Tetrahedron. Vol. 25, pp. 1911 to 1920. Pergamon Press 1969. Printed in Great Britain

THE NEO-HOLOTHURINOGENINS—III[1]

NEO-HOLOTHURINOGENINS BY ENZYMATIC HYDROLYSIS OF DESULFATED HOLOTHURIN A

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(*Received in USA 27 August 1968; Received in the UK for publication 3 December 1968*)

Abstract—The enzymatic hydrolysis of desulfated holothurin A has been investigated. The products 12α-methoxy-7,8-dihydro-17-desoxy-22,25-oxidoholothurinogenin (IVA), 12β-methoxy-7,8-dihydro-22,25-oxidoholothurinogenin (III), 12α-hydroxy-7,8-dihydro-24,25-dehydroholothurinogenin (V), 12β-methoxy-7,8-dihydro-22-hydroxyholothurinogenin (VII), and the 3β-xyloside of 12β-methoxy-7,8-dihydro-24,25-dehydroholothurinogenin (VI) have been identified. Their structures and relationships to the new-holothurinogenins, the aglycone moieties as they exist in holothurin A and the holothurinogenins, obtained on strong acid hydrolysis of holothurin A, are discussed. The NMR spectra of the neo-genins in chloroform and pyridine are reported.

THE holothurinogenins,[2] derived from the glycosidic mixture holothurin A[3] by strong acid hydrolysis, differ from the neo-holothurinogenins,[1] the aglycone moieties as they exist in the glycoside. To obtain the neo-holothurinogenins we have investigated the enzymatic hydrolysis of the glycoside, Holothurin A, for reasons given below, was unsatisfactory as a substrate. Desulfated holothurin A, DHA, proved to be the suitable substrate. It should be mentioned at this point that the sulfate moiety is attached to the xylose of holothurin's sugar residue.[4]

Holothurin A contains no OMe group other than the one associated with the 3-methoxyglucose in its sugar residue.[3] Desulfated holothurin A was to a considerable extent additionally methoxylated, in spite of the extremely mild conditions (MeOH–HCl, 0·2N, 37°, 7 hr) used for its preparation. Two of the enzymatic products (acetylated) 12β-methoxy-7,8-dihydro-22,25-oxidoholothurinogenin-3-acetate (III) and 12β-methoxy-7,8-dihydroholothurinogenin-3,22-diacetate (VII) had been obtained[1] by MeOH–HCl hydrolysis of holothurin A under more stringent conditions. Enzymatic hydrolysis also gave 12α-methoxy-17-desoxy-7,8-dihydro-22,25-oxidoholothurinogenin-3-acetate (IVA), 12α-acetoxy-7,8-dihydro-24,25-dehydroholothurinogenin-3-acetate (V), and the 3β-xyloside of 12β-methoxy-7,8-dihydro-24,25-dehydroholothurinogenin (VI). Of particular significance was the fact that in both compounds. IVA and V, the oxygen on C_{12} occupied the α (axial) position. We discuss below the enzymatic hydrolysis and products, compounds IVA, V (the first neoholothurinogenin isolated), and VI.

Enzymatic hydrolysis of holothurin A, employing the enzyme mixture "glusulase" (glycosidases, β-glucuronidase and sulfatase) from *Helix pomatia*, gave little, if any,

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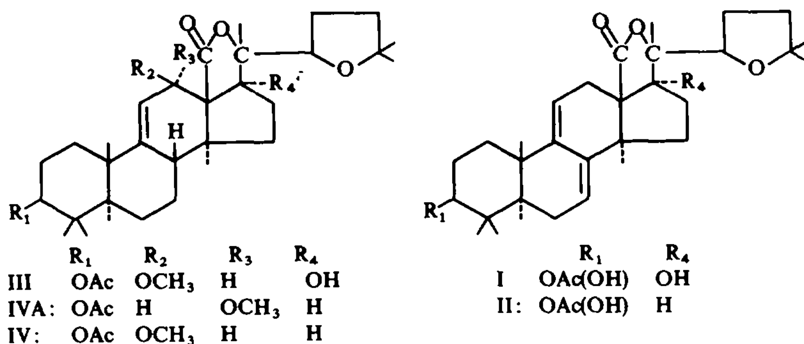
aglycones. The product was primarily a mixture of the insoluble xyloside sulfates of the neo-holothurinogenins. The sugars, glucose, 3-methoxy glucose, and quinovose, which together with xylose sulfate comprise the sugar residue of holothurin A, are removed easily. Apparently, the sulfatase present in the enzyme mixture is ineffective, while the sulfate moiety inhibits the enzymatic cleavage of the xyloside by the glycosidases. As previously mentioned, desulfated holothurin A, prepared by methanol–HCl hydrolysis of holothurin A was a suitable substrate, although additionally methoxylated to a considerable extent.* It was separated into two fractions, DHA I and DHA II, on the basis of their different solubilities in methanol–ether mixtures. Enzymatic hydrolysis of both fractions proceeded in small yield to the aglycone stage. Compounds III, V, and VI were obtained from DHA I (the fraction insoluble in a 1:3 mixture of methanol–ether); DHA II (insoluble in a 1:6 mixture) yielded compounds III, IVA, and VII.

