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Labdane diterpenes with a new oxidation pattern from the marine pulmonate *Trimusculus peruvianus*

Ana R. Díaz-Marrero,[a] Enrique Dorta,[a] Mercedes Cueto,[a] Juana Rovirosa,[b] Aurelio San-Martín,[b] Alberto Loyola[c] and José Darias[a,*]

[a]*Instituto de Productos Naturales y Agrobiología del CSIC, Avda. Astrofísico F. Sánchez 3, Apdo 195, 38206 La Laguna, Tenerife, Spain*
[b]*Departamento de Química, Facultad de Ciencias, Universidad de Chile, Santiago de Chile, Chile*
[c]*Departamento de Química, Facultad de Ciencias, Universidad de Antofagasta, Antofagasta, Chile*

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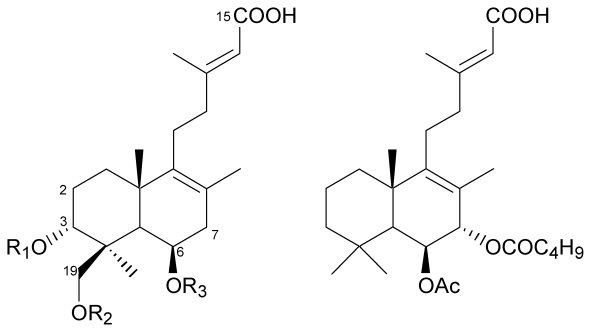
Abstract—Four new labdane diterpenes, **1**-**4**, with an unusual oxidation pattern have been isolated from the marine pulmonate *Trimusculus peruvianus*. The structure and stereochemistry of these compounds were determined by spectroscopic data and chemical transformations. The absolute stereochemistry of **4** was assigned by application of the modified Mosher method. These compounds exhibit in vitro moderate cytotoxic activity against human colon carcinoma cell lines.

1. Introduction

Whereas shell-less marine opisthobranch and nudibranch molluscs in particular, have the ability to sequester a wide variety of natural compounds from dietary source,[1] as well as to produce some de novo metabolites[2–4] that are presumably involved in their defence against predators, shell-bearing molluscs such as intertidal pulmonate limpets of the family Trimusculidae seem only to produce de novo secondary metabolites.[5] These sessile limpets, that live with unusual constraints, produce diterpenes which appear to be repellent to predatory fish,[6] and also toxic to larvae settling around them,[7] in order to avoid the presence of other invertebrates.

At the present time, four species of *Trimusculus* have been studied and all of them have a common feature: they produce diterpenes belonging to a unique class of labdane skeleton revealing preferred oxidation sites along the framework. All naturally occurring diterpenes from *T. reticulatus*,[5,6] *T. peruvianus*,[8,9] *T. conica*[6] and *T. costatus*,[7] are oxidized at carbons C-6, C-7 and C-15, except one from *T. peruvianus*[8] which lacked oxidation at C-7. Only two metabolites, one isolated from *T. reticulatus*[5] and the other from *T. costatus*, are additionally oxidized at C-2. The labdane diterpenes from *Trimusculus* differ in the degree and type of esterification with acetoxy and isovaleroxy ester predominantly.

In this work we report on the structure elucidation of four new diterpenes **1**-**4** featuring a novel oxidation pattern at C-3 and C-19, unlike other metabolites from this genus. These compounds exhibit in vitro moderate cytotoxic activity against human colon carcinoma cell lines.



1: $R_1 = R_2 = R_3 = H$
2: $R_1 = COC_4H_9$, $R_2 = R_3 = H$
3: $R_1 = R_3 = COC_4H_9$, $R_2 = H$
4: $R_1 = R_2 = COC_4H_9$, $R_3 = H$

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2. Results and discussion

From the crude ethyl acetate extract of *T. peruvianus*, collected near Antofagasta coast (Chile), compounds **1**-**4** were obtained after flash chromatography followed by gel filtration and successive HPLC.

Keywords: labdane diterpenes; pulmonate; carcinoma.
* Corresponding author. Tel.: +34-922-25-21-44; fax: +34-922-26-01-35; e-mail: jdarias@ipna.csic.es

