Lactocortexone acetate"] (XXV). To a solution of 40 mg. of XXV, m.p. 183–184°, in 1 cc. of methylene chloride was added 2.5 cc. of 0.05*M* potassium carbonate in 75% methanol.[38] After 5 min., 1 cc. of water and a piece of Dry Ice were added, and the mixture was concentrated *in vacuo* to about 1 cc., yielding a crop of colorless needles. After filtering and washing the crystals (28.2 mg.), the filtrate was extracted with ethyl acetate, yielding 7.5 mg. of crystalline residue. These two portions were combined and chromatographed over 5 g. of Florisil (10 × 115 mm.). Chloroform containing from 2% to 25% of acetone eluted a total of 31.6 mg. of crystalline material which was recrystallized from acetone-water and acetone-hexane to give 24.2 mg. of XXVI as clusters of minute needles, m.p. 199–201°, depressed on admixture with XVI. $[\alpha]_D^{21}$ +158° ± 3°; M_D^{21} +566° ± 10° (9.95 mg., α + 1.57°). λ_{max}^{alc} 244 mμ, ϵ 14,000.

Anal. Calcd. for $C_{21}H_{26}O_5$ (358.42): C, 70.37; H, 7.31. Found[39]: C, 70.54; H, 7.50. Residue, 1.38.

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Isolation, Purification, and Structural Identity of an Alfalfa Root Saponin

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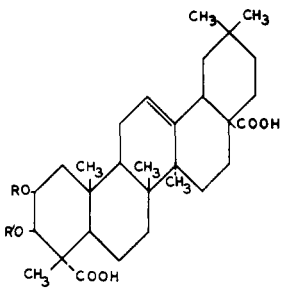
The isolation and structural identity of a pure alfalfa root saponin nucleus was accomplished. The probable structural formula was shown by experimentation to be a β-linked D-glucoside of 2β-hydroxy-Δ^{12}-oleanene-23,28-dioic acid; the aglycone is known as medicagenic acid. Specific enzyme hydrolysis was used to establish the linkage involved in the glucose attachment. Other evidence for the reported structure was prepared from chemical constants, elemental analysis, hydroxyl determinations, infrared interpretations and comparisons, and an NMR study.

In the last fifteen years the water soluble saponins isolated from alfalfa (Medicago sativa) have been indicated as a factor in inhibiting growth of chicks[2] and contributing to ruminant bloat.[3,4] From current structural investigations of these saponins isolated from alfalfa tops has come valuable information confirming the presence of a number of hexose and pentose sugars attached in some order to a triterpenoid nucleus.[5,6] The final absolute structure of the aglycone was reported in 1957 and is now known as medicagenic acid.[7]

Previous structural investigations[5–7] have not included the isolation and structural determination of a pure saponin but have concentrated on the individual identity of the monosaccharides and the triterpenoid nucleus following hydrolysis. It was therefore considered expedient to attempt the isolation, purification, and structural determination of a compound representative of a composite of carbohydrate and sapogenin from alfalfa. Such a compound would help establish the proper sequence of hexoses and pentoses in some saponin formations, furnish information on the glycosidic linkage and permit the complete structural proof of component parts. The information obtained should be useful in a better understanding of saponin formation and suggest a useful method for synthesis of these products.



I R,R'= H
II R,R'= CH_3CO
III R=H, R'= β-D-GLUCOSE

Figure 1

EXPERIMENTAL

Saponin isolation and purification. Preliminary investigations of the occurrence of saponins in alfalfa plants showed a potentially rich concentration in the roots. This portion of the plant body was therefore selected for this study.

Samples of alfalfa roots from Ranger and Lahontan varieties were gathered as plowed, cleaned of leaf material, and dried in a forced-draft oven until the roots became brittle. These dried roots were then ground to a fine powder and stored until needed.

The saponin isolation from root powder follows:

Four hundred and fifty grams of this powder was placed in a 4-l. steel beaker together with 1300 ml. of water and 1500 ml. of 95% ethanol. The slurry was maintained at 80° on a hot plate under constant agitation for 6 hr. This mixture was filtered with gentle suction through a large Buchner funnel so packed that it retained about a quarter of an inch

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bed of Hyflo-supercel on a good grade qualitative paper. The filtrate was then brought just under a boil (90°) and acidified with 360 ml. of concentrated hydrochloric acid. This approximately 12% solution was maintained near boiling for 20 min. Upon cooling the filtrate to room temperature with constant stirring, a light brown gelatinous precipitate formed. Complete precipitation was effected by allowing the mix to stand quietly at room temperature for 24 hr. The precipitate was collected by gentle suction on a filter paper. Care was taken to remove the filter cake from the paper while still moist.

The partially dried residue was completely dissolved in 125 ml. of hot 95% ethanol and treated repeatedly with activated carbon and filtered through a carbon retentive paper until the filtrate assumed a pale yellow color. To this ethanol solution was added 150 ml. of distilled water resulting in the formation of a white gelatinous precipitate. Following this treatment the gelatinous product was removed by filtration and the water precipitation process repeated. Two water treatments gave a nearly white gelatinous residue. The last traces of color were removed by taking the precipitate up in a minimum of 95% ethanol and treating twice as before with activated carbon. The clear colorless alcoholic solution was evaporated under a dry air stream. The resulting product was a fine white powder that was easily ground to any desired mesh.

