New polyhydroxylated steroids from the marine pulmonate *Trimusculus peruvianus*

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Dedicated to Professor Edmundo A. Rúveda on his 70th birthday and Professor Roberto A. Rossi on his 60th birthday

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Abstract

Two new sterols, **1**-**2**, unusually hydroxylated at C-21 have been isolated from the marine pulmonate *Trimusculus peruvianus*. Compound **2** possesses a Δ^7-3α,5α,6β-triol nucleus unprecedented in marine organisms. The structure and stereochemistries were determined on the basis of spectral studies, particularly NMR and MS spectroscopic data. **1** and **2** are the entire sterol content of the mollusk and both exhibit moderate *in vitro* cytotoxic activity against human colon carcinoma cell lines.

Keywords: Marine mollusk, *Trimusculus peruvianus*, Δ^5- and Δ^7-polyhydroxylated steroids, cytotoxic activity

Introduction

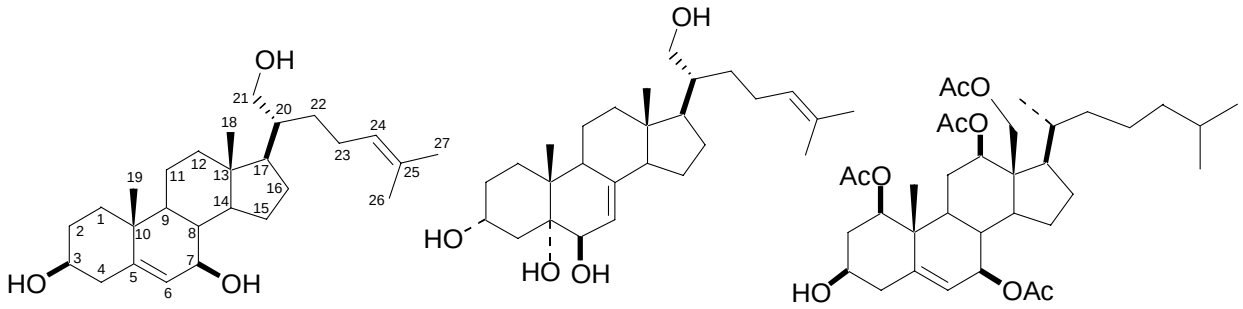
Although shell-bearing mollusks such as intertidal pulmonate limpets of the family Trimusculidae are common marine invertebrates, only a limited number of chemical investigations on their metabolites have been reported.[1] These sessile limpets, that live with unusual constraints, seem to produce *de novo* secondary metabolites, mostly diterpenes which appear to be involved in their defense against predators.[2]

In this work we report on the structure elucidation of two new polyhydroxylated steroids **1** and **2**. Both compounds possess an identical side chain, unusually oxidized at C-21, and differ in the Δ^5- and Δ^7-steroidal nucleus, respectively. Interestingly, although the Δ^7-3β,5α,6β-triol nucleus is widespread in marine invertebrates[3] and terrestrial plants,[4] as well as in basidiomycete

fungi,[5] the Δ^7-3α,5α,6β-triol nucleus of **2** is unprecedented in naturally occurring marine steroidal metabolites.

Polyhydroxylated sterols are frequently found in marine invertebrates,[3] such as alcyonarians, sponges, gorgonians, briozoos, starfish and bivalves, but they seem more rare in trimusculide mollusks. Four species of genus *Trimusculus* has been studied[1] and, until now, only a sole steroidal metabolite, **3**, isolated from *Trimusculus conica*, has been recently described.[6]

Compounds **1** and **2** exhibit moderate *in vitro* cytotoxic activity against human colon carcinoma cell lines.



