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CHEMICAL STUDIES OF MARINE INVERTEBRATES—XXI[1]

SIX TRITERPENE GENINS ARTIFACTS FROM THELOTHURINS A AND B, TOXIC SAPONINS OF THE SEA CUCUMBER *THELONOTA ANANAS* JAEGER (ECHINODERMATA). BIOSYNTHESIS OF THE THELOTHURINS

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Abstract—Aqueous hydrochloric acid hydrolysis of the mixture of thelothurins A and B, the saponins from the sea cucumber *Thelonota ananas* Jaeger, gave a complex mixture from which six sapogenins were isolated. Chemical and spectroscopic evidence led to the assignment of structures **3–8** to these genins which are correlated to the known seychellogenin acetate (**9**). It is also shown that compounds **1** and **2**, obtained by mild aqueous acetic acid hydrolysis of the thelothurins, are the genuine aglycones, whereas **3–8** are artifacts. Their formation during the hydrolysis of the saponins is discussed. The aglycone moiety of thelothurins A (20_A) and B (20_B) was biosynthesized from the acetate.

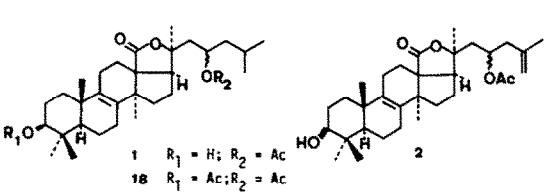
In the previous paper[1] we reported the structures of the two sapogenins 23ξ - acetoxy - Δ^8 - holostene - 3β - ol (**1**) and 23ξ - acetoxy - $\Delta^{8,25}$ - holostadiene - 3β - ol (**2**), derived from thelothurins A and B by mild aqueous acetic acid hydrolysis.

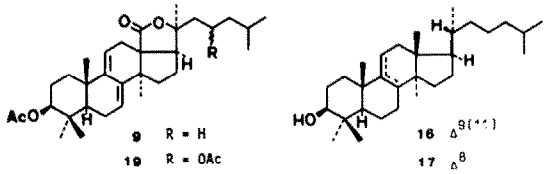
We now report the structures and intercorrelation of six genins **3–8** obtained by the classical method of aqueous hydrochloric acid hydrolysis of the parent saponins, namely 23ξ - acetoxy - $\Delta^{9(11)}$ - holostene - 3β - ol (**3**), $\Delta^{9(11)}$ - holostene - 3β,23ξ - diol (**4**), $\Delta^{9(11),25}$ - holostadiene - 3β,23ξ - diol (**5**), 23ξ - acetoxy - $\Delta^{9(11),25}$ - holostadiene - 3β - ol (**6**), $\Delta^{9(11)}$ - holostene - 3β,23ξ,25 - triol (**7**), and 23ξ - acetoxy - $\Delta^{9(11)}$ - holostene - 3β,25 - diol (**8**). Compounds **3** and **4** have already been described,[2] whereas **5–8** are new sapogenins.

The empirical formula of sapogenin **3**, established by MS (M^+ 514), is $C_{32}H_{50}O_5$. The mass spectrometric fragmentation pattern and the IR spectrum (ν_{OH} at 3450 cm^{-1}, $\nu_{C=O}$ 1765 and 1740 cm^{-1}) of **3** are very similar to those of 23ξ - acetoxy - Δ^8 - holostene - 3β - ol (**1**)[1] from which it is undistinguishable by TLC on silica gel plates impregnated with silver nitrate. Its NMR spectrum, however, (Table 1) differs from that of **1** by the presence of two 1H m at 5.22 (vinylic H) and at 2.93 ppm and of two 3H s at 0.88 and 1.16 ppm assigned to the C-30 and C-19 methyls respectively. The C-30 Me is thus shielded by 0.14 ppm and the C-19 Me deshielded by 0.08 ppm from their respective positions in **1** (Table 1). All these data suggest that **1** and **3** are double bond isomers. The only positions compatible with a trisubstituted C=C in the holostane skeleton[4] are Δ^5, Δ^7 and $\Delta^{9(11)}$. The latter has been found to occur in 23ξ - acetoxy - 17 - deoxy - 7,8 - dihydroholothurinogenin, recently isolated from *Stichopus chloronotus*.[2,3] Indeed, **3** is identical with the latter by IR, MS, m.p., [α] and NMR. Using the more convenient nomenclature based on the holostane skeleton, **3** is thus 23ξ - acetoxy - $\Delta^{9(11)}$ - holostene - 3β - ol.

Compound **4** is a diol of empirical formula $C_{30}H_{48}O_4$ (M^+ 472). The spectral data of **4** (IR: ν_{OH} 3450 cm^{-1}, $\nu_{C=O}$ 1760 cm^{-1}; NMR: Table 1) suggest it to be the corresponding diol of **3**. Indeed, acetylation of **3** and **4**, under usual conditions, affords the same diacetate **10**; moreover, base treatment of **3** yields pure diol **4** which is identical with the already described $\Delta^{9(11)}$ - holostene - 3β,23ξ - diol.[2]

Quite recently, Elyakov *et al.* have reported the isolation of genin **3** from *Thelonota ananas*[5,6] and *Astichopus multifidus*.[6] In both holothurians, **3** is accompanied by another sapogenin whose structure has not been discussed by Elyakov. From the spectroscopic data published by the Russian group and from our own results (*vide infra*) it seems most likely that the second compound of *Thelonota ananas* is diol **4**.





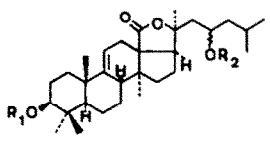
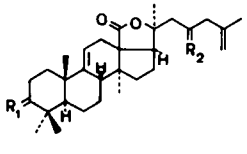
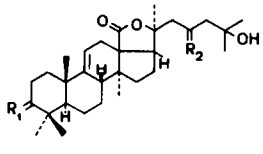
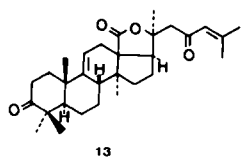


Table 1. NMR spectra of sapogenins **1–8**

	Compound	C-31	C-32	C-19	C-30	C-21	C-26,27	8βH	3αH	23-H	11-H	23ξOAc
1		0.83s	1.02s	1.09s	1.02s	1.44s	0.93 d J=6Hz		3.23m	5.25m		2.05s
3	*Thelonota ananas*	0.83s	1.00s	1.16s	0.88s	1.40s	0.94 d J=7Hz	2.94m	3.19m	5.22m	5.22m	2.04s
	Stichopus chloronotus[2]	0.83s	0.98s	1.15s	0.87s	1.40s	0.91 d J=6Hz	2.95m	3.19m	5.17m	5.17m	2.03s
4	*Thelonota ananas*	0.83s	1.00s	1.16s	0.88s	1.56s	0.94 d J=7Hz	2.95m	3.20m	3.90m	5.23m	
	Stichopus chloronotus[2]	0.82s	0.96s	1.15s	0.87s	1.50s	0.91 d J=6Hz	2.95m	3.20m	?	5.17m	
2		0.84s	1.03s	1.05s	1.00s	1.44s	4.80m;1.77bs		3.20m	5.20m		2.03s
5		0.83s	0.98s	1.14s	0.88s	1.52s	4.85m;1.77bs	2.95m	3.20m	3.96m	5.20m	
6		0.83s	1.00s	1.15s	0.87s	1.44s	4.83m;1.78bs	2.95m	3.19m	5.23m	5.23m	2.05s
7		0.83s	1.00s	1.16s	0.89s	1.54s	1.25s 1.32s	2.92m	3.23m	4.24m	5.23m	
8		0.83s	1.00s	1.15s	0.89s	1.44s	1.25s 1.25s	2.90m	3.22m	5.25m	5.25m	2.04s