The isolation of the genin, compound V, established the fact that at least for some of the holothurins the disposition of the C_{12}-OH group in the aglycone moiety is α (axial). The apparent ease with which this OH is replaced by a OMe radical confirms this (cf. methoxylation of 7α vs 7β-hydroxylcholesterol)[5] and suggests that in the intact holothurins the C_{12}—OH is axial, the 12β substituent arising via a facile acid catalyzed equilibration, $\alpha \leftrightarrow \beta$, in favor of the β orientation. The almost exclusive formation of 12β-methoxylated neo-holothurinogenins[1] from the more vigorous hydrolysis (MeOH–HCl, 0·2N, 75 hr) of holothurin A is consonant with this suggestion.

The assigned structures for compounds IVA, V, and VI were based on an examination of their UV, IR, and NMR spectra and confirmed by their conversion by strong acid treatment to the known holothurinogenins as discussed below.

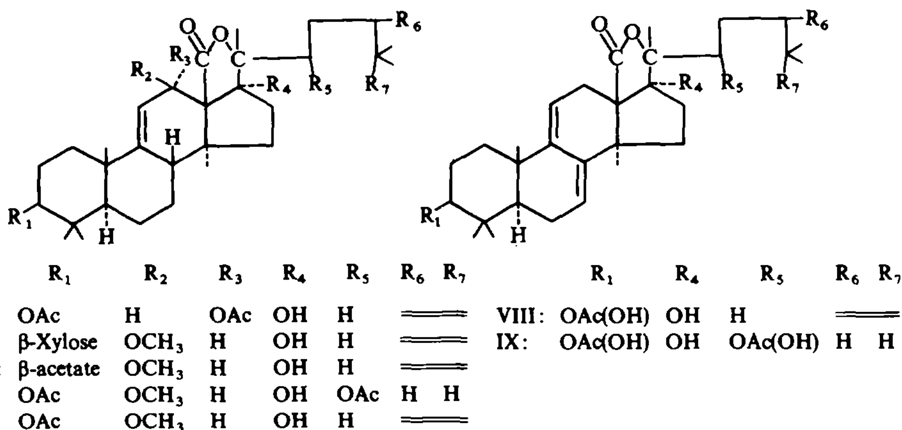
12α-Methoxy-17-desoxy-7,8-dihydro-22,25-oxidoholothurinogenin-3-acetate (IVA). $C_{33}H_{50}O_6$, m.p. 205–208°, exhibited only end absorption in the UV. On treatment with strong acid, it gave a product identical in all respects with the known holothurinogenin II. The IR (CS_2) showed absorption bands for a γ-lactone (1760); acetyl (1733); side-chain ether (1128); and OMe group (1089 cm^{-1}) accounting for the six oxygens in the molecule. No absorption for an OH was noted. Corresponding IR bands are observed for the 12β-OMe isomer (IV)[1] as well as (except for the OMe group) in holothurinogenin (II).[2] The NMR spectra showed the presence of seven tertiary methyls, one vinyl hydrogen (5·61, doublet, $J = 6$ c/s, C_{11}—H), and one proton on the C atom bearing the OMe group (3·74, doublet, $J = 6$ c/s, C_{12}—H). The mutual splitting, 6 c/s, of the protons on C_{11} and C_{12} are compatible, as previously discussed,[1,6] only with the assigned configuration, β (equatorial) for the proton on C_{12}, and may be contrasted with the 12β-OMe isomer, (IV), which exhibits[1] two broad singlets at 3·97 and 5·37 for the C_{12} and C_{11} protons, respectively. The chemical shift (4·17) and splitting (triplet, $J = 7$ c/s) of the proton on C_{22} corresponded to that observed for this proton in both the 12β-OMe analog (IV)[1] and holothurinogenin (II).[2]

* Desulfated material prepared by dioxan-HCl hydrolysis was obtained in a modification scarcely soluble in water and therefore unsuitable for enzymatic hydrolysis. After the completion of this work, it was found that this product could be solubilized by repeated lyophilizations of its suspension in water.



