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Cytotoxic Sesquiterpenes from *Aplysia dactylomela*

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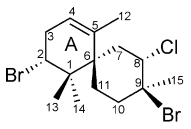
Three new chamigrenes, compound **1**, acetyldeschloroelatol **2** (2-bromo-1,1,9-trimethyl-5-methylenespiro-[5.5]undec-8-en-3-yl acetate), and acetylelatol **4** (2-bromo-8-chloro-1,1,9-trimethyl-5-methylenespiro[5.5]-undec-8-en-3-yl acetate), and the known metabolites deschloroelatol **3**, elatol **5**, 8-acetylcaespitol **6**, and caespitol **7** have been isolated from the sea hare *Aplysia dactylomela*. The structures of **1**, **2**, and **4** were determined on the basis of spectroscopic evidences. The in vitro cytotoxicity of six of these compounds against two cancer cell lines (HeLa and Hep-2) and nontumoral Vero cells was evaluated. The results support the hypothesis that the acetate derivatives decrease the toxicity of the corresponding alcohols in a strategy to store toxic metabolites acquired through the diet.

There is a close relationship between compounds stored in the digestive gland of certain opisthobranchs of the genus *Aplysia* and the chemical constituents of the algae that form a major portion of their diet.[1] A possible role of the metabolites acquired through the diet in the defense system of the sea hares has been proposed,[1] although the fact that most of these compounds are stored in the digestive gland of the animal, where they are not optimally located for defense, has caused that hypothesis to be questioned.[2,3] It has also been shown that the acquired metabolites from algae are occasionally chemically transformed by the sea hares,[4] and they are frequently converted into less toxic compounds.[5]

We report here on the isolation and identification of three new chamigrenes from *Aplysia dactylomela* from the Canary Islands, compound **1**, acetyldeschloroelatol **2**, and acetylelatol **4**, which were isolated together with the known deschloroelatol **3**,[6,7] elatol **5**,[8] 8-acetylcaespitol **6**,[9] and caespitol **7**,[9,10] after Sephadex LH-20 chromatography followed by successive HPLC. In vitro toxicity of **2**−**7** was evaluated against two selected cancer cell lines, HeLa (human cervix carcinoma) and Hep-2 (human larynx carcinoma), and nontumoral Vero (African green monkey kidney) cells.



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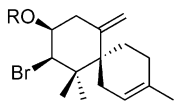
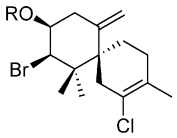
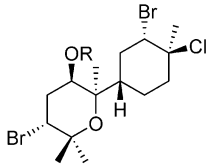


Acetyldeschloroelatol **2**, R=Ac
Deschlorelatol **3**, R=H



Acetylelatol **4**, R=Ac
Elatol **5**, R=H



8-Acetylcaespitol **6**, R=Ac
Caespitol **7**, R=H

Compound **1** was obtained as an oil whose EIMS spectrum showed peaks at *m/z* 396/398/400/402, with relative intensities suggestive of two bromine atoms and one chlorine, which correspond to the empirical formula $C_{15}H_{23}ClBr_2$ $[M]^+$ (HREIMS). The ^{13}C NMR and DEPT spectra of **1** (Table 1) showed the presence of 15 carbon signals assigned to 4 × CH_3, 4 × CH_2, 3 × CH (one olefinic), and 4 quaternary carbons (one olefinic and one bearing a heteroatom). The following ^1H NMR signals were observed: one olefinic proton at δ 5.24 (m); two protons geminal to a heteroatom [δ 4.88 (dd, J = 6.4, 11.8), 4.52 (dd, J = 6.8, 10.8)]; eight methylene protons at δ 2.69−1.55; and four methyl groups [δ 1.94 (br s), δ 1.84 (s), δ 1.20 (s), δ 0.94 (s)].

A ^1H−^1H COSY experiment established the presence of three discrete spin systems: H-2−H-4; H_2-7−H-8; and H_2-10−H_2-11. HMQC and HMBC data were used to confirm these fragments and to establish their connectivity. The three-bond correlation of H_3-13 and H_3-14 to the opposite carbons C-14 and C-13, and the correlations of both to C-1, permitted placement of the gem-dimethyl group at C-1. Correlation of H_3-12 with C-4 together with correlation of H_3-12, H_3-13, and H_3-14 with C-6 established ring A, whereas the H_3-15/C-8, C-9, C-10 and H-8 and H_2-11/C-6 correlations established the chamigrene structure of compound **1**. The chemical shifts of C-2 (δ 60.9), C-8 (δ 69.5), and C-9 (δ 67.8) indicate that C-2 and C-9 bear bromine and C-8 chlorine. The relative configuration of compound **1** was assigned on the basis of the coupling constants and a 2D NOESY experiment. Coupling constants of H-2 (J = 6.8, 10.8) and H-8 (J = 6.4, 11.8) indicate that both protons must have axial dispositions. The NOEs observed between H-8 and H_3-12 and between H-2 and H-11β established the relative configuration of C-2, C-8, and the spiro carbon C-6. Finally, the NOEs observed between H-8 and H-10β and between H_3-15 and H-10α indicated that the chlorine and the bromine at carbons C-8 and C-9 are on different sides of the molecule.

