Anal. Calcd. for $C_{17}H_{20}O_8S_2$: C, 49.0; H, 4.8; S, 15.4. Found: C, 49.2; H, 4.9; S, 15.0.

1,2-*O*-Isopropylidene-3-*O*-(*p*-tolylsulfonyl)-α-D-glucofuranose 5,6-Carbonate (VI). **A. From V.**—To a solution of 100 mg. of V in 20 ml. of acetone was added 200 mg. of silver nitrate. As in IIIA, silver sulfide formed immediately upon addition of water. The solution was treated as described in IIIa to yield 79 mg. (83%) of a crystalline product, m.p. 100–103°. Haworth and Porter[16] report m.p. 103–105° for VI.

Anal. Calcd. for $C_{17}H_{20}O_9S$: C, 51.1; H, 5.0; S, 8.0. Found: C, 50.8; H, 5.1; S, 8.3.

B. From IV.—A solution of 100 mg. of IV in 2 ml. of pyridine was treated with 0.5 g. of *p*-toluenesulfonyl chloride as described in A for V. The crystalline product had m.p. 98–101° and 98–102° in admixture with the compound prepared in A above.

Barium Hydroxide Hydrolysis of III.—To a solution of 200 mg. of III in 4 ml. of acetone was added 4 ml. of barium hydroxide solution (2%) and the mixture was heated on a steam bath for 30 min. After cooling to room temperature, carbon dioxide was passed through the suspension to remove any excess barium hydroxide. Additional acetone (20 ml.) was added and the suspension was filtered. The clear filtrate was evaporated to give 150 mg. of a colorless sirup that contained no sulfur.

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Acknowledgment.—We are grateful to Mrs. Clara McGrew, Mrs. Bonita Heaton, and Mrs. Anita Dirks for microanalysis and molecular weight determinations by vapor pressure osmometry.

A Natural Glycoside of Medicagenic Acid. An Alfalfa Blossom Saponin[1]

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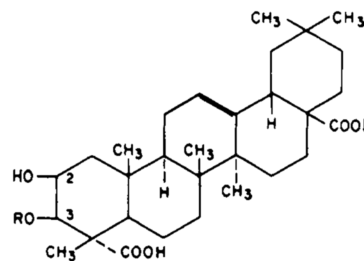
Received March 23, 1964

The structural identification of a pure homogeneous saponin obtained from an alfalfa blossom concentrate has been completed. The crystalline triterpenoid aglycone was identified as medicagenic acid. Experimental evidence showed the probable structure of the saponin to be 2β-hydroxy-3β-(β-rhamnopyranosyl-β-glucopyranuronosyl-β-glucopyranosyloxy)-Δ^{12}-oleanene-23,28-dioic acid.

During recent years there has been interest in determining the relationship of saponin content in alfalfa and clover to a number of physiological and biological effects. Growth depressing effects,[2] ruminant bloat,[3,4] respiratory inhibition,[5,6] and the retarding of seed germination[7] are among the phenonena studied which may involve saponins.

Along with these pharmacological and biophysical studies, isolation and structural identity of the saponins and sapogenins found in alfalfa have been reported. From initial studies of some of these compounds,[8,9] the exact structure of medicagenic acid was deduced,[10] and some valuable information about total saponin content[11] accompanied by a characterization of individual sugars was obtained. Other investigators have recently reported chromatographic separations of these compounds.[12-15] These studies, although they have presented considerable detailed information about the individual sugars and aglycones found in saponins, have not been specifically concerned with purification and structural determination procedures applicable to individual saponins.