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Compound **1** was isolated as an oil, $[\alpha]_D^{25}=+40$ (*c* 0.45, MeOH). NMR data coupled with a $[M-Me]^+$ peak at *m/z* 337.2091 in the HREIMS of **1** suggested a molecular formula of $C_{20}H_{32}O_5$, indicating five degrees of unsaturation. ^{13}C NMR spectrum of **1** (Table 1), together with the information from a DEPT spectrum, showed the presence of 20 carbon signals assigned to four methyls, six methylenes (one bearing oxygen), four methines (one olefinic, and two geminal to oxygen) and six nonprotonated carbons (one carbonyl and three olefinic). The IR absorptions at 3450, 3440–3100 and 1699 cm^{-1} were consistent with both oxygenated functionalities: hydroxyls and one acid carbonyl. Since the IR spectrum revealed no absorption for additional unsaturations, the molecule must be bicyclic. As the molecule possesses only four methyl groups, one of the five methyl groups characteristic of a diterpene skeleton must be oxidized to a primary alcohol [H_{2a}-19: δ 3.18 (d, *J*=11.2 Hz), H_{2b}-19: δ 4.09 (d, *J*=11.2 Hz); C-19: δ 66.6] allowing us to consider now the possibility that compound **1** could belong to the labdane skeletal class characteristic of the trimusculid diterpenes above mentioned.

Besides the primary alcohol, the ^1H NMR showed signals for two protons geminal to the remaining alcoholic oxygens at δ 3.36 (dd, *J*=2.7, 2.7 Hz) and δ 4.24 (d, *J*=5.2 Hz), completing, with that of the carboxylic acid, the five oxygens present in the molecular formula. In addition, the ^1H NMR spectrum displayed a signal for one olefinic proton at δ 5.67 (s) and at high field a signal for two olefinic and two angular methyl groups at δ 2.17, δ 1.65, δ 1.37, δ 1.10, respectively. The HMBC correlation of the C-19 methylene alcohol with an angular methyl group, C-18, indicated that the oxidized methyl group is part of the typical *gem*-dimethyl group of ring A of a diterpene structure.

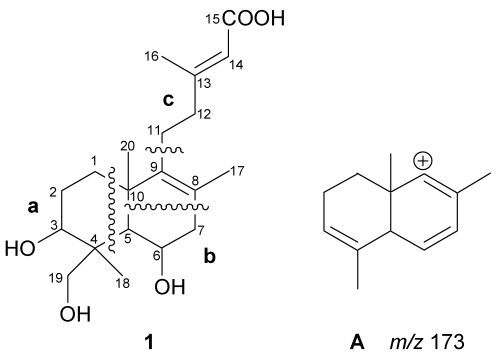
Chemical shift arguments, $^1H-^1H$ COSY and HMBC correlations allowed the assignment of fragments **a**-**c** in a labdane skeleton as shown in **1**. From the $^1H-^1H$ COSY NMR spectrum it was clearly possible to differentiate three discrete spin systems (H-3–H_2-2–H_2-1; H-5–H-6–H_2-7 and H_2-11–H_2-12). The HMBC correlations: H_3-18/C-3, C-4, C-5, C-19; H_2-19/C-3, C-4, C-5, C-18 as well as the correlation H-6/C-8, C-10 established the connectivity of fragments **a** and **b** through C-4. The decaline ring system was confirmed by: (a) the HMBC long-range correlation of H_3-20/C-1, C-5, (b) the HMBC long-range correlation of the two H_3-17 and H_3-20 methyl groups with C-9, and (c) the HMBC correlation H_3-17/C-7. This was corroborated by

Table 1. ^1H and ^{13}C NMR data of compounds **1-4** (500 MHz, δ, (*J*) Hz, $CDCl_3$)