Compound **2** was isolated as a colorless oil. Its EIMS spectrum showed peaks at *m/z* 340/342 with relative intensities suggestive of one bromine atom, which correspond to the empirical formula $C_{17}H_{25}O_2Br$ $[M]^+$ (HREIMS). Absorption for a carbonyl group at 1743 cm^{-1} was observed in its IR spectrum. The ^{13}C NMR and DEPT spectra of **2** (Table 1) showed the presence of 17 carbon signals assigned to 4 × CH_3 (one acetate group), 5 × CH_2 (one olefinic), 3 × CH (one geminal to acetate and one olefinic), and five quaternary carbons (one carbonyl and two olefinic). The ^1H NMR spectrum showed signals for

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Table 1. ^{1}H, ^{13}C, and HMBC NMR Data of Compounds **1**, **2**, and **4** [500 MHz, δ ppm, (J) Hz, $CDCl_3$]

	1			**2**			**4**	
no.	δ_H	δ_C	HMBC	δ_H	δ_C	HMBC	δ_H	δ_C
1		43.0			43.5			43.4
2	4.52 dd (6.8, 10.8)	60.9	C-13	4.59 d (3.3)	64.0	C-1, C-3, C-13, C-14	4.52 d (3.4)	63.0
3	α: 2.69 m	36.3		5.26 ddd (3.2, 3.2, 3.2)	74.0	C-5	5.24 ddd (3.2, 3.2, 3.2)	73.7
	β: 2.55 m							
4	5.24 m	123.0		β: 2.65 dddd (1.5, 1.5, 3.1, 14.9)	36.7	C-12	β: 2.57 m	36.8
				α: 2.39 dd (2.8, 14.9)		C-2, C-3, C-6, C-12	α: 2.40 dd (2.8, 15.0)	
5		139.7			141.0			140.6
6		47.3			46.9			49.0
7	β: 2.08 m	38.0	C-5, C-6, C-8, C-9	β: 2.24 m	30.2	C-5, C-8, C-9	β: 2.61 m	38.6
	α: 2.08 m			α: 2.09 m		C-5, C-8, C-9	α: 2.35 m	
8	4.88 dd (6.4, 11.8)	69.5	C-9	5.28 m	119.4	C-6, C-10, C-15		124.1
9		67.8			132.7			128.1
10	α: 2.41 ddd (4.4, 4.4, 14.1)	42.3	C-9	α: 1.82 m	27.8		α: 1.97 m	29.4
	β: 2.55 m			β: 1.62 m			β: 1.79 m	
11	β: 1.55 m	31.7	C-5, C-6, C-9	α: 1.79 m	26.0		α: 1.80 m	25.6
	α: 2.00 m			β: 1.61 m			β: 1.63 m	
12	1.94 br s	26.0	C-4, C-5, C-6	α: 4.95 dd (1.6, 1.6)	115.7	C-4, C-5, C-6	α: 4.99 s	115.8
				β: 4.76 s		C-4, C-5, C-6	β: 4.76 s	
13	0.94 s	17.1	C-1, C-2, C-6, C-14	1.03 s	20.1	C-1, C-2, C-6, C-14	1.05 s	20.1
14	1.20 s	24.6	C-1, C-2, C-6, C-13	1.07 s	24.3	C-1, C-2, C-6, C-13	1.09 s	24.2
15	1.84 s	24.0	C-8, C-9, C-10	1.57 s	23.1	C-8, C-9, C-10	1.69 s	19.4
CH_3*CO*					170.2			170.1
CH$_3$CO				2.06 s	21.0	CH_3*CO*	2.09 s	21.0

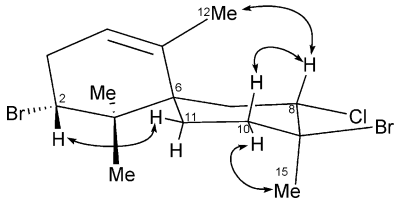

Figure 1. Selected NOEs of **1**.

three olefinic protons at δ 5.28 (m), 4.95 (dd J = 1.6, 1.6), and 4.76 (s); the proton geminal to acetate at δ 5.26 (ddd, J = 3.2, 3.2, 3.2); the proton geminal to bromine at δ 4.59 (d, J = 3.3); eight methylene protons at δ 2.65−1.61; and four methyl groups [δ 2.06 (s), 1.57 (s), 1.07 (br s), 1.03 (s)]. All these data indicated that compound **2** is a chamigrene sesquiterpene derivative. The chemical shifts of C-2 (δ_H 4.59 d, δ_C 64.0) and C-3 (δ_H 5.26 ddd, δ_C 74.0) indicated that these carbons bear bromine and an *O*-acetyl group, respectively.