I, medicagenic acid, R = H
II, alfalfa root saponin, R = β-glucopyranosyloxy-
III, alfalfa blossom saponin, R = β-rhamnopyranosyl-β-glucopyranuronosyl-β-glucopyranosyloxy-

In a former communication, the isolation, purification, and structural identity of an alfalfa root saponin was reported.[16] The present work is an extension of alfalfa saponin studies and presents the isolation and structural identification of a more complex saponin. The sequence and individual identity of the hexoses in the structure were primarily determined by the use of controlled acid and enzymatic hydrolysis in conjunction with descending paper chromatography. Individual linkages to the aglycone as well as those between the hexoses were established by specific enzymatic hydrolysis. Evidence for the homogeneity of the sapogenin and saponin structures was provided by column separation followed by thin layer chromatography, while structural proof was obtained by the use of physical constants, elemental analysis, neutralization equivalent, and hydroxyl determinations, derivative studies, and infrared interpretations. Structural studies on this saponin showed glucose forming the primary attachment to medicagenic acid as observed earlier for an alfalfa root saponin.[16] It is postulated that this attachment is completed through the equatorial 3-hydroxyl

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position on the aglycone as was previously discovered to be the case when the synthesis of the natural root saponin was completed.[17] The greater reactivity of the equatorial hydroxyl group toward substitution together with the attendant bulk of the relatively large sugar moiety would be expected to give preference to an attachment at the 3-position on the aglycone.[18]

Experimental

Isolation, Purification, and the Establishment of Saponin Homogeneity.—The procedure parallels one reported previously for the isolation of an alfalfa root saponin[16] with the following variations.

The hand-picked blossoms were dried at 80° for 24 hr. immediately after harvesting and ground to a fine meal prior to further chemical processing. Following addition of hydrochloric acid, the precipitate was allowed to stand quietly at room temperature no longer than a 24-hr. period to reduce the possibility of further degradation of the carbohydrate chain. Purification of this saponin proved to be more complicated than that required for the root saponin; hence, this procedure will be more completely described.

As all attempts to crystallize the product failed, initial purification of the compound was begun as follows.

The precipitate was dissolved in hot 95% ethanol and treated repeatedly with activated carbon until no further color could be removed. At this point the red-brown solution was precipitated with water and filtered, the precipitate was dissolved in ethanol, and the process was repeated. It was found that the fewer water precipitations, the higher the yield, owing to a loss of the product from water solubility. After a colorless solution in ethanol was obtained, the solvent was evaporated under a dry air stream, dried overnight at 80 ± 5° in a vacuum oven, and ground in an agate mortar. From a 0.5% yield by dry weight, 2.5 g. of pure white powder was obtained which melted in a range from 253 to 255° with considerable decomposition and has $[\alpha]^{23}D$ +24 ± 1° (*c* 0.02, absolute ethanol).

A thin layer chromatogram of this isolate resulted in the development of two color spots. The impurity observed was shown to be identical with the root saponin[16] by using the pure root compound as a reference. The solvent systems employed were formic acid–chloroform (1:4.4), and glacial acetic acid–cyclohexane (1:2). Chromatography was effected on silica gel G with iodine for detection. This material was then chromatographed on 30 times its weight of Davison silica gel (28–200 mesh) and eluted with 95% ethanol. The product, m.p. 257–258°, $[\alpha]^{23}D$ +24° (*c* 0.02, absolute ethanol), exhibited only a single spot when thin layer chromatograms were completed in the solvent systems previously mentioned.

Determination of the Saponin Structure.—Structural investigation of the homogeneous saponin began with an infrared investigation of the purified white powder isolated. An interpretation of an infrared spectrogram from potassium bromide pellet using a Perkin-Elmer Infracord showed a marked increase in the hydroxyl loadings and lactone occurrence due to enhanced absorbance in the 2.85- and 8–8.5-μ region, respectively. The absorbances were considerably stronger than those exhibited for either medicagenic acid or its glucoside.[16] The curve also indicated the presence of a new carboxyl loading as it showed two definite absorption peaks, one at 5.9 μ common to medicagenic acid and its glucoside, and a second at 5.8 μ suggestive of a different carboxyl loading.

In addition to the melting point and rotational data reported in the previous section, neutralization equivalent data along with elemental analysis and hydroxyl determinations were used to establish the molecular weight and final purity of the homogeneous saponin. The method used to determine the neutralization equivalent follows.

