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THE HOLOTHURINOGENINS—II[1]

METHOXYLATED NEO-HOLOTHURINOGENINS

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Abstract—Holothurin A, on hydrolysis in methanol-HCl (0·2N; 50°) yielded the methoxylated neo-holothurinogenins: 12β-methoxy-7,8-dihydro-22,25-oxidoholothurinogenin (III), its desoxy analog (IV), 12β-methoxy-7,8-dihydro-24,25-dehydro (V), 12β-dimethoxy-7,8-dihydro (VI), and 12β-methoxy-22-hydroxy-7,8-dihydroholothurinogenin (VII). Their structures and relationships to the neo-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 methoxylated neo-holothurinogenins in benzene, chloroform and pyridine are reported.

MANY species of sea-cucumbers in the family of holothuroidea of the phylum Echinodermata contain a poisonous principle, presumably for their defense against predators. Holothurin, the toxic saponin principle, obtained from the Carbibean species, *Actinopyga agassizi*, has been the subject of a number of physiological[2] and chemical investigations.[3] Recently, we have reported[1] on the structures of a few of the holothurinogenins, 22,25-oxidoholothurinogenin (I), its 17-desoxy derivative (II), and holothurinogenin U, the latter a mixture of closely related genins possessing the same annular portion as compounds I or II, but with open side-chains. The holothurinogenins, derived from the glycoside holothurin by strong acid hydrolysis, differ, however, from the corresponding aglycone moieties, neo-holothurinogenins, as they exist in native holothurin. The holothurinogenins contain the 7:8, 9:11 heteroannular conjugated diene system, holothurin does not. This diene system is formed during the strong acid hydrolysis. Additionally, the question has been raised as to whether other substantial changes in the triterpenoid moiety, e.g. closure of the oxide ring, lactone ring rearrangement, had resulted from strong acid treatment.

To obtain the unmodified aglycones we have investigated the mild non-aqueous acid hydrolysis of holothurin A[3], the principal glycosidic constituent of holothurin. In this paper we report on the methoxylated neo-holothurinogenins,[4] 12β-methoxy-7,8-dihydro-22,25-oxidoholothurinogenin (III), its 17 desoxy analog, compound IV; 12β-methoxy-7,8-dihydro-24,25-dehydro (V), 12β, 25-dimethoxy-7,8-dihydro (VI), and 12β-methoxy-22-acetoxy-7,8-dihydroholothurinogenin (VII) (Fig. 1) obtained from holothurin A by methanol–HCl hydrolysis. Their structural relationships to the neo-holothurinogenins, the known holothurinogenins, and NMR spectra are discussed.

Hydrolysis of holothurin A in CH_3OH–HCl (0·2N; 50°) effected the removal of the entire sugar residue without formation of the conjugated diene system and gave

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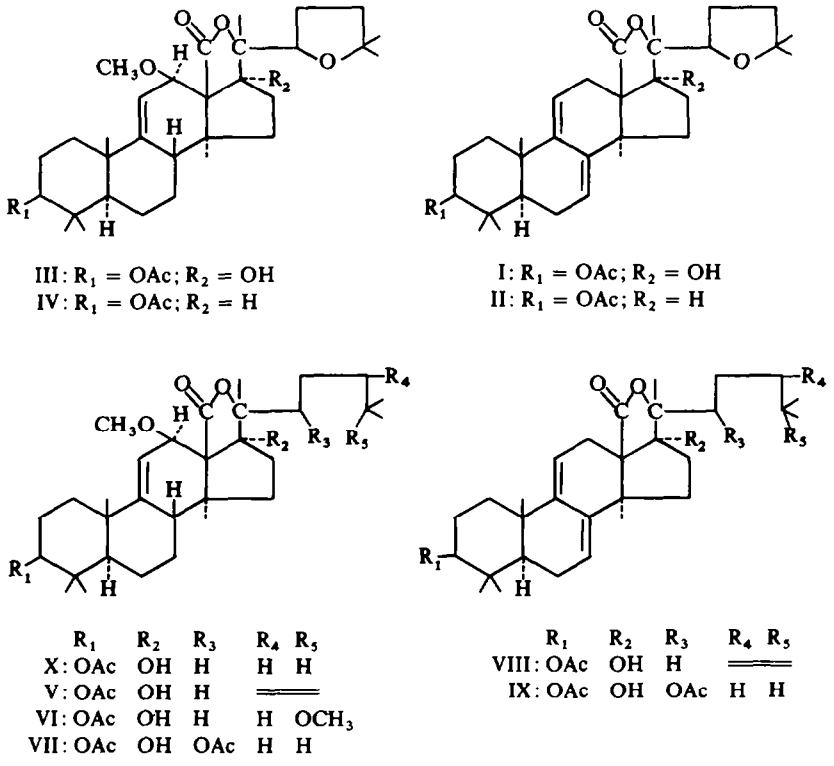


	R_1	R_2	R_3	R_4	R_5
X:	OAc	OH	H	H	H
V:	OAc	OH	H	═	═
VI:	OAc	OH	H	H	OCH_3
VII:	OAc	OH	OAc	H	H

	R_1	R_2	R_3	R_4	R_5
VIII:	OAc	OH	H	═	═
IX:	OAc	OH	OAc	H	H

FIG. 1

a mixture of mono- and dimethoxylated neo-holothurinogenins. Holothurin A contains no OMe group other than the one associated with 3-methoxyglucose[3] in its sugar residue, which additionally contains xylose, glucose, and quinovose. The separation of the individual components was achieved by chromatography of their acetates. Their identification (described in detail below), was confirmed by their conversion on strong acid treatment to their corresponding known holothurinogenins. The monomethoxylated compounds III, IV, V and VII gave the known holothurinogenins I, II, VIII[5] and IX (griseogenin),[6] respectively (Fig. 1). The dimethoxylated neo-holothurinogenin VI, the principal constituent of the mixture, also gave VIII.

