Steroidal Sapogenins.   III.   Structure of Steroidal Saponins[2]

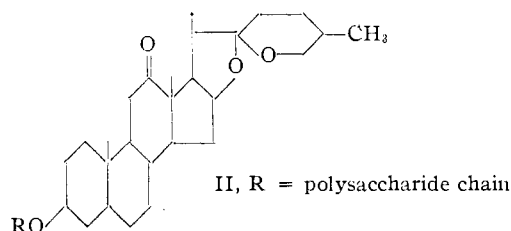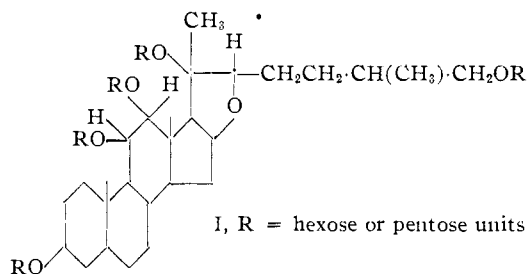BY EDWARD S. ROTHMAN, MONROE E. WALL AND C. ROLAND EDDY

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Infrared absorption spectra show that steroidal saponins have the closed-ring spiroketal side chain in the same stereochemical configuration as do their sapogenin hydrolysis products. Infrared spectra also show that the C-12 carbonyl function found in certain sapogenins is not an artifact but occurs already formed in the native saponin.

Steroidal sapogenins with a C-12 carbonyl function are promising starting materials for cortisone synthesis.[3,4] Marker and Lopez,[5] on the basis of rather limited evidence, proposed that the C-12 carbonyl function in certain sapogenins, e.g., hecogenin, manogenin and kammogenin, is an artifact formed during the hydrolytic cleavage of the parent saponins. They further proposed that the spiroketal side chain in the isosapogenins is also produced during hydrolysis, and formulated I as a saponin structure. They postulated that the carbonyl group arises through intermediary C-11, C-12 dihydroxy compounds, which give rise to the carbonyl function by dehydration to an enol followed by ketonization; a somewhat similar process supposedly gives rise to the isospiroketal side chain.

Djerassi, Martinez and Rosenkranz[6] have cast some doubt on the validity of such a course of C-12 carbonyl formation using C-11, C-12 dihydroxylated sapogenins in model experiments. We here present unequivocal evidence, based on infrared studies of native steroidal saponins, that both the C-12 carbonyl group and the spiroketal side chain occur in the unhydrolyzed saponin, and are not hydrolysis artifacts. Our suggested formulation for ketonic steroidal saponins is shown in II.

We have previously reported[2,7] that the spiroketal side chain in sapogenins has a highly specific infrared absorption spectrum, with maxima occurring near 866, 900, 922 and 982 cm.$^{-1}$ (11.55, 11.1, 10.85 and 10.18 μ) in "iso" sapogenins, and near 852, 900, 922 and 987 cm.$^{-1}$ (11.75, 11.1, 10.85, and 10.14 μ) in "normal" sapogenins. Cholesterol and dihydrosapogenins, such as dihydrotigogenin (allofurostan-3β,26-diol),[8] lack this characteristic system of maxima.[2,7] We have found this system of four absorption bands to be a property of all native saponin preparations examined. The same differences in infrared absorption which distinguish iso from nor-

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. This·work was done as part of a coöperative arrangement between the Bureau of Plant Industry, Soils and Agricultural Engineering and the Bureau of Agricultural and Industrial Chemistry, United States Department of Agriculture, and the National Institutes of Health, Federal Security Administration. Article not copyrighted.

(2) Paper II, M. E. Wall, C. R. Eddy, M. L. McClennan and M. A. Klumpp, submitted for publication in Anal. Chem.

(3) R. E. Marker and N. Aplezweig, Chem. Eng. News, 27, 3348 (1949).

(4) C. Djerassi, H. J. Ringold and G. Rosenkranz, THIS JOURNAL, 73, 5513 (1951).

(5) R. E. Marker and J. Lopez, ibid., 69, 2390 (1947).

(6) C. Djerassi, H. Martinez and G. Rosenkranz, J. Org. Chem., 16, 1278 (1951).

(7) M. E. Wall, M. M. Krider, E. S. Rothman and C. R. Eddy, submitted for publication in J. Biol. Chem.

(8) Nomenclature in accordance with system of G. Rosenkranz and C. Djerassi, Nature, 166, 104 (1950).

