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New Ketosteroids from the Red Alga *Hypnea musciformis*

V. Bultel-Poncé,[a,*] S. Etahiri[b] and M. Guyot[a]

[a]*Laboratoire de Chimie des Substances Naturelles associé au CNRS, Muséum National d'Histoire Naturelle, 63 rue Buffon, 75005 Paris, France*

[b]*Laboratoire de Biochimie Marine, Faculté des Sciences, Université Chouaib Doukkali, BP 20, El Jadida, Maroc*

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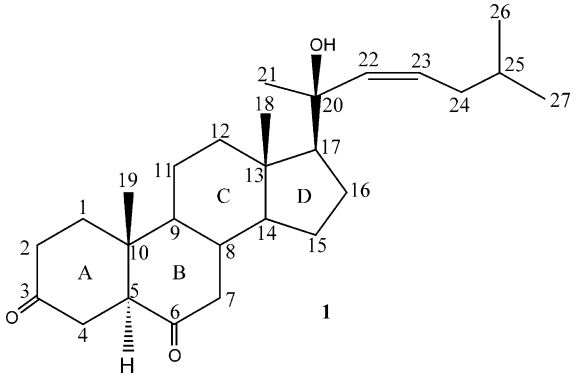
Abstract—The dichloromethane/methanol extract from the red alga *Hypnea musciformis* exhibited PPE elastase inhibition. A diketosteroid, the 20-hydroxy-5α-cholest-22-ene-3,6-dione was responsible for this activity. Two new steroids were isolated, **2** was assigned as the 6α-hydroxy-cholest-4-ene-3-one and **3** as the 6α-hydroxy-cholest-4,22-diene-3-one. The structures were assigned mainly on the basis of ^{1}H and ^{13}C NMR experiments.

In the course of a program aimed to the isolation and characterization of bio-active compounds from marine algae collected along the Atlantic coast of Morocco, 25 algae were tested for biological activities. The organic extract of the red alga *Hypnea musciformis* (Wulfen) Lamouroux Rhodophyceae/Florideophycidae,[1] a cosmopolitan red alga, was found to possess anti-elastase activity against porcine pancreas elastase (PPE). We wish to describe herein the isolation from this alga of a new 3,6-diketosteroid (**1**), which is responsible for the activity of the extract along with two new steroids **2** and **3**. Babu et al. have reported the presence, in an alga also identified as *H. musciformis* of several 7,11-diketosteroids.[2–4]

Hypnea musciformis was collected in March 1999, at El Jadida on the Atlantic coast of Morocco. After air-drying in darkness, samples were ground and exhaustively extracted with dichloromethane/methanol (v/v).

The extract was concentrated in vacuo to yield 5 g of crude material. Purification of the active compound was monitored by measuring the inhibition of the amidolysis of *N*-succinyl-alanyl-alanyl-prolyl-leucyl *p*-nitroanilide (Sigma) by the elastase (EC 3.4.21.36 Type II-A) from porcine pancreas (Sigma).[5] Chromatography over a silica gel column (CH_2Cl_2 to MeOH) of the crude extract led to the active fraction eluted by CH_2Cl_2–EtOAc (9:1).



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Repeated chromatography of this active fraction on silica gel column yield 1.2 mg of pure **1**, (CH_2Cl_2–EtOAc (9:1), R_f 0.62) which was isolated as a white powdery solid, $[\alpha]_D$ +17 (*c* 0.09 CH_2Cl_2).

The IR absorption of **1** indicated the presence of hydroxyl group (ν_{max} 3428 cm^{-1}) and carbonyl function (ν_{max} 1701 cm^{-1}).

High resolution mass spectrometry established the molecular formula $C_{27}H_{42}O_3$ ($[M+H-H_2O]^+$ measured 397.3102, calculated 397.3096), which indicates 7 double-bond equivalents in the molecule.

