chloric acid, reëxtraction with ether, and removal of the ether, a gummy solid was obtained weighing 9 g. This was extracted with benzene. The benzene insoluble part, after several crystallizations from a benzene–methyl alcohol mixture, melted at 276–277°, and unchanged when mixed with a sample of diphenyl-2,5-dicarboxylic acid.[2] The benzene-soluble part after several crystallizations from benzene–petroleum ether, melted at 248–249°, and unchanged when mixed with a sample of 2-bromodiphenyl-5-carboxylic acid.[2] From the ether solution extracted by alkali, 1.1 g. of diphenyl was isolated.

4 - Bromodiphenyl - 3 - carboxylic Acid.—Seventy - one grams of 4-bromo-3-methylaniline, obtained by brominating 3-acettoluide, was treated with 110 cc. of water and 76 cc. of concd. hydrochloric acid. The suspension was then diazotized with 27.5 g. of sodium nitrite in saturated solution, treated with 300 cc. of benzene and gradually with 110 cc. of 5 *N* sodium hydroxide keeping the temperature below 5°. After standing overnight the benzene was removed, yielding 18 g. of a liquid boiling at 165–170° (3 mm.). On oxidation with aqueous permanganate and crystallization from benzene, 4-bromodiphenyl-3-carboxylic acid was obtained, m. p. 194–195°.

Anal. Calcd. for $C_{13}H_9O_2Br$: Br, 28.85. Found: Br, 28.70.

3-Bromodiphenyl-4-carboxylic Acid.—4-Nitro-2-aminotoluene was prepared by the nitration[6] of *o*-toluidine. It was converted to 4-nitro-2-bromotoluene by the Gattermann reaction. From this, by reduction with stannous chloride in hydrochloric acid, was prepared 2-bromo-4-aminotoluene (acetyl derivative, m. p. 111–112°. Beilstein gives 113°). Sixty-five grams of 2-bromo-4-aminotoluene was treated with 70 cc. of concd. hydrochloric acid and 60 cc. of water, and diazotized with 26 g. of sodium nitrite in 50 cc. of water. The resulting solution was treated with 300 cc. of benzene and gradually with 100 cc. of 5 *N* sodium hydroxide solution. After standing overnight, the benzene solution was separated and the benzene removed by distillation, yielding 19.5 g. of an oil boiling at 165–170° (7 mm.). On oxidation with aqueous potassium permanganate solution and crystallization from methyl alcohol–petroleum ether, 3-bromodiphenyl-4-carboxylic acid, m. p. 179–180° was obtained.

Anal. Calcd. for $C_{13}H_9O_2Br$: Br, 28.85. Found: Br, 29.05.

(6) Schiff and Vanni, *Ann.*, **268**, 322 (1892).

The author acknowledges the assistance of Mr. Max Gabis in the preparation of certain intermediates involved in this work.

Summary

1. The "3,4-dibromodiphenyl" of Scarborough has been shown to be a mixture containing largely the 2,5-isomer, and some of the 3,4.

2. 3,4-Dibromodiphenyl has been synthesized.

3. The bromination product of 3-acetaminodiphenyl is believed to be a constant melting mixture of 4-bromo-3-acetamino- and 2-bromo-5-acetaminodiphenyls.

4. Both of these pure products have been synthesized.

5. The syntheses of 4-bromodiphenyl-3-carboxylic acid and of 3-bromo-diphenyl-4-carboxylic acid are described.

PHILADELPHIA, PENNA. RECEIVED MAY 18, 1936

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF STANFORD UNIVERSITY]

Saponins and Sapogenins. IV. The Isolation of Amolonin and Determination of the Products of Hydrolysis

BY P. C. JURS AND C. R. NOLLER

The hydrolysis of crude methyl alcoholic extracts of the bulbs of *Chlorogalum pomeridianum*, Kunth, commonly known as California soap plant or amole, has been shown to yield two crystalline sapogenins.[1] One of these was assigned the formula $C_{26}H_{40}O_2(OH)_2$ and the name *chlorogenin*, it being isomeric with but different than gitogenin, while the other was found to be identical with tigogenin, then believed to have the formula $C_{26}H_{41}O_2(OH)$. Recent work[2] indicates that gitogenin and tigogenin have the formulas $C_{27}H_{42}O_2(OH)_2$ and $C_{27}H_{43}O_2(OH)$, respectively, and chlorogenin is now known to have the formula $C_{27}H_{42}O_2(OH)_2$.[3]

