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SECONDARY METABOLITES OF THE VIETNAMESE NUDIBRANCH MOLLUSK *Phyllidiella pustulosa*

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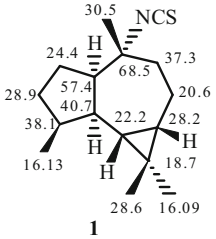
The chemical composition of the EtOH extract of the nudibranch mollusk Phyllidiella pustulosa *was studied. Six sesquiterpenoids including the novel secondary metabolite (+)-10(*R*)-isothiocyanoalloaromodendrane were isolated. The steroid composition of the extract was studied. The structures of three previously known ketosteroids and six sterols were identified.*

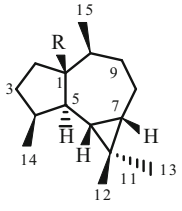
Keywords: *Phyllidiella pustulosa*, isocyanates, isothiocyanates, thiocyanates, sesquiterpenoids, steroids.

Nudibranch mollusks of the family Phyllidiidae are commonly known to be sources of various toxic sesquiterpenoids containing cyanate, isocyanate, thiocyanate, and isothiocyanate groups [1]. Secondary metabolites from populations of these animals collected along the shores of China [2], Fiji [3], the Philippines [4], Japan [5–9], and Australia [10] have been previously studied. These soft mollusks lack shells and feed on sponges. They use certain compounds ingested with their food for chemical protection against predators. Several compounds isolated from them exhibit various types of biological activity in addition to a deterrant action. These include antimicrobial [11], antifouling [9], antifungal [5], and antimalarial [3]. The qualitative and quantitative contents of secondary metabolites in extracts of nudibranch mollusks are known to depend on the time, site, and food source of the animals [1, 12].

Seven sesquiterpenoids including the new isothiocyanate **1** were isolated by chromatographic methods from the extract of *Phyllidiella pustulosa* collected during cruise O34 of RS Akademik Oparin off the shores of Vietnam.

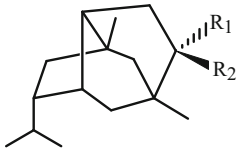


1



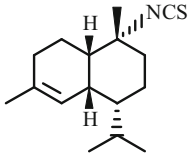
2, 3

2: R = NC; **3:** R = NCS



4a, 4b

4a: R_1 = SCN, R_2 = H
4b: R_1 = H, R_2 = SCN



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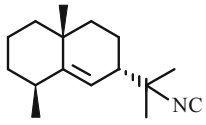

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The structure and stereochemistry of **1** were determined by PMR and ^{13}C NMR spectroscopy (including NOESY, HMBC, and HMQC experiments), mass spectrometry, and comparision of the results with the literature. NMR spectra of **1** were identical to those of the previously known semisynthetic (–)-1*S*,4*R*,5*R*,6*S*,7*R*,10*S*-isothiocyanoalloaromadendrane [13]. However, its optical rotation $[\alpha]_D^{20}$ +6.0° (*c* 0.15, $CHCl_3$) turned out to be close but opposite in sign to that of (–)-alloaromadendrane {$[\alpha]_D^{20}$ –5.9° (*c* 2.10, $CHCl_3$)}. Therefore, **1** had the absolute configuration 1*R*,4*S*,5*S*,6*R*,7*S*,10*R* and was an enantiomer of the synthetic product.

In addition to **1**, we isolated and identified the structures of previously known sesquiterpenoids. The major metabolite was the isocyanate **2**; a minor one, isothiocyanate **3**, which is related to it. Both metabolites were found earlier in the sponge *Acanthella acuta* [14]. The inseparable mixture of **4a–4b** (1:1) obtained by us turned out to be identical to a mixture of these