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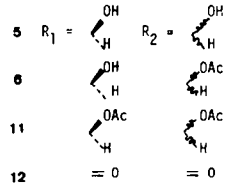
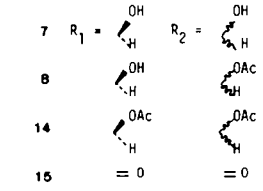
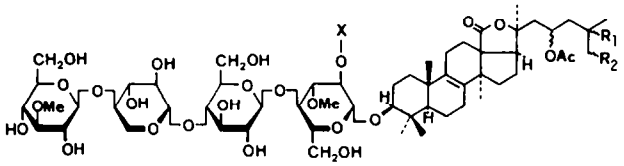




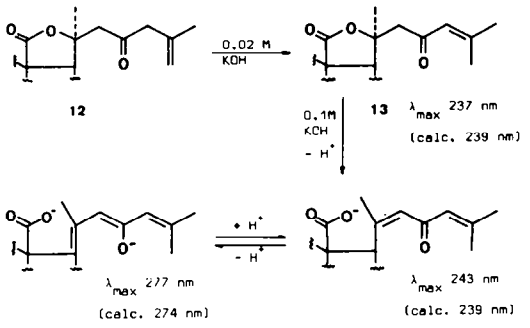


20_A $R_1 = R_2 = H$

20_B $R_1, R_2 = C{=}C$

X: unidentified substituent

The spectral data of genin **5**, $C_{30}H_{46}O_4$ (M^+ 470), establish the presence of a γ-lactone ($\nu_{C=O}$ 1765 cm^{-1}), of two secondary OH groups (ν_{OH} 3500 cm^{-1}, two 1H m at 3.20 and 3.96 ppm H–C–OH) which could be acetylated, under usual conditions, to give diacetate **11** (no ν_{OH} band, $\nu_{C=O}$ 1735 cm^{-1}), of a Me on a double bond (3H bs at 1.77 ppm C=C–CH$_3$), of a vinylidene group ($\nu_{C=C}$ 1650 cm^{-1} and $\delta_{C=CH_2}$ 895 cm^{-1}, 2H m at 4.85 ppm C=CH$_2$) and of a trisubstituted double bond (1H m at 5.20 ppm C=C–H). The absence of an isopropyl group in NMR (Table 1) together with the presence of a Me on a double bond and of a vinylidene group strongly suggest **5** to contain an isopropenyl group in the side chain. This hypothesis is proved by Jones oxidation of **5** into dione **12** (no ν_{OH} band, $\nu_{C=O}$ 1755 and 1715 cm^{-1}) whose NMR spectrum displays two 2H s at 3.07 and 3.14 ppm attributable to the C-22 and C-24 methylenes respectively, two 1H bs at 4.90 and 5.02 ppm due to the C-26 protons and a 1H m at 5.32 ppm (C=C–H). The low field singlet of the C-24 methylene suggests it to be vicinal to two deshielding groups, namely the C-23 carbonyl and a double bond in C-25. This interpretation is further confirmed by the following observations. Dione **12**, showing only end-absorption in neutral methanol, absorbs at 237 nm (ϵ = 10000) in MeOH/KOH 0.02 M. This may be interpreted as a base catalysed double bond migration into conjugation with the C-23 CO function. By increasing the concentration of KOH to 0.1 M, a double absorption is observed at 243 nm (ϵ = 6700) and 277 nm (ϵ = 5600). A tentative explanation is shown in Scheme 1.



Scheme 1.

Moreover, on refluxing dione **12** in pyridine, an isomeric dione **13** is obtained resulting from the isomerisation of the Δ^{25}-double bond in conjugation with the CO at C-23. The spectral data of **13** (UV: λ_{max} 238 nm, ϵ = 13500; IR: $\nu_{C=O}$ 1690 cm^{-1} and $\nu_{C=C}$ 1620 cm^{-1}; NMR: two 3H bs at 1.93 and 2.16 ppm CH_3-27 and CH_3-26 respectively, 1H bs at 6.17 ppm CH-24, disappearance of the 2H s at 3.14 ppm of **12**) are in complete agreement with the published data for 23-oxo-Δ^{24} chromophores.[7] This definitely establishes the 23 - hydroxy - Δ^{25} functionality of the side chain of compound **5**, which was shown to be $\Delta^{9(11),25}$ - holostadiene -3β,23ξ - diol (**5**) by catalytic hydrogenation of **5** into diol **4**, identified by TLC on silver nitrate impregnated silica gel plates, m.p., MS, IR and NMR.

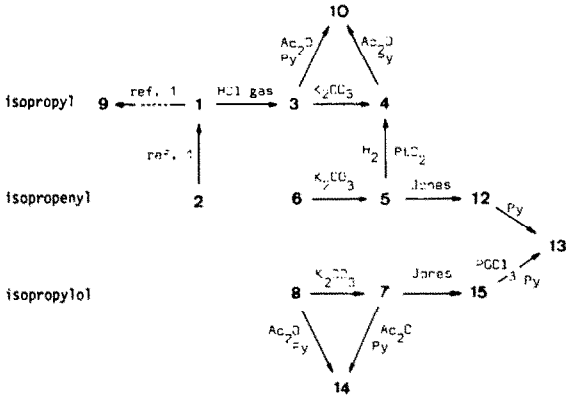
The IR, NMR and MS of compound **6** ($C_{32}H_{48}O_5$, M^+ 512) suggest the presence of a γ-lactone ($\nu_{C=O}$ 1760 cm^{-1}), of a secondary OH group (ν_{OH} 3540 cm^{-1}, 1H m at 3.19 ppm H–C–OH), of an acetoxyl group ($\nu_{C=O}$ 1720 cm^{-1}, 3H s at 2.05 ppm CH_3–COO, loss of acetic acid from molecular ion in MS), of a Me on a double bond (3H bs at 1.78 ppm C=C–CH_3) and of a vinylidene group ($\nu_{C=C}$ 1660 and $\delta_{C=CH_2}$ 895 cm^{-1}, 2H m at 4.83 ppm C=CH_2). The 2H m at 5.23 ppm is attributed to the vinylic proton at C-11 and to the proton on the carbon bearing the acetoxyl function. These assignments are confirmed by base hydrolysis of **6** into its corresponding diol identified with **5** by UV, IR, MS and NMR. Hence **6** is a monoacetylated derivative of **5**. The location of the acetoxyl in C-23, suggested by analogy with genins **1–3**, comes from the NMR of **6** in which the proton on the carbon vicinal to the acetoxyl function appears more deshielded (5.23 ppm) than expected for a 3αH (about 4.55 ppm[8]). This conclusion is also supported by the similarities in the NMR spectra of **2** and **6**, the latter thus being 23ξ - acetoxy - $\Delta^{9(11),25}$ - holostadiene - 3β - ol.