	R_1	R_2	R_3	R_4
III	OAc	OCH_3	H	OH
IVA:	OAc	H	OCH_3	H
IV:	OAc	OCH_3	H	H

	R_1	R_4
I	OAc(OH)	OH
II:	OAc(OH)	H



	R_1	R_2	R_3	R_4	R_5	R_6	R_7
V:	OAc	H	OAc	OH	H	═══	
VI	β-Xylose	OCH_3	H	OH	H	═══	
VIA:	β-acetate	OCH_3	H	OH	H	═══	
VII:	OAc	OCH_3	H	OH	OAc	H	H
X:	OAc	OCH_3	H	OH	H	═══	

	R_1	R_4	R_5	R_6	R_7
VIII:	OAc(OH)	OH	H	═══	
IX:	OAc(OH)	OH	OAc(OH)	H	H

12α-*Acetoxy*-7,8-*dihydro*-24,25-*dehydroholothurinogenin*-3-*acetate* (V). $C_{34}H_{50}O_7$, m.p. 240–243° (dec). In the UV compound V exhibited only end absorption; IR (CCl_4) absorption bands attributable to an OH (3618, 3480); γ-lactone (1762); acetyls (1737 cm^{-1}) accounted for the seven oxygens present in the molecule. The NMR spectrum ($CDCl_3$) showed the presence of seven methyls; five tertiary (0·90, 0·93, 1·21, 1·28, 1·43) and two vinyl methyls (1·65, 1·72); two vinyl protons, one a doublet (5·66, J = 6 c/s, C_{11}—H), the other a multiplet (5·15, C_{24}—H); one proton for each of the C atoms bearing an acetyl group (5·46, doublet, J = 6 c/s, C_{12}—H; 4.53, multiplet, C_3—H); and two acetyl Me singlets (2·07, C_3—OCOMe; 2·12, C_{12}—OCOMe). The mutual splitting, 6 c/s, of the protons on C_{11} and C_{12} is compatible only with the assigned β (equatorial) configuration for the proton on C_{12}. Treatment of compound V with strong acid gave a product identical in all respects with compound VIII.[7]

3β-*Xyloside of* 12β-*methoxy*-7,8-*dihydro*-24,25-*dehydroholothurinogenin* (VI, VIA). Compound VI showed only end absorption in the UV and yielded on strong acid treatment the known holothurinogenin (VIII) and xylose. The IR (KBr) spectrum showed the presence of a lactone (1742) and a OMe group (1085 cm^{-1}). Following acetylation VI gave the triacetate (VIA). The NMR spectrum ($CDCl_3$) of VIA showed the presence of seven methyls; five tertiary (0·82, 0·93, 1·07, 1·23, and 1·54); and two vinyl methyls (1·64, 1·70); one methoxyl Me (3·40); one vinyl proton (5·34, broad singlet, C_{11}—H); and one proton attributable to the C atom bearing the OMe group

TABLE 1. NMR SPECTRA (δ VALUES, J VALUES IN c/s IN PARENTHESES)

Comp.	Solv.	Me groups			21	26, 27	3H	11H	12H	22H	12-OMe	3-OAc	
		at 4,4	at 10β	at 14α									
IVA	$CDCl_3$	0·91, 0·93	1·23	0·91	1·35	1·25, 1·28	4·56 m	5·61 (d, 6)	3·74 (d, 6)	4·17 (t, 7)	3·45	2·08	
	C_6H_5N	0·94, 0·97	1·35	1·02	1·42	1·22, 1·28	4·71 m	5·71 (d, 6)	3·80 (d, 6)	4·21 (t, 7)	3·47	2·08	
IV	$CDCl_3$	0·88, 0·93	1·26	0·93	1·47	1·26, 1·26	4·57 m	5·37 b	3·93 b	3·99 (t, 7)	3·45	2·08	
	C_6D_5N	0·95, 0·97	1·37	0·97	1·60	1·23, 1·30	4·60[e]	5·46 b	4·05 b	4·15 (t, 7)	3·47	2·08	
													22-OAc
VII	$CDCl_3$	0·88, 0·93	1·25	1·08	1·55	0·88 (d, 6)	4·56 m	5·37 b	4·13 b	5·08 m	3·45	2·07	2·12
	C_6D_5N	0·95, 0·97	1·35	1·37	1·97	0·83 (d, 6)	4·72 m	5·55 b	4·50 b	5·24 m	3·53	2·05	2·11
													24H
VIA	$CDCl_3$	0·82, 0·93	1·23	1·07	1·54	1·64, 1·70	[f]	5·34 b	4·03 b	n.i.	3·40	—	[a]
	C_6H_5N	0·92, 1·07	1·35	1·40	1·90	1·57, 1·68	[f]	5·54	4·48 b	n.i.	3·50	—	[a]
VI	C_6H_5N	1·06, 1·35	1·35	1·42	1·90	1·55, 1·68	[f]	5·58 b	4·48 b	n.i.	3·50	—	5·22
X	$CDCl_3$	0·88, 0·93	1·27	1·08	1·55	1·67, 1·73	4·56 m	5·37 b	4·12 b	n.i.	3·45	2·08	5·16 m[b]