I, R = hexose or pentose units

II, R = polysaccharide chain

mal sapogenins are also present in saponin curves. Thus the reversal of the transmittance values of the 900 and 922 cm.$^{-1}$ bands and the pronounced shift of the first band from 866 cm.$^{-1}$, for iso, to 852 cm.$^{-1}$, for normal compounds, are clearly shown (Figs. 1 and 2). The alignment of the characteristic four bands is obvious in spite of the greater background absorption in the glycosides than in the sapogenins, and sarsasaponin, like sarsasapogenin, is shown to have the C-22 normal configuration, whereas yucconin and dioscin, like their sapogenins, have the iso configuration. Similar curves have been observed with other saponin preparations.

To ensure the minimum degree of alteration of the native saponins, they were examined as crude preparations obtained by using only solvent extraction procedures. Further we acetylated and purified, by chromatography on alumina columns, samples of sarsasaponin, dioscin, yucconin, digitonin, chloronin and heconin–manonin mixtures (identified by hydrolysis and isolation of the sapogenin present). When these acetylated saponins were examined in the infrared region, they showed the same four characteristic absorption bands as the untreated saponins (Figs. 1a and 2b).

A pure sample of digitonin, after acetylation, chromatographic "purification" and alkaline hydrolysis to regenerate the starting material, showed only slightly diminished hemolytic power, so that fundamental molecular alteration by these processes seems improbable.

The carbonyl function in the saponins was similarly determined using both crude, native glycosides (Figs. 3b and 3c) and glycosides purified by acetylation, chromatography and de-acetylation
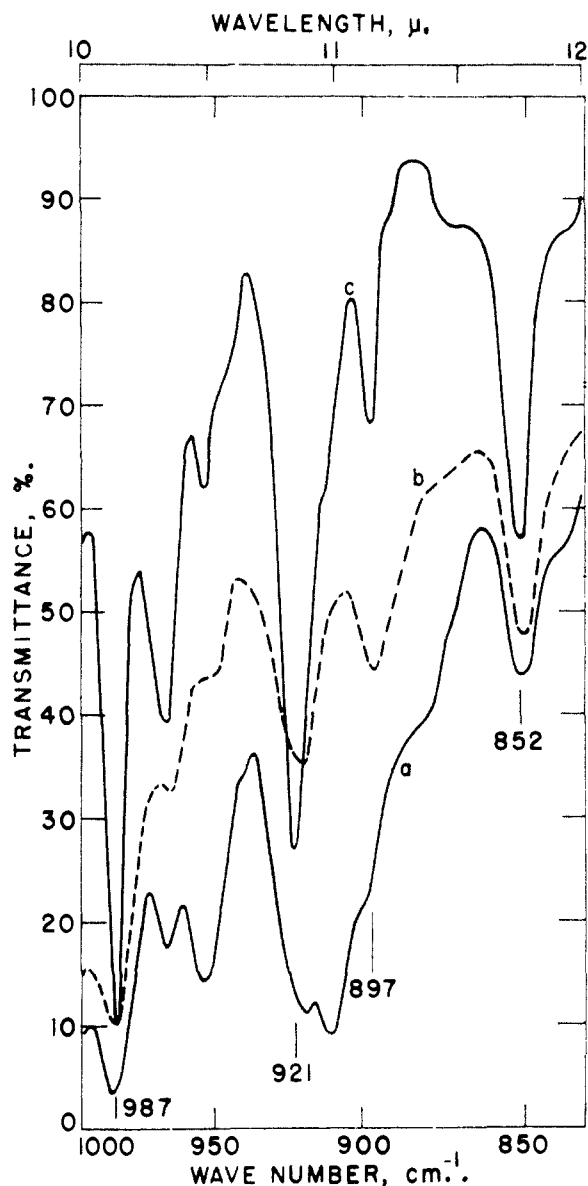
Fig. 1.—Infrared absorption spectra of: a, sarsasaponin acetate, 40 g./l. in CS_2, 1.0-mm. cell; b, sarsasaponin mull in mineral oil; c, sarsasapogenin acetate, 10 g./l. in CS_2, 1.0-mm. cell.
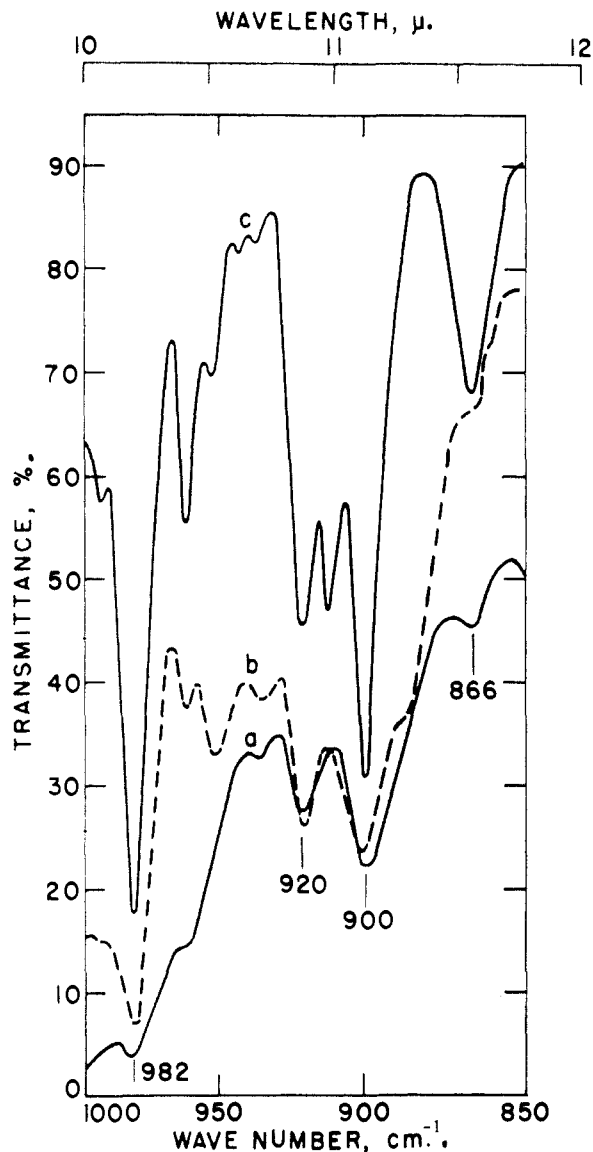
Fig. 2.—Infrared absorption spectra of: a, yucconin mull in mineral oil; b, dioscin acetate, 10.2 g./l. in CS_2. 1.0-mm. cell; c. yuccagenin diacetate. 10.4 g./l. in CS_2, 1.0-mm. cell.

