Acospectoside A II: The Structure of the Cardenolide Glycoside

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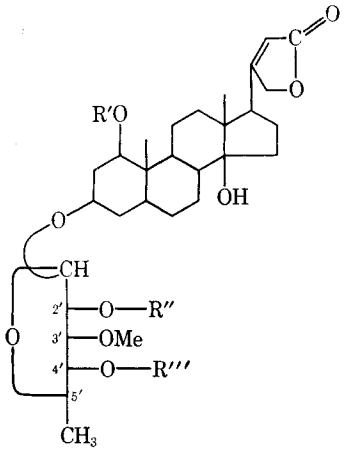
Abstract □ The structure of acospectoside A, a new cardenolide bioside isolated from *Acokanthera oblongifolia*, was elucidated by studying the various hydrolysis products. Anhydroacovenosigenin A, acovenosigenin A, D-glucose, and L-acovenose resulted from mineral acid hydrolysis. Enzymatic hydrolysis furnished acovenosides A and B, acobioside A, and D-glucose. These and other results showed that acospectoside A is 1-*O*-acetyl-acobioside A. The findings were confirmed by acetylating acospectoside A and acobioside A to yield the same hexa-*O*-acetyl-acobioside A which with mild alkaline hydrolysis afforded acospectoside A.

Keyphrases □ Acospectoside A—structure determination □ Hydrolysis products, formation—acospectoside A structure determination □ Cardiotoxicity—acospectoside A □ Cytotoxicity—acospectoside A □ TLC—separation, identification □ IR spectrophotometry—structure □ NMR spectroscopy—structure

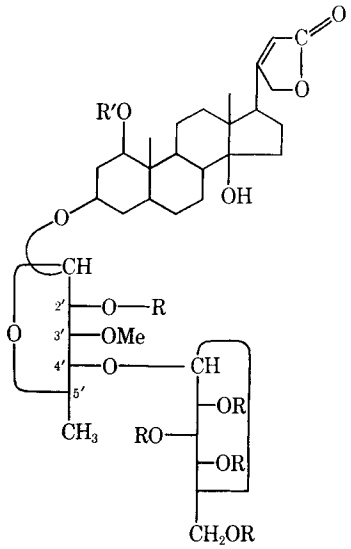
In studies on the cardiac-active glycosides of *Acokanthera oblongifolia* (syn. *Acokanthera spectabilis*) (1, 2) the isolation of a new cardenolide glycoside, acospectoside A, was reported in addition to the known monosides acovenosides A (I) and B (II) and the bioside acobioside A (III or IV). Evidence is now presented which proves acospectoside A to be 1-*O*-acetyl-acobioside A, thereby leading to the structure V (or VI) for the glycoside. The point of attachment of D-glucose to 2′ or 4′ of L-acovenose in the two biosides (III or IV and V or VI) remains to be established (4). The evidence for the proposed structure V (or VI) of acospectoside A is as follows.

