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INVESTIGATIONS ON STEROIDS. XXII. STUDIES ON OUABAGENIN. I*

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Of the therapeutically important cardiac glycosides, ouabain is the only one for which the exact structure of the aglycone (ouabagenin) still has to be established. Ouabain was discovered and so named by Arnaud (1) who in 1888 isolated from the bark and roots of the ouabaio tree (*Acokanthera ouabaio*) a toxic glycoside which was used by the Somalis of East Africa as an arrow poison. Later (2) he found the same glycoside in another arrow poison, the inée or onaye of the Pahouins which was prepared from the seeds of *Strophanthus gratus* (*S. glaber*). The name g-strophanthin, introduced by Thoms (3, pp. 114, 119) to distinguish it from the strophanthins of other species, is frequently used in the German literature for ouabain. Of all cardiac active principles, ouabain is the most easily obtainable since it can be isolated and crystallized from the seeds of *Strophanthus gratus* in 5% yield (4).

As early as 1898 Arnaud (5) identified rhamnose as the sugar moiety of ouabain. Efforts to prepare the true aglycone (ouabagenin) remained fruitless for a long period of time. Various modifications of acid and enzymatic hydrolysis had yielded only resins which were the result of dehydration and possibly simultaneous polymerization. It was a major accomplishment when in 1942 Mannich and Siewert (4) succeeded in preparing the true crystalline aglycone by hydrolysis of its acetonide which in turn had been obtained by treatment of the glycoside with small amounts of concentrated hydrochloric acid in acetone.

The early investigators, therefore, studied the crystalline glycoside rather than the altered aglycone. Jacobs (6) established for ouabain the formula $C_{29}H_{44}O_{12}$. He was able to show its close relationship to the other cardiac active steroids, since ouabain gives a positive Legal color test and forms an iso-compound as well as a dihydro derivative, both of which are negative in the Legal test.

When Arnaud (7) treated ouabain with acetic anhydride and zinc chloride, he obtained an anhydroheptaacetate. Mannich and Siewert (4) observed that acetylation of ouabain without addition of zinc chloride proceeds without formation of an anhydro linkage and gives a hexaacetate. In both cases three acetyl groups are in the sugar residue. A tertiary hydroxyl group at carbon atom 14

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[1] Merck & Co. Predoctoral Fellow (1951–1952). This paper is based on a section of a thesis submitted by Klaus Georg Florey to the Graduate School of Arts and Sciences of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry (June 1954).

which is responsible for the iso-reaction is lost in the formation of Arnaud's anhydroheptaacetate. Another tertiary hydroxyl group is probably at carbon atom 5 in analogy to strophanthin. It is acylable under the forcing conditions of Arnaud but not under the milder conditions of Mannich (*cf.* 8, p. 549). The sugar residue is probably linked to a secondary hydroxyl group at carbon atom 3, in analogy to the other known cardiac glycosides. In addition, the aglycone contains three other acylable hydroxyl groups, two secondary ones which were postulated by Mannich (4, 9) at carbon atoms 1 and 11, and one primary hydroxyl group at carbon atom 19. The last assumption is based on an observation by Jacobs and Bigelow (6) who subjected the tetrahydro derivative of heptaacetylanhydroouabain (heptaacetyldesoxydihydroouabain) to acetolysis with glacial acetic acid containing 3% of hydrogen chloride. The rhamnose-free crystalline reaction product represented the acetate of a trianhydromonohydroxylactone, resulting from the genin moiety with the loss of a carbon atom as formaldehyde. Analogous results were obtained in experiments conducted with isoouabain as starting material (10). The absorption spectrum of the acetoxytrianhydrolactone derived from isoouabain indicated the presence of a benzenoid ring (λ_{max} 270 mμ). This finding led Fieser and Newman (11) to suggest that during the course of the reaction a hydroxymethyl group at C_{10} is eliminated as formaldehyde leading to aromatization of ring B. *In toto* this reaction involves the loss of four hydroxyl groups originally present in the genin moiety. Marker and associates (12) presented evidence in support of aromatization of ring A, rather than ring B. As will be shown, the results of the present investigation are in agreement with the latter view.

An important consideration in the tentative designation of the positions of the six hydroxyl groups is the fact that ouabagenin is not attacked by lead tetraacetate (4) or periodic acid (13), two reagents which oxidize 1,2-glycols. Therefore no two hydroxyl groups in the ouabagenin molecule can be in adjacent positions.

On the basis of this analysis of the literature, the working hypothesis appears permissible that the hydroxyl groups at carbon atoms 3, 5, and 14 are in the β-position, in analogy to strophanthidol. The hydroxyl group at carbon atom 1 would also have to be in the β-position to allow for the formation of a 1,3-acetonide (4, 13). The α-positions at carbon atoms 1 and 3 in the etiocholane series are equatorial and do not permit the formation of an acetonide. A hydroxyl group in position 11, if present, should possess the α-configuration, since 11 β-hydroxyl groups are known to resist acetylation (*cf.* 14).

By acetylation of ouabagenin Mannich and Siewert (4) obtained a tetraacetate, a finding which is consistent with the postulation of one primary, three secondary, and two tertiary hydroxyl groups in the aglycone molecule. More recently Meyrat and Reichstein (15) repeated the acetylation of ouabagenin by treatment at room temperature overnight followed by heating to 60° for 4 hours. Careful chromatography of the reaction mixture afforded two acetylation products. The less polar compound, designated ouabagenin acetate A, m.p. 291–294°, was identical with the tetraacetate obtained by Mannich and Siewert.

The more polar compound, m.p. 253–257°, was designated ouabagenin acetate B. The analytical values of both acetates were the same within the limit of error. Further acetylation of acetate B failed to yield acetate A and, moreover, acetate B was stable toward oxidation by chromic acid. On the basis of this evidence, Meyrat and Reichstein ruled out their original surmise that acetate B was a triacetate and considered the possibilities that acetate B was a pentaacetate or was derived from an impurity present in ouabagenin or that ouabagenin formed two isomeric acetates.

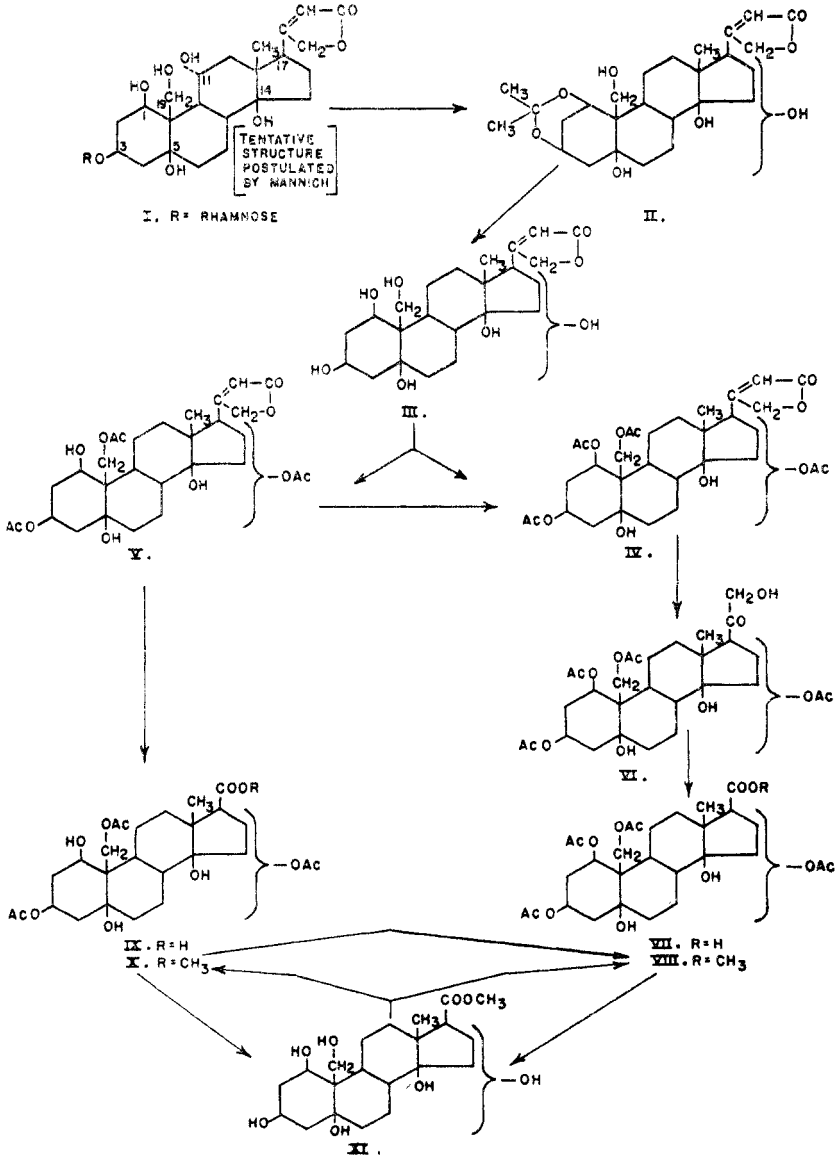


TABLE I

COMPARISON OF ACETATES, OUABAGENIN SERIES

	M.P., °C.	$[\alpha]_D$	ANALYSES	
			Acetyl	
			Calc'd	Found
Ouabagenin tetraacetate "A" (IV)	a. 291–294 b. 290–291	+7.1° (dioxane) +6.5° (chloroform)	— 28.38	— 28.31, 28.01
Ouabagenin triacetate "B" (V)	a. 253–257 b. 239–241	+10.5° (chloroform) +9.2° (chloroform)	— 22.87	— 20.85, 21.02
Methyl 1β,3β,(11?),19-tetraacetoxy-5,14-dihydroxy-14β-etianate (VIII)	a. 250–256 b. 252–256	+5.2° (chloroform) +6.2° (chloroform)	— 29.56	— 27.56
Methyl 3β,(11?),19-triacetoxy-1β,5,14-trihydroxy-14β-etianate (X)	a. 150–152 b. 155–158	+12.6° (acetone) +26.9° (chloroform)	— 23.94	— 24.78, 24.93

[a] Meyrat and Reichstein (15).
[b] This investigation.