The ^{1}H and ^{13}C NMR spectra were typical of a sterol. A tertiary alcohol function was indicated by the ^{13}C NMR spectrum (*J* mod), with a quaternary carbon resonating at δ 75.2, in addition with two carbonyls resonating at 209.2 and 211.3 ppm, and five methyl groups at 12.6, 13.9, 22.5, 22.5, 29.5 ppm. The ^{1}H NMR spectrum

*Corresponding author. Tel.: +33-1-4079-3144; fax: +33-1-4079-3135; e-mail: bultel@mnhn.fr

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($CDCl_3$, 400 MHz, Table 1) of **1** showed the presence of two protons involved in a double bond; three tertiary methyl groups resonating as singlets and two others resonating as doublets, involved in an isopropyl group.

Starting from the H_3-19, HMBC correlations allowed to identify C-1, C-5, C-9 and C-10 and therefore CH_2-2 (COSY). The HMBC correlations of H_2-2 and H_2-1 with the carbonyl at 211.3 clearly assigned C-3 at 211.3 ppm. H-5 and the deshielded methylene group (δ 2.30–2.59 ppm) assigned to H_2-4 gave HMBC correlations with the two carbonyl groups which allow to place the second carbonyl at C-6. Another deshielded methylene δ 1.98–2.38 ppm gave a long range correlation with the carbonyl at 209.2 ppm and was assigned to CH_2-7.

The second six-membered ring was built with the correlations observed for H_2-7 with C-6, C-8, C-9, C-14 and those of H-8 with C-7 and C-9. In the same way, the connectivities of ring C were clarified by the HMBC correlations of H_2-11 with C-13 and those of H-9 and H-8 with C-11.

The chemical shift of C-19 (12.6 ppm in **1**) was compared to C-19 in: 5β-cholesta-3-one (22.7 ppm); 5β-cholesta-6-one (24.4 ppm); 5α-cholesta-3-one (11.4 ppm) and 5α-cholesta-6-one (13.1 ppm). These values suggested the H-5 was located in the α or axial position.[6]

Owing to the HMBC correlations observed for H_3-18 (Table 1) we were able to identify C-12, C-13, C-14, C-17. These long range correlations allowed to clearly deduced the chemical shift of H_2-12, which in turn showed correlations in the COSY spectrum with H_2-11.

Table 1. ^1H and ^{13}C assignments (δ ppm) of **1** in $CDCl_3$ on a Bruker Advance 400 at 298 K

No.	^1H (m, *J* Hz)	^{13}C	HMBC	COSY
1	1.61 (m, 2H)	38.2	2, 3, 5, 10	2
2	2.34–2.41 (m, 2H)	37.8	3, 5, 10	
3	—	211.3	—	
4	2.30–2.59 (m, 2H)	37.6	2, 3, 5, 6, 10	
5	2.56 (m, 1H)	57.5	3, 6, 9, 10	
6	—	209.2	—	
7	1.98–2.38 (m, 2H)	46.4	6, 8, 9, 14	
8	1.83 (m, 1H)	37.5	7, 9	7
9	1.33 (m, 1H)	53.6	10, 11, 19	
10	—	41.5	—	
11	1.45–1.65 (m, 2H)	21.8	13	12
12	2.15 (m, 2H)	40.0		11
13	—	43.5	—	
14	1.24 (m, 1H)	56.9	16	
15	1.12–1.58 (m, 2H)	23.8	13	16
16	1.26 (m, 2H)	29.5	13	17
17	1.56 (m, 1H)	60.1	12, 18, 20	
18	0.81 (s, 3H)	13.9	12, 13, 14, 17	
19	0.94 (s, 3H)	12.6	1, 5, 9, 10	
20	—	75.2	—	
21	1.33 (s, 3H)	29.5	17, 20, 22	
22	5.56 (d, 8.4, 1H)	139.3	20	
23	5.47 (m, 1H)	125.6	24	22
24	1.89 (m, 2H)	42.1	22, 23, 25, 26, 27	23
25	1.65 (m, 1H)	28.7	26, 27	24
26	0.86 (d, 6.6, 3H)	22.5	24, 25, 27	25
27	0.89 (d, 6.6, 3H)	22.5	24, 25, 26	25