PROCEDURES

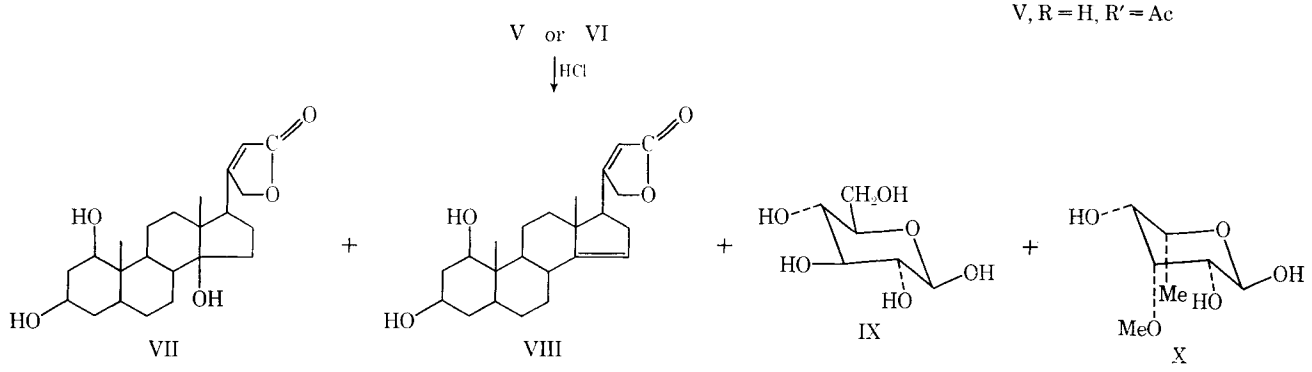
Hydrolysis of the isolated glycoside with hydrochloric acid afforded a crystalline compound which was identified as Δ^{14}-anhydroacovenosigenin A (VIII). That this product was an artifact resulting from acid induced dehydration of the true genin, acovenosigenin A (VII), was apparent as the latter was actually identified by TLC in the mother liquors of VIII. The water-soluble fraction of the acid hydrolyzate was found by thin-layer and paper chromatograms to contain two sugars identified as D-glucose (IX) and L-acovenose (X) (see Scheme I).



I, R′ = R″ = R‴ = H
II, R′ = Ac, R″ = R‴ = H
IV, R′ = H, R″ = β-D-glucosyl, R‴ = H
IVa, R′ = R‴ = Ac, R″ = tetraacetyl-β-D-glucosyl
VI, R′ = Ac, R″ = β-D-glucosyl, R‴ = H

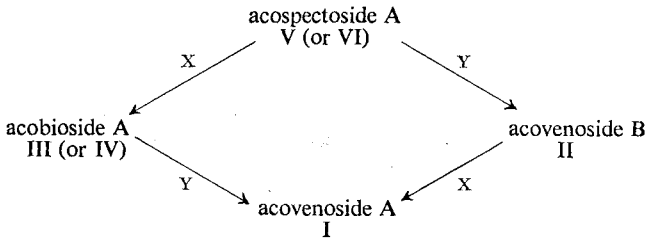


III, R = R′ = H
IIIa, R = R′ = Ac
V, R = H, R′ = Ac



Scheme I

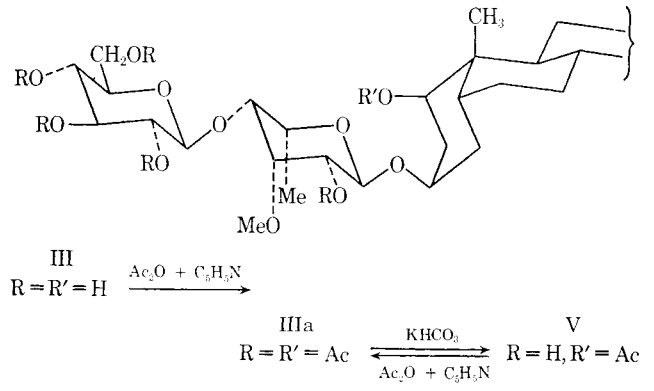
Additional evidence for the assigned structure V (or VI) of acospectoside A was obtained by treatment of the glycoside with snail (*Helix pomatia*) enzyme. Thus it was shown chromatographically that the aqueous hydrolyzate contained IX and that the chloroform–ethanol-soluble portion of the enzymic hydrolyzate contained a mixture of III (or IV) and the monosides I and II. The formation of II was significant in that it constituted a proof for the presence of a C-1 acetoxyl group in acospectoside A, suggesting that III (or IV) and I arise by reactions involving deacetylation at C-1. The reactions involved in the hydrolysis with the snail enzyme could be depicted as shown in Scheme II.



