PHOSPHATED AND SULPHATED MARINE POLYHYDROXYLATED STEROIDS FROM THE STARFISH *Tremaster novaecaledoniae* (*)(**)

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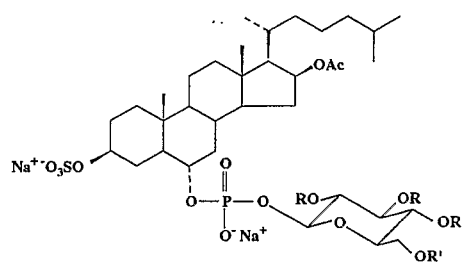
Summary — Beside tremasterols A-C (**1-3**), first phosphated steroid glycosides to be found from a natural source, the starfish *Tremaster novaecaledoniae* contains nine more novel steroid constituents. One, **4**, is related to the previous tremasterols by having the same 6-*O*-phosphated function. A second group of compounds (**5-8**) possesses the same 3,6-disulphated 3β,6α,(22*R*)-trihydroxycholestane structure, differing for the presence of the $\Delta^{9(11)}$ double bond in **5** and **6** and for the acetate conjugation of the 22-hydroxyl group in **6** and **8**. The remaining compounds **9-12** are highly hydroxylated steroids, among which the steroid **11** features the *cis* A/B ring junction, never encountered before among steroids derived from starfishes.

It is known that there are three groups of steroid oligoglycosides in starfishes: sulphated steroidal penta- and hexa-glycosides («asterosaponins»), steroidal cyclic glycosides (so far found in two species of the genus *Echinaster*), and glycosides of polyhydroxylated steroids consisting of a polyhydroxylated steroid aglycone with one or two sugar units, which are found in both sulphated and non sulphated form. Often these glycosides are accompanied by small amounts of polyhydroxylated steroids, also occurring in both sulphated and non sulphated form[1,2]. In our continuing investigation of the New Caledonian marine species we had the occasion to examine the polar extracts of the starfish *Tremaster novaecaledoniae*, Jangoux 1982, which is a «living fossil» species[3], collected at a depth of 530 m off New Caledonia, and to isolate a fourth group of steroidal glycosides, tremasterols A - C (**1-3**), characterized by the presence of sulphated and phosphated groups in the steroidal aglycone with the phosphate residue further linked to 1'-glucose in **1**, 1'-glucose-tetra-acetate in **2** and 1'-glucose-6-acetate in **3**. To our knowledge, this was the first isolation of phosphated sterols from a natural source[4]. Continuing with the analysis of the polar extracts of *Tremaster novaecaledoniae* we have now isolated nine more polyhydroxylated steroids and in this paper we report the isolation and the structure elucidation.

The steroid compound **4** is related to the previous tremasterols A - C (**1-3**) by having the same 6-*O*-phosphate grouping.

A group of four steroids (**5-8**) possess the same 3β,6α-disulphated 3β,6α,(22*R*)-trihydroxycholestane structure and differ for the presence of the $\Delta^{9(11)}$ double bond in **5** and **6** and for the acetate conjugation of the 22-hydroxyl group in **6** and **8**.

The remaining four compounds isolated are highly hydroxylated steroids. The steroid **9** and its 15-sulphated analogue **10** were assigned the 25*R* configuration, encountered before in pavonins, 26-hydroxysteroidal glycosides from the sole *Pardachirus pavonius*[5] and in 26-hydroxysteroids from ophiuroids[6], but never found among 26-hydroxysteroids from starfishes. Besides the 25*R* configuration, the steroid **11** features the *cis* A/B ring junction, never encountered before among steroids derived from starfishes.



1 R = R' = H
2 R = R' = Ac
3 R = H, R' = Ac

RESULTS AND DISCUSSION

All compounds were isolated and purified by chromatography on Sephadex LH-60 of the methanol soluble portion of the acetone extracts, followed by droplet counter current chromatography (DCCC),

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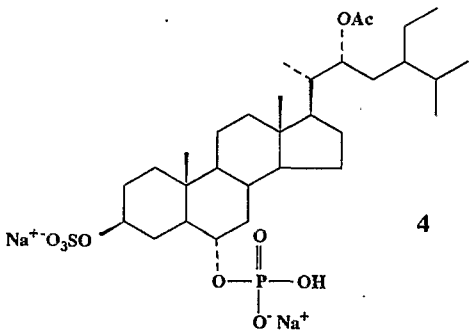

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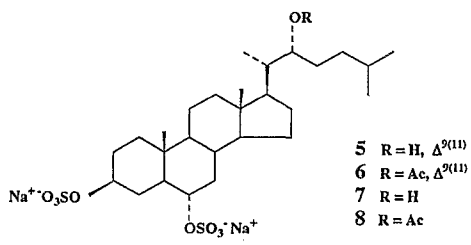
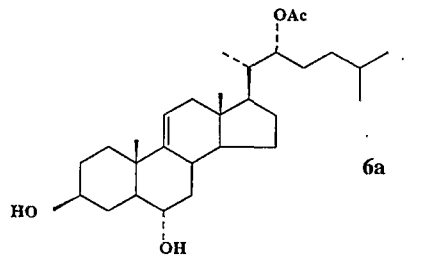
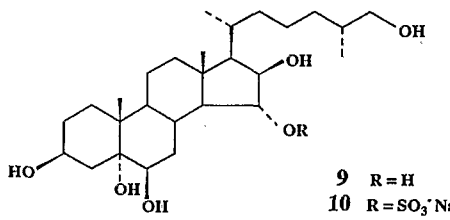