Examination of the more polar sapogenin fraction obtained through hydrochloric hydrolysis of the thelothurins leads us to isolate two products **7** and **8**.

The IR and NMR spectra of genin **7** ($C_{30}H_{48}O_5$, M^+-18: 470) establish the presence of a γ-lactone ($\nu_{C=O}$ 1755 cm^{-1}), of two secondary hydroxyl groups (ν_{OH} 3440 cm^{-1}, two 1H m at 3.23 and 4.24 ppm H–C–OH) and of one trisubstituted double bond (1H m at 5.23 ppm C=C–H). The fifth O atom of **7** must belong to a tertiary OH group since acetylation of **7** under usual conditions affords diacetate **14** whose IR still possesses an OH absorption at 3500 cm^{-1}. Moreover, the absence of an isopropyl group in the NMR of **7** (Table 1) as well as the presence of two Me's attached to quaternary carbons probably vicinal to an oxygen (two 3H s at 1.26 and 1.32 ppm) strongly suggest the existence of an isopropylol group in **7**. This is proved by Jones oxidation of **7** into dione **15** (ν_{OH} 3500 cm^{-1}, $\nu_{C=O}$ 1760 and 1708 cm^{-1}) whose NMR spectrum displays two 2H s at 2.63 (CH_2-24) and 3.02 ppm (CH_2-22), a 1H s at 3.48 ppm disappearing on addition of D_2O and one vinylic proton at 5.28 ppm (C=C–H). The chemical shift of the C-24 methylene suggests it to be vicinal to two deshielding groups, the C-23 carbonyl and the isopropylol. The β-hydroxyketone moiety of **15** is also indicated by the intense ion in MS at m/e = 426 (M^+-58), interpreted as a loss of acetone via a retro-aldol fragmentation. Finally, dehydration of dione **15** into the known **13**, identified by m.p., UV, IR, MS and NMR, not only establishes the 1,3-relationship between the secondary and the tertiary hydroxyls of the side chain in **7**, but also constitutes a chemical correlation of $\Delta^{9(11)}$ - holostene - 3β,23ξ,25 - triol (**7**) with $\Delta^{9(11),25}$ - holostadiene - 3β,23ξ - diol (**5**).

The spectral data of sapogenin **8** ($C_{32}H_{50}O_6$, M^+-18: 512) indicates the presence of a γ-lactone ($\nu_{C=O}$ 1760 cm^{-1}), of a secondary OH group (ν_{OH} 3480 cm^{-1}, 1H m at 3.22 ppm H–C–OH) and of an acetoxyl function ($\nu_{C=O}$ 1740 cm^{-1}, 3H s at 2.04 ppm CH_3–COO, loss of acetic acid from the ion at m/e = 512 in MS). Moreover, a 2H m 5.25 ppm is attributed to one vinylic proton and to one proton on the carbon bearing the acetoxyl function, since acetylation of **8** under usual conditions yields a diacetate identified with **14** by comparison of their IR and MS. Moreover, alkaline hydrolysis of **8** gives pure **7** (R_f, IR). These results show **8** to be a monoacetylated derivative of **7**. As in the case of **6**, the C-23 position of the acetoxyl, suggested by analogy with compounds **1–3**, is deduced from the 1H m at 5.25 ppm in the NMR of **8**, which is thus 23ξ - acetoxy - $\Delta^{9(11)}$ - holostene - 3β,25 - diol.

In order to prove unambiguously the skeleton of compounds **3–8** which is still based on spectroscopic evidence only,[2] we tried to correlate chemically sapogenin **3** with compound **1**. However, when **10** (diacetyl-3) is treated with dry gaseous hydrochloric acid in anhydrous chloroform, a reaction used to isomerise 24,25 - dihydroparkeol (**16**) into 24,25 - dihydrolanosterol (**17**),[9] no double bond migration from the $\Delta^{9(11)}$ into the Δ^8 position takes place. On the contrary, bubbling dry HCl in a chloroformic solution of **1** transforms the latter into its $\Delta^{9(11)}$-isomer **3**. The sapogenins **1–8** are thus interrelated and also correlated[1] to seychellogenin acetate (**9**),[10] as summarized in Scheme 2. The structures of **3–8** are thus completely established except for the absolute configuration at C-23.



Scheme 2.

We shall now discuss the formation of these compounds by acid catalysed hydrolysis (2N HCl, reflux during 4 hr). Milder hydrolysis conditions (reflux in 0.5 N aqueous–methanolic HCl or H_2SO_4 for 3.5 hr) greatly reduce the yield of compounds **7** and **8**. On shortening the hydrolysis period (idem 2 hr, 1 hr, 0.5 hr), one can also observe a progressive decreasing production of diols **4** and **5**, and nearly no more **7** and **8**. These results seem to indicate that compounds **4**, **5**, **7** and **8** are hydrolysis artifacts, which is confirmed by acid treatment of pure **3** and **6**. When pure **3** is refluxed in aqueous sulfuric t-BuOH for 2 hr, **4** is obtained in a good yield, confirming that it occurs via an acid catalysed hydrolysis of the side chain acetate of **3**. Diol **4** appears to be stable in acid conditions since it is recovered unchanged after a 3 hr reflux in 1.5N aqueous sulfuric acetone.

On the other hand, when pure **6** is treated in acid media

(aqueous sulfuric acid/solvent), genin **5** is formed alone or accompanied with **7** and **8**, depending on the solvent used. With dioxane, only diol **5** is obtained, even after a 20 hr reflux, while with t-BuOH or acetone, **6** yields, in addition to **5**, small amounts of **7** and **8**.

Thus acid treatment of **6** allows two competing reactions to occur: the hydrolysis of the acetoxyl function affording **5** or the addition of water on the isopropenyl group yielding **8**, the former being predominant. Subsequently, **7** can be formed either from **5**, by addition of water, or from **8**, by hydrolysis of the C-23 acetoxyl function. At least the existence of the former pathway was proved by treatment of **5** in aqueous sulfuric acetone which gives a small amount of **7**.

Hence, the primary products of the hydrochloric acid hydrolysis of the thelothurins A and B are **3**, from which **4** is derived, and **6** which gives rise to **5**, **7** and **8**. However, since the acetic acid hydrolysis affords as sole products the Δ^8 isomers **1** and **2** and since it is known[11] by the MS of the glycosides A and B that their aglycones have molecular ions at $m/e = 514$ and 512 respectively, it remains to assess whether the double bond isomerisation takes place in the acetic or in the hydrochloric medium. Unfortunately, no olefinic proton can be detected in the NMR spectra of the saponins or of their permethylated derivatives since too many signals appear between 4.50 and 6.00 ppm.