The five-membered ring from C-13 to C-17 and from C-14 to C-15 was established and confirmed by the COSY correlations between H_2-15 and H_2-16 and H_2-16 with H-17. The H_3-21, resonating as a singlet, showed HMBC correlations with the C-20 bearing the tertiary alcoholic function at δ 75.2 ppm and with the olefinic carbon at δ 139.3 ppm. The stereochemistry at C-20 was assumed to be *S* as postulated by Nes and Varkey,[7] which indicated the two epimers can be distinguished by the ^1H NMR chemical shifts of H_3-18 and H_3-21 being 0.87 and 1.28 ppm respectively in the 20*S*-hydroxycholesterol and 0.87 and 1.13 ppm in the 20*R*-hydroxycholesterol. We observed the first set of chemical shift values for these protons in **1**.

The side chain linked to C-17 was assigned by the ^1H–^1H COSY spectrum showing cross peaks due to coupling between the olefinic H-23 and two protons H_2-24 resonating at 1.89 ppm, which in turn were coupled to a methine H-25 involved in an isopropyl group. The nature of the double bound was assumed to be *cis* by the coupling constant value $J = 8.4$ Hz observed between H-22 and H-23.

Interpretation of NMR experiments (^1H–^1H COSY, HMQC, HMBC) (Table 1) permitted to establish the structure of **1** as the 20-hydroxy-5α-cholest-22-ene-3,6-dione. This compound inhibited the porcine pancreatic elastase (PPE) with an ED_{50} of 0.1 mM.

We did not isolate any 7,11-diketosteroids in the extract of *H. musciformis* as Babu et al. reported in previous papers.[2–4]

Diketo-3,6 steroids are not common as natural products. To date, only three examples were found in the literature: 5α-cholestane-3,6-dione and 11-hydroxy-5α-cholestane-3,6-dione were isolated from the red alga *Acantophora spicifera*[8,9] and 16β-hydroxy-5α-cholestane-3,6-dione from the red alga *Jania rubens* which exhibited cytotoxic activity.[10]

The new steroids **2** and **3** were obtained in small amount (1.2 mg) as a mixture 4:1 (CH_2Cl_2–EtOAc (8:2), R_f 0.54), $[\alpha]_D$ +10.4 (*c* 0.15 CH_2Cl_2), and were not separated even on a RP18 column. These compounds contained an hydroxyl group (IR ν_{max} 3636 cm^{-1}) and a conjugated carbonyl group (ν_{max} 1685 cm^{-1}). This was confirmed by the strong absorption at 237 nm (log ε 4.01) in the UV spectrum.

The ^1H NMR spectrum of the mixture clearly showed a steroid structure with two tertiary methyl groups Me-18 at δ 0.67 and Me-19 δ 1.36 ppm, three secondary methyl groups resonating as doublets Me-21 at 0.81 ppm (1.02 ppm in **3**), Me-26 and Me-27.

A sharp singlet at δ 5.80 was due to an olefinic proton and another one at δ 4.33 to a proton geminal with an hydroxyl group. The ^{13}C spectral data (Table 2) showed the presence of 27 carbons including one carbonyl at 200.1 ppm and a carbon bearing an hydroxyl group at δ 73.3 ppm.

The molecular formula of compound **2** was shown to be $C_{27}H_{44}O_2$ by HRCIMS ($[M+NH_4]^+$ measured 418.3679 calculated 418.3673 for $C_{27}H_{48}O_2N$).

The rings A and B were assigned by the HMBC correlations observed for H_3-19 with C-1, C-5, C-9, C-10; those of H_2-2 with C-1 and C-3; those of the olefinic proton H-4 with C-2, C-6 and C-10; moreover H_2-7 correlated with C-5, C-6 and C-9.

