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Isolation and Synthesis of the First Natural 6-Hydroximino 4-en-3-one- Steroids from the Sponges *Cinachyrella* spp.

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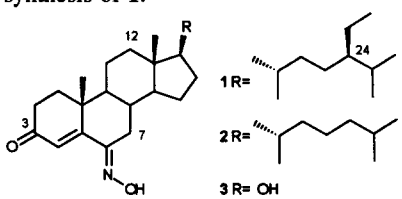
Abstract: Two new 6-hydroximino-4-en-3-one steroids: (24*R*, 6*E*)-24-ethylcholest-6-hydroximino-4-en-3-one (**1**) and (6*E*) cholest-6-hydroximino-4-en-3-one (**2**), accompanied by the known cholest-4-en-3-one were isolated from a mixture of two morphospecies of the sponge *Cinachyrella* (*C. alloclada* and *C. apion*). Use of spectroscopic methods (NMR and MS) was key to establish their structures which were confirmed by synthesis. Described in this report are the first hydroximino steroids derived from a natural source. © 1997 Elsevier Science Ltd. All rights reserved.

A great number of steroids have been isolated from marine sponges having a very unusual and interesting structures. As far as we know, yet not any marine steroid with an oxime group has so far been reported in the literature.[1]

Steroids from sponges belonging to the Tetillidae family (subclass Tetractinomorpha, order Spirophorida) have been little investigated to date.[2] In the course of our chemotaxomic investigations to differentiate two morphospecies of the *Cinachyrella* (*C. alloclada* versus *C. apion*),[3] we were able to isolate the first natural 6-hydroximino-3-oxo-4-en steroids: (24*R*, 6*E*)-24-ethylcholest-6-hydroximino-4-en-3-one (**1**) and cholest-6-hydroximino-4-en-3-one (**2**), along with cholest-4-en-3-one[4]. Their structures were deduced by extensive use of 1D and 2D-NMR, FABMS and HREIMS, and corroborated by synthesis of **1**.



The MeOH extract of a specimens mixture (60.5 g, wet) of the two morphospecies (collected at the Pituba beach in Salvador de Bahia, Brazil) were partitioned into *n*-hexane, CH_2Cl_2 and *n*-BuOH. The hexane fraction (4.5 g) was fractionated by SiO_2 gel flash chromatography eluting in a gradient mode with hexane/AcOEt mixtures. The hexane/AcOEt (9:1) fraction gave, after repeated chromatographic separations on SiO_2 gel column and normal phase HPLC, 565 mg of 4-cholesten-3-one as the major metabolite present in the sponge.[5] The fraction eluted with hexane/AcOEt (8:2) was further submitted to several HPLC separations (normal phase in a μ-Porasil column, hexane/AcOEt (8:2) and then reversed phase (1 ml/min., MeOH/H_2O 9:1 in μ-Bondapack C18 and C8 columns)) to give 6 mg of compound **1** (C8 column, rt. = 69.3 min.) and 2 mg of compound **2** (C8 column, rt. = 53 min.).

The molecular formula of **1** was established as $C_{29}H_{47}NO_2$ by HREIMS (*m/z* 441.3604, Δ 0.3 mmu of calcd). Positive test on TLC to an 0.5 % aqueous cupric chloride solution pointing to a nitrogenous metabolite possessing a hydroximino group. A further indication of an oxime moiety was the presence of bands at 3340 (NO-H) and 1647 (C=N-O) cm^{-1} in the IR spectrum.

A combination of ^{1}H, ^{13}C and DEPT-135 NMR data suggested a C_{29} steroid which also showed the presence of a ketone (δ_C 201.0), a trisubstituted double bond [δ_H 6.43 (1H, s); δ_C 122.6 (d) and 162.3 (s)], an OH group [δ_H 9.88 (1H, bs), interchanged with D_2O], a quaternary carbon [δ_C 156.0 (s)] and two diasterotopic protons at δ_H 3.42 (1H, dd, J = 16.0 and 4.7 Hz) and 1.55 (1H, m). In order to overcome a great deal of overlapping signals in the δ_H 2.00-0.70 region, HMQC, ^{1}H-^{1}H COSY and HMBC experiments were made. Careful analysis of COSY and HMQC data for **1** revealed the two diasterotopic protons (H7α and β) showing correlations with a ^{13}C signal at δ_C 29.6 (t). The long-range heteronuclear correlations (HMBC, figure 1) between pairs δ_H 6.43 (H4) and δ_C 201.0 (C3), δ_H 6.43 (H4) and δ_C 156.0 (C6), δ_H 3.42 (H7β) and δ_C 162.3 (C5), δ_H 1.55 (H7α) and δ_C 156.0 (C6), strongly suggested the presence of a 6-hydroximino-4-en-3-one group in this C_{29} steroid. The strong NOESY correlation between H-7α at δ_H 1.55 and the OH proton and the downfield shift of the H-7β due to the proximity of the oxime OH demonstrate the *E* geometry in the oxime group. The remaining proton and carbon chemical shifts were coincident with those of a 24-ethylcholesterol structure and allowed us to identify compound **1** as (24*R*, 6*E*)-24-ethylcholest -6-hydroximino-4-en-3-one.[6]

