Deoxymanoalides from the Nudibranch *Chromodoris willani*

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Two sesterterpenes, deoxymanoalide (1) and deoxysecomanoalide (2), were isolated from the nudibranch *Chromodoris willani* collected in Okinawa and their structures determined on the basis of spectroscopic data and chemical conversions. The mollusk feeds on a sponge containing manoalide (3) and secomanoalide (4) and is likely to biotransform them into 1 and 2. Both 1 and 2 showed moderate antimicrobial activity against *Escherichia coli* and *Bacillus subtilis* and inhibited snake venom phospholipase A2 at 0.2 to 0.5 μM.

Key words manoalide; *Chromodoris willani*; phospholipase A2

Nudibranchs are colorful, slow-moving, and shell-less mollusks. Their aposematic coloration and possession of toxic substances are believed to function synergetically for their protection from predators and microorganisms.[1,2)]

In our survey for the metabolites of nudibranchs collected along the coast of Okinawa, we often isolate exactly the same molecules obtained from their prey sponges. Some examples are isocyanoterpenes, latrunculin A, a spongian diterpene,[3)] and reticulidins[4)] which have been isolated from both sponges and nudibranchs. However, when we examined the contents of *Chromodoris willani*, the extract appeared to be different from the metabolites of their prey sponge, which contained two sesterterpenes, manoalide (**3**) and secomanoalide (**4**).[5)] Manoalide was first discovered as an antimicrobial constituent of the sponge *Luffariella variabilis*[5)] and later shown to have anti-inflammatory activity against phospholipase A2 (PLA2)[6)] in addition to cytotoxicity.[7)] In this note, we report the structures and biological activities of two manoalide analogs isolated from *C. willani*.

Results and Discussion

Five specimens of *C. willani* and its prey sponge were collected at the same spot in Okinawa and were separately extracted. The lipophilic extract of *C. willani* was subjected to conventional chromatography to give two molecules **1** and **2** as major metabolites. The sponge extract was similarly treated to give manoalide (**3**) and secomanoalide (**4**) which have been identified by the same spectral data as those reported.[5,7)]

HR-electrospray ionization (ESI)-MS determined the molecular formula of compound **1** as $C_{25}H_{36}O_4$ indicating one oxygen atom short of **3** and **4**. Inspection of IR and NMR spectra confirmed the presence of two vinyl methyls (δ 1.65 s, 1.60 s), two equivalent aliphatic methyls (δ 0.99 s), an acetal (δ 5.32 br s, δ 91.4 d), and a lactone (δ 173.7 s, 1783 cm^{-1}) as in **3** and **4**. However, the NMR data of **1** for the lactone portion [oxymethylene (δ 4.87 dd, 4.92 dd; δ 71.1 t)] were different from that [hemiacetal (δ 6.15 s, δ 97.8 d)] of **3**, while the remaining portion (C7—C24) showed nearly identical ^{13}C-NMR data. Therefore, the structure can be elucidated as 25-deoxymanoalide. To determine the configuration at C-4 of **1**, both deoxymanoalide (**1**) and manoalide (**3**) were treated with $NaBH_4$ to furnish the same diol **5** as confirmed by NMR and chromatographic behavior. Both samples of the diol showed virtually the same specific rotational value (+6.8, +6.4) indicating the 4*R* configuration in **1**.

The isomeric nature of compound **2** to **1** was deduced from HR-ESI-MS, suggesting a similar relationship between **3** and **4**. Characteristic signals for an aldehyde (δ 9.43 s, δ 194.2 d, 1681 cm^{-1}) and a secondary alcohol (δ 4.85 br t, δ 67.5 d, 3417 cm^{-1}) were observed, while two aliphatic methyls (δ 0.98 s, δ 28.6) and vinyl methyls (δ 1.59, 1.60, δ 16.1, 19.8) were quite reminiscent of those in **4**. As the lactone moiety (δ 172.9, 1747 cm^{-1}) of **2** contained oxymethylene signals (δ 4.91 br s, δ 70.9 t) instead of oxymethine signals (δ 6.10 s, δ 98.3 d) in **4**, compound **2** was elucidated as 25-deoxysecomanoalide. The stereochemistry at C-4 position of **2** was determined by preparing α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) esters **6** and **7**.[8)] The results indicated negative $\Delta\delta$ values for H-2 (−0.07) and H-4 (−0.09, −0.04) and positive values for H-5 (+0.03), H-6 (+0.11) and H-24 (+0.09) concluding the 4*R* configuration.