The long-range correlations of H_2-2 and H-4 with the carbonyl at 200.1 ppm indicated that the ketone function was located at C-3.

The long-range correlations of H_2-7 and H-4 with the methine resonating at 73.3 ppm supported the location of the secondary alcoholic function at C-6. H-6 resonating as a sharp singlet implies a weak coupling constant value with H_2-7 so H-6 is in the α or equatorial position.

The ring C and D were assigned by the HMBC correlations of H_3-18 with C-12, C-13, C-14 and C-17; those of H_2-11 with C-8, C-9, C-12; those of H_2-15 with C-8.

The side chain was assigned by the correlations observed for H_3-21 and H-20 in the COSY spectrum and by the long range correlations of H_2-22 with C-20 and C-24 and those of H_2-24 with C-26 and C-27. The structure of compound **2** was assigned as the 6α-hydroxy-cholest-4-ene-3-one.

The molecular formula of compound **3** was shown to be $C_{27}H_{42}O_2$ by HRCIMS ($[M+NH4]^+$ measured 416.3523 calculated 416.3517 for $C_{27}H_{46}O_2N$). The same skeleton as **2** was assumed for **3** but the signal of the Me-18 protons shifted downfield to δ 0.73 (0.67 in **2**) and the Me-21 protons shifted downfield to δ 1.02 (0.85 in **2**) due to the presence of the double bond.

The side chain was assigned through the ^1H–^1H COSY from H-22 to H-24. HMBC correlations of the olefinic methine proton H-22 (δ 5.20 ppm) with C-20; C-23 and C-24 and those of H-23 (δ 5.26 ppm) with C-20; C-22; C-24; confirmed these connectivities. The nature of the double bound was assumed to be *trans* by the coupling constant value $J = 14.8$ Hz observed between H-22 and H-23.

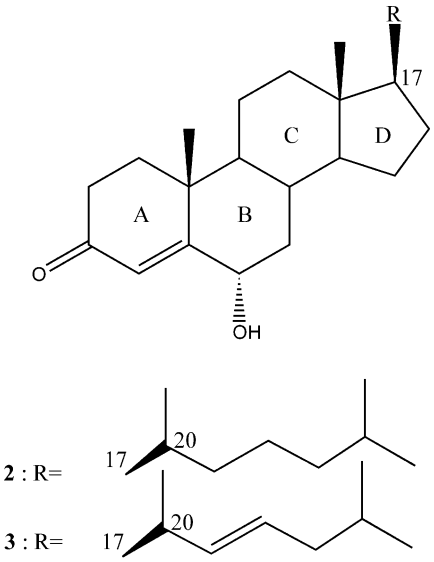

Table 2. ^1H and ^{13}C assignments (δ ppm) of compound **2** and **3**[a] in $CDCl_3$ on a Bruker Advance 400 at 298 K