	C_6D_5N	0·95, 0·98	1·37	1·40	1·90	1·57, 1·65	4·72 m	5·55 b	4·50 b	n.i.	3·52	2·08	5·23 m[b]
											12-OAc		
V	$CDCl_3$	0·90, 0·93	1·21	1·28	1·43	1·65, 1·72	4·53 m	5·66 (d, 6)	5·46 (d, 6)	n.i.	2·12	2·07	5·15 m

		Protons for Xylose				
		C_1'—H	$(C_2'$–$C_4')$H	C_5'–H_e[c]	C_5'–H_a	C_2', C_3', C_4'–OAc
VIA	$CDCl_3$	4·55 (d, 6)	4·66–5·25) m	4·12 (dd 5, 12)	3·2–3·6	2·04
	C_6H_5N	4·85 (d, 6)	(5·00–5·50) m	4·33 (dd 5, 12)	3·60 (dd 8, 12)[d]	2·00, 2·05, 2·13
VI	C_6H_5N	4·86 (d, 6)	3·40 ————	————	———— 4·38[f]	—

In the Table the following abbreviations are used: b, broad; d, doublet; dd, double doublet; t, triplet; m, multiplet; n.i., line not identified.

[a] Overlapped by protons on C_2', C_3', and C_4'.

[b] Cf. Ref. 1

[c] Of the anticipated double doublets in $CDCl_3$ *for the* C_5'–H_e, *B* portion of the ABX spectrum, only the lower field doublet centered at ca. 4·23 (J = 5 c/s) is clearly visible; the upper field doublet centered at ca. 4·03 is overlapped by the proton on C_{12}. In pyridine the C_{12}–H signal moves significantly downfield, thereby uncovering the upper field doublet, centered at 4·23 (J = 5 c/s), but now overlaps the lower field doublet centered at ca. 4·45 (cf. Ref. 8).

[d] Of the anticipated double doublet for the C_5'–H_a, A portion of the ABX spectrum, the lower field doublet centered at 3·71 (J = 8 c/s) is clearly visible in pyridine; the upper field doublet centered ca. 3·50 is overlapped by the signal of the OMe (cf. Ref. 8).

[e] Appears as two doublets with splittings of 5 cps and separation of ca. 8 c/s; center given.

[f] See text.

(4·03, broad singlet, C_{12}—H). The overlapping signals (2·04), in $CDCl_3$, for the acetyl methyls were resolved in pyridine and three sharp singlets (2·00, 2·05, and 2·13 ppm) were observed. A complex series of bands, 4·7–5·2 ppm, representing four hydrogens, may be ascribed to the vinyl proton on C_{24} and the protons on C'_2, C'_3, and C'_4 of the xylose moiety. The multiplets at 3·10–4·30 representing the three hydrogens associated with the protons on C_3 and C'_5 were obscured by the superimposition of the methoxyl Me and C_{12}—H protons. A doublet centered at 4·55 (J = 6 c/s) may be ascribed to the proton on C'_1. Its chemical shift and splitting established the β-orientation[8] of the xylose. The latter assignment was confirmed by the NMR spectrum of VIA in pyridine, where the doublet was shifted downfield to 4·85 and the splitting (6 c/s) remained unchanged. The NMR spectrum of the unacetylated xyloside (VI) in pyridine confirmed the presence of the second vinyl proton (5·22, multiplet, C_{24}—H). In compound VI the protons attached to C'_2, C'_3, and C'_4 are found relatively upfield (3·4–4·4), thereby revealing the vinyl proton on C_{24}. The doublet for the proton on C'_1 was again found at 4·86 (J = 6 c/s). The various assignments are summarized in Table 1. A comparison of the NMR spectra of (VIA) and its corresponding aglycone 12β-methoxy-7,8-dihydro-24,25-dehydroholothurinogenin-3-acetate (X) in both pyridine and chloroform confirm the assigned structure.

The NMR spectra of compounds IVA, V, VI, VIA, and VII in $CDCl_3$ and pyridine are given in Table 1. The previously prepared methoxylated neo-holothurinogenins X and IV the 12β-OMe isomer of IVA are included for comparison. The various assignments for the Me groups (for other proton assignment see text) are based on analogy, comparison with the known holothurinogenins and on the reasonable assumption that an internally consistent picture should obtain. The influence, downfield shift, of the 17α-OH group on the C_{14}—Me has been discussed[1,2] (compare ($CDCl_3$) VIA, VII, and X vs IVA (IV)). Of particular interest is the large downfield shift for the C_{21}—Me, ca. 0·40 ppm on solvent change (pyridine → $CDCl_3$) in all the compounds which contain the 17α-OH group (compare VIA, VII, and X vs IVA (IV)). A similar shift has been noted for the C_{21}—Me in a number of other methoxylated neo-holothurinogenins[1] and holothurinogenins.[7] An analogous finding[9] has been reported for the C_4-methyls of lanosterol, presumably also due to the proximity of the C_3—OH. The enhancement of the downfield shift of the C_{14}—Me group of compound V may be ascribed to the 1:3 diaxial interaction between it and the $C_{12}\alpha$—OAc. A comparable downfield displacement of the $C_8\alpha$—Me (axial) has been observed[10] in fusidic acid and also ascribed to its interaction with the $C_{11}\alpha$—OH-group (axial) present in the molecule.