The hydrolysis of dihydroholothurin A in MeOH–HCl was investigated, since the possibility existed that even under these mild conditions the 22,25-oxido ring common to compounds III and IV was formed via cyclization of a precursor with an hydroxyl at C_{22} and a double bond issuing from C_{25}. The 22,25-oxido compounds III and IV were again obtained, as was compound VII. Neither the dimethoxylated holothurinogenin VI, nor the related unsaturated monomethoxylated holothurinogenin V were found. In their place the dihydromonomethoxylated neo-holothurinogenin X was obtained. Clearly, the second OMe group in compound VI resulted from the

addition of methanol to a double bond. A comparison of the NMR spectra (cf. Table 1) of compounds V, VI, VII and X, demonstrated that this OMe group was situated on C_{25} and obviously derived by addition of methanol to its immeduate precursor, compound V.

The fact that each of the methoxylated neo-holothurinogenins gave, on strong acid treatment, the conjugated 7:8, 9:11-diene system, characteristic of the holothurinogenins, and the ease with which the one OMe common to all these compounds was substituted, suggested that an OH group, α to a double bond, present in the parent holothurin, was replaced. Confirmation was obtained by hydrolysis of dihydroholothurin A in dioxan–HCl. The mixture of mono-unsaturated aglycones (as acetates) exhibited a broad singlet at δ 5·6 ppm (not present in the methoxylated compounds), which may be attributed to the proton on the C atom bearing the replaceable OH group, since by subsequent treatment of this mixture with methanol–HCl this singlet was eliminated with the concomitant appearance of a broad singlet at ca. δ 3·5, characteristic of the proton on the C atom bearing the aforementioned OMe group (Table 1). The NMR spectra and spin decoupling experiments (discussed below) of the various methoxylated precursors, were in accord with the assigned structures and established both the position, C_{12}, and stereochemistry, β, of the OMe group in each of the aforementioned precursors. The IR spectra of the precursors, especially with respect to the position of the lactone absorption, corresponded closely to that observed for their derived holothurinogenins.

These findings strongly support the presumption that, except for the replacement of an OH on C_{12} by a OMe, the methoxylated neoholothurinogenins correspond in structure to the aglycone moieties as they exist in holothurin A. The question as to the stereochemistry, α, and/or β, of the C_{12}–OH in holothurin A is discussed in the following paper. We discuss below the identification of compounds III, IV, V, VI and VII.

12β-*Methoxy*-7,8-*dihydro*-22,25-*oxidoholothurinogenin*-3-*acetate* (III). $C_{33}H_{50}O_7$, m.p. 273°, $[\alpha]_D^{25}$ −60°. The compound exhibited no significant absorption in the UV above 215 mμ. The IR (CS_2) spectrum showed the presence of an OH (3570), γ-lactone (1766), acetyl (1730), and side-chain ether (1124 cm^{-1}). These bands correspond to those observed[1] for 22,25-oxodoholothurinogeninacetate (I). The seventh oxygen was accounted for in the OMe group (1089 cm^{-1}). The NMR spectra (60 Mc/s, $CDCl_3$) showed the presence of one vinyl hydrogen (5·37, broad singlet); and two hydrogens (overlapping) centered at 4·12 (C_{12}—H and C_{22}—H). In benzene and pyridine a clear separation into a triplet and a broad singlet was achieved. The triplet, centered at 4·08 (J = 7 c/s) and 4·26 (J = 7 c/s) in benzene and pyridine, respectively, corresponded in both chemical shift and coupling constant to the observed for the C_{22}—H proton in 22,25-oxidoholothurinogenin (I). The second signal, which appeared as a broad singlet at 4·37 and 4·50 in benzene and pyridine, respectively, may be ascribed to the proton on the C atom bearing the OMe group. Its disposition, α to the double bond, was indicated by its relative downfield chemical shift as compared to such protons in saturated molecules.[7] This conclusion was confirmed by spin decoupling experiments at 100 Mc/s. Irradiation ($CDCl_3$) at 4·12 resulted in narrowing of the broad singlet (5·37) of the vinylic hydrogen. Of the various possibilities for the position of the double bond, 5:6, 7:8, 9:11, with the OMe group at C_7, C_6 or C_{12}, respectively, the following considerations clearly establish β sub-