Scheme II—Products of the hydrolysis of acospectoside A with Helix pomatia *enzymes (X = deactylation with esterase; Y = removal of glucose with β-glucosidase)*

In one course V (or VI) may be hydrolyzed to III (or IV) by the esterase which the snail enzyme is known to contain. Subsequent removal of a glucose molecule from III (or IV) by the β-glucosidase component of the enzyme mixture would result in I. In another sequence glucose may first be split from V (or VI) to yield II which ultimately undergoes ester cleavage to furnish I. That the esterase and glucosidase reactions were both operative was demonstrated by subjecting II and III (or IV) separately to the action of the enzyme whereupon I was shown to result in each case.

The presence of acetoxyl group in V (or VI) was completely supported by analysis (acetyl value determination) and by spectral data (IR and NMR). Its location and configuration follow from transformation to known compounds (*vide supra*) and from the observed difficulty of its saponification which might be expected for an axially bound acetoxyl group. Thus hexa-*O*-acetyl acobioside A (IIIa or IVa) (4), obtained as the same derivative from both V (or VI) and III (or IV), furnished V (or VI) upon mild saponification (which would only affect the sugar equatorial groups) using potassium bicarbonate (Scheme III) in the manner recommended by



Scheme III

Reichstein *et al.* (6) for a parallel transformation, namely that of tri-*O*-acetyl acovenoside A to II.

Finally, the above transformations embody a proof for the identical mode of linkage of the sugars (IX and X) in both V (or VI) and III (or IV). Experiments are now being conducted in this laboratory to enable a decision between the two alternative structures of acospectoside A and consequently those of acobiosiue A.

The results of biological testing for cardiotoxicity in cats and cytotoxicity against KB (Table I) are inclined to show that the C-1 acetoxylated compounds are less active than the corresponding hydroxylated ones. It also appears that the monosides are more cytotoxic than the corresponding biosides.

Table I—Cardiotoxicity and Cytotoxicity of the *Acokanthera oblongifolia* Cardenolides

Compound	Cardiotoxicity MLD, mg./kg.	Cytotoxicity[a] ED_{50}, mcg./ml.
Acovenoside A (I)	0.2357 (4, 7)[b]	0.031
Acobioside A (III or IV)	0.1535 (4)	0.15
Acovenoside B (II)	2.144 (7)	0.22
Acospectoside A (V or VI)	1.806 (1)	0.30

[a] Cytotoxicity was determined by tests with cell cultures of human carcinoma of the nasopharynx (KB), and a compound is considered active if the ED_{50} (dose that inhibits growth of 50% of control growth) is equal to 4 mcg./ml. or less. These data were provided by the Cancer Chemotherapy National Service Center, and the results reported earlier (8). [b] Numbers in parentheses refer to references.

EXPERIMENTAL

Acid Hydrolysis of Acospectoside A (V or VI)—A mixture of acospectoside A (43.9 mg.) and 0.6 *N* hydrochloric acid acetone solution (8 ml.) was allowed to stand at room temperature for 14 days. After dilution with water (10 ml.) and removal of the acetone (*in vacuo*), the aqueous concentrate was extracted with chloroform. The residue from the latter extract was washed thoroughly with petroleum ether (b.p. 30–60°) then crystallized from acetone–petroleum ether to give 8.4 mg. of colorless crystals, m.p. 253–255°. The compound was shown on silica gel G chromatoplates to be identical with Δ^{14}-anhydroacovenosigenin A (VIII) (5) [R_f 0.44, 0.28, 0.56, and 0.58 using the systems ethyl acetate–ethanol (95:5), chloroform–tetrahydrofuran (3:1), chloroform–ethanol (9:1), and chloroform-dioxane (3:1), respectively]. The IR spectrum (KBr) contained bands at 3400 cm.$^{-1}$ (OH), 1787 and 1755 cm.$^{-1}$ (with the 1755 cm.$^{-1}$ stronger) (αβ-unsaturated γ-lactone) (9) and 1632 cm.$^{-1}$ (C=C) and was superimposable on that measured for authentic VIII. The NMR spectrum (60 mc., TMS/$CDCl_3$) contained signals at δ 0.83 and 1.14 (C-18 and C-19 CH_3, respectively), 4.78 (C-21 CH_2), and at δ 5.25 and 5.92 (vinyl protons on C-15 and C-22, respectively).