1 **2** **3**

Figure 1

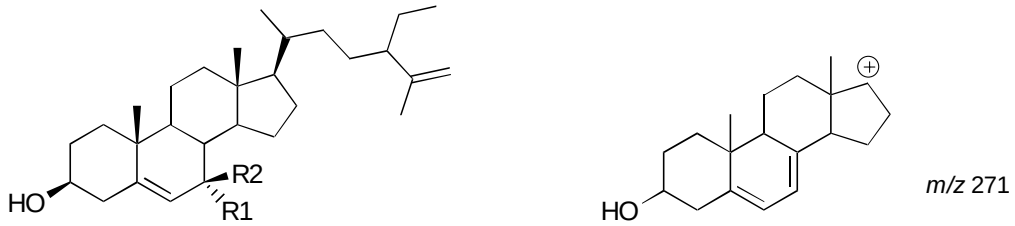
Results and Discussion

General Procedures. From the crude ethyl acetate extract of *T. peruvianus*, collected near Antofagasta coast (Chile), compounds **1** and **2** were obtained after flash chromatography followed by gel filtration and successive HPLC.

Structural analysis of 1: (20R)-cholest-5,24-diene-3β,7β,21-triol

Compound **1** was isolated as an oil. NMR data coupled with a M^+ peak at *m/z* 416.3325 in the HREIMS of **1** suggested a molecular formula of $C_{27}H_{44}O_3$, indicating six degrees of unsaturation. The ^{13}C NMR spectrum of **1** (Table 1), together with the information from a DEPT spectrum, showed the presence of 27 well-resolved signals of which four were methyls (two olefinic), ten methylenes (one bearing oxygen), nine methines (two olefinic, and two geminal to oxygen) and four nonprotonated carbons (two olefinic). The ^{13}C NMR data and DEPT NMR experiment were consistent for an di-unsaturated C-27 sterol having three hydroxyl groups when compared with the reported values[7] for a tetracyclic chlolestane nucleus (e. g., **4a**, **4b**, Figure 2 and Table 1).

The striking absence of the resonance of the C-21 methyl group, together with the presence of an oxymethylene carbon (δ_H: 3.68 and 3.72; δ_C 62.7), suggested that the typical C-21 methyl group was oxidized to a primary hydroxyl function.



4b: R_1 = H, R_2 = OH
4a: R_1 = OH, R_2 = H

A

Figure 2

Besides the primary alcohol, the ^{1}H NMR showed resonances for two protons geminal to the remaining alcoholic oxygens at δ 3.85 (ddd, *J* = 2.2, 2.2, 8.0 Hz) and δ 3.55 (dddd, *J* = 4.4, 4.4, 11.3, 11.3 Hz) completing, with that of the C-21 hydroxymethylene, the three oxygens present in the molecular formula. In addition, the ^{1}H NMR spectrum displayed signals for two olefinic protons [δ 5.29 (t, *J* = 2.0 Hz), δ 5.11 (tt, *J* = 1.3, 7.1 Hz)] and for two olefinic and two angular methyl groups at δ 1.61, δ 1.69, δ 0.71 (s) and δ 1.05 (s), respectively. The HMBC correlation of both the H_2-21 methylene alcohol and the H_3-18 methyl group with C-17 corroborated the position of the primary alcohol in the C-21 side chain.

The location of the remaining functionalities was deduced by a combination of COSY NMR, HMQC and HMBC experiments. From the ^{1}H-^{1}H COSY NMR spectrum the isolated H_2-2–H-3–H_2-4 and H-6–H-7–H-8 spin systems were inferred, and by the HMBC correlations (Table 1) hydroxylic functions at C-3 and C-7, and trisubtituted double bonds at $\Delta^{5(6)}$ and $\Delta^{23(24)}$ were placed. The gross steroidal nucleus was corroborated by the MS spectrum of **1**, which showed a peak at *m/z* 271 corresponding to fragment A, Figure 2. These data support the structure proposed for **1**.

Chemical shift arguments and 2D NOESY experiments established the relative configurations of the chiral centers of the rings. The chemical shifts and the vicinal coupling H-3/H_2-2, H_2-4 and H-7/H-6, H-8 are in agreement with the respective protons of a Δ^5-3β,7β-diol nucleus[7] (**4a**, Table 1). This was corroborated by the NOEs observed between H-4α/H-3, H-6; H-6 with H-7; and H-7/H-9, H-14. The NOE observed between H-4β and H_3-19 placed the methyl group at C-10 in the β axial position. The β stereochemistry of the lateral side chain linked to C-17 was consistent with the NOE observed between H_3-18 and H_2-21.

