Dietary Derived Sesquiterpenes from *Phyllodesmium lizardensis*

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The recently discovered new aeolidean species *Phyllodesmium lizardensis* Burghardt, Schrödl and Wägele, 2008 was investigated concerning its secondary metabolite profile. *P. lizardensis* so far has only been found on Lizard Island. Analysis of *P. lizardensis* led to the isolation of the new sesquiterpenes (+)-3β-hydroxy-α-muurolene (**1**) and (+)-3β-acetoxy-α-muurolene (**2**). GC-MS analysis of the host coral, identified as *Heteroxenia* sp., also showed the presence of compounds **1** and **2**, whereas a sympatric *Xenia* species lacks these products. These results indicate that *P. lizardensis* specifically sequesters these compounds from *Heteroxenia* sp.

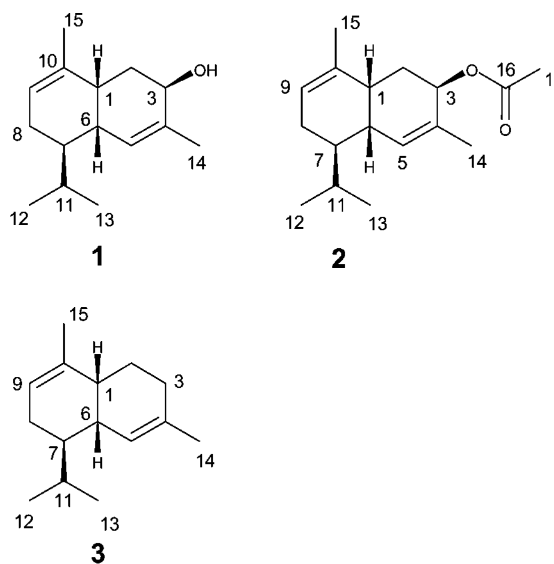
Secondary metabolites as defensive mechanisms are a common feature of shell-less opisthobranchs.[1] Contrary to other groups, like Sacoglossa, or Doridoidea, however almost nothing is known about chemically based defensive systems in Aeolidoidea,[2] since members of this taxon usually discharge stored nematocysts sequestered from their typical hydrozoan prey.

The genus *Phyllodesmium* is a group of cryptic aeolid nudibranchs. Their members are known to feed on certain octocorals,[3] this way taking up symbiotic zooxanthellae from the corals and storing them in their digestive gland cells, where they are still photosynthetically active.[4−6] In doing so, the slugs are able to survive a certain time without feeding by living only on the photosynthesis products of the zooxanthellae. However, in contrast to other aeolidoideans, the soft coral feeding *Phyllodesmium* species do not take up cnidocysts from their prey, therefore lacking this typical defensive system.[3,4] Apart from the genus *Phyllodesmium,* the only aeolids that do not incorporate cnidocysts from their prey are *Phestilla lugubris, Phestilla minor, Cuthona poritophages,* and *Pinufius rebusand*,[7−9] which feed on hexacorals. One alternative for those species lacking cnidocysts for their defense is the accumulation of secondary metabolites from their prey.

Although at least 20 described and about five undescribed species of the genus *Phyllodesmium* are known, so far only two of them have been investigated for the presence of secondary metabolites. Coll et al. investigated *P. longicirrum*, which accumulates the terpenes (+)-thunbergol and (+)-epoxythunbergol from the soft coral *Sarcophyton trocheliophorum*.[10] Slattery et al. showed that *P. guamensis* specifically sequesters the diterpene 11-β-acetoxypukalide from its preferred prey, the soft coral *Sinularia maxima*.[11] The latter diterpene has been positively tested for feeding deterrence against reef fishes that are potential predators for *P. guamensis*.

In the current study, the isolation and structure elucidation of two new sesquiterpenes (**1**, **2**), besides the known metabolite (+)-α-muurolene (**3**) from *P. lizardensis* Burghardt, Schrödl and Wägele, 2008 are described. This species has been recorded only from Lizard Island, Great Barrier Reef (Australia).[12] GC-MS analyses of extracts obtained from the soft coral *Heteroxenia* sp. on which *P. lizardensis* was found regularly indicated that this coral produces these compounds, whereas a sympatric occurring *Xenia* species is lacking these metabolites.