It has been suggested[1] that in the neo-holothurinogenins the C_{21}—Me group is β-oriented (behind the plane of the lactone ring) as in lanosterol.[11] This proposal was based, in part, on the observation that the C_{21}—Me signal for the C_{12}—β-methoxylated neo-holothurinogenins was significantly displaced in pyridine downfield as compared to their corresponding holothurinogenins, because of the proximity of the Me and OMe groups. No such effect would be anticipated for the C_{12}—α-methoxylated neo-holothurinogenins, in which the OMe group (as shown by Dreiding model) would be too distant from the Me group. The observed signal for the C_{21}—Me in compounds IVA, II (no substitution on C_{12}), and IV appear in $CDCl_3$ at 1·35, 1·35,[2] and 1·47,[1] and in pyridine 1·42, 1·37,[7] and 1·60,[1] respectively. This result is in agreement with the aforementioned postulate.

EXPERIMENTAL

The UV in 95% EtOH and IR spectra were recorded with a Beckman D.U. and Perkin–Elmer IR 421 spectrophotometer, respectively. NMR spectra were determined at 60 Mc/s with a Varian Associates spectrometer, A-60A. The chemical shifts δ are given in ppm relative to an internal TMS standard. M.p's were determined on a Fisher–Jones m.p. block. Alumina IIA, prepared by deactivation of Woehlm neutral alumina activity I with 4% by wt of a 10% AcOH soln, was employed for column chromatography. TLC (20 × 20 cm glass plates) were made on silica gel G and Woehlm neutral alumina, water–sat BuOH–pyridine (3%), and benzene–EtOH (9 : 1), respectively were employed as developing agents. Triple developments were generally made. For detection of spots the plates were heated to 120°, sprayed with a 1 : 1 mixture of anisaldehyde–AcOH–H_2SO_4 (1 : 50 : 0·5) and AcOH–$HClO_4$ (1 : 1) and reheated at 120° for 5 min. The enzyme mixture, "glusulase", was obtained from Endo Products, Incorp., Richmond Hill 18, New York, N.Y.

Desulfated holothurin A

DHA I *and DHA* II. Holothurin A[1] (30 g, R_f = 0·17 (silica gel)), finely powdered, was suspended in MeOH-HCl (0·2N, 1500 ml) (prepared by diluting conc HCl (25·5 ml) with MeOH to 1500 ml) and the mixture stirred and heated at 37° for 7 hr. The hydrolysis was followed by removing aliquots for TLC (silica gel): At the end of the aforementioned period no starting material was present and a chromatographically homogeneous product (R_f = 0·37) was obtained. The sulfate moiety had been removed as its methyl ester (no ppt with $BaCl_2$). Ether (4·5 l.) was added and the mixture allowed to stand in the cold (4°) over night. The ppt was separated by centrigugation, washed with MeOH–ether (1 : 4) (2·5, 1·25, and 0·91 l., centrifuging after each wash), ether (2 ×, 0·9 l.), collected by filtration, and dried *in vacuo* over night over P_2O_5. The product (13·7 g), which retained ether, was suspended in H_2O (100 ml) and the mixture, under slight vacuum for 4 hr, was stirred for 24 hr. The resulting slightly opalescent soln was diluted with H_2O (100 ml) and centrifuged (25,000 rpm) for 45 min. The clear supernatant was taken to dryness by lyophylization, yield: 12·4 g; $[\alpha]_D^{25}$ −36°; R_f = 0·37 (silica gel). DHA I contained no sulfur (Na fusion) and was completely soluble in H_2O. Its properties will be discussed more fully in a separate communication.[4]

DHA II. The original mother liquor and washes of DHA I (see above) were combined and evaporated *in vacuo* at room temp to one l., excess Ag_2CO_3 added, the mixture stirred for 3 hr, and the precipitated AgCl removed. The filtrate was evaporated *in vacuo* to 300 ml and ether (1800 ml) added. The ppt was washed with ether, dried over night *in vacuo* over P_2O_5, and treated in the same manner as described above for DHA I, yield: 12·0 g. DHA II contained no sulfur (Na-fusion) and, though indistinguishable from DHA I, on TLC gave a slightly different mixture of products on enzymatic hydrolysis (see below).