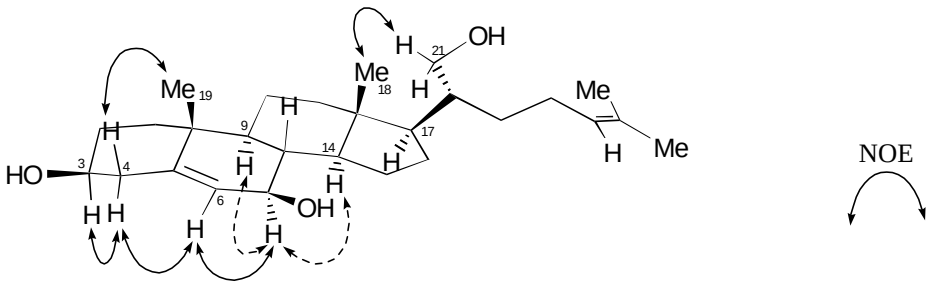


Figure 3

Table 1. ^{1}H, ^{13}C NMR and HMBC Data of compound **1** [500 MHz, δ ppm, (*J*) Hz, $CDCl_3$] and ^{13}C NMR data of compounds **4a** and **4b**

#	1			4a	4b
	δ_H	δ_C	HMBC	δ_C	δ_C
1	1.06 m 1.84 m	36.9	C-5	36.8	36.9
2	1.55 m 1.85 m	31.5	C-3, C-4	31.5	31.2
3	3.55 dddd (*4.4, 4.4, 11.3, 11.3*)	71.4		71.4	71.0
4	α: 2.26 ddd (*2.0, 2.0, 11.3*) β: 2.34 ddd (*2.1, 4.9, 13.2*)	41.7	C-2, C-3, C-5, C-6, C-10	41.6	41.6
5		143.5		143.4	142.3
6	5.29 t (*2.0*)	125.4	C-4, C-10	125.3	124.1
7	3.85 ddd (*2.2, 2.2, 8.0*)	73.2	C-5, C-6, C-14	73.3	65.4
8	1.45 m	40.9		40.8	37.2
9	1.04 m	48.2		48.2	42.1
10		36.4		36.2	36.2
11	1.53 m	21.0	C-9	21.0	20.9
12	1.21 m 1.94 m	38.9		39.5	39.8
13		42.7		42.8	42.8
14	1.17 m	55.8		55.9	49.4
15	1.95 m	24.9		26.4	26.4
16	1.45 m	27.7		29.4	29.3
17	1.40 m	49.7		55.3	55.7
18	0.71 s	12.1	C-12, C-13, C-14, C-17	11.7	11.6
19	1.05 s	19.1	C-5, C-9, C-10	19.1	19.8
20	1.45 m	42.0		35.4	36.3
21	3.68 dd (*4.5, 11.2*) 3.72 dd (*3.3, 11.2*)	62.7	C-17, C-22	18.6	18.6
22	1.45 m	29.4		33.6	33.6
23	1.90 m and 2.09 m	26.2	C-22, C-24, C-25	29.9	29.6
24	5.11 tt (*1.3, 7.1*)	124.7	C-26, C-27	49.4	49.4
25		131.5		17.7	17.7
26	1.69 s	25.7	C-24, C-25	147.6	147.6
27	1.61 s	17.7	C-24, C-25	111.3	111.3

28	26.3	26.3
29	11.8	11.9

Structural analysis of 2: (20R)-cholest-7,24-diene-3α,5α,6β,21-tetrol

Compound **2**, Figure 1, was isolated as an oil. NMR data coupled with a M^+ peak at *m/z* 414.3181 in the HREIMS suggested a molecular formula of $C_{27}H_{44}O_4$, indicating six degrees of unsaturation**.** The ^{13}C NMR spectrum of **2** (Table 2), together with the information from a DEPT spectrum, showed the presence of 27 signals of which four were methyls (two olefinic), ten methylenes (one bearing oxygen), eight methines (two olefinic, and two geminal to oxygen) and five nonprotonated carbons (two olefinic and one bearing oxygen).