This procedure prepared 1 g. of pure white dry saponin for every 100 g. of dry root powder used. It should be noted that this yield was significantly higher than that obtained from alfalfa tops.[5] The prepared saponin was vacuum dried at 50° for 8 hr. A sample of this product melted sharply at 255° ± 1° and gave an $(\alpha)_D^{25}$ of +70° in absolute ethanol.

The isolation and purification procedure for the aglycone. Continued hydrolysis under reflux using 12% hydrochloric acid resulted in the isolation of the sapogenin. The procedure for this hydrolysis follows:

Three grams of purified saponin were placed in a round bottom flask fitted with a reflux condenser. To this residue was added 400 ml. of a 50% ethanol-water solution containing a 12% concentration of hydrochloric acid. Boiling chips were added and the solution refluxed for 60 hr. Following this hydrolysis the solution was concentrated by means of a clean dry air stream to 50 ml., added to 100 ml. of hot ethanol, and treated repeatedly with activated carbon until a light straw yellow solution was obtained. Following a single water precipitation, the partially dried white gelatinous precipitate was taken up in 150 ml. of hot ethanol and treated twice more with activated carbon, whereupon a clear colorless solution resulted. The pure aglycone was recovered by evaporation, air dried, and redissolved in a minimum of hot 95% ethanol. Recovery from cold ethanol gave 1.2 g. of white microcrystals, the crystalline nature being confirmed by an x-ray diffraction study. A sample of these crystals selected for analysis was dried at 100° for 4 hr. They exhibited a sharp melting point at 352–353° and gave an $(\alpha)_D^{25}$ of +106° in absolute ethanol.

Determination of sapogenin structure. As preliminary constants obtained for the purified aglycone indicated the nucleus to be medicagenic acid, it was first chosen for further analyses.

Anal. Calcd. for $C_{28}H_{42}(OH)_2(COOH)_2$: C, 71.7; H, 9.16; O, 19.12; neut. equiv., 251.2. Found: C, 71.7; H, 9.17; O, 19.08; neut. equiv., 253, 255.7.

The value 253 was obtained by adding an excess of 0.1*N* sodium hydroxide under reflux and back titrating with standard 0.05*N* hydrochloric acid; the value of 255.7 by direct titration in aqueous ethanol using 0.1*N* sodium hydroxide.

A diacetate derivative of medicagenic acid was also prepared. The preparation of the diacetate duplicated a procedure already reported.[5] A sample of the crystalline diacetate dried at 100° for 2 hr. melted sharply at 207° and gave an $(\alpha)_D^{27}$ of +92° from chloroform.

To aid in confirming the number and position of functional groups on these nuclei, infrared and NMR spectrograms were prepared for the sapogenin and its diacetate.

Infrared spectrograms for these compounds were obtained from potassium bromide pellets using a Perkin-Elmer Infracord spectrophotometer. The graph obtained for the acid showed strong absorbances at 3.9, 5.9, and 2.85 μ indicative of carboxyl and hydroxyl loadings. Interpretation of the curve also suggests that the hydroxyl groups are axial because of the absorptive band in the 9–10 μ region. A double bond is most probably located such that there is only one hydrogen attached to the double bonded carbon as suggested by significant absorption bands between 11.5 and 12.5 μ.[8] The spectrogram obtained for the diacetate showed a significant decrease in hydroxyl absorbance and increased absorbance at 7.3 and 8 μ brought about by the acetate loadings.

The above infrared interpretations for the sapogenin nucleus loadings were properly confirmed when the infrared spectrograms of these compounds were shown to be exactly identical with the infrared charts of medicagenic acid and its diacetate supplied by WURDD[9].

A nuclear magnetic resonance spectrogram of the diacetate of medicagenic acid was prepared in deuterochloroform with a sweep rate of 1×10^{-5} and a chart rate of 4″/min., using a frequency of 60 mc. Characteristically sharp bands at 116 and 124 cps. were immediately evident and were indicative of the two acetate loadings. A broad signal was obtained at 500 cps. typical of carboxylic acid protons. Signals centering around 317 cps. were classified as characteristic of one olefinic proton and two protons attached in equatorial configuration with the acetate groups on the six-membered ring. Electronic integration of this spectrogram showed that the two sets of low field signals for the five protons considered above are in ratio of 2 to 3. The assignment of the two carboxyl protons, one olefinic proton, and two protons adjacent to acetate loadings is offered as supporting evidence in confirming the absolute structure for both medicagenic acid and its diacetate.

Determination of saponin structure. Following confirmation of the sapogenin, the saponin structure was further investigated. An infrared spectrogram from a potassium bromide pellet was prepared. An interpretation of the curve showed an increase in hydroxyl loadings evident from an increased absorbance at 2.85 μ over that exhibited by medicagenic acid. The curve also suggested the presence of a lactone structure due to enhanced absorbance at 8 and 8.5 μ, with a glycosidic attachment predicted because of the strong absorption in the 9–10 μ region.

