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A New Labdane Diterpene from the limpet *Trimusculus peruvianus*

[Un Nuevo Diterpeno labdano aislado del molusco *Trimusculus peruvianus*]

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Abstract
The family Trimusculidae produces labdane diterpenes, which differ in the degree and type of esterification with acetoxy and isovaleroyl ester predominantly. Here we describe the isolation from the marine pulmonate *Trimusculus peruvianus*, collected on intertidal rocks of Chilean coasts, of a new diterpene closely related to the above mentioned characteristics. It structure was determined by spectroscopic data. The compounds were subjected to toxicity tests using nauplii and cysts of *Artemia salina*. The known compounds isolated in this study have shown an ability to inhibit egg hatch of *A. salina*.

Keywords: marine mollusc, diterpene, *Trimusculus peruvianus, Artemia salina*

Resumen
La familia Trimusculidae produce diterpenos tipo labdano, que difieren en el grado y tipo de esterificación con esteres acetato e isovalérico predominantemente. En este trabajo describimos el aislamiento de un nuevo diterpeno con las características ya mencionadas y de otros ya conocidos desde el molusco marino pulmonado *Trimusculus peruvianus*, recolectado en la zona intermareal del litoral chileno. Su estructura fue determinada a través de métodos espectroscopicos. Los compuestos fueron sometidos a ensayos de toxicidad y eclosión de los huevos de *Artemia salina*.

Palabras Clave: Molusco marino, diterpeno, *Trimusculus peruvianus*, *Artemia salina*

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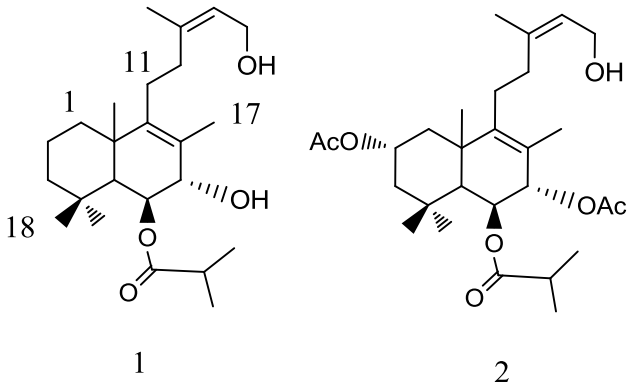
INTRODUCTION

Many marine molluscs as Nudibranchia and Opisthobranchia, have been shown to contain secondary metabolites and there have been numerous studies investigating the role of these compounds. Unlike most intertidal limpets, *Trimusculus reticulatus* filter feeds by producing mucus net which traps phytoplankton (Manker, 1996); the mucus is also produced when this sessile marine organism is disturbed. Since *T. reticulatus* is prey of few predators and as the mucus has been shown to repel starfish (Rice, 1985), the role of its secondary metabolites as protective agents has been suggested. Two diterpenes, **1** and **2** (Figure 1), were isolated from the extracts of both the whole animal and the mucus but no ecological correlation between the mucus properties and chemical composition had been established (Manker, 1987). At the present time four species of *Trimusculus* have been studied and all of them produce diterpenes belonging to a unique class of labdane skeleton. The labdane diterpenes from *Trimusculus* differ in the degree and type of esterification with acetoxy and isovaleroxy ester predominantly. In previous works (San-Martín, 1996; Diaz-Marrero, 2003; Diaz-Marrero, 2008) we reported the isolation and structure elucidation of many diterpenes from *T. peruvianus*. From a new collection, now we describe the isolation of a new diterpene **3** with a labdane skeleton, together with two known (Diaz-Marrero, 2003) diterpenes, **4** and **5** (Figure 1), which differ in the position of esterification. The compounds **3**, **4** and **5** were evaluated with the brine shrimp hatchability test and brine shrimp lethality test (Migliore, 1997; Carballo, 2002).

Figure 1



1 2

	R_1	R_2	R_3
3	$C_4H_9CO_2$	C_4H_9CO	H
4	$C_4H_9CO_2$	H	$C_4H_9CO_2$
5	$C_4H_9CO_2$	C_4H_9CO	OH

EXPERIMENTAL

IR spectra were recorded on a Perkin Elmer System 2000 FTIR spectrophotometer in $CHCl_3$ solutions. EIMS and HRMS data were taken on a Micromass Autospec spectrometer. The ^1H, ^{13}C-NMR spectra as well as ^1H-H COSY, ^{13}C (DEPT), HMQC (optimized for J_{H-C} = 140 Hz), HMBC (mixing time of 75 msec) and ROESY (mixing time of 250 msec) data were obtained on Bruker AM-400. All chemical shifts are reported with respect to TMS (δ=O). Two-dimensional NMR spectra were obtained with the standard Bruker software. The gel filtration column (Sephadex LH-20) used *n*-hexane-MeOH-CH_2Cl_2 (3:1:1) as solvent. Merck Si gels 7734 and 7729 were used in column chromatography. Spray reagent for TLC was H_2SO_4-MeOH (1:10). Solvents were of analytical grade.