This is the first report on compounds **1** and **2** from a natural source, although **1** has been known as a synthetic entity.[6)] Both compounds are believed to be biotransformed by the

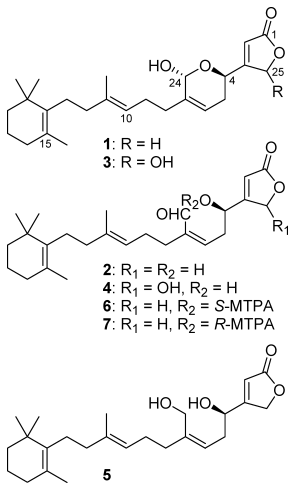

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Table 1. ^{1}H- and ^{13}C-NMR Data for Compounds **1** and **2** in $CDCl_3$

C#	Deoxymanoalide (**1**)		Deoxysecomanoalide (**2**)	
	δ ^{13}C	δ^{1} H mult. (*J* in Hz)	δ^{13}C	δ^{1} H mult. (*J* in Hz)
1	173.7 s		172.9 s	
2	115.0 d	6.03 br q (1.9)	115.9 d	6.04 dt (3.4, 1.9)
3	169.8 s		170.3 s	
4	63.5 d	4.94 dd (10.5, 5.4)	67.5 d	4.85 br t (6.4)
5	32.6 t	2.23 m, 2.17 m	35.7 t	2.80 t (6.7)
6	122.8 d	5.71 m	146.3 d	6.51 t (7.0)
7	137.6 s		146.4 s	
8	29.8 t	2.16 m	24.6 t	2.32 t (7.5)
9	27.9 t	2.03 m	27.8 t	2.09 q (7.5)
10	120.7 d	5.14 br t (6.5)	122.2 d	5.12 br t (7.2)
11	137.5 s		137.7 s	
12	39.8 t	2.03 m	39.8 t	1.97 m
13	25.9 t	2.16 m	26.7 t	2.03 m
14	137.0 s		136.9 s	
15	127.0 s		127.1 s	
16	32.7 t	1.90 t (6.5)	32.7 t	1.90 t (6.3)
17	19.5 t	1.56 m	19.5 t	1.57 m
18	40.2 t	1.42 m	40.2 t	1.41 m
19	34.9 s		35.0 s	
20	28.6 q	0.99 s	28.6 q	0.98 s
21	28.6 q	0.99 s	28.6 q	0.98 s
22	19.8 q	1.60 s	19.8 q	1.59 s
23	16.1 q	1.65 s	16.1 q	1.60 s
24	91.4 d	5.32 br s	194.2 d	9.43 s
25	71.1 t	4.87 dd (17.8, 1.9) 4.92 dd (17.8, 1.9)	70.9 t	4.91 br s (2H)

nudibranch from **3** and **4** which have been sequestered from the sponge. A similar relationship has been reported between the metabolites luffariellins-C and D from the nudibranch *Chromodoris funerae* and lufarriellins-A and B from the sponge *L. variabilis*.[9)]

Compounds **1**—**5** showed moderate cytotoxicity (IC_{50} at 30, 8, 32, 14, 36 μg/ml) against NBT-T2 rat bladder epithelial cells. Although we could not obtain any meaningful data on the inhibitory activity against sPLA2 using a commercial kit (Cayman), both **1** and **2** inhibited PLA2 of the venomous snake *Protobothrops flavoviridis* with IC_{50} values at 0.5 and 0.2 μM, which are less potent than those (0.03, 0.03 μM) of **3** and **4**. Minimum inhibitory concentration (MIC) values of compounds **1**, **2**, and **5** were determined as: 1.7, 1.7 and 3.3 μg/ml against *Escherichia coli*, and 1.7, 6.7, and 6.7 μg/ml against *Bacillus subtilis*, respectively.

Experimental

General Procedures Optical rotations were measured on a Jasco P-1010 digital polarimeter. UV and IR spectra were taken on Hitachi U-2000 and Varian FTS-3000 instruments. ESI-MS and EI-MS were obtained on PE QSTAR and on Hitachi M-2500 mass spectrometers. NMR data were measured at 500 MHz for ^{1}H and 125 MHz for ^{13}C on a Jeol A500 instrument using TMS and $CDCl_3$ as internal standards.