A ^{1}H−^{1}H COSY experiment established the presence of three discrete spin systems: H-2−H_2-4; H_2-7−H-8; and H_2-10−H_2-11. HMQC and HMBC data were used to confirm these fragments and to establish the connectivity between them. The three-bond correlation of H_3-12, H_3-13, and H_2-14 (see Table 1) allowed ring A to be established, whereas the correlations of H_3-15 completed the structure of compound **2**, thus establishing that **2** is the *O*-acetyl derivative of deschlorelatol **3**, which has also been isolated in this study.

Compounds **2** and **4** have very similar ^{1}H and ^{13}C NMR spectra, the most significant difference being the disappearance of the endocyclic-olefinic proton of **2**. The EIMS spectrum of **4** indicates the presence of a chlorine atom in the molecule, indicating that **4** was the *O*-acetyl derivative of elatol **5**. This supposition was confirmed by the acetylation of elatol to produce a compound that possessed spectra identical to those of compound **4**.

The optical activities of deschloroelatol **3** and elatol **5** are coincident with those established previously,[6,9] and if we admit that *Aplysia dactylomela* is responsible for the acetylation of the dietary-derived metabolites **3** and **5**, the absolute configuration of **2** and **4** can be defined as 2*R*, 3*S*, and 6*R*.

The cytotoxic activity data (Table 2) showed that elatol **5** was the most active compound under the two conditions assayed. It is important to emphasize that when the cells are exponentially grown, the activity increases considerably in the case of elatol and acetylelatol against HeLa (IC_{50} 1.3 and 13.7 μM, respectively) but not against Hep-2 cells. Furthermore, both compounds show selective cytotoxic activity (IC_{50} 25.0 and 44.6 μM) against Vero cells. Deschloroelatol **3** and its acetyl derivative, compound **2**, were inactive (IC_{50} > 67 and > 58 μM), which indicates the relevance of the chlorine atom in the molecule, while caespitol **7** was slightly active. The results support the hypothesis that acetyl derivatives decrease the toxicity of the corresponding alcohols and that sea hares use acetylation as a strategy to store toxic metabolites acquired through diet.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer model 343 Plus polarimeter using a Na lamp at 20 °C. IR spectra were obtained with a Perkin-Elmer 1600/FTIR spectrometer. EIMS and HRMS spectra were recorded on a Vg-Micromass Zab 2F spectrometer. ^{1}H NMR, ^{13}C NMR, HSQC, HMBC, COSY, and NOESY spectra were measured employing a Bruker AMX 500 instrument operating at 500 MHz for ^{1}H NMR and at 125.7 MHz for ^{13}C NMR, using $CHCl_3$ as internal standard. Two-dimensional spectra were obtained with the standard Bruker software. HPLC separations were performed with a Hewlett-Packard HP 1050 (Jaigel-Sil semipreparative column, 10 μm, 20 × 250 mm) with hexane−EtOAc mixtures. The gel filtration

Table 2. Cytotoxicity of Compounds **2**−**7** against HeLa, Hep-2, and Vero Cells

	IC_{50} (μM)					
	HeLa		Hep-2		Vero	
compound	lag phase	log phase	lag phase	log phase	lag phase	log phase
acetyldeschloroelatol **2**	>58	>58	>58	>58	>58	>58
deschloroelatol **3**	>67	>67	>67	>67	>67	>67
acetylelatol **4**	50.3	13.7	28.0	22.4	>53	44.6
elatol **5**	4.1	1.3	2.4	2.0	2.3	25.0
8-acetylcaespitol **6**	>42	>42	>42	>42	31.9	33.6
caespitol **7**	26.9	30.6	>46	>46	25.5	25.8
actinomycin D[a]	0.011	0.001	0.191	0.006	0.071	0.001

[a] Actinomycin D was used as a positive control.

column (Sephadex LH-20) used hexane−MeOH−$CHCl_3$ (3:1:1) as solvent. Merck Si gels 7734 and 7741 were used in column chromatography. The spray reagent for TLC was H_2SO_4−H_2O−AcOH (1:4:20).