The isolation of two sapogenins indicated that at least two saponins were present and early attempts by Liang to isolate the saponins proved this to be the case.[4] On cooling hot aqueous solutions of the mixed saponins or hot methyl alcoholic extracts of the roots, a product deposited that on hydrolysis yielded pure tigogenin, while hydrolysis of the more soluble fractions in the filtrates yielded largely chlorogenin. It appeared

(1) Liang and Noller, THIS JOURNAL, **57**, 525 (1935).

(2) Simpson and Jacobs, *J. Biol. Chem.*, **109**, 573 (1935); Tschesche and Hagedorn, *Ber.*, **68**, 1412 (1935).

(3) Private communication from Dr. L. F. Fieser. The results of extremely accurate combustions on chlorogenin at the Chemical Laboratory of Harvard University gave C, 74.956, 74.982; H, 10.249, 10.253. Calcd. for $C_{27}H_{44}O_4$: C, 74.955; H, 10.251.

(4) Liang, Thesis, Stanford University, 1934.

that it should be possible to isolate the less soluble saponin in a pure state and the present paper deals with the isolation of a pure crystalline saponin and with the quantitative determination of the products formed on hydrolysis.

At first an attempt was made to purify the less soluble saponin fraction by a triangular fractional crystallization from methyl and ethyl alcohol. This was partially successful and resulted in the isolation of two saponins, one of which was crystalline and the other amorphous, and which differed in specific rotation and solubility in methyl and ethyl alcohols. Both of these saponins yielded tigogenin on hydrolysis, a fact that increases the minimum number of saponins present in the plant.

Fractional crystallization was tedious but it was found that these two saponins possessed a marked difference in solubility in *t*-butyl alcohol and this gave a rapid method for obtaining the less soluble fraction in a pure form and in quantity. The mixed saponins were repeatedly extracted with hot *t*-butyl alcohol to remove most of the amorphous saponin, and the insoluble portion repeatedly crystallized from ethyl and methyl alcohol until the product was crystalline (Fig. 1)

Fig. 1.—Photomicrograph of crystalline amolonin.

and had a constant rotation. These crystals apparently contain alcohol of crystallization since on drying they fall to an amorphous powder. Although this saponin is only slightly soluble in cold water, its aqueous solutions show all the typical properties of a saponin such as the formation of a stable foam on shaking, toxicity to fish and hemolysis of red blood corpuscles.

The pure crystalline saponin, which has been named "*amolonin*," was hydrolyzed and the sugars identified by the usual procedures. Positive tests were obtained for *d*-glucose, *d*-galactose, *l*-rhamnose and ketoses. Tests for pentoses, mannose and uronic acids were negative. Quantitative estimations showed that the hydrolysis could be represented by the equation

$$\underset{\text{amolonin}}{C_{63}H_{87}O_{14}(OH)_{17}} + 6H_2O \longrightarrow C_{27}H_{43}O_2(OH) + 3C_6H_{12}O_6 + C_6H_{12}O_6 + 2C_6H_{12}O_5$$

tigogenin; *d*-glucose; *d*-galactose; *l*-rhamnose

The ketoses are present only in small amounts in the hydrolytic products and probably arise from the aldohexoses by a Lobry de Bruyn–van Ekenstein[5] interconversion.

The formula $C_{63}H_{87}O_{14}(OH)_{17}$ was confirmed by the preparation of a septadeca-acetyl derivative.