Biological material

Specimens of *T. peruvianus* were collected on intertidal rocks bahía de Pichidangui, Chile (33º11', S; 71º43', W) during March 2008. The organism was identified by Prof. C. Osorio, Universidad de Chile. A voucher specimen has been deposited at the Facultad de Ciencias, Universidad de Chile collection.

Extraction and isolation

700 freeze dried specimens of *T. peruvianus* were stored in ethyl acetate for one week and then the

solvent was decanted and evaporated to obtain an aqueous suspension. The aqueous phase was extracted with ethyl acetate (4 x 100 mL) and the extracts were combined, dried over anhydrous sodium sulphate and evaporated to obtain brown oil (34.0 g). The crude extract was chromatographed by flash chromatography on silica gel, using (1:1) *n*-hexane: EtOAc as eluant, followed by filtration column to give a complex mixture that was further separated by HPLC using *n*-hexane: EtOAc (3:7) as eluant, to obtain the compound **3**. The fraction eluted with *n*-hexane: EtOAc (2:8) was rechromatographed on Sephadex LH-20 column, affording a fraction that was purified by HPLC to give the known compounds **4** and **5.**

Compound 3

Colourless oil; $[\alpha]_D^{25}$ +101º (*c* 0.15, $CHCl_3$)**.** IR **(**film**)** ν_{max}**:** 3450, 3440-3120, 1699, 1642, 1420,1280 cm^{-1}; ^1H and ^{13}C NMR, see Table 1. EIMS *m/z*: 504 [M^+, $C_{30}H_{48}O_6$] (2), 459 [M^+- CHO_2] (10), 419 [M^+-$C_5H_9O_2$] (12), 301[M^+-2 $C_5H_{10}O_2$-H]$^+$ (100).

Brine shrimp hatchability test

The brine shrimp hatchability test is based on Migliore (Migliore, 1997). They calculated the hatch, harvesting the free nauplii from 1 g of cysts on a Millipore 45 µm filter, weighed and placed in a desiccator at 60º C for 24 h to obtain the dry weight. In our case, the percentages of hatchability were calculated by comparing the number of free nauplii in each treatment with the number of free nauplii in the control and the whole procedure was standardized. Following the procedure, 0.5 g of dried cysts was separated from their shells using the commercial brine shrimp hatchers solution. After that, the cysts were hatched in seawater (1 g cyst per liter) at 28° C, under conditions of continuous illumination and strong aeration. After 2 h aliquots measuring 250 µL were placed in each well where the extracts had previously been deposited, and they were incubated at the same conditions of temperature and illumination under gentle shaking. After 12, 24 and 48 h of exposure the free nauplii were counted under a stereoscopic microscope. The percentages of hatchability were calculated by comparing the number of free nauplii in each treatment with the number of free nauplii in the control. Later the percentage of hatch inhibition (%HI) was calculated as:

% HI = % hatchability in the control - % hatchability in each treatment.

Brine shrimp lethality test

Dried cysts were performed as indicated above, and incubated (1 g cyst per liter) in a hatcher at 28–30°C with strong aeration, under a continuous light regime. Approximately 12 h after hatching the phototropic nauplii were collected with a pipette from the lighted side and concentrated in a small vial. Ten brine shrimps were transferred to each well using adequate pipette. Each test consisted of exposing groups of 10 *Artemia* aged 12 h to various concentrations of compounds **3**, **4**, **5**) compound. The toxicity was determined after 12 h (mainly nauplii in instar I/II), 24 h (nauplii in instar II/III) and 48 h (mainly nauplii in instar III/IV) of exposure.

The numbers of survivors were counted and percentages of deaths were calculated. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation.The larvae did not receive food. To ensure that the mortality observed in the bioassay could be attributed to bioactive compounds and not to starvation; we compared the dead larvae in each treatment to the dead larvae in the control. In any case, hatched brine shrimp nauplii can survive for up to 48 h without food because they still feed on their yolk-sac. However, in cases where control deaths were detected, the percentage of mortality (% M) was calculated as:

% M = percentage of survival in the control - percentage of survival in the treatment

