Steroid Sapogenins VII. Identification and Origin of 25D-Spirosta-3,5-diene Among the Fenugreek Sapogenins

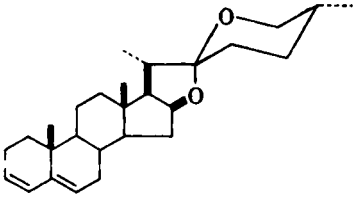
By M. S. BEDOUR, D. EL-MUNAJJED, M. B. E. FAYEZ, and A. N. GIRGIS

A reinvestigation of a certain fraction of the fenugreek sapogenin mixture led to the isolation of diosgenin, tigogenin, and gitogenin along with a fourth product identified with 25D-spirosta-3,5-diene. No evidence was found for trigonellagenin, which has been reported previously to occur in the same fraction. Evidence is presented which suggests that the diene is actually an artifact derived from diosgenin and produced early during the acid hydrolysis of the natural saponins.

THE STEROID sapogenins of fenugreek, *Trigonella foenum graecum*, seeds have had a few sporadic investigations. In 1943, Soliman and Mustafa (1) first alluded to the presence of a steroid sapogenin in the alcoholic extract hydrolysate of the seeds, shortly afterward Marker *et al.* (2) identified it with diosgenin. In a later communication (3), Marker *et al.* reported that the sapogenin mixture was made up mostly of diosgenin along with a small quantity of gitogenin and a trace of tigogenin. Soliman and Mustafa (4) then reported once again on the steroid sapogenins of fenugreek seeds and confirmed Marker's findings with respect to the presence of diosgenin and gitogenin, they did not mention tigogenin. Moreover, Soliman described another steroid sapogenin which he isolated in appreciable amounts from the mixture and, assuming it to be new, named it "trigonellagenin."

In the present search for steroid sapogenins in various sources from the local flora, fenugreek seeds were considered on account of their reputed (3) attractive diosgenin content. The processing of the seeds was conducted essentially according to the description of Wall *et al.* (5), where saponin-rich concentrates were prepared using *n*-butanol, and hydrolysis was effected by boiling in 4 *N* hydrochloric acid solution for 3 hours. The sapogenin mixture was next solvent-fractionated in the manner described by Soliman *et al.* (4) (*cf. Experimental*); the present report is concerned with that fraction from which the latter authors isolated trigonellagenin. A careful resolution of this fraction (of acetylated material) on alumina gave us tigogenin, diosgenin, and gitogenin along with a fourth adjunct having low polarity. The latter was purified with difficulty because of much contaminant matter and evidently possessed properties, m.p. 162–163°, $[\alpha]_D$ − 179°, different from those reported by Soliman *et al.* for trigonellagenin, m.p. 188–190°, $[\alpha]_D$ − 72.9°, acetate, m.p. 156–158° (4). No other products were isolated in appreciable amounts from this fraction.

The new product was soon realized to be nonhydroxylic, for it was recovered unchanged after treatment with acetic anhydride under normal acetylation conditions as well as after treatment with alkali. It also exhibited no apparent hydroxyl absorption in the infrared spectrum. However, its color reaction with the tetranitromethane reagent, taken together with the characteristic absorption (triplet max. at 229, 234, and 245 mμ) exhibited in the ultraviolet region of the spectrum, indicated the presence of a heteroannular conjugated diene system of the type present in cholesta-3,5-diene. That the compound possessed a steroid sapogenin nucleus with an intact spiroketal side chain was evident from the infrared spectrum which contained pronounced absorptions at 985, 920, 900, and 868 cm.$^{-1}$, with the 900 cm.$^{-1}$ peak stronger than that at 920 cm.$^{-1}$, an indication of 25D configuration. The substance was finally established to be 25D-spirosta-3,5-diene (I) by direct comparison with the synthetic product.



