Anal. Calcd. for $C_{24}H_{21}N$: C, 89.2; H, 6.50. Found: C, 89.0; H, 6.40.

CANCER RESEARCH LABORATORY
UNIVERSITY OF FLORIDA
GAINESVILLE, FLA.

Alfalfa Saponin[1]

BY E. D. WALTER, G. R. VAN ATTA, C. R. THOMPSON AND W. D. MACLAY

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Investigation of the water-soluble fraction of alfalfa (*Medicago sativa*), undertaken to test the hypotheses that it contains saponin capable of inhibiting growth of chicks[2] and contributing to ruminant bloat[3,4] confirmed earlier reports of the presence of at least two saponins.[5-8] Mixed saponins were recovered from dried alfalfa and have since been shown in a coöperative study by Heywang[9] to be inhibitory to the growth of chicks. In other recent coöperative experiments[10] the feeding of alfalfa saponin to ruminants caused typical symptoms of bloat.

Recovery of the mixed saponins from the plant material was effected through formation of their water-insoluble cholesterides. Because the cholesterides are split by alcohols,[7,8] it was necessary to form them by heating aqueous plant extract solutions containing an excess of cholesterol in suspension.

Partial resolution of the mixed saponins gave two fractions which differed from each other in optical rotation and mobility on paper and which by acid hydrolysis were also found to differ significantly from one another in both their sugar and aglycone components.

One of the sapogenins, its diacetate and diacetate dimethyl ester were prepared in crystalline form. Its dimethyl ester and monobromolactone were obtained as non-crystalline products. Properties of the sapogenin and its derivatives indicate that it is a monounsaturated dihydroxy dicarboxylic acid having the molecular formula $C_{30}H_{46}O_6$. The specific rotation of the sapogenin, +111°, and of its diacetate, +87°, suggests a triterpenoid, since the steroid-sapogenin side chain usually confers pronounced levorotation.[11]

The acidic character of the sapogenin and the fact that it contains 30 carbon atoms further support the idea that it belongs to the triterpenoid rather than the steroid class.[12]

A search of the literature disclosed no description of a sapogenin coinciding in all respects with that of the present substance. Castanogenin, a dihydroxy dicarboxylic acid sapogenin obtained by Simes[13] from the wood of *Castanospermum australe* apparently has the same molecular formula, $C_{30}H_{46}O_6$, as the sapogenin derived from alfalfa. However, melting points and specific rotations reported for the diacetate and the diacetate dimethyl ester differ from those of the corresponding substances prepared from alfalfa.

Experimental

Recovery of Saponin.—Dehydrated alfalfa meal (91 kg.) was extracted with 3 portions of hot water to yield 1,163 liters of solution, which was concentrated in a rising-film evaporator to yield 73 kg. of sirupy liquid. Ethanol (95%) was added to the concentrate to form an 80% alcohol mixture. The resulting precipitate was drained, suspended in 26.7 liters of water, and reprecipitated by adding 142.5 liters of 95% ethanol. This precipitate was drained and discarded. The combined alcoholic mother liquors were evaporated *in vacuo* to produce 26.4 kg. of aqueous concentrate. The concentrate was washed twice by mixing with $^1/_9$ its volume of chloroform and separating the chloroform solutions in a continuous centrifuge, leaving 24.3 kg. of washed concentrate.

The chloroform washed concentrate, in 3-kg. portions, was boiled with 720-g. portions of cholesterol and then mixed with filter aid and suction filtered. The filter cakes were washed with warm water until no more color was removed, then dried at 40°. Dried cake from each 3-kg. portion was leached with about 3 liters of anhydrous pyridine. Four volumes of anhydrous ether was added to the pyridine solution. The precipitated crude saponin was collected on a filter with light suction and washed with ether to remove pyridine and cholesterol, and then the product was dried to an amorphous white powder; yield 431 g.