	1		**2**		**3**		**4**	
	δ_H[a]	δ_C[a]	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.53 ddd (3.5, 3.5, 12.3) 1.72 ddd (3.4, 13.0, 13.0)	32.5	1.50 ddd (3.3, 13.3, 13.3) 1.59 ddd (3.3, 3.3, 12.4)	33.9	1.53 ddd (3.3, 13.4, 13.4) 1.62 m	32.9	1.51 ddd (3.3, 13.4, 13.4) 1.58 m	32.9
2	1.65 m, 1.93 dddd (3.5, 3.5, 13.8, 13.8)	26.1	1.72 m, 1.84 m	24.5	1.77 dddd (3.5, 3.5, 3.5, 15.3), 1.97 m	23.3	1.75 m, 1.96 m	23.5
3	3.36 dd (2.7, 2.7)	71.6	4.69 dd (2.5, 2.5)	74.5	5.18 dd (2.7, 2.7)	72.7	5.00 dd (2.7, 2.7)	73.1
4		43.8		43.6		43.4		41.6
5	1.79 br s	47.0	1.73 br s	48.8	1.87 br s	48.1	1.74 br s	48.8
6	4.24 d (5.2)	63.8	4.32 d (4.8)	64.6	5.43 d (5.4)	67.6	4.39 d (5.1)	64.6
7	2.05 d (18.2), 2.39 dd (5.2, 18.2)	41.2	2.10 m, 2.47 dd (2.5, 18.5)	42.6	2.05 m, 2.46 dd (4.8, 18.5)	39.7	2.06 d (18.5), 2.49 dd (5.1, 18.5)	43.6
8		122.8		123.5		123.2		123.3
9		138.2		138.6		138.2		138.1
10		38.3		38.9		38.7		38.3
11	2.13 ddd (4.0, 7.0, 14.0), 2.25 m	26.0	2.12 m, 2.26 m	26.5	2.23 m	26.1	2.22 m	26.1
12	2.26 m	41.2	2.25 m	42.0	2.24 m	41.6	2.25 m	41.6
13		160.5		163.3		163.0		163.1
14	5.67 s	114.9	5.72 s	114.9	5.72 br s	114.7	5.72 br s	114.5
15		168.9		171.2		171.3		171.3
16	2.17 d (1.1)	17.5	2.21 d (0.7)	19.5	2.21 s	19.2	2.21 d (0.7)	19.1
17	1.65 s	18.5	1.67 s	20.1	1.61 s	19.5	1.66 s	19.7
18	1.10 s	22.1	1.05 s	22.7	1.05 s	22.1	1.02 s	22.3
19	3.18 d (11.2), 4.09 d (11.2)	66.6	3.29 d (11.2), 4.19 d (11.2)	67.4	3.64 d (11.6), 3.92 d (11.6)	65.7	4.43 d (11.8), 4.60 d (11.8)	67.4
20	1.37 s	19.7	1.38 s	20.9	1.28 s	21.6	1.33 s	21.1
1′				172.8		172.6[b]		172.8[b]
2′			2.21 m	44.0	2.19 m	43.8[c]	2.20 m	43.3
3′			2.12 m	26.1	2.09[d] m	25.6[d]	2.11 m	25.7[d]
4′			0.96 d (6.6)	22.8	0.95[e] d (6.7)	22.4	0.96[e] d (6.7)	22.3
5′			0.96 d (6.6)	22.8	0.94[e] d (6.7)	22.4	0.96[e] d (6.7)	22.3
1″						172.7[b]		172.3[b]
2″					2.19 m	44.1[c]	2.20 m	43.3
3″					2.15[d] m	25.8[d]	2.11 m	25.6[d]
4″					0.95[e] d (6.7)	22.4	0.96[e] d (6.7)	22.3
5″					0.95[e] d (6.7)	22.4	0.95[e] d (6.7)	22.3

[a] CD_3OD.
[b] Interchangeable signals.
[c] Interchangeable signals.
[d] Interchangeable signals.
[e] Interchangeable signals.

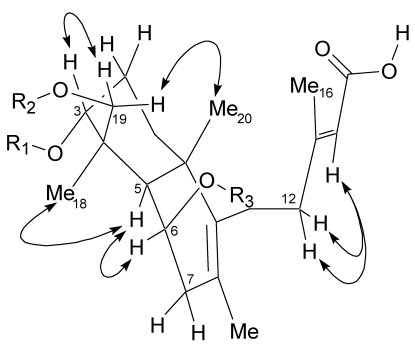
the HRMS of **1**, which showed a peak at *m/z* 173 (base peak) corresponding to fragment A.



The terminal α,β-unsaturated carboxylic acid side chain fragment **c** linked to C-9 was determined by: (a) HMBC correlation of H-14/C=O, C-13, C-16; H_3-16/C-12, C-13, C-14, and (b) HMBC correlation of H-11/C-8. These data support the structure proposed for **1**. Although a related compound **5** with an identical linear side chain to **1** has recently been described,[6] attempts to compare spectroscopic data of this moiety on stereochemical grounds were not reliable due either to incomplete or unassigned ^{1}H NMR and ^{13}C NMR data.

Chemical shift arguments and 2D NOESY experiments established the relative configurations of the chiral centres of the rings. The small values observed for the coupling constants of H-3 with the adjacent methylene as well as those of H-6 with H-5 and H_2-7 (Table 1) indicated that both hydroxyl groups on C-3 and C-6 must be axial. The NOE effects observed between H-5 with H_3-18 and H-6 and between H_2-19 with H-3 and H_3-20 supported a *trans* ring junction for **1**, as shown in Figure 1.