The missing C-21 methyl group, the presence of an oxymethylene carbon (δ_H: 3.66 and 3.71; δ_C 62.7), and the ^{13}C chemical shifts of the respective C-20–C-27 fragments of **1** and **2 (**Tables 1, 2) suggested that both compound possessed an identical side chain. Taking into account the extra oxygen of the molecular formula and the DEPT spectrum, showing one additional quaternary carbon bearing oxygen and one methine less than **1**, an unsaturated trihydroxylated nucleus for **2** was deduced. The almost identical ^{13}C NMR chemical shifts of C-17 and C-18 of **1** and **2** suggested that their differences are limited to ring A-C.

The rather high-field position of H_3-18 methyl resonance at δ 0.56, relative to the corresponding methyl group (δ 0.71) of **1**, is in good agreement[8] with a Δ^7- rather than a Δ^5-sterol suggesting, at first glance, that the $\Delta^{7(8)}$-3β,5α,6β-triol nucleus, common in marine invertebrates,[9] may be present in **2.** Assuming that those are really the relative positions of these functionalities, the hydroxyl group at C-3 must possesses an α-stereochemistry in virtue of the chemical shift and the small coupling constants (J = 5.5 Hz) of H-3 with the adjacent methylene protons (Table 2). The alternative 3β-hydroxyl in a *cis*-A/B ring fusion, Figure 4, is also compatible with the coupling constants, but is in disagreement with a ^{13}C chemical shift observed at δ 18.6, which is the value to be expected for a C-19 methyl group in a 5α-steroid rather than in a 5β-steroid, where it should be deshielded about 11 ppm by the absence of γ-gauche interaction.[10]

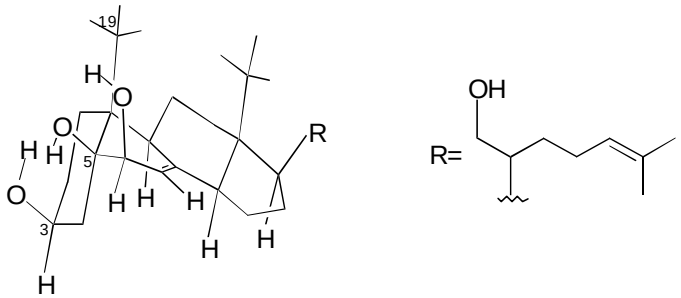


Figure 4

The location of these functionalities on the rings, as mentioned above, was corroborated by a combination of COSY NMR, HMQC and HMBC experiments. From the COSY spectrum the

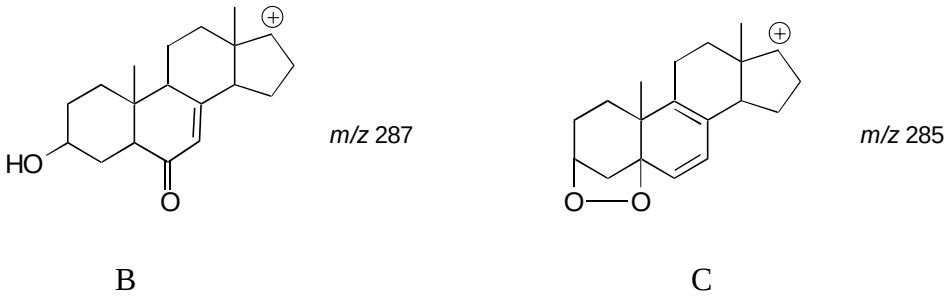
H_2-2–H-3–H_2-4 and H-6–H-7 spin systems were established, and by HMBC correlations (Table 2) a $\Delta^{7(8)}$ endocyclic olefin and hydroxylic functions were placed at C-3, C-5 and C-6.