Purification of Crude Saponin.—A 25-g. portion of the crude material was leached with 7 successive portions of boiling 95% ethanol. An undissolved dark brown residue that remained was discarded. Each solution was decanted and filtered hot. The first leach solution was found to contain more impurities than the others and was set aside. The remaining solutions were combined and evaporated to a volume of 1,250 ml. Thirty ml. of water was added to the hot mixture to redissolve the portion of material that came out of solution by concentration. Saponin was precipitated from the concentrate by adding 2 volumes of ether, filtered off with light suction, washed with ether and vacuum dried at 50°; yield 7.2 g.

Anal: sulfated ash, 0.04%; N, 0.09%; $[\alpha]^{26}D$ −13.2° (*c* 1.0, water, *l* 4).

The product was moderately soluble in water and dilute ethanol but almost insoluble in absolute ethanol. Like the crude, it was strongly sternutatory and gave stable foams in water. Its 1% solution was faintly straw colored. The purified material hemolyzed defibrinated rat blood diluted with normal saline solution at slightly faster rates than did equal concentrations of a commercial saponin (J. T. Baker Chemical Co. lot No. 61543). Mosquito fish (*Gambuzia affinis*) placed in a solution of 1 g. of the product per liter died within 15 minutes, but a solution of $^1/_{10}$ of that concentration produced no effect on fish within a 8-hour test period.

Fractionation of Saponin.—Purified saponin (10.8 g.) was dissolved in 60 ml. of warm water. One liter of boiling 95% ethanol and 1.12 liters of boiling absolute ethanol were added to the solution. The precipitate, formed during addition of the absolute ethanol (fraction A), was filtered from the hot mixture, washed with hot 95% ethanol and dried; yield 3.3 g. The filtrate from fraction A was allowed to cool and stand overnight at room temperature. During this time a further quantity of precipitate formed. This was filtered off and 3 volumes of ether was added to the remaining solution. The resulting precipitate (fraction B), was recovered on a suction filter, washed with ether and dried; yield 4.55 g.

When tests showed that both fractions contained appreciable amounts of ash, 500-mg. portions of each were dissolved in water and demineralized by passage through columns of cation-exchange resin (2 g. each of Analytical Grade Amberlite IR-120 (H) and Duolite A-4 (OH)). The

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(12) C. R. Noller, *Ann. Rev. Biochem.*, **14**, 383 (1945).

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demineralized fractions were recovered by freeze drying the solutions and washings.

Fraction A: yield 460 mg. *Anal.:* sulfated ash, 0.03%; N, 0.08%; $[\alpha]^{26.9}D$ −14.6° (*c* 1.006, water, *l* 4).

Fraction B: yield 487 mg. *Anal.:* sulfated ash, 0.03%; N, 0.07%; $[\alpha]^{26.9}D$ −4.1° (*c* 1.141, water, *l* 4).

A series of 65-hour descending paper chromatograms of the two fractions and reference sugars were irrigated with *n*-butanol:ethanol:water (10:2:1), and sprayed with Tollens reagent, resorcinol, dinitrosalicylic acid and aniline solutions.[14] They failed to show the presence in the fractions of 5- or 6-carbon aldoses, ketoses, higher ketose-type polysaccharides or uronic acids. Both fractions produced stains only with the silver spray. In both instances uniform, weakly stained spots which began at the origin extended short distances down the paper. The spot given by fraction A was about 2.5 cm. long while that due to fraction B was three and one-half times as long.

In dilute solutions both fractions were lethal to fish and strongly hemolytic.

Carbohydrates from Saponin Fractions.—Portions of saponin fractions A and B (100 mg. each) mixed with 2-ml. portions of 2 *N* sulfuric acid were held in steam at atmospheric pressure for 1 hour. The mixtures were neutralized with barium carbonate, mixed with acid-washed diatomaceous earth and suction filtered. The filtrates and washings were passed through columns of mixed ion-exchange resins (1 g. Amberlite IR-120 (H) and 1.2 g. Duolite A-4 (OH) each). The deionized solutions and washings were freeze-dried. A series of 48-hour descending paper chromatograms of the 2 residues and of reference sugars were developed with *n*-butanol:ethanol:water (10:2:1) and sprayed with Tollens reagent, dinitrosalicylic acid and aniline solutions. Strong spots, all of equal size and intensity, indicated the presence of arabinose and xylose in the hydrolyzate from fraction A and of glucose, arabinose and xylose in the hydroyzate from fraction B. The hydrolyzate from fraction A yielded only a very faint spot corresponding to glucose. Both hydrolyzates also gave 3 faint spots, which were assumed to be from aldehydic substances, possibly hydrolysis by-products, having fewer than 5 carbon atoms per molecule.