The work reported in this paper[2] started with a reinvestigation of this problem. Ouabagenin (III), obtained by the procedure of Mannich and Siewert (4) from ouabain (I), was acetylated at room temperature overnight. In agreement with the findings of Meyrat and Reichstein (15) chromatography of the reaction mixture yielded both, acetates A (IV) and B (V). Since the conditions of acetylation were somewhat milder[3] than those employed by Reichstein, the ratio of yields of the two acetates was shifted in favor of acetate B.[4] Acetate B (V) could be converted to acetate A (IV) in 55 % yield in this laboratory by treating V with acetic anhydride in pyridine at room temperature for seven days and then heating the reaction mixture to 60° for six hours. Acetyl determinations of the acetates IV and V clearly demonstrated (see Table I) that, as Reichstein had originally suspected, ouabagenin acetate A is a tetraacetate and ouabagenin acetate B, a triacetate.

Reichstein and associates degraded ouabagenin tetraacetate (IV) to the corresponding tetraacetoxydihydroxy-14β-etianic acid (VII) both by a one step

[2] The formulas are presented on the basis of the conclusions reached in this investigation. One of the substituents in some compounds has been arbitrarily placed in the 11-position, *i.e.* (11?). It is to be understood, however, that this is only a convenient means of naming those compounds in which the positions of all other substituents are known. To emphasize this uncertainty, in the corresponding structural formulas the questionable position is not designated.

[3] Heating the reaction mixture to 60° was omitted.

[4] To exclude the possibility that the formation of V was due to partial deacetylation of IV by the action of sulfuric acid, routinely used in working up the reaction mixture, the use of sulfuric acid was avoided in one experiment. Again IV and V were obtained in the same ratio of yields.

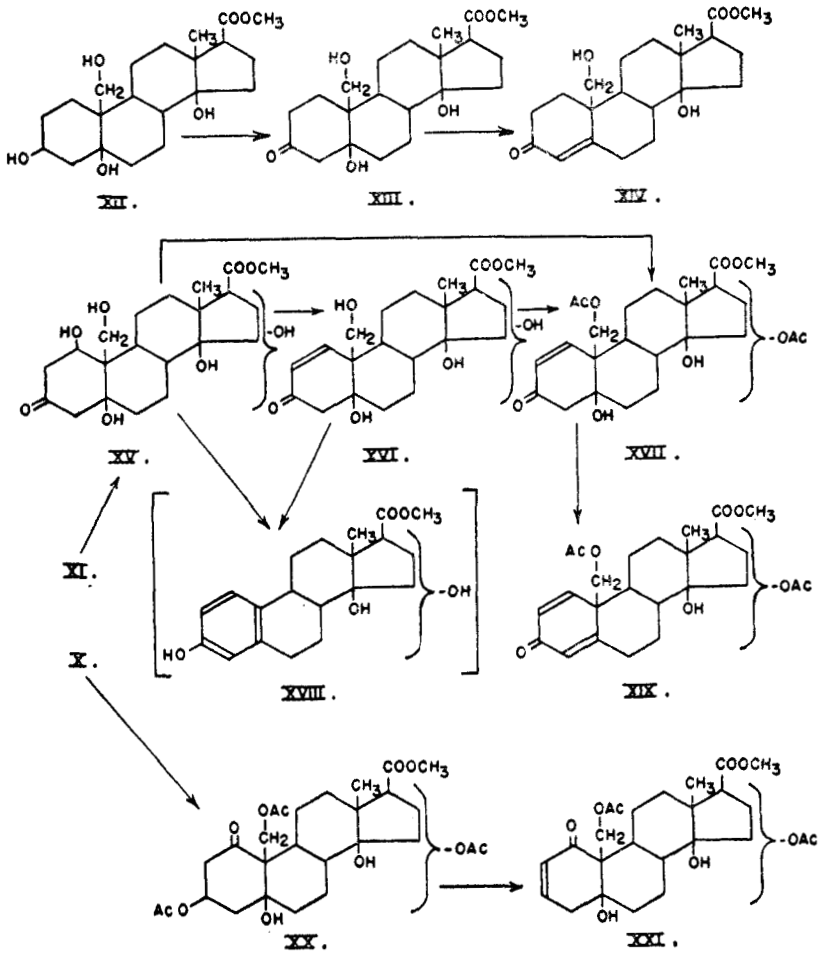
oxidation with potassium permanganate (15) and by treatment of IV with ozone followed by oxidation with periodic acid of the ketol VI (16). The acid (VII) was characterized as its methyl ester (VIII). By oxidizing ouabagenin triacetate (V) with potassium permanganate Meyrat and Reichstein (15) obtained an amorphous acid (IX) which was characterized as its crystalline methyl ester (X). By following Reichstein's procedures we obtained acid VII by ozone degradation of IV, with VI as an intermediate, and acid IX by oxidation of V with potassium permanganate. All attempts to crystallize the chromatographically uniform amorphous acid IX were unsuccessful. Treatment of the acids VII and IX with diazomethane furnished the corresponding methyl esters VIII and X. The physical constants of both VIII and X were in good agreement with the data given by Reichstein (15) (*cf.* Table I). Acetyl determinations again showed that VIII, derived from ouabagenin tetraacetate (IV), contained four acetyl groups and X, derived from ouabagenin triacetate (V), three acetyl groups. Reacetylation of X under the same conditions used for the reacetylation of V, gave VIII in 50% yield together with 25% of unchanged X. Saponification of VIII with sodium carbonate in methanol-water furnished the very water-soluble, crystalline, methyl 1β,3β,5,(11?),14,19-hexahydroxy-14β-etianate (XI) in 47% yield. A resinous by-product which yielded a crystalline acetylation product, awaits further investigation. As was expected, saponification of X under the same conditions used with VIII also yielded XI in 43% yield. Reacetylation of XI furnished a mixture of VIII (43%) and X (25%).

The conversion of the esters VIII and X, derived from ouabagenin tetraacetate (IV) and triacetate (V) respectively to the same product (XI) is additional proof that ouabagenin acetate B is a triacetate of ouabagenin and not the acetylation product of an impurity (*cf.* 15, p. 2105).

In a previous investigation from this laboratory (17) it was established that the primary hydroxyl group at carbon atom 19 is inert to oxidation with N-bromoacetamide. Thus ethyl 3β,5,19-trihydroxyetianate was oxidized with 2 equivalents of N-bromoacetamide to ethyl 3-oxo-5,19-dihydroxyetianate in good yield. The latter compound was dehydrated to ethyl 3-oxo-19-hydroxy-Δ^4-etienate by treatment with Girard's reagent T.

This reaction was studied with a model compound even more closely related to XI than ethyl 3β,5,19-trihydroxyetianate. Methyl 3β,5,14,19-tetrahydroxy-14β-etianate (XII) (18) was oxidized with two equivalents of N-bromoacetamide to methyl 3-oxo-5,14,19-trihydroxy-14β-etianate (XIII) which by treatment with Girard's reagent T was dehydrated to methyl 3-oxo-14,19-dihydroxy-Δ^4-14β-etienate (XIV). XIII was not completely stable, when chromatographed on alumina. A total of 23% of the invested material was dehydrated to XIV, the rest was recovered as unchanged XIII.

By applying this oxidation method to the ouabagenin series, methyl 1β,3β,5,(11?),14,19-hexahydroxy-14β-etianate (XI) was reacted with an excess (four equivalents) of N-bromoacetamide at room temperature for 16 hours. A crude crystalline reaction product [methyl 3-oxo-1β,5,(11?),14,19-pentahydroxy-14β-etianate (XV)] was isolated which exhibited no specific absorption between 226



and 300 mμ.[5] When chromatographic purification on neutral alumina was attempted, XV was converted to a crystalline dehydration product (λ_{max}^{alc} 234 mμ; ε 10,000), to which the structure of methyl 3-oxo-5,(11?),14,19-tetrahydroxy-Δ^1-14β-etienate (XVI) has been assigned. By treatment of XVI with acetic anhydride in pyridine at room temperature overnight and heating to 60° for 3 hours, a chromatographically uniform crystalline acetylation product (λ_{max}^{alc} 231 mμ; ε 11,500) was obtained which must possess the structure of methyl 3-oxo-(11?),19-diacetoxy-5,14-dihydroxy-Δ^1-14β-etienate (XVII), since the presence of two acetyl groups was demonstrated. XVII was also obtained from XV by simultaneous acetylation and dehydration.

When the diacetate XVII was refluxed with glacial acetic acid for one hour,

[5] By treatment of this material with Girard's reagent T a small amount (10%) of resinous non-ketonic material was obtained. Only insignificant amounts of ketonic material could be isolated from the acidified aqueous reaction mixture by repeated extractions over a period of ten days.

further dehydration took place yielding an amorphous chromatographically uniform product (XIX) (λ_{max}^{alc} 240 mμ; ϵ 12,500). The shift of the absorption maximum from 231 mμ for XVII to 240 mμ for XIX indicates loss of the tertiary hydroxyl group at carbon atom 5. It is in good agreement with the shifts of ultraviolet maxima of Δ^1-androsten-17β-ol-3-one, λ_{max}^{alc} 230 mμ (19) to $\Delta^{1,4}$-androstadien-17β-ol-3-one, λ_{max}^{alc} 244 mμ (20) and of Δ^1-cholesten-3-one, λ_{max}^{alc} 230 mμ (21) to $\Delta^{1,4}$-cholestadien-3-one, λ_{max}^{ether} 236 mμ (*cf.* 8, p. 193). Therefore the structure of methyl 3-oxo-(11?),19-diacetoxy-14-hydroxy-$\Delta^{1,4}$-14β-etiadienate was assigned to XIX.

The infrared spectrum of XIX was measured in chloroform solution.[6] There were absorption bands at 1667, 1627, and 1608 cm^{-1} characteristic of the presence of a $\Delta^{1,4}$-diene-3-ketone system (22, 23). These bands exhibited only minor shifts from those expected (C=O stretching band—1666 to 1660 cm^{-1}, C=O stretching bands—1621 and 1606–1603 cm^{-1}). The correlation of the other bands with the structure assigned on chemical evidence is under investigation at the Sloan-Kettering Institute and will be reported in detail by this group.

In an exploratory experiment XVI was refluxed with acetic acid for one hour. An amorphous product (XVIII) was obtained with an ultraviolet absorption spectrum similar to that of estradiol (*vide* Figure 1). Evidently aromatization with loss of the angular hydroxymethyl group at carbon atom 10 had taken place, since in contrast to the dehydration experiment with XVII, XVI was not protected by acetylation. A similar, though less characteristic ultraviolet spectrum was obtained by refluxing of XV with acetic acid. With the aromatization of ring A proven in the present instance, it appears very probable that the aromatization of ring A is also involved in the old experiments of Jacobs and Bigelow (6, 10), discussed earlier in this paper.