stitution at C_{12} and the double bond in the 9:11 position. The significantly further downfield position of this proton as compared to its 17-desoxy analog (IV), (Table 1) indicated the profound influence of the 17-α—OH group. The proximity of an α (axial) oriented hydrogen on C_{12} to the $C_{17\alpha}$—OH group (1:3, diaxial type interaction) could well account for the observed differences.[8] No such effect would be anticipated for the protons (C_6—H, C_7—H) of the alternative possibilities. The NMR investigations of Greene *et al.*[9] on a number of 9:11 dehydrotigogenins substituted on C_{12} with an OH, OAc or OMe group have demonstrated that in all those derivatives, in which the substituent is α (axial). both the vinylic proton, C_{11}—H, and the $C_{12}\beta$—H proton appear as doublets with a coupling constant of ca. 6 c/s, while for the 12β-oxygenated (equatorial) derivatives both signals appear as broad singlets. The broadness of these signals in compound III results from the small coupling between the C_{11} and C_{12} protons as well as allylic and homoallylic coupling of the C_8-proton (β, axial) with the vinylic and C_{12}—H protons,[7α, 10] respectively (see below). Confirmatory evidence for the 9:11-dehydro-12β-OMe assignment was forthcoming from the comparison of the chemical shift for the vinylic hydrogen (5·38 ppm), which corresponds to that reported for the C_{11} proton of 12β-methoxy-9:11-dehydrotigogenin acetate (5·34)[9] and not the 12α-OMe analog (5·64).[9] The vinylic proton for 7α-methoxy-cholest-5-ene-3β-diol[11] appears at 5·73 ppm ($J_{6,7\beta} = 5{\cdot}5$ c/s). The C_{12}—H proton in both compounds, III and IV, we may note, appears significantly further downfield than that reported for 12β-methoxy-9-dehydrotigogenin acetate (3·42). We presume that the lactone moiety (C_{18}—C_{20}) is in part responsible for this.

17-*Desoxy*-12β-*methoxy*-7,8-*dihydro*-22,25-*oxidoholothurinogenin*-3-*acetate* (IV) $C_{33}H_{50}O_6$, m.p. 281–282°, $[\alpha]_D^{25}$ −45°. In the UV, the compound showed no significant absorption above 215 mμ. IR (CS_2) absorption bands attributable to a γ-lactone (1761), acetyl (1730), side-chain ether (1132, 1107), and OCH_3 (1080 cm^{-1}), accounted for all the oxygens present in the molecule. No IR absorption band for an OH group was noted. Corresponding IR absorption bands (except for the OMe group) are observed[1] in its derived holothurinogenin (II). The NMR ($CDCl_3$) spectrum showed the presence of one vinyl hydrogen (5·37, broad singlet; C_{11}—H); a broad singlet at 3·93 (C_{12}—H) partially overlapped by a just discernible triplet centered at 3·99 (J = 7 c/s, C_{22}—H). In benzene the signals for the C_{12} and C_{22} protons were clearly separated and appeared at 3·81 (broad singlet), and 4·07 (triplet, J = 7 c/s), respectively. The position and splitting of the triplet corresponded to that for the C_{22}—H proton of its derived holothurinogenin (II).[1] The 12β-assignment for the OMe grouping was deduced following the same line of argument presented above for the 12β-OMe substituted neo-holothurinogenin (III). Confirmation of these assignments was obtained in the 100 Mc/s spectra and spin decoupling experiments. A clear separation of the C_{12}—H proton (3·88, broad singlet) and the C_{22}—H proton (3·95, triplet, J = 7 c/s) was achieved. Irradiation at 3·88 resulted in the transition of the broad signal for the vinylic hydrogen to a doublet (J = ca. 1·5 c/s); conversely, irradiation of the C_{11} proton (5·29) transformed the broad signal for the C_{12} proton to a doublet (J = ca. 1 c/s). These decoupling experiments clearly demonstrated that allylic and homoallylic coupling of the C_8 proton with the C_{11} and C_{12} protons, respectively, was also present. Homoallylic coupling would only be anticipated, if both the C_{12}—H and C_8—H bonds were approximately perpendicular to the plane of the 9:11 bond.[10] An examination of Drieding models

demonstrated that this condition is fulfilled, when the protons on C_8 and C_{12} are β (axial) and α (axial), respectively.

12β-*Methoxy*-7,8-*dihydroholothurinogenin*-3-22, *diacetate* (VII) $C_{33}H_{54}O_8$, m.p. 310° (dec). Compound VII showed no absorption in the UV above 215 mμ. IR ($CHCl_3$) absorption bands attributable to an OH (3604, 3500 bonded); γ-lactone (1753); 2-acetyls (1720–1710) and OMe group (1086 cm^{-1}) accounted for the eight oxygens present in the molecule. The NMR ($CDCl_3$) spectrum showed the presence of seven methyls (0·88, 0·93, 1·25, 1·08, 1·55, 0·88 (2), J = 6 c/s); two acetyl methyls (2·08, 2·12); OMe (3·45); one vinyl hydrogen (5·37; C_{11}—H); one proton on C_{12} bearing the OMe (4·13); and two protons on the C atoms bearing the acetyl groups (4·56, 5·08 ppm C_3—H and C_{22}—H, respectively). The broad singlet nature of the protons on C_{11} and C_{12} established the C_{12} proton as axial (*vide supra*).