Aglycones from Saponin Fractions.—Portions of saponin fractions A and B were hydrolyzed by autoclaving in 3 *N* sulfuric acid for 6 hours at 15 p.s.i. The aglycone fractions were parted from other hydrolysis products and acid by repeated washings conducted in separatory funnels. Proportions of ether, ethanol and water used in the washings were adjusted so that the aglycones remained dissolved in an upper phase and the other substances, some of which did not dissolve, passed into the lower phase. When no more colored material or acid could be removed with the lower phase the upper phase was dried with sodium sulfate, decolorized with carbon, filtered and evaporated. The amorphous residues were weighed, dissolved in 95% ethanol, made to volume and examined for optical activity.

Aglycone fraction from saponin fraction A: $[\alpha]^{26.3}D$ +45.9° (*c* 0.166, 95% ethanol, *l* 1).

Aglycone fraction from saponin fraction B: $[\alpha]^{26.3}D$ +72.9° (*c* 0.375, 95% ethanol, *l* 1).

Previous workers have observed that hydrolysis of saponins containing both hexoses and pentoses tends to proceed in such a fashion that most of the hexoses may be split off before the pentoses.[15] Accordingly, in the absence of further information, detection of glucose in the hydrolyzate from one saponin fraction and not from the other could be accounted for with the assumptions that only one saponin was initially present in the plant and that the fraction lacking glucose was a partial hydrolysis product, or "prosapogenin," derived from the original saponin. However, the differing rotations of the aglycones give more positive support to the view that two saponins are present in alfalfa.

Recovery of a Crystalline Sapogenin.—Crude saponin (50 g.) was dissolved in 65% ethanol (1 liter). Hydrochloric acid was then added to make the solution 1.0 *N*. The solution was boiled under reflux for 60 hours, preliminary experiments having shown that, under these conditions, refluxing for this length of time was necessary to remove all carbohydrate. Addition of water to the alcohol solution gave a pasty gel which was washed free of acid, alcohol and sugars by repeated suspension in water and centrifugation. The sapogenin was filtered and dried *in vacuo* at 80°. The dried material was dissolved in ethyl ether, decolorized with activated carbon, and the ether was evaporated. The sapogenin crystallized incompletely and with difficulty from absolute alcohol. It was dried at 130°; yield 10 g.

Crystalline sapogenin was best obtained by preparation of the acetate (see below) and saponification of this followed by crystallization of sapogenin from ether. When recrystallized 3 times, this product melted without decomposition in an evacuated, sealed, Pyrex tube at 349–350°. For analysis the sample was dried *in vacuo* at 130°; $[\alpha]^{26}D$ +111°, +111° (*c* 0.192, 0.116, abs. alc., *l* 4).

Anal. Calcd. for $C_{28}H_{42}(OH)_2(CO_2H)_2$: C, 71.7; H, 9.22; neut. equiv., 251.3. Found: C, 71.9; H, 9.06; neut. equiv., 252, 256, 258.

(The value 256 was obtained by refluxing with alkali and back titrating with 0.1 *N* hydrochloric acid. The other values were direct titrations with 0.1 *N* NaOH).

The sapogenin was soluble in methanol, ethanol, 2-propanol, acetone, ether and dioxane but sparingly soluble in chloroform. Red colors were obtained with concentrated sulfuric acid, the Liebermann–Burchard reagent and also with antimony pentachloride. With thionyl chloride containing a small amount of stannic chloride it gave in succession yellow → red → purple → blue colors. In this behavior it was similar to certain triterpenoid sapogenins studied by Noller, *et al.*[16] With tetranitromethane, the sapogenin in acetic acid solution gave a weak yellow color. When burned it yielded white fumes with a resinous odor.

