Cytotoxic Lissoclimide-Type Diterpenes from the Molluscs *Pleurobranchus albiguttatus* and *Pleurobranchus forskalii*†

Xiong Fu,‡ Alana J. Palomar,‡ Eugene P. Hong,‡ Francis J. Schmitz,*,‡ and Frederick A. Valeriote§

Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma 73019, and Josephine Ford Cancer Center, Detroit, Michigan 48202

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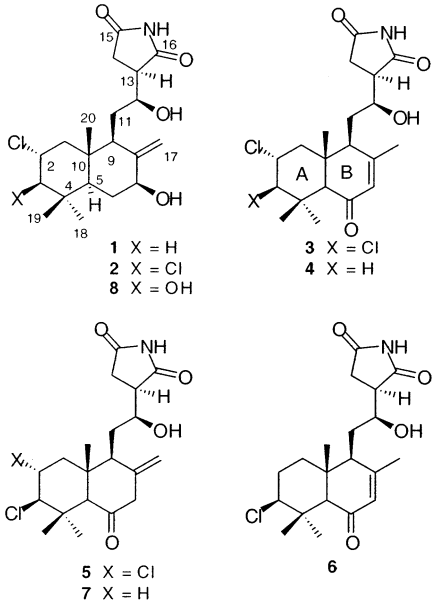
Three new chlorinated diterpenes, **6**−**8**, along with five known ones, **1**−**5**, were isolated from the molluscs *Pleurobranchus albiguttatus* and *P. forskalii* collected in the Philippines. These diterpenes are presumably metabolites of a *Lissoclinum* species of ascidian on which the molluscs have fed. The structures of the new compounds were determined by interpretation of their spectral data. Compounds **1** and **2** were found to be potent cytotoxins in the National Cancer Institute's screening panel of 60 tumor cell lines and showed some selectivity for melanomas. Two other samples exhibited solid tumor selectivity in a soft agar disk diffusion assay.

Since they lack physical protection against predators, the shell-less opisthobranch molluscs are considered to defend themselves through chemical secretions.[1] As a result, they have been regarded as sources of biologically active compounds. Earlier studies from our laboratory on the opisthobranchs have focused on sea hares,[2] nudibranchs,[3] and sacoglossan molluscs.[4] We have now investigated two species of pluerobranchs collected in the Philippines, and this has resulted in the isolation of three new chlorinated diterpenes together with five known ones, all of which we describe herein. The two molluscs are *Pleurobranchus albiguttatus* (Bergh 1905) and *P. forskalii* (Ruppell & Leuchart, 1828) (family Pleurobranchidae, order Notaspidea). Polypropoinates,[5] a cyclic peptide,[6] and triterpenoids[7] have been previously reported from molluscs of the genus *Pleurobranchus*. The discoveries reported here indicate that the molluscs examined in this study had grazed on a *Lissoclinum* sp. of ascidian, which has been the source of related chlorinated diterpenes.[8−12] This appears to be the first report of the isolation of *Lissoclinum* metabolites from a predator species.

Both *P. albiguttatus* and *P. forskalii* were collected near St. Rosa in the Philippines. Specimens of *P. albiguttatus* were processed as outlined in the Experimental Section and yielded compounds **1**−**8**. Extracts of *P. forskalii* were fractionated similarly and yielded compounds **1**−**6**.

Chlorolissoclimide (**1**) and dichlorolissoclimide (**2**) were first isolated in 1991 from a New Caledonian ascidian *Lissoclinum voeltzkowi* Michaelson[8−10] and were identified here by comparison of their ^{1}H and ^{13}C NMR data with literature values.[8,9] Compounds **3**−**5**, haterumaimide D, H, and B, respectively, were discovered more recently from an unidentified species of *Lissoclinum* collected at Hateruma Island off Okinawa by Uemura's group. To date this group has reported the isolation of 11 diterpene imides of this type.[11] Our isolates were identified by comparison of ^{1}H and ^{13}C NMR data with the reported values.[11a,b] Our samples of **5** were contaminated with varying amounts of **3**. This can be attributed to facile isomerization of **5** to the more stable conjugated form, **3**, by acid catalysis.[11a] Haterumaimide B (**5**) was identified from spectral data of a sample comprised of ∼2.3 parts **5** and one part **3**.