Animal Material. The 28 specimens of *Aplysia dactylomela* were collected off the southwest coast of La Palma Island at −1.5 m depth. Specimens were dissected and their digestive system along with the mantle were separated and analyzed independently.

Extraction and Isolation. *A. dactylomela* digestive glands were extracted with acetone at room temperature. The extract was concentrated to give a dark green residue (31.0 g), which was partitioned with H_2O−CH_2Cl_2. The resulting fraction of CH_2Cl_2 (7.5 g) was then submitted to a gel filtration column to give fraction A (822.9 mg), which after flash chromatography on Si gel and HPLC yielded the new sesquiterpenes **1** (0.4 mg), acetyldeschloroelatol **2** (10.9 mg), and acetylelatol **4** (4.7 mg) together with the known compound 8-acetylcaespitol **6** (35.0 mg). From fraction D after flash chromatography on Si gel the known compounds deschloroelatol **3** (2.7 mg), elatol **5** (50.6 mg), and caespitol **7** (73.6 mg) were isolated. *A. dactylomela* mantles were extracted and processed following the same scheme. From the extract of the mantle deschloroelatol **3** (1.1 mg), elatol **5** (36.9 mg), caespitol **7** (3.1 mg), and 8-acetylcaespitol **6** (3.3 mg) were isolated. None of the new chamigrenes **1**, **2**, and **4** were detected.

Cytotoxic Activity. HeLa (human carcinoma of the cervix), Hep-2 (human carcinoma of the larynx), and Vero (African green monkey kidney) cell lines were each grown as a monolayer in Dulbecco's modified Eagle's medium, DMEM (Sigma), supplemented with 5% fetal calf serum (Gibco) and a 1% penicillin−streptomycin mixture (10.000 UI/mL). The cells were maintained at 37 °C in 5% CO_2 and 98% humidity. Cytotoxicity was assessed using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay.[11] A total of 2×10^4 cells in lag and log-phase of growth were incubated in a microtiter well plate (96-well Iwaki) with the compounds at different concentrations predissolved in DMSO. After 48 h the opical density was measured using a microELISA reader (Multiskan Plus II) at 550 nm after dissolving the MTT formazan with DMSO (100 μL). The percentage viability (IC_{50}) was calculated from the curve. All the experiments were repeated three times.

Compound 1: colorless oil; $[\alpha]^{20}_D$ −210.2 (*c* 0.033, $CHCl_3$); IR ν_{max} (film) 2917, 1654 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 396/398/400/402 $[M]^+$ (<1, <1, <1, <1), 361/363/365 $[M - Cl]^+$ (<1, <1, <1), 317/319/321 $[M - Br]^+$ (43, 58, 14), 145 (100); EIHRMS $[M]^+$ 395.9818 (calcd for $C_{15}H_{23}ClBr_2$, 395.9855), $[M - Br]^+$ 317.0660 (calcd for $C_{15}H_{23}ClBr$, 317.0672).

Acetyldeschloroelatol 2: colorless oil; $[\alpha]^{20}_D$ +53 (*c* 0.300, $CHCl_3$); IR ν_{max} (film) 1743 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m/z* 340/342 $[M]^+$ (1, 1), 201 $[M - HOAc - Br]^+$ (100); EIHRMS $[M]^+$ 340.1044 (calcd for $C_{17}H_{25}O_2{}^{79}Br$, 340.1037), 342.0977 (calcd for $C_{17}H_{25}O_2{}^{81}Br$, 342.1017), $[M - HOAc - Br]^+$ 201.1640 (calcd for $C_{15}H_{21}$, 201.1643).

Acetylelatol 4: colorless oil; $[\alpha]^{20}_D$ +173 (*c* 0.086, $CHCl_3$); IR ν_{max} (film) 1740 cm^{-1}; ^1H and ^{13}C NMR, see Table 2; EIMS *m/z* 374/376/378 $[M]^+$ (<1, <1, <1), 235/237 $[M - Br - HOAc]^+$ (100, 34), 199 $[M - HBr - HOAc - Cl]^+$ (49); EIHRMS $[M]^+$ 376.0696 (calcd for $C_{17}H_{24}O_2{}^{81}Br^{35}Cl$, 376.0627).

Acetylation of Elatol. A solution of elatol (4.7 mg) in C_5H_5N (1.5 mL) was treated with Ac_2O (1.0 mL) and stirred at room temperature for 5 h. The reaction was quenched with H_2O, and the mixture was extracted twice with EtOAc. The organic layer was washed with H_2O and 5% aqueous HCl, dried (Na_2SO_4), concentrated, and weighed (1.3 mg).

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