RESULTS AND DISCUSSION

From the crude ethyl acetate extract of *T. peruvianus* collected on intertidal rocks near Pichidangui, IV Region (Chile), compound **3** was obtained after flash chromatography followed by gel filtration and successive HPLC. Compound **3** was isolated as a colourless oil, whose mass spectrum showed a molecular ion $[M]^+$ m/z 504, in accordance with NMR data suggest the molecular formula $C_{30}H_{48}O_6$ indicating that there are seven insaturations. The ^1H-NMR spectrum of the compound **3** showed one vinyl proton signal at δ 5.75 ppm (1H, bs). Two vinylic

methyl signals at δ 2.24 ppm (3H, bs) and 1.66 ppm (3H, s) together with four signals in the ^{13}C-NMR spectra belonging to sp^2 carbons at δ 163.0(s), 138.7(s), 123.1 (s) and 114.8 (d) ppm established the presence of two double bonds. The ^{13}C NMR spectrum together with the information from a DEPT spectrum, showed signals for 30 carbon atoms; besides to four olefinic sp^2 carbons, three carbonylic carbons at δ 171.5, 172.5 and 172.9 ppm were observed. Also were observed five methines, two of them were attached to oxygens at δ 69.2 and 68.4 ppm. Seven methylenes, nine methyls and two sp^3 quaternary carbons completed the ^{13}C-NMR spectra. (See table 1). So the molecule must be a bicyclic diterpenoid esterified of the labdane type with two olefinic double bonds. The E configuration of the trisubstituted double bond was deduced from the ^{13}C NMR upfield chemical shift for C-16 (d 19.2) due to the shielding effect of the *cisoid* relationship to a carboxyl group, and the downfield chemical shift for C-12 (d 41.7). A 7 ppm shielding of C-12 or C-16 *cisoid* to the polar group at C-15 in the ^{13}C NMR spectrum should be expected (Bastard, 1984) .The stereochemistry of the double bond was corroborated by the NOE effect observed between H-14 and H2-12. The IR absorptions at 1699, 1642, 1420 and 1280 cm^{-1} were consistent with both oxygenated functionalities: carboxylic acid and ester. The nature of the ester groups as secondary was confirmed by the presence of ^{13}C-NMR signals at δ 69.2 (d) and 68.4 (d) ppm. In addition, the ^{1}H-NMR spectrum showed signals at δ 4.69 (1H, bs) and 5.54 (1H, d J = 5.4 Hz) ppm. That the secondary ester was attached to C-6 was shown by ^{13}C and ^{1}H-NMR data: δ 68.4 (d) and 5.54 ppm respectively. The other ester was assigned to C-3 by detailed analysis of ^{1}H and ^{13}C-NMR spectra plus ^{1}H-^{1}H COSY, HMQC and HMBC experiments. The nature of ester group was determined by comparison of the NMR data of compound **3** with those isolated previously from the same source (Díaz-Marrero et al., 2003). The relative configuration was assigned by comparison with related compounds (**4** and **5**) and ROESY data. The structure of compound **3** is assigned as 3α-6 ß diisovaleroxylabda-8, 13-dien-15-oic acid. To our knowledge this is the first natural occurrence of this compound. The compounds **4** and **5** were identified by comparison with authentic samples and spectroscopic data.

Table 1

Nº	δ_H (in ppm)	δ_C
1	1.53 ddd (3.4;12.4;12.4)	29.1
2	1.79 m	25.8
3	4.69 brs	69.2
4		77.8
5	1.74 brs	47.1
6	5.54 d (5.2)	68.4
7	2.10 m	39.8
8		123.1
9		138.7
10		38.8
11	2.01 m	28.0
12	2.04 m	41.7
13		163.0
14	5.75 s	114.8
15		171.5
16	2.24 s	19.2
17	1.66 s	19.5
18	1.12 s	22.4
19	0.98 s	23.3
20	1.31 s	21.0
1’		172.5[a]
2’	2.19 m	43.8[b]

3’	2.09 m[d]	37.3[c]
4’	0.95 d (6.7)[e]	29.1
5’	0.94 d (6.7)[e]	22.4
1’’		172.9[a]
2’’	2.19 (m)[b]	46.8[b]
3’’	2.15 (m)[d]	33.1[c]
4’’	0.95 d (6.7)[e]	29.2
5’’	0.95 d (6.7)	22.4

[a, b, c, d, e] interchangeable signals

In the experiments using eggs and nauplii of *Artemia salina*, is determined the ability to inhibit hatching of *A. salina* and toxicity of the compounds tested. After the respective statistical analysis, it is possible to obtain the inhibitory concentration for both tests, (see Table 2), were for the hatch assay, we can see significant differences between compound **4** and the other two compounds**.** Compound **4** has a greater ability to inhibit the hatching of eggs of *A. salina*. In the toxicity assay, **5** present a high inhibititory capacity of the nauplii.

Table 2
IC_{50} of Hatching and Toxicity Assays

Compound	Hatching IC_{50}(µg mL^{-1})	Toxicity IC_{50}(µg mL^{-1})
3	**1.28 ± 0.09**	**12.7 ± 0.53**
4	**1.04± 0.38**	**9.49 ± 0.38**
5	**4.47± 0.50**	**6.91±0.50**

CONCLUSION

The mollusc *T. peruvianus* remains as a rich source of labdane diterpenoids.The known compounds isolated in this study has demonstrated their ability to inhibit egg hatch of *A. salina.* This result suggests a protective function of compounds avoiding larval fouling.

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