In the model experiment chromatography of XIII on alumina caused a small amount of dehydration to XIV, *i.e.* elimination of the hydroxyl group at carbon atom 5. Therefore, the possibility might be considered that by chromatography of XV on alumina the hydroxyl group at carbon atom 5 was eliminated rather than the one at carbon atom 1. A Δ^4-instead of the postulated Δ^1-compound (XVI) should have the absorption maximum at approximately 240 mμ rather than at the observed 234 mμ. Furthermore, acetylation of a Δ^4-compound under the conditions that led to a mixture of tri- and tetra-acetates in the case of ouabagenin (III) and methyl 1β,3β,5,(11?),14,19-hexahydroxy-14β-etianate (XI) respectively, should have given a mixture of a di- and a tri-acetate, yet the only acetylation product obtained from XVI was a diacetate (XVII).

Therefore, the N-bromoacetamide oxidation of XI to the monoketone XV, the subsequent dehydration of XV to the α,β-unsaturated ketone XVI, the further dehydration of the diacetate XVII to the dienone XIX, and the ready

[6] A Perkin-Elmer Model 21 double beam spectrometer was used; we wish to express our appreciation to Friederike Herling and Dr. Thomas F. Gallagher of the Division of Steroid Metabolism of the Sloan-Kettering Institute for Cancer Research for the determination and interpretation of the infrared spectra.

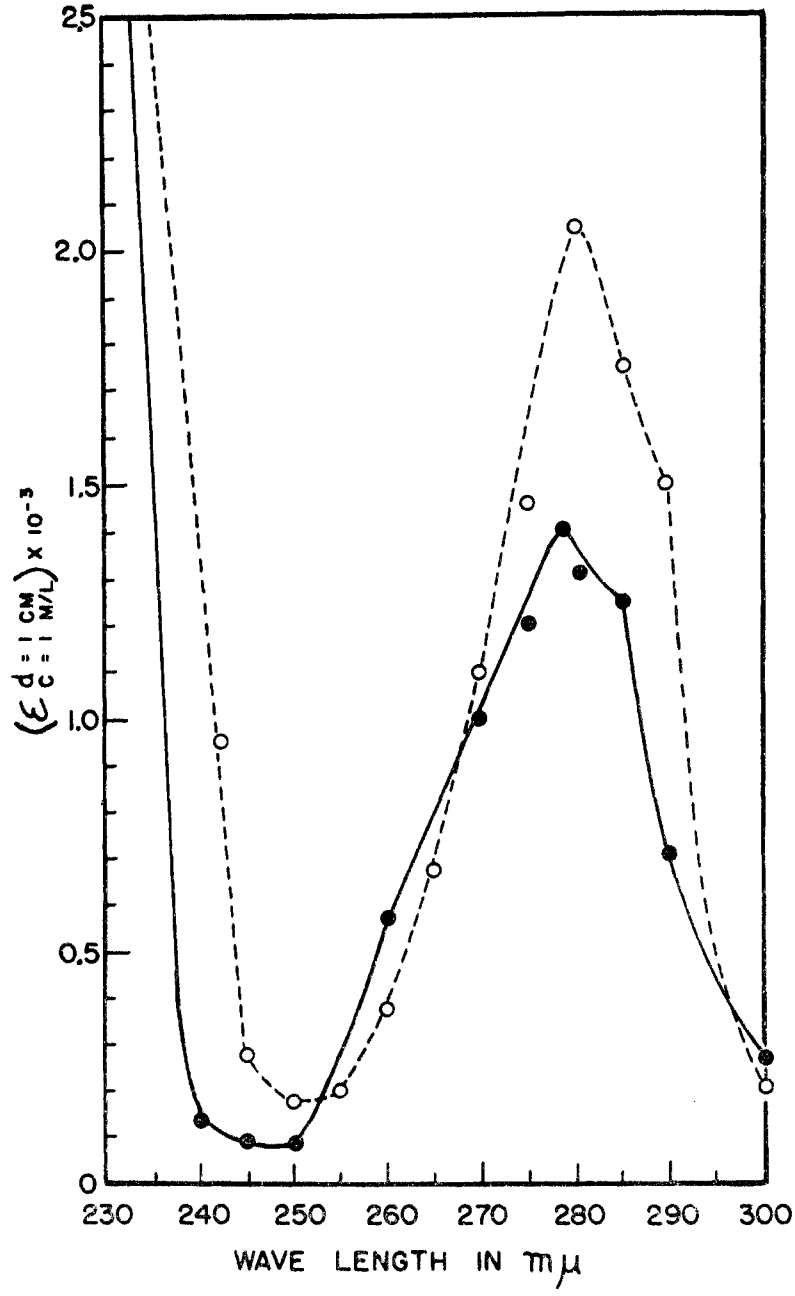


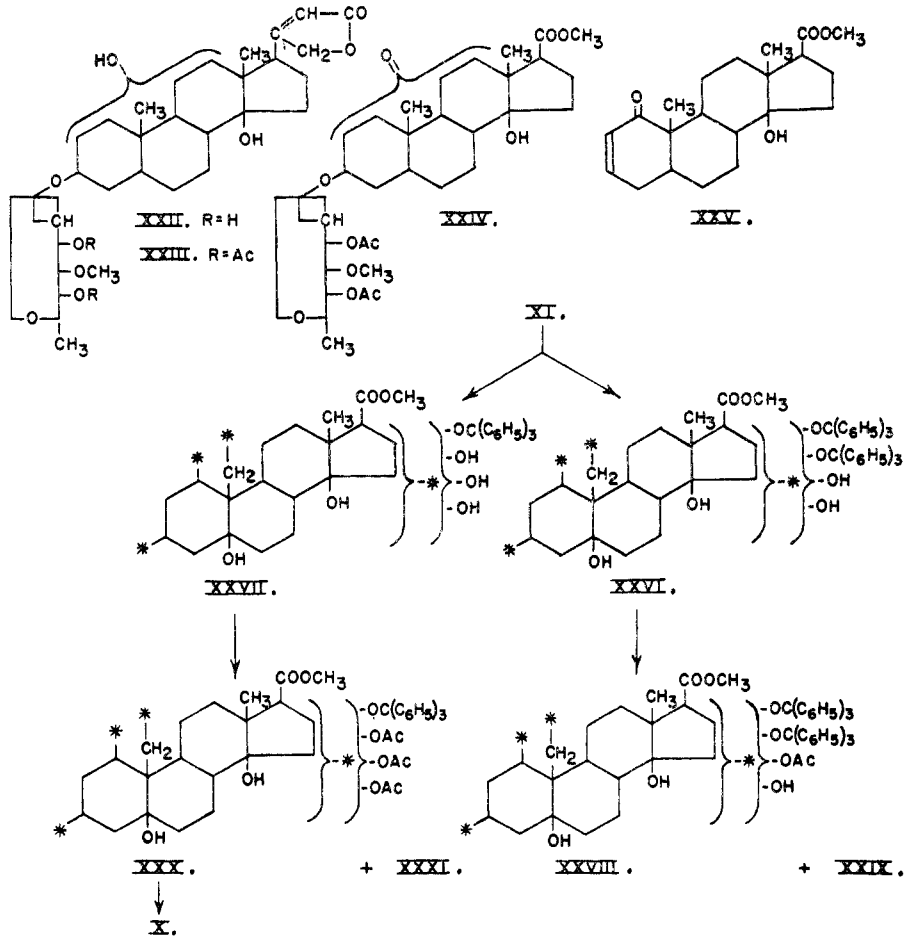
FIGURE 1. BROKEN CURVE, ESTRADIOL, U.S.P. Reference Standard, m.p. 174.5–176°. λ_{max}^{alc} 282 mμ (ε 2067); λ_{min}^{alc} 250 mμ (ε 171). SOLID CURVE, XVIII. λ_{max}^{alc} 278 mμ (ε 1400); λ_{min}^{alc} 245–250 mμ (ε 70).

aromatization of XVI constitute proof for the presence of hydroxyl groups at carbon atoms 1,3,5, and 19 of ouabagenin.

As has been stated, both Mannich and Siewert (4) and Sneeden and Turner (13) postulated a 1,3-bridge for ouabagenin acetonide (II). Acetylation of II furnishes ouabagenin monoacetonide diacetate (4, 13, 16). By treatment of this substance with dilute aqueous alcoholic sulfuric acid, Mannich and Siewert (4) obtained a ouabagenin diacetate, m.p. 240°, and Raffauf and Reichstein (16) obtained two isomeric substances, designated α-ouabagenin diacetate, m.p. 262–265°, and β-ouabagenin diacetate, m.p. 193–196°. By using aqueous acetic acid as hydrolytic agent, Sneeden and Turner (13) obtained β-ouabagenin diacetate, m.p. 193–196°, as the only product. These investigators attribute the formation of the α-isomer to acyl migration promoted by the strongly acidic medium employed by the former workers (4, 16). By further acetylation of β-ouabagenin diacetate, Raffauf and Reichstein (16) obtained a mixture of ouabagenin triacetate (V) and tetraacetate (IV). Therefore in V and consequently also in X the hydroxyl group at carbon atom 1 or at carbon atom 3 should have remained unacetylated. It was hoped that an oxidation of X would shed some light on this question.

X reacted rather slowly when treated with chromium trioxide (2.2 equivalents). There remained a large excess of the oxidizing agent after two days standing at room temperature. Only a trace of acidic material resulted from the reaction mixture. Since under identical conditions ethyl 3β-acetoxy-5,19-dihydroxyetianate was oxidized to the corresponding 10-carboxylic acid (17), the presence of a free hydroxyl group at carbon atom 19 in X can be ruled out. The crude neutral fraction of the oxidation product was amorphous. Chromatography on alumina yielded two crystalline substances (XX and XXI). The analytical data for the major product agreed with the structure of methyl 1-oxo-3β,(11?),19-triacetoxy-5,14-dihydroxy-14β-etianate (XX). Small amounts of the other compound (XXI) which had an ultraviolet absorption maximum at 222 mμ (ϵ 12,500) were isolated. Possibly under the influence of the alumina an acetyl group (at C_3) of XX was eliminated with formation of an α,β-unsaturated ketone. Both the ultraviolet absorption maximum at 222 mμ and the microanalytical data are compatible with the structural assignment of methyl 1-oxo-(11?),19-diacetoxy-5,14-dihydroxy-Δ^2-14β-etienate for XXI. The formulation of XXI as a 3-keto-Δ^1 compound can be ruled out because it is not identical with XVII.

