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Testudinariol A and B, Two Unusual Triterpenoids from the Skin and the Mucus of the Marine Mollusc *Pleurobrancus testudinarius*

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Abstract: The skin of the Notaspidea mollusc *Pleurobranchus testudinarius* contains a very unusual triterpenoid, testudinariol-A (**1**), which is also the main liposoluble component of the defensive mucous secretion. The structure, and the absolute stereochemistry, of **1** has been assigned on the basis of extensive spectral and chemical studies. The structural work on a minor co-occurring metabolite, testudinariol-B (**2**), led to the epimer of **1** at C-10. © 1997 Elsevier Science Ltd.

Chemical studies of the molluscan subclass Opisthobranchia species have been stimulated by the observation that, in the course of their evolution, these marine invertebrates have gradually replaced the mechanical defence due to the shell with more sophisticated defensive strategies including the use of chemicals as deterrent against predators.[3] Several reports summarize the excellent results obtained during these studies and highlight how opisthobranch molluscs have proved to be a rich source of bioactive and structurally intriguing compounds.[4] In the course of our study on the chemical ecology of opisthobranch molluscs we have analyzed the metabolites from the skin and the mucus of *Pleurobrancus testudinarius* (Cantraine, 1835), a large mollusc belonging to the Pleurobranchidae family of the Notaspidea order.[5] Pleurobranchidae molluscs are characterized by possessing only a reduced internal shell and their defensive strategy has generally been described as due to acid secretion discharged when they are molested.[6] This probably was considered, for a long time, to exclude other chemical defensive strategies making these molluscs a scarcely interesting target for chemical studies. Only recently few notaspidean molluscs have been object of chemical investigations.[7] In this paper we report isolation and structure elucidation of two novel triterpenoids, testudinariol-A (**1**) and testudinariol-B (**2**), from the skin and the mucus of the Pleurobranchidae *Pleurobrancus testudinarius*.

A specimen of *P. testudinarius* was collected in the Gulf of Salerno (South of Italy) in June 1993. The animal, kept for few hours in a big beaker filled with marine water, discharged a copious white mucus. Then the mollusc was frozen and diethyl ether was added to the marine water. After extraction, the solvent was evaporated from the organic phase giving an oil. The frozen mollusc was immersed in acetone and sonicated. The acetone extract, after concentration, was partitioned between water and diethyl ether. The mollusc was then carefully dissected separating the skin (mantle) from the internal organs. Diethyl ether soluble fractions were separately obtained from the acetone extracts of both mantle and internal parts of the animal. All the extracts were compared by TLC analysis (silica gel; light petroleum/diethyl ether, 1:1). The ether soluble extract obtained after sonication showed three spots (*Rf* 0.45, 0.25, 0.20) which were completely absent in the extract from the internal organs. After chromatography (silica gel; light petroleum ether /diethyl ether, 8 :2) testudinariol A (**1**) , testudinariol B (**2**) and a third compound, whose structure has