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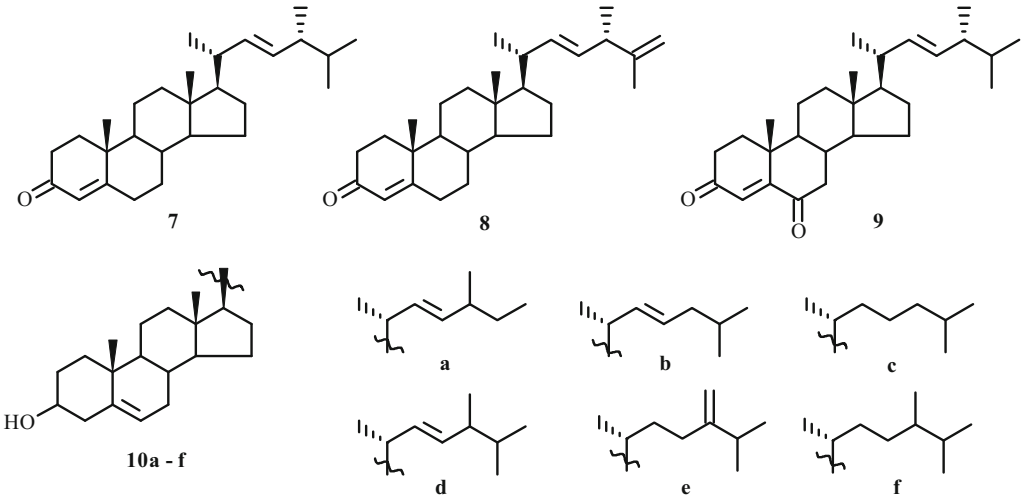
same products that was isolated earlier from the nudibranch mollusk *Phyllidia varicosa* and its food source, the sponge *Axinyssa aculeate* [11].

Compound **5** is known as a metabolite of sponges from the genus *Halichondria* [15]. Compound **6** was isolated previously from the sponge *Axinella cannabina* [16]. The structures and relative stereochemistries of **2–6** were established by comparing NMR and mass spectra and physical constants with those published for these compounds [11, 14–16].

The steroid composition of the collected animals was studied. The steroidal ketones (24*R*)-24-methylcholesta-4,22-dien-3-one (**7**) [17, 18], (24*S*)-24-methylcholesta-4,22,25-trien-3-one (**8**) [11, 19], and (24*R*)-24-methylcholesta-4,22*E*-dien-3,6-dione (**9**) [20] were isolated pure. Their structures were determined using NMR and mass spectra. However, spectral data in the literature for **8** and **9** were incomplete so that careful analysis of their spectra and additional experiments were needed in order to refine their structures.

The structure and stereochemistry of **7**, which was isolated earlier from the sponges *Stelleta clorella* [17] and *Haliclona* spp. [21], were established by comparing the spectral data with the literature [17, 18, 21]; the structure of **8**, by comparing the spectral data with those of **7**. The resonance for Me-26 (1.676) was shifted to weak field and appeared as a singlet. A resonance of Me-27 was not observed. However, the spectrum of **8** contained resonances for additional exomethylene protons at 4.71 (2H, m). Compound **8** was previously prepared synthetically and was isolated as a natural product from the nudibranch mollusk *P. varicosa* [11]. Ketones **7** and **8** were reduced over Adams catalyst in order to resolve the issue of the absolute configuration of C-24. In both instances (24*S*)-24-methyl-5β-cholestan-3α-ol (side chain f) was obtained. The cis-fusion of rings A and B in the hydrogenation product was established based on the chemical shift of Me-19 (0.919) [22]. The chemical shift and multiplicity of the C-3 resonance indicated that the hydroxyl was in the 3α-position. Calculation of the chemical shifts using the Zurcher rule [23] confirmed that the product had the 5β,3α-configuration. Resonances in the PMR spectrum for Me-26 (0.852), Me-27 (0.783), and Me-28 (0.775) in the side chain agreed with those for dihydrobrassicosterol, which has the (24*S*)-configuration [24] and confirmed that **8** had the (24*S*)-configuration. As far as we know, (24*S*)-24-methyl-5β-cholestan-3α-ol has not been previously reported.



The sterochemistry of the 22(23) double bond in **9** was also established as *trans* by taking into account the spin–spin coupling constant $J_{22,23}$ = 15.0 Hz. Catalytic hydrogenation of **9** produced (24*S*)-24-methyl-5α-cholestan-3β,6β-diol, the structure and absolute configuration of which were established by comparison of its PMR spectrum with the literature for the chemical shifts of Me-26 (0.855), Me-27 (0.781), and Me-28 (0.774) [24, 25]. Thus, natural product **9** had 24*R* stereochemistry.

Free steroid alcohols were isolated by column chromatography over silica gel using hexane:EtOAc (10:1) and were purified by normal-phase HPLC using the same solvent system. Components were identified by GC–MS of their acetates. All identified compounds including 27-nor-24-methylcholesta-5,22-dien-3β-ol (**10a**), cholesta-5,22-dien-3β-ol (**10b**), cholest-5-en-3β-ol (**10c**), 24-methylcholesta-5,22-dien-3β-ol (**10d**), 24-methylcholesta-5,24(28)-dien-3β-ol (**10e**), and 24-methylcholest-5-en-3β-ol (**10f**) were Δ^5 sterols.