I

With regard to the origin of 25D-spirosta-3,5-diene, it is safe to say that it could not have possibly existed as a glycoside, engaging the hydroxyl of the open ring F in the corresponding furostane, in the natural fenugreek saponins—not only because of the experimental evidence reported here but also because of the established (6) fact that saponins, like their aglycones, possess intact spiroketal side chains. The isomerizing effect of mineral acids on the configuration at C-25 has long been recognized and is now well established (7). The susceptibility of steroid sapogenins to the dehydrating action of mineral acid does not, however, seem to have been sufficiently appreciated. We are in possession of evidence which strongly suggests that the 25D-spirosta-3,5-diene was an artifact produced from diosgenin, the principal sapogenin of fenugreek, during the acid hydrolytic processing of the saponins. This was sought by the direct treatment of chromatographically pure diosgenin with hydrochloric acid. The treatment (1 hour reflux, 4 *N* HCl) was milder than that normally employed for the hydrolysis of the saponins (3 hours reflux, 4 *N* HCl). Chromatographic resolution of the reaction mixture gave a crop constituting 18%, which was shown to be 25D-spirosta-3,5-diene and which was identical in all aspects with the fourth product isolated from the sapogenin mixture of fenugreek seeds. This reaction might also have led to some isomerization at C-25 as well as other unpredictable complications. This was evident from the continued analysis of the reaction mixture on thin layers of silica gel, although the period of reaction (1 hour) was far short of the time known (7) to be necessary for the attainment of equilibration. In a study of the hydrochloric acid-catalyzed equilibration of 25D and 25L spirostanes, Wall *et al.* (7) described an impure product, m.p. 130–140°, which was the sole crystalline substance

Received January 3, 1964, from the Drug Research Institute, National Research Centre, Cairo, Egypt.
Accepted for publication April 1, 1964.

resulting in low yield from refluxing yamogenin, 25L-spirost-5-en-3β-ol, with concentrated hydrochloric acid in ethanol for 96 hours. The product, which they rightly designated 25D-spirosta-3,5-diene, must evidently have resulted from the isomerization of the side chain to the more stable 25D configuration preceded by dehydration in ring A. The circumstances of its formation and its survival under such conditions are evidence to the durability of its structure, an observation which we endorse and which finds support in the results in this report.

We have also prepared the diene (I) by treatment of diosgenin methanesulfonate with pyridine under reflux, and the product was identical with the diene preparations already discussed.

In the literature (8) 25D-spirosta-3,5-diene had been previously identified among the sapogenin mixtures isolated from *Heloniopsis orientalis*, Thunb. and *Mianthemum dilatatum*, Wood. In both sources diosgenin was a prominent constituent of the mixture; therefore, it is likely that the diene was not a genuine natural product. Wall and Serota (9) have also described its synthesis by treatment of diosgenin tosylate with sodium iodide in acetone under pressure.

EXPERIMENTAL

The melting points were determined on a Kofler block and are uncorrected; the optical rotations were measured in chloroform in a 1-dm. tube at room temperature. The infrared spectra were determined with a Perkin-Elmer Infracord 137 spectrophotometer in sodium chloride cells in carbon disulfide solution.

Processing of Plant Material.—The powdered seeds of fenugreek were defatted by extraction with light petroleum, and a quantity (10 Kg.) was exhausted with hot ethanol. The extract was then concentrated and diluted with water (2 L.). The solution was repeatedly washed with light petroleum and again concentrated to 4 L. The saponins [1] were taken up in *n*-butanol saturated with water. This was done after the aqueous solution had first been treated with sodium chloride (5% w/v) and adjusted to pH 4 with hydrochloric acid. Concentration of the *n*-butanol extracts to a small volume was followed by treatment with hydrochloric acid and adjustment of the solution to 4 *N*. After a reflux period of 3 hours, water was added and the mixture worked up through chloroform. The dark resin so obtained was freed from much phenolic matter by refluxing in benzene (1 L.) with an alcoholic solution (300 ml.) of sodium hydroxide (50 Gm.) for 20 minutes. Working up the product gave 39 Gm. of crude sapogenin matter.

Isolation of 25D-Spirosta-3,5-diene.—The crude material mentioned above was fractionated using solvents according to the prescription of Soliman and Mustafa (4). Extraction of the mixture with light petroleum (b.p. 70–80°) in a Soxhlet apparatus removed 30 Gm. of brownish semisolid (*A*); then extraction with ether removed 3.9 Gm. of partly crystalline material (*B*). Fraction *A* was acetylated (acetic anhydride-sodium acetate, 2 hours of heating at 100°), and the product (32 Gm.) then was treated with 150 ml. of cold methanol. The insoluble part was again treated with boiling methanol (400 ml.) for 30 minutes and filtered while hot from insoluble matter. The hot filtrate was allowed to cool and again freed from deposited matter. The filtrate thus obtained was evaporated to dryness to give 8.58 Gm. of the fraction from which Soliman and Mustafa (4) isolated trigonellagenin acetate by repeated crystallization. Chromatographic resolution of this fraction on an alumina (210 Gm.) column gave the following characterized products.[2]

(*a*) A fraction (0.72 Gm.) eluted with light petroleum which after repeated crystallization from ethanol gave 25D-spirosta-3,5-diene, m.p. 162–163° (undepressed), $[\alpha]_D$ − 179°. [Reported m.p. 164°, $[\alpha]_D$ − 175° (9)]. The infrared absorption spectrum was identical with that determined for an authentic sample prepared from diosgenin (*vide infra*). The ultraviolet absorption spectrum (ethanol) exhibited triplet maximal absorption with peaks at 229, 234, and 245 (sh) mμ (ε 28000, 28600, and 18800, respectively). The substance gave a strong orange color reaction with tetranitromethane in chloroform.