It is interesting to compare these findings with investigations on the structure of acovenoside A (XXII), the main glycoside of *Acokanthera venenata* (24, 25) which has also been found in *Acokanthera longiflora* (26). When acovenoside A (XXII) was acetylated (excess of acetic anhydride in pyridine; 20 hours at 34°) a diacetate (XXIII) resulted which possessed a free secondary hydroxyl group in the genin moiety. Subsequent treatment with chromic acid gave the corresponding keto compound. Degradation of the latter (O_3, Zn; $KHCO_3$; HIO_4; CH_2N_2) gave the methyl ester of the etio acid (XXIV), accompanied by a small amount of a compound to which the structure of a 1-keto-Δ^2 ester (XXV) was



CARBON ATOMS BELIEVED TO CARRY SUBSTITUENTS ARE MARKED BY ASTERISKS.

tentatively assigned (25). This compound had an ultraviolet absorption maximum at 225 mμ, comparable to the low maximum of XXI.

Although the chromic acid oxidation products of X need further study, the evidence obtained is in support of a keto group in position 1 in the oxidation products XX and XXI and consequently of a hydroxyl group in the same positions in the triacetates V and X.

As is known from sugar chemistry, primary alcohol groups react more readily with triphenylchloromethane than do secondary ones, to form ethers which are stable towards alkali but are easily hydrolyzed by acids. As was shown in this laboratory (27), treatment of ethyl 3β,5,19-trihydroxyetianate, a degradation product of strophanthidol, with a stoichiometric amount (1.1 equivalents) of triphenylchloromethane yields as the main reaction product 19-tritoxy-3β,5-dihydroxyetianate, accompanied by a small amount of ethyl 3β,19-ditritoxy-5-hydroxyetianate. By acetylation and subsequent detritylation of the main

product, ethyl 3β-acetoxy-5,19-dihydroxyetianate was obtained. In this compound the hydroxyl group at C_3 was protected by acetylation and thus the primary alcohol group at C_{19} could be selectively oxidized to a carboxyl group with chromic acid. An attempt was made to apply this method of selective acetylation and oxidation to the ouabagenin series.

Methyl 1β,3β,5,(11?),14,19-hexahydroxy-14β-etianate (XI) by treatment with 1.1 equivalents of triphenylchloromethane furnished after chromatography a crystalline methyl ditritoxytetrahydroxy-14β-etianate (XXVI) in 6% yield and an amorphous methyl monotritoxypentahydroxy-14β-etianate (XXVII) in 43% yield. By using two or three equivalents of triphenylchloromethane the yield of the ditritoxy compound (XXVI) was raised to 40%. Apparently the amorphous monotritoxy compound (XXVII) is somewhat resistant to further tritylation, but this needs further study.

Acetylation of the ditrityl ether (XXVI) gave after chromatography an amorphous product, possibly methyl ditritoxydiacetoxydihydroxy-14β-etianate (XXIX) and the slightly more polar crystalline methyl ditritoxymonoacetoxytrihydroxy-14β-etianate (XXVIII).

The amorphous monotrityl ether (XXVII) resisted all attempts at crystallization. The ether is unstable to chromatography on silica gel which effected cleavage to triphenylcarbinol and XI. Rechromatography of XXVII on alumina gave a single chromatographic peak. In spite of the apparent chromatographic uniformity the possibility remains that this amorphous product is a mixture of different monotrityl ethers of XI. The first, middle, and last fractions of the chromatographic peak of XXVII were acetylated separately. In all three cases chromatography yielded a higher-melting fraction, m.p. 207–237°, and a slightly more polar lower-melting fraction, m.p. 115–190°. By rechromatography of the pooled higher-melting fractions a crystalline methyl monotritoxytriacetoxydihydroxy-14β-etianate (XXX), m.p. 269–270°, was obtained. Repeated rechromatography of the pooled lower-melting fractions yielded more rather crude XXX and a crystalline product XXXI, melting with effervescence between 140 and 145°, which could not be purified to a well defined entity.

The crystalline monotritoxytriacetoxy compound XXX was rather resistant to cleavage by acid. Whereas in the case of ethyl 19-tritoxy-3β-acetoxy-5-hydroxyetianate treatment with 95% acetic acid at room temperature accomplished quantitative cleavage of the ether linkage within 20 hours (27, p. 289), XXX either had to be kept at room temperature for seven days or had to be refluxed for 1½ hours to achieve complete cleavage. Isolated as hydrolysis products were triphenylcarbinol and a crystalline substance, m.p. 150–152°, which proved to be identical with methyl 3β,(11?),19-triacetoxy-1β,5,14-trihydroxy-14β-etianate (X) as to melting point, rotation, microanalysis, and infrared spectrum. When X was reacted with triphenylchloromethane in an attempt to reintroduce the trityl group into the molecule, only triphenylcarbinol and unchanged X could be isolated from the reaction mixture.

The infrared spectra of three samples of X, obtained by (a) degradation of V, (b) acetylation of XI, and (c) detritylation of XXX, were determined in chloro-

form solution.[6] In the region from 1150 to 800 cm^{-1}, all three products were essentially identical, both as to position and intensity of the principal bands. There were minor differences in the absorption spectrum from 1040 to 1020 cm^{-1} which were ascribed to impurities or other minor differences in the samples.

The identity of the product of detritylation of XXX with X is rather unexpected. Two different interpretations may be advanced: (a) Contrary to expectations the secondary hydroxyl group in the 1β-position was preferentially tritylated. Subsequent acetylation gave a 1β-tritoxy-3β,(11?),19-triacetoxy compound which on detritylation furnished X in a normal fashion. (b) The primary hydroxyl group in the 19-position was preferentially tritylated. Subsequent acetylation gave a 19-tritoxy-1β,3β,(11?)-triacetoxy compound. The detritylation of the latter was accompanied by the migration of an acetyl group from the difficulty acetylable 1β-position to the more easily acetylable 19-position, thus leading to X. Experiments will be conducted to differentiate between these two possibilities.

The spectra of compounds IV, V, VIII, XV, XVI, XVII, XX, and XXI, in general terms, were consistent with the structures assigned on chemical evidence. Further studies on these and related compounds with infrared spectrometry are in progress at the Sloan-Kettering Institute and will be reported in detail elsewhere by these workers.

EXPERIMENTAL

Melting points. Unless stated otherwise, the m.p.'s were determined with the Fisher-Johns melting point apparatus. The readings are sufficiently near the true melting points so that no corrections have been made.

Absorption spectra. Ultraviolet spectra were determined in absolute ethanol with a Beckman Model DU spectrophotometer.

Chromatography. The alumina (Alumina Adsorption, 80–200 Mesh, Fisher Scientific Company) was washed with dilute acetic acid, methanol, and water, dried at 180° for 48 hours, and standardized according to Brockmann (28). The silica gel (100–200 Mesh, The Davison Chemical Corporation, Baltimore, Md.) was washed with water and methanol and dried at 180° for 48 hours. The solvents used were reagent grade and freshly distilled.

Analyses. Unless stated otherwise, the microanalyses were performed by Dr. E. W. D. Huffman, Wheatridge, Colorado, on samples which were dried to constant weight *in vacuo* over phosphorus pentoxide at 80–90° according to Milner and Sherman (29). The percentage loss of weight on drying and gain of weight on exposure of the sample to the atmosphere are recorded.

Optical rotations. No corrections for crystal solvent have been made. Unless stated otherwise, the sample was dissolved in chloroform to make a 2-cc. solution and the rotation was determined in a 2-dm. semi-micro tube.

Ouabagenin (III). Preparation essentially by the method of Mannich and Siewert (4). To 10.0 g. of ouabain U.S.P.-Penick in 500 cc. of acetone was added 5 cc. of conc'd hydrochloric acid and the solution was kept at room temperature. The crystals of ouabagenin acetonide (II), total 4.8 g., m.p. range 235–260°, were collected over a period of two weeks and were then refluxed in ethanol containing a trace of hydrochloric acid. Concentration of the solution *in vacuo* gave 4.0 g. of crude crystalline ouabagenin (III), m.p. range 178–190°. A sample of the crude III was chromatographed on alumina (activity V). With ethyl acetate-methanol uniform crystalline material was eluted; recrystallization from methanol-ether; m.p. 237–246°.

Ouabagenin tetraacetate "A" (IV) *and ouabagenin triacetate "B"* (V). A solution of 7.4 g. of crude ouabagenin (III), m.p. 178–180°, in 50 cc. of pyridine and 25 cc. of acetic anhydride was kept at room temperature (28°) for 17 hours and then was poured into 700 cc. of 3% sulfuric acid. After refrigerating for several hours the white precipitate was extracted with six 200-cc. portions of chloroform. After washing the extract with *N* sodium bicarbonate and water, drying over sodium sulfate, and evaporating to dryness, 9.21 g. of resin resulted which was chromatographed on 130 g. of alumina (activity III). Two major fractions were obtained:

A. With benzene-chloroform (1:1) 2.546 g. of crystalline material was eluted which from acetone gave 2.292 g. of crystalline IV, m.p. 288–291°. Recrystallization from methanol raised the m.p. to 290–291°; $[\alpha]_D^{26}$ +6.5° (14.72 mg.; α +0.10°). Meyrat and Reichstein (15): m.p. 291–294°; $[\alpha]_D^{18}$ +7.1° (dioxane).

Anal. Calc'd for $C_{31}H_{42}O_{12}$ (606.64): C, 61.37; H, 6.98; Acetyl, 28.38.

Found: C, 61.50, 61.63; H, 7.07, 7.17; Acetyl, 28.31, 28.03. No weight loss.

B. With benzene-chloroform (1:3), chloroform, and chloroform-methanol (49:1) 5.435 g. of crystalline material was eluted which from acetone yielded 4.173 g. of V, m.p. 238–240°. Rechromatography of the crystalline material on 90 g. of alumina gave only one, uniform, series of fractions, eluted with chloroform and chloroform-methanol (49:1); total: 4.140 g. Recrystallization of the individual fractions from acetone gave a total of 3.775 g. of crystals, m.p. range 235–237°. By repeated recrystallization from methanol the m.p. was raised to 239–241°; $[\alpha]_D^{26}$ +9.2° (12.45 mg.; α +0.12°). Meyrat and Reichstein (15): m.p. 253–257°; $[\alpha]_D^{18}$ +10.5° (chloroform).