In addition to the melting point and rotation data the following analytical data were recorded for the saponin nucleus:

Anal. Calcd. for $C_{28}H_{42}(COOH)_2(OH)(OC_6H_{11}O_5)$: C, 65.0; H, 8.4; O, 26.5. Hydroxyl det'm.: 12.4 corres. to 5 OH groups. Calcd. for $C_{28}H_{42}(COOH)_2(OH)(OC_6H_9O_4)$: C, 66.2; H, 8.5; O, 25.2. Hydroxyl det'm.: 10.4 corres. to 4 OH groups. Found: C, 64.88; H, 8.40; O, 26.63. Hydroxyl det'm.: 12.11.

The essential features of the hydroxyl acetylation is described in the literature.[10] One exception was the use of an indicator for end point detection prepared from one part by weight of Cresol Red with 3 parts by weight of Thymol Blue.

Determination of the linkage and specific sugar. In order to establish the linkage and determine the exact sugar present, the following procedures were used:

One gram of very finely ground saponin and 25 milligrams of pure β-D-glucosidase suspended in 25 ml. of water in a large test tube was mounted in a water bath at 37° ± 0.5° under constant agitation for 12 hr. The mixture was removed

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and centrifuged at 30,000 G for 15 min. The clear supernatant solution was deionized by passing it through a mixed resin bed composed of amberlite IR-120 and amberlite IR-4B. The deionized filtrate was reduced under vacuum to 5 ml. A 2-ml. portion of this concentrate was tested by a standard orcinol method for hexose.[11] Although the test was not quantitative, it was clearly evident that the sugar was a hexose.

As noted in the literature[12] pure β-D-glucosidase is generally considered very specific for hydrolyzing normal β-linked D-aglucones. Therefore, the confirmation of a hexose by the orcinol test coupled with the fact that β-D-glucosidase will not hydrolyze β-linked D-fructose or D-mannose might be considered sufficient to indicate the presence of β-D-glucose. However, as some doubt exists as to whether this enzyme may also hydrolyze β-D-galactose under favorable conditions, the following confirmatory experiment to identify the sugar was completed:

To the remaining 3 ml. of filtrate were added 0.4 g. of phenylhydrazine, 0.6 g. of sodium acetate and 0.5 ml. of a saturated bisulfite solution. The volume was made up to 5 ml. and the solution immersed in a boiling water bath. An osazone of the sugar formed between 4 and 5 min. The time for osazone formation coincided exactly with that established for glucosazone,[13] as well as with time of osazone formation in a known glucose solution treated simultaneously in an identical manner. A galactose solution run under similar conditions did not form osazone crystals until 19 min. had elapsed.

Microscopic examination of the osazone crystals indicated that the unknown gave crystals that were identical with glucosazone and differed significantly from those formed for galactose.[14] As a result of these experimentations, the presence of glucose was confirmed in the saponin.

CONCLUSIONS

On the basis of the results recorded, it is expected that the pure root saponin isolated was a β-linked glucoside of 2β-hydroxy-Δ^{12}-oleanene-23,28-dioic acid (Medicagenic acid). Because of the difficulty encountered in the acid hydrolysis of glucose it is predicted that glucose is initially attached to the triterpene nucleus in a majority of alfalfa root saponins of this type regardless of the sugar chain complexity. This conclusion was suggested in that several different impure water soluble saponins isolated in initiating this research were all reduced by controlled hydrolysis to the same saponin nucleus reported in this paper.

Surface contact enzyme hydrolysis using pure β-D-glucosidase not only proved that a β-linkage for D-glucose was involved, but that the glucosidic attachment was probably free of further substitution or unusual binding to the aglycone.[12] This latter evidence was strengthened when the hydroxyl group determination presented evidence of five free hydroxyl groups in the saponin which is expected when a normal glucoside linkage exists.

Alfalfa roots have been found to be an excellent source for the isolation of saponins of this type or for isolating pure medicagenic acid. Should these compounds prove valuable as intermediates, alfalfa root powder would be a practical source material.

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[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

Steroidal Sapogenins. L.[2,3] Conversion of 12-Ketosapogenins to 11β,12β-Epoxypregnanes

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Hecogenin was converted to 11β,12β-epoxytigogenin (IV), and then to 3β-acetoxy-11β,12β-epoxy-5α-pregnane-20-one (X). Gentrogenin or gentrogenin-correllogenin mixtures were converted to 11β,12β-epoxydiosgenin (XV) and then to 11β,-12β-epoxy-3β-acetoxy-5-pregnene-20-one (XXI).

The elegant researches of Fried and his associates have demonstrated that 12-halosteroids have physiological activities comparable to those of corresponding 9-halosteroids.[4] Probably the most available route to such compounds are *via* the

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(2) Previous paper in this series, Steroidal Sapogenins. XLIX, *J. Org. Chem.*, **23**, 1741 (1958).