Experimental

Isolation of Saponins by Fractional Crystallization.—The bulbs were collected in March, the hairy covering and leaves discarded and the remainder ground in a food chopper. The liquid portion of the ground pulp was removed by a cider press and the press cake washed twice with cold water and twice with methyl alcohol, pressing each time to remove the liquid. The damp press cake was repeatedly extracted with boiling methyl alcohol until the extract no longer gave a precipitate on cooling. The gelatinous brown precipitates were filtered and evaporated to dryness under a vacuum. Further crops were obtained by concentrating the alcoholic filtrates.

When the crude saponin obtained in this way was subjected to a fractional crystallization with sufficient treatments with Norite to remove the color completely, two main fractions were obtained. The less soluble fraction consisted of definitely crystalline needles, $[\alpha]_{546}$ −74.2°, in pyridine. One gram on hydrolysis in 50% aqueous methyl alcohol containing 5% hydrogen chloride gave 0.304 g. of tigogenin, m. p. 197–201° (corr.); after crystallization from isopropyl alcohol and then from benzene it melted at 200–207°. This saponin fraction had a solubility in methyl alcohol of 0.36 g. and in ethyl alcohol of 0.24 g. per 100 g. of solvent at 25° and was practically insoluble in hot *t*-butyl alcohol. The more soluble fraction was usually amorphous although sometimes hair-like crystals were observed under the microscope. The specific rotation in pyridine varied from −68.7 to −63.8° and apparently became less as purification proceeded. One gram on hydrolysis gave 0.411 g. of sapogenin, m. p. 189–204° (corr.) which after crystallization from isopropyl alcohol and from benzene melted at 197–202°. A mixed melting point determination with the sapogenin from the needles showed no depression. The amorphous saponin had a solubility in methyl alcohol of 1.62 g. and in ethyl alcohol of 2.45 g. per 100 g. of solvent at 25° and was quite soluble in hot *t*-butyl alcohol. Both saponins had a low solubility in benzene, acetone, carbon tetrachloride and ether and a high solubility in pyridine, dioxane and glacial acetic acid.

Isolation of Amolonin by Extraction.—The crude dried saponin obtained as above was ground to a powder and

(5) Lobry de Bruyn and van Ekenstein, *Rec. trav. chim.*, **14**, 156, 204 (1895); Spoehr and Wilbur, *J. Biol. Chem.*, **69**, 421 (1926).

extracted with ten times its weight of boiling *t*-butyl alcohol. The drying, grinding and extraction of the insoluble portion was repeated three or four times until the loss in weight on an extraction was less than 2%. The light brown dried powder was recrystallized from ethyl alcohol and then repeatedly from methyl alcohol until the product was colorless, completely crystalline under the microscope and had a constant rotation. The recrystallizations from methyl alcohol were best performed by dissolving in a minimum of boiling solvent and then concentrating to from one-half to one-fifth of the original volume and cooling rapidly. The product was deposited as amorphous spheres which on standing for some time changed into microscopic needles (Fig. 1). During the first crystallization the crystals appeared to be mixed with small, spherical, gelatinous globules when examined under the microscope but these disappeared as the purification proceeded.

The crystals obtained in this way fell to an amorphous powder on drying. This product gave no perceptible ash on ignition; $[\alpha]^{28}_{546}$ −75.5° in pyridine, −67.5° in dioxane.

Qualitative Detection of Hydrolytic Products.—Pure amolonin was hydrolyzed completely by heating with twenty times its weight of 50% aqueous methyl alcohol containing 5% sulfuric acid. The mixture was heated in a pressure bottle at 100–110° with shaking for two days. The tigogenin was filtered and the filtrate analyzed qualitatively by a procedure essentially as recommended by van der Haar[6] unless otherwise noted.

The usual naphthoresorcinol color reaction for uronic acids, when applied to both the original saponin and to the barium uronate fraction[7] of the sugars, gave doubtful positive tests. Spectrographic examination of benzene extracts compared with controls, especially after the extracts had stood for several hours, showed definitely the absence of uronic acids.