12β,25-*Dimethoxy*-7,8-*dihydroholothurinogenin*-3-*acetate* (VI) $C_{34}H_{54}O_7$, m.p. 272–273°, $[\alpha]_D^{25}$ −51° ($CHCl_3$). The observed mol ion peak at *m/e* 574 as well as peaks at *m/e* 542, 510 (loss of one and two moles of methanol respectively) and *m/e* 559 (loss of a Me group) confirmed the molecular formula and the presence of two OMe groups. The compound showed no significant absorption in the UV above 215 mμ. The IR ($CHCl_3$) showed absorption bands for an OH (3600, 3480 bonded); γ-lactone (1750); acetyl (1720 broad), and OMe group (1090 cm^{-1}); ν_{max} (KBr) 1743 (lactone) and 1722 (acetate). In CCl_4 the band for the acetate was split into two, 1728 and 1710 (small and sharp). This splitting of the acetyl in CCl_4 appears to be characteristic of the holothurinogenins belonging to the open side-chain series. We have noted the same behavior with holothurinogenin (VIII)[5], obtained from VI or holothurin A following strong acid treatment. The very limited solubility of compounds VI and VIII in CCl_4 suggests that in this solvent gels rather than true solutions are obtained. Hydrolysis of compound VI with alkali furnished the corresponding alcohol, (R_1 = OH), whose IR showed only one band at 1751 cm^{-1} ($CHCl_3$) and 1733 cm^{-1} (KBr) attributable to the lactone. The NMR ($CDCl_3$) spectrum showed the presence of one vinyl hydrogen (5·41; C_{11}—H); two OMe groups (3·45, 3·20; C_{12} and C_{25}, respectively); and one proton (4·12, C_{12}—H) on the C atom bearing a OMe group (Table 1). The broad singlets, attributable to the protons on C_{11} and C_{12}, indicated that the C_{12} proton (*vide supra*) was axial. This was confirmed by spin decoupling experiments at 100 Mc/s. Irradiation of the vinyl proton at 5·37 resulted in the collapse of the broad singlet at 4·12 to a doublet (J = 1·5 c/s); alternatively irradiation at 4·12 ppm resulted in a sharpening of the signal at 5·41. As with compound V (*vide supra*) the broadness of the signals for the protons on C_{11} and C_{12} arises from their coupling with each other and allylic and homoallylic coupling,[10] respectively, with the proton (β, axial) on C_8.

12β-*Methoxy*-7,8-*dihydro*-24,25-*dehydroholothurinogenin*-3-*acetate* (V) $C_{37}H_{50}O_6$; m.p. 245–247° (dec) $[\alpha]_D^{25}$ −53° ($CHCl_3$). IR (CCl_4), absorption bands, attributable to an OH (3600, 3500); γ-lactone (1764); acetyl (1730), and OMe (1091 cm^{-1}) accounted for the six oxygens present in the molecule. The NMR spectrum ($CDCl_3$) showed seven methyls: five tertiary (0·88, 0·93, 1·08, 1·27, 1·55) and two on a double bond (1·67, 1·73); one OMe (3·45); two vinyl hydrogens (5·37, C_{11}—H; 5·16, C_{24}—H); and a proton on the C atom bearing the OMe group (4·12, C_{12}—H). The chemical shift and broad singlet character of the signals attributable to the C_{11} and C_{12} protons corresponded in three solvents ($CDCl_3$, pyridine and benzene) with those observed

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

Comp.	Solv.	Me groups at 4,4	Me groups at 10β	Me groups at 14α	21	26,27	3H[1]	11H	12αH	22H	12β-OMe	3-OAc	
IV	$CDCl_3$	0·88, 0·93	1·26	0·93	1·47	1·24, 1·26	4·57	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	1·22, 1·28	4·68	5·46 b	4·05 b	4·15 (t, 7)	3·47	2·08	
	C_6D_6	0·92, 0·95	1·37	0·78	1·57	1·13, 1·22	4·72	5·35 b	3·81 b	4·07 (t, 7)	3·22	1·80	
III	$CDCl_3$	0·88, 0·93	1·26	1·10	1·47	1·26, 1·26	4·55	5·37 b	4·12 b	4·12[2]	3·45	2·08	
	C_6D_5N	0·93, 0·97	1·35	1·40	1·79	1·10, 1·18	4·72	5·55 b	4·50 b	4·26 (t, 7)	3·57	2·08	
	C_6D_6	0·92, 0·97	1·38	1·08	1·63	1·12, 1·20	4·72	5·43 b	4·32 b	4·08 (t, 7)	3·38	1·79	
													24H
V	$CDCl_3$	0·88, 0·93	1·27	1·08	1·55	1·67, 1·73	4·56	5·37 b	4·12 b	n.i.	3·45	2·08	5·16 m[3]
	C_6D_5N	0·95, 0·98	1·37	1·40	1·90	1·57, 1·65	4·72	5·55 b.	4·50 b	n.i.	3·52	2·08	5·23 m
	C_6D_6	0·93, 0·97	1·37	1·12	1·63	1·60, 1·72	4·75	5·45 b	4·20 b	n.i.	3·27	1·78	5·25 m
													25-OCH_3
VI	$CDCl_3$	0·88, 0·93	1·27	1·10	1·55	1·17, 1·17	4·56	5·41 b	4·12 b	n.i.	3·45	2·08	3·20
	C_6D_5N	0·95, 0·97	1·35	1·40	1·90	1·08, 1·08	4·72	5·55 b	4·50 b	n.i.	3·52	2·08	3·14
	C_6D_6	0·93, 0·97	1·38	1·13	1·63	1·10, 1·10	4·73	4·45 b	4·20 b	n.i.	3·28	1·78	3·09