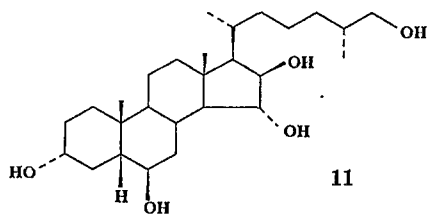
5 R = H, $\Delta^{9(11)}$
6 R = Ac, $\Delta^{9(11)}$
7 R = H
8 R = Ac



6a



9 R = H
10 R = $SO_3^- Na^+$



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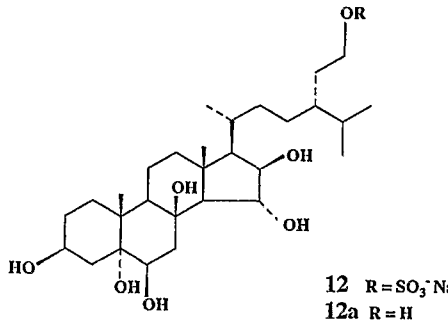

12 R = $SO_3^- Na^+$
12a R = H

and HPLC. The results of our analysis are shown in table 1.

STRUCTURE ELUCIDATION OF THE PHOSPHATED STEROID **4**

The negative FAB mass spectrum of **4** exhibited molecular anion peaks at *m/z* 671 $[M_{Na}]^-$ and 649 $[M_H]^-$. Examination of its spectral properties (tables 2 and 3) indicated that 4 contains a 3β,6α-dihydroxy-5α-steroid skeleton with a sulphate at C-3 and a phosphate at C-6, already found in tremasterols A - C (**1-3**). In the ^1H NMR spectrum the multiplet at δ 4.24 ppm had the complexity normally seen for a 3β-oxygenated group and its downfield chemical shift, virtually identical with that seen in the spectrum of the 5α-cholestane-3β-yl sulphate, suggested a sulphate group located there. The shape of the signal at δ 3.95 ppm, a dddd, J = 9.5, 9.5, 7.5 and 4.5 Hz, assigned to the axial proton at C-6, was suggestive for the presence of the phosphate[7], as in **1-3**. The axial proton associated with the 6α-hydroxyl group is usually seen as a double triplet (J = 4.5, 9.5 Hz)[8]. The presence of the phosphate at C-6 was confirmed by the proton noise-decoupled ^{13}C NMR spectrum, in which the signals for carbons-5 and -6 appear as doublets with $J_{P\text{-}O\text{-}C}$ of 5.5 Hz and $J_{P\text{-}O\text{-}C\text{-}C}$ of 7.4 Hz.

Continuing now with the examination of the 500 MHz ^1H NMR data of compound **4**, signals characteristically well separated at δ 0.84 (3 H, d) and 0.91 (3 H, d) ppm for the isopropyl methyl protons and the 3 H triplet at δ 0.92 ppm, partially overlapped with the methyl doublet at δ 0.91 ppm, indicated the presence of an ethyl group at the C-24 position. This was supported by the ^{13}C NMR signals at 23.6 (methylene, DEPT) and 12.7 ppm (methyl, DEPT) for the ethyl group and at 20.6 and 17.8 ppm for the isopropyl methyl carbons, characteristically well separated because of the substitution at C-24. In the 24-methyl and 24-ethyl steroids the slight differences in the chemical shift of 26- and 27-methyl protons between epimers, can be used for differentiating 24*R* and 24*S* epimers[9]. In the 24-ethyl steroid series, the chemical shift differences between epimers are too

TABLE 1 - COMPOSITION OF THE POLAR STEROIDS OF *Tremaster novaecaledoniae* (0.9 kg OF WET WEIGHT)

Steroid	Amount	Rotations $[\alpha]_D{}^a$
Tremasterol A, **1**	15 mg	+ 40.0
Tremasterol B, **2**	6.5 mg	+ 33.1
Tremasterol C, **3**	5.8 mg	+ 35.2
Tremasterol D, **4**	4.0 mg	+ 28.2
Sterol **5**	9.0 mg	+ 25.0
Sterol **6**	5.7 mg	+ 27.2
Sterol **7**	6.4 mg	+ 36.4
Sterol **8**	3.7 mg	+ 21.2
Sterol **9**	3.5 mg	+ 17.2
Sterol **10**	4.2 mg	+ 21.5
Sterol **11**	18 mg	+ 32.1
Sterol **12**	7.3 mg	+ 25.0

(*a*) From solutions in MeOH (*c* ranging from 0.4 to 1.0). Units of [α] deg cm^2 dag^{-1}.