Both types of reactions recommended by van der Haar[6] for the detection of ketoses gave positive tests. Later quantitative work, however, showed that the ketoses were present in such small amount that their presence may be explained by a Lobry de Bruyn–van Ekenstein interconversion[5] during the preparation of the sugar solution. It is possible that fructose does not occur in other saponins where it has been reported as a hydrolytic product.[8]

Nitric acid oxidation of the mixed sugars gave mucic acid, identified by its melting point (209–210°) and mixed melting point, crystal form and the crystal form of its insoluble thallous salt, and saccharic acid identified by the crystal form of its acid potassium salt and by the preparation and analysis of the silver salt. This indicates the presence of galactose and of glucose in the saponin molecule. That these sugars are the *d*-forms is shown by the fact that they are fermented by the yeasts, *Torula lactosa* and *Torula alactosa*, which do not ferment the *l*-forms. The non-formation of a phenyl-hydrazone was taken to indicate the absence of mannose.

For the detection of pentoses and methyl pentoses the tests depending on their conversion to furfural and methylfurfural and on the color reactions for these compounds were used. Both visual and spectroscopic examination of the color reactions showed the absence of pentoses and the presence of methyl pentoses. To identify the methylpentose, the glucose was removed by fermentation with baker's yeast and the solution of galactose and methyl pentose treated with α-methylphenylhydrazine. The formation and identification of the methylphenylhydrazone of galactose, m. p. 187.8° (corr.) further proved the presence of this sugar. After the removal of galactose as the hydrazone, the methylpentose was regenerated and the solution freed of methylphenylhydrazine by treatment with formaldehyde as recommended by van der Haar.[6] On subsequent reaction with *p*-bromophenylhydrazine an osazone formed which appeared to be identical with *p*-bromophenylrhamnosazone as determined by melting point (213–214° corr.) and mixed melting point with an authentic specimen. This identification was not considered entirely satisfactory because the product could not be recrystallized, and the melting points were really decomposition points. Examination under the polarizing microscope did not yield further information since *p*-bromophenylrhamnosazone needles show parallel extinction. The β-naphthylhydrazone was a much more satisfactory derivative and proved to be identical (m. p. 190–191°, $[\alpha]_{546}$ −12.6° in pyridine) with the β-naphthylhydrazone of *l*-rhamnose. The extinction angle of the crystals is 42.3°.

No other products of hydrolysis were detected and the following quantitative work shows that no other components can be present in the saponin molecule.

Estimation of Tigogenin.—Samples of pure amolonin weighing from 0.3 to 3.0 g. were hydrolyzed under different conditions and the amount of tigogenin formed was determined by filtering, washing and drying to constant weight at 100°. Hydrolyses in 50% aqueous methyl alcohol containing 5% hydrogen chloride at the boiling point for seventy hours gave 30.4 and 29.4% tigogenin. Using 5% sulfuric acid and shaking in a sealed tube at 90° for sixty-five hours gave 28.4 and 28.0% tigogenin. Using water containing 4% sulfuric acid and shaking in a sealed tube at 90° for five days gave 30.4%. The average of these five determinations is 29.3%.

Preparation of Sugar Solutions for Analysis.—The filtrates after removal of tigogenin were treated with freshly prepared pure barium carbonate, made from barium hydroxide and carbon dioxide, care being taken to avoid an excess of barium carbonate and to maintain the *p*H at 6.0 ± 0.5. In this way appreciable conversion of the aldohexoses to ketoses is avoided. The solutions were evaporated in a vacuum desiccator at 40–50° until a thick sirup was obtained. Any precipitate that formed during the evaporation was filtered and the *p*H of the filtrate adjusted before further evaporation. Solutions of this sirup failed to ferment and it was thought at first that this might be due to the traces of barium salts that always remained. Solutions neutralized with sodium bicarbonate, however, behaved in the same way. It was found that non-fermentation was due to the presence of barium or of sodium methyl sulfate, an appreciable amount of methyl hydrogen sulfate having been formed during the hydrolysis in 50%

(6) Van der Haar, "Anleitung zum Nachweis, zur Trennung und Bestimmung der Monosaccharide und Aldehydsaüren," Gebrüder Borntraeger, Berlin, 1920.