The molecular formula of compound **1** was deduced by accurate mass measurement (HREIMS) to be $C_{15}H_{24}O$, implying four degrees of unsaturation. The ^{13}C NMR spectrum contained 15 resonances resulting from four methyl, two methylene, five sp^3 methine, two sp^2 methine, and two quaternary carbons. Due to the molecular formula and 23 protons evident from ^1H NMR and DEPT135 spectra, one proton had to be present as a hydroxyl group. With two elements of unsaturation attributed to carbon−carbon double bonds a bicyclic structure was in agreement with the four degrees of unsaturation. After assignment of all protons to their directly bonded carbon atoms via a $^1H-^{13}C$ HSQC experiment, it was possible to deduce two major fragments of the molecule from the results of a $^1H-^1H$ COSY experiment. Analysis of the COSY spectrum of **1** gave evidence for connectivities from CH-3 to CH-5 through CH_2-2, CH-1, and CH-6 (Figure 1, fragment A). Methyl groups CH_3-12 and CH_3-13 both coupled to H-11, which further coupled to H-7. H_2-8 showed couplings to both H-7 and H-9, thus completing the second spin system B (Figure 1). A $^1H-^{13}C$ HMBC experiment permitted the planar structure of **1** to be further elaborated. HMBC correlations from H_3-15 to C-1, C-9, and C-10 indicated CH_3-15 to be positioned at C-10 and connected COSY fragments A and B via C-10. CH_3-14 was bonded to carbon 4, as evident by cross-peaks from H_3-14 to C-3, C-4, and C-5. The HMBC cross-peak from H_3-14 to C-5 also delineated a first ring closure between C-4 and C-5 to give a cyclohexene ring. A second ring closure was provided by an HMBC correlation between H-6 and C-7, resulting in an α-cadinene skeleton. The missing hydroxyl group had to be connected to C-3 because of its low-field ^{13}C NMR chemical shift (δ_C 68.5).



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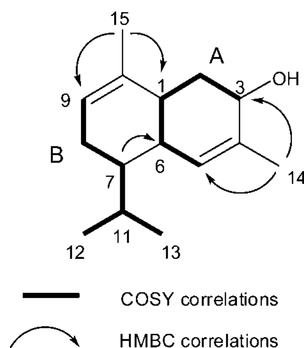

Figure 1. $^{1}H-^{1}H$ COSY and key $^{1}H-^{13}C$ HMBC correlations of compound **1**.

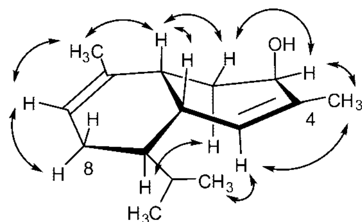

Figure 2. $^{1}H-^{1}H$ NOESY correlations of compound **1**.

A diagnostic NOE correlation between the resonances of H-1 and H-6 provided a *cis*-fused decalin ring system. Additional NOE correlations between H-2α and H-7, between H-1 and H-2β, between H-5 and H_3-13, and between H-3 and both H-2α and H-2β as well as $^{1}H-^{1}H$ coupling constants of H-3 (t, $J = 3.4$ Hz; showing an equatorial position of H-3) were indicative for the relative configuration of **1** as 1*R**, 3*R**, 6*S**, 7*R** (Figure 2). After derivatization with α-methoxyphenylacetic acid (MPA) the difference in the δ_H values of the (*R*)-MPA and (*S*)-MPA adducts indicated C-3 possessed an *R* absolute configuration (see Figure S16).[13] Thus, the absolute configuration of compound **1** was established as 1*R*, 3*R*, 6*S*, 7*R*. A comparison of the optical rotation value of **1** (+131) with the literature value of (−)-3β-hydroxy-α-muurolene (−147) confirmed this result.[14] Thus, compound **1** is the enantiomer of (−)-3β-hydroxy-α-muurolene, a constituent of the essential oil of *Teucrium yemense* Deflers.[15] We propose the trivial name (+)-3β-hydroxy-α-muurolene for compound **1**.