TABLE 2 - SELECTED ^1H NMR CHEMICAL SHIFTS OF THE STEROIDS **4-8**[a]

Compound	3α-H	6β-H	11-H	22-H	18-H_3	19-H_3	21-H_3	26,27-H_3	Other signals
4	4.24 m	3.95 dddd (9.5, 9.5, 7.5, 4.5)	–	5.04 br d (10.8)	0.72 s	0.92 s	1.00 d (7)	0.84 d, 0.91 d (7)	0.92 t (29-H_3)[b] 2.04 s, 3 H
5	4.23 m	4.35 td (10.5, 4)	5.40 br d	3.60 br d	0.69 s	1.07 s	0.94 d (6.8)	0.93 d (6.8)	–
6	4.23 m	4.35 td (10.5, 4)	5.40 br d	4.90 br d	0.66 s	1.07 s	0.98 d	0.91 d, 0.92 d (7) (7)	2.06 s, 3 H
7	4.25 m	4.17 td (10.5, 4)	–	3.59 br d (8.5)	0.75 s	0.94 s	0.93 d (7)	0.94 d (7)	–
8	4.24 m	4.17 td (10.5, 4)	–	4.90 br d	0.72 s	0.94 s	0.97 d (7)	0.92 d (7)	2.05 s, 3 H
6a	3.58 m	3.61 td (10.5, 4)	5.31 br d	4.88 dt (10, 3.5)	0.60 s	0.96 s	0.92 d (7)	0.89 d (7)	2.05 s, 3 H

([a]) The spectra were run at 250.13 MHz in CD_3OD except that of **6a** which was run in $CDCl_3$; the coupling constants are given in Hertz and are enclosed in parenthesis. ([b]) Partially overlapped.

small for differentiation, therefore we propose to leave the stereochemistry at C-24 unassigned. The presence of an acetoxy group was shown from ^1H NMR (δ 2.04 ppm, s, 3 H) and ^{13}C NMR (δ_C 178.7 and 21.2 ppm) data. The use of the COSY experiment showed the broad doublet at δ 5.04 ppm to be coupled to a signal at δ 1.78 ppm (H-20), which in turn gave a crosspeak with the 21-methyl proton signal at δ 1.00 ppm, thus suggesting to locate the acetoxy group in the side chain at C-22. This was confirmed by the ^{13}C NMR spectrum, which showed the signals for C-17, C-21 and C-24 shifted upfield by 1.7, 5.2 and 1.6 ppm, respectively (γ effects), and the signals for C-20 and C-23 shifted downfield by 6 and 3 ppm, respectively (β effects), with respect to the reference stigmasterol[10]. The magnitude of the β effects of the acetoxy group on C-20 and especially on C-23 signals are totally different for the 22*R* and 22*S* compounds[11]. The large effect on C-20 (6 ppm) and the small one on C-23 (3 ppm) observed in the spectrum of **4** relative to the reference stigmasterol[10] indicated the 22*R* configuration.

STRUCTURE ELUCIDATION OF THE SULPHATED STEROIDS **5-8**

The negative-ion FAB mass spectrum of the major steroid of this group, **5**, exhibited molecular anion species at *m/z* 599 and 577 corresponding to $[M_{Na}]^-$ and $[M_H]^-$, respectively. Next to the molecular ion species, the spectrum displayed an intense fragment at *m/z* 497 which corresponds to the loss of SO_3 from *m/z* 577. The ^{13}C NMR spectrum was consistent with the presence of 27 carbon atoms (table 3) and DEPT measurements revealed the presence of five methyl groups, nine methylene, six methine, two quaternary carbons, three -OCH and one -HC=C. Taken together, these data indicated a disulphated trihydroxycholestene structure. The assignments for the NMR signals associated with the tetracyclic nucleus showed similarity to those reported for thornasterol A[12] and the many thornasterol A 3β-sulphated saponins[13]. The ^{13}C NMR spectrum featured signals at 118.0 and 146.6 ppm in the downfield region, indicative of the presence of an endocyclic double bond at the 9,11 positions, and two low-field resonances at δ 79.5 and 78.4 ppm assigned to C-3 and C-6, both bearing a sulphoxy group. The ^1H NMR spectrum (table 2) showed signals at δ 4.23 (m) and 4.35 ppm (td, $J = 4$ and 10.5 Hz) with the characteristic shapes of 3β- and 6α-oxygenated methines, respectively. The use of the COSY experiment allowed the remaining hydroxyl function to be determined at the position 22. Further support comes from the upfield shifts exhibited by C-17, C-21 and C-24 signals (3.0, 6.3 and 3.4 ppm, γ effect) and the downfield shifts of the signals corresponding to C-20 and C-23 (+6.8 and +3.6 ppm, β effect) observed in the ^{13}C NMR spectrum of **5** with respect to the reference cholesterol[10]. The large β-effect on C-20 (6.8 ppm) and the smaller one on C-23 (3.6 ppm) indicated the 22*R* configuration[14]. In confirmation, **5** was acetylated (acetic anhydride and pyridine at room temperature for 8 h) followed by solvolysis in a pyridine-dioxan mixture to afford (22*R*)-5α-cholest-9(11)-ene-3β,6α-22-triol-22-acetate, **6a**, whose ^1H NMR spectrum in $CDCl_3$ showed the 21-methyl doublet signal at δ 0.92 ppm, in close agreement with that published[11] for (22*R*)-cholest-5-ene-3β-22-diol-3,22-diacetate (δ 0.92 ppm) and far away from that of the 22*S* isomer (δ 0.97 ppm).

The natural steroid **6** is the 22-acetate derivative of **5**, as confirmed by conversion of **5** into **6** by acetylation, while the compounds **7** and **8** are the 9,11-dihydro derivatives of **5** and **6**, respectively, as conclusively shown by their NMR spectra (tables 2 and 3) and by comparison with those reported in the literature[8,10,11], taking into account the effects of shifts due to the presence of sulphate at C-3 and C-6[15].

