Spongian Diterpenes from Australian Nudibranchs: An Anatomically Guided Chemical Study of *Glossodoris atromarginata*

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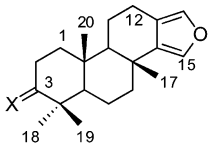
An Australian population of the nudibranch mollusk *Glossodoris atromarginata* has been found to contain furanoditerpenes of the spongian series. Spongia-13(16),14-dien-3-one (**1**) and 3β-acetoxy-19-hydroxyspongia-13(16),14-dien-2-one (**2**) were isolated for the first time from a natural source, along with a series of known diterpenes (**3**−**7**). Anatomical dissection of the animals revealed the relative distribution and chemical variation of secondary metabolites. Structural studies have provided a basis for chemical comparisons between populations from different geographic locations.

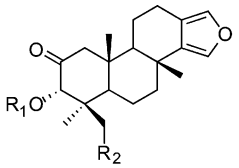
Opistobranch mollusks (Mollusca: Gastropoda: Opistobranchia) have been the subject of much research in the effort to discover new marine natural products.[1−3] The rich chemical diversity of the spongivorous species belonging to the order Nudibranchia has been attributed to their alimentary habits.[4] This sponge diet provides nudibranchs with secondary metabolites, which are then either sequestered or further metabolized and are believed to play a role in the chemical protection of an otherwise defenseless marine mollusk. Defensive compounds are often stored in mantle glands distributed around the mantle edge. Belonging to the family Chromodorididae, two populations of the species *Glossodoris* (=*Casella*) *atromarginata* have been studied so far.[2,5] These previous studies were on animals from Sri Lanka and India. Those from Sri Lanka were found to contain furanoditerpenes of the spongian series, while the Indian samples contained scalarane sesterterpenes. Spongian diterpenoids have also been isolated from a nudibranch first identified as *Glossodoris atromarginata* and subsequently revised as *Glossodoris cincta*, collected along the Eyptian coast of the Red Sea.[5,6] The chemical variation of the spongian compounds is typically due to a varied pattern of substitution at the methyl groups and at C-3.

In this paper we report findings from the first chemical study on an Australian population of *G. atromarginata* (Cuvier, 1804) (Chromodorididae), which confirms the association of this species with spongian diterpenes. The acetone extracts of the nudibranch afforded an array of furanoditerpene compounds (**1**−**7**) of the spongian series. These compounds were isolated from a study of extracts of dissected or whole animals. Structural assignment of all compounds was based on spectroscopic methods.

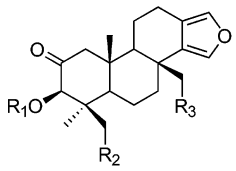
The nudibranchs were collected from Gneerings Reef, Moolooloaba, South-East Queensland, at a depth 10−15 m, together with a sample of sponge prey in January 2005. Five of the nudibranchs were dissected to allow the chemical comparison of various anatomical compartments, specifically the internal organs, mantle tissue, and the mantle glands. Each compartment was extracted exhaustively with acetone in accordance with previous studies of *G. atromarginata*.[5] Purification by reversed-phase HPLC of the ether-soluble fractions of the acetone extracts gave compounds **1**−**4** from the internal organs, **1**, **5**, and **6** from the mantle, and **7** from the mantle glands. Compounds **1** and **2** are new natural products, while it is the first time that **4** has been isolated from *G. atromarginata*.