The ^{1}H and ^{13}C NMR spectra of compounds **6**−**8** contained features closely resembling those of the spectra of **1**−**5** and this facilitated the structure elucidations. Compounds **6**−**8** all have the same side chain structure (C-11 to C-16) as that of **1**−**5** judging from a comparison of the NMR data for this unit (Tables 1 and 2) in all these compounds. Hence the structural differences between these diterpenes consist of variations of double-bond position and substituents in the decalin portion of the structures. The new compounds have been assigned names based on correspondence to structures already established in the literature.[8−11]

Haterumaimide L (**6**) has the same molecular formula, $C_{20}H_{28}ClNO_4$, as that of **4**, indicating that they are isomers. The difference between these two compounds was traced to the location of the chlorine substituent. The chlorine atom was placed at C-3 because of HMBC correlations between C-3 (δ 73.0) and both H-18 (δ 1.32) and H-19 (δ 1.22). The chemical shifts for H/C-3 in **6** were also close to those in **2** and **3**. The coupling constants for H-3 (dd, J = 4.4, 11.4) indicated an axial hydrogen at C-3. Therefore, structure **6** was assigned to haterumaimide L.

† Dedicated to the late Dr. D. John Faulkner (Scripps) and the late Dr. Paul J. Scheuer (Hawaii) for their pioneering work on bioactive marine natural products.

* To whom correspondence should be addressed. Tel: (405) 325-5581. Fax: (405) 325-6111. E-mail: fjschmitz@ou.edu.

‡ University of Oklahoma.

§ Josephine Ford Cancer Center.

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Table 1. ^{1}H NMR Data for Compounds **4** and **6**−**8**[a]

position	4	6	7	8
1α	1.56 (m)	1.45 (m)	1.38 (m)	1.42 (t, 12.3)
1β	2.27 (m)	1.83 (dt, 13.2, 3.5)	1.91 (dt, 13.2, 3.5)	2.23 (dd, 4.4, 12.3)
2	4.20 (tt, 4.0, 12.0)	1.98 (m)	2.01 (m)	4.11 (dt, 4.4, 11.0)
3	1.50 (m); 1.95 (m)	3.71 (dd, 4.4, 11.4)	3.67 (dd, 7.0, 9.7)	3.16 (d, 10.5)
5	2.15 (s)	2.23 (s)	2.28 (s)	1.30 (m)
6				1.30 (m)
				2.04 (m)
7	5.83 (br s)	5.83 (br s)	3.05 (d, 15.1)	3.93 (br d, 9.5)
			3.18 (br d, 15.1)	
9	2.46 (br m)	2.37 (br m)	2.09 (br d, 9.6)	1.52 (m)
11	1.54 (m)	1.53 (m)	1.68 (m)	1.52 (m)
	1.75 (m)	1.72 (m)	1.82 (ddd, 6.1, 10.5, 14.9)	1.82 (m)
12	4.38 (t, 7.0)	4.35 (m)	4.40 (t, 6.1)	4.23 (m)
13	3.09 (m)	3.03 (m)	2.98 (m)	2.79 (m)
14	2.73 (dd, 9.5, 18.0)	2.71 (dd, 9.6, 17.5)	2.70 (dd, 9.7, 17.5)	2.60 (dd, 8.8, 17.5)
	2.90 (dd, 5.5, 18.0)	2.87 (dd, 5.2, 17.5)	2.87 (dd, 5.3, 17.5)	2.79 (m)
17	2.01 (s)	2.00 (s)	4.91 (br s)	4.89 (br s)
			5.04 (br s)	5.31 (br s)
18	1.21 (s)	1.32 (s)	1.17 (s)	1.08 (s)
19	1.21 (s)	1.22 (s)	1.27 (s)	0.81 (s)
20	0.92 (s)	0.89 (s)	0.71 (s)	0.74 (s)

[a] Spectra were recorded at 500 MHz in CD_2Cl_2 for **4**, **6**, **7**, and CD_2Cl_2−CD_3OD (2:1) for **8**; referenced to residual solvent CD_2Cl_2 (δ 5.32); δ (mult. J in Hz); assignments were aided by COSY experiments.