(*b*) A fraction (1.04 Gm.) eluted with benzene/light petroleum (1:1) which afforded tigogenin acetate, m.p. 204–208°, undepressed by authentic sample, $[\alpha]_D$ − 71.63°. [Reported m.p. 204°, $[\alpha]_D$ − 73° (5)]. The infrared absorption spectrum was superimposible on that of authentic material.

(*c*) A fraction (0.35 Gm.) eluted with the same solvent mixture which after repeated crystallization gave diosgenin acetate, m.p. 190–194° (undepressed), $[\alpha]_D$ − 107°. The identity was further confirmed by comparison of the infrared absorption spectra. [Reported m.p. 202°, $[\alpha]_D$ − 115° (10)].

(*d*) A fraction (0.77 Gm.) removed with the same eluent which afforded gitogenin diacetate, melting point and mixed m.p. 233–236°. The infrared absorption spectrum was identical with that of authentic material.

(*e*) A fraction (0.75 Gm.) eluted with 3% chloroform in benzene which afforded tigogenin, melting point and mixed m.p. 201–204°, $[\alpha]_D$ − 78°. [Reported m.p. 206°, $[\alpha]_D$ − 67° (5)]. The identity was further confirmed by comparison of the infrared spectra and by acetylation to give a product identical in all respects with tigogenin acetate.

(*f*) A fraction (0.32 Gm.) removed with 3% ethanol in benzene which afforded gitogenin, m.p. 268–270° (undepressed), $[\alpha]_D$ − 80.6°. [Reported m.p. 268°, $[\alpha]_D$ − 63° (11)]. Acetylation of this material gave gitogenin diacetate, m.p. 233–237°, $[\alpha]_D$ − 91.7°. [Reported m.p. 237–242°, $[\alpha]_D$ − 98° (11)]. Infrared absorption spectra were identical with those of authentic samples.

Preparation of 25D-Spirosta-3,5-Diene.—A solution of chromatographically pure diosgenin (0.5 Gm.) in alcohol (150 ml.) was refluxed for 1 hour with enough concentrated hydrochloric acid to attain a 4 *N* mixture. After working up the reaction product, the residue (0.47 Gm.) was chromatographed on 35 Gm. of activated alumina. The course of fractionation was followed on thin layers of silica gel[3] using a mixture of acetone/benzene (1:5) for development and chlorosulfonic acid reagent for spraying the plates. Elution with dry benzene removed 0.086 Gm.

[1] The method of Wall *et al.* (5) for the enrichment of saponins was preferred to that described by Soliman and Mustafa (4) which involved the use of barium hydroxide and gave no satisfactory results in this investigation.

[2] The chromatography, having lasted several days, must have involved partial hydrolysis of the acetylated material.

[3] Actually it was adduced from various experiments that 1 hour reflux was optimal for the formation of the diene, R_f 0.95; for diosgenin, R_f 0.55 (same indicated solvent system).

of a crystalline product, m.p. 162–163°, which did not depress the melting point of the product isolated from the sapogenin mixture of fenugreek seeds. The infrared spectra were identical in all details. Elution with 2% methanol in benzene recovered most of the unchanged diosgenin.

Diosgenin Methanesulfonate.—This was prepared by treatment of a solution of diosgenin (0.34 Gm.) in pyridine (5 ml.) with methanesulfonyl chloride (2 ml.) at room temperature for 18 hours. Working up in the usual manner afforded 0.22 Gm. of the desired product which, after repeated crystallization from methanol, gave a pure sample, m.p. 162–163°, $[\alpha]_D$ − 108°.

Anal.—Calcd. for $C_{28}H_{44}O_5S$: C, 68.30; H, 8.94. Found: C, 68.27; H, 9.00.

A solution of this substance (0.1 Gm.) in pyridine (3 ml.) was refluxed for 10 hours. After dilution with water and isolation of the product, chromatography on a column of 4 Gm. of alumina gave a fraction (30 mg.) recovered with light petroleum. This was repeatedly crystallized from light petroleum-methanol to give a pure sample of 25D-spirosta-3,5-diene, melting point and mixed m.p. 160–162°, which was identical in all respects with the preparations described previously.