(Figs. 3a and 4a). Jones and co-workers [9-11] have shown that the C-11 and C-12 carbonyl groups have an infrared absorption band in the region of 1710 cm.$^{-1}$ (5.85 μ). Ketosteroids with a conjugated structure, such as a $\Delta^{(9,11)}$-C-12 carbonyl, show a shift of the carbonyl band to the 1675 cm.$^{-1}$ (5.97 μ) region and a band near 1605 cm.$^{-1}$ (6.23 μ) due to the conjugated olefinic bond.

We have reported similar data for sapogenins[7] and now extend these findings to the saponins. Saponin precursors of non-ketonic sapogenins, e.g., dioscin (Fig. 3c) fail to show any evidence of carbonyl absorption bands in the 1710 cm.$^{-1}$ region.

(9) R. N. Jones. P. Humphries and K. Dobriner, THIS JOURNAL. 71, 241 (1949).

(10) Ibid., 72, 956 (1950).

(11) R. N. Jones. P. Humphries, E. Packard and K. Dobriner. ibid., 72, 86 (1950).

On the other hand, all saponin preparations giving rise to hecogenin or manogenin invariably show steroidal carbonyl or conjugated carbonyl infrared absorption bands (Figs. 3a, 3b and 4a). Further proof of the existence of a carbonyl function in certain saponins is shown by reaction with reducing agents. Figure 4 shows the infrared carbonyl absorption bands of: a, original saponin preparation; b, the same saponin after attempted reduction with sodium and alcohol; c, the saponin after reduction with lithium aluminum hydride. Infrared carbonyl absorption bands of the corresponding sapogenin hydrolysis products are shown in Fig. 5. Although sodium in alcohol was an ineffective reducing agent, lithium aluminum hydride resulted in approximately 85% reduction.

