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## The First Occurrence of Polyhydroxylated Steroids with Phosphate Conjugation from the Starfish *Tremaster novaecaledoniae*

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**Abstract:** Three steroid constituents have been isolated from the starfish *Tremaster novaecaledoniae* (Jangoux 1982) collected at a depth of 530 m off New Caledonia. Compounds 1 - 3, designated as tremasterol A - C, are characterized by the presence of 3 $\beta$ -O-sulphated, 6 $\alpha$ -O-phosphated and 16 $\beta$ -O-acetylated groupings on a steroidal skeleton. In compound 1 the monophosphate residue is further linked to 1'-glucose (1'-glucose tetracetate in 2 and 1'-glucose-6'-acetate in 3).

It is known that there are three groups of steroidal oligoglycosides in starfishes: sulphated steroidal penta- and hexa-glycosides ("asterosaponins"), steroidal cyclic glycosides (so far only found in two species of the genus *Echinaster*), and glycosides of polyhydroxysteroids consisting of a polyhydroxysteroid with one or two sugar units, which are found in both sulphated and non sulphated form. Often these saponins are accompanied by small amounts of polyhydroxylated steroids, also occurring in both sulphated and non-sulphated form<sup>2,3</sup>. In our continuing investigation of the New Caledonian marine species we have examined the polar extracts of the starfish *Tremaster novaecaledoniae* (Jangoux 1982), which is a "living fossil" species<sup>4</sup>, collected at depth of 530 m off New Caledonia, and wish to report the first isolation of phosphated polyhydroxysteroids from a natural source. Compounds 1 - 3 are also sulphated.

Compounds were isolated and purified by chromatography on Sephadex LH-60 (methanol-water 2:1 as eluant) of the methanol soluble portion of the acetone extracts, followed by droplet counter current chromatography [DCCC, butan-1-ol : acetone : water (3:1:5); descending mode] and HPLC ( $\mu$ -Bondapak C<sub>18</sub>, 30 cm x 7.8 mm i.d.; methanol-water 1:1 as eluant).

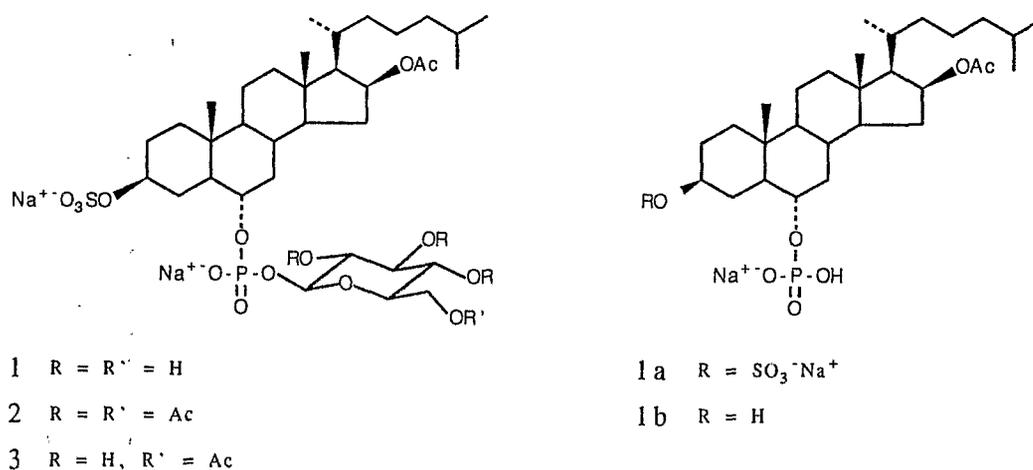
Tremasterol A (1),  $[\alpha]_D^{25} = +40^\circ$  (c 0.5, MeOH) exhibited, in its negative-ion FAB mass spectrum, molecular ion species at m/z 803 (80%) and 781 (40%), corresponding to  $[M_{Na}]^-$  and  $[M_H]^-$ , respectively, and intense fragment ion peaks at m/z 641 (100%) and 619 (90%) arising by loss of the glucosyl residue (= 162 m. u.). Fragment ion peaks at m/z 539 (20%) and 521 (50%) were interpreted as loss of SO<sub>3</sub> from m/z 619 and NaHSO<sub>4</sub> from m/z 641. The presence of a steroidal skeleton was deduced from <sup>1</sup>H NMR methyl proton signals (Table 1) and one sugar unit (identified as glucose; anhydrous acid methanolysis) would be also considered for the signals in the 3.28 - 3.68 region and the anomeric one at  $\delta$  4.89.