Therefore, **3** is treated with aqueous acetic acid at 100°. In addition to a small amount of its diacetate **10**,[12] **3** is recovered unchanged as attested by its NMR spectrum (the only reliable method to distinguish **1** and **3**). On the other hand, when **18** (acetyl-**1**) is refluxed in aqueous dioxane containing sulfuric or hydrochloric acid, a mixture of compounds is obtained from which the major less polar component is isolated and identified as pure **10** by NMR. This leads to the conclusion that the double bond migrates from the Δ^8 position (as in **18**) into the $\Delta^{9(11)}$ (as in **10**) in the hydrochloric medium and that the reverse does not occur.

Moreover, when the mixture of thelothurins A and B is hydrolysed in acetic acid for a longer period (e.g. 10 hr), the sapogenins obtained are then mainly a mixture of **3** and **6**, still containing a small amount of **1** and **2**.† These experiments show that, in aqueous acetic acid, the formation of **1** and **2** are under kinetic control and that of **3** and **6** under thermodynamic control.

Accordingly, **1** and **2** are the genuine aglycones of the parent toxins, thelothurins A and B respectively represented by $\mathbf{20_A}$ and $\mathbf{20_B}$. Hence, all of the $\Delta^{9(11)}$-compounds described in this paper are artifacts.

Since aqueous or alcoholic mineral acid (HCl, H_2SO_4) promotes rapid isomerization of the Δ^8 into the more stable $\Delta^{9(11)}$-genins, aqueous acetic acid may be considered much safer than the classical HCl method for saponin hydrolysis.

Biosynthesis of the thelothurins

Until recently the origin of the holothurins was not clearly established. Barbier *et al.*,[14] studying the biosynthesis of sterols in *Stichopus japonicus*, could only observe an incorporation of acetate-1,2-^{14}C in squalene. The holothurians thus seem unable to transform squalene into lanosterol and to synthesize their own sterols. Consequently, it was suggested that the triterpene glycosides should entirely originate from the diet. In contrast with these results, Goad[15] and Voogt[16] established the existence of a sterol biosynthesis in the sea cucumbers.

Therefore, in order to investigate the glycoside biosynthesis in holothurians, we injected into the coelomic cavity of *Thelonota ananas* a sea water solution of acetate-1-^{14}C (0.1 mCi). After a three day incubation, both animals (blank and labelled) were eviscerated and the sun-dried skins extracted as usual to obtain the mixture of thelothurins A and B ($\mathbf{20_{A/B}}$). This mixture appeared to be radioactive, the yield of incorporation being however extremely low (29 dec/mg/min), that is an incorporation of $5 \cdot 10^{-2}$% (Table 2). Aqueous hydrochloric acid hydrolysis of the saponins isolated from animals Nos. 1 and 2 afforded, on silica gel column chromatography, the mixture of genins **3** and **6** which were not separated. After repeated crystallisation, products **3** and **6** from the blank showed no activity whereas the labelling of genins **3** and **6** from specimen No. 2 amounted to a constant value of ±52 dec/mg/min. This establishes that the activity of the glycosides is significant and thus that *Thelonota ananas* is able to biosynthesize its defensive toxins.

†The obtention of **10**, in addition to the expected $\Delta^{7,9(11)}$ conjugated diene **19**, by selenium dioxide treatment of **18** in aqueous acetic acid during 17 hr (see previous paper[1]), is now understood as a competition between the SeO_2 oxidation of the Δ^8 into the $\Delta^{7,9(11)}$-conjugated system and the slow acid catalyzed isomerisation of the Δ^8- into the $\Delta^{9(11)}$-double bond, inert to SeO_2 in this series.[13]

Table 2. Biosynthetic data

Animal n°	Skin dry weight	Nominal tot. act.	Incubation period		dec/mg/min	purification	% incorp.	specific activity
1	144 g	0 mCi	72h	thelothurins :				
				4900 mg	18	crude		
					2	2 crystall.	-	-
				genins 3 and 6	0	chromatogr. + 2 crystall.	-	-
2	90 g	0.1 mCi	72h	thelothurins :				
				3860 mg	85	1 crystall.		
					28	3 crystall.	5.10^{-2}%	$2.3\ 10^{+4}$ dec/mM
				genins 3 and 6	135	crude		
					51	chromatogr.		
					52	+ 1 crystall.		
					50	+ 2 crystall.		
					54	+ 3 crystall.		$2.7\ 10^{+4}$ dec/mM

Moreover, the data collected in Table 2 show that the thelothurins A and B are almost exclusively labelled in the aglycone moiety whose specific activity (in decompositions/mmole) is equal, in the limits of experimental errors, to that of the saponins. The sugars are thus not, or nearly not radioactive. This may be in agreement with the hypothesis of an exogeneous origin of the carbohydrates, but a rapid exchange of the sugars cannot be excluded. The latter however, would not explain that the thelothurins A ($\mathbf{20_A}$) and B ($\mathbf{20_B}$) obtained from animals collected in the Seychelles archipelago and near Singapore differ in their carbohydrate composition.[11] This is not surprising for it has been reported[5] that the saponins of a given species of holothurian may show important fluctuations in carbohydrate composition, probably associated with seasonal variations or with a geographic distribution of the animals.

During the preparation of this paper, Elyakov *et al.*[17] reported the biosynthesis, from acetate-1,2-^{14}C, of the glycosides obtained from *Stichopus japonicus*. The labelling was mainly concentrated in the aglycone of the saponin, whereas the sugar moiety was nearly not labelled.

Our reults, obtained on another species, completely confirm the observations of Elyakov.

Since the aglycones are synthesized *de novo* and since the sugars of the saponins may originate from the diet, only the former should be taken into account, for chemotaxonomic interpretations.

EXPERIMENTAL

The equipment is described in the previous paper except for the radioactivity measurements carried out, by liquid scintillation counting, on a Packard tri CARB 3375 instrument. Samples are dissolved in 1 cm^3 methanol and the solution is dispersed in 15 cm^3 of insta-gel emulsifier (Packard). The labelled CH_3-^{14}COONa is a commercial product available from the Institut National des Radioéléments.

The isolation of the mixture of thelothurins A and B ($\mathbf{20_{A/B}}$) has been previously described, see Refs. 11 and 1.

Drastic hydrolysis of thelothurins A and B. Compound $\mathbf{20_{A/B}}$ (3 g) was treated at 100° during 4 hr in 2N HCl (100 ml). The reaction medium was worked up in the usual way. The crude extract (1 g) was subjected to silica gel column chromatography using a gradient of acetone (from 5 to 50%) in light petroleum. The fractions 9–11 contained 15 mg of an unidentified compound. The fractions 12–22 contain 600 mg of a mixture of **3** and **6** as it appeared by MS (two molecular ions at *m/e* = 514 and 512). The fractions 24–28 contained 140 mg of the mixture of **4** and **5** (two molecular ions in MS at *m/e* = 472 and 470). The fractions 30–36 contained 65 mg of the mixture of **7** and **8** unseparable in this solvent system.