Table 2. ^{1}H, ^{13}C NMR and HMBC Data of compound **2** [500 MHz, δ ppm, (*J*) Hz, $CDCl_3$] and ^{13}C NMR data of compound **5**

#	**2**			**5**
	δ_H	δ_C	HMBC	δ_C
1	1.23 m 1.91 m	35.3	C-2, C-3, C-5, C10, C-18	32.8
2	1.55 m 1.80 m	29.7	C-3, C-10	30.4
3	4.40 t (*5.5*)	76.9	C-1, C-5	67.2
4	1.31 m 1.97 m	41.1		39.3
5		78.1		75.9
6	4.06 d (*5.2*)	79.0	C-5, C-7, C-8, C-10	73.0
7	5.38 d (*5.2*)	111.6	C-5, C-9, C-14	117.3
8		145.4		143.2
9	1.55 m	52.3	C-1, C-5, C-7, C-8, C-10, C-11, C-14	43.2
10		38.1		37.0
11	1.55 m	25.0	C-8, C-9, C-12, C-13	22.0
12	1.38 m 1.90 m	40.3	C-9, C-13, C-19	38.9
13		46.4		43.6
14	2.06 m	56.6	C-7, C-8, C-9, C-12, C13, C-15, C-16, C-19	54.7
15	1.91 m	21.1		22.9
16	1.60 m	26.7		28.0
17	1.62 m	50.3	C-13, C-19, C-20 C-21, C-22	55.9
18	0.56 s	12.4	C-12, C-13, C-14, C-17	12.3
19	1.10 s	18.6	C-1, C-5, C-9, C-10	18.3
20	1.45 m	42.4	C-13, C-21	40.4
21	1.50 m 1.60 m	29.4	C-20, C-23, C-24, C-27	19.6
22	3.66 dd (*4.6, 11.2*) 3.71 dd (*3.2, 11.2*)	62.7	C-17, C-20, C-21	131.9
23	1.90 m 2.06 m	27.7	C-24, C-25	135.3
24	5.11 t (*7.1*)	124.7	C-26, C-27	42.8

25		131.5		33.1
26	1.68 s	25.7	C-24, C-25, C-27	19.9
27	1.61 s	17.7	C-24, C-25, C-26	21.1

MS spectra has been a useful tool in steroidal structures elucidation. For example, in a series of eight $\Delta^{7(8)}$-3β,5α,6β-trihydroxysterols, isolated from a sponge,[8] the molecular ion was absent. However, the presence in the MS spectrum of each compound of fragment B peak at *m/z* 287, which illustrated characteristic losses of water and side-chain, allowed to determine the gross steroidal nucleus. Thus, the two a.m.u less peak at *m/z* 285 of **2** compared with the former diagnostic peak at *m/z* 287 is consistent with a peroxy fragment C (Figure 5) and, interestingly, it supported the 3α,5α stereochemistry of an Δ^{7}-3α,5α,6β-triol nucleus. Therefore, there are two easily distinguished fragment peak in each of the MS spectra which provide a straightforward assignment of an $\Delta^{7(8)}$-3β,5α,6β- and a $\Delta^{7(8)}$-3α,5α,6β-trihydroxysterol, for example in a routine GC-MS analysis.