The presence of an acetoxy group was shown from IR ( $\nu_{max}$  1735 cm<sup>-1</sup>), <sup>1</sup>H NMR ( $\delta_H$  2.02 s, 3H) and <sup>13</sup>C NMR ( $\delta_C$  172.4 and 21.2 ppm) data. In addition to the sugar moiety and the acetoxy group, the <sup>13</sup>C NMR spectrum revealed carbon signals in agreement with a trioxxygenated cholestane structure (Table 1). 2D-COSY experiment allowed the sequential assignment of the resonances for the glucosyl residue and also the establishment of the connectivities C-1 to C-8 and C-15 to C-21 within the steroidal framework. The multiplet at  $\delta$  4.24 had the complexity normally seen for a 3 $\beta$ -oxygenated group and its downfield chemical shift, virtually identical to that

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seen in the spectrum of the 5 $\alpha$ -cholestan-3 $\beta$ -yl sulphate, suggested a sulphate group located there. The multiplet at  $\delta$  4.06 appearing as a dddd ( $J = 9.5, 9.5, 7.5$  and  $4.5$  Hz), was assigned to an axial proton (H-6 $\beta$ ) in a cholestane skeleton. The axial proton associated with the 6 $\alpha$ -hydroxyl group is usually seen as a triple doublet ( $J = 9.5$  and  $4.5$  Hz), and the shape of the 6 $\beta$ -proton signal in **1** was suggestive for the presence of a phosphate (typical  $J_{\text{H-C-O-P}} = ca\ 8.4$  Hz)<sup>6</sup>, to which the  $\beta$ -glucopyranosyl residue is glycosidally linked. This was confirmed by the proton noise decoupled <sup>13</sup>C NMR spectrum, in which 1', 2', 5 and 6 carbons appear as doublets with  $J_{\text{P-O-C}} = 5.5$  Hz and  $J_{\text{P-O-C}} = 7.4$  Hz. The presence of a phosphate group linking C-6 of the steroid and C-1' of glucose was definitively confirmed by <sup>31</sup>P NMR spectrum, which showed a triplet ( $J = 7.5$  Hz) signal at 3.54 ppm downfield from external standard ( $\text{H}_3\text{PO}_4$  85% in  $\text{D}_2\text{O}$ ), converted into doublets on irradiation at  $\delta_{\text{H}}$  4.06 (H-6) and at  $\delta_{\text{H}}$  4.89 (H-1'). The <sup>13</sup>C chemical shifts of the glucosyl residue were also compatible with those of reference  $\beta$ -O-glucopyranose-1-phosphate<sup>7</sup>. Under very mild acid treatment (reverse phase HPLC using 0.1%  $\text{CH}_3\text{CO}_2\text{H}$  in 60% *aq.* methanol) glucose was removed giving **1a**<sup>8</sup>, negative-ion FAB mass spectrum,  $m/z$  643 [ $\text{M}_{\text{Na}}^-$ ] and  $m/z$  621 [ $\text{M}_{\text{H}}^-$ ]. The presence of the sulphate group at C-3 was confirmed by solvolysis of **1** in a dioxane-pyridine mixture, affording the desulphated derivative **1b**<sup>9</sup>, negative-ion FAB mass spectrum,  $m/z$  541 [ $\text{M}_{\text{H}}^-$ ],  $\delta_{\text{H-3}}$  3.50 m (4.24 in **1**);  $\delta_{\text{C-3}}$  72.0 ppm (79.9 ppm in **1**). The remaining acetoxy function was located at the 16 position from the chemical shift of the H-16 signal ( $\delta$  5.27 m), which showed coupling to signals at  $\delta$  2.42 (H-15), 1.32 (H-17) and 1.15 (H-15) ppm in the 2D-COSY spectrum. The  $\beta$ -configuration was deduced from the downfield shift of the 18-methyl protons to



$\delta$  0.93, requiring the presence of a 1,3-*syn* interaction, and confirmed by the <sup>13</sup>C NMR signal at  $\delta$  31.3 ppm assigned to C-20, significantly upfield relative to the model 5 $\alpha$ -cholestan-3 $\beta$ -ol<sup>10</sup> ( $\delta_{\text{C}}$  36.0 ppm), as expected upon introduction of a substituent at 16 $\beta$ -position.