From accurate mass measurement, compound **2** was found to have a molecular formula of $C_{17}H_{26}O_2$. The spectroscopic data of **2** were very similar to those of **1**, suggesting a related planar structure (Tables 1 and 2). The major differences between the two data sets were two additional resonances in the ^{13}C NMR spectrum of **2** (δ_C 21.2 and 172.8) indicative of an acetyl group. An $^{1}H-^{13}C$ HMBC correlation from H-3 to C-16 allowed connection of this acetyl group to C-3. The absolute configuration of compound **2** was deduced to be the same as for compound **1** because of nearly identical optical rotation values and comparable NOESY correlations. In the literature compound **2** so far has only been published without configuration as a transformation product of khusinol.[16] For compound **2** the trivial name (+)-3β-acetoxy-α-muurolene is proposed.

The structure of (+)-α-muurolene (**3**) was identified by comparing its NMR spectroscopic data and optical rotation with published values.[17]

Compounds **1** and **2** were evaluated in antibacterial (*Escherichia coli*, *Bacillus megaterium*), antifungal (*Mycotypha microspora*, *Eurotium rubrum*, and *Microbotryum violaceum*), and antialgal (*Chlorella fusca*) assays at the 50 μg/disk level, but did not show any activities.

GC-MS investigation of the host coral of *P. lizardensis*, an unidentified species of the genus *Heteroxenia*, gave evidence that

Table 1. 1D and 2D NMR Spectroscopic Data for (+)-3β-Hydroxy-α-muurolene (**1**)

atom no.	δ_C[a,b,e]	δ_H[a,b] (mult., J in Hz)	COSY[a,c]	HMBC[a,d]	NOESY[a,c]
1	35.2, CH	2.34, m	2α, 2β, 6		2β, 6, 15
2	35.1, CH_2	α: 1.66, m	1, 3		2β, 3, 7
		β: 1.93, m	1, 3	1, 3, 6	2α, 3
3	68.5, CH	3.93, t (3.4)	2α, 2β, 5	2, 4, 5, 14	2α, 2β, 14
4	136.4, C				
5	129.1, CH	5.68, dd (1.1, 4.6)	6, 14	1, 3, 4, 6, 14	6, 7, 11, 13, 14
6	38.2, CH	2.10, m	1, 5, 7	1, 4, 5, 7	1, 5, 13
7	41.0, CH	1.47, m	6, 8, 11	13	2α, 5, 8, 11, 12
8	25.5, CH_2	1.88, m	7, 9	9	9
9	122.6, CH	5.45, brs	8, 15		8, 15
10	136.9, C				
11	28.1, CH	1.98, m	7, 12, 13	8, 12, 13	5, 12, 13
12	21.7, CH_3	0.94, d (7.0)	11	7, 11	11
13	16.3, CH_3	0.90, d (6.6)	11	7, 11	6, 11
14	21.4, CH_3	1.84, brs	5	3, 4, 5	3, 5
15	21.6, CH_3	1.73, brs	9	1, 9, 10	1, 9

[a] MeOH-d_4, 300/75.5 MHz. [b] Assignments are based on extensive 1D and 2D NMR experiments (HMBC, HSQC, COSY). [c] Numbers refer to proton resonances. [d] Numbers refer to carbon resonances. [e] Implied multiplicities determined by DEPT.