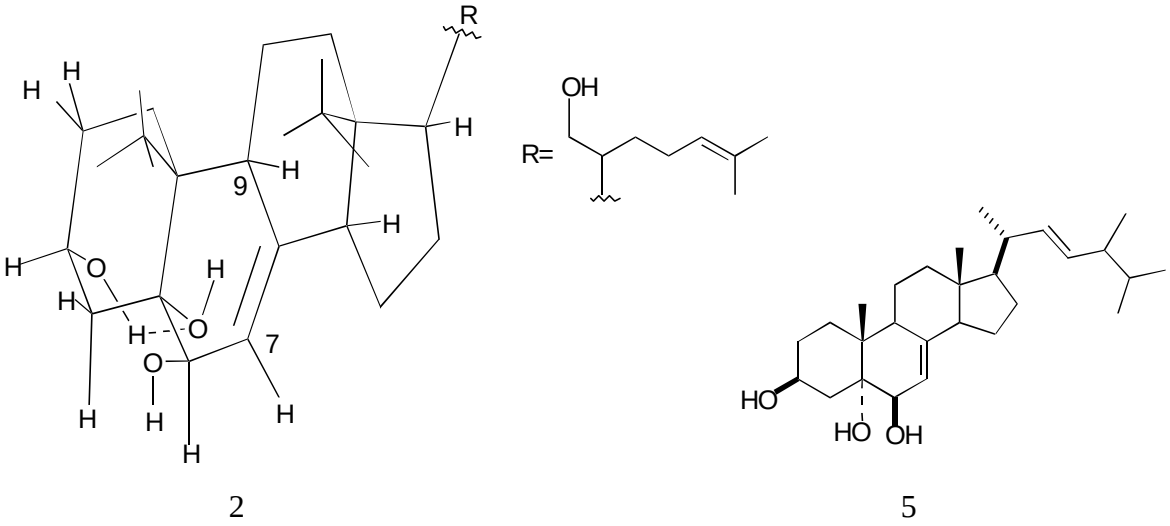


B C

Figure 5

We were intrigued by the prominent strong shielding resonances of carbons C-7 and C-9 of **2** when compared with the respective carbons of the $\Delta^{7(8)}$-3β,5α,6β-nucleus of cerevisterol **5**,[5] Figure 6, Table 2. Although they are epimeric at C-3 this did not seem sufficient to justify insight differences of Δδ ~6 and Δδ ~9 ppm, respectively. Thus, molecular mechanics calculations were performed to gain insight about the spatial disposition of the hydroxylic functions. The minimization[11] of the structure revealed a most favorable conformation **2**, Figure 6, involving a H-bond interaction between 3α- and 5α-hydroxyls, with the alcoholic proton of the C_5-OH oriented towards the centre of the α face of ring B. This arrangement would induce some stereoelectronic conformational changes that nicely explain certain shielded carbon resonances observed. The combination of both MS and ^{13}C NMR spectral studies provides an accurate method to distinguish a C-3 epimeric $\Delta^{7(8)}$-3,5α,6β-steroidal nucleus.

Chemical shift arguments and 2D NOESY experiments confirmed the above proposed relative configurations of the chiral centers of the rings. The small coupling constants observed for H-3 with the adjacent methylene indicate that it must be equatorial with a 3α-OH stereochemistry. As the chemical shift and coupling constant of H-6 are in agreement with the corresponding proton of a 6β-hydroxysteroid such as **5**, Figure 5, compound 2 possesses a Δ^{7}-3α,5α,6β-trihydroxy nucleus. The β stereochemistry of the lateral side chain linked to C-17 was consistent with the NOE observed between H_3-18 and H_2-21.



2 5

Figure 6

C-20 configuration of 1 and 2

Although the angular methyl proton shielding permits stereochemical assignment in many systems, there are cases where it is not possible, marked ^{13}C shielding differences being a better way to resolve stereochemical problems.[12] It has been reported[13] that synthetic 21-hydroxysterols of the 20 R series (natural configuration) exhibit a proton resonance at δ 3.70 as a singlet (-C**H$_2$**OH), while those of the 20 S configuration show a multiplet centered at δ 3.62. Consequently, the chemical shift (δ 3.66 dd and 3.71 dd), but not the multiplicities of the signals, of the hydroxymethylene of **1** and **2** are in agreement with a 20 R configuration. Unfortunately, that report is devoid of any ^{13} C NMR data for additional comparison.

Further considerations of ^{13}C NMR data showed that the C-17 chemical shift (δ ~ 50 ppm) of compounds **1** and **2** is shielding by 5 ppm when compared with the corresponding C-17 in a reduced C-21 carbon (21-CH$_3$) of compounds **4a, 4b** (Tables 1 and 2) and **5**, Figure 7 (depicted as ring D and side-chain only). Also, in both 21-CH$_2$OH and 21-CH$_3$ compounds **7a** and **7b**, respectively, isolated from a basidiomycete fungus[14] and with a known 20R configuration, similar shielding differences $\Delta\delta_{ROH-RH}$ ~ 6 were also found. This may be rationalized as C-17, in compounds **1, 2,** and **7a**, are in a γ-gauche, 1,3 synperiplanar orientations, with the polar C-21-hydroxyl substituent, absorbing at significantly higher field than their reduced counterparts of compounds **5** and **7b**. Thus, compounds **1** and **2** have a 20R configuration.

