_{42}H_{60}O_{12}$: C, 66.65; H, 7.99. Found: C, 66.78; H, 8.16.

Copper Pyrolysis of Gymnemagenin (I)—A 200-mg. quantity of gymnemagenin (I) was mixed with finely powdered copper (1.4 g.) and heated to 270–290° for 1 hr. in a stream of nitrogen, passing the evolved gases into a saturated aqueous solution of dimedone. The precipitate formed in dimedone solution was collected (12.4 mg.) and recrystallized from methanol to give colorless fine needles, m.p. 188–189°. This was identified as a formaldehyde dimedone derivative [lit. (25), m.p. 189°] upon comparison with an authentic sample, undepressed mixed melting point and superimposable IR spectra being the criteria for identity.

Gymnemic Acid A Methyl Ester (X)—A solution of 100 mg. of gymnemic acid A (6) in 30 ml. of an ether–methanol mixture was treated with excess of diazomethane (26) at 0°. The reaction product was recrystallized twice from ethyl acetate to yield 87.1 mg. of methyl ester X, m.p. 250–255° dec.; $\nu_{max.}^{KBr}$ 3450 (O—H), 2950 (C—H), 1720 (C=O), 1635 (trisubstituted C=C), 1440, 1360 (side-chain methylene and methyl, respectively, of —CH_2—O—CO—CH_3), 1260 (H-bonded O—H), 1070, 1040 (C—OH), and 915 cm.$^{-1}$ (β-pyranose ring vibration).

Anal.—Calcd. for $C_{50}H_{76}O_{16}$: C, 64.36; H, 8.21. Found: C, 63.98; H, 8.38.

Compound X was found to be homogeneous in the TLC Systems III and IV previously reported for the gymnemic acids (6).

Diborane Reduction Product of Gymnemic Acid A (XI)—Diborane (4 mmoles) was generated (20) over a period of 1 hr. by the addition of a solution of 1.9250 g. of boron trifluoride etherate (10 mmoles) in 3.3 ml. of diglyme to 0.2550 g. of sodium borohydride (6.75 mmoles) in 6.25 ml. of diglyme and was passed into a flask containing 100 mg. of gymnemic acid A (10 mmoles) in 7.5 ml. of diglyme. The reaction mixture was allowed to stand at room temperature for 1 hr. About 5 ml. of ethanol was then added to destroy excess diborane and evaporated *in vacuo* to yield 89.6 mg. of a white residue. This residue was found upon TLC analysis [silica gel G, chloroform–methanol (10:3)] to be a mixture of unreacted gymnemic acid A and its reduction product. Isolation of reduction product XI was achieved by chromatography on a column (12 × 250 mm.) of activated silica gel G (25 g., activated at 105° for 1 hr.) with elution by chloroform–methanol (10:3). A yield of 38.6 mg. of XI was obtained. Recrystallization from ethyl acetate gave XI as white microcrystals, m.p. 173–176° dec.; $\nu_{max.}^{KBr}$ 3450 (O—H), 2980 (C—H), 1720 (C=O), 1640 (trisubstituted C=C), 1440, 1380 (side-chain methylene and methyl, respectively, of —CH_2—O—CO—CH_3), 1070 and 1045 cm.$^{-1}$ (C—OH).

Anal.—Calcd. for $C_{49}H_{76}O_{15}$: C, 65.02; H, 8.46. Found: C, 64.76; H, 8.71.

Reduction of the glucuronic acid moiety in gymnemic acid A to glucose in the diborane reduction product (XI) was confirmed by its acidic hydrolysis (3 *N* ethanolic hydrochloric acid, 12 hr.) and detection of glucose in the hydrolysate by paper chromatography and by glucostat test. Method 1-A of the reagent kit[6] was employed.

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[6] Worthington Biochemical Corp., Freehold, N. J.

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Note added in proof: An isolation and antisweet evaluation of the gymnemic acids which includes their methyl derivatives was recently reported [K. Kurihara, Y. Kurihara, and L. M. Beidler, in "Olfaction and Taste," C. Pfaffmann, Ed., Rockefeller University Press, New York, N. Y., 1969, p. 450; K. Kurihara, *Life Sci.*, **8**, 537(1969)].