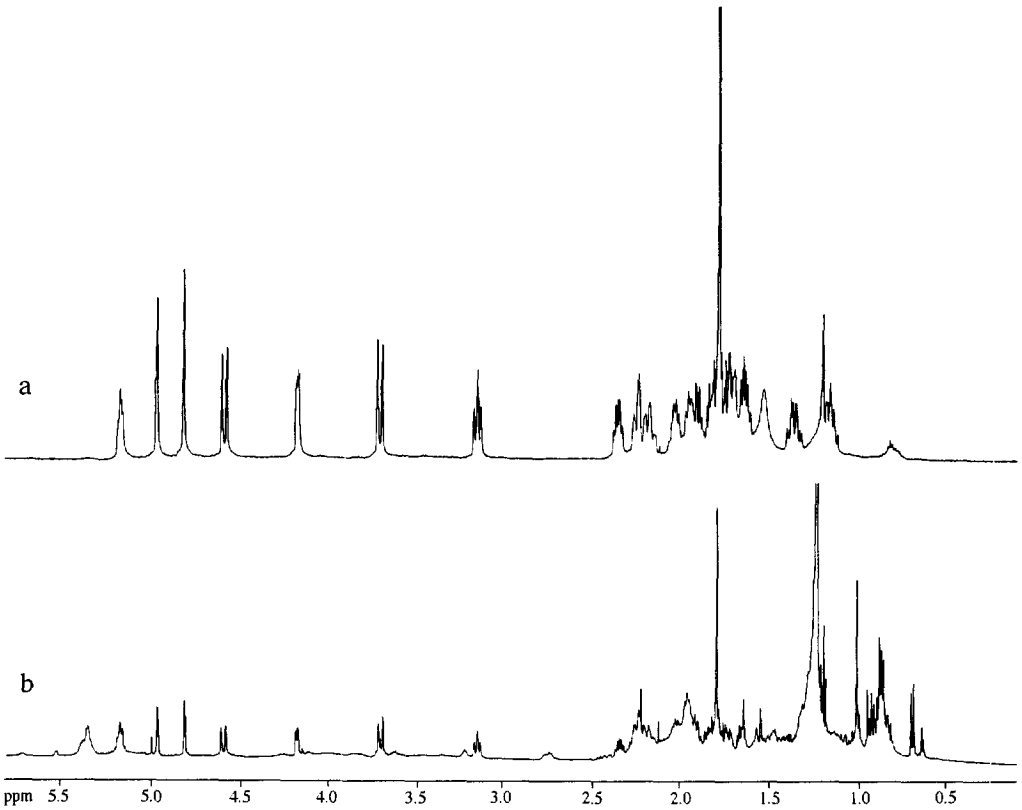
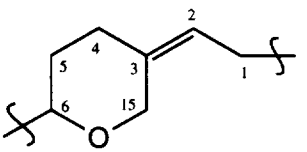


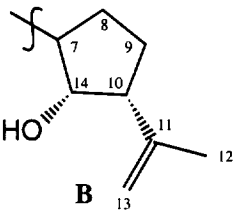
Figure 1: ^{1}H-NMR Spectra of Testudinariol A (a) and Mucus Extract (b).

not yet determined, were obtained in order of decreasing polarity, respectively. TLC of the ether extract from the skin also showed the presence of the more polar compound. Interestingly, both TLC and ^{1}H-NMR spectrum (Figure 1) showed that testudinariol A (**1**) is almost the unique component of the mucus extract .

Testudinariol A (**1**) gave a molecular ion in the HRMS corresponding to the molecular formula $C_{30}H_{46}O_4$. However, its ^{13}C-NMR spectrum showed only 15 signals: seven triplets, five doublets, one quartet and two singlets. This suggested that **1** was a symmetrical compound. The IR spectrum ($CHCl_3$) exhibited a weak broad absorption band corresponding to a hydroxyl group (3446 cm^{-1}). Since no typical carbonyl absorption bands were observed in the IR spectrum, the remaining oxygen function might be either an ether or a hydroxyl group. The half structure of testudinariol A (**1**) was deduced by careful analysis of the 1D-NMR (including ^{1}H-^{1}H decoupling) and 2D-NMR (^{1}H,^{1}H COSY, HMQC, HMBC) spectra. The highfield half of the ^{1}H-NMR spectrum contained the signals of two oxymethines at δ 3.18 (m; H-6) and δ 4.18 (m; H-14), of a CH_2O group at δ 3.73 (d, *J*= 12.5 Hz; H-15) and δ 4.59 (bd, J=12.5 Hz; H-15). The oxymethylene protons did not show any direct coupling with other protons while the oxymethine at δ 3.18 (H-6) was directly coupled to the protons at δ 1.76, 1.41 (H_2-5) which in turn were coupled with the protons at δ 2.24 and 2.29 (H_2-4). 2D ^{1}H-^{13}C NMR experiments showed that the carbon resonating at δ 66.7 (C-15)