The sapogenin, isolated after acid hydrolysis of a sample of the original saponin, has been shown[7] to be essentially hecogenin and its $\Delta^{9.(11)}$-dehydro
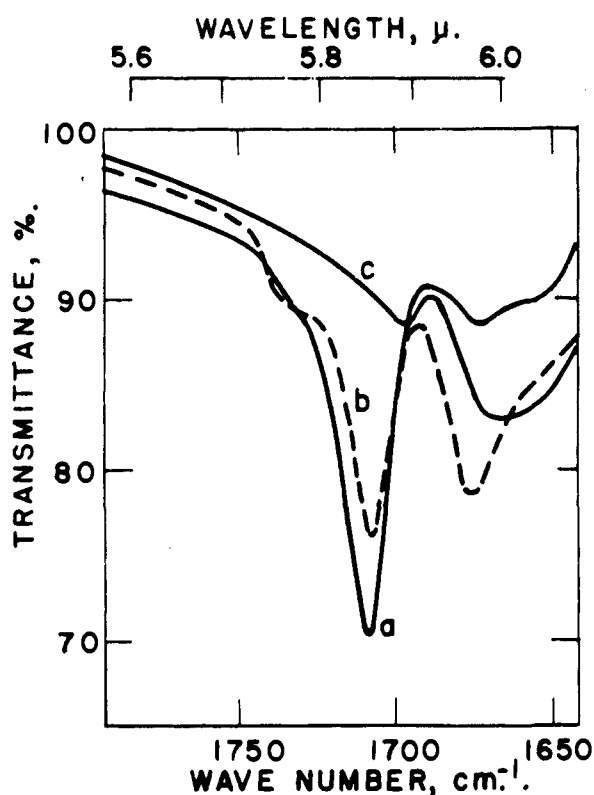
Fig. 3.—Infrared absorption spectra of: a, heconin, 48.4 g./l. in pyridine, 0.1-mm. cell; b, heconin, 46 g./l. in pyridine, 0.1-mm. cell; c, dioscin, 46 g./l. in pyridine, 0.1-mm. cell.
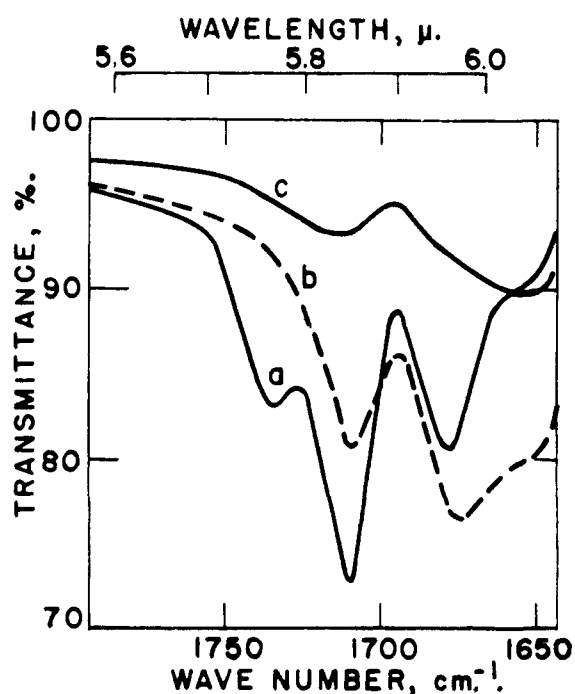

Fig. 4.—Infrared absorption spectra of: a, mixed carbonyl-containing saponins (heconin, $\Delta^{9(11)}$-heconin) from *Agave Nelsoni*, 50.7 g./l. in pyridine, 0.1-mm. cell; b, same as 4a after Na/alcohol reduction, 51.1 g./l. in pyridine, 0.1-mm. cell; c, same as 4a after LiAlH₄ reduction, 49.9 g./l. in pyridine, 0.1-mm. cell.
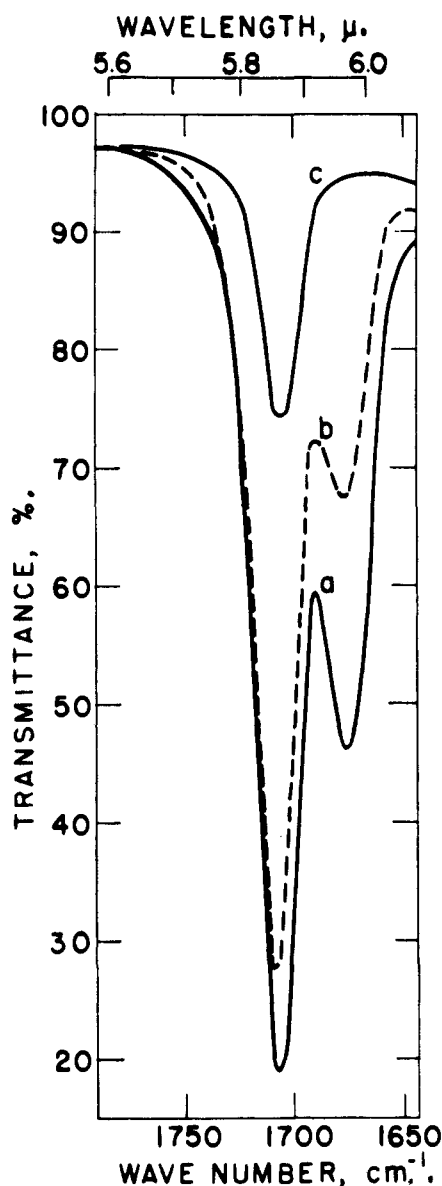