Isolation of pure **3** *and* **6**. The mixture of **3** and **6** (600 mg) was chromatographed several times on silica gel columns impregnated with 10% $AgNO_3$. Elution occurred with a gradient of acetone (from 3 to 40%) in light petroleum. This gave 400 mg of pure **3** and 160 mg of pure **6**.

Compound **3**: m.p. 221–222°; $[\alpha]_D = -18°.0$ ($c = 1.01$ in $CHCl_3$); UV: end absorption; IR_{film}: ν_{OH} 3540 cm^{-1}, $\nu_{C=O}$ 1765 and 1740 cm^{-1}, ν_{C-O} 1245 cm^{-1}, $\delta_{C=CH}$ 945 cm^{-1}; MS: M$^+$ 514 (19, $C_{32}H_{50}O_5$), 499 (2, M$^+$–CH_3), 496 (1, M$^+$–H_2O), 481 (1, M$^+$–CH_3, H_2O), 454 (4, M$^+$–CH_3COOH), 439 (14, M$^+$–CH_3, CH_3COOH), 421 (25, M$^+$–CH_3, H_2O, CH_3COOH), 395 (56, M$^+$–CH_3, CH_3COOH, CO_2), 393 (10), 377 (5, M$^+$–CH_3, CO_2, CH_3COOH, H_2O); NMR: Table 1.

Compound **6**. m.p. 225–227°; $[\alpha]_D = -4°.0$ ($c = 0.88$ in $CHCl_3$); UV: end absorption; IR_{film}: ν_{OH} 3540 cm^{-1}, $\nu_{C=O}$ 1760 and 1720 cm^{-1}, $\nu_{C=C}$ 1660 cm^{-1}, ν_{C-O} 1260 cm^{-1}, $\delta_{C=CH}$ 945 cm^{-1} and $\delta_{C=CH_2}$ 895 cm^{-1}; MS: M$^+$ 512 (12, $C_{32}H_{48}O_5$), 497 (4, M$^+$–CH_3), 494 (2, M$^+$–H_2O), 479 (2, M$^+$–CH_3, H_2O), 452 (9, M$^+$–CH_3COOH), 437 (7, M$^+$–CH_3, CH_3COOH), 419 (10, M$^+$–CH_3, CH_3COOH, H_2O), 393 (14, M$^+$–CH_3, CH_3COOH, CO_2), 375 (5, M$^+$–CH_3, CH_3COOH, H_2O, CO_2), 365 (4); NMR: Table 1.

Isolation of pure **4** *and* **5**. The separation of **4** and **5**, extremely poor on the diols, was achieved on their diacetylated derivatives **10** and **11** on $AgNO_3$ impregnated silica gel columns. The pure diols **4** and **5** were then recovered by base hydrolysis of **10** and **11** respectively.

Acetylation of the mixture of **4** *and* **5**. The mixture of **4** and **5** (140 mg) was treated with Ac_2O (3 ml) in pyridine (3 ml) at room temp. for 17 hr. The medium was worked up in the usual way to yield 145 mg of the crude mixture of **10** and **11** which were separated by column chromatography on silica gel impregnated with 10% $AgNO_3$. Elution with a gradient of acetone (from 2 to 5%) in pentane gave in the fractions 11–21 90 mg of pure **10**.

Compound **10**. m.p. 189–191°; UV: end absorption; IR_{film}: no ν_{OH}, $\nu_{C=O}$ 1765 and 1735 cm^{-1}, ν_{C-O} 1240 cm^{-1}, $\delta_{C=CH}$ 940 cm^{-1}; MS: M$^+$556 (36, $C_{34}H_{52}O_6$), 541 (4, M$^+$–CH_3, metastable ion at 527.2: 556→541), 510 (3), 496 (20, M$^+$–CH_3COOH, metastable ion at 442.8: 556→496), 481 (26, M$^+$–CH_3, CH_3COOH, metastable ion at 466.3: 496→481 and metastable at 427.5: 541→481), 457(4), 451 (10), 437 (73, M$^+$–CH_3, CH_3COOH, CO_2), 421 (58, M$^+$–CH_3COOH, CH_3, CH_3COOH), NMR ($CDCl_3$): 0.88 (s, 3H, C-30) −0.90 (s, 3H, C-31) −0.94 (d J = 7 Hz, 6H, C-26,27) −0.97 (s, 3H, C-32) −1.17 (s, 3H, C-19) −1.40 (s, 3H, C-21) −2.05 (s, 6H, 3β and 23ξ CH_3–COO) −2.95 (m, 1H, 8β H) −4.52 (m, 1H, 3α H) and 5.22 ppm (m, 2H, 11-H and 23 H-C-OAc).

The fractions 25–36 contain 40 mg of pure **11**.

Compound **11**. UV: end absorption; IR_{film}: no ν_{OH}, $\nu_{C=O}$ 1765 and 1735 cm^{-1}, $\nu_{C=C}$ 1650 cm^{-1}, ν_{C-O} 1245 cm^{-1}, $\delta_{C=CH}$ 940 cm^{-1} and $\delta_{C=CH_2}$ 895 cm^{-1}; MS: M$^+$ 554 (29, $C_{34}H_{50}O_6$), 539 (8, M$^+$–CH_3), 512 (3, M$^+$–C_2H_2O), 494 (48, M$^+$–CH_3COOH, metastable ion at 440.5: 554→494), 479 (43, M$^+$–CH_3, CH_3COOH), 457 (18), 449 (25), 435 (84, M$^+$–CH_3COOH, CH_3, CO_2), 419 (100, M$^+$–CH_3COOH, CH_3, CH_3COOH), 391 (18), 389 (15), 375 (36, M$^+$–CH_3COOH, CH_3, CH_3COOH, CO_2), 373 (21), 367 (35, M$^+$–CO_2, H_2, $C_8H_{13}O_2$ side chain), 365 (35), 353 (21, M$^+$–$C_8H_{13}O_2$ side chain, CH_3COOH); NMR ($CDCl_3$): 0.90 (s, 6H, C-30,31) −1.00 (s, 3H, C-32) −1.11 (s, 3H, C-19) −1.42 (s, 3H, C-21) −1.77 (bs, 3H, C-27) −2.03 (s, 6H, 3β and 23ξ CH_3–COO) −3.00 (m, 1H, 8βH) −4.50 (m, 1H, 3αH) −4.80 (m, 2H, C-26) and 5.22 ppm (m, 2H, 11H and 23 H-C-OAc).

Alkaline hydrolysis of **10** *into* **4**. Diacetate **10** (25 mg) was stirred at room temp. for 17 hr, in sat methanolic K_2CO_3 aq. The mixture was neutralized by careful addition of a 10% HCl aq and treated in the usual way to give 24 mg of pure **4**.