Therefore, based on the above results, tremasterol A possesses structure **1**, that is 5 $\alpha$ -cholesta-3 $\beta$ ,6 $\alpha$ ,16 $\beta$ -triol-3-sulphate,6-(glucose-1')-phosphate,16-acetate.

Tremasterol B (**2**) is the 2',3',4',6'-tetraacetate derivative of **1**. The negative-ion FAB mass spectrum showed molecular anion species at  $m/z$  971 (100%) [ $\text{M}_{\text{Na}}^-$ ] and 949 (20%) [ $\text{M}_{\text{H}}^-$ ] accompanied by a fragment ion peak at  $m/z$  641 (25%), corresponding to the loss of a glucosyl tetraacetate residue (330 m.u.) from  $m/z$  971. In addition to the signals for the aglycone already observed in the spectrum of **1**, the <sup>1</sup>H NMR spectrum of **2**<sup>11</sup> contained four more methyl singlets in the 1.99-2.09 region (four acetate groups) and the sugar signals were seen downfield shifted to  $\delta$  3.97 (m, H-5'),  $\delta$  4.22 (dd,  $J = 12.5$  and  $2.5$  Hz) - 4.36 (dd,  $J = 12.5, 5.0$  Hz,  $-\text{CH}_2-\text{OAc}$ ) and as overlapping signals in the 4.9-5.3 ppm region (H-1', H-2', H-3' and H-4'). The <sup>13</sup>C NMR chemical shifts of the glucosyl tetraacetate residue in **2** were compatible with those in references<sup>7</sup>. Reverse phase HPLC using 0.1%  $\text{CH}_3\text{CO}_2\text{H}$  in 60% *aq.* methanol resulted in the removal of glucose tetraacetate affording **1a** and, upon solvolysis in a dioxane-pyridine mixture, **2** afforded the desulphated steroid **1b**.

Table 1. NMR data (CD<sub>3</sub>OD) of tremasterol A (1).

Carbon	<sup>13</sup> C NMR <sup>a</sup> (ppm)	Proton	<sup>1</sup> H NMR <sup>b</sup> (ppm)
1	37.0	1 $\alpha$ , 1 $\beta$	1.12 m, 1.82 m
2	29.5	2 $\alpha$ , 2 $\beta$	2.07 m, 1.61 m
3	79.9	3 $\alpha$	4.24 m
4	31.0	4 $\alpha$ , 4 $\beta$	2.60 m, 1.50 m
5	52.0*		1.30 m
6	76.0*	6 $\beta$	4.06 dddd (9.5,9.5,7.5,4.5)
7	40.9	7 $\alpha$ , 7 $\beta$	1.08 m, 2.35 m
8	35.1		1.65 m
9	55.5		
10	37.6		
11	22.0		
12	41.1		
13	44.1		
14	55.1		
15	35.8	15 $\alpha$ , 15 $\beta$	2.42 m, 1.15 m
16	76.6	16 $\alpha$	5.27 m
17	61.6		1.32 m
18	13.2		0.93 s, 3H
19	13.9		0.92 s, 3H
20	31.3		1.88 m
21	18.6		1.00 d (7), 3H
22	38.4		
23	25.3		
24	40.6		
25	29.0		
26	23.0		0.91 d (7), 3H
27	22.8		0.91 d (7), 3H
CH <sub>2</sub> CO-	21.2	CH <sub>2</sub> CO-	2.02 s, 3H
CH <sub>3</sub> CO-	172.4		
1'	99.3*		4.89 t (7.5)
2'	76.0*		3.28 dd (9.5,7.5)
3'	77.8		3.45 t (9.5)
4'	71.8		3.27 t (9.5)
5'	78.4		3.44 m
6'	63.0		3.68 dd (11.2,6.2)-3.91 dd (11.2,2.5)

<sup>a</sup> At 62.9 MHz; values relative to <sup>13</sup>CD<sub>3</sub>OD ( $\delta_c = 49$  ppm).