Anal.—Calcd. for $C_{23}H_{32}O_4$: C, 74.17; H, 8.66. Found: C, 74.63; H, 8.62.

The crystallization mother liquors of VIII were shown on silica gel G chromatoplates to contain another product which was identical with acovenosigenin A (VII) (5) (R_f 0.36, 0.17, 0.30, and 0.32 using the same solvent systems given above, respectively).

Identification of the Sugars—The aqueous layer remaining after chloroform extraction (above) was treated with silver carbonate (350 mg.), filtered then evaporated *in vacuo*. The residue (14.4 mg.) was shown to comprise two sugars which were identified as D-glucose (IX) and L-acovenose (X) (5) by direct comparison on papergrams [R_f 0.69 and 0.84, 0.37 and 0.75, 0.13 and 0.59 and 0.18 and 0.68 using systems ethyl acetate–pyridine–water (8:3:8), *n*-butanol–pyridine–water (9:5:8), *n*-butanol–acetic acid–water (20:1:20), and *n*-butanol–ethanol–water (10:1:2), respectively] and on silica gel G chromatoplates [R_f 0.29 and 0.39 using system ethyl acetate–isopropanol–methanol (14:3:1)]; aniline hydrogen phthalate was used as a spray reagent.

Enzymatic Hydrolysis of Acospectoside A (V or VI)—A solution of acospectoside A (72 mg.) in acetate buffer (pH 5.4, 20 ml.) was treated with 72 mg. of snail enzyme preparation[1] and a few drops of toluene and the mixture left for 5 days at 37°. After extraction with chloroform–ethanol (7:3), the aqueous and organic layers of the hydrolyzate were evaporated and separately processed. The residue from the aqueous layer was shown by thin-layer and paper chromatography (using the solvent systems given above) to contain one sugar identified as glucose. The residue from the organic phase was crystallized from ethanol–ether to give 7 mg. of a product (m.p. 248–258°) identified as acobioside A (III or IV) by direct comparison [chromatographically (*cf.* Table II), mixed m.p. and IR spectra]; reported (4) m.p. 248–258°. The mother liquors were evaporated and the residue crystallized from acetone–ether to give 6.4 mg. of a compound with double m.p. 160–162° → 229–232°, which was identified as acovenoside A (I) by direct comparison [chromatographically (*cf.* Table II), mixed m.p. and IR spectra]

[1] Helicase, a commercial product of Industrie Biologique Francaise S.A., Gennivilliers (Seine), France.

Table II—Chromatographic Data on Monosides and Biosides Identified[a] in the Enzymatic Hydrolyzate of Acospectoside A

Solvent system	Chromatographic Matrix	Acovenoside A	Acovenoside B	Acospectoside A (unreacted)	Acobioside A
EtOAc–EtOH (7:3)	Silica Gel G (TLC)	0.56	0.65	0.37	0.22
$C_6H_5CH_3$–*n*-BuOH–H_2O (193:7:20)	Paper with[b] ~35% H_2O	0.25	0.52	—	—
n-BuOH–$C_6H_5CH_3$–H_2O (1:1:1)	Paper equil. overnight	0.76	0.81	0.60	0.56
n-BuOH–$C_6H_5CH_3$–H_2O (4:1:2)	Paper with[b] ~35% H_2O	0.92	0.95	0.52	0.39
n-BuOH–$C_6H_5CH_3$–H_2O (2:1:1)	Paper with[b] ~35% H_2O	0.91	0.93	0.55	0.43
n-BuOH–$C_6H_5CH_3$–H_2O (1:1:1)	Paper with[b] ~35% H_2O	0.93	0.95	0.87	0.80

[a] Chromogen-Kedde reagent. [b] Procedure followed for the paper chromatographic technique is that reported by Reichstein *et al.* (10, 11).

reported (5) m.p. 160–163° → 230–232°. The mother liquor remaining from this compound was shown on thin-layer and paper chromatograms to contain, in addition to I, another product which was chromatographically identical to acovenoside B (II) (*cf.* Table II).