Compound **4**. m.p. 229–232°; $[\alpha]_D = +6°.2$ ($c = 0.69$ in $CHCl_3$); UV: end absorption; IR_{KBr}: ν_{OH} 3450 cm^{-1}, $\nu_{C=O}$ 1760 cm^{-1}, ν_{C-O} 1260 cm^{-1} and $\delta_{C=CH}$ 945 cm^{-1}; MS: M$^+$ 472 (45, $C_{30}H_{48}O_4$), 457 (11, M$^+$–CH_3), 454 (4, M$^+$–H_2O), 439 (12, M$^+$–CH_3, H_2O), 421 (15, M$^+$$CH_3$, H_2O, H_2O), 415 (5, M$^+$–C_4H_9 cleavage at C_{23}/C_{24}), 413 (5, M$^+$–CH_3, CO_2), 411 (3, M$^+$–CH_3, CO_2, H_2), 395 (19, M$^+$–CH_3, H_2O, CO_2), 386 (6, M$^+$–$C_5H_{10}O$ cleavage at C_{22}/C_{23}), 371 (14, M$^+$–$C_6H_{13}O$ side chain), 353 (48, M$^+$–H_2O, $C_6H_{11}O$); NMR ($CDCl_3$–CD_3OD): Table 1.

Alkaline hydrolysis of **11** *into* **5**. Diacetate **11** (17 mg) was treated as described to yield 14 mg of pure **5**.

Compound **5**. m.p. 202–203° (poor crystals); $[\alpha]_D = +10°.5$ ($c = 1.35$ in $CHCl_3$); UV: end absorption; IR_{KBr}: ν_{OH} 3480 cm^{-1}, $\nu_{C=O}$ 1760 cm^{-1}, $\nu_{C=C}$ 1650 cm^{-1}, $\delta_{C=CH}$ 940 cm^{-1} and $\delta_{C=CH_2}$ 895 cm^{-1}; MS: M$^+$ 470 (80, $C_{30}H_{46}O_4$), 455 (38, M$^+$–CH_3), 452 (19, M$^+$–H_2O), 437 (38, M$^+$–CH_3, H_2O), 419 (40, M$^+$–CH_3, H_2O, H_2O), 415 (40, M$^+$–C_4H_9 cleavage at C_{23}/C_{24}), 411 (27, M$^+$–CH_3, CO_2), 399 (36), 393 (55, M$^+$–CH_3, H_2O, CO_2), 381 (50, M$^+$–C_4H_9, H_2O, CH_3, H), 375 (25, M$^+$–CH_3, H_2O, H_2O, CO_2), 371 (45, M$^+$–$C_6H_{11}O$ side chain), 353 (100, M$^+$–H_2O, $C_6H_{11}O$), 337 (35), 335 (40), 327 (60, M$^+$–CO_2, $C_6H_{11}O$); NMR ($CDCl_3$–CD_3OD): Table 1.

Isolation of pure **7** *and* **8**. The mixture of **7** and **8** (65 mg) was separated by repetitive column chromatographies on silica gel using a gradient elution of AcOEt (from 10 to 50%) in benzene. 15 mg of pure **8** and 35 mg of pure **7** were obtained.

Compound **7**. m.p. 227–228°; $[\alpha]_D = -1°.3$ ($c = 0.80$ in $CHCl_3$); UV: end absorption; IR_{film}: ν_{OH} 3440 cm^{-1}, $\nu_{C=O}$ 1755 cm^{-1}, $\delta_{C=CH}$ 945 cm^{-1}; MS: no molecular ion at 488 ($C_{30}H_{48}O_5$), 470 (84,

M^+-H_2O), 455 (22, M^+-H_2O, CH_3), 452 (19, M^+-H_2O, H_2O), 437 (18, M^+-H_2O, H_2O, CH_3), 419 (19, M^+-H_2O, CH_3, H_2O, H_2O), 414 (27, $M^+-C_4H_{10}O$ cleavage at C_{23}/C_{24} and rearrangement of one H), 399 (39), 393 (59, M^+-H_2O, CH_3, H_2O, CO_2), 381 (46, $M^+-C_4H_{10}O$, CH_3, H_2O), 371 (23, $M^+-C_6H_{13}O_2$ side chain), 367 (28), 353 (64, $M^+-C_6H_{13}O_2$, H_2O), 327 (48, $M^+-C_6H_{13}O_2$, CO_2); NMR ($CDCl_3$): Table 1.

Compound **8**. m.p. 203–205°C; $[\alpha]_D = -10°.0$ ($c = 0.33$ in $CHCl_3$); UV: end absorption; IR_{film}: ν_{OH} 3480 cm^{-1}, $\nu_{C=O}$ 1760 and 1740 cm^{-1}, ν_{C-O} 1250 cm^{-1}, $\delta_{C=CH}$ 940 cm^{-1}; MS: no molecular ion at 530 ($C_{32}H_{50}O_6$), 512 (48, M^+-H_2O), 510 (28), 497 (13, M^+-H_2O, CH_3), 486 (28, M^+-CO_2), 468 (60, M^+-H_2O, CO_2), 453 (45, M^+-H_2O, CO_2, CH_3), 452 (86, M^+-H_2O, CH_3COOH), 437 (55, M^+-H_2O, CH_3COOH, CH_3), 419 (69, M^+-H_2O, CH_3COOH, CH_3, H_2O), 409 (53), 407 (48), 393 (85, M^+-H_2O, CH_3COOH, CH_3, CO_2), 371 (33, $M^+-C_8H_{15}O_3$ side chain), 365 (43), 353 (42, $M^+-C_8H_{15}O_3$, H_2O); NMR ($CDCl_3$): Table 1.

Acetylation of **3**. Genin **3** (22 mg) was treated with Ac_2O (1 ml) in pyridine (1 ml) at room temp. during 17 hr. The mixture was treated in the usual way to yield 22 mg of pure **10** (spectral data, see above).

Oxidation of **5** *into dione* **12**. Sapogenin **5** (35 mg) was dissolved in acetone (3 ml) and oxidized with Jones reagent at room temp. during 3 min. The mixture was worked up in the usual way and the crude extract purified by silica gel column chromatography using gradient elution of acetone (From 3 to 10%) in light petroleum to afford 30 mg of **12**.

Compound **12**. UV: end absorption in neutral MeOH, in MeOH 0.02 M in KOH λ_{max} 237 nm ($\epsilon = 10000$) and in MeOH/KOH 0.1 M λ_{max} 243 ($\epsilon = 6700$) and 277 nm ($\epsilon = 5600$); IR_{film}: no ν_{OH}, $\nu_{C=O}$ 1755 and 1715 cm^{-1}, $\nu_{C=C}$ 1660 cm^{-1}, $\delta_{C=CH}$ 945 cm^{-1} and $\delta_{C=CH_2}$ 895 cm^{-1}; MS: M^+ 466 (34, $C_{30}H_{42}O_4$), 451 (6, M^+-CH_3), 448 (4), 433 (4, 448-CH_3), 420 (12, M^+-CO_2, H_2), 407 (27, M^+-CH_3, CO_2), 405 (56, M^+-CH_3, CO_2, H_2), 389 (9), 383 (10, $M^+-C_5H_7O$ cleavage at C_{22}/C_{23}), 377 (3), 369 (8, $M^+-C_6H_9O$ side chain), 366 (12), 351 (10), 349 (8), 337 (38), 325 (58, $M^+-C_6H_9O$, CO_2), 323 (100, $M^+-C_6H_9O$, CO_2, H_2); NMR ($CDCl_3$): 0.87 (s, 3H, C-30) −1.10 (s, 6H, C-31,32) −1.35 (s, 3H, C-19) −1.48 (s, 3H, C-21) −1.77 (bs, 3H, C-27) −3.00 (m, 1H, 8βH) −3.07 (s, 2H, C-22) −3.14 (s, 2H, C-24) −4.90 (bs, 1H, C-26H cis) −5.02 (bs, 1H, C-26H trans) and 5.32 ppm (m, 1H, 11H).