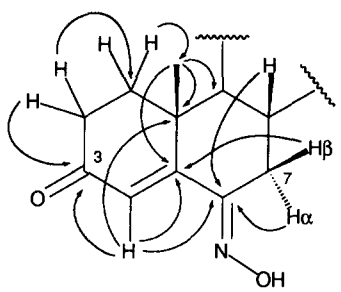


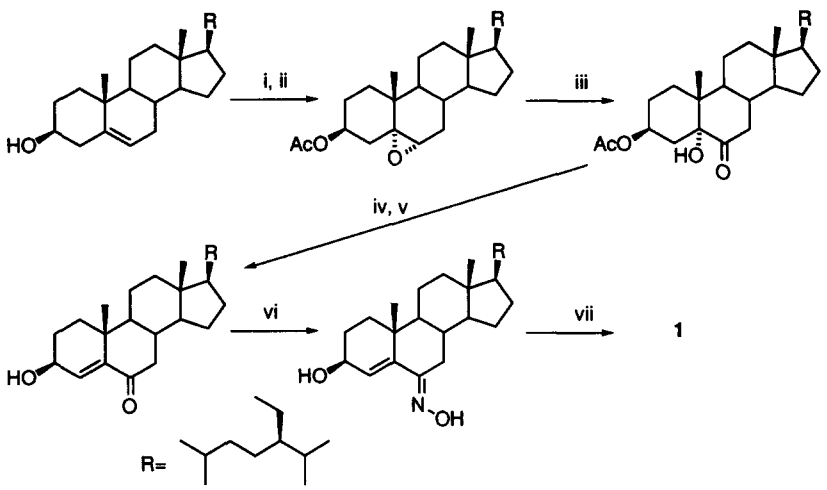
Figure 1. *Selected HMBC correlations of 1.*

Based on the above results and to confirm the presence of the hydroximino group (during the separation process we noticed an isomerization *E-Z* in the oxime group),[7] we decided to synthesize **1** for its full characterization. Our retrosynthetic strategy was built on the methodology developed by Holland[8] group who complete the synthesis of **3**. As shown in Scheme 1, β–sitoesterol was protected, epoxydized, and oxidized to give an α-β unsaturated ketone. The treatment of this compound with hydroxylamine hydrochloride afforded a key precursor of the (6*E*)-stigma-6-hydroximino-4-en-3β-ol. Finally, MnO_2 oxidation gave **1** which showed identical chromatographic (TLC and rt in HPLC) and spectroscopic data as the natural compound.

Compound **2** was isolated in small amount. Its molecular ion at *m/z* 413 in the EIMS, the NMR data (Table 1) and the comparison to those of compound **1** allowed us to identify compound **2** as (6*E*)-cholest-6-hydroximino-4-en-3-one.[9]

Although 6-hydroximino-4-en-3-one steroids, which constitute a new class of steroid, have been recently synthesized, compounds **1** and **2** are the first examples occurring in nature. This group of steroids were reported to show a high affinity for human placental aromatase, and function as competitive inhibitor of this enzyme.[8] Several 4-cholesten-3, 6-diones have been isolated from marine sponges, including species belonging to the *Cinachyrella* genus (formerly named *Cinachyra*), such as *C. tarantina* where is present (24*R*)-ethylcholest-4-en-3,6-dione and cholest-4-en- 3,6-dione.[10] The present species of *Cinachyrella* has not only the ability to oxidize the C-3 and C-6 positions in the steroid but also to obtain selectively the hydroximino group at C-6.

Compound **1** did not show any cytotoxic activity against several tumor cells (P-388, A-549, HT-29, MEL-28).