STRUCTURE ELUCIDATION OF STEROIDS **9** AND **10**

The negative-ion FAB mass spectrum of **9** showed a molecular ion species at *m/z* 467, $[M-H]^-$. The

TABLE 3 - ^{13}C NMR SPECTRAL DATA (62.9 MHz IN CD_3OD)

C	4	5	6	7	11	12
1	38.5	37.0	36.9	37.1	35.2	34.4
2	29.6	29.6	29.6	29.2	30.9	31.2
3	80.0	79.5	79.5	79.4	72.1	68.3
4	30.9	31.3	31.3	30.6	34.8	40.5
5	52.0[a]	49.0	49.0	50.9	49.2	76.5
6	74.6[a]	78.4	78.4	78.3	73.9	78.0
7	41.3	40.9	40.8	40.9	37.3	41.2
8	35.6	36.9	37.0	35.4	31.7	77.1
9	55.1	146.6	146.6	54.8	42.2	49.1
10	37.5	39.6	39.6	37.1	35.5	39.3
11	22.3	118.0	118.0	22.4	21.4	19.5
12	41.3	43.1	43.0	39.8	42.0	43.2
13	43.1	42.6	42.7	43.8	44.8	45.3
14	57.4	54.8	54.6	57.0	61.4	63.9
15	25.4	26.4	26.4	25.0	85.1	81.0
16	28.9	28.6	28.3	28.0	83.1	82.9
17	54.4	54.6	54.5	54.4	60.0	60.7
18	12.4	11.9	11.9	12.1	15.0	16.8
19	13.8	19.6	19.6	13.5	26.1	18.0
20	41.2	43.6	40.4	43.5	30.9	31.0
21	13.4	12.6	13.1	12.6	18.6	18.5
22	76.7	74.8	78.6	74.3	37.1	35.0
23	28.3	28.7	26.1	28.2	24.7	28.8
24	44.1	37.4	36.8	38.2	34.8	42.4
25	30.0	29.3	28.9	29.0	36.9	31.1
26	20.6	23.2	23.1	23.0	68.7	19.3
27	17.8	22.8	22.6	22.5	17.0	19.9
28	23.6	-	-	-	-	31.8
29	12.7	-	-	-	-	68.3
$\underline{C}H_3CO$-	21.2	21.1	-	-	-	-
>C=O	178.7	172.8	-	-	-	-

([a]) In the proton-noise decoupled spectrum signals are doublets with $J_{P\text{-}O\text{-}C}$ = 5.5 Hz and $J_{P\text{-}O\text{-}C\text{-}C}$ = 7.4 Hz.

assignments of the NMR signals showed close similarity tho those reported for the 5α-cholestane-3β,5,6β,15α,16β,26-hexaol first isolated from the starfish *Luidia maculata*[16], and then from *Myxoderma platyacanthum*[18]. The steroid **10** is the 15-sulphate derivative of **9**; it showed in the FAB mass spectrum (negative ion mode) a molecular anion peak at *m/z* 547 $[M]^-$, and NMR signals very close to those of 5α-cholestane-3β,5,6β,15α,16β,26-hexaol-15-sulphate previously isolated from the genus *Rosaster*[17] and later from *Myxoderma platyacanthum*[18]. An accurate

TABLE 4 - 125 MHz- ^{13}C NMR CHEMICAL SHIFTS (CD_3OD) OF SIDE-CHAIN CARBONS OF **9** AND **10** (25*R* ISOMERS) AND THEIR CORRESPONDING 25*S* ISOMERS

C	9		10	
	25*R*	25*S*[a]	25*R*	25*S*[a]
22	37.3	37.4	37.2	37.3
23	24.8	24.9	24.8	24.9
24	34.7	34.9	34.7	34.9
25	36.9	37.0	36.9	37.0
26	68.6	68.4	68.6	68.5
27	17.1	17.3	17.1	17.3

([a]) The 25*S*-isomer samples used were isolated from the starfish *Myxoderma platyacanthum*[18].

inspection of the ^1H NMR spectrum of **9** revealed very small differences in the chemical shifts of the 27-methyl and 26-H signals relative to those of a sample of 5α-cholestane-3β,5,6β,15α,16β,26-hexaol isolated from *Myxoderma platyacanthum*[18] (figure 1).

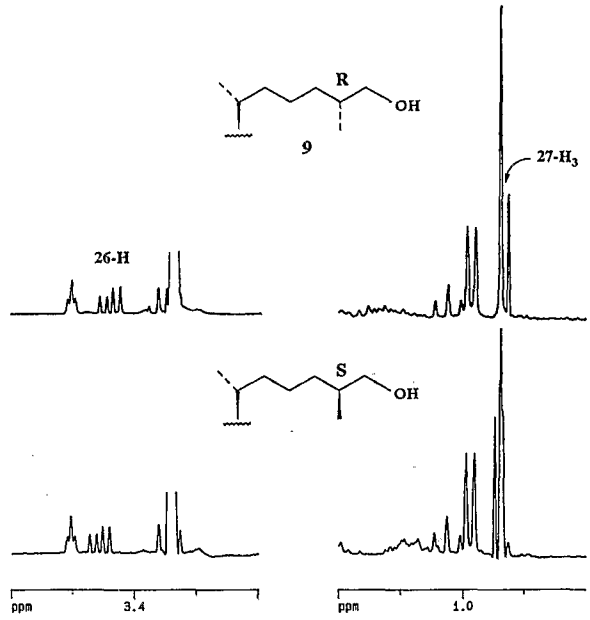

Fig. 1 - 500 MHz ^1H NMR spectra of **9** (25*R* isomer) and its 25*S* isomer.