The 20 R configuration of the naturally occurring 21-hydroxysteroid **6a**, Figure 7, isolated from a starfish,[15] was also established on the basis of the chemical shift of H$_2$-21. In this and other 21-hydroxy related steroids, isolated from the same source, the corresponding C-17 resonated at ~ δ 50.5 ppm also, providing additional evidence of the utility of the C-21 shielding effect on C-17 to establish the configuration at C-20.

Compounds **1** and **2** exhibit moderate in vitro cytotoxicity against H-116 and HT-29 (human colon carcinoma) cell lines, displaying IC$_{50}$ values of 2.5 and 12.5 μg/mL, respectively, in each case.

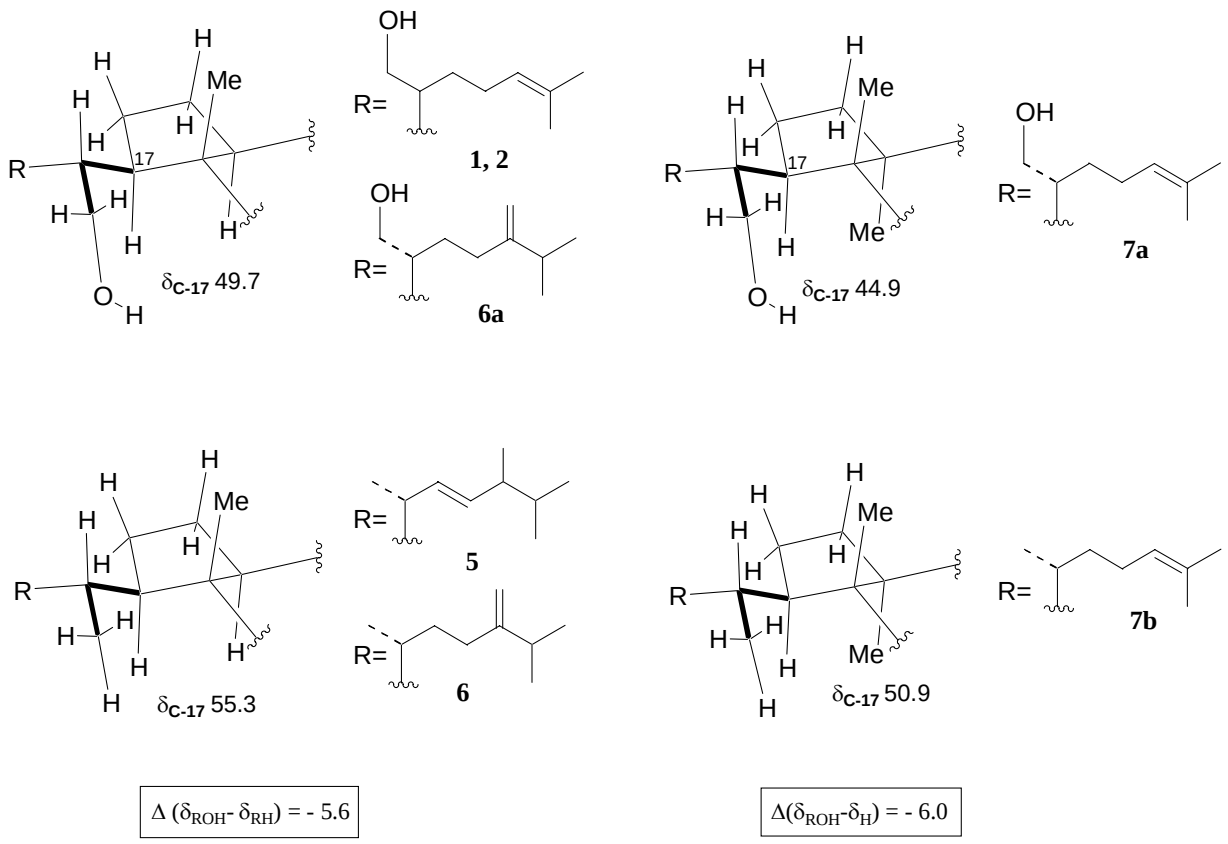


Figure 7

Conclusions

Two new sterols **1-2**, unusually hydroxylated at C-21, have been isolated from the marine pulmonate *Trimusculus peruvianus*, **2** being an unprecedented Δ^7-3α,5α,6β-triol nucleus. The *cis* stereochemistry of the 3α,5α-diol moiety of nucleus produced a characteristic *m/z* peak in the MS spectrum and the ^{13}C shielding resonances of C-7 and C-9 that provided a straightforward and accurate method to distinguish at the C-3 epimeric $\Delta^{7(8)}$-3,5α,6β-steroidal nucleus. C-21 substituents shielding effect on C-17, due to γ-gauche interaction, provided a method to establish the configuration at C-20.

Experimental Section

General Procedures. Optical rotations were measured on a Perkin–Elmer model 343 Plus using a Na lamp at 25°C. IR spectra were obtained with a Perkin-Elmer 1650/FTIR spectrometer in $CHCl_3$ solutions. ^{1}H NMR and ^{13}C NMR, HMQC, HMBC and COSY spectra were measured at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR. Two-dimensional NMR spectra were

obtained with the standard Bruker software. EIMS and HRMS data were taken on a Micromass Autospec spectrometer. HPLC separations were performed with a Hewlett Packard 1050 (Jaigel-Sil preparative column 10μ 20x250 mm) with hexane-EtOAc mixtures. The gel filtration column (Sephadex LH-20) used hexane-MeOH-CH_2Cl_2 (3:1:1) as solvent. Merck Si gels 7734 and 7729 were used in column chromatography. The spray reagent for TLC was H_2SO_4-H_2O-AcOH (1:4:20).