Fig. 5.—Infrared absorption spectra of: a, sapogenin derived from original carbonyl-containing saponin 4a, 25.9 g./l. CHCl₃, 0.5-mm. cell; b, sapogenin derived from Na/ alcohol reduced saponin 4b, 24.2 g./l. in CHCl₃, 0.5-mm. cell; c, sapogenin derived from LiAlH₄ reduced saponin 4c, 24.4 g./l. in CHCl₃, 0.5-mm. cell.

analog, 22-isoallospirostan-3β-ol-12-one, and $\Delta^{9,(11)}$-22-isoallospirosten-3β-ol-12-one.[8] Identification of the sapogenin produced by acid hydrolysis of the saponin reduced with lithium aluminum hydride as rockogenin   (22-isoallospirostan-3β,12-diol)   was based on the following facts: (a) Infrared carbonyl assay indicated essentially non-ketonic material. (b) Mild chromic acid oxidation of this material[12] produced hecogenone (22-isoallospirostan-3,12-dione) as the sole product. Since hecogenone can come only from oxidation of hecogenin or rockogenin, the foregoing evidence is regarded as satisfactory proof that the crude sapogenin was essentially rockogenin.

(12) R. E. Marker, *et al.*, THIS JOURNAL, **69**, 2176 (1947).

Experimental

Isolation and Purification of Saponin Preparations.—In a typical experiment, 14 lb. of *Agave Nelsoni* leaves was extracted with hot 95% ethanol. The concentrated extract was defatted, and the saponin was transferred to *n*-butanol.[7] The butanol was evaporated in a resin flask under diminished pressure to 200 ml. Trituration of the sirup with acetone gave 59 g. of tan, granular powder, which was washed repeatedly with fresh acetone, air-dried, and vacuum-dried for one hour at 95°. Spectra of materials at this stage of purification are given in Figs. 1b, 2a, 3b and 3c.

The preparation was dissolved in 100 ml. of pyridine, 100 ml. of acetic anhydride was added, and the solution was refluxed for two hours. After cooling, the solution was poured into 2 liters of water and stirred until a powder formed. This product was filtered off, washed with water, air-dried overnight and vacuum dried for one hour at 95°. The yield of acetate was 70 g. The acetate was dissolved in 300 ml. of benzene and placed on a weakly activated alumina adsorbent packed in a column 33 cm. long and 7 cm. in diameter. Elution with 4 liters of benzene removed a small amount of a greenish, glassy impurity. Elution with a benzene–chloroform mixture of increasing chloroform content up to 100% gave 44 g. of nearly colorless, purified saponin acetate. Spectra of materials at this stage of purification are given in Figs. 1a and 2b. Further elution with chloroform containing increasing concentrations of ethanol gave additional fractions, believed to be incompletely acetylated material.

An 11.6-g. sample of the acetate from the benzene–chloroform eluate was dissolved in a mixture of 150 ml. of methanol and 50 ml. of H_2O. Thirty ml. of KOH-saturated methanol was added, and the mixture was refluxed for two hours. The solution was cooled, diluted with water, the methanol was evaporated, and the aqueous solution, after slight acidification (pH 6.5) with hydrochloric acid, was extracted with three 100-ml. portions of *n*-butanol (water-saturated). The butanol, after being washed with two 50-ml. portions of water saturated with butanol was evaporated to give 8 g. of saponin consisting almost entirely of hecogenin glycosides and 9-dehydro analogs. Spectra of materials at this stage of purification are shown in Figs. 3a and 4a.