Hydrolysis (HCl, 3N, 100°, 3 hr) of DHA I and DHA II gave qualitatively equivalent mixtures of the holothurinogenins (TLC, alumina, of the acetates) as well as the four sugars, xylose, glucose, 3-O-methylglucose, and quinovose (paper chromatography, Whatman No. 1, phenol–H_2O).[3] The mother liquor from DAH II precipitation (see above) on strong acid treatment (HCl, 3N, 100°, 3 hr) gave sulfuric acid ($BaSO_4$) in addition to the holothurinogenins and the four sugars.

Enzymatic hydrolysis of DHA I *to compounds* III, V, *and* VI

DHA I (5·4 g) was dissolved in NaAc–AcOH buffer (pH 5·2, 0·2N, 500 ml) and "glusalase" (10 ml, Lot No. 61202) added. The mixture was gently stirred and incubated at 37° for 320 hr. The course of the hydrolysis was followed by TLC (silica gel) and reducing value (Nelson's reagent)[12] of the mixture. After 280 hr no starting material remained and the reducing value was constant. Chromatography (Whatman paper No. 1, phenol–H_2O) of a centrifuged aliquot revealed the presence in the centrifugate of, approximately equal quantities of glucose, quinovose, and 3-methoxyglucose and a significantly smaller amount of xylose. The viscous mixture was centrifuged (25,000 rpm) for 1 hr at 4°. The gelatinous ppt was washed with H_2O (4 ×, recentrifuging, 23,000 rpm, following each wash) and dried *in vacuo* over night over P_2O_5. The product (4·5 g), a mixture of proteins, aglycones and xylosides, was extracted with warm MeOH (3 × 20 ml), and the combined extracts, evaporated to dryness at room temp, gave ppt A (2 g). The material, insoluble in MeOH, was extracted thoroughly with warm $CHCl_3$ (4 × 25 ml), and the combined extracts evaporated to dryness *in vacuo* gave ppt B (510 mg).

Ppt A (see above) showed no significant absorption in the UV above 215 mμ and gave three spots on TLC (silica gel, R_f = 0·61, 0·70, 0·76), each of which was in reality a mixture. Hydrolysis of an aliquot in strong acid (HCl, 3N, 100°, 3 hr) gave a mixture of holothurinogenins (TLC of acetates on Woehlm neutral alumina) and xylose only. Ppt A (1·5 g) was dissolved in pyridine (10 ml) and Ac_2O (5 ml) and the mixture allowed

to stand at room temp 24 hr. The acetylated product (1·7 g), precipitated with H_2O, was dried *in vacuo* over P_2O_5, dissolved in warm benzene (5 ml), and chromatographed on alumina IIA (30 g, column prepared in Skelly B-benzene (1:1)). Elution with Skelly B–benzene (1:1; 200 ml) gave a mixture of aglycone acetates (230 mg). All following fractions eluted with benzene–EtOAc mixtures, chloroform and hot MeOH were primarily xyloside mixtures (1·16 g) and not further investigated. The acetylated aglycone mixture (230 mg) was dissolved in benzene (1 ml) and Skelly B (8 ml) and rechromatographed on alumina IIA (9 g, column prepared in Skelly B). Elution was affected with Skelly B–benzene ethyl acetate mixtures and fractions (25 ml) were collected and combined according to their TLC (Woehlm neutral alumina) and NMR spectra as shown in Table 2.

Table 2.

Fraction	Eluent	Vol. (ml)	TLC (cm)[a]	Material (mg)
A	Skelly B–benzene (95·5)	250	11·1, (15·5)	Oil
B	Skelly B–benzene (85:15)	200	12·4, (15·5, 14·0, 13·0)	V (28)
C	Skelly B–benzene (3:7) Skelly B–benzene (1:1)	150 50	12·4' 10·2, (14·0, 13·0)	V + (?) (40)
D	Skelly B–benzene (1:1)	100	7·0, (7·3)	III (21)
E	Skelly B–benzene (1:1)	400	7·0	III (36)
F	Benzene Benzene–EtOAc (4:1) Benzene–EtOAc (1:1) Ethylacetate	300 100 200 100	2·0, 5·1	non-methoxylated mixture (100)

[a] TLC on Woehlm neutral alumina; distance given in cm from origin following triple development; minor products, not identified, are given in ( ).