Anal. Calc'd for $C_{29}H_{40}O_{11}$ (564.61): C, 61.69; H, 7.14; Acetyl, 22.87.

Found: C, 61.83, 61.65; H, 7.32, 7.20; Acetyl, 20.85, 21.02. No weight loss.

To exclude the possibility that the formation of the triacetate was due to partial deacetylation of the tetraacetate by the action of the sulfuric acid used in working up the preceding experiment, 83 mg. of pure III, m.p. 237–245°, was kept in a solution of 2 cc. of pyridine and 1 cc. of acetic anhydride at room temperature overnight. The reaction mixture was concentrated to near dryness *in vacuo* and the solution of the residue in 100 cc. of ethyl acetate was washed quickly with *N* hydrochloric acid, *N* sodium bicarbonate, and water. After drying over sodium sulfate and evaporating, 73.3 mg. of resin was obtained. Chromatography on alumina and recrystallization of the two major fractions eluted gave 15.8 mg. of ouabagenin tetraacetate (IV), m.p. 290–291°, and 18.4 mg. of ouabagenin triacetate (V), m.p. 238–240°.

Ouabagenin tetraacetate "A" (IV) *by reacetylation of ouabagenin triacetate "B"* (V). A solution of 137 mg. of pure V, m.p. 239–241°, in 2 cc. of pyridine and 1 cc. of acetic anhydride was kept at room temperature (27–30°) for 7 days and was then heated to 60° for 6 hours. It was poured into 150 cc. of 3% sulfuric acid and the mixture was worked up as described above. Evaporation of the solvent gave 145 mg. of resin which was chromatographed on 10 g. of alumina (activity III). With benzene-chloroform (1:1) 102 mg. of crystals, m.p. 289–291°, (69% yield) was eluted. The mixture m.p. with ouabagenin tetraacetate (IV) gave no depression. With chloroform-methanol (49:1) 9.5 mg. of starting material (V), m.p. 244–246°, (7% yield) was recovered.

1β,3β,(11?),19-Tetraacetoxy-5,14-dihydroxy-14β-etianic acid (VII). VII was prepared from ouabagenin tetraacetate (IV) on the basis of the procedure of Raffauf and Reichstein (16). A solution of 800 mg. of IV, m.p. 288–294°, in 100 cc. of ethyl acetate was treated with ozone for one hour at −80° (violet color). After removal of the solvent *in vacuo* (0–10°), the residue was taken up in 40 cc. of glacial acetic acid and was reduced with zinc dust. The reaction mixture was filtered, diluted with water, and extracted with chloroform. The extract was washed with *N* sodium carbonate and water, dried over sodium sulfate, and brought to dryness *in vacuo*. The resinous residue, dissolved in 40 cc. of methanol, was treated with 1.2 g. of potassium bicarbonate in 20 cc. of water at room temperature overnight. The methanol was evaporated *in vacuo* and the aqueous solution was extracted with chloroform. After washing the solvent with water, drying over sodium sulfate, and con-

centrating to dryness *in vacuo*, the resinous residue (595 mg.) gave from ethyl acetate-ether 264 mg. of crystalline 1β,3β,(11?),19-tetraacetoxy-14β-pregnane-5,14,21-triol-20-one (VI), m.p. 202–206°. [Raffauf and Reichstein (16), anal. sample, m.p. 215–220°.] The yield of crude crystalline VI in several experiments ranged from 60–80%.

To 250 mg. of VI, m.p. 202–206°, in 6 cc. of methanol was added 96 mg. of periodic acid in 3 cc. of water. The solution was kept at room temperature overnight. After evaporating the methanol *in vacuo* at room temperature, 208 mg. of crystalline VII, m.p. above 280°, (87% yield from VI) was collected. By recrystallization from acetone the m.p. was raised to 297–298° (uncorr.).

Titration: 4.3 mg. of VII in 2 cc. of methanol and 5 cc. of water; 0.01 *N* NaOH: calc'd 0.75 cc.; found 0.79 cc.

Methyl 1β,3β,(11?),19-tetraacetoxy-5,14-dihydroxy-14β-etianate (VIII). To 135 mg. of crude VII, m.p. above 280°, in 15 cc. of methanol was added a slight excess of ethereal diazomethane. After keeping the solution at room temperature for ½ hour, it was worked up as usual, yielding a crystalline neutral fraction. Recrystallization from methanol gave 85 mg. of VIII, m.p. 252–256°; $[\alpha]_D^{26}$ +6.2° (6.76 mg.; α +0.04°). Meyrat and Reichstein (15): m.p. 250–256°; $[\alpha]_D^{19}$ +5.2° (chloroform).

Anal. Calc'd for $C_{29}H_{42}O_{12}$ (582.63): C, 59.78; H, 7.27; Acetyl, 29.56.

Found: C, 59.95; H, 7.29; Acetyl, 27.56; Residue, 0.13. No weight loss.

3β,(11?),19-Triacetoxy-1β,5,14-trihydroxy-14β-etianic acid (IX). IX was prepared from ouabagenin triacetate (V) on the basis of the procedure of Meyrat and Reichstein (15). To 2.768 g. of V, m.p. 239–241°, in 300 cc. of acetone (redist. over $KMnO_4$) was added 2.8 g. of powderized potassium permanganate and the mixture was stirred at room temperature for six hours. The acetone was evaporated *in vacuo* and the residue was slurried with 200 cc. of 0.5 *N* sulfuric acid. The slurry was extracted with 5 portions of 100 cc. of chloroform-ethanol (4:1). The extract was washed in the cold repeatedly with 0.5 *N* sodium carbonate. Evaporation of the chloroform yielded 771 mg. of resinous neutral material. The combined sodium carbonate extracts were immediately acidified with 10% sulfuric acid and repeatedly were extracted with chloroform. The extract was washed with water, dried over sodium sulfate, and then was evaporated to dryness, leaving 1.083 g. of resinous IX (40% yield). A 202 mg. portion of the crude resinous acid was chromatographed on 20 g. of silica gel. With chloroform-ethyl acetate (3:7) several consecutive fractions were eluted, totalling 110.9 mg. All attempts to crystallize the individual resinous fractions failed.

Methyl 3β,(11?),19-triacetoxy-1β,5,14-trihydroxy-14β-etianate (X). To 330 mg. of the crude resinous IX in 10 cc. of methanol and 20 cc. of ether was added a slight excess of ethereal diazomethane. After standing at room temperature for ½ hour, the solution was worked up as customary. The neutral fraction yielded 264 mg. of resin which from ethyl acetate gave 221.0 mg. of crystalline X, m.p. 152–154° (softening of the crystals at 130–140°). A portion of the crystalline product (162 mg.) was chromatographed on 4 g. of alumina (activity III). With ether-ethyl acetate (9:1) 131.6 mg. of crystalline material was eluted which upon recrystallization of the individual fractions from ethyl acetate gave a total of 111.6 mg. of X, m.p. 155–158° (crushed crystals melt at 130–140°). The m.p. was not raised further by recrystallization. $[\alpha]_D^{24}$ +26.9° (15.53 mg.; α +0.42°). Meyrat and Reichstein (15): m.p. 150–152°; $[\alpha]_D^{17}$ +12.6° (acetone). For infrared data *cf.* theoretical part.

Anal. Calc'd for $C_{27}H_{40}O_{11}$ (540.59): C, 59.98; H, 7.46; Acetyl, 23.94.

Found: C, 59.77, 59.61, 59.58; H, 7.65, 7.63, 7.61; Acetyl, 24.78, 24.93.

Weight loss, 2.38, 2.04, 1.95; Weight gain, 1.90, 0.72, 0.86.

A 23.8 mg. sample of IX which had been purified by chromatography (*vide supra*), was treated with diazomethane and worked up as above. Evaporation of the solvent yielded 23.7 mg. of resin which crystallized from ethyl acetate, m.p. 150–152°. Recrystallization gave 16.2 mg. of X, m.p. 151–153° (crushed crystals melt at 130°).

Methyl 1β,3β,(11?),19-tetraacetoxy-5,14-dihydroxy-14β-etianate (VIII) *by reacetylation of methyl 3β,(11?),19-triacetoxy-1β,5,14-trihydroxy-14β-etianate* (X). A solution of 28.0 mg. of X, m.p. 147–149°, in 1 cc. of pyridine and 0.5 cc. of acetic anhydride was kept at room

temperature for seven days and then was heated to 65–70° for six hours. After evaporating to dryness *in vacuo*, the residue was taken up in ethyl acetate and the solution was washed with *N* hydrochloric acid, *N* sodium bicarbonate, and water, and dried over sodium sulfate. Evaporation *in vacuo* gave 29.5 mg. of resin which was chromatographed on 3.3 g. of alumina (activity III). With ether-ethyl acetate (19:1) 15.6 mg. of crystalline material (50% yield), m.p. 251–255° was eluted. Recrystallization from ethyl acetate-petroleum ether gave 10.5 mg. of VIII, m.p. 255–256°. The mixture m.p. with a sample of VIII, as obtained by degradation of ouabagenin tetraacetate (IV) gave no depression. With ether-ethyl acetate (3:2) 6.9 mg. of crystalline material (25% yield), m.p. 147–148°, was eluted which from ethyl acetate gave 3.9 mg. of unchanged starting material (X), m.p. 149–150°, identified by mixture m.p.

Methyl 1β,3β,5,(11?),14,19-hexahydroxy-14β-etianate (XI). *A. By saponification of methyl 1β,3β,(11?),19-tetraacetoxy-5,14-dihydroxy-14β-etianate* (VIII). To 10 g. of VIII, m.p. 252–255°, as obtained by degradation of IV, in 200 cc. of methanol was added 300 cc. of 0.5 *N* sodium carbonate. After keeping the mixture at room temperature overnight, the methanol was evaporated *in vacuo* and the aqueous solution was extracted with 8 portions of 500 cc. of chloroform-ethanol (3:1). The combined extracts were washed with a conc'd sodium chloride solution, dried over sodium sulfate, and evaporated to dryness. The resinous residue (3.75 g.) from methanol gave three crops of crystalline XI, m.p. 236–238°, totalling 3.30 g. (47% yield). By repeated recrystallization the m.p. was raised to 238–242°. $[\alpha]_D^{24}$ −5.1° (ethanol; 13.15 mg.; α −0.07°).