<sup>b</sup> At 500.13 MHz by <sup>1</sup>H-<sup>1</sup>H correlation spectroscopy; chemical shifts referred to CHD<sub>2</sub>OD ( $\delta_H = 3.34$ ), J (in Hz) are given in parenthesis.

\*Signals appear as doublets in the proton noise decoupled spectrum.

Tremasterol C (3) [negative-ion FAB mass spectrum: m/z 845 (30%) [ $M_{Na}$ ]<sup>-</sup>, 823 (100%) [ $M_H$ ]<sup>-</sup> and 619 (loss of the glucosyl-O-acetate residue from m/z 823)] is a monoacetate derivative of the steroid 1. On very mild acid treatment 3 afforded 1a whereas on solvolysis in a dioxane-pyridine mixture it gave 1b. The acetate group on 3 was located at C-6 of the glucopyranose moiety from the <sup>1</sup>H NMR spectrum<sup>12</sup>, which showed the C-6 H<sub>2</sub> signals downfield shifted to  $\delta_H$  4.23 (dd, J = 12.5 and 5 Hz) and 4.49 (dd, J = 12.5, 2.5 Hz).

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  - 9 Spectral data of 1b:  $^1\text{H NMR}$  ( $\text{CD}_3\text{OD}$ );  $\delta_{\text{H}}$  = 0.89 (6H, d,  $J$  = 7 Hz,  $\text{CH}_3$ -26 and  $\text{CH}_3$ -27), 0.91 (6H, s,  $\text{CH}_3$ -18 and  $\text{CH}_3$ -19), 1.00 (3H, d,  $J$  = 7 Hz,  $\text{CH}_3$ -21), 2.03 (3H, s,  $\text{CH}_3\text{CO}$ -), 3.50 (1H, m, H-3), 3.98 (1H, dddd,  $J$  = 9.5, 9.5, 7.5 and 4.5 Hz, H-6) and 5.25 (1H, m, H-16) ppm.  $^{13}\text{C NMR}$  ( $\text{CD}_3\text{OD}$ );  $\delta_{\text{C}}$  = C-1: 38.6, C-2: 31.4, C-3: 72.0, C-4: 33.3, C-5: 52.1\*, C-6: 76.7\*, C-7: 41.0, C-8: 35.1, C-9: 55.6, C-10: 37.0, C-11: 22.1, C-12: 41.4, C-13: 44.0, C-14: 55.3, C-15: 35.8, C-16: 76.7, C-17: 61.7, C-18: 13.2, C-19: 13.9, C-20: 31.8, C-21: 18.6, C-22: 38.5, C-23: 25.4, C-24: 40.6, C-25: 29.0, C-26: 23.0, C-27: 22.8,  $\underline{\text{CH}_3\text{CO}}$ : 21.2,  $\underline{\text{CH}_3\text{CO}}$ : 172.3.
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  - 12 Spectral data of 3:  $^1\text{H NMR}$  ( $\text{CD}_3\text{OD}$ );  $\delta_{\text{H}}$  = 0.90 (6H, d,  $J$  = 7 Hz,  $\text{CH}_3$ -26 and  $\text{CH}_3$ -27), 0.91 (3H, s,  $\text{CH}_3$ -19), 0.93 (3H, s,  $\text{CH}_3$ -18), 1.00 (3H, d,  $J$  = 7 Hz,  $\text{CH}_3$ -21), 2.02 (3H, s,  $\text{CH}_3\text{CO}$ -), 2.10 (3H, s,  $\text{CH}_3\text{CO}$ -), 4.05 (1H, dddd,  $J$  = 9.5, 9.5, 7.5 and 4.5 Hz, H-6), 4.24 (1H, m, H-3) and 5.26 (1H, m, H-16) ppm.
- \* Signals appear as doublets in the proton noise decoupled spectrum.

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