The two samples differ also by the different chemical shifts of the side-chain carbons (table 4). The Δδ values of the corrisponding carbons are so small that the differences are better appreciated in the spectrum of their mixtures. The same different chemical shifts are also observed in the spectra of the 15-sulphated samples. The samples from *Rosaster sp.*[17] and *Myxoderma platyacanthum*[18] were assigned the 25*S* configuration and, accordingly, the samples now isolated from *Tremaster novaecaledoniae* are suggested to be the 25*R* isomers. Similar small chemical shift differences have been reported for the (25*S*)- and (25*R*)-26-hydroxycholesterol[19].

The chemical shift differences of the C-26 methylene protons in the 26-(+)-MTPA [α-methoxy-

TABLE 5 - SELECTED ^1H NMR (CD_3OD) SIGNALS OF THE STEROIDS **9-12**. *J* (Hz) ARE SHOWN IN PARENTHESIS

H at C⁻	**9**	**10**	**11**	**12**
3	4.04 m	4.03 m	3.54 m	4.08 m
6	3.50 br s	3.51 br s	3.74 br s	3.60 br t (3)
15	3.76 dd (11, 2.5)	4.38 dd (11, 2.5)	3.77 dd (11, 2.5)	4.15dd (10, 2.5)
16	4.00 dd (8, 2.5)	4.33 dd (8, 2.5)	4.01 dd (8, 2.5)	4.04 dd (7.5, 2.5)
18	0.94 s	1.00 s	0.94 s	1.13 s
19	1.20 s	1.20 s	1.15 s	1.34 s
21	0.99 d (7)	0.98 d (7)	0.99 d (7)	0.96 d (7)
26	3.44 dd (10.4, 5.5) 3.35 dd[a]	3.44 dd (11, 6.1) 3.33 dd (11, 6.0)	3.45 dd (10.5, 5.8) 3.35 dd (10.5, 4)	0.89 (6.8)
27	0.93 d (7)	0.93 d (7)	0.93 d (7)	0.92 (6.8)
29	-	-	-	4.05 m

([a]) Partially overlapped with the CHD_2OD signal.

α-(trifluoromethyl)phenylacetic acid, Mosher's reagent[20]; the term (+) or (–) MTPA ester refers to an ester obtained using the acid chloride prepared from (+)-(*R*)- and (–)-(*S*)-MTPA acid, respectively] and 26-(–)-MTPA esters were used to determine the C-25 *S* configuration of the previous starfish-derived 26-hydroxysteroids[17,18,21]. In the spectra of the MTPA esters of a 25*S* isomer, the 26-methylene proton signals appear much closer in the spectrum of the (+)-MTPA ester than in that of the (–)-MTPA derivative, while the reverse occurs for MTPA esters of a 25*R* isomer (figure 2).

Thus, we have treated **9** with (+)- and (–)-α-methoxy-α(trifluoromethyl)phenylacetyl chloride affording the 3,26-di-(+)-MTPA and the 3,26-di-(–)-MTPA esters, respectively, and we have recorded their ^1H NMR spectra. The methylene protons signals of the (+)-MTPA ester appeared as well separated dd's at δ 4.14 (*J* = 6.6, 10.8 Hz) and 4.28 ppm (*J* = 6.4, 10.8 Hz), while in the spectrum of the (–)-MTPA ester they appear as closer dd's at δ 4.19 and 4.23 ppm, in agreement with the 25*R* configuration.

The same reaction with (+)- and (–)-MTPA chloride was performed with **10**. The presence of the bulky sulphoxy group at C-15α makes the differences of the 26-methylene proton signals in the spectra of the (+)-MTPA (δ 4.15 and 4.25 ppm) and (–)-MTPA (δ 4.15 and 4.23 ppm) very small[18]. When **10** was solvolysed, the removal of the sulphate group produced the appearance of the expected double doublets at δ 4.14 and 4.27 ppm in the ^1H NMR spectrum of the (+)-MTPA ester and at δ 4.19 and 4.23 ppm in that of the (–)-MTPA ester.

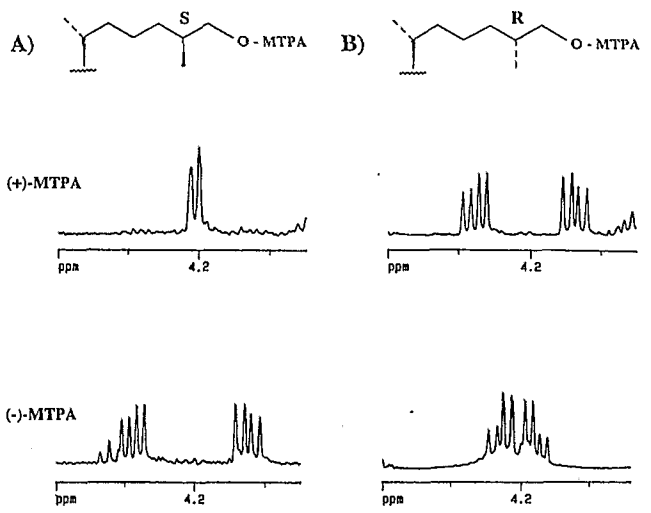

Fig. 2 - Chemical shifts of the 26-methylene protons of (+)-MTPA and (–)-MTPA esters of the 25*S* steroid from *Myxoderma platyacanthum* (A) and **9**, 25*R* isomer (B).