	Compound **2**				Compound **3**			
No.	^1H (m, *J* Hz)	^{13}C	HMBC	COSY	^1H (m, *J* Hz)	^{13}C	HMBC	COSY
1	1.75–2.01	37.1	2, 9, 10, 19	2	1.75–2.01	37.1	2, 9, 10, 19	2
2	2.35–2.48	34.3	1, 3		2.35–2.48	34.3	1, 3	
3	—	200.1	—		—	200.1	—	
4	5.80	126.4	2, 6, 10		5.80	126.4	2, 6, 10	
5	—	168.0	—		—	168.0	—	
6	4.33	73.3	—	7	4.33	73.3	—	7
7	1.22–1.98	38.5	5, 6		1.22–1.98	38.5	6, 5	
8	1.93	29.7	9, 14	7	1.93	29.7	9, 14	7
9	0.91	53.6	11, 14, 19	11	0.91	53.6	11, 14, 19	
10	—	37.9	—		—	37.9	—	
11	1.50	21.0	8, 9, 12		1.50	21.0	8, 9, 12	
12	1.12	39.4	13, 17	11	1.12	39.4	13, 17	11
13	—	42.2	—		—	42.2	—	
14	1.02	56.2	8, 18		1.02	56.2	8, 18	
15	1.24	24.8	8		1.24	24.8	8	
16	1.28	28.5			1.30	28.5		
17	1.12	55.9	15, 16	16	1.12	55.9	15, 16, 20, 21, 22	16
18	0.67	12.0	12, 13, 14, 17		0.73	12.2	12, 13, 14, 17	
19	1.35	19.5	1, 5, 9, 10		1.35	19.5	1, 5, 9, 10	
20	1.35	32.8	13		1.98	40.1	13, 21	
21	0.81	18.7	17, 20, 22	20	1.02 (d, 6.6, 3H)	20.8	17, 20	20
22	1.24	37.3	20, 24		5.20 (dd, 8.2, 14.8, IH)	137.9	20, 23, 24	23
23	1.16	24.5	22	22	5.26	126.4	20, 22, 24	
24	1.04	39.3	26, 27	25	1.83	41.9	22, 23, 25, 26, 27	23
25	1.51	28.1	26, 27		1.53	28.1	26, 27	
26	0.84 (d, 6.6, 3H)	22.5	24, 25, 27	25	0.84 (d, 6.6, 3H)	22.5	24, 25, 27	25
27	0.87 (d, 6.6, 3H)	22.5	24, 25, 26	25	0.87 (d, 6.6, 3H)	22.5	24, 25, 26	25

[a]The values were measured from the 4:1 mixture of **2** and **3**.

The isopropyl group is linked to C-24 as shown by the COSY and HMBC experiments. A complete proton and carbon assignment in $CDCl_3$ is given in Table 2.

The structure of compound **3** was assigned as the 6α-hydroxy-cholest-4, 22-diene-3-one.

Such natural 6-hydroxy-4-ene-3-ketosteroids from marine origin have been discovered previously from marine algae.[11,12] Some of them were described as cytotoxic compounds (KB cells) but this mixture was atoxic even at 10^{-5} M and did not inhibit the porcine pancreatic elastase (PPE).

Acknowledgements

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References and Notes

1. A voucher specimen no. HM2000 was deposited at P.C. (Laboratoire de Cryptogamie, Muséum National d'Histoire Naturelle, 12 rue Buffon, Paris, France).
2. Babu, J. M.; Trivedi, G. K.; Mathur, H. H. *Phytochemistry* **1989**, *28*, 3237.
3. Babu, J. M.; Mathur, H. H.; Trivedi, G. K. *Phytochemistry* **1990**, *29*, 2029.
4. Babu, J. M.; Sridharan, R.; Mathur, H. H.; Trivedi, G. K. *Phytochemistry* **1990**, *29*, 3965.
5. La Barre, S.; Longeon, A.; Barthélémy, M.; Guyot, M.; Le Caer, J. P.; Bargibant, G. *C. R. Acad. Sci. Paris* **1996**, *319*, 365.
6. Blunt, J. W.; Stothers, J. B. *Org. Magn. Res.* **1977**, *9*, 439.
7. Nes, W. R.; Varkey, T. E. *J. Org. Chem.* **1976**, *41*, 3429.
8. Prakash, O.; Roy, R.; Bhakuni, D. S.; Wahidulla, S.; Kamat, S. Y. *J. Nat. Prod.* **1989**, *52*, 686.
9. Wahidulla, S.; D'Souza, L.; Patel, J. *Phytochemistry* **1987**, *26*, 2864.
10. Ktari, L.; Blond, A.; Guyot, M. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2563.
11. Sheu, J. H.; Liaw, C. C.; Duh, C. Y. *J. Nat. Prod.* **1995**, *58*, 1521.
12. Sheu, J. H.; Huang, S. Y.; Duh, C. Y. *J. Nat. Prod.* **1996**, *59*, 23.