Sapogenin Diacetate.—Sapogenin (2.0 g.) obtained directly from hydrolyzed saponin, fused sodium acetate (0.5 g.) and acetic anhydride (20 ml.) were boiled under reflux for 2 hours and poured into water, with stirring. After several hours a white, granular solid was filtered off and dried. This material was dissolved in ether and decolorized with activated charcoal. The ether was evaporated and the product was crystallized by cooling the hot methanol solution to which water had been added. For analysis it was crystallized 3 times from methanol and water. When dried *in vacuo* at 130° it lost 7.75% of solvate; m.p. 206°, $[\alpha]^{26.7}D$ +87 ± 3° (*c* 0.166 chloroform, *l* 4).

Anal. Calcd. for $C_{34}H_{50}O_8$: C, 69.6; H, 8.59; CH_3CO, 14.67; mol. wt., 586.7. Found: C, 69.0; H, 8.73; CH_3CO,[17] 14.5; mol. wt. (Rast), 591.

A molecular weight determination was made by means of Weisenberg photographs of single crystals and a density measurement obtained with a gravity gradient tube. Air-dried crystals (plus solvate) gave a value of 647.7. When corrected for solvate, the value was 597.5.

The air-dried crystals were monoclinic and had a density of 1.190 g. per ml. lying flat in parallel polarized light (between crossed nicols) the crystals showed parallel extinction with the slow ray crosswise of the blades. The common view gives only hazy interference figures when viewed in convergent polarized light.

The refractive indices determined by the immersion method were $\alpha = 1.507$ for vibrations lengthwise of the blade and $\gamma' = 1.523$ for vibrations crosswise of the blades.

Dimethyl Ester.—Sapogenin recovered directly from saponin hydrolyzate was treated with diazomethane in ether yielding a product, presumably the dimethyl ester, but which failed to crystallize. When precipitated from methanol solution with water and dried at 110° in a vacuum, it was obtained as a white powder. It was recovered unchanged when 100 mg. was refluxed with 5 ml. of 0.1 *N* sodium hydroxide for 2 hours.

Anal. Calcd. for $C_{30}H_{44}O_4(OCH_3)_2$: CH_3O, 11.70. Found: CH_3O, 10.9.[18]

Sapogenin Diacetate Dimethyl Ester.—The above methylated product was acetylated as described previously, to give the diacetate dimethyl ester, which was crystallized from methanol after addition of a little water. Recrystallized

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three times, it melted at 220–225°, $[\alpha]^{27.3}D$ +73 ± 3.5° (*c* 0.2, chloroform, *l* 4). For analysis it was dried *in vacuo* at 130°.

Anal. Calcd. for $C_{36}H_{54}O_8$: C, 70.3; H, 8.85; CH_3CO, 14.00; CH_3O, 10.10; mol. wt., 614.8. Found: C, 69.9; H, 8.73; CH_3CO,[17] 14.0; CH_3O, 10.1; mol. wt. (Rast), 620.

Sapogenin Bromolactone.—Sapogenin obtained directly from saponin hydrolyzate was brominated in methanol–carbon tetrachloride solution as described by Winterstein and Egli[19] for the preparation of a bromolactone of siaresinolic acid. The product was dissolved in ether and dried over anhydrous sodium sulfate. Attempts to crystallize it from the usual solvents were not successful. It was obtained in form of a white powder by adding water to the methanol solution, and filtering.

Anal. Calcd. for $C_{30}H_{45}O_6Br$: Br, 13.74; mol. wt., 581.6. Found: Br, 14.3; equiv. wt., 570 (titration of remaining carboxyl).

In acetic acid solution the product gave no color with tetranitromethane.

Acknowledgments.—We wish to acknowledge the assistance of K. J. Palmer, D. R. Black and Francis T. Jones for crystallographic data on the sapogenin diacetate, L. M. White for elemental analyses, and A. Bevenue for identification of sugars from the sapogenins. We also acknowledge the technical assistance of J. Guggolz and I. V. Ford.

WESTERN REGIONAL LABORATORY[20]
ALBANY, CALIFORNIA

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(20) Agricultural Research Service, U. S. Department of Agriculture. Article is not copyrighted.