Samples weighing approximately 0.2 g. were weighed directly into a 300-ml., three-necked round-bottomed flask. The flask was then equipped with a magnetic stirrer and a nitrogen delivery tube and placed in an ice-salt mixture. One hundred milliliters of methanol was added and the mixture was stirred until dissolution occurred. The system was flushed with nitrogen and 25.0 ml. of 0.05 *N* methanolic sodium hydroxide was added from a buret. Back titration was effected with 0.05 *N* methanolic hydrochloric acid to a phenolphthalein end point. Satisfactory duplication of neutralization equivalent values was obtained from three separate determinations. Carbon and hydrogen data together with neutralization equivalent and hydroxyl determinations are reported as follows.

Anal. Calcd. for $C_{45}H_{71}O_{15}(COOH)_3$: C, 58.7; H, 7.6; neut. equiv., 329.0; hydroxyl, 15.5. Found: C, 58.2, 58.6, 58.5; H, 8.0, 8.0, 7.8; neut. equiv., 329.4, 332.7, 327.1; hydroxyl, 15.3, 15.1.

The procedure for the hydroxyl determination was essentially that described in the literature.[19]

Further structural evidence corroborating the recorded analytical data was obtained through the preparation of the trimethyl ester of the purified blossom saponin. The compound was prepared by adding ethereal diazomethane[20] to a methanolic solution of 1.0 g. of blossom saponin in 100 ml. of methanol. The ether was removed by distillation and water added to the methanol solution. The resulting precipitate was collected and vacuum dried: m.p. 169–171°, $[\alpha]^{30}D$ + 17.7° (*c* 0.01, absolute ethanol). An infrared spectrogram of this compund showed only an ester band in the carboxyl region at 5.72 μ. Neutralization with standard base followed by back titration with standard acid revealed no carboxyl groups; hence, esterification was complete. Chromatography of this compound on silica gel G with formic acid–chloroform (1:4.4) and glacial acetic acid–cyclohexane (1:2) yielded single spots in both cases.

Anal. Calcd. for $C_{45}H_{71}O_{15}(COOCH_3)_3$: C, 59.3; H, 7.83; methoxyl, 9.04. Found: C, 59.04, 59.21; H, 8.49, 8.40; methoxyl, 8.50, 8.70.

Determination of the Aglycone.—The aglycone was isolated, purified, and identified as follows. The saponin (2 g.) was placed in a round-bottomed flask with 200 ml. of 3 *N* ethanolic hydrochloric acid and the mixture refluxed for 90 hr. Water was added and the resulting precipitate was collected on a Büchner funnel, dissolved in 19% ethanol, and decolorized with activated carbon. Water was again added and the resulting precipitate was dissolved in hot *p*-dioxane yielding 70 mg. of white crystalline product: m.p. 348–349°, $[\alpha]^{23}D$ + 110° (*c* 0.01, absolute ethanol); lit.[8] (for medicagenic acid) m.p. 349–350°, $[\alpha]^{26}D$ +111° (*c* 0.192, 0.116, absolute ethanol). A mixture melting point with authentic material was undepressed.

By a procedure previously reported,[8] 50 mg. of pure white crystalline diacetate was obtained: m.p. 206–207°, $[\alpha]^{24}D$ +91.5° (*c* 0.01, chloroform); lit.[16] m.p. 207°, $[\alpha]^{27}D$ +92.0°. A mixture melting point of this material with an authentic sample was undepressed. The infrared spectrograms of these products were identical with those prepared from authentic samples, which had previously been identified by infrared and n.m.r. interpretations.[8,16]

Determination of Carbohydrates Present.—Because of the small concentrations involved, paper chromatography seemed the most suitable method for identifying the individual sugars present. Descending paper chromatography was selected as most applicable and the system ethyl acetate–pyridine–water (12:5:4) was chosen because of the rapidity with which the solvent front moved and the marked differences in R_g values for the carbohydrates suspected.[21] Whatman 3-mm. filter paper was used in all chromatographic separations. The procedure used for separation follows.