Attempted Sodium–Alcohol Reduction of Saponin.—To the 8 g. of saponin dissolved in 1 liter of hot ethanol 58 g. of sodium was added rapidly. A precipitate, which formed initially, dissolved on heating, so that the reduction was conducted in a homogeneous medium. After the sodium had dissolved, the solution was cooled, diluted with 1 liter of water, and evaporated to 600 ml. The residue was made slightly acid (pH 6.5) with hydrochloric acid, and the saponins were extracted as before with wet butanol; the yield was 7.5 g. A sample was used for infrared absorption (Fig. 4b). The remainder was hydrolyzed to sapogenin (Fig. 5b).

Reduction of Saponin with Lithium Aluminum Hydride.—To 11 g. of LiAlH₄ in 300 ml. of dry, peroxide-free tetrahydrofuran was added an 11-g. sample of the chromatographed saponin acetate. The mixture was stirred for two hours under reflux and let stand overnight. After most of the solvent was distilled off under diminished pressure, water was added, the hydroxides were dissolved by lowering the pH to 6.5 with hydrochloric acid, and the saponin was extracted with butanol as described above. The yield of glycoside was 8 g. A small portion was used to obtain the spectrum shown in Fig. 4c; the remainder, after hydrolysis to sapogenin, was used for Fig. 5c.

Identification of Reduction Product.—A 0.65-g. aliquot of the hydrolyzed sapogenin was chromatographed on alumina. The product, 0.56 g., eluted with 100% chloroform, m.p. 196–201°, contained about 10% of ketosapogenin, representing unreduced starting material. There was no depression of melting point (mixed m.p. 199–201°) with an authentic sample of rockogenin (m.p. 208°) obtained from LiAlH₄ reduction of hecogenin. Chromic acid oxidation of this material gave only hecogenone, m.p. 235°. The infrared spectrum, 2 to 15 μ, was identical with that of an authentic specimen prepared by oxidation of hecogenin.

Hydrolysis of the Saponins.—The saponins were dissolved in 200 ml. of a 1:1 ethanol–water mixture (by volume) 2 N in hydrogen chloride. One hundred ml. of benzene equilibrated with 1:1 ethanol–water was added and the resultant two-layered mixture was refluxed for 16 hours. After cooling, the benzene layer containing the sapogenins was drawn off, and the aqueous residue was reextracted several times with benzene. The united benzene layers were treated with methanolic KOH,[6] and the benzene containing the purified sapogenins was evaporated to dryness. The sapogenins as such were crystallized from methanol prior to infrared carbonyl assay (Fig. 5). The sapogenins were acetylated prior to examination of the fingerprint region of the spectrum (Figs. 1c and 2c).

Infrared Spectra.[13]—The infrared absorption spectra were obtained with a Beckman IR-3 spectrophotometer; NaCl prisms were used. For spiroketal determinations, free saponins were examined as mulls in mineral oil, sapogenin acetates were examined in carbon disulfide solution, and saponin acetates were examined in either chloroform or carbon disulfide solution, depending on solubility. Carbon disulfide has the advantage of greater transparency, allowing the entire fingerprint region of the spectrum to be obtained; chloroform has the advantage of greater solubility for most saponin acetates. Concentrations from 10 to 100 g. per liter were used, in cells 0.5 and 1.0 mm. thick. One ml. of solution sufficed for the determination.

For carbonyl determination, the free glycosides were dissolved in pyridine at a concentration of approximately 50 g. per liter using a 0.1-mm. cell. Carbonyl in sapogenins was determined in chloroform solutions at a concentration of 15 to 25 g. per liter with a 0.5-mm. cell.

Carbonyl determinations: Fig. 5a: Sapogenin from untreated saponin contained approximately 65% unconjugated, approximately 20% conjugated carbonyl. Total, approximately 85% carbonyl-containing sapogenins. Fig. 5b: Sapogenin from sodium–alcohol treated saponin contained approximately 55% unconjugated carbonyl, 10% conjugated carbonyl. Total, 65% carbonyl-containing sapogenins. Fig. 5c: Sapogenin from LiAlH₄ treated saponin contained approximately 13% unconjugated carbonyl, no conjugated carbonyl. Total approximately 13% carbonyl-containing sapogenins.

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