Nudibranch and Sponge Five specimens of the nudibranch (6 g, wet) and the unidentified prey sponge (218 g, wet) were collected at a depth of 20—25 m at cape Zampa, Okinawa by hand using SCUBA and kept frozen until use.

Extraction and Isolation All the mollusk specimens were extracted with acetone (100 ml) and then with MeOH (100 ml) for three times each. The combined extracts were concentrated, and the residue was partitioned between EtOAc and water. The organic extract (89.7 mg) was applied onto a silica gel column to give eight fractions. The fourth fraction (67.4 mg) eluted with CH_2Cl_2–EtOAc (4–1) was separated on HPLC (silica, *n*-hexane–EtOAc, 1–2) to give compounds **1** (39.1 mg) and **2** (9.4 mg). Neither manoalide (**3**) nor secomanoalide (**4**) was detected in the mollusk extract.

The sponge was cut into pieces and steeped in acetone (500 ml). This extraction process was repeated for three times. The acetone solution was concentrated under vacuum, and the resulting material was partitioned between EtOAc and water. The organic layer was taken to give 3.17 g of a crude oil, which was subjected to silica gel column chromatography to give eight fractions. The fourth fraction (1.33 g) was purified on HPLC (silica, *n*-hexane–EtOAc, 1–2) to give manoalide (**3**, 350.0 mg) and secomanoalide (**4**, 46.8 mg), both of which showed identical NMR data to those reported.[5,7)]

Deoxymanoalide (**1**): $[\alpha]_D$ +33.8 (c=2.87, CH_2Cl_2). ^{1}H- and ^{13}C-NMR: Table 1. IR (neat) 3417, 2943, 2866, 1783, 1732, 1645 cm^{-1}. UV (MeOH) 227 nm (ε 3.4×10^3). HR-ESI-MS *m*/*z*: 423.2493 (Calcd for $C_{25}H_{36}O_4Na$, 423.2511).

Deoxysecomanoalide (**2**): $[\alpha]_D$ +11.7 (c=0.77, CH_2Cl_2). ^{1}H- and ^{13}C-NMR: Table 1. IR (neat) 3448, 2932, 2866, 1783, 1732, 1645 cm^{-1}. UV (MeOH) 227 nm (ε 9.6×10^3). HR-ESI-MS *m*/*z*: 423.2475 (Calcd for $C_{25}H_{36}O_4Na$, 423.2511). EI-MS *m*/*z*: 400 (M^+), 382, 137. HR-EI-MS *m*/*z*: 382.2104 (Calcd for $C_{25}H_{34}O_3$, 382.2142).

$NaBH_4$ Reduction of Compounds 1 and 3 A solution of **1** (9.3 mg) in MeOH (2 ml) was treated with $NaBH_4$ (1.6 mg) and the whole mixture was stirred under N_2 atmosphere for 2 h. The reaction mixture was partitioned between EtOAc and water, and the organic layer was taken. After drying and concentration, the crude product was separated on HPLC (RP-18, MeOH–H_2O, 9–1) to give diol (**5**) in the amount of 5.3 mg (57%). Similar treatment of **3** with $NaBH_4$ gave **5** in 56% yield.

Manoalide Diol (**5**): ^{1}H-NMR ($CDCl_3$) δ: 5.98 (1H, br s, H-2), 5.39 (1H, t, J=8.0 Hz, H-6), 5.13 (1H, m, H-10), 4.88 (2H, br s, H-25), 4.64 (1H, br t, J=5.5 Hz, H-4), 4.19 (1H, d, J=11.5 Hz, H-24a), 4.10 (1H, d, J=11.5 Hz, H-24b), 2.54 (2H, m, H-5), 2.20 (2H, m, H-9), 2.16 (2H, m, H-8), 2.03 (4H, m, H-12, 13), 1.90 (2H, t, J=6.5 Hz, H-16), 1.64 (3H, s, H-23), 1.60 (3H, s, H-22), 1.57 (2H, m, H-17), 1.41 (2H, m, H-18), 0.99 (6H, s, H-20, 21). ^{13}C-NMR ($CDCl_3$) δ: 174.0 s, 172.8 s, 144.1 s, 137.1 s, 137.0 s, 127.0 s, 122.8 d, 122.6 d, 114.8 d, 71.5 t, 67.3 d, 60.0 t, 40.2 t, 39.8 t, 36.8 t, 35.0 t, 35.0 s, 32.7 t, 28.6 q (2C), 27.8 t, 26.7 t, 19.8 q, 19.5 t, 16.1 q. HR-ESI-MS *m*/*z*: 425.2654 (Calcd for $C_{25}H_{38}O_4Na$, 425.2668).