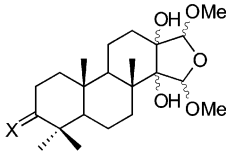
1 X = O
8 X = H_2



2 R_1= Ac; R_2= OH
3 R_1= OH; R_2= OAc
4 R_1= OH; R_2= OH
7 R_1= Ac; R_2= OAc



5 R_1= H; R_2= OH; R_3= H
6 R_1= H; R_2=R_3= OH



9 X = O
10 X = H_2

Spongia-13(16),14-dien-3-one (**1**), isolated from both the internal organs and the mantle tissue, is closely related to spongia-13(16),-14-diene (**8**), first isolated from *Spongia officinalis*.[7] The molecular formula of **1** was deduced to be $C_{20}H_{28}O_2$ by the HREIMS parent ion at *m*/*z* 300.2096. The presence of four methyl singlets (δ_H 0.99, H_3-20; 1.06, H_3-19; 1.09, H_3-18; 1.23, H_3-17) together with two broad signals typical of a β,β'-disubstituted furan ring (δ_H 7.05 and 7.09) hinted at a structure similar to the spongian-based furanoditerpenes. The ^{13}C NMR data presented 14 protonated and six nonprotonated carbons. Of the 20 carbon signals, those at δ_C 119.4, 135.0, 136.9, and 137.0 closely matched previously reported values assigned for the furan moiety of the spongianditerpenes.[7,8] In contrast to the reported literature for *Glossodoris* compounds,[2,6] there was a distinct lack of signals characteristic of oxygenated carbons, except for a signal at δ_C 217.3, indicating carbonyl functionality. The HMBC spectrum revealed two methyl groups at δ_H 1.06 and 1.09, both correlating to a quarternary carbon at δ_C 47.3, consistent with a geminal dimethyl substituent. Correlations from the *gem*-dimethyl protons to the carbonyl at δ_C 217.3 showed that the carbonyl was positioned at C-3. COSY and HMBC data were used to infer the position of the two tertiary methine protons, placing the δ_H 1.52 signal at C-5 (δ_C 54.8) and the δ_H 1.26 signal at C-9 (δ_C 55.3). HSQC data allowed the two furan protons to be assigned to their corresponding carbons. The assignment of these protons as H-15 or H-16 was based on HMBC correlations. The furan signal at δ_H 7.05 (H-15) and the C-17 methyl at δ_H 1.23 both

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Table 1. NMR Data of Compound **1**[a]

C	δ_C[b]	δ_H, m, *J* (Hz)	DQF-COSY correlations	HMBC correlations[c]
1a	39.0	2.02, m	H-1b, H-2a,b	H-2a
1b		1.49, m	H-1a, H-2b, H_3-20	
2a	33.9	2.53, m	H-1a	H-1a
2b		2.48, m	H-1a, b	
3	217.3			H-1a, H_3-18, H_3-19, H-2a
4	47.3			H_3-18, H_3-19
5	54.8	1.52, m	*d*	H-1a, H_3-18, H_3-19, H_3-20
6	19.7	1.62, m	*d*	
7a	40.0	2.12, dd, (2, 9.5)	H-7b	H_3-17
7b		1.60, m	H-7a	
8	33.9			H-11a, H_3-17
9	55.3	1.26, dd, (1.5, 10)	H-11a, b, H_3-20	H-11b, H-12a, H_3-17
10	36.8			H-1a, H-9, H_3-20
11a	18.7	1.74, m	H-9, H-12b	
11b		1.66, m	H-9, H-12a,b	
12a	20.5	2.78, dd, (6, 16)	H-11b, H-12b	H-11b
12b		2.45, ddd, (5, 8, 13)	H-11a,b, H-12a	
13	119.4			H-11a, H-12a,b, H-16
14	136.9			H-15, H_3-17
15	137.0	7.05, q, (1.5)	*e*	
16	135.0	7.09, d, (1.5)	*e*	H-12a
17	25.5	1.23, s		
18	26.8	1.09, s		H_3-19
19	20.8	1.06, s		H_3-18
20	16.0	0.99, s	H-1b, H-9	

[a] Chemical shifts (ppm) refer to $CHCl_3$ (δ 7.25) for proton and $CDCl_3$ (δ 77.0) for carbon. [b] Assignments by HMBC experiments. [c] Optimized for $^1J_{C-H}$ of 135 Hz and $^nJ_{C-H}$ of 8 Hz. [d,e] Not resolved.

correlated to C-14 at δ_C 136.9. In contrast, the signal at δ_H 7.09 (H-16) correlated to the carbon signal at δ_C 119.4 (C-13), as did methylene protons on carbons 11 and 12. These data confirmed the structure to be spongia-13(16),14-dien-3-one (**1**). A literature survey revealed that this compound had been previously synthesized during a program to make spongian diterpenes for use in biological assays. The ^{1}H and ^{13}C NMR data reported for the synthetic compound matched very closely to that of the newly isolated compound.[9]

Compound **2**, isolated from the digestive tissue of the nudibranch, had a molecular formula of $C_{22}H_{30}O_5$ inferred from HRESIMS. The ^{1}H and ^{13}C NMR presented three methyls (δ_H 1.24, H_3-20; δ 0.94, H_3-18; 1.23, H_3-17), a β,β'-disubstituted furan ring (δ_H 7.09 and 7.05), and an acetoxymethyl group (δ_H 2.16; δ_C 20.6 and 170.6). An isolated AB system (δ_H 2.59, H-1a; 2.17, H-1b) showing HMBC correlations to δ_C 205.0, 39.4, and 19.3 confirmed the ring A substitution pattern, in particular the carbonyl functionality at C-2. A downfield singlet (δ_H 5.62) showed HMBC correlations to resonances at δ_C 205.0, 64.4, 45.5, and 20.7 and was assigned to H-3. The chemical shift of H-3 (δ_H 5.62) indicated that the acetoxy group orientation was α, as it had shifted downfield in comparison to its previously reported β-epimer, which has a chemical shift of δ 5.01 for this proton.[10] A low-field AB system (δ_H 3.65 and 3.39) correlated to the signal at δ_C 64.4 was consistent with hydroxylation at C-19 since this carbon showed HMBC correlations from both H-3 and H_3-18. The final structure was confirmed by 2D data with the C and D ring substitution pattern established by the correlations of the C-17 protons to C-7, C-8, C-9, and C-14 and the protons of C-12 to C-9, C-13, C-14, and C-15.