The *E* configuration of the trisubstituted double bond was deduced from the ^{13}C NMR upfield chemical shift for C-16 (δ 17.5) due to the shielding effect of the *cisoid* relationship



1: $R_1 = R_2 = R_3 = H$
2: $R_1 = C_5H_{10}O$, $R_2 = R_3 = H$
3: $R_1 = R_3 = C_5H_{10}O$, $R_3 = H$
4: $R_1 = R_2 = C_5H_{10}O$, $R_3 = H$

Figure 1. Selected NOE of compounds **1**-**4**.

to a carboxyl group, and the downfield chemical shift for C-12 (δ 41.2). 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.[10] The stereochemistry of the double bond was corroborated by the NOE effect observed between H-14 and H_2-12, as is represented in the minimized structure **1**, Figure 1.

The NMR spectra of **2** were very similar to those of compound **1** (Table 1). The most significant differences were the presence of five more carbons in compound **2** at δ 172.8, δ 44.0, δ 26.1, and δ 22.8 (2C), and the proton and carbon chemical shifts at C-3 from δ 3.36 (dd, *J*=2.7, 2.7Hz) and δ 71.6 in compound **1** to δ 4.69 (dd, *J*=2.5, 2.5Hz) and δ 74.5 in compound **2**. These variations, together with the loss of a $C_5H_{10}O_2$ fragment in the MS spectrum can be rationalized by the presence of an isovaleric ester at C-3.

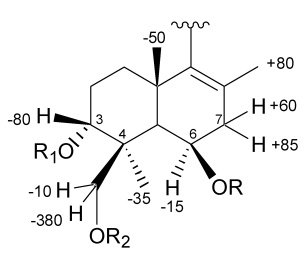
The hydrolysis of **2** with potassium hydroxide in methanol gave **1**, indicating that the relative stereochemistries at the chiral centres of both **1** and **2** were identical, which were also corroborated by 2D NOESY experiments, Figure 1.

Compounds **3** and **4** were the corresponding isovaleric diester derivatives of **1** at C-3, and C-6 and at C-3, and C-19, respectively. Each compound was hydrolyzed to **1** by treatment under the same conditions indicated above. Thus, all four compounds **1**-**4** possess the same relative stereochemistry as shown in Figure 1.

The absolute stereochemistry of compound **4** was established by application of the modified Mosher method.[11,12] *R*- and *S*-Mosher esters of the alcohol at C-6 were prepared. Subsequent NMR analysis of the Δδ values for the two MTPA esters gave clean evidence to assign the absolute stereochemistry at C-6 as *R* (Fig. 2). These information together with the NOESY data of compounds **1**-**4** indicates that C-3 and C-5 are *R* and C-4 and C-10 are *S*, so these compounds belong to the labdane series.

Compounds **3**-**4** exhibit moderate in vitro cytotoxicity against H-116 and HT-29 (human colon carcinoma) cell lines, displaying IC_{50} values of 12.5 μg/mL, in each case.

Marine pulmonates of genus *Trimusculus* are unusual not only in both habitat and behavior[6] but also in economizing the biosynthesis of diterpenes to a single type of labdane skeleton. Neither carbon rearrangement nor any functional group that induces significant structural modification has



4a: R = MTPA-(S), $R_1 = R_2 = COC_4H_9$
4b: R = MTPA-(R), $R_1 = R_2 = COC_4H_9$

Figure 2. Δδ values ($\delta_S - \delta_R$) in Hz (500 MHz) of the two MTPA esters derived from compound **4**.

been observed in the diterpenes metabolites they produce. The selectivity at the oxidation site along the skeleton resembles the mode of action of certain fungi on diterpene substrata,[13,14] suggesting that some symbiotic micro-organism interaction should not be excluded.

Related compounds to **1**-**4** have been found in terrestrial plants such as *Amphiachyris amoena*.[15]

3. Experimental

3.1. General procedures

IR spectra were obtained with a Perkin–Elmer 1650/FTIR spectrometer in $CHCl_3$ solutions. ^1H NMR and ^{13}C NMR, HMQC, HMBC and COSY spectra were measured at 500 MHz for ^1H 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 20×250 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).

3.2. Biological material

Specimens of *T. 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.

3.2.1. 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 compounds **1** (9 mg), and **2** (6 mg). The fraction eluted with hexane–EtOAc (80:20) (718 mg) was chromatographed on a Sephadex LH-20 column, affording a fraction that was chromatographed by HPLC to give compounds **3** (7.5 mg), and **4** (5.5 mg).

3.2.2. Compound 1. White powder; $[\alpha]_D^{25}=+40$ (*c* 0.45, MeOH); IR ν_{max} (film) 3450, 3440–3100, 1699 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 352 $[M]^+$ (<1), 337 $[M-CH_3]^+$ (1), 319 $[M-CH_3-H_2O]^+$ (3), 187 (57), 185 (72), 173 (100); HREIMS 337.2091 (calcd for $C_{19}H_{29}O_5$, 337.2015), 319.1986 (calcd for $C_{19}H_{27}O_4$, 319.1909), 187.1422 (calcd for $C_{14}H_{19}$, 187.1487), 173.1285 (calcd for $C_{13}H_{17}$, 173.1330).