A



B

was directly coupled with the protons at δ 4.59 and 3.73 (HMQC) and long range coupled (HMBC) with the protons at δ 2.29 (H-4) and δ 5.16 (H-2). The coupling of the vinylic proton (H-2, δ 5.16) with the protons at δ 2.00 and 2.07 (H_2-1) completed the substructure **A**. Starting from the oxomethine signal at δ 4.18 (H-14), the COSY spectrum signals were assigned to H-7 (δ 1.95), H-8 (δ 1.22; 1.85), H-9 (δ 1.80; 1.67) and H-10 (δ 2.40). The substructure **B** was completed adding, to the obtained cyclopentanol moiety, a 2-propenyl substituent at C-10. In fact, the quaternary carbon C-11 (δ 144.4) was long range coupled in the HMBC experiment with H_2-13 (δ 4.82; 4.97), H-9 (δ 1.80) and H-10 (δ 2.40). Although only a weak ^{1}H,^{1}H COSY correlation was observed between ^{1}H-NMR resonances of H-6 and H-7, three bond HMBC correlations from C-6 to H-14 (δ 4.18) and H-8 (δ 1.22) and two bond correlation between C-6 and H-7 (δ 1.95) provided conclusive evidence for the connectivity of substructures A and B through the C-6 / C-7 bond. 1D NOE difference experiment easily allowed assignment of the *Z* stereochemistry of the double bond. Irradiation of H-2 (δ 5.16) and H-15 (δ 4.59) enhanced H-4 (δ 2.28) and H-1 (δ 2.07) respectively. Others stereochemical details were obtained recording further NOE experiments. In fact, irradiation at δ 3.73 (H-15) resulted in enhancements at δ 3.18 (H-6) and δ 4.59 (H-15). Irradiation of H-6 proton (δ 3.18) induced NOE in H-14 (δ 4.18) and in H-8 (δ 1.22). The strong NOE effect between two protons, H-6 and H-14, belonging to different rings was justified by the presence of a hydrogen bond between the hydroxy group at C-14 and the oxygen of the pyran ring. On the basis of both the absence of a NOE effect between H-7 and H-14 and the presence of the hydrogen bond, the relative stereochemistry at C-6, C-14 and C-7 was tentatively assigned. Finally, irradiation at H-14 caused a strong enhancement of the signal at δ 2.40 (H-10). Absolute configuration at the carbinolic center C-14 was determined applying the modified Mosher method.[8] Testudinariol A (**1**) was submitted to esterification with (*R*) and (*S*) -MTPA chlorides. Analysis of the ^{1}H-NMR spectra of the two MTPA esters showed that the chirality of C-14 (C-14') was *S*.

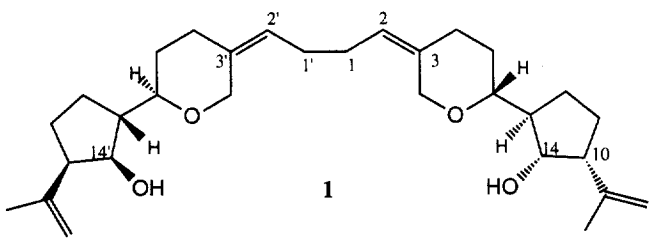


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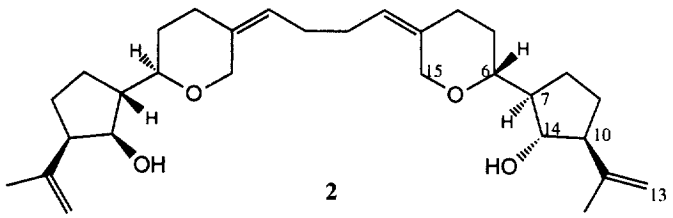
Table 1: NMR Data [a] for Testudinariol A (**1**) and B (**2**).