Dissection of the nudibranchs prior to analysis enabled the anatomical distribution of the secondary metabolites to be determined. Structural determination of compounds **3**−**7** was ascertained by comparison of NMR and MS with previously reported spectral

Table 2. NMR Data of Compound **2**[a]

C	δ_C[b]	δ_H, m, *J* (Hz)	HMBC correlations[c]
1a	54.1	2.59, d, (14.8)	H_3-20
1b		2.17, d, (14.8)	
2	205.0		H_2-1, H-3
3	77.2	5.62, s	H_3-18
4	45.5		H-3, H_3-18
5	53.8	1.51, m	H-1a, H_3-18
6	20.0	1.68, m	
7a	40.2	2.15, m	H_3-17
7b		1.59, m	
8	33.9		H_3-17
9	56.3	1.26, m	H_3-17, H_2-12
10	39.4		H_2-1, H_3-20
11a	19.2	1.72, m	
11b		1.62, m	
12a	20.6	2.79, dd, (6,16)	
12b		2.43, m	
13	119.0		H_2-12, H-16
14	136.0		Ha-12, H_3-17
15	136.8	7.05, q, (1.5)	Ha-12
16	135.2	7.09, d, (1.5)	
17	25.3	1.23, s	
18	20.7	0.94, s	H-3,
19a	64.4	3.65, d, (9)	H-3, H_3-18
19b		3.39, d, (9)	
20	19.3	1.24, s	H-1a, H-9
CH$_3$CO	20.6	2.16, s	
CH$_3$*C*O	170.6		H-3

[a] Chemical shifts (ppm) refer to $CHCl_3$ (δ 7.25) for proton and $CDCl_3$ (δ 77.0) for carbon. [b] Assignments by HMBC experiments. [c] Optimized for $^1J_{C-H}$ of 135 Hz and $^nJ_{C-H}$ of 8 Hz.

data,[2,6,8] with the isolation of spongiadiol (**4**) being the first reported for the genus *Glossodoris*. Compounds of the C-3 α epimeric series were identified in the digestive tissue (**2**−**4**) and the mantle dermal formations (compound **7**), whereas compounds with a C-3 β-configuration (**5** and **6**) were found only in the mantle tissue. In this study, no acetylated metabolites were identified in the mantle tissue, which was found to contain only the new compound **1** together with the hydroxylated compounds **5** and **6**. One explanation for the difference in chemistry is that the compounds found in the digestive tissue may represent the chemistry of the sponge prey, while those found in the mantle tissue either have been selectively requisitioned by the nudibranch or are the outcome of selective enzymatic transformation. Alternatively the axially oriented hydroxyl present in the 3α-series may convert to the more stable equatorial position when stored in the mantle tissue. Epimerization at the C-3 position could occur through keto−enol tautomerism. The selective compartmentalization of compound **7** in the mantle glands suggests an ecological role that necessitates reservoirs of specific metabolites for these anatomical structures. Some analogies were observed with the Egyptian *Glossodoris* since both mollusks contain compound **6** in the mantle. Indeed compound **6** and its acetyl derivative at C-3 were the only metabolites found in the mucous secretion of the Egyptian mollusk.

Previous reports pertaining to the chemistry of *G. atromarginata* have examined populations from Sri Lanka and India.[2,5] These studies showed the nudibranch population from India to contain sesterterpene scalaranes, while those from Sri Lanka contained diterpenes of the spongian series. Although these authors lacked information about the preferred food of the Sri Lankan nudibranchs, a dietary origin was inferred given the isolation of diterpenes from an Australian sponge *Spongia* sp.[8] The results from the current study have shown that Australian specimens of *G. atromarginata*, containing spongian diterpenes, resemble the Sri Lankan rather than the Indian population in their accumulation of secondary metabolites. It further represents a divergence from the generalization that most of the nudibranchs belonging to the genus *Glossodoris* contain sesterterpenoids.[3,11] The geographical variation in chemical composition most likely arises as a consequence of the choice of sponge

prey available to the nudibranch and, therefore, the availability of sponge-derived secondary metabolites.