Table 2. ^{13}C NMR Data for Compounds **4** and **6**−**8**[a]

position	4	6	7	8
1	49.5	39.0	39.1	47.7
2	54.5	29.1	28.8	63.2
3	53.3	73.0	72.2	82.5
4	35.5	38.7	38.9	40.8
5	62.4	64.0	66.4	52.5
6	198.4	197.9	205.2	33.1
7	129.2	129.1	56.0	73.2
8	158.9	159.0	143.4	149.6
9	52.6	52.6	53.3	51.1
10	45.5	43.3	41.6	41.1
11	32.1	32.1	30.2	30.0
12	70.2	70.4	69.2	68.1
13	47.6	47.6	47.1	46.8
14	30.1	30.0	29.8	29.6
15	177.5[b]	177.1[b]	176.6[b]	179.1[b]
16	179.9[b]	179.5[b]	179.0[b]	181.4[b]
17	22.9	22.8	111.4	105.3
18	33.2	29.3	30.0	29.0
19	22.1	16.0	16.1	16.4
20	15.4	14.8	15.9	14.9

[a] Spectra were recorded at 125 MHz in CD_2Cl_2 for **4**, **6**, **7**, and CD_2Cl_2−CD_3OD (2:1) for **8**, referenced to CD_2Cl_2 (δ 53.8); assignments were made using HMQC and HMBC data. [b] Signals may be interchanged.

The molecular formula $C_{20}H_{28}ClNO_4$ determined by HRFABMS for haterumaimide M (**7**) was identical to that of haterumaimide L (**6**). Comparison of NMR data for **7** and **6** revealed that they were a pair of isomers differing in the C−C double-bond position. The ^{1}H and ^{13}C NMR spectra of **7** contained signals for an exocyclic methylene group [δ 4.91 (br s), and 5.04 (br s); δ_C 111.4 (t) and 143.4 (s)], an isolated methylene [δ_H 3.05 (d, J = 15.1 Hz) and 3.18 (br d, J = 15.1 Hz); δ_C 56.0 (t)], and an isolated ketone (δ_C 205.2) which resonated downfield from the ketone group of **6**. In the COSY spectrum of **7**, both protons at C-17 showed allylic couplings to H-9 and to one of the H-7 (δ 3.18) protons. The above data in combination with HMBC correlations between C-6 and H-5, Hs-7, and between H-17 and C-7, C-9, suggested structure **7** for haterumaimide M. The 3β-chloro configuration was assigned from NOESY correlations between H-3/H-5 and H-18, H-5/H-9 and H-18, and H-19/H-20. However, the coupling constants for H-3 (J = 7.0, 9.7 Hz) of **7** were quite different from those of **6** (J = 4.4, 11.4 Hz), indicating a difference in ring A conformation between these isomers.

3β-Hydroxychlorolissoclimide (**8**), the most polar compound in the series, was obtained in trace amounts and about 90% pure. An attempt to purify this compound by HPLC was unsuccessful because neither UV nor RI could effectively detect the compound. The molecular formula $C_{20}H_{30}ClNO_5$ for **8** was deduced from FABMS {m/z 422 [M + Na]$^+$ (1.5%)}, EIMS {m/z 381/383 (3:1) [M − H_2O]$^+$}, and NMR data. The latter was unambiguously assigned by COSY, HMQC, and HMBC experiments. Compounds **8** and **2** not only had the same proton spin systems, as was evident from their COSY spectra, but also had identical connectivities, as judged from HMBC data. The ^{1}H and ^{13}C NMR data of **8** matched closely those of **2** except for the chemical shifts of C-3 and H-3. After two out of the three hydroxyl groups had been assigned to C-7 and C-12 from COSY and HMQC data, the third hydroxyl group was assigned to C-3 to account for the downfield chemical shift of C-3 (δ 82.5), although H-3 resonated further upfield (δ 3.16) than expected. The coupling constant for H-3 (J = 10.5 Hz) indicated that H-3 and H-2 were both axial, and hence the 3-OH and 2-Cl were *trans* to each other. Thus, **8** was determined to be 3β-hydroxychlorolissoclimide.

It has been reported[12] that chlorolissoclimide (**1**) and dichlorolissoclimide (**2**) are potent cytotoxins against four cell-lines: NSCLC, KB, P388, and doxorubicin-resistant P388. Consistent with this, we found compounds **1** and **2** were potent cytotoxins in the NCI screening panel of 60 human tumor cell lines[13] with some selectivity for melanomas. Haterumaimide D (**3**) was significantly less cytotoxic than **1** or **2** and showed less selectivity for melanomas. The mean graph midpoint data for compound **1**−**3** versus the NCI panel of cell lines was as follows: **1**, log GI_{50} −8.24 (0.06, 1.48); log LC_{50} −6.89 (1.42, 4.00); log LC_{50} −4.78 (2.53, 3.01); **2**, log GI_{50} −8.26 (0.04, 1.51); log TGI −6.91 (1.39, 4.00); log LC_{50} −4.70 (3.29, 3.69); **3**, log GI_{50} −6.01 (2.06, 3.48); log TGI −4.91 (2.55, 3.16); log LC_{50} −4.35 (1.67, 1.72). The remaining compounds were not evaluated in the NCI human tumor panel.