Table 2. 1D and 2D NMR Spectroscopic Data for (+)-3β-Acetoxy-α-muurolene (**2**)

atom no.	δ_C[a,b,e]	δ_H[a,b] (mult., J in Hz)	COSY[a,c]	HMBC[a,d]	NOESY[a,c]
1	35.8, CH	2.28, m	2α, 2β, 6		2β, 6, 15
2	31.9, CH_2	α: 1.76, m	1, 3		2β, 3, 7
		β: 1.94, m	1, 3	3	2α, 3, 15
3	71.7, CH	5.21, t (3.3)	2α, 2β, 5	1, 4, 14, 16	2α, 2β, 14, 17
4	132.9, C				
5	132.0, CH	5.83, brd (4.1)	6, 14	1, 3, 14	6, 7, 11, 13, 14
6	38.1, CH	2.21, m	1, 5, 7	5	1, 5, 13
7	41.1, CH	1.50, tt (9.8, 4.9)	6, 8	6	2α, 5, 8, 11
8	25.4, CH_2	1.90, m	7, 9		9
9	123.1, CH	5.47, brs	8, 15		8, 15
10	136.1, C				
11	28.2, CH	1.96, m	12, 13	12, 13	5, 7,12, 13
12	21.6, CH_3	0.96, d (7.0)	11	7, 11	7, 11
13	16.5, CH_3	0.92, d (6.6)	11	7, 11, 12	5, 6, 11
14	20.9, CH_3	1.73, brs	5	3, 4, 5	3, 5
15	21.5, CH_3	1.70, brs	9	9, 10	1, 2β, 9
16	172.8, C				
17	21.2, CH_3	2.10, s		16	

[a] MeOH-d_4, 300/75.5 MHz. [b] Assignments are based on extensive 1D and 2D NMR experiments (HMBC, HSQC, COSY). [c] Numbers refer to proton resonances. [d] Numbers refer to carbon resonances. [e] Implied multiplicities determined by DEPT.

this xeniid synthesizes compounds **1** and **2** (Figures S12−S15). Analysis of another sympatric occurring *Xenia* species did not reveal the presence of compounds **1** and **2**. This could be one reason to explain the preference of the slugs for *Heteroxenia* sp. over *Xenia* sp. In natural habitats, the slugs were found associated exclusively with the former coral, although the number of *Xenia* colonies was higher in the observed area around Lizard Island. Additionally, in experiments the slugs also preferred to sit on *Heteroxenia*. A species specificity association induced by chemotaxis was observed in *P. longicirrum* with *Sinularia maxima*.[10]

Separate analyses of cerata and body resulted in higher quantities of compounds in the former (data not shown). During preparation of the living slugs, a sticky fluid was exuded in the upper half of the cerata, which also autotomized very easily. A higher concentration of compounds in the cerata was also described for *P. longicirrum*.[10]

Heteroxenia spp. are known to produce sesquiterpenes of the muurolene type.[17,18] Proksch and co-workers described (+)-6-hydroxy-α-muurolene to be active against the phytopathogenic fungus *Cladosporium cucumerinum* and active in the brine shrimp

lethality test.[18] Thus, the uptake of secondary metabolites such as the muurolenes and other terpenes could be an alternative defense strategy of *P. lizardensis* and congeners instead of the uptake of functional cnidocysts.

Experimental Section

General Experimental Procedures. All NMR spectra were recorded in MeOH-d_4 employing a Bruker Avance 300 DPX spectrometer. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ 3.35/49.0 (MeOH-d_4). IR spectra were obtained employing a Perkin-Elmer Spectrum BX instrument. GC-MS analyses were performed with a Perkin-Elmer AutoSystem XL and a TurboMass spectrometer. HREIMS were recorded on a Finnigan MAT 95 spectrometer. ESIMS measurements were recorded employing an API 2000, Applied Biosystems/MDS Sciex. HPLC was carried out using a Merck-Hitachi system equipped with an L-6200A pump, an L-4500A photodiode array detector, a D-6000A interface with D-7000 HSM software, and a Rheodyne 7725i injection system. Optical rotation was measured on a Jasco DIP 140 polarimeter.