MTPA Esters (6, 7) from Deoxysecomanoalide (2) To dried deoxysecomanoalide (**2**, 1.1 mg) were added *S*-MTPA (1.3 mg in 10 μl of CH_2Cl_2), DCC (1.02 mg in 20 μl of CH_2Cl_2), and DMAP (0.20 mg in 20 μl of CH_2Cl_2). The mixture was allowed to stand in a desiccator for 14 h at room temperature. After passing through a short silica gel column (CH_2Cl_2–EtOAc, 9–1) and concentration, 1.0 mg (70%) of *S*-MTPA (**6**) ester was obtained. *R*-MTPA ester (**7**) was similarly prepared (59%).

Deoxysecomanoalide *S*-MTPA Ester (**6**): ^{1}H-NMR ($CDCl_3$) δ: 9.32 s (H-24), 7.45 m (5H), 6.24 t (H-6), 5.99 t (H-4), 5.95 s (H-2), 5.08 br t (H-10), 4.77 d (H-25a), 4.61 d (H-25b), 3.48 s (3H), 2.93 m (2H, H-5), 2.24 m (2H, H-8), 1.59 s (3H, H-23), 1.57 s (3H, H-22), 0.98 s (6H, H-20, 21).

Deoxysecomanoalide *R*-MTPA Ester (**7**): ^{1}H-NMR ($CDCl_3$) δ: 9.23 s (H-24), 7.45 m (5H), 6.13 t (H-6), 6.02 s (H-2), 5.97 t (H-4), 5.07 br t (H-10), 4.81 d (H-25a), 4.70 d (H-25b), 3.48 s (3H), 2.90 t (2H, H-5), 2.21 m (2H, H-8), 1.59 s (3H, H-23), 1.57 s (3H, H-22), 0.98 s (6H, H-20, 21).

Antimicrobial and Cytotoxicity Assay The MIC of compounds against two bacteria *E. coli* and *B. subtilis* was determined with a series of diluted solutions of compounds in MeOH using modified Andrews method. Compounds were dispensed (75 μl) into the wells of 96-well microtitre plates in duplicate followed by the addition of the appropriate bacterial inoculum (75 μl). A MeOH control was included in all assays. The plates were incubated at 37 °C for 12 h for *E. coli* and 16 h for *B. subtilis* and the MIC's were recorded as the lowest concentration at which no growth was observed. This was facilitated by the addition of 20 μl of a methanolic solution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, 5 mg/ml) to each well and incubation for 30 min. A dark blue coloration indicated bacterial growth.

NBT-T2 cells (BRC-1370, purchased from Riken BioResource Center) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and antimicrobials under standard protocol and seeded in 200 μl wells. After preincubation (37 °C, 24 h), cells were exposed to graded concentrations of compounds in duplicate (37 °C, 48 h). Then, the cells were treated with MTT solution (15 μl, 5 mg/ml in PBS) after removal of the medium and incubated for 3 h. The residual formozan was dissolved in dimethyl sulfoxide (DMSO) (100 μl) and the absorbance was measured with a Tecan sunrise microplate reader at 560 nm. The IC_{50} values were estimated by plotting the absorbance values against concentrations.

Phospholipase Assay Purified phospholipase A2 ([Asp^{49}]PLA2, pI 7.9,

highly lipolytic and myolytic) of the snake *P. flavoviridis* was purified from the milked crude venom as previously reported.[10] A mixture of 30 μl of phospholipase A2 (4.8 ng/μl) in a buffer (0.1 M Tris–HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0) and 1 μl of a test compound (50 mM—500 nM) was incubated in a 100 μl well at room temperature for 15 min. Then, a solution (69 μl) of PED6 (Molecular Probe D23739, 0.01 mg/ml in the same buffer) was added to each well, and the fluorescence was measured at Ex 488 nm and Em 530 nm for 2 min using a Thermo Varioskan microplate reader. After obtaining average rates, the data were plotted against concentrations, and approximate IC_{50} values were estimated.

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