12α-Acetoxy-7,8-dihydro-24,25-dehydroholothurinogenin-3-acetate (V) *and conversion to* VIII

Fraction B (28 mg) was recrystallized from Skelly B and gave compound V, m.p. 240–243° (dec). For IR (CCl_4) see text; ν_{max} (KBr) 3520 (OH); 1756, 1052, 1042 (γ-lactone); 1733, 1705, 1250 cm^{-1} (C_3 and C_{12} acetates). For NMR spectra see Table 1. Compound V was dissolved in dioxan (0·5 ml) and HCl (3N, 0·25 ml) and the mixture heated on the steam bath for 3 hr. The product, after addition of H_2O, was separated by centrifugation, dried *in vacuo* over P_2O_5, and redissolved in pyridine (0·5 ml) and Ac_2O (0·25 ml). The mixture, after standing at room temp for 48 hr, was decomposed with H_2O and the acetate, separated by centrifugation, was dried over P_2O_5 *in vacuo*, yield: ca. 8 mg, λ_{max} 244 mμ, ε 12,800, λ_{sh} 237, ε 11,000, λ_{sh} 255, ε 9300. A comparison of IR and NMR spectra of this impure product with the known holothurinogenin (VIII) left little doubt as to their identity.

12β-Methoxy-7,8-dihydro-22,25-oxidoholothurinogenin-3-acetate (III) *and conversion to* I

Fraction E (36 mg) was identical in all respects, IR (CS_2, $CHCl_3$), NMR ($CDCl_3$) with authentic material previously prepared.[1] Fraction E (30 mg) was heated in dioxan (0·5) and HCl (3N 0·25 ml) on a steambath for 3 hr. The product was isolated and acetylated following the procedure given above. The acetate proved identical, UV, IR (CS_2, $CHCl_3$), NMR, in all respects with an authentic sample of 22,25-oxidoholothurinogenin-3-acetate (I).

3β-Xyloside of 12β-*methoxy*-7,8-*dihydro*-24,25-*dehydroholothurinogenin* (VI) *and triacetate* (VIA)

Precipitate B (510 mg; see above) was refluxed in MeOH (200 ml) for 0·75 hr, the mixture cooled to room temp and the insoluble material, compound VI (171 mg), was collected and dried *in vacuo* over P_2O_5 over night. It was now insoluble in both $CHCl_3$ and MeOH. Hydrolysis of a portion (3N, HCl, 100°, 3 hr) and paper chromatography (Whatman No. 1, phenol–H_2O) of the hydrolysate showed xylose to be the only sugar present. In the UV the compound exhibited only end absorption above 215 mμ, ν_{max} (KBr) 1742 cm^{-1} (γ-lactone). For NMR spectra see Table 1. The xyloside (100 mg) was dissolved in dioxan (4 ml) and HCl (3N, 2 ml), and the mixture heated on a steam bath for 1 hr. The cooled mixture was diluted with H_2O (40 ml), the ppt (see below) removed and the filtrate, collected, and diluted volumetrically to 100 ml. Aliquots

were removed for duplicate determinations of the xylose present,(Nelson's reagent). Standards of xylose, subjected to the same treatment as the xyloside, were concurrently run. The xyloside (100 γ) gave 20·0 γ of xylose. $C_{35}H_{56}O_9 . 4H_2O$ requires: 20·2 γ of xylose.

The ppt of the dioxan–HCl hydrolysis (70 mg; see above) was dissolved in hot benzene (25 ml) and chromatographed on alumina IIA (3 g, column prepared in benzene). The eluates with benzene (250 ml), benzene–EtOAc (50:1, 250 ml) and benzene–EtOAc (9:1, 200 ml) each gave compound VIII (R_1 = OH) identical in all respects, NMR, IR, UV, with an authentic sample, yield: 30 mg. Elution with higher percentages of EtOAc gave material of decomposition. An authentic sample of compound VIII is also destroyed in large measure by refluxing in the aforementioned dioxan–HCl mixture. Compound VI (40 mg) was dissolved in pyridine (4 ml) and Ac_2O (2 ml) and the mixture allowed to stand at room temp for 24 hr. The triacetate (VIA), obtained in the usual manner, gave a single spot, 1·0 cm from origin, (TLC, Woehlm neutral alumina, triple development). For NMR spectra see Table 1.

The MeOH mother liquor of ppt B (see above) was evaporated to dryness and gave material (340 mg), which was primarily compound VI. Hydrolysis with dioxan–HCl and chromatography (as above) gave again compound VIII and a small quantity of I, identified by its NMR spectrum.

Enzymatic hydrolysis of DHA II *to compounds* IVA, III, *and* VII

DHA II (5·0 g) was dissolved in NaAc–AcOH buffer (pH 5·2, 0·2N, 500 ml) and "glusalase" (20 ml, Lot No. 61259) was added. The mixture was gently stirred and incubated at 37° for 254 hr. The course of the enzymatic hydrolysis was followed as described above and the resulting gelatinous suspension was treated in the same manner as DHA I. The dried product was extracted thoroughly with dioxan (4 × 50 ml), the dioxan evaporated at room temp *in vacuo* to dryness, and the residue (1·9 g) reextracted with ether (3 × 50 ml). The ether extract, on evaporation to dryness gave a mixture of aglycones (485 mg). (The material (1·3 g), not soluble in ether, was a mixture of xylosides and not further investigated.) The aglycone mixture (485 mg), dissolved in pyridine (4 ml) and Ac_2O (2 ml), was allowed to stand at room temp 20 hr. The acetylated product (498 mg), recovered in the usual manner, was redissolved in a mixture of warm benzene (3 ml) and Skelly B (2 ml) and chromatographed on alumina IIA (20 g, column prepared in Skelly B). Elution was effected with Skelly B–benzene mixtures and finally EtOAc–MeOH. Fractions (25 ml) were collected and combined according to TLC (Woehlm neutral alumina) and their NMR spectra.