All pure samples and the 2.3:1 mixture of **5**/**3** were evaluated for differential cytotoxicity in the Corbett−Valeriote soft agar disk diffusion assay.[14] The assay detects differences in zones of inhibition between a solid tumor cell

(Colon38, ColonH116, LangH125) and either leukemia cells (L1210 or CEM) or normal cells (CFU-GM). A sample is assessed as solid tumor selective if (zone units of solid tumor − normal or leukemia cells) is greater than 250 units. Compounds **1** and **2** were the most active in this assay as expected (zones of ~550−650 for all cell lines at 1.5 and 1.8 μg/mL, respectively), but they showed no solid tumor selectivity. All the remaining compounds and the mixture of **5**/**3** showed cytotoxicity against all cell lines used in the soft agar assay, but at 10−100-fold higher doses. Compound **8** and the **5**/**3** mixture exhibited solid tumor selectivity. The zone sizes for these latter two samples were as follows: [μg/disk: L1210/C38/CFU] **5**/**3** mixture (2.3/1) 113: 250/650/350; **8** 63: 500/400/100. Pure **3** showed no selectivity. Further follow-up testing was not possible due to lack of material.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Rudolph Autopol III automatic polarimeter, and IR spectra were taken on a Bio-Rad 3240-SPC FT instrument. NMR experiments were conducted with a Varian VXR-500 instrument equipped with a 3 mm ^{1}H/^{13}C switchable gradient microprobe (MDG-500-3) and a pulsed field gradient driver; signals are reported in parts per million (d), referenced to the solvent used. FABMS were measured on a VG ZAB-E mass spectrometer. Merck Si gel 60 (230−240 mesh) was used for vacuum flash chromatography. HPLC was conducted using a RI detector and a Spherex 5 C_{18} column. All solvents were redistilled.

Animal Material. The molluscs were collected in February 1994 at St. Rosa, Philippines, and frozen shortly after collection. They were identified by Dr. Terry Gosliner as *Pleurobranchus forskalii* (Ruppell & Leuchart 1828) and *P. albiguttatus* (Bergh 1905). Voucher specimens of the tissues (2PH94 and 3PH94, respectively) are available at the University of Oklahoma.

Extraction and Isolation. Frozen specimens of *P. albiguttatus* (3PH94: 2.1 kg wet wt; 103 g dry wt after extraction) were freeze-dried and extracted with MeOH (1.5 L × 2) followed by MeOH−CH_2Cl_2 (1:1) (1.5 L × 2). All extracts were combined and the solvents evaporated under vacuum. The residue was dissolved in 10% aqueous MeOH (700 mL), and the solution extracted with hexane (2 × 700 mL) to give, after evaporation of solvent, 5.9 g of hexane-soluble material. The aqueous MeOH phase was diluted with H_2O (200 mL) to 30% H_2O in MeOH and extracted with CH_2Cl_2 (2 × 700 mL) to yield 6.0 g of CH_2Cl_2-soluble residue. The CH_2Cl_2 extract was fractionated by vacuum liquid chromatography over Si gel using increasing amounts of MeOH in CH_2Cl_2 as eluent (5%−50% MeOH−CH_2Cl_2). Thirteen fractions were collected. The known and major compounds chlorolissoclimide (**1**) and dichlorolissoclimide (**2**) were obtained from the fourth and fifth fractions by reversed-phase C_{18} HPLC using 40% H_2O−MeOH as eluent. Reversed-phase C_{18} HPLC of the third fraction using the same solvent system afforded, in order of elution, **1**, **4**, **6**, **3**, **7**, and **5**. Fractions 6−8 were pooled and rechromatographed on a reversed-phase C_{18} open column using 50% H_2O−MeOH and then 40% H_2O−MeOH as eluents to give **8** (3β-hydroxylissoclimide).