	1			**2**		
	δ ^{1}H (m, *J* Hz)	δ ^{13}C (m)	long-range connectivities[b]	δ ^{1}H (m, *J* Hz)	δ ^{13}C (m)	long-range connectivities[b]
1	2.07 (m) 2.00 (m)	27.3 (t)		2.07 (m) 2.00 (m)	27.3 (t)	H-2
2	5.16 (m)	123.4 (d)	H_2-1; H_2-15; H-4	5.17 (m)	123.8 (d)	H-15 (3.77)
3	-	134.3 (s)	H-4; H_2-1;	-	134.0 (s)	H_2-15
4	2.29 (m) 2.24 (m)	33.0 (t)		2.29 (m) 2.24 (m)	32.7 (t)	H-15 (4.59)
5	1.76 (m) 1.41 (m)	32.0 (t)		1.75 (m) 1.39 (m)	32.6 (t)	H-15 (4.59)
6	3.18 (m)	80.7 (d)	H-5; H-7; H-8; H-14; H-15	3.35 (dd; *9.9, 10.1*)	83.9 (d)	H-15 (4.59)
7	1.95 (m)	53.1 (d)		1.86 (m)	50.9 (d)	H-9 (1.87)
8	1.85 (m) 1.22 (m)	26.7 (t)		1.45 (m) 1.25 (m)	26.7 (t)	
9	1.80 (m) 1.67 (m)	27.2 (t)		1.87 (m) 1.50 (m)	27.2 (t)	
10	2.40 (ddd; *11.5, 5.8, 5.5*)	52.0 (d)		2.47 (bdd; *11.5, 5.8*)	53.2 (d)	H_2-13; H_3-12
11	-	144.4 (s)	H-9; H-10; H_2-13	-	145.9 (s)	H_3-12
12	1.84 (s)	23.3 (q)	H-10; H-13	1.75 (s)	23.8 (q)	H_2-13;
13	4.82 (bs) 4.97 (bs)	112.1 (t)	H-10; H_3-12	4.80 (bs) 4.87 (bs)	110.4 (t)	H_3-12
14	4.18 (m)	74.9 (d)	H-7; H-9; H-10;	3.85 (dd; *9.0, 8.7*))	80.2 (d)	H-10
15	4.59 (bd; *12.5*) 3.73 (d; *12.5*)	66.7 (t)	H-2; H-4	4.59 (bd; *12.5*) 3.77 (d; *12.5*)	66.7 (t)	
1'-15'	as 1-15			as 1-15 of **1**		

(a)Bruker 500 AMX; $CDCl_3$; chemical shifts referred to $CHCl_3$ at 7.26 ppm and to $CDCl_3$ at 77.0 ppm. (b) from HMBC, *J*=10 Hz.

HRMS data yielded also for testudinariol B (**2**) a molecular formula $C_{30}H_{46}O_4$. Furthermore, testudinariol B showed in its ^{1}H-NMR spectrum the set of signals observed for testudinariol A (**1**) together with another set of signals close to the former but slightly shifted. Analysis of proton-proton and proton-carbon 2D spectra (COSY, HMQC, HMBC) led for **2** to a planar structure identical to that of **1**. Therefore a stereochemical change is responsible of the twin set of signals observed in ^{1}H-NMR spectrum of **2**. Looking at the structure **1**, changes can occur on C-6, C-7, C-14 or C-10. However, the analysis of the ^{1}H-NMR spectrum of **2** evidentiates that H-6 and H-6' signals (δ 3.18 and 3.35) have the same molteplicity while the couples of signals H-14/H-14' and H-10/H-10' showed very different patterns. These data allowed us to consider likely a change of configuration on one of these two latter centers. In order to test this hypothesis we subjected **1** to some chemical manipulations. In fact, testudinariol A (**1**) was oxidised with pyridinium chlorochromate in dichloromethane giving a ketone which was subjected to reduction with $NaBH_4$ in ethanol. These trasformations were done with the hope to recover both testudinariols. However, after reduction, we obtained **1** and its epimer at C-14 whose ^{1}H-NMR spectrum was different from that of **2**. Therefore we concluded that the stereochemical change effected C-10. Furthermore, this was confirmed by some NOE experiments which helped, also in this case, to obtain information on the relative stereochemistry. In fact, irradiation of H-15 (δ 3.77) caused an enhancement of H-6 (δ 3.35) signal.