Three grams of saponin was subjected to hydrolysis for 90 hr. with 3 *N* ethanolic hydrochloric acid; the resulting solution was treated once with activated carbon and concentrated under air, the sapogenin was removed, and the filtrate was neutralized with a saturated solution of sodium hydroxide to a pH of 5–6. This solution was further concentrated under a dry air stream to a volume of about 15 ml. After the salt was removed by use of an electric desalter, the solution was passed through 2-ml. columns of Dowex 1 and Dowex 50 resins, and concentrated to approximately 10 ml. prior to spotting the paper. Descending chromatography with silver nitrate for a developer produced four spots

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with average R_g values of 0.16, 1.00, 1.70, and 2.67. Three of these spots were quite dark while a fourth was relatively light. When pure reference sugars were used, R_g values from this solvent system indicated that the spots were indicative of glucuronic acid, glucose, rhamnose, and glucuronolactone, respectively. Although the R_g values are the same in this solvent system for galacturonic acid as glucuronic acid, only glucuronic acid would form the lactone structure observed. In all, five different determinations were made from different concentrates and each gave nearly identical concentration patterns and R_g values. The three dark spots observed for the hydrolytic mixture from the saponin resulting from glucuronic acid, glucose, and rhamnose were always accompanied by a much weaker colored spot for glucuronolactone. The occurrence of a low concentration of glucuronolactone undoubtedly results from the glucuronic acid–glucuronolactone equilibrium accompanying the hydrolysis. A similar equilibrium was observed even when pure gluruonic acid was allowed to equilibrate in water for 24 hr. at room temperature.

Determination of Order of Attachment of Carbohydrates.—The three carbohydrate fractions were successfully cleaved from the aglycone and one another by hydrolysis with 3 N ethanolic hydrochloric acid. It was therefore considered expedient to attempt a controlled degradation of the carbohydrate chain with mild acid hydrolysis to effect a systematic removal. One gram of saponin, 45 ml. of ethanol (95%), 25 ml. of water, and 0.5 ml. of 12 N hydrochloric acid were placed in a water bath at 78°. Ten-milliliter samples were removed periodically beginning with 15 min. These samples were immediately neutralized, freed of ethanol by use of a dry air stream, and centrifuged; the supernatant liquid was desalted. The samples, further concentrated to a volume of 5 ml. under a dry air stream, were chromatographed by the method already described with the results shown in Table I.

TABLE I

CHROMATOGRAPHIC DATA RESULTING FROM 0.1 N HYDROCHLORIC ACID HYDROLYSIS

Sample	Hydrolysis time, min.	Carbohydrates obsd.	Relative concn. based on color intensity
1	15	Rhamnose	Strong
		Glucuronic acid	Weak
2	55	Rhamnose	Strong
		Glucuronic acid	Moderate
		Glucose	Weak
3	95	Rhamnose	Strong
		Glucuronic acid	Moderate
		Glucose	Moderate
4	48 hr.	Rhamnose	Strong
		Glucuronic acid	Strong
		Glucose	Strong
		Glucuronolactone	Weak

Determination of Specific Linkages.—As the occurrence of a β-linkage was observed for a previously isolated root saponin,[16] it was of interest to examine the nature of the specific linkages in the blossom saponin to discover if they were of a similar type. A spot identical with that of glucose and one later shown to be due to a combination of rhamnose and glucuronic acid first appeared when either β-glucosidase or β-glucuronidase were employed.[22,23] This result indicates a β-link in the attachment of glucose to the aglycone and to glucuronic acid. Since individual spots identical with those for rhamnose and glucuronic acid were obtained when the isolated rhamnose–glucuronic acid complex was subjected to further acid hydrolysis, a rhamnose specific enzymatic system was sought to attack the linkage between these two sugars. As this enzyme was commercially unavailable, a concentrate prepared from an extract of partially ripened berries from the *Rhamnus rubra* Greene bush was used. The extraction procedure followed one previously described.[24] Seventeen milligrams of this extract was digested with 1 g. of saponin and 20 ml. of water for 18 hr. in a constant-temperature bath at 37° and the resulting mixture was centrifuged at 20,000 g for 10 min. Chromatography of the supernatant liquid produced three spots corresponding to glucose, glucuronic acid, and rhamnose. Rhamnodiastase has been postulated to be a β-glycosidase,[24] and the accompanying hydrolysis of other established β-linkages in the saponin confirmed this hypothesis. These results indicate that a β-link also exists between rhamnose and glucuronic acid which is apparently not so readily severed enzymatically as the other β-links found in the hexose chain.

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