The composition of low-molecular-weight metabolites of nudibranch mollusks of the family Phyllidiidae inhabiting the shores of Vietnam was studied for the first time. Apparently the isolated sesquiterpenoids from *P. pustulosa* are exogenous metabolites and originate in sponges. The mollusk studied by us obviously feeds on sponges of the genera *Axinella*, *Acanthella*, *Halichondria*, and *Axinyssa*, in which many of the compounds isolated by us were previously found.

EXPERIMENTAL

General Methods. PMR and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker DPX 300 spectrometer at 300 and 75 MHz, respectively. Chemical shifts in NMR spectra are reported on the δ-scale in ppm relative to TMS as an internal standard ($\delta_{TMS} = 0$); SSCC, in Hz. Optical rotation was measured on a Perkin–Elmer 343 Polarimeter. GC–MS was carried out in a Hewlett–Packard HP6890 GC chromatograph using an HP-5MC capillary column (30.0 m × 250 μm × 0.25 μm) at 150–230°C with He carrier gas and 70 eV ionizing potential. Direct-probe mass spectra were taken in an AMD-604 S double-focusing high-resolution mass spectrometer at 70 eV. Column chromatography was performed over silica gel (KSK, Russia, 0.05–0.16 mm); HPLC, on Ultrasphere^{TM} Si (5 μm, 250 × 4.6 mm) and YMC-Pack ODS-A (5 μm, 250 × 4.6 mm) columns on an Agilent 1100 Series chromatograph.

Biological Material. Animals were collected by diving on May 19, 2007, in a bay of Long Vi Island in the South China Sea (20°09, 030N; 107°44, 359E, Vietnam) at a depth of 6 m and were preserved in EtOH. The species was confirmed by Dr. Ernesto Mollo (Istituto di Chimica Biomolecolare-CNR, Naples 200071, Italy).

Extraction and Isolation. Animals (4, each 7.2 g dry weight) were extracted successively with EtOH (150 mL) and $CHCl_3$ (50 mL). The combined extracts were concentrated in vacuo to obtain a dark oil (1.3 g) that was fractionated by column chromatography over KSK silica gel using gradient elution by hexane:EtOAc. The fraction eluting with hexane was separated by HPLC over silica gel (hexane eluent) and afforded **1**, **3**, and **5**.

The fraction eluting with hexane:EtOAc (20:1) yielded pure **2**.

The fraction eluting with hexane:EtOAc (10:1) was separated by HPLC over silica gel (hexane:EtOAc 80:1) and afforded **2** in addition to an inseparable mixture of **4a** and **4b** (it could not be separated by HPLC over Ultrasphere^{TM} Si, 5 μm, 250 × 4.6 mm; YMC-Pack ODS-A, 5 μm, 250 × 4.6 mm; or a ChiraDex® chiral column) and **6**.

HPLC over silica gel (hexane:EtOAc 15:1) afforded ketosteroids **7** and **8** from the fraction eluting with hexane:EtOAc (20:1).

Diketosteroid **9** and an inseparable mixture of several sterols **10a–f** were isolated by normal-phase HPLC (hexane:EtOAc 10:1) of the fraction obtained from column chromatography using hexane:EtOAc (5:1). The fraction containing the sterols (3.9 mg) was analyzed by GC after preliminary acetylation by acetic anhydride in pyridine (1:1) for 24 h at room temperature and removal of the reagents in vacuo. Sterol acetates were purified by column chromatography over silica gel using hexane:EtOAc (80:1) (0.6 mg).

(+)-10(*R*)-Isothiocyanoalloaromodendrane (1). Colorless oil, $[\alpha]_D^{20}$ +6.0° (*c* 0.1, $CHCl_3$), 3.5 mg (0.05% of dry weight). PMR and ^{13}C NMR spectra ($CDCl_3$) agreed with those reported [13].

GC–MS (*m*/*z*, I_{rel}, %): 263 (35) $[M]^+$, 248 (41), 230 (29), 220 (15), 204 (21), 189 (35), 181 (24), 174 (12), 161 (65), 149 (47), 135 (32), 119 (44), 107 (79), 93 (68), 81 (65), 69 (100), 55 (50).

High-resolution mass spectrum (EI, 70 eV): calcd for $C_{16}H_{25}NS$ 263.1778; found 263.17161.

Compound 2. Colorless oil, $[\alpha]_D^{20}$ –12.5° (*c* 0.65, $CHCl_3$), 17.3 mg (0.24% of dry weight); lit. $[\alpha]_D^{25}$ –13.7° (*c* 1.4, $CHCl_3$). PMR and ^{13}C NMR spectra ($CDCl_3$) agreed with those published [14].