Action of Diazoalkanes on *o*-Quinones and Other Carbonyl (Thiocarbonyl) Compounds with Special Reference to the Nature of the Intermediate Compounds

BY ALEXANDER SCHÖNBERG, AHMED MUSTAFA, WILLIAM IBRAHIM AWAD AND (in part) GAMAL EL-DIN MOHAMED MOUSSA

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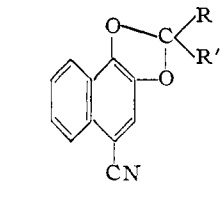
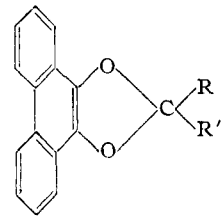
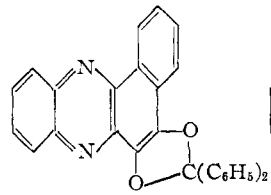
Formation of methylene ethers (such as I, II and III) by the reaction of *o*-quinones with diazoalkanes seems to be general.[1] In one case, however, the reaction of phenanthraquinone with diazomethane, both the ether IIa[2] and the ethylene oxide V[3] are obtained. We have examined the reactions of phenanthraquinone, 4-cyano-β-naphthoquinone and 1,2-benzophenazine-3,4-quinone with a few diazoalkanes, and in all cases obtained a single product (Ia-Ic, IIb, III, see Table I). The substances are assigned the ether structures as shown, not only on analogy with similar products, but also because they show greater stability to acid hydrolysis than would be expected if they contained an ethylene oxide ring. Under more drastic acid hydrolysis, however, they are cleaved to the corresponding dihydroxy compounds. Thus we obtained 1,2-dihydroxy-4-cyanonaphthalene from Ic; the product from hydrolysis of III is IV, a tautomeric form of the expected 3,4-dihydroxy-1,2-benzophenazine.[4] Similar hydrolysis of the diphenylmethylene ether of 9,10-dihydroxyphenanthrene has been noted.[5]



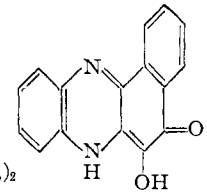
Ia, R = H; R′ = CH_3
Ib, R = R′ = 9-fluorenylidene ($C_{13}H_8$)
Ic, R = R′ = C_6H_5



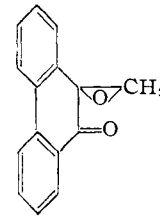
IIa, R = R′ = H
IIb, R = CH_3, R′ = H
IIc, R = R′ = C_6H_5



III



IV



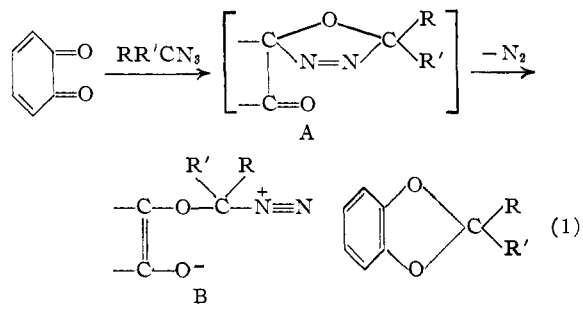
V

TABLE I

LIST OF THE NEW DERIVATIVES OF METHYLENE ETHERS

4-Cyano-1,2-(methylmethylenedioxy)-naphthalene (Ia)
4-Cyano-1,2-(diphenylmethylenedioxy)-naphthalene (Ic)
(4-Cyano-1,2-naphthylenedioxy)-9-fluorene (Ib)
9,10-(methylmethylenedioxy)-phenanthrene (IIb)
3,4-(Diphenylmethylenedioxy)-1,2-benzophenazine (III)

We consider the reaction of diazoalkanes with *o*-quinones follows a course similar to that of the diazoalkanes with olefins and can be formulated as shown in (1); the nitrogen-containing intermediate A can also be written as B. An analogous reaction occurs in the case of the reaction of diazoalkanes with *o*-quinoneimines and *o*-quinone monoximes.[6]



A

B

(1)

A slightly different mechanism originally proposed by Arndt is generally accepted[7] for the reaction of diazoalkanes with monoketones and aldehydes.

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