												22-OAc
VII	$CDCl_3$	0·88, 0·93	1·25	1·80	1·55	0·88 (d, 6)	4·56	5·37 b	4·13 b	5·08 m	3·45	2·08	2·12
	C_6D_5N	0·95, 0·97	1·35	1·37	1·97	0·83 (d, 6)	4·72	5·55 b	4·50 b	5·24 m	3·53	2·05	2·11
	C_6D_6	0·93, 0·93	1·37	1·12	1·73	0·88 (d, 6)	4·73	5·43 b	4·23 b	5·08 m	3·31	1·76	1·68
X	$CDCl_3$	0·88, 0·93	1·25	1·08	1·53	0·88 (d, 6)	4·56	5·37 b	4·11 b	n.i.	3·45	2·08	
							100 Mc/s						
IV	$CDCl_3$	0·86, 0·91	1·22	0·91	1·42	1·23, 1·25	4·46	5·29 b	3·88 b	3·95 (t, 7)	3·39	2·04	
III	$CDCl_3$	0·86, 0·91	1·23	1·08	1·44	1·23, 1·25	4·49	5·30 b	4·05 b	4·05[2]	3·39	2·04	
													24H
V	$CDCl_3$	0·88, 0·92	1·24	1·08	1·52	1·63, 1·69	4·49	5·31 b	4·06 b	n.i.	3·40	2·05	5·08 m[3]
													25-OCH_3
VI	$CDCl_3$	0·86, 0·91	1·23	1·07	1·52	1·16, 1·16	4·58	5·32 b	4·07 b	n.i.	3·40	2·04	3·15

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

[1] This proton appeared as a multiplet or two doublets with splitting of 5 c/s and separation of ca. 8 c/s.

[2] Overlapping with C_{12}–H obscures triplet (see text).

[3] The signal ($CDCl_3$) integrates for 0·85 hydrogens. A broad singlet ca. 4·75 equivalent to ca. 0·3 hydrogen is also found, indicating the presence of the C_{22}=CH_2 isomer (15%). Recrystallization did not significantly alter the ratio of these two signals. In benzene and pyridine the latter signal was overlapped by the C_3—H proton.

with compounds VI, VII and clearly demonstrated that the OMe group was 12β (Table 1).

12β-*Methoxy*-7,8-*dihydroholothurinogenin*-3-*acetate* (X) $C_{33}H_{52}O_6$; m.p. 263–267°. Compound X showed no absorption in the UV above 215 mμ. The NMR spectrum showed the presence of seven methyls (0·88, 0·93, 1·08, 1·25, 1·53, 0·88(2), J = 6 c/s); one acetyl methyl (2·08); one OMe (3·45); one vinyl hydrogen (5·37, C_{11}—H); and one proton on the C atom bearing the OMe group (4·11, C_{12}—H). The similar disposition of these signals as compared to griseogenin (IX) and compound V, coupled with the observation that compound X is only obtained from the hydrolysis of dihydroholothurin A, leaves little doubt as to the assigned structure.

The NMR spectra of the methoxylated neo-holothurinogenins have been examined in chloroform, benzene and pyridine. The results are summarized in Table 1. The various assignments follow from both the discussions given above, analogy with the holothurinogenins, and the reasonable assumption that an internally consistent picture should obtain for these closely related compounds. The Me groups on C_4 and C_{10} are essentially in the same environment in all these compounds and would have similar chemical shifts in all the solutions, as indicated in Table 1. As previously discussed[1] for the holothurinogenins I and II, the deshielding effect of the 17α—OH group (1,3-diaxial interaction) causes a downfield shift of the C_{14} Me in compound III as compared to compound IV. Thus, with the C_{21} assigned[1,12] the farthest downfield position and the C_{26} and C_{27} methyls equivalent ($CDCl_3$), the assignments for compounds III and IV are uniquely defined. The assignments of the Me groups of the "open side-chain" methoxylated neo-holothurinogenins, all of which contain a 17α—OH, follow by comparison with the compounds III and IV and the known structural interrelationships of the compounds within the group.

A number of interesting chemical shifts observed on solvent change ($CDCl_3 \rightarrow C_6D_5N$ and/or C_6D_6) deserve comment. The chemical shifts for the methyls on C_4 are relatively little affected in the various solvents. Particularly noteworthy is the non-equivalence, observed in benzene and pyridine, of the C_{26} and C_{27} methyls of the 22,25-oxidoholothurinogenins (III and IV) as well as the relatively large downfield shift noted for one of these methyls. The C_{14}—Me is displaced significantly downfield in pyridine only and in only those compounds which contain the 17α—OH group, while the C_{10} and C_{21} methyls are displaced downfield in both benzene and pyridine. The magnitude of the shift for the C_{21} methyls in pyridine is again clearly dependent on whether the compound contains a 17α—OH group. In pyridine the C_{21}—Me group of compounds III, V and VI are displaced downfield 20 ± 1 c/s as compared to $CDCl_3$; in compound IV (no 17α—OH) the displacement is 8 c/s downfield. A similar comparison[5] of the C_{21} Me in holothurinogenin II with the holothurinogenins I, VIII and IX showed the same effect of pyridine. No downfield shift was noted in compound II, while for compounds I, VIII and IX the signal appeared ca. 12 c/s downfield in pyridine as compared to chloroform. Clearly then the 12β—OMe group also contributes to the downfield shift of the C_{21}—Me. It appears reasonable to postulate that for pyridine to have such an effect, both, 17α—OH and 12β—OMe must be close to the C_{21}—Me. In which case it may be argued that the C_{21}—Me is oriented β (behind the plane of the lactone ring) as in the lanosterol series.[13] The α-orientation of the Me group places it perpendicular to the 17α—OH and at much too great a distance from the 12β—OMe for pyridine molecules, pre-

sumably preferentially bound to these groups, to have any substantial magnetic effect (see following paper).