STRUCTURE ELUCIDATION OF STEROID **11**

The negative-ion FAB mass spectrum of **11** showed a molecular ion species at *m/z* 451 $[M-H]^-$. The ^{13}C NMR spectrum was consistent with the presence of 27 carbon atoms (table 3) and DEPT measurements revealed the presence of four methyl groups, nine methylene, seven methine, two quaternary carbons, four-OCH and one-OCH_2. Taken together, these data indicated one pentahydroxycholestane structure with one of the five methyl groups oxidised to hydroxymethylene. Examination of the ^1H- and ^{13}C NMR spectra immediately indicated the presence of a 15α,16β-dihydroxy moiety (double doublets at δ 3.77, *J* = 11, 2.5 and 4.01 ppm, *J* = 8, 2.5 Hz), and of a 26-hydroxyl group [two 1 H double doublets at δ 3.45 (*J* = 10.5, 5.8 Hz) and 3.35 ppm (*J* = 10.5, 4 Hz), the latter overlapping with the CHD_2OD signal]. Also present in the ^1H NMR spectrum is a 1 H multiplet ($W_{1/2}$ = 20 Hz) at δ 3.54 ppm with the shape of a 3β-hydroxymethine in a 5α-stanol or a 3α-hydroxymethine in a 5β-stanol and 1 H narrowing signal at δ 3.74 ppm for an equatorial proton. The chemical shifts of the angular methyl carbons at 15.0 and 26.1 ppm were noteworthy. The lowfield shielding of one of them was indeed strongly indicative of the *cis*-A/B ring fusion[22]. In addition, since the relative rigidity of the steroidal skeleton is such that the C-18 shielding may be expected to be essentially unaffected by a change of the A/B ring fusion, the shift of 15.0 ppm agreed with that expected for a C-18 carbon in a structural environment such as **11** (*cfr.* **9** and **10**). Thus, the 3α,15α,16β,26-tetrahydroxy-5β-cholestane

structure could be established and the fifth hydroxyl group was located at the 6β-position, in agreement with the downfield shift of the 19-methyl proton signal to δ 1.15 ppm in the ^{1}H NMR (calcd. for 5β-cholestane-3α,6β-diol, δ_H 1.13 ppm)[23]. The alternative 11β-position could be rouled out from the ^{1}H NMR chemical shift of the 18-methyl protons at δ 0.94 ppm, unshifted relative to that of 5α-cholestane-3β,6β,15α,16β,26-pentol[23], and from the ^{13}C NMR signal at 21.4 ppm assigned to C-11. Complete assignments of the carbon signals in the spectrum of **11** (table 3) by using 5β-cholestan-3α-ol[10] and 5α-cholestane-3β,6β,15α,16β,26-pentol[24] as reference compounds, confirmed the 5β-cholestane-3α,6β,-15α,16β,26-pentol formulation for the new natural steroid. The stereochemistry at C-25 is suggested to be 25*R* likewise the steroids **9** and **10**, by using the MTPA method. In the 3,26-di-(+)-MTPA ester, the 26-methylene protons appear as well separated double doublets at δ 4.28 and 4.14 ppm, as in the (+)-MTPA ester of **9**.

While steroids with the *cis*-A/B ring junction are commonly found in ophiuroids[6,25], the steroid **11** appears the first example with such a structural feature isolated from starfishes.

Structure elucidation of steroid **12**

The negative-ion FAB mass spectrum of steroid **12** exhibited molecular anion species at *m/z* 591 $[M]^-$. The ^{13}C NMR spectrum (table 3) indicated the presence of 29 carbon atoms and DEPT measurements revealed the presence of five methyl groups, nine methylene, six methine, two quaternary carbons, two-O-C, four -O-CH and one -O-CH_2. Taken together, these data indicated a saturated C_{29} sterol with six hydroxy and one sulphoxy groups. The presence of a sulphate group was confirmed by solvolysis of **12**, affording the desulphated derivative **12a**, which gave a molecular ion species in the FAB mass spectrum at *m/z* 511 $[M-H]^-$. The assignments of the NMR signals associated with the tetracyclic moiety showed similarity with those reported for 5α-cholestane-3β,6β,8,15α,16β-26-hexol, **9**, and 5α-cholestane-3β,6β,8,15α,16β-26-hexol isolated from the starfish *Sphaerodiscus placenta*[26]. The ^{1}H NMR spectrum (table 5) featured 1 H multiplet downfield shifted to δ 4.08 ppm with the complexity normally seen for a 3β-hydroxyl group, and 1 H apparent triplet (J = 3 Hz) at δ 3.60 ppm, characteristic for an equatorial proton, indicative for the presence of a 3β,5α,6β-trihydroxy moiety and signals at δ 4.04 ppm (dd, J = 7.5, 2.5 hz) and 4.15 ppm (dd, J = 10, 2.5 Hz) assigned to 15β- and 16α-protons in an 8,15α,16β-trihydroxy steroidal structure. The hydroxyl group at position 8 is a very common element in polyhydroxysteroids from starfishes and its presence in **12** is confirmed from the chemical shift of the 19-methyl protons downfield shifted to δ 1.34 ppm in **12** relative to **9** (δ 1.20 ppm). A 24-(β-sulphoxyethyl) side chain, already found in a steroid from the starfish *Poraster superbus*[27], accounts for the remaining ^{1}H- and ^{13}C NMR data. The location of the sulphate group at C-29 received confirmation from the comparison of the ^{1}H NMR spectra of the native **12** (δ_{CH_2O} 4.05 ppm, br t) and the desulphated **12a** (δ CH_2OH 3.60 ppm, m). The 24*R* configuration is suggested from the signals due to the isopropyl methyls, at 19.3 and 19.9 ppm in the ^{13}C NMR spectrum and at δ 0.89 and 0.92 ppm, in the ^{1}H NRM spectrum[28].