Isomerisation of dione **12** *into conjugated dione* **13**. Dione **12** (30 mg) was refluxed in pyridine (5 ml) for 20 hr. The pyridine was evaporated under reduced pressure and the crude extract chromatographed on a silica gel column using as eluent a gradient of acetone (from 2.5 to 10%) in hexane. 23 mg (yield 80%) of pure conjugated diketone **13** was obtained.

Compound **13**. m.p. 208–211°; UV: 238 nm ($\epsilon = 13500$) in neutral methanol, 244 nm ($\epsilon = 9570$) and 276 nm ($\epsilon = 9360$) in MeOH/KOH 0.1 M; IR_{film}: no ν_{OH}, $\nu_{C=O}$ 1760–1708 and 1690 cm^{-1}, $\nu_{C=C}$ 1620 cm^{-1}, $\delta_{C=CH}$ 940 cm^{-1}, disappearance of the $\delta_{C=CH_2}$ band at 895 cm^{-1}; MS: M^+ 466 (28, $C_{30}H_{42}O_4$), 451 (2, M^+-CH_3), 448 (1), 433 (3), 420 (4, M^+-CO_2, H_2), 407 (20, M^+-CH_3, CO_2), 405 (38, M^+-CH_3, CO_2, H_2), 389 (4), 383 (4, $M^+-C_5H_7O$ cleavage at C_{22}/C_{23}), 369 (5, $M^+-C_6H_9O$ side chain), 366 (11), 351 (6), 337 (25), 323 (70, M^+-CO_2, C_6H_9O, H_2); NMR ($CDCl_3$): 0.88 (s, 3H, C-30) −1.10 (s, 6H, C-31,32) −1.36 (s, 3H, C-19) −1.59 (s, 3H, C-21) −1.93 (bs, 3H, C-26 cis) −2.16 (bs, 3H, C-27 trans) −2.95 (m, 1H, 8βH) −3.02 (s, 2H, C-22) −5.33 (m, 1H, 11H) and 6.17 ppm (bs, 1H, 24-H).

Hydrogenation of **5** *into* **4**. Sapogenin **5** (14 mg) in AcOEt (10 ml) was hydrogenated on PtO_2 at room temp. during 19 hr, under atmospheric pressure of H_2. The medium was evaporate to dryness and the residue is filtered on a silica gel column (elution: hexane–acetone 8/2) to eliminate the catalyst and the greases. This yielded 13 mg of a compound identified with **4** by TLC on silica gel plates impregnated with $AgNO_3$, UV, IR (disappearance of the $\delta_{C=CH_2}$ band at 895 cm^{-1}), MS (M^+, fragmentation and ions intensities) and NMR (no methyl on a double bond, no signal at 4.80 ppm and apparition of an isopropyl signal at 0.94 ppm (d J = 7 Hz).

Alkaline hydrolysis of **6** *into* **5**. Sapogenin **6** (23 mg) was treated as diacetate **10**. The crude extract was purified by column chromatography on silica gel using gradient elution of acetone (from 8 to 20%) in hexane to yield 15 mg of a compound identical with **5** by UV, IR, MS and NMR.

Acetylation of **7** *into* **14**. Sapogenin **7** (3 mg) was treated as genin **3**. The crude extract was purified by filtration on a silica gel column (elution with hexane–acetone 8/2). 2 mg of **14** were obtained.

Compound **14**. UV: end absorption; IR_{film}: ν_{OH} 3500 cm^{-1}, $\nu_{C=O}$ 1760 and 1740 cm^{-1}, ν_{C-O} 1245 cm^{-1}; MS: M^+ 572 (3, $C_{34}H_{52}O_7$), 554 (33, M^+-H_2O), 512 (25, M^+-CH_3COOH), 494 (46, M^+-H_2O, CH_3COOH), 479 (12, M^+-H_2O, CH_3COOH, CH_3), 456 (18), 449 (16), 435 (48, M^+-H_2O, CH_3COOH, CH_3, CO_2), 419 (38, M^+-H_2O, CH_3COOH, CH_3, CH_3COOH), 381 (41, $M^+-C_7H_{15}O_3$ cleavage at C_{22}/C_{23}, CO_2, H_2), 353 (29, $M^+-C_8H_{15}O_3$ side chain, CH_3COOH), 339 (25), 325 (48), 313 (27), 309 (23, $M^+-C_8H_{15}O_3$, CH_3COOH, CO_2).

Oxidation of **7** *into diketone* **15**. Sapogenin **7** (35 mg) was oxidized with Jones reagent as described for **5**. The crude extract was chromatographed on silica gel; elution was carried out with a gradient of acetone (from 10 to 20%) in hexane. This afforded 30 mg of diketone **15**.

Compound **15**. UV: end absorption; IR_{film}: ν_{OH} 3500 cm^{-1}, $\nu_{C=O}$ 1760 and 1710 cm^{-1}, ν_{C-O} 1270 cm^{-1} and $\delta_{C=CH}$ 945 cm^{-1}; MS: M^+ absent (484 = $C_{30}H_{44}O_5$), 466 (3, M^+-H_2O), 426 (41, $M^+-CH_3CO-CH_3$ retro-aldolisation), 411 (20, $M^+-CH_3COCH_3$, CH_3), 405 (3, M^+-H_2O, CH_3, CO_2, H_2), 394 (12, $M^+-C_4H_9O$ cleavage at C_{23}/C_{24}, CH_3, H_2), 381 (10, $M^+-C_5H_9O_2$ cleavage at C_{22}/C_{23}, H_2), 366 (43, M^+-CH_3, $C_5H_9O_2$, H_2), 349 (22), 341 (12), 338 (15), 327 (57), 325 (45, $M^+-C_6H_{11}O_2$ side chain, CO_2), 311 (39), 309 (55); NMR ($CDCl_3$): 0.88 (s, 3H, C-30) −1.08 (s, 6H, C-31,32) −1.26 (s, 6H, C-26,27) −1.33 (s, 3H, C-19) −1.50 (s, 3H, C-21) −2.63 (s, 2H, C-24) −2.90 (m, 1H, 8βH) −3.02 (s, 2H, C-22), −3.48 (s, 1H, 25-OH disappearing on addition of D_2O), and 5.28 ppm (m, 1H, 11-H).