TABLE 3.

Fraction	Eluent	Vol. (ml)	TLC (cm)[a]	Material (mg)
A	Skelly B–benzene (95:5 3:1)	500		Oil (traces)
B	Skelly B–benzene	75	16·5	IVA (16)
C	Skelly B–benzene (1 1)	300	16·5, (13·5, 15·5*, 8·5*)	mixt. (34)
D	Skelly B–benzene (1:2) Skelly B–benzene (1:4)	500 200	7·8	III (82)
E	Skelly B–benzene (1:4) Benzene	600 100	4·5, (3·4)*	VII (26)
F	Benzene–EtOAc(4:1)–EtOAc	1000	(2·8, 1·6)	non-methoxylated aglycone mixt. (132)
G	$EtOAc–CHCl_3$ (1:1) $EtOAc–CH_3OH$ (1:1)	200 600	0·0	? unacetylated material (120)

[a] TLC on Woehlm neutral alumina; distance given in cm from origin following triple development. Unidentified products ( ), those starred ( ),* present in trace amounts.

12α-Methoxy-7,8-dihydro-22,25-oxidoholothurinogenin-3-acetate (IVA) *and conversion to compound* II

Fraction B was recrystallized from MeOH and gave compound IVA, m.p. 205–208°. For IR (CS_2) see text, for NMR spectra see Table 1. Compound IVA (10 mg) in dioxan (0·4 ml) and HCl (3N, 3·2 ml) was heated on a steam bath for 3 hr. The product was isolated (as described for compound V) and recrystallized from MeOH. It was identical in all respects, m.p., IR (KBr), NMR ($CDCl_3$) and UV, with an authentic sample of II.

12β-Methoxy-7,8-dihydro-22,25-oxidoholothurinogenin-3-acetate (III).

Fraction D (82 mg) was identical in all respects, m.p., IR (CS_2), NMR ($CDCl_3$) with an authentic sample.[1]

12β-Methoxy-7,8-dihydroholothurinogenin-3,22-diacetate. (VII)

Fraction E (26 mg) was identical in all respects, IR (KBr), NMR ($CDCl_3$), with an authentic sample.[1]

Acknowledgements—The authors wish to express their thanks to the National Science Foundation (Grant GB-6645), Office of Naval Research (Contract NR 107 543), and the National Institutes of Health (Grant AM 06180) for their generous support. We gratefully acknowledge the help of Prof. C. Djerassi, Stanford University, Stanford, Calif., who so kindly furnished the mass spectroscopy measurements. We also wish to express our appreciation to the Lerner Marine Laboratory, Bimini, Bahamas, who made their facilities available for the collection of Actinopyga agassizi.

REFERENCES

[1] J. D. Chanley and C. Rossi, *Tetrahedron* **25**, 1897 (1969).
[2] J. D. Chanley, T. Mezzetti and H. Sobotka, *Ibid.* **22**, 1857 (1966).
[3] J. D. Chanley, R. Ledeen, T. Wax, R. F. Nigrelli and H. Sobotka, *J. Am. Chem. Soc.* **81**, 5180 (1959); J. D. Chanley, J. Perlstein, Ross F. Nigrelli and H. Sobotka, *Ann. N.Y. Acad. Sci.* **90**, 902 (1960).
[4] J. D. Chanley, E. Feageson, T. Mezzetti and C. Rossi, to be communicated.
[5] C. W. Shoppee and B. C. Newman, *J. Chem. Soc.* (C), 981 (1968).
[6] G. F. H. Green, T. E. Page and Susan E. Stanisforth, *Ibid.* (B) 807 (1966).
[7] J. D. Chanley and C. Rossi, to be communicated.
[8] R. U. Lemieux, R. K. Kullnig, H. J. Bernstein and W. G. Schneider, *J. Am. Chem. Soc.* **80**, 6098 (1958).
[9] B. Hampel and J. M. Kraemer, *Tetrahedron* **22**, 1601 (1966).
[10] D. Arigoni, W. von Daehne, W. E. Godfredsen, A. Melera and S. Vangedahl, *Experientia* **20**, 344 (1964).
[11] L. F. Fieser and M. Fieser, *Steroids* pp. 334–340. Reinhold, New York, N.Y. (1959).
[12] N. Nelson, *J. Biol. Chem.* **153**, 375 (1944).