Scheme 1. i Ac_2O, Py; ii MCPBA/CH_2Cl_2; iii CrO_3/H_2O; iv $SOCl_2$/Py; v KOH/MeOH vi NH_2OH-HCl; vii $MnO_2/CHCl_3$

	1		2	
C	δ_C mult	δ_H mult (*J* in Hz)	δ_C mult	δ_H mult (*J* in Hz)
1	34.8 t	2.05 m / 1.75 m	34.8 t	2.05 m
2	33.6 t	2.53 m	33.7 t	2.54 m
3	201.0 s		200.8 s	
4	122.6 d	6.43 s	122.7 d	6.41 s
5	162.3 s		162.1 s	
6	156.0 s		156.2 s	
7	29.6 t	(β) 3.42 dd (16.0, 4.7) / (α) 1.55 m	29.6 t	(β) 3.41 dd (16.0, 4.7)
8	32.7 d	1.65 m	32.8 d	
9	51.2 d	1.15 m	51.3 d	
10	38.7 s		38.8 s	
11	20.8 t	1.55 m	20.8 t	
12	39.3 t	2.05 m / 1.25 m	39.3 t	
13	42.5 s		42.6 s	
14	56.6 s	1.18 m	56.6 s	
15	24.0 t	1.70 m /1.15 m	24.0 t	
16	28.1 t	1.90 m / 1.33 m	28.1 t	
17	55.9 d	1.14 m	56.0 d	
18	12.0 q	0.70 s	11.9 q	0.70 s
19	16.6 q	1.13 s	16.6 q	1.21 s
20	36.2 d	1.35 m	35.7 d	
21	18.7 q	0.93 d (6.3)	18.6 q	0.90 d (6.3)
22	33.8 t		36.1 t	
23	26.4 d	1.18 m	23.8 t	
24	46.1 d	0.92 m	39.5 t	
25	29.0 q	1.65 m	28.0 q	
26	19.0 q	0.84 d (6.6)	22.8 q	0.90 d (6.6)
27	19.6 q	0.81 d (6.6)	22.5 q	0.84 d (6.6)
28	23.1 t	1.22 m / 1.30 m		
29	12.3 q	0.86 t		
OH		9.88 brs		9.71 brs

Table. *^{13}C (75 MHz) and ^{1}H-NMR (500 MHz) Chemical Shifts (ppm) for* **1** *and* **2** *in Cl_3CD.*

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3. Two morphospecies from Pituba Beach (Salvador) presented the same variation of external morphology and only few differences in spicular characters were found. *C. alloclada* have one size class of tetractines whereas *C. apion* have two raphids as microscleres.
4. In a second study we also detected the presence of these three compounds on individual specimens of *Cinachyrella alloclada* and *C. apion* collected at the same place.
5. Pouchert, C. J. and Behnke, J., *The Aldrich Library of the ^{13}C and ^{1}H NMR Spectra* Ed. I. Spectrum **3**, 578B, **1993**. This compound was first encountered in the sponge *Stelleta clarella*: Sheikh, Y. M.; Djerassi, C. *Tetrahedron*, **1974**, *30* , 4095-4103.
6. **1**: Amorphous white solid. $[\alpha]_D$ = +99° (*c* 0.065). UV λ_{max}: 271 nm. IR ν_{max}.: 3880, 2923, 1647, 1307, 1131 cm^{-1}. ^{1}H and ^{13}C-NMR see Table 1. HREIMS: $C_{29}H_{47}O_2N$ found 441.3604: calc. 441.3607. LREIMS *m/z* (%): 441 (55), 424 (10), 398 (6), 300 (12), 188 (4), 169 (100), 174 (6), 125 (9). (+) FABMS, *m/z*, (%): 442 ($[M+H]^+$, 100).
7. This was deduced by the appearance of the signals at δ 6.13 ppm (s) and 2.55 (m) assignable to H-4 and H-7β of the *Z* isomer. The conversion of *(E)*-cholest-4-en-6-hydroximino to its *Z* isomer in MeOH under irradiation is known: Suginome, H.; Ohshima, K.; Ohue, Y.; Ohki, T.; Senboku, H. *J. Chem. Soc. Perkin Trans.* 1, **1994**, 3239-3249.
8. Holland, H. L.; Kumaresan, S.; Tan, L.; Njar, V. C. O. *J. Chem. Soc. Perkin Trans.* 1, **1992**, 585-587.
9. **2**: Amorphous white solid. $[\alpha]_D$ = +136° (*c* 0.225). UV λ_{max}: 271 nm. ^{1}H and ^{13}C-NMR see Table 1. EIMS *m/z* (%): 413 (14), 396 (30), 370 (80), 174 (45), 125 (70), 55 (100).
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