(7) Link and Dickson, *J. Biol. Chem.*, **86**, 491 (1930); Link and Nedden, *ibid.*, **94**, 307 (1932); Hopkins, Peterson and Fred, THIS JOURNAL, **53**, 306 (1931).

(8) Kofler, "Die Saponine," Julius Springer, Vienna, 1927, p. 96.

methyl alcohol. These salts were removed either by repeated extraction of the sugars with absolute alcohol and evaporation to dryness, or by boiling the sirup with dilute sulfuric acid for several hours and precipitating the sulfuric acid with barium carbonate. The sugar sirups prepared in this way were diluted to a known volume and aliquot portions analyzed according to procedures recommended by van der Haar.[8]

Rhamnose.—In order to avoid undue decomposition of the rhamnose the determinations were made on sugar solutions from which barium methyl sulfate had not been removed completely. The sugars from 1.8835 g. of amolonin were diluted to 10 cc. and 3.67 cc. of this solution yielded 0.1204 g. of methylfurfural phloroglucide. From Ellet's tables as given by van der Haar this corresponds to 0.1826 g. (0.00100 mole) of rhamnose hydrate. The corresponding amount of amolonin would have yielded 0.00048 mole of tigogenin so that the molecular ratio is 2 to 1.

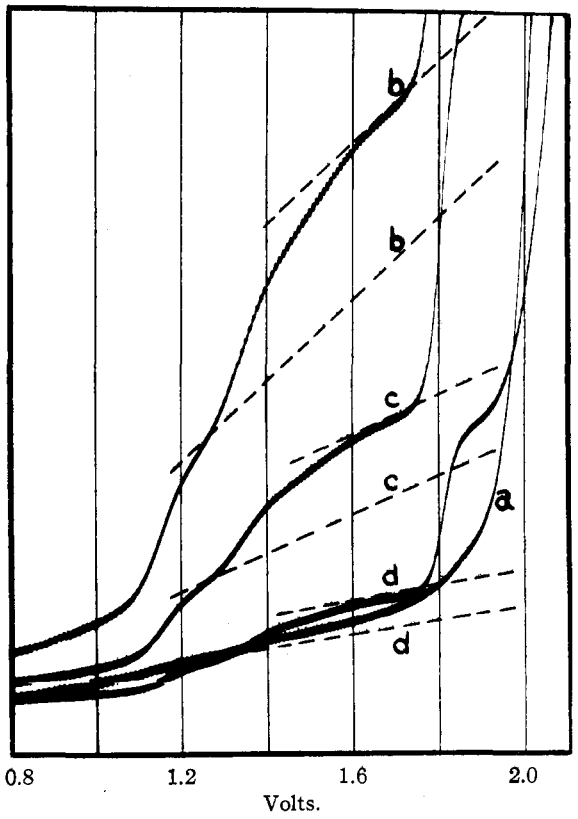

Fig. 2.—Polarigram of sugar solution from the hydrolysis of amolonin.

Ketoses.—The simplest procedure for the estimation of ketoses in the presence of aldoses is by means of the polarigraph since ketoses are reduced at the dropping mercury cathode whereas aldoses are not.[9] If a polarigram is made of a solution of a ketose, the potential at which the wave develops depends on the sugar, the temperature and somewhat on the concentration, while the height of the wave, that is, the vertical distance between two lines tangential to the curve at the points of inflection, is directly proportional to the concentration. The test solution was made by diluting the sugars from 2.9081 g. of amolonin to 25 cc. Figure 2 shows four curves: (a) 5 cc. of 0.2 *M* calcium chloride in the cell, hydrogen bubbled through for twenty minutes and the curve taken at $s = 0.1$ (s = sensitivity of the galvanometer); (b) 0.1 cc. of test solution added to the cell, hydrogen passed through for ten minutes and the curve taken at $s = 0.1$; (c) repeat of (b) at $s = 0.05$; (d) repeat of (b) at $s = 0.02$. Figure 3 gives curves for a solution of fructose of known concentration which gave curves similar to the unknown when run under the same conditions. The correct concentration to accomplish this was arrived at by trial and was 0.0111 mg. of fructose per cc. in approximately 0.2 *M* calcium chloride solution. The letters have the same significance as in Fig. 2. It is seen that the fructose wave occurs at approximately the same voltage as the unknown. Height bb in Fig. 3 is 3.16 cm. while height cc at half the sensitivity is 1.51 cm., which checks the reproducibility of separate runs. Taking the height of 1.51 cm. as corresponding to a concentration of 0.0111 mg. of fructose per cc., the height cc in Fig. 2 of 1.01 cm. corresponds to 0.00743 mg. per cc. of calcium chloride solution or 0.379 mg. (0.0000021 mole) per cc. of original sugar solution. The corresponding amount of amolonin would have yielded 0.000081 mole of tigogenin, or one mole of fructose for 38 moles of tigogenin, an impossible ratio.