Anal. Calc'd for $C_{21}H_{34}O_8$ (414.48): C, 60.85; H, 8.27.

Found: C, 60.71; H, 8.81 (Dried without special precautions)

The residual alkaline aqueous phase (*vide supra*) was stirred with ion exchange resin IR-120 (Rohm & Haas, activated with *N* hydrochloric acid and washed acid-free) to remove sodium ions. The now weakly acid solution (acetic acid) was brought to dryness *in vacuo*, leaving 1.6 g. of very water-soluble resin which could not be made to crystallize. It was acetylated with acetic anhydride in pyridine at room temperature for 4 days and after chromatography (alumina; activity III) and recrystallization gave 411 mg. of a crystalline product, m.p. 295–297° (uncorr.). The mixture m.p. with VII, m.p. 297–298° (uncorr.), showed a depression (m.p. 275–278°). $[\alpha]_D^{24}$ −37.4° (15.93 mg.; α −0.60°).

Anal. Calc'd for $C_{28}H_{38}O_{11}$ (550.58): C, 61.08; H, 6.96.

Found: C, 60.86, 60.97; H, 6.87, 6.79. No weight loss.

B. By saponification of methyl 3β,(11?),19-triacetoxy-1β,5,14-trihydroxy-14β-etianate (X). To 1.584 g. of X, m.p. 139–143°, as obtained by degradation of V, in 150 cc. of methanol was added 150 cc. of 0.5 *N* sodium carbonate. The solution was kept at room temperature overnight and worked up as described under *A*. The chloroform-ethanol extract gave 517 mg. of crystalline XI, m.p. 232–236° (43% yield). By repeated recrystallization from methanol-ethyl acetate, the m.p. was raised to 242–243°. The mixture m.p. with a sample of XI, as obtained by saponification of VIII (*vide* under *A*) gave no depression. $[\alpha]_D^{28}$ −5.8° (ethanol; 18.20 mg.; α −0.11°).

Methyl 1β,3β,(11?),19-tetraacetoxy-5,14-dihydroxy-14β-etianate (VIII) *and methyl 3β,(11?),19-triacetoxy-1β,5,14-trihydroxy-14β-etianate* (X) *from methyl 1β,3β,5,(11?),14,19-hexahydroxyetianate* (XI). A solution of 30.2 mg. of XI, m.p. 242–243°, in 1 cc. of pyridine and 0.5 cc. of acetic anhydride was kept at room temperature for 6 days and then was poured into 30 cc. of 3% sulfuric acid. After extracting with ethyl acetate, the extract was washed with *N* sodium bicarbonate and water and dried over sodium sulfate. Evaporation of the solvent yielded 43.7 mg. of resin which was chromatographed on 4 g. of alumina (activity III). Two major fractions were eluted: (a) with ether-ethyl acetate (9:1) 17.7 mg. of VIII, m.p. 255–256° (43% yield), identified by mixture m.p.; (b) with ether-ethyl acetate (1:1) 9.7 mg. of X, m.p. 148–150° (25% yield), identified by mixture m.p.; for infrared data *cf.* theoretical part.

Methyl 3-oxo-5,14,19-trihydroxy-14β-etianate (XIII) *and methyl 3-oxo-14,19-dihydroxy-Δ^4-14β-etienate* (XIV) *from methyl 3β,5,14,19-tetrahydroxy-14β-etianate* (XII). To 75 mg.

of XII (18), m.p. 160–162°, in 5 cc. of *tert*-butanol and 2 cc. of water was added 59 mg. (2 equivalents) of *N*-bromoacetamide (m.p. 103–105°; 96% by thiosulfate titration). The solution was kept at room temperature (30°) for 16 hours and was then poured into 30 cc. of water, containing 200 mg. of sodium sulfite. The aqueous mixture was extracted 6 times with ether and 4 times with ethyl acetate. The extracts were washed with water, dried over sodium sulfate, and evaporated to dryness. The ether extract yielded 34.7 mg. of resin, the ethyl acetate extract 35.7 mg. of resin. To the pooled crude XIII (70.4 mg.) in 3 cc. of absolute ethanol was added 150 mg. of Girard's reagent T and 0.1 cc. of acetic acid. The solution was refluxed for one hour, then cooled to 0° and poured into 6 cc. of ice-water containing 65 mg. of sodium carbonate. The solution was extracted with 4 portions of ice-cold ether. The ether extract, after the usual manipulations, gave 6.0 mg. of yellowish resin (non-ketonic fraction). The residual aqueous solution was acidified to Congo Red with *N* hydrochloric acid and, after standing at room temperature for one hour, was extracted with 6 portions of 50 cc. of ether and 6 portions of ethyl acetate. Both extracts were washed with *N* sodium bicarbonate and water, dried over sodium sulfate, and evaporated to dryness. Yields: 40.3 mg. and 5.7 mg. of resin respectively. The material resulting from the ether extract (40.3 mg.) gave from ethyl acetate-ether 30 mg. of crystalline XIV, m.p. 173–175°. The product was chromatographically uniform and by recrystallization from ethyl acetate the m.p. was raised to 174–176°. $\lambda_{max.}^{alc.}$ 242 mμ; ε 13,800. $[\alpha]_D^{28}$ +109.3° (9.85 mg.; α +1.08°).

Anal. Calc'd for $C_{21}H_{30}O_5$ (363.42): C, 69.58; H, 8.34.

Found: C, 69.50; H, 8.18; Residue, 0.27. No weight loss.

In another experiment 35 mg. of XII, m.p. 165–168°, treated with 27 mg. of N-bromoacetamide at room temperature overnight and worked up as described above, but omitting the Girard separation, yielded 34 mg. of crude resinous XIII. This material, which showed no ultraviolet absorption maximum between 226 and 300 mμ, was chromatographed on 3 g. of alumina (activity III). With ether-ethyl acetate (3:7) and ethyl acetate 8.0 mg. of crystalline material, m.p. 170–172°, was eluted. Recrystallization from ethyl acetate-acetone gave 5.3 mg. of crude XIV, m.p. 177–178°, λ_{max}^{alc} 240 mμ; ε 9,200. With ethyl acetate and ethyl acetate-methanol (24:1) 18.7 mg. of crystalline material was eluted. Recrystallization from acetone-ether yielded 15.0 mg. of XIII, m.p. 190–192°. This product had no ultraviolet absorption maximum between 228 and 280 mμ. By recrystallization the m.p. of XIII was raised to 191–193°. $[\alpha]_D^{21}$ +54.6° (9.00 mg.; α +0.49°).

Anal. Calc'd for $C_{21}H_{32}O_6$ (380.47): C, 66.29; H, 8.48.

Found: C, 66.38; H, 8.44. No weight loss.

Methyl 3-oxo-5,(11?),14,19-tetrahydroxy-Δ¹-14β-etienate (XVI) *from methyl 1β,3β,5,(11?),14,19-hexahydroxy-14β-etianate* (XI). To 747 mg. of XI, m.p. 236–238°, in 25 cc. of redistilled *tert*-butanol and 10 cc. of water was added 1.05 g. (4 equivalents) of N-bromoacetamide (m.p. 103–105°; 96% by titration). The solution was kept at room temperature for 16 hours. Titration of a small sample indicated that 2 equivalents of N-bromoacetamide had been consumed. The solution was poured into 60 cc. of water containing 1 g. of sodium sulfite and the mixture was extracted with 20 portions of 50 cc. of ethyl acetate. The combined extracts were washed with water, dried over sodium sulfate, and evaporated to dryness, yielding 624 mg. of solid material. A second extraction of the aqueous phase with 10 portions of 60 cc. of ethyl acetate yielded 60 mg. of additional solid material. The pooled product (684 mg.) was crystallized from acetone and gave 405.6 mg. of crude methyl 3-oxo-1β,5,(11?),14,19-pentahydroxy-14β-etianate (XV), m.p. 193–195°. By recrystallization the m.p. was raised to 208–210°. The product showed no absorption in the ultraviolet between 226 and 300 mμ.

Refluxing 146.4 mg. of the crude XV in 6 cc. of ethanol with 0.2 cc. of acetic acid and 280 mg. of Girard's reagent T yielded 15.3 mg. as a discolored resinous non-ketonic fraction. The aqueous (ketonic) solution was acidified to Congo Red. Extraction with ethyl acetate after one hour, two hours, and ten days yielded only insignificant amounts of resinous material.

Crude XV (175 mg.) was chromatographed on 10 g. of alumina (activity V). With ethyl

acetate-methanol (49:1) 112.9 mg. of solid material was eluted which upon recrystallization from acetone yielded 40.3 mg. of crystalline methyl 3-oxo-5,(11?),14,19-tetrahydroxy-Δ^1-14β-etienate (XVI), m.p. 235–239°. By repeated recrystallization from methanol the m.p. was raised to 242–244°. $\lambda_{max.}^{alc.}$ 234 mμ; ϵ 10,000. $[\alpha]_D^{27}$ +54.0° (10.55 mg.; α +0.57°).

Anal. Calc'd for $C_{21}H_{30}O_7$ (394.47): C, 63.94; H, 7.67.

Found: C, 63.69; H, 7.82; Residue, 0.25.

Weight loss, 1.39; Weight gain, 0.0.

Methyl 3-oxo-(11?),19-diacetoxy-5,14-dihydroxy-Δ^1-14β-etienate (XVII). *A. By treatment of methyl 3-oxo-1β,5,(11?),14,19-pentahydroxy-14β-etianate* (XV) *with acetic anhydride.* To 157 mg. of crude XV, m.p. 193–195°, in 2 cc. of pyridine was added 1 cc. of acetic anhydride. The solution was kept at room temperature overnight, heated to 60° for 3 hours, and then poured into 30 cc. of 3% sulfuric acid. After one hour the mixture was extracted repeatedly with ethyl acetate and the extract was washed with *N* sodium bicarbonate and water. Evaporation of the solvent *in vacuo* left 178 mg. of resin which from ethyl acetate gave 105.0 mg. of crystalline XVII, m.p. 232–234°. $\lambda_{max.}^{alc.}$ 231 mμ; ϵ 9,000. The compound was chromatographically uniform and by repeated recrystallization from acetone the m.p. was raised to 240–242°. $\lambda_{max.}^{alc.}$ 231 mμ; ϵ 11,500. $[\alpha]_D^{27}$ +60.3° (9.57 mg.; α +0.58°).