3.2.3. Compound 2. Colorless oil; $[\alpha]_D^{25}=-23$ (*c* 0.48, $CHCl_3$); IR ν_{max} (film) 3230, 2945, 1699, 1641 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 418 $[M-H_2O]^+$ (1), 403 $[M-H_2O-CH_3]^+$ (2), 385 $[M-2H_2O-CH_3]^+$ (7), 334 $[M-C_5H_{10}O_2]^+$ (<1), 316 $[M-C_5H_{10}O_2-H_2O]^+$ (6), 187 (90), 173 (95), 57 (100); HREIMS 418.2679 (calcd for $C_{25}H_{38}O_5$, 418.2719), 403.2431 (calcd for $C_{24}H_{35}O_5$, 403.2485), 385.2355 (calcd for $C_{24}H_{33}O_4$, 385.2379), 334.2111 (calcd for $C_{20}H_{30}O_4$, 334.2144), 316.2044 (calcd for $C_{20}H_{28}O_3$, 316.2038), 187.1499 (calcd for $C_{14}H_{19}$, 187.1487), 173.1355 (calcd for $C_{13}H_{17}$, 173.1330).

3.2.4. Compound 3. Colorless oil; $[\alpha]_D^{25}=-36$ (*c* 0.97, $CHCl_3$); IR ν_{max} (film) 2955, 2870, 1726, 1642 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 418 $[M-C_5H_{10}O_2]^+$ (1), 403 $[M-C_5H_{10}O_2-CH_3]^+$ (2), 385 $[M-C_5H_{10}O_2-CH_3-H_2O]^+$ (18), 316 $[M-2C_5H_{10}O_2]^+$ (7), 283 $[M-2C_5H_{10}O_2-CH_3-H_2O]^+$ (24), 271 (66), 203 (100), 185 (82), 173 (30); HREIMS 418.2716 (calcd for $C_{25}H_{38}O_5$, 418.2719), 403.2557 (calcd for $C_{24}H_{35}O_5$, 403.2485), 316.2090 (calcd for $C_{20}H_{28}O_3$, 316.2038), 283.2207 (calcd for $C_{19}H_{23}O_2$, 283.2273), 173.1284 (calcd for $C_{13}H_{17}$, 173.1330).

3.2.5. Compound 4. Colorless oil; $[\alpha]_D^{25}=-21$ (*c* 0.85, $CHCl_3$); IR ν_{max} (film) 3514, 2956, 2872, 1725, 1636 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 502 $[M-H_2O]^+$ (1), 418 $[M-C_5H_{10}O_2]^+$ (1), 400 $[M-C_5H_{10}O_2-H_2O]^+$ (4), 385 $[M-C_5H_{10}O_2-CH_3-H_2O]^+$ (15), 316 $[M-2C_5H_{10}O_2]^+$ (7), 283 $[M-2C_5H_{10}O_2-CH_3-H_2O]^+$ (25), 185 (60), 173 (14), 57 (100); HREIMS $[M-H_2O]^+$ 502.3272 (calcd for $C_{30}H_{46}O_6$, 502.3294), 418.2735 (calcd for $C_{25}H_{38}O_5$, 418.2719), 400.2636 (calcd for $C_{25}H_{36}O_4$, 400.2614), 283.1662 (calcd for $C_{19}H_{23}O_2$, 283.1698), 173.1290 (calcd for $C_{13}H_{17}$, 173.1330).

3.3. Conversion of 2-4 to 1

Treatment of **2**-**4** (1.6 mg each) with KOH–MeOH (35%) afforded **1** after work-up of the respective reaction mixtures followed by purification. The ^1H NMR spectrum of each synthetic derivative was identical with the natural compound **1**.

3.4. Preparation of Mosher esters derivatives 4a and 4b

A mixture of compound **4** (2.2 mg, 4.23 μM), (*R*)-MTPA-Cl (4.8 mg, 19.1 μM), pyridine (75 μL), and 4-(dimethyl-amino)-pyridine (5.0 mg, 40.9 μM) was stirred at room temperature for 96 h and then it was poured into water and extracted with EtOAc. The organic layer was dried with Na_2SO_4 and concentrated. The residue was purified by silica gel chromatography to give the (*S*)-MTPA ester **4a** (1.4 mg). The same experimental procedure was followed for the production of the corresponding (*R*)-MTPA ester **4b**.

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