Reactions of Germine-3,16-diacetate in Aqueous Solution

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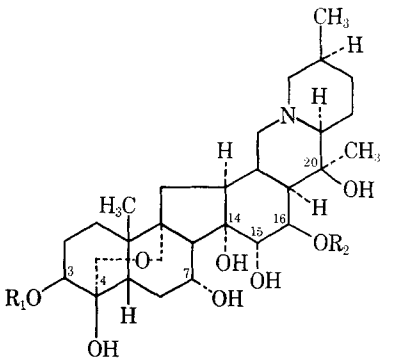
Abstract □ Germine-3,16-diacetate degrades rapidly in aqueous solution above pH 7 at room temperature ($t_{90\%} \sim 1$ hr. at pH 7.2 and $t_{1/2} < 1$ hr. at pH 9.5). The compound undergoes ester hydrolysis to form germine monoacetate and germine, as well as conversion to two as yet unidentified germine acetate esters. TLC was used to monitor these changes. A kinetic model describing the degradative behavior is proposed. Directions are given for preparing an aqueous solution stable for at least 3 days at room temperature.

Keyphrases □ Germine-3,16-diacetate reactions—aqueous solution □ Degradation—germine-3,16-diacetate in aqueous solution □ Stability profile and pH effect—germine-3,16-diacetate □ TLC—degradation monitoring □ IR spectrophotometry—identification

Recent work in this laboratory has revealed that germine-3,16-diacetate (GDA-3,16) degrades rapidly in aqueous solution at its natural pH, 9–10. The relatively rapid rate of hydrolysis of GDA-3,16 was first detected when titrating the compound as a base with standard acid. Consistently low results were obtained as the result of liberation of acetic acid during the dissolution of the compound in water prior to titration (about 40 min.). A concurrent observation of significance, reflecting this hydrolysis, was that the pH of a 0.1% GDA-3,16 solution decreased from an initial value of about 9.5 to about 6.5 in 3.5 days at room temperature.

These findings are of considerable importance because several publications have described the pharmacology and clinical experience obtained with unbuffered aqueous solutions of the drug, presumed to be GDA-3,16 (1). The significance of GDA-3,16 instability in connection with previously reported biological data is given by Torchiana *et al.* (2). The purposes of this publication are fourfold:

1. To alert investigators using GDA-3,16 about the chemical instability of this compound and recommend a method for preparing solutions that avoid the initial decomposition.
2. To describe a TLC system to assess the integrity of GDA-3,16 in solution.
3. To report preliminary findings about the influence of pH and solvent composition on the stability of GDA-3,16.
4. To report the conversion of GDA-3,16 into Unknowns 1 and 2.



structure of germine compounds

I

Compound	R_1	R_2
G	—H	—H
GDA-3,16	—$COCH_3$	—$COCH_3$
GMA-3	—$COCH_3$	—H
GMA-16	—H	—$COCH_3$

EXPERIMENTAL

Chemicals—GDA-3,16, germine-3-monoacetate (GMA-3), and germine-16-monoacetate (GMA-16) were synthesized,[1] starting from commercially available germine (G). The structure of germine and the acetate esters discussed is given in Structure I. The physical and chemical constants obtained for these compounds were in accord with either literature or expected values (3, 4). All of the germine compounds were reduced in particle size to less than 100-mesh to facilitate dissolution of the solids. All other chemicals used in these studies were reagent grade or better and were not further purified.

TLC—Glass plates were coated by standard techniques (5) with a 250-μ thick layer of a commercial adsorbent.[2] The TLC plates were air dried for about 15 min. and then further dried at 105° for 30 min.; they were stored in a desiccator prior to use.

An ethyl acetate–methanol–concentrated ammonia (80:15:5) mobile solvent resolved all of the germine compounds. Some typical R_f values observed by ascending TLC in a vapor-saturated developing chamber follow:

Compound	R_f
Unknown 1	0.80
GDA-3,16	0.75
GMA-3	0.60
Unknown 2	0.45
GMA-16	0.40
Germine	0.25

[1] Merck Sharp & Dohme Research Laboratories.
[2] Absorbosil-1, Applied Science Labs., Inc., State College, Pa.