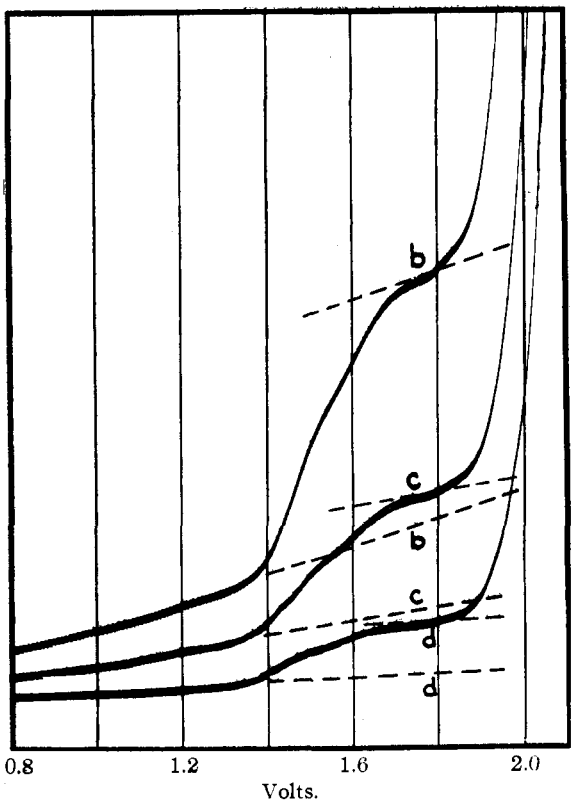

Fig. 3.—Polarigram of standard fructose solution.

It should be noted that two minor waves occur in Fig. 2 beginning at approximately 1.1 and 1.8 volts that do not

(9) Heyrovský and Smoleř, *Coll. Czechoslov. Chem. Comm.*, **4**, 521 (1932); *C. A.*, **27**, 1828 (1933). We are indebted to Dr. O. H. Mueller of the Physiology Department for the use of his polarigraph and for his assistance in this investigation.

occur in Fig. 3. The wave at 1.8 v. is due to traces of barium ion but the impurity causing that at 1.1 v. is unknown.

Glucose and Galactose.—These sugars were determined by fermentation in van Iterson–Kluyver tubes according to the general procedure given by van der Haar. Special yeasts, different from those recommended by van der Haar, were obtained in pure culture through the courtesy of Professor T. Skogsberg of the Hopkins Marine Station. These yeasts were *Torula lactosa* and *Torula alactosa*, both of which ferment *d*-glucose and *d*-galactose, and *Torula datilla* and *Torula monosa*, which ferment glucose but not galactose. None of these yeasts ferments *l*-rhamnose.[10] Controls were run on 1, 2 and 4% solutions of glucose and galactose with each yeast in order to determine the carbon dioxide evolved and the amount of carbon dioxide remaining in solution. The latter volume depended on the type of yeast and the sugar, and for the mixture of sugars a weighted average was used and added to the volume read. Table I gives the average values for the data obtained on the controls. The gas volumes are for 0° and 760 mm.