Enzymatic Conversion of Acobioside A (III or IV) and Acovenoside B (II) to Acovenoside A (I)—Acobioside A and acovenoside B (1 mg. of each) were separately shaken in 0.5 ml. of acetate buffer (pH 5.4) and 0.5 ml. of water, respectively, containing snail enzyme (1 mg.) and a drop of toluene for 5 days at 37°. After evaporation *in vacuo* the residue from each hydrolyzate was extracted with chloroform–ethanol (7:3) and the extract inspected chromatographically (*cf.* Table II). It was shown that acovenoside A (I) was a product in each experiment in addition to the unchanged starting compound.

Hexa-O-acetyl acobioside A (IIIa or IVa)—A solution of acobioside A (III or IV) (36 mg.) in pyridine (0.4 ml.) was treated with acetic anhydride (0.4 ml.) at 37° for 13 days. After the usual work-up the acetylated product (36.3 mg. of a resin) was percolated in chloroform solution through a silicic acid (10 g.) column which afforded 28.5 mg. of a colorless foam, $[\alpha]_D^{27} = -54°$ ($CHCl_3$). This material, unamenable to crystallization, was chromatographically pure as shown on Silica Gel G plates [R_f 0.93, 0.47, 0.30, and 0.37 using solvents ethyl acetate–ethanol (7:3), chloroform–ethanol (39:1), chloroform–dioxane (83:17), and chloroform-tetrahydrofuran (3:1), respectively] and spraying with Kedde's reagent. The IR spectrum showed peaks at 3000, 2890, and 1740 cm.$^{-1}$ (C=O), 1625 cm.$^{-1}$ (C=C), 1372 and 1244–1210 cm.$^{-1}$ (broad) (C—O of acetate), 1128, 1070, and 1040–1030 cm.$^{-1}$ (broad) (acetate), and 995 cm.$^{-1}$. Reported (4) as colorless foam $[\alpha]_D^{27} = -56.1°$ ($CHCl_3$).

Acetylation of acospectoside A (V or VI) and purification of the product as described above gave an acetate which was identical with hexa-*O*-acetyl acobioside A (above) as judged by direct comparison (R_f values and IR spectra).

Mild Saponification of III a (or IVa)—A solution of hexa-*O*-acetyl acobioside A (IIIa or IVa, prepared from either sources, above) (440 mg.) in methanol (30 ml.) was treated with 30 ml. of a solution of potassium bicarbonate (0.6 g.) in water and the mixture left to stand at 37° for 9 days. The solution was reduced to half its volume *in vacuo* then extracted with chloroform–ethanol (7:3). The residue from this extract afforded, upon crystallization from ethanol, a product (326 mg.) which was identical (mixed m.p., R_f values on thin-layer and paper chromatograms and IR spectra) with acospectoside A (V or VI) which was obtained from the natural source.

In addition to previous data (including those reported in *Reference* 1) regarding the constitution of acospectoside A, the following are given in evidence for the C-1 acetoxyl group: the IR spectrum (KBr) contained peaks at 1745 and 1240 cm.$^{-1}$ (acetate C=O and C—O, respectively) in addition to ones at 3430 cm.$^{-1}$ (OH) and 1724 cm.$^{-1}$ (lactone C=O). The NMR spectrum (60 mc., TMS/$CDCl_3$) of the polytrimethylsilyl ether contained an acetyl group signal at δ 2.03 in addition to others at δ 0.90 and 0.98 (C-18 and C-19 CH_3, respectively), 3.53 (OCH_3), 4.94 (CH_2 at C-21) and 5.92 (vinyl proton on C-22).

Anal.—Calcd. for $C_{38}H_{58}O_{15}$: C, 60.46; H, 7.74; acetyl, 5.70. Found: C, 60.67, 60.71; H, 7.97, 7.87; acetyl, 5.36.

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