Biological material. Specimens of *Trimusculus peruvianus* were collected on intertidal rocks near Antofagasta, III Region of Chile, in November, 2001. A voucher specimen has been deposited at the Facultad de Ciencias, Universidad de Antofagasta, collection.

Extraction and isolation. 600 Freeze-dried specimens of *T. peruvianus* were extracted with ethyl acetate at room temperature and concentrated to give a dark residue (10.7 g). The extract was chromatographed by flash chromatography on silica gel. The fraction eluted with hexane: EtOAc (70:30) (804 mg) was chromatographed on a LH-20 column to give a complex mixture that was further separated by HPLC to give compound **2** (14.0 mg). The fraction eluted with hexane: EtOAc (50:50) (718 mg) was chromatographed on a Sephadex LH-20 column, affording a fraction that was chromatographed by HPLC to give compound **1** (3.8mg).

Compound (1). Colorless oil, $[\alpha]_D^{25}$ -20 (*c* 0.25, $CHCl_3$), IR (film) ν_{max} 3344, 2930, 2864 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* (%) 416 $[M]^+$ (7), 398 $[M-H_2O]^+$ (100), 380 $[M-2H_2O]^+$ (19), 287 (7); HREIMS *m/z* $[M]^+$ 416.3325 (calcd for $C_{27}H_{44}O_3$, 416.3290), 398.3220 (calcd for $C_{27}H_{42}O_2$, 398.3185).
Compound (2). Colorless oil; $[\alpha]_D^{25}$ +53 (*c* 0.58, $CHCl_3$), IR (film) ν_{max} 3381, 2940, 2864, 1440, 1374, 1276 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* (%) 414 $[M-H_2O]^+$ (32), 396 $[M-2H_2O]^+$ (27), 285 (82), 69 (100); HREIMS *m/z* $[M-H_2O]^+$ 414.3181 (calcd for $C_{27}H_{42}O_3$, 414.3134), 396.3098 (calcd for $C_{27}H_{40}O_2$, 396.3028), 285.1902 (calcd for $C_{19}H_{25}O_2$, 285.1855).

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