TABLE I

CONTROL FERMENTATIONS OF GLUCOSE AND GALACTOSE

Sugar	Vol. of CO_2 dissolved per cc. of solution: Glucose	Galactose	Mg. of hexose per cc. of CO_2 formed: Glucose	Galactose
T. datilla	0.51	...	4.65	..
T. monosa	.42	...	4.75	..
T. alactosa	.51	0.48	4.70	4.61
T. lactosa	.65	.14	4.37	5.10

In a typical run the purified sugars from 2.9081 g. of amolonin were diluted to 25 cc. and 3 cc. of this solution mixed with 6 cc. of nutrient broth. The solution was passed through a Chamberland filter and 1 cc. placed in each of four fermentation tubes and inoculated under sterile conditions with the four yeasts. The volumes of carbon dioxide evolved after three days at 32° were corrected to 0° and 760 mm. and the estimated amount of dissolved carbon dioxide added. This gave for *T. datilla*, 3.28 cc., *T. monosa*, 3.24 cc.; *T. alactosa*, 4.22 cc., *T. lactosa*, 4.12 cc. The first two volumes can be converted to glucose directly and correspond to 15.3 and 15.4 mg. per cc. The volumes of carbon dioxide from galactose can be obtained by subtracting each of the volumes from *T. datilla* and *T. monosa* from each of the volumes from *T. alactosa* and *T. lactosa*, giving, respectively, 0.94, 0.98, 0.84, and 0.88 cc. When the first two volumes are multiplied by 4.61 and the last two by 5.1, one obtains 4.3, 4.5, 4.3 and 4.5 mg. of galactose per cc. Multiplying by the aliquot factor, 75, one obtains 1.1512 g. (0.00639 mole) of glucose and 0.330 g. (0.00183 mole) of galactose from 2.9081 g. of amolonin which yields 0.00203 mole of tigogenin. The ratio of tigogenin to glucose to galactose is therefore 1 to 3 to 1.

The results of the determinations of rhamnose, glucose and galactose were checked by comparison with total aldoses by iodine oxidation.[11] Thus a solution of the mixed sugars gave rhamnose 12.6 mg. per cc. and total hexoses by fermentation 29.5 mg. per cc. or a total of 42.0 mg. per cc. The average aldose content found by iodine oxidation on the assumption of 2 moles of methylpentose to 4 moles of hexose was 40.1 mg. per cc.

Septadeca-acetylamolonin.—Amolonin was fully acetylated by dissolving 0.970 g. in 5.4 cc. of pyridine and 4.9 cc. of acetic anhydride. After standing for one day at room temperature, the solution was poured into water and filtered. The precipitate was crystallized from dilute methyl alcohol and dried in vacuum at 100°; $[\alpha]^{20}_{546}$ −33.65° in pyridine; recrystallized and dried, $[\alpha]^{20}_{546}$ −33.53°. Analysis for acetyl:[12] Calcd. for $C_{63}H_{87}O_{14}(OCOCH_3)_{17}$: % acetyl, 35.32. Found: 35.67, 35.14.

Combustion Analyses.—Amolonin Calcd. for $C_{68}H_{104}O_{31}$: C, 55.71; H, 7.74. Found: C, 55.39, 55.38, 55.56, 55.32; H, 7.99, 7.98, 8.02, 7.94. Amolonin Septadeca-acetate. Calcd. for $C_{97}H_{138}H_{48}$: C, 56.23; H, 6.71. Found: C, 55.85 55.78; H, 6.90, 6.82.

Summary

Amolonin, a crystalline saponin obtained from *Chlorogalum pomeridianum* has the formula $C_{63}H_{87}O_{14}(OH)_{17}$. On hydrolysis it yields for each mole of tigogenin, one mole of *d*-galactose, two moles of *l*-rhamnose and three moles of *d*-glucose. The septadeca-acetyl derivative of amolonin has been prepared.

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(10) Harrison, *Trans. Roy. Soc. Can.*, **21**, Sec. 5, 341 (1927); **22**, Sec. 5, 187 (1928).

(11) Cajori, *J. Biol. Chem.*, **54**, 617 (1922); Macleod and Robison, *Biochem. J.*, **23**, 517 (1929).

(12) Gattermann–Wieland, "Laboratory Methods of Organic Chemistry," Macmillan Co., New York, 1932, p. 70.