Dehydration of **15** *into dione* **13**. Diketone **15** (25 mg) in anhyd pyridine (1.5 ml), was allowed to react with $POCl_3$ (1 ml). The mixture was stirred at room temp. during 2½ hr, then worked up in the usual way. The crude residue was chromatographed on a silica gel column (elution: hexane–acetone 10%). This gave 14 mg (yield 60%) of a compound identical with the conjugated dione **13** by TLC, UV (λ and ϵ), IR_{film}, MS (M^+, fragmentation pattern and ions intensities) and NMR.

Acetylation of **8** *into diacetate* **14**. Sapogenin **8** (3 mg) was acetylated in the usual way (see acetylation of **5**). The crude extract was chromatographed on a silica gel column (eluent: hexane–acetone 20%) to yield 2 mg of a diacetate identical with **14** by UV, IR and MS (M^+, fragmentation and ions intensities).

Alkaline hydrolysis of **8** *into* **7**. Sapogenin **8** (10 mg) was treated as diacetate **10**. This afforded 9 mg of pure **7** (TLC, IR).

Treatment of **10** *with dry gaseous* HCl. Diacetate **10** (30 mg) dissolved in anhyd $CHCl_3$ (5 ml) was submitted at room temp., during 3 hr, to a gentle bubbling of dry gaseous HCl. The chloroform was evaporated under reduced pressure. The crude residue was purified by column chromatography on silica gel using a gradient elution of acetone (from 3 to 20%) in hexane. Diacetate **10** was recovered unchanged (NMR).

Treatment of **1** *with dry gaseous* HCl. Sapogenin **1** (40 mg) was treated with dry gaseous HCl as described. Silica gel column chromatography of the crude extract, using a gradient elution of acetone (from 4 to 30%), afforded 19 mg of an homogeneous compound identified with **3** by NMR.

Mild hydrolysis of thelothurins A and B. (a) The mixture $\mathbf{20}_{A/B}$ (5 mg) was refluxed in 0.5 N aqueous–methanolic (1/1, v/v) HCl (4 ml) for 3½ hr. Treatment of the mixture in the usual way afforded a crude extract whose examination in TLC showed the presence mainly of **3**, **6**, **4** and **5** accompanied with a small amount of **7**. (b) Under the same conditions, after a reflux of 2 hr, 1 hr or 0.5 hr, **7** and **8** were considerably less abundant and the amount of **4** and **5** seriously decreased (TLC).

Acid treatment of **3** *in* t-BuOH. Compound **3** (2 mg) dissolved in t-BuOH (2 ml) was refluxed during 2 hr with 3N H_2SO_4 (2 ml). The medium, treated in the usual way, gave a mixture of unreacted **3** and of diol **4** (TLC).

Acid treatment of **4** *in acetone*. Compound **4** (3 mg), dissolved in acetone (2 ml) was refluxed for 3 hr with 3N H_2SO_4 (2 ml).

Treatment of the mixture in the usual way afforded pure unchanged diol **4** (TLC).

Acid treatment of **6** *in dioxane.* Compound **6** (2 mg), dissolved in dioxane (1 ml) was refluxed during 20 hr with 3N H_2SO_4 (2 ml). TLC examination of the mixture showed the presence of pure **5**.

Acid treatment of **6** *in acetone.* Sapogenin **6** (30 mg), dissolved in acetone (10 ml) was refluxed for 5 hr with 7N H_2SO_4 (10 ml). The medium was worked up in the usual way. The crude extract was purified by silica gel column chromatography (elution: gradient of AcOEt, from 4 to 40%, in benzene). The fractions 5–10 contained 9 mg of an unidentified compound resulting probably from the addition of acetone on the isopropenyl group. The fractions 12–15 contained 3 mg of unreacted **6** (TLC, IR). The fractions 20–23 contained 10 mg of **5** (TLC, IR). The fractions 29–31 contained 1 mg of **8** (TLC) and the fractions 32–36 contain 4 mg of **7** (TLC, IR).

When t-BuOH was used, the former unidentified compound was not obtained, but another artifact was isolated which may have been the 3β - t - butoxy - ether.

Acid treatment of **5** *in acetone.* Treating **5** (24 mg) in acetone as described for **6**, yielded a mixture of three compounds. Chromatography of the crude extract on silica gel column (see acid treatment of **6** in acetone) afforded in the fractions 9–12 5 mg of an unidentified compound. The fractions 17–20 contained 10 mg of unreacted **5**. The fractions 24–26 contained 3 mg of **7** identified by TLC and IR.

Acetic acid treatment of **3**. Compound **3** (20 mg) was treated with 80% aqueous AcOH (15 ml) at 90–100° during 3 hr. The crude extract, obtained in the usual way, was purified by silica gel column chromatography (elution: gradient of acetone from 5 to 10% in hexane). The fractions 4–7 contained 2 mg of **10** (TLC, IR). The fractions 13–16 contained 16 mg of unreacted **3**.

Acid treatment of **18** *in dioxane.* Diacetate **18** (33 mg) dissolved in dioxane (10 ml) and water (2 ml), was refluxed during 3 hr with conc H_2SO_4 (0.5 ml) or conc HCl (1 ml). The medium, treated in the usual way, afforded a mixture of three compounds. The less polar (13 mg) was identified as **10** by NMR. The other compounds, identical in TLC with **3** and **4** respectively, resulted from acid hydrolysis of the acetyl function(s) and gave on acetylation an homogeneous diacetate also identified as pure **10**.

Acetic acid treatment of $\mathbf{20}_{A/B}$. See previous paper Ref. 1.

Administration of labelled sodium acetate to Thelonota ananas. The animals were collected in February 1974 off Albatros Rocks (Seychelles Islands) at depths from 6 to 14 m. One animal, used as blank, was injected with 2 cm^3 sea water and another specimen was injected with a 2 cm^3 sea water solution of CH_3-^{14}COONa (0.1 mCi) and kept in a nursepond at 6 m deep for the incubation period (72 hr). The experiments were carried out entirely under water, from the collection until the end of the incubation period, in order to avoid any handling out of water which could damage the animals. After 72 hr, the holothurians were taken out of the nurse-pond and eviscerated. The inner organs were kept in a 50% aqueous methanolic solution and the skins were carefully sun-dried. The latter were then treated separately.

Isolation of thelothurins A and B. See Refs. 1 and 11.

Acid hydrolysis of $\mathbf{20}_{A/B}$. (a) Compounds $\mathbf{20}_{A/B}$ (524 mg) from animal No. 2 (fraction 85 dec/mg/min) were treated with HCl aq as described. The medium was extracted as usual to yield 18 mg of crude genin mixture (135 dec/mg/min). This crude fraction was purified by silica gel column chromatography using a gradient elution of acetone (from 10 to 20%) in hexane. 113 mg (yield: 50%) of the mixture of genins **3** and **6** were obtained (51 dec/mg/min). This mixture was then crystallized three times from MeOH–hexane until constant activity (±52 dec/mg/min). (b) The unseparated mixture of **3** and **6** were obtained, in the same way, from animal No. 1. After silica gel column chromatography and one crystallisation from MeOH–CH_2Cl_2, the genin fraction was shown to contain no activity.

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