EXPERIMENTAL

Spectra were obtained on the following instruments: Bruker WM-250 and AMX-500 (^{1}H and ^{13}C NMR); VG-ZAB equipped with an FAB source (FAB mass spectra in glycerol matrix; Xe atoms of 2-6 kV); Perkin-Elmer polarimeter mod. 141 (optical rotations); Water model 6000 A pump equipped with a U6K injector and a differential refractometer model 401 (HPLC); DCCC-A apparatus manufactured by Tokyo Rikakikai Co. equipped with 250 tubes and Büchi apparatus equipped with 300 tubes (DCCC); Bruker FT-IR IFS-48 spectrophotometer (IR spectra).

Extraction and isolation

The animals, *Tremaster novaecaledoniae* Jangoux, 1982, were collected off Nouméa (New Caledonia) in 1987 at a depth of 530 m. The identification was done by Professor M. Jangoux of the Université Libre de Bruxelles, Belgique, and a voucher specimen is preserved there. The animals (0.9 kg, wet weight) were then cut in small pieces and soaked in water (1 l, 4 h) and then treated with 1.5 l of acetone (residue after concentration, 5.3 g). The aqueous extracts were centrifuged and passed through a column of Amberlite XAD-2 resin (1 kg.). This column was washed with water and eluted with methanol to give a partially purified steroid mixture (0.62 g). The residue from the acetone extration was partitioned between methanol and *n*-hexane. The methanol extracts (2.8 g) were combined with the above partially purified steroid mixture (0.62 g) and chromatographed on a column of Sephadex LH-60 (4 × 100 cm) with methanol-water (2:1) as eluant. Fractions (4 ml) were collected and analysed by TLC on SiO_2 in butan-1-ol-acetic acid-water (60:15:25) and chloroform-methanol-water (80:18:2). Fractions 9-31 (1.1 g) mainly contained the phosphated steroid metabolites **1-4**, while fractions 32-47 (620 mg) mainly contained the polyhydroxylated steroids **9-12** and fractions 48-60 (80 mg) contained a crude mixture of the disulphated steroids **5-8**.

The residue from the first eluted fractions was submitted to droplet counter corrent chromatography (DCCC) with butan-1-ol-acetone-water (3:1:5) [descending mode; the upper phase was used as stationary phase; flow rate 10 ml h^{-1}; fractions (5 ml) were collected] to give, in the first more polar fractions, small amounts of a mixture of the disulphated steroids **5-8** (27 mg) and the phosphated metabolites **1-4**. These compounds were further purified by reverse-phase HPLC on a C_{18} μ-Bondapak column (30 cm ×8 mm *i.d.*) with methanol-water (1:1) as the eluant. The results of our analysis are summarised in table 1. ^{1}H- and ^{13}C NMR spectra of the new compounds are in tables 2, 3 and 5; FAB-MS spectral data are in the text.

Fractions 32-47 (0.62 g) from the Sephadex LH-60 column were submitted to DCCC in chloroform-methanol-water (7:13:8) in the ascending mode (4 ml fractions were collected) to give, in the polar eluted fractions (more polar), the complex mixture of sulphated steroids (310 mg) and then the polyhydroxysteroids **9** and **11** in that order, which were further purified by reverse-phase HPLC on a C_{18} μ-Bondapak column with methanol-water (7:3). The mixture of the sulphated steroids were again submitted to DCCC in butan-1-ol-acetone-water (3:1:5) in the ascending mode (3-ml fractions were collected) to give, in the fractions 49-66, a mixture of the monosulphated steroids **12** and **10**, which were then separated by HPLC on a C_{18} μ-Bondapak column with methanol-water (55:45), and in the subsequent more polar fractions (67-104) a mixture of the disulphated steroids **5** and **7** (32 mg). ^{1}H- and ^{13}C NMR spectra of the new compounds **11** and **12** are in tables 3 and 5; FAB-MS data are in the text. Finally, the fractions 48-60 (80 mg) from the chromatography on the column of Sephadex LH-60 were sub-

mitted to HPLC on a C_{18} μ-Bondapak column with methanol-water (4:6) to give the steroids **5, 7, 6** and **8** in that order. ^{1}H- and ^{13}C NMR spectra of the new steroids **5-8** are in tables 2 and 3. FAB-MS spectra are in the text.

ACETYLATION OF **5** GIVING **6**

The steroid **5** (2.5 mg) was acetylated in pyridine and acetic anhydride and stirred at room temperature for 8 h. ^{1}H NMR signals were identical to those reported for compound **6**.

SOLVOLYSIS OF **6** AND **12**

A solution of **6** (3 mg) in dioxan (0.1 ml) and pyridine (0.1 ml) was heated at 140 °C for 1 h in a stoppered reaction vial. After the solution was cooled, H_2O was added and the solution was extracted two times with butan-1-ol affording the corresponding desulphated derivative **6a**; FAB-MS (negative ion), *m/z* 459 $[M-H]^-$, ^{1}H NMR see table 2.