REFERENCES

(1) Soliman, G., and Mustafa, Z., *Nature*, **151**, 196(1943).
(2) Marker, R. E., *et al.*, *J. Am. Chem. Soc.*, **65**, 1247 (1943).
(3) Marker, R. E., *et al.*, *ibid.*, **69**, 2242(1947).
(4) Soliman, G., and Mustafa, Z., *Repl. Pharm. Soc. Egypt.*, **31**, 117(1949).
(5) Wall, M. E., *et al.*, *J. Biol. Chem.*, **198**, 533(1952).
(6) Rothman, E. S., Wall, M. E., and Eddy, C. R., *J. Am. Chem. Soc.*, **74**, 4013(1952).
(7) Wall, M. E., Serota, S., and Withauer, L. P., *ibid.*, **77**, 3086(1955).
(8) Okanishi, T., Akahori, A., and Yasuda, F., *Ann. Repl. Shionogi Res. Lab.*, **10**, 137, 143(1960).
(9) Wall, M. E., and Serota, M., *J. Am. Chem. Soc.*, **78**, 1747(1956).
(10) Wall, M. E., and Walens, H. A., *ibid.*, **77**, 5661(1955).
(11) Sannié, C., and Lapin, H., *Bull. Soc. Chim. France*, **19**, 1080(1952).

Study of the Polyvinyl Alcohol-Borate-Iodine Complex III. Detection of Borates in Urine

By ANTHONY J. MONTE-BOVI, JOHN J. SCIARRA, and VINCENT DE PAUL LYNCH

A survey of the prevalence of boric acid toxicity indicates the difficulty encountered in detecting cases of poisoning by this commonly used substance. Because of this situation, an investigation was carried out utilizing a previously reported method for the detection of borates by the polyvinyl alcohol-borate-iodine reaction. The test was extended in this study to permit the detection of boric acid in the urine of poisoned animals. Rabbits were fed varying quantities of boric acid, and urine samples were collected. After concentration of the urine sample, the polyvinyl alcohol-iodine reagent was applied to the residue. Results of both *in vitro* and *in vivo* studies indicate that this method is capable of detecting as little as 0.3 mg. of boric acid (0.05 mg. B).

THE CHARACTERISTIC blue produced when solutions of polyvinyl alcohol (PVA) and iodine were brought into contact with boric acid has been reported in several diverse areas (1–5). Since most of these applications involved macroquantities of boric acid, there was need to determine the suitability of this method for the detection of smaller quantities and to extend the investigation further for the purpose of determining the possibility of utilizing this test for the detection of boric acid in animal fluids and tissues.

That such a test is desirable becomes apparent when one surveys the literature concerned with case histories of poisoning by boric acid. As early as 1904 (6) and as recently as March 1963 (7), attention was called to the toxic nature of boric acid. In a previous study, Sciarra (8) summarized the prevalence of poisonings due to boron compounds. Most cases reported in the literature involved children ranging in age from a few weeks to 12 years. In many of these instances it was noted that several days elapsed between the time of admission to the hospital and the time at which boric acid poisoning was first suspected. This problem is further complicated by the fact that the afflicted children usually were not given medical attention during the critical early stages of poisoning—apparently because the toxicity symptoms are not characteristic.

Many investigators have called attention to this problem. McNally and Rukstinat (9) commented on 58 cases, 28 of which proved fatal. Pfeiffer (10) revealed 86 cases, of which 42 were fatal. Goldbloom and Goldbloom (11) reported on 109 poisonings by boric acid. Valdes-Dapena and Arey (12) analyzed 175 cases, of which 86 resulted in death. It may be that there is some duplication in reporting these cases. Nevertheless, the records clearly indicate the prevalence of boric acid poisoning; and in spite of the many articles and warnings relative to the toxic nature of the boron compounds, poisoning continues. As late as 1962 (12) and 1963 (13, 18), accidental deaths due to the erroneous use of these compounds have been reported. It should also be noted that many of these investigators mention the probability that many such cases of poisoning go undetected, since the symptoms produced may be confused with those produced by other illnesses. This is especially true in cases of chronic toxicity; for acute poisoning usually demands rapid medical treatment which, while it may not be specific for boron compounds, will at least counteract the poison and prevent further damage to the victim. The problem is complicated further because, through the ingestion of certain foods boron can be found in the blood. A range of 0–0.72 mg./100 ml. of blood was reported (12). In addition, the expanding use of boron industrially

Received May 16, 1963, from the Departments of Pharmaceutical Chemistry and Pharmacology, College of Pharmacy, St. John's University, Jamaica, N. Y.
Accepted for publication March 3, 1964.
Presented to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.
The authors express their thanks to Mr. Nicholas G. Camarinos and Mr. Lawrence Sell for their aid and technical assistance during this investigation.