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 and 100 Mc/s with Varian Associates spectrometers A-60A and HA 100. Spin decoupling experiments were performed at 100 Mc/s. The chemical shifts δ are given in ppm relative to an internal TMS standard. M.ps were determined on a Fisher–Jones m.p. block. Alumina IIA, prepared by the deactivation of Woehlm neutral alumina I with 4% by wt of a 10% AcOH soln, was employed for column chromatography. TLC (20 × 20 cm glass plates) on silica gel G and Woehlm neutral alumina with water–sat BuOH–pyridine (3%) and benzene–EtOAc (9:1), respectively, as developing agents. Triple developments were generally employed and distance travelled given in cm from origin. 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.

Starting materials

Holothurin A. Holothurin (50 g) prepared, as previously described,[1] from Cuvier's gland of *Actinopyga agassizi* was dissolved in boiling MeOH (3 l.) and filtered from insoluble material (3·5 g). The filtrate was boiled down to ½ of its volume and allowed to stand overnight in the cold. The ppt (25 g) was recrystallized from MeOH containing H_2O (0·2–0·5%) and gave chromatographically homogeneous holothurin A (15 g) indistinguishable from material previously prepared via cholesterol precipitation,[3] $(\alpha)_D^{25} = -16°$ (H_2O), $R_f = 0·17$ (silica gel). Additional recrystallization did not alter its properties. The NMR and IR spectra will be discussed in a separate communication.[14] *Dihydroholothurin A* was prepared[14] by hydrogenation, at atmospheric pressure, of holothurin A in H_2O, employing PtO_2 as catalyst.

Methanol–HCl hydrolysis of holothurin A

Compounds III, IV, V, VI and VII. Holothurin A (5·0 g) was suspended in MeOH–HCl (0·2 N, 250 ml) (prepared by diluting conc HCl (4·24 ml) to 250 ml with MeOH and the mixture stirred and heated at 50° for 76 hr. A clear soln was obtained in 0·5 hr. The hydrolysis was followed by removing aliquots for TLC (silica gel) and UV absorption. After 76 hr no glycoside remained and little conjugated diene (λ_{max} 244 mμ, ε 1,500) was present in the hydrolysate. The product (1·9 g), $R_f = 0·77$ (silica gel), precipitated with H_2O (1·5 l.), was dried *in vacuo* over P_2O_5, dissolved in a mixture of pyridine (8 ml) and Ac_2O (4 ml)

TABLE 2

Fraction	Eluent	Vol. (ml)	TLC (cm)[a]	Material (mg)
A	Benzene–Skelly B (1:3)	1000	18·5	Oil (19)
B	Benzene–Skelly B (1:1)	235	14·0	IV (75)
C	Benzene–Skelly B (1:1)	250	14, 7·5, (9),* (10),* (12)*	IV + III (110)
D	Benzene–Skelly B (1:1)	650	7·5' (9)*, (10)*, (12)*	III (397)
	Benzene–Skelly B (3:2)	600		
E	Benzene–Skelly B (3:2)	1100	7·0, (7·5)	V (86)
F	Benzene–Skelly B (2:3:1)	500	7·0, 4·0	VI + VII (32)
G	Benzene–Skelly B (2:3:1)	1200	4·0, (3·0)	VII + VI (105)
	Benzene–Skelly B (6:1)	400		
H	Benzene	700	3·0, 4·0	VI + VII (58)
	Benzene–AcOEt (20:1)	150		
I	Benzene–AcOEt (20:1)	250	3·0	VI (439)
J	Benzene–AcOEt (10:1)	200	3·0, 1·0	VI + ? (93)

[a] TLC on Woehlm neutral alumina; distance given in cm from origin following triple development at room temp; subsidiary spots in ( ); those starred ( )* represent minor constituents not identified.

and the mixture allowed to stand at room temp for 3 days. The acetate mixture (1·95 g), precipitated with H_2O, was dried over P_2O_5 *in vacuo*, redissolved in benzene–Skelly B (10:1, 11 ml) and chromatographed on alumina IIA (70 g; column (50 × 2 cm) prepared in Skelly B). Elution was effected with Skelly B–benzene and benzene–EtOAc mixtures. Fractions (25–30 ml) were collected and combined according to their TLC (alumina) and NMR spectra, as shown in Table 2.

17-*Desoxy*-12β-*methoxy*-7,8-*dihydro*-22,25-*oxidoholothurinogenin*-3-*acetate* (IV)

Fraction B (75 mg) was recrystallized (2 ×) from MeOH and gave compound IV, m.p. 281–282° (change in form at 265°); $[\alpha]_D^{25} = -45°$ ($CHCl_3$), end absorption λ 213 mμ (ε 4900). For IR absorption in CS_2 see text; ν_{max} (KBr) 1760 (lactone); 1720, 1248, 1030 (acetate); 1129, 1122 (side-chain ether); 1084 (OCH_3); 1061, 1051, 847 cm^{-1} (lactone and side-chain ether). No IR absorption in the OH region. For NMR spectra see Table 1. (Found: C, 73·54; H, 9·16. $C_{33}H_{50}O_6$ requires: C, 73·03; H, 9·29%).