A solution of **12** (2 mg) was similarly solvolysed affording the corresponding desulphated derivative **12a**, purified by reverse-phase HPLC with methanol-water (65:35), FAB-MS (negative ion) *m/z* 511 $[M-H]^-$; ^{1}H NMR (CD_3OD), δ_H: 0.87 and 0.91 (each 3 H, J = 6.8 Hz, 26,27 H_3), 0.96 (3 H, d, J = 6.8 Hz, 21-H_3), 1.14 (3 H, s, 18-H_3), 1.34 (3 H, s, 19-H_3), 3.59 (3 H, m, 6α-H and 29-H_2), 4.01 (1 H, dd, J = 8, 2.5 Hz, 16α-H), 4.08 (1 H, m, 3α-H) and 4.16 ppm (1 H, dd, J = 10, 2.5 Hz, 15β-H).

MTPA ESTERS OF THE POLYHYDROXYSTEROID **9**

Compound **9** (2 mg) was esterified with (+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (5 μl) in dry pyridine (0.15 ml) for 1 h at room temperature to give, after removal of the solvent, the 3,26-di-(+)-MTPA ester. ^{1}H NMR (CD_3OD), δ_H: 0.94 (3 H, s, 18-H_3), 0.95 (3 H, d, J = 7 Hz, 27-H_3), 0.99 (3 H, d, J = 6.8 Hz, 21-H_3), 1.20 (3 H, s, 19-H_3), 3.76 (1 H, dd, J = 11, 2.5 Hz, 15β-H), 3.99 (1 H, dd, J = 8, 2.5 Hz, 16α-H), 4.14 and 4.28 (each 1 H, dd, J = 10.8, 6.6 and 10.8, 6.4 Hz, respectively, 26-H_2), 5.5 ppm (1 H, m, 3α-H).

The 3,26-di-(–)-MTPA ester of **9** (1.5 mg) was prepared using (–)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride: ^{1}H NMR (CD_3OD) identical with values reported for (+)-MTPA except for signals of 26-H_2 split at δ 4.19 (1 H, dd, J = 10.7, 5.6 Hz) and 4.23 (1 H, dd, J = 10.7, 6.3 Hz) ppm.

MTPA ESTER OF THE SULPHATED POLYHYDROXYSTEROID **10**

Compound **10** (2 mg) was esterified with (+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride as above to give the 3,26-di-(+)-MTPA ester. ^{1}H NMR (CD_3OD), δ_H: 0.93 (1 H, d, J = 7 Hz, 27-H_3), 0.98 (3 H, d, J = 6.8 Hz, 21-H_3), 1.00 (3 H, s, 18-H_3), 1.20 (3 H, s, 19-H_3), 4.15 and 4.25 (each 1 H, dd, J = 10.7, 6.4 and J = 10.7, 5.6 Hz, respectively, 26-H_2), 4.32 (1 H, dd, 16α-H), 4.36 (1 H, dd, 15β-H), 5.5 ppm (1 H, m, 3α-H). The 3,26-di-(–)-MTPA ester of **10** (2 mg) was prepared using (–)-α-methoxy-a-(trifluoromethyl)phenylacetyl chloride in an identical manner. ^{1}H NMR spectra (CD_3OD) were identical with values reported for (+)-MTPA, except for the signals of 26-H_2 at δ 4.15 and 4.23 ppm (each 1 H, dd, J = 10.8, 5.6 and J = 10.8, 6.0 Hz, respectively). A solution of 3,26-di-(+)-MTPA ester of **10** (2 mg) was heatead in dioxan (0.1 ml) and pyridine (0.1 ml) at 130 °C for 2 h in a stoppered reaction vial. After the solution had cooled, H_2O was added, and the solution was extracted three times with *n*-butanol. Removal of the solvent left the glassy material of the desulphated (+)-MTPA ester. ^{1}H NMR spectra (CD_3OD) were almost identical with that of **9**-(+)-MTPA ester, signals of 26-H_2 at δ 4.14 (1 H, dd, J = 10.8, 6.6 Hz) and 4.27 ppm (1 H, dd, J = 10.8, 6.4 Hz). Compound 3,26-di-(–)-MTPA ester **10** (2 mg) was solvolysed as described above to the corresponding desulphated (–)-MTPA ester, ^{1}H NMR (CD_3OD) almost identical with that of **9**-(–)-MTPA ester; signals of 26-H_2 appearing at δ 4.19 and 4.23 ppm (each 1 H, dd, J = 10.7, 5.6 and J = 10.7, 6.3 Hz respectively).

MTPA ESTER OF POLYHYDROXYSTEROID 11

The steroid **11** (2 mg) was treated with freshly distilled (+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride as above to give the 3,26-di-(+)-MTPA ester. ^{1}H NMR (CD_3OD), δ_H: 0.94 (3 H, s, 18-H_3), 0.95 and 0.99 (each 3 H, d, J = 7 Hz, 27 and 21-H_3), 1.20 (3 H, s, 19-H_3), 3.76 (1 H, dd, J = 11, 2.5 Hz, 15β-H), 3.99 (1 H, dd, J = 8, 2.5 Hz, 16α-H), 4.14 and 4.28 (each 1 H, dd, J = 10.8, 6.6 and J = 10.8, 6.4 Hz respectively, 26-H_2), 4.90 (1 H, m, 3β-H) ppm.

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