Specimens of the *P. forskalii* (2PH94: 2.2 kg wet wt; 63 g dry wt after extraction) were extracted and the extracts partitioned in a manner similar to that described above for *P. albiguttatus* (3PH94) to give hexane-soluble (3.3 g) and CH_2Cl_2-soluble (3.1 g) fractions. Using the same isolation protocol as described above, the CH_2Cl_2-solubles were fractionated to give 13 fractions. The third fraction was subjected to C_{18} reversed-phase C_{18} HPLC (40% H_2O−MeOH) to yield **1**−**3**. Compounds **1** and **2** were also obtained from the fourth and fifth fractions using the same column and eluent. Reversed-phase HPLC of the second fraction using 40% H_2O−MeOH as eluent furnished compounds **1** and **3**−**6** (order of elution: **1**, **4**, **6**, **3**, **5**). Percent yields were not calculated because not all of the fractions were exhaustively processed.

Chlorolissoclimide (1): glass (45 mg); $[\alpha]_D$ +119.3° (*c* 1.59, MeOH); NMR data identical to literature.[9]

Dichlorolissoclimide (2): glass (48 mg); $[\alpha]_D$ + 23.7° (*c* 0.92, MeOH), lit.[10] +30° (*c* 0.2, MeOH); NMR data identical to literature.[9]

Haterumaimide D (3): amorphous solid (38 mg); $[\alpha]_D$ −29.9° (*c* 0.167, MeOH), lit.[11] −27.7° (*c* 0.165, MeOH); IR (neat) ν_{max} 3455, 3256, 3065, 1772, 1716, 1659, 1362, 1186 cm^{-1}; ^{1}H and ^{13}C NMR, identical to literature;[11a] HRFABMS *m*/*z* 416.1380 $[M + H]^+$ (calcd for $C_{20}H_{28}{}^{35}Cl_2NO_4$, 416.1395).

Haterumaimide H (4): amorphous solid (21.8 mg); $[\alpha]_D$ +6.5° (*c* 0.35, MeOH); lit.[12] +47.6° (*c* 0.31, MeOH); ^{1}H and ^{13}C NMR, identical to literature;[11b] HRFABMS *m*/*z* 382.1763 (calcd for $C_{20}H_{29}{}^{35}ClNO_4$, 382.1785).

Haterumaimide L (6): amorphous solid (6.0 mg); $[\alpha]_D$ −7.2° (*c* 0.43, CH_2Cl_2); IR (neat) ν_{max} 3429, 3250, 3070, 1777, 1715, 1665, 1360, 1187 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 1 and 2, respectively; selected HMBC correlations H-20/C-1, -5, -9, -10; H-19/C-3, -4, -5; H-18/C-3, -4, -5; H-17/C-7, -8, -9; H-14/C-15, -16; H-13/C-16; H-7/C-5(w), -9, -17; H-5/C-4, -6, -9, -10, -19, -20; HRFABMS *m*/*z* 382.1764 (calcd for $C_{20}H_{29}{}^{35}ClNO_4$, 382.1785).

Haterumaimide M (7): amorphous solid (5.1 mg); $[\alpha]_D$ +14.4° (*c* 0.36, CH_2Cl_2); IR (neat) ν_{max} 3447, 3255, 3082, 1772, 1714, 1653, 1362, 1187 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 1 and 2, respectively; selected HMBC correlations H-20/C-1, -5, -9, -10; H-19/C-3, -4, -5, -18; H-18/C-3, -4, -5, -19; H-17/C-7, -9; H-14/C-15, -16; H-7/C-6, -8, -9, -17; H-7′/C-6, -8; H-5/C-1, -4, -6, -9, -18, -20; HRFABMS *m*/*z* 382.1792 (calcd for $C_{20}H_{29}{}^{35}ClNO_4$, 382.1785).

3β-hydroxylissoclimide (8): amorphous solid (8.0 mg, approximately 90% pure), CH_2Cl_2−MeOH (7:1)]; IR (neat) ν_{max} 3391, 1771, 1710, 1653, 1362, 1189 cm^{-1}; ^{1}H and ^{13}C NMR, see Tables 1 and 2, respectively; selected HMBC correlations H-20/C-1, -5, -9, -10; H-19/C-3, -4, -5, -18; H-18/C-3, -4, -5, -19; H-17(δ 4.89)/C-7, -9; H-17(δ 5.31)/C-7, -9, -8(wk); H-14/C-15, -16; H-9(δ 1.52)/C-7, -8, -10, -12, -17; H-5/C-4, -6, -20; H-1β/C-2, -3, -5, -10; FABMS *m*/*z* 422 $[M + Na]^+$; EIMS (12 eV) *m*/*z* 381/383 (3:1) $[M - H_2O]^+$.

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