17-*Desoxy*-22,25-*oxidoholothurinogenin*-3-*acetate* (II) *from compound* IV

Compound IV (10 mg) dissolved in a mixture of dioxan (2 ml) and HCl (3 N; 1·0 ml) was heated on a steambath for 3 hr. The product precipitated with H_2O was dried *in vacuo* over P_2O_5 and treated at 37° for 7 hr with a mixture of pyridine (1 ml) and Ac_2O (0·5 ml). The acetylated product, obtained in the usual manner, was identical, NMR, TLC with an authentic sample[1] of compound II.

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

Fraction D (390 mg) was recrystallized (3 X) from MeOH and gave compound III, m.p. 273–274° (transition at ca. 250°), $[\alpha]_D^{25} -60°$ ($CHCl_3$). For IR (CS_2) see text; ν_{max} (KBr) 3568 (OH); 1752, (γ-lactone); 1722, 1252, 972 (acetate); 1088 (OCH_3) 1138 (side-chain ether); 1072, 1046, 841 cm^{-1} (side-chain ether + lactone). For NMR spectra see Table 1. (Found: C, 70·98; H, 8·87. $C_{33}H_{50}O_7$ requires: C, 70·94; H, 9·02%).

22,25-*Oxidoholothurinogenin*-3-*acetate* (I) *from compound* III

Compound III (25 mg) was heated in dioxan–HCl and acetylated following the same procedure given above for compound IV. The final product proved identical in all respects, IR, NMR, m.p., TLC with an authentic sample[1] of compound I.

12β-*Methoxy*-7,8-*dihydro*-24,25-*dehydroholothurinogenin*-3-*acetate* (V)

Fraction E (86 mg) was recrystallized (2 ×) from EtOAc and yielded compound V, m.p. 245–247°, $[\alpha]_D^{25}$ −53° ($CHCl_3$). For IR (CCl_4) see text; ν_{max} (KBr) 3520 (OH); 1745, 1069, 1044 (γ-lactone); 1723, 1250, 979 (acetate); and 1089 cm^{-1} (OCH_3). For NMR spectra see Table 1. (Found: C, 72·55; H, 9·36. $C_{33}H_{50}O_6$ requires: C, 73·03; H, 9·29%).

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

Fraction G (105 mg) was recrystallized from EtOAc and gave compound VII, m.p. 310°. For IR ($CHCl_3$) see text, for NMR see Table 1.

Griseogenin diacetate (IX) *from compound* VII

Compound VII (19 mg) was heated with dioxan–HCl and acetylated following the same procedure as given above. The product, recrystallized from MeOH, m.p. 256–257°, was identical in all respects, IR, UV, NMR, with an authentic sample[6] kindly provided by Prof. C. Djerassi.

12β,25-*Dimethoxy*-7,8-*dihydroholothurinogenin*-3-*acetate* (VI)

Fraction I (213 mg) was recrystallized (3 × from MeOH and gave compound VI, m.p. 272–273° (transition at ca. 260°), $[\alpha]_D^{25} -52°$ ($CHCl_3$). For IR spectra in $CHCl_3$ and CCl_4 see text; ν_{max} (KBr) 3519 (OH); 1743, 1067, 1042 (γ-lactone); 1722, 1250, 973 (acetate); 1088 cm^{-1} (OCH_3); for NMR spectra see Table 1. (Found: C, 70·80; H, 9·51; mol.wt. 574 (mass spectroscopy). $C_{34}H_{54}O_7$ requires: C, 71·05; H, 9·47%; mol. wt. 574). The mass spectrum revealed the presence of two contaminants, *m/e* 572 and 568. The first probably a dehydro derivative of VI, the second presumably a fragment from a higher mol.wt. material, possibly the 12-acetate derivative of dehydro-VI (M_{600}—CH_3OH).

24,25-*Dehydroholothurinogenin*-3-*acetate from compound* VI

Compound VI was heated in dioxan–HCl and acetylated following the same procedures described

above. The product was chromatographed on alumina IIA (2 g) and the material (10 mg), eluted with benzene (300 ml), was identical, NMR, UV, IR, with an authentic sample[5] of compound VIII (R_1 = OH), prepared directly by strong acid hydrolysis of holothurin A.

Methanol–HCl hydrolysis of dihydroholothurin A → compounds III, IV, VII *and* X

Dihydroholothurin A (4 g) was suspended in MeOH–HCl (0·2 N, 200 ml) and the mixture stirred and heated at 50° for 66 hr. The course of the hydrolysis was followed as described above for holothurin A. The product, precipitated by the addition of H_2O, contained ca. 15% of conjugated dienes (λ_{max} = 244 mμ, ε 3,000), was extracted with ether. The ether soluble residue (0·53 g) was dissolved in pyridine (6 ml) and Ac_2O (3 ml) and the mixture allowed to stand one day at room temp. The acetylated product precipitated with H_2O was dried over P_2O_5 *in vacuo*, redissolved in benzene (3 ml) Skelly B (2) and chromatographed on a column of alumina IIA (20 g, prepared in Skelly B). Elution was effected with Skelly B–benzene–EtOAc mixtures. Fractions (25–30 ml) were collected and combined according to their TLC (alumina) and NMR spectra, as indicated in Table 3.