Anal. Calc'd for $C_{25}H_{34}O_9$ (478.53): C, 62.74; H, 7.16; Acetyl, 17.97.

Found: C, 62.67; H, 7.15; Acetyl, 19.78, 20.15. No weight loss.

B. By acetylation of methyl 3-oxo-5,(11β),14,19-tetrahydroxy-Δ^1-14β-etienate (XVI). To 18.0 mg. of XVI, m.p. 232–235°, in 1 cc. of pyridine was added 0.5 cc. of acetic anhydride. The solution was kept at room temperature overnight, heated to 60° for 3 hours, and then was worked up as described above. Evaporation of the solvent yielded 22.1 mg. of solid material which from ethyl acetate gave 18.8 mg. of crystalline XVII, m.p. 225–227°. The substance was chromatographically uniform. By recrystallization from ethyl acetate the m.p. was raised to 235–237°. The mixture m.p. with a sample of XVII, as obtained by treatment of XV with acetic anhydride, gave no depression. $\lambda_{max.}^{alc.}$ 231 mμ; ϵ 12,000. $[\alpha]_D^{27}$ +60.7° (5.93 mg.; α +0.36°).

Methyl 3-oxo-(11?),19-diacetoxy-14-hydroxy-$\Delta^{1,4}$-14β-etiadienate (XIX) *by dehydration of methyl 3-oxo-(11?),19-diacetoxy-5,14-dihydroxy-Δ^1-14β-etienate* (XVII). A total of 55.0 mg. of XVII, m.p. 232–234°, was refluxed in 3 cc. of glacial acetic acid for one hour. After evaporating the solvent *in vacuo*, the residue was dissolved in 50 cc. of ethyl acetate which was then washed with *N* sodium bicarbonate and water, dried over sodium sulfate, and brought to dryness. This yielded 52.4 mg. of resinous XIX which was chromatographed on 3 g. of alumina (activity III). With petroleum ether-benzene (1:4) a total of 27.4 mg. was eluted which resisted all attempts at crystallization. $\lambda_{max.}^{alc.}$ 240 mμ; ϵ 12,500. $[\alpha]_D^{28}$ +77.6° (2.0 mg.; α +0.16°). For infrared data *cf.* theoretical part.

Anal. Calc'd for $C_{25}H_{32}O_8$ (460.51): C, 65.20; H, 7.01; Acetyl, 18.69.

Found: C, 65.29; H, 7.13; Acetyl, 18.5; Residue, 0.1.

Weight loss, 1.00; Weight gain, 0.

Treatment of methyl 3-oxo-5,(11?),14,19-tetrahydroxy-Δ^1-14β-etienate (XVI) *with acetic acid.* A total of 3.8 mg. of XVI, m.p. 237–238°, was refluxed with 1 cc. of glacial acetic acid for one hour and then worked up as described in the preceding experiment. There resulted 3.6 mg. of resin. The ultraviolet absorption spectrum showed strong end absorption, a minimum between 245 mμ and 250 mμ; ϵ 70 and a maximum at 278 mμ; ϵ 1,400 (ϵ calc'd for M.W. 346.45) (See Figure 1).

Oxidation of methyl 3β,(11?),19-triacetoxy-1β,5,14-trihydroxy-14β-etianate (X) *with chromium trioxide: Methyl 1-oxo-3β,(11?),19-triacetoxy-5,14-dihydroxy-14β-etianate* (XX) *and methyl 1-oxo-(11?),19-diacetoxy-5,14-dihydroxy-Δ^2-14β-etienate* (XXI). To 114.2 mg. of X (obtained by degradation of V), m.p. 155–157°, in 3 cc. of glacial acetic acid was added 3.2 cc. of 90% acetic acid containing 32 mg. (2.2 equivalents) of chromium trioxide. The solution was kept at room temperature for 40 hours, after which time its color had not completely changed to green. A few drops of ethanol were added and after 30 minutes the acetci acid was evaporated *in vacuo* at room temperature. The residue was partitioned between 50 cc. of water and 50 cc. of ethyl acetate, and the aqueous phase was extracted with three

portions of 20 cc. of ethyl acetate. The combined ethyl acetate extracts were washed repeatedly with ice-cold *N* sodium carbonate and with water. From the carbonate phase there was obtained, after acidification with ice-cold 10% sulfuric acid and extraction with chloroform, 5.4 mg. of brownish resin (acid fraction). The ethyl acetate extract (neutral fraction) was dried over sodium sulfate and was then evaporated to dryness, leaving 93.7 mg. of resin which did not absorb in the range 228–280 mμ.

The resin was chromatographed on 6 g. of alumina (activity III). A total of 37 fractions of 50 cc. each was eluted. Fractions 9 through 14 [ether-ethyl acetate (9:1)] gave 29.7 mg. of crystalline material, m.p. 80–100°. Recrystallization from ethyl acetate-ether gave 20.5 mg. of crystalline XX, constant m.p. 100–105° (effervescence). $[\alpha]_D^{27}$ +18.0° (11.59 mg.; α +0.21°). No absorption of ultraviolet light in the range 226–280 mμ.

Anal. Calc'd for $C_{27}H_{38}O_{11}$ (538.57): C, 60.21; H, 7.11.

Found: C, 59.92; H, 7.36.

Weight loss, 2.74; Weight gain, 1.75 (Dried at 60°).

Fractions 15 through 24 [ether-ethyl acetate (4:1)] yielded 33.8 mg. of resin which from ethyl acetate-ether gave 16.4 mg. of crystals, m.p. 100–110° (effervescence).

Fractions 25 through 32 [ether-ethyl acetate (1:1 and 2:3)] gave 16.2 mg. of crystalline XXI, m.p. 183–185°. By repeated recrystallization the m.p. was raised to 187–188°. The tetranitromethane test was negative. $\lambda_{max.}^{alc.}$ 222 mμ; ε 12,500. $[\alpha]_D^{27}$ −36.3° (8.44 mg.; α −0.31°).

Anal. Calc'd for $C_{25}H_{34}O_9$ (478.52): C, 62.75; H, 7.16.

Found: C, 62.54; H, 6.98; Residue, 0.17. No weight loss.

Methyl ditritoxytetrahydroxy-14β-etianate (XXVI) *and methyl monotritoxypentahydroxy-14β-etianate* (XXVII). To 1.00 g. of methyl 1β,3β,5,(11?),14,19-hexahydroxy-14β-etianate (XI), m.p. 236–238°, in 10 cc. of pyridine (freshly distilled over barium oxide) was added 730 mg. (1.1 equivalents) of triphenylchloromethane (m.p. 106–108°; 97% by titration). The solution was heated to 105° for six hours and kept at room temperature overnight. It was then poured into 60 cc. of ice-water and the mixture was extracted exhaustively with ethyl acetate and with chloroform-ethanol (3:1). The respective extracts were washed quickly with 100 cc. of ice-cold *N* hydrochloric acid, 30 cc. of *N* sodium bicarbonate, repeatedly with water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. The ethyl acetate extract yielded 1.3 g. of resin. Chromatography of this resin on 26 g. of alumina (activity III) yielded 3 major fractions: (*a*) With petroleum ether-benzene (1:1) 270.9 mg. of triphenylcarbinol, m.p. 162–163°, was eluted.

(*b*) With benzene-ether (9:1) 126.5 mg. of crude crystalline XXVI, m.p. 228–230°, (6% yield) was eluted which from acetone-petroleum ether yielded 97.4 mg. with constant m.p. 243–245°. $[\alpha]_D^{27}$ +7.3° (12.36 mg.; α +0.09°).

Anal. Calc'd for $C_{59}H_{62}O_8$ (899.09): C, 78.79; H, 6.95.

Found: C, 78.53; H, 7.00 (Dried without special precautions).

The yield of crude crystalline XXVI in several experiments under these conditions was 6–11%.

(*c*) With ethyl acetate several consecutive resinous fractions were eluted which did not crystallize from various solvent combinations. They were combined (689 mg. total; 43% yield) and rechromatographed on 18 g. of alumina. With benzene-chloroform (2:3) 12 consecutive resinous fractions were eluted, totalling 586 mg. which again resisted all attempts at crystallization. For acetylation the first 2 fractions (product A, 103.7 mg.), the next 4 fractions (product B, 396 mg.) and the last 6 fractions (product C, 85.6 mg.) were combined (*vide infra*). These resinous products obviously represented methyl monotritoxypentahydroxy-14β-etianate (XXVII). The analytical data were secured from product B. $[\alpha]_D^{26}$ +2.3° (12.36 mg.; α +0.03°).

Anal. Calc'd for $C_{40}H_{48}O_8$ (656.78): C, 73.14; H, 7.37.

Found: C, 73.39, 73.59; H, 7.32, 7.43.

Weight loss, 3.58, 2.91; Weight gain, 0.0, 0.0.

The chloroform-ethanol extract gave 110 mg. of resin which crystallized from methanol; 86.8 mg.; m.p. 190–192°; probably representing impure starting material (XI).

Preparation of methyl ditritoxytetrahydroxy-14β-etianate (XXVI) *from methyl*

1β,3β,5,(11?),14,19-hexahydroxy-14β-etianate (XI). (*a*) *With 2 moles of triphenylchloromethane.* To 101 mg. of XI, m.p. 235–236°, in 5 cc. of pyridine was added 140 mg. (2.2 equivalents) of triphenylchloromethane. The solution was heated to 106° for 6 hours, then kept at room temperature overnight, and worked up as described above. The resulting resin (225 mg.) was chromatographed on 8 g. of alumina (activity III). With benzene-ether (7:1) 89.8 mg. of ditritoxy compound (XXVI) (41% yield) was eluted; m.p. after recrystallization 238–241°. With ether-methanol (7:1) 80.2 mg. (50% yield) of resinous monotritoxy compound (XXVII) was obtained.

(*b*) *With 3 moles of triphenylchloromethane.* A solution of 49.7 mg. of XI, m.p. 235–236°, in 3 cc. of pyridine was treated with 100 mg. (3.3 equivalents) of triphenylchloromethane under the stated conditions. Chromatography of the resulting resin (148.1 mg.) yielded 54.4 mg. (31%) of crystalline XXVI and 32.7 mg. (40%) of resinous XXVII.