2

Irradiation of this last signal caused a clear NOE effect on signals at δ 3.85 (H-14), δ 3.77 (H-15) and 1.25 (H-8) while irradiation of signal at δ 3.85 caused NOE on signals at δ 3.35 (H-6) and 4.87 (H-13). A ROESY experiment confirmed the NOE observed in 1D experiments and showed also a cross peak between H-10 and H-7. All these data allowed us to assign to testudinariol B the structure **2**.

The presence of **1** in the most exposed part of the animal and in the mucus is strongly indicative that this compound could act as defensive allomone of *Pleurobrancus testudinarius*. Furthermore, testudinariol A (**1**) was ichthyotoxic in the test against *Gambusia affinis* at the concentration of 10 ppm.[9]

Finally a particular consideration deserves the fact that this kind of triterpenes is quite rare and only two related compounds, limatulone (a defensive allomone from the limpet *Collisella limatula*)[10] and neurols from a sponge,[11] have been isolated until now from marine sources.

EXPERIMENTAL SECTION

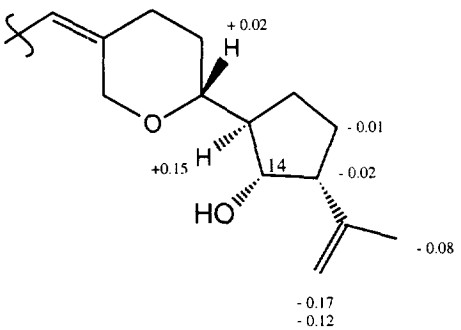
General Methods: NMR spectra were measured at 500 MHz, 400 MHz and 250 MHz for ^1H and 125 MHz and 100 MHz for ^{13}C.The signals of deuterated solvent ($CDCl_3$) were taken as reference (the singlet at 7.26 ppm for ^1H NMR and the triplet at 77.0 ppm for ^{13}C NMR data). One and two dimensional NMR experiments were performed by using standard Bruker software. Mass spectra were run in the electron impact mode (70 eV). HREIMS were obtained on a Kratos MS50 spectrometer. Column chromatography was done on silica gel Merck (60-200 μm). Optical rotations were measured on a Jasco DIP-370 polarimeter.

Isolation of testudinariol A (1) and B (2): A large specimen (15 cm long) of *Pleurobrancus testudinarius* was collected in the Gulf of Salerno in June 1993. The mollusc, put in a beaker filled with marine water, secreted a copious white mucus. Then the mollusc was removed and frozen at -80°C while the marine water was extracted with ethyl ether. The animal was sonicated in acetone in order to remove metabolites from the most external part of it (mantle). The acetone extract was concentrated *in vacuo* to an aqueous suspension and then partitioned between water and diethyl ether. The mollusc was then carefully dissected separating the skin (mantle) from the internal organs and diethyl ether soluble fractions were separately obtained from the acetone extracts of the mantle and the internal parts of the animal. All the extracts were compared by analysis by TLC (silica gel; light petroleum/diethyl ether, 1:1). The skin extract (28 mg) obtained after sonication showed three spots (*Rf* 0.45, 0.25, 0.20) which were absent in the extract from the internal organs (69 mg). The most intense spot (*Rf* 0.20) was observed also in the extract from the mucus (0.5 mg) and from the mantle (150 mg). The ether soluble extract obtained after sonication was chromatographed (silica gel; light petroleum ether : diethyl ether 8 :2) to give testudinariol A (**1**) (3.2 mg), testudinariol B (**2**) (0.9 mg) and a third compound (0.5 mg) whose structure has not yet been determined. Testudinariol A (**1**) (2 mg) was also obtained, after chromatography, from the mantle extract.