Extraction of the small sample of sponge prey available to the Australian nudibranch provided an extract that contained ^{1}H NMR signals diagnostic of β,β'-disubstituted furanoditerpenes. On HPLC purification, the major product isolated, **9**, appeared to be a methanolic adduct of **1** on the basis of the presence of two −OMe signals at δ_H 3.50 and 3.45 together with four methyl signals at δ_H 1.09, 1.06, 1.03, and 0.95. No furan signals were present, but there were two acetal protons at δ_H 4.69 and 4.90, respectively. In the HMBC spectrum, the δ_H 4.69 signal correlated to a carbon at δ_C 112.0. These data supporting a dihydroxylated, dimethoxylated furan ring are in very close agreement with the data for a compound (**10**) isolated from *Spongia matamata*.[12] Compound **9** is likely a keto analogue of **10** since HMBC correlations from the methyl groups at δ_H 1.09 and 1.03 pinpointed a carbonyl carbon at δ_C 217.8. Although a low-resolution molecular ion at 419 corresponding to (M + Na) was obtained, there was insufficient material for further characterization or for high-resolution mass measurement. The presence of compounds with the same carbon skeleton as **1** strongly supports the prey/predator metabolite transfer theory commonly associated with nudibranchs and their dietary sponges.

In conclusion, the most intriguing aspects of this study are the selective compartmentalization of the fully acetylated diterpenoid **7** in the mantle glands and the selective anatomical distribution of the diterpenoids. The difference in stereochemistry at C-3 of the metabolites in the internal organs compared with those in the mantle suggests there may be distinct enzymatic systems able to reduce selectively C-3, thus leading to two series of hydroxyl derivatives. *G. atromarginata* is an excellent candidate for further work aimed at proving the ability of nudibranchs to modify dietary diterpenoids.[4,5,11]

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 241-MC polarimeter. One- and two-dimensional NMR spectra were acquired using Bruker AMX-400, Bruker DRX-500, or Bruker DMX-750 instruments. NMR spectra were obtained in deuterochloroform or deuterated methanol at room temperature. Samples were internally referenced to either $CHCl_3$ at δ_H 7.25 and δ_C 77.0 or MeOH at δ_H 3.30. High- and low-resolution electron impact mass spectrometry (EIMS) was recorded on a Kratos MS25RFA mass spectrometer with an ionizing voltage of 70 eV.

Animal Material. Specimens of *Glossodoris atromarginata* were collected from the Gneerings Reef, Mooloolaba (Australia), while scuba diving at a depth of 10−15 m during January of 2005. A sample of a dark gray, rubbery sponge on which one of these nudibranchs was feeding was also collected. The size of the sponge sample was insufficient for preparation of a voucher specimen. The samples were transported to the University of Queensland on ice, where they were stored at −20 °C until analysis.

Extraction and Isolation. Five specimens of *G. atromarginata* were dissected into three portions (internal organs 3.59 g, mantle 7.30 g, mantle glands, weight not recorded owing to small sample size). Each portion was crushed and sonicated while submerged in a minimum volume of Me_2CO. The extract was removed, filtered through cotton, then evaporated under reduced pressure to give an aqueous residue, which was then partitioned with Et_2O. The organic layer was removed, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure to give 80.3 mg from the internal organs (yellow oil), 28.3 mg from the mantle (yellow oil), and 3.3 mg from the mantle glands (colorless oil). Each extract was analyzed by ^{1}H NMR with all of them showing signals associated with β,β'-disubstituted furan compounds. Each extract was separately fractioned by semipreparative reversed-phase HPLC using a MeOH/H_2O gradient (Agilent 1100; 1100 series variable-wavelength UV detector; Waters 10 μm μBondapak 300 × 7.8 mm column; flow rate 1.5 mL/min). Fractionation of the internal organs extract (70−100% MeOH gradient over 40 min) yielded compounds **1**−**4**, the mantle extract (50−100% MeOH gradient over 40 min) afforded **1**, **5** and **6**, while the mantle dermal formations gave **7**.

Extraction of Sponge Material. Extraction of the sponge sample (28.2 g, wet weight) with DCM/MeOH (1:1) gave an extract (149 mg), which was subjected to gradient elution Si flash chromatography (hexanes → DCM → EtOAc → MeOH), then reversed-phase HPLC using a MeOH/H_2O gradient as for the *G. atromarginata* samples to give compound **9** (<0.05 mg).

Spongia-13,(16),14-dien-3-one (1): clear oil (1.5 mg); $[\alpha]_D$ +4.2 (*c* 0.63, $CHCl_3$); ^{1}H and ^{13}C NMR ($CDCl_3$, 500 MHz), see Table 1; HREIMS *m*/*z* 300.2096, calcd for $C_{20}H_{28}O_2$ 300.2089.

3β-Acetoxy-19-hydroxyspongia-13,16(14)-dien-2-one (2): clear oil (<0.05 mg); ^{1}H and ^{13}C NMR ($CDCl_3$, 500 MHz), see Table 2; HRESIMS *m*/*z* 397.1983, calcd for $C_{22}H_{30}NaO_5$ 397.1991.

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Supporting Information Available: Figures S1−S4. ^{1}H NMR data for compounds **1** and **2** and photographs of animal material. This material is available free of charge via the Internet at http://pubs.acs.org.

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