Acetylation of methyl ditritoxytetrahydroxy-14β-etianate (XXVI): *Methyl ditritoxymonoacetoxytrihydroxy-14β-etianate* (XXVIII). To 86 mg. of XXVI, m.p. 243–245°, in 1.5 cc. of pyridine was added 0.75 cc. of acetic anhydride. The solution was kept at room temperature (30°) overnight, heated to 60° for 3 hours, and concentrated to dryness *in vacuo*. The solution of the residue in ethyl acetate was washed with ice-cold *N* hydrochloric acid, *N* sodium bicarbonate, and water and was dried over sodium sulfate. The solvent was evaporated *in vacuo* and the resinous residue (99.8 mg.) was chromatographed on 5 g. of alumina (activity III). With petroleum ether-benzene (2:3) 49.1 mg. of resinous material (XXIX) was eluted which resisted all attempts at crystallization. With petroleum ether-benzene (3:7) 44.3 mg. of resinous material was eluted which from ether gave 15.6 mg. of crystalline XXVIII, m.p. 166–168°. By repeated recrystallization from acetone-petroleum ether the m.p. was raised to 169–171°. $[\alpha]_D^{24}$ +12.1° (13.95 mg.; α +0.17°).

Anal. Calc'd for diacetate $C_{63}H_{66}O_{10}$ (983.15): C, 76.96; H, 6.77; Acetyl, 8.76.
for monoacetate $C_{61}H_{64}O_9$ (941.13): C, 77.84; H, 6.85; Acetyl, 4.57.
Found: C, 77.36, 77.36, 77.44; H, 7.02, 7.06, 6.91; Acetyl, 5.0.
Weight loss, 2.33, 0.46; Weight gain, 0.0, 0.22.

The resinous XXIX was rechromatographed on alumina. From the later eluates a small amount of additional crystalline XXVIII, m.p. 167–170°, was obtained. However, the main fractions again resisted all attempts at crystallization.

Acetylation of methyl monotritoxypentahydroxy-14β-etianate (XXVII): *Methyl monotritoxytriacetoxydihydroxy-14β-etianate* (XXX). *A. Acetylation of chromatographic "product A" (vide supra).* To 103.7 mg. of resinous XXVII ("product A") in 2 cc. of pyridine was added 1 cc. of acetic anhydride. The mixture was kept at room temperature (32°) for 48 hours, heated to 60° for 4 hours, and then evaporated to dryness *in vacuo*. The solution of the residue in ethyl acetate was washed with ice-cold *N* hydrochloric acid, *N* sodium bicarbonate, and water, and dried over sodium sulfate. Evaporation of the solvent left 110.5 mg. of semi-solid material which was chromatographed on 5 g. of alumina (activity III). With petroleum ether-benzene (1:4) several consecutive fractions of crude XXX, totalling 42.9 mg. (35% yield), m.p. range 207–237°, were obtained and pooled. Several more crystalline fractions, eluted with benzene, totalling 40.6 mg., had a m.p. range from 167–190°. This lower-melting material was pooled separately.

B. Acetylation of chromatographic "products B and C" (vide supra). A part of "product B" (341 mg.) and the total of "product C" (85.6 mg.) of resinous XXVII were acetylated and worked up as described under *A*. After chromatography 184 mg. (45%) and 24.7 mg. (24%) respectively of the higher-melting material (m.p. range 200–238°) and 134 mg. and 25.3 mg. respectively of the lower-melting material (m.p. range 115–190°) were obtained.

C. Rechromatography of the higher-melting fractions. The higher-melting fractions of all three acetylation experiments were combined (252 mg.) and rechromatographed on 12 g. of alumina (activity III). Three consecutive fractions eluted with petroleum ether-benzene (1:4) were combined (71.7 mg.; m.p. range 243–257°). By repeated recrystallization from ethyl acetate and from acetone-ether the m.p. of methyl monotritoxytriacetoxydihydroxy-14β-etianate (XXX) was raised to 269–270°. $[\alpha]_D^{25}$ −26.5° (16.98 mg.; α −0.45°).

Anal. Calc'd for $C_{46}H_{54}O_{11}$ (782.89): C, 70.57; H, 6.95; Acetyl, 16.5.
Found: C, 70.44; 70.35; H, 7.08, 7.03; Acetyl, 16.7. No weight loss.

With benzene more of the lower-melting acetylation product (59.5 mg.), m.p. range 150–190°, was eluted from the column.

D. Rechromatography of the lower-melting acetylation product. The combined lower-melting fractions after rechromatography yielded more rather crude XXX and a more polar crystalline material (XXXI), m.p. 140–145° (effervescence), which could not be purified to a well defined entity.

Methyl 3β,(11?),19-triacetoxy-1β,5,14-trihydroxy-14β-etianate (X) *by detritylation of methyl monotritoxytriacetoxydihydroxy-14β-etianate* (XXX). *A. Detritylation at room temperature for seven days.* A solution of 28.3 mg. of XXX, m.p. 265–267°, in 2 cc. of 95% acetic acid was kept at room temperature for seven days and was then diluted with 100 cc. of ethyl acetate which was washed acid-free with *N* sodium bicarbonate and water and dried over sodium sulfate. After evaporation of the solvent 26.6 mg. of resin was obtained which was chromatographed on 3 g. of alumina (activity III). With petroleum ether-benzene (1:1) 8.4 mg. of crystalline material, m.p. 152–154° was eluted which after recrystallization from acetone-petroleum ether yielded 6.2 mg. of triphenylcarbinol, m.p. 161–162°. With ether-ethyl acetate (3:2) 11.1 mg. of crystalline material, m.p. 140–142° was eluted which upon recrystallization from ethyl acetate-ether gave 7.4 mg. of methyl 3β,(11?),19-triacetoxy-1β,5,14-trihydroxy-14β-etianate (X), m.p. 145–148°. By further recrystallization the m.p. was raised to 150–152° (crushed crystals melt at 135–140°). The mixture m.p. with a sample of X, as obtained by methylation of the etio acid IX resulting from the oxidation of ouabagenin triacetate (V), showed no depression. $[\alpha]_D^{24}$ +27.4° (12.44 mg.; α +0.34°). For infrared data *cf.* theoretical part.

Anal. Calc'd for $C_{27}H_{40}O_{11}$ (540.59): C, 59.98; H, 7.46.
Found: C, 59.68; H, 7.68.
Weight loss, 2.30; Weight gain, 1.89.

B. Detritylation under various conditions. (*a*) After treatment of XXX with 95% acetic acid at room temperature overnight unchanged starting material (XXX) was recovered in almost quantitative yield.

(*b*) Treatment of XXX with 95% acetic acid at room temperature for 4 days gave after chromatography triphenylcarbinol, 43% of unchanged starting material (XXX), and 55% of X.

(*c*) Refluxing of XXX with 95% acetic acid for 1½ hours yielded triphenylcarbinol and 80% of X.

Attempted tritylation of methyl 3β,(11?),19-triacetoxy-1β,5,14-trihydroxy-14β-etianate (X). To 147 mg. of X, m.p. 154–156°, (made water-free by repeated evaporation to dryness *in vacuo* from a benzene solution and drying over P_2O_5 *in vacuo*) in 5 cc. of absolute pyridine was added 230 mg. (3 equivalents) of triphenylchloromethane (m.p. 106–109°; 96% by titration). The solution was heated to 100° for 7½ hours, kept at room temperature overnight, and was then worked up as described earlier (*vide supra*). The ethyl acetate extract yielded 352 mg. of solid material which was chromatographed on 16 g. of alumina (activity III). In the order of elution 214 mg. of triphenylcarbinol, 95.2 mg. of starting material (X), m.p. 146–150°, and 17 mg. of non-identified resinous material were isolated.

SUMMARY

1. The experiments reported in this paper establish the presence of hydroxyl groups in the ouabagenin molecule at carbon atoms 1,3,5, and 19. On the basis of the evidence reported in the literature another hydroxyl group can be assumed to be in position 14. Evidence is also presented in support of the β-configuration of the hydroxyl groups at carbon atoms 1,3,5, and 14. The location of the sixth hydroxyl group, postulated by Mannich (4, 9) to be at carbon atom 11, still has to be determined.

2. The ouabagenin derivatives described by Reichstein (15, 16) as "ouabagenin acetate A" and "ouabagenin acetate B" were recognized as the tetraacetate IV and the triacetate V respectively. The degradation products of IV and V (15, 16) were found to be methyl 1β,3β,(11?),19-tetraacetoxy-5,14-dihydroxy-14β-etianate (VIII) and methyl 3β,(11?),19-triacetoxy-1β,5,14-trihydroxy-14β-etianate (X) respectively. Both, VIII and X, were converted into methyl 1β, 3β,5,(11?),14,19-hexahydroxy-14β-etianate (XI).

3. XI was selectively oxidized with N-bromoacetamide to methyl 3-oxo-1β,5,(11?),14,19-pentahydroxy-14β-etianate (XV) which by the action of alumina was dehydrated to methyl 3-oxo-5,(11?),14,19-tetrahydroxy-Δ^1-14β-etienate (XVI). The latter was characterized as the (11?),19-diacetate (XVII) which by refluxing with acetic acid was further dehydrated to methyl 3-oxo-(11?),19-diacetoxy-14-hydroxy-$\Delta^{1,4}$-14β-etiadienate (XIX). By refluxing with acetic acid, XVI was aromatized to a compound believed to possess structure XVIII.

4. The oxidation of the triacetate X with chromic acid yielded mainly methyl 1-oxo-3β,(11?),19-triacetoxy-5,14-dihydroxy-14β-etianate (XX) and, in addition, a small amount of a compound interpreted to be methyl 1-oxo-(11?),19-diacetoxy-5,14-dihydroxy-Δ^2-14β-etienate (XXI).

5. XI has been subjected to tritylation studies resulting in the isolation of a number of compounds which require further study.

6. As a model experiment, methyl 3β,5,14,19-tetrahydroxy-14β-etianate (XII) was selectively oxidized with N-bromoacetamide to methyl 3-oxo-5,14,19-trihydroxy-14β-etianate (XIII) which by the action of alumina is converted only to a minor extent to methyl 3-oxo-14,19-dihydroxy-Δ^4-14β-etienate (XIV). Treatment of XIII with Girard's reagent T effected complete dehydration leading to XIV.

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