Animal Material. *Phyllodesmium lizardensis* was collected on Lizard Island, the Great Barrier Reef, Australia, during low tide in July 2006 and stored in methanol until workup. Live specimens of the soft corals *Heteroxenia* sp. and *Xenia* sp. were collected at the same place and preserved in methanol. Voucher specimens of *P. lizardensis* have been deposited at the Zoologische Staatssammlung, Munich, voucher number ZSM Mol 20060654. Specimens of *Heteroxenia* and *Xenia* are in the collection of HW.

Extraction and Isolation. After removal of the preservative MeOH, two nudibranchs (wet wt 3.2 g) were extracted once with MeOH. The MeOH extracts were combined and evaporated to dryness to yield 220 mg of a yellow extract. This extract was fractionated by vacuum liquid chromatography (VLC) over Polygoprep 60-50 C_{18} material (Macherey-Nagel) using gradient elution from H_2O (100%) to MeOH (100%), to yield 12 fractions. ^{1}H NMR investigations indicated VLC fractions 10, 11, and 12 to contain pure compounds **1** (20.0 mg), **2** (4.6 mg), and **3** (73.4 mg), respectively.

GC-MS Analysis. A 1.0 mg amount of compounds **1**, **2**, and **3** and 2.0 mg of MeOH extracts of the soft corals were each dissolved in 1 mL of acetone. A 1 μL sample of these solutions was analyzed using a Perkin-Elmer PE-1 column (30 m × 0.32 mm; 0.25 μm; program rate: column temperature held at 50 °C for 5 min; 50−180 °C at 5 °C/min; flow: 2.0 mL/min; inj.: 220 °C).

Preparation of the Acid Chlorides of (*R*)- and (*S*)-MPA. Oxalyl chloride (52 μL, 0.6 mmol) was added to a solution of the corresponding MPA (10 mg, 0.06 mmol) and DMF (0.47 μL, 0.006 mmol) in 1 mL of hexanes at room temperature. After 3 h, the solution was dried under nitrogen.

Preparation of the (*R*)- and (*S*)-MPA Esters. The corresponding MPA-Cl (10 mg, 54 μmol) was dissolved in 2 mL of CH_2Cl_2 and added to a solution of compound **1** (2.5 mg, 11 μmol), Et_3N (18 μL, 130 μmol), and DMAP as catalyst. After 18 h reaction time the obtained products were dried under vacuum and further purified by HPLC using the Merck-Hitachi system. The separation was performed with a RP18 column (Macherey-Nagel Nucleodur ISIS RP, 5 μm, 250 × 4.6 mm) and a mobile phase (1.0 mL/min) consisting of MeCN/H_2O, 80/20.

(+)-3β-Hydroxy-α-muurolene (1): colorless solid; $[\alpha]^{24}_D$ +131 (*c* 1.67 $CHCl_3$); IR (ATR) ν_{max} 3408, 2956, 2927, 1714, 1671, 1367, 1222 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS *m/z* 221 $[M + H]^+$; HREIMS *m/z* 220.1825 (calcd for $C_{15}H_{24}O$, 220.1827).

(+)-3β-Acetoxy-α-muurolene (2): colorless solid; $[\alpha]^{24}_D$ +122 (*c* 0.38 $CHCl_3$); IR (ATR) ν_{max} 2925, 2858, 1731, 1713, 1437, 1365, 1238 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; ESIMS *m/z* 263 $[M + H]^+$; HREIMS *m/z* 262.1933 (calcd for $C_{17}H_{26}O_2$, 262.1933).

Biological Assays. Compounds **1** and **2** were tested in agar diffusion assays[19] against the bacteria *Bacillus megaterium* and *Escherichia coli*, the fungi *Microbotryum violaceum*, *Eurotium rubrum*, and *Mycotypha microspora*, and the green microalga *Chlorella fusca*. The pure compounds showed no activity in these assays.

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Supporting Information Available: ^{1}H and ^{13}C NMR, ^{1}H−^{1}H COSY, ^{1}H−^{13}C HMBC, and ^{1}H−^{1}H NOESY spectra of compounds **1** and **2** as well as GC-MS chromatograms. Figures with results of modified Mosher's method. This material is available free of charge via the Internet at http://pubs.acs.org.

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