TABLE 3

Fraction	Eluent	Vol. (ml)	TLC (cm)[a]	Material (mg)
A	Benzene–Skelly B (1:6)	100	17·0	Oil (32)
B	Benzene–Skelly B (1:1)	100	14·0, (10)*	IV (24)
C	Benzene–Skeely B (1:1)	150	9·5, (10)*	I (33)
D	Benzene–Skelly B (1:1)	300	9·5 (9)	I + (X) (45)
E	Benzene–Skelly B (1:1)	1700	9, 8, (7)*	X + III (147)
	Benzene–Skeely B (2:5:1)	300		
	Benzene–Skeely B (6:1)	300		
	Benzene	250		
F	Benzene–AcOEt (20:1)	100	8, (4)	III + (VII) (14)
G	Benzene–AcOEt (20:1)	175	4	VII (42)
H	Benzene–AcOEt (1:1)	200	2, (0·5)	? (40)
	AcOEt	250		

[a] TLC on Woehlm neutral alumina at distance given in cm from origin following triple development; ( ) indicate minor constituents, ( )* unidentified material present in traces.

17-Desoxy-12β-methoxy-7,8-dihydro-22,25-oxidoholothurinogenin-3-acetate (IV)

Fraction B was identified as compound IV by comparison, NMR, TLC, with the authentic sample prepared from holothurin A (see above).

22,25-Oxidoholothurinogenin-3-acetate (I)

Fraction C (33 mg) was recrystallized from MeOH and gave compound I (14 mg), identical in all respects, m.p., UV, IR, NMR, R_f with an authentic sample,[1] prepared from holothurin A by strong acid hydrolysis.

12β-Methoxy-7,8-dihydroholothurinogenin-3-acetate (X)

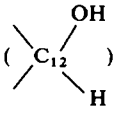
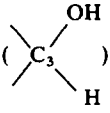
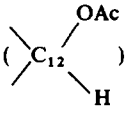
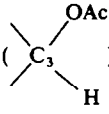
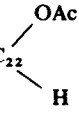
12β-Methoxy-7,8-dihydro-22,25-oxidoholothurinogenin-3-acetate (III). Fraction E (147 mg) was subjected to preparative TLC (Woehlm neutral alumina). Following triple development the material, migrating 9·0 and 8·0 (observed with UV), was extracted from the alumina with $CHCl_3$ and rechromatographed (TLC, triple development). Recrystallization of the slower moving compounent, 8·0, from MeOH gave compound X (17 mg), m.p. 263–267°. For NMR see text. (Found: C, 72·53; H, 9·68. $C_{33}H_{52}O_6$ requires: C, 72·76; H, 9·62%).

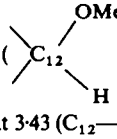
Recrystallization of the faster moving component (9·0) from MeOH gave compound III (20 mg) identical in all respects, m.p., NMR, with that prepared above.

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

Fraction G (42 mg) was recrystallized from EtOAc and gave material identical in all respects, m.p., IR, NMR, with the sample (VII), prepared from holothurin A (see above).

Hydrolysis of dihydroholothurin A in dioxan–HCl (0·2 N), *followed by* MeOH–HCl (0·2 N)

Dihydroholothurin A (0·8 g), suspended in a mixture of dioxan (36 ml), H_2O (3·2 ml) and conc HCl (0·65 ml) was stirred and heated at 55° for 45 hr. A clear soln was obtained after 7 hr. The hydrolysis was followed by removing aliquots for TLC (silica gel) and UV absorption. After 45 hr no glycoside remained and ca. 20% of conjugated dienes (λ_{max} 244 mμ, ε 3,500) were present in the hydrolysate. The product (0·25 g, R_f = 0·69, silica gel) was precipitated with H_2O (250 ml) and dried *in vacuo* over P_2O_5 overnight. The NMR ($CDCl_3$) spectrum of the mixture showed broad singlets at δ 5·20 (C_{11}—H, vinyl proton), and 4·57 (); overlapping multiplets, 4·3–3·6, representing the C_{22} proton of the oxido and OH substituted neo-holothurinogenins; a multiplet at 3·25 () and no signal attributable to Me groups on a double bonded C atom. The crude product (60 mg), dissolved in pyridine (2 ml) and Ac_2O (1 ml), was allowed to stand at room temp for several hr. The acetate mixture (40 mg), precipitated with H_2O, was dried *in vacuo* over P_2O_5. The NMR ($CDCl_3$) spectrum showed broad singlets at 5·75 () and 5·12 (C_{11}—H), a multiplet at 4·57 (); a broad triplet at 4·12 (C_{22}—H of oxido ring), and singlets at 2·08 (C_3 and C_{12} acetyls), and 2·12 (C_{22} acetyl). A multiplet corresponding to the  was covered by the broad singlet at 5.12.

The aforementioned aglycone mixture (50 mg), obtained from dioxan–HCl hydrolysis (see above), suspended in methanol (5 ml) and conc. HCl (0·08 ml), was stirred and heated at 53° for 24 hr. The product (45 mg), precipitated with H_2O, was dried in vacuo over P_2O_5. The NMR ($CDCl_3$) spectrum, which showed broad singlets at δ 5·37 (C_{11}—H, vinyl proton) and 4·12 () (the latter signal covers the triplet for the C_{22}—H proton of the oxido ring), and a singlet at 3·43 (C_{12}—O<u>C</u>H_3) was identical with the NMR spectrum of the mixture obtained directly from dihydroholothurin A on hydrolysis in MeOH–HCl (0·2N) at 55° for 24 hr.

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