Testudinariol A (1): oil; $[\alpha]_D^{25}$ = + 15.2° (*c* = 0.3 , $CHCl_3$) ; IR ($CHCl_3$) 3446 cm^{-1} (br) ; EIMS (70eV) *m/z* 470, 452, 442, 434, 424, 370, 343, 234, 219; HREIMS obsd 470.3392 calcd for $C_{30}H_{46}O_4$ 470.33959. See Table 1 for ^1H and ^{13}C NMR spectral data.

Testudinariol B (2): oil; $[\alpha]_D^{25}$ = + 15.0° (*c* = 0.05 , $CHCl_3$) ; IR ($CHCl_3$) 3445 cm^{-1} (br) ; EIMS (70eV) *m/z* 470, 452, 442, 434, 424, 370, 343, 234, 219; HREIMS obsd 470.3394 calcd for $C_{30}H_{46}O_4$ 470.33959. See Table 1 for ^1H and ^{13}C NMR spectral data.

Preparation of Mosher esters of Testudinariol A (1): Two sample of **1** (1 mg) were dissolved in anhydrous pyridine, and then separately treated with an excess of (*S*) and (*R*)-2-methoxy-2-(trifluoromethyl)-phenylacetyl chloride (MTPACl). After 24 hours the reactions were stopped and pyridine was evaporated under a stream of N_2. The residues were purified by chromatography on silica gel (petroleum ether/diethyl ether, 1:1) yielding the *R* and *S* Mosher's esters of **1**. (*S*)- MTPA ester of **1:** selected ^{1}H-NMR data (500 MHz; $CDCl_3$,assignment aided by ^{1}H-^{1}H COSY experiment;) δ 3.27 (H-6), 2.14 (H-7), 1.76 (H-9), 2.46 (H-10), 1.68 (H_3-12), 4.66 and 4.63 (H_2-13).(*R*)- MTPA ester of **1:** selected ^{1}H-NMR data (500 MHz; $CDCl_3$,assignment aided by ^{1}H-^{1}H COSY experiment;) δ 3.25 (H-6), 1.99 (H-7), 1.77 (H-9), 2.48 (H-10), 1.76 (H_3-12), 4.83 and 4.75 (H_2-13). The $\Delta\delta$ (δ_S-δ_R) values are indicated in the following figure. From these results, it was determined[8] that the absolute configuration of the C-14/C-14' carbons was *S*.



Epimerization of 1 by oxidation followed by reduction: Testudinariol A (2 mg) was dissolved in anhydrous dichloromethane with an excess of pyridinium chlorochromate. The reaction mixture was stirred for three hours. The mixture was filtered, the filtrate was concentrated and then chromatographed on silica gel (petroleum ether-diethyl ether 9:1) to give 1.0 mg of ketone: (500 MHz; $CDCl_3$; assignment aided by ^{1}H-^{1}H COSY experiment) δ 5.13 (m, 1H, H-2), 4.92 (s, 1H, H-13), 4.79 (s, 1H, H-13), 4.56 (d, *J* = 12.8 Hz, 1H, H-15), 3.76 (m,1H, H-6), 3.75 (d, *J* = 12.8 Hz, 1H, H-15), 2.75 (dd, *J* = 8.8, 10.1 Hz, 1H, H-10), 2.41 (m, 1H, H-7), 2.24 (m, 2H, H_2-4), 2.14 (m, 1H, H-9), 2.12 (m, 1H, H-8), 2.06 (m, 1H, H-1), 1.97 (m, 1H, H-1), 1.80 (m, 2H, H-8 and H-9), 1.71 (s, 3H, H_3-12), 1.69 (m, 1H, H-5), 1.49 (m, 1H, H-5). The ketone was then subjected to reduction with $NaBH_4$ in ethanol. After usual work-up, a mixture containing testudinariol A and its C-14 epimer was isolated: ^{1}H-NMR (500 MHz; $CDCl_3$; selected values) δ 4.97, 4.82, 4.77, 4.74, 4.59, 4.29, 4.18, 3.72, 3.18.

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