GC–MS (*m*/*z*, I_{rel}, %): 231 (7), 216 (39), 203 (5), 189 (27), 174 (25), 161 (27), 148 (18), 135 (27), 121 (30), 107 (50), 94 (36), 8 (100), 67 (28), 53 (25).

Compound 3. Colorless oil, $[\alpha]_D^{25}$ –61.8° (*c* 0.33, $CHCl_3$), 6.9 mg (0.10% of dry weight); lit. $[\alpha]_D$ –32.8° (*c* 0.70, $CHCl_3$). PMR and ^{13}C NMR spectra ($CDCl_3$) agreed with those reported [14].

GC–MS (*m*/*z*, I_{rel}, %): 363 (100) $[M]^+$, 348 (100), 230 (36), 220 (14), 204 (41), 189 (18), 180 (9), 161 (43), 149 (50), 135 (25), 122 (86), 107 (82), 95 (64), 81 (68), 69 (50), 55 (52).

Mixture of 4a and 4b. Colorless oil, 11.7 mg (0.16% of dry weight). PMR and ^{13}C NMR spectra ($CDCl_3$) agreed with the literature [11].

GC–MS (*m*/*z*, I_{rel}, %): 263 (tr.) $[M]^+$, 257 (7), 205 (11), 187 (36), 159 (43), 145 (25), 135 (18), 121 (54), 109 (36), 95 (54), 81 (32), 69 (46), 55 (29).

Compound 5. Colorless oil, $[\alpha]_D^{25}$ –168.0° (*c* 0.10, $CHCl_3$), 1.0 mg (0.01% of dry weight); lit. $[\alpha]_D^{25}$ –63.0° (*c* 7.4, $CHCl_3$). PMR and ^{13}C NMR spectra ($CDCl_3$) agreed with those reported [15].

GC–MS (*m*/*z*, I_{rel}, %): 263 (84) $[M]^+$, 248 (20), 230 (45), 220 (5), 204 (9), 189 (9), 178 (7), 161 (100), 147 (9), 133 (11), 119 (68), 105 (67), 95 (45), 81 (32), 69 (17), 55 (25).

Compound 6. Colorless oil, $[\alpha]_D^{25}$ –20.0° (*c* 0.05, $CHCl_3$), 3.5 mg (0.05% of dry weight); lit. $[\alpha]_D$ –63.0° (*c* 7.4, $CHCl_3$). PMR and ^{13}C NMR spectra ($CDCl_3$) agreed with those reported [16].

GC–MS (*m*/*z*, *I*rel, %): 231 (4) $[M]^+$, 216 (5), 204 (6), 189 (7), 174 (2), 163 (11), 147 (3), 133 (5), 119 (6), 105 (11), 93 (23), 81 (100), 67 (9), 55 (7).

(24*R*)-24-Methylcholesta-4,22*E*-dien-3-one (7), 1.7 mg (0.02% of dry weight). PMR and ^{13}C NMR spectra ($CDCl_3$) agreed with those reported [17, 18].

(24*S*)-24-Methylcholesta-4,22*E*,25-trien-3-one (8), 0.8 mg (0.01% of dry weight).

PMR spectrum (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 5.72 (1H, br.d, J = 1.5), 5.25 (2H, m), 4.70 (2H, m), 2.35-2.39 (4H, m), 2.00–2.06 (4H, m), 1.676 (3H, s, Me-26), 1.182 (3H, s, Me-19), 1.081 (3H, d, J = 6.9, Me-28), 1.008 (3H, d, J = 6.6, Me-21), 0.723 (3H, s, Me-18).

^{13}C NMR spectrum (75.5 MHz, $CDCl_3$, δ, ppm): 12.2, 17.4, 18.9, 20.6, 20.7, 21.0, 24.2, 28.5, 29.7, 32.0, 32.9, 34.0, 35.6, 35.7, 38.6, 39.5, 40.0, 42.3, 43.6, 53.8, 55.9 (2C), 108.7, 123.7, 131.9, 135.6, 171.7, 199.7.

Mass spectrum (EI, 70 eV, *m*/*z*, I_{rel}, %): 394 (11) $[M]^+$, 311 (7), 298 (32), 269 (100), 253 (20), 245 (10), 229 (17), 215 (8), 203 (10), 187 (11), 175 (20), 161 (22), 147 (32), 135 (18), 124 (48), 109 (22), 95 (41), 81 (36), 69 (17), 55 (28).

(24*R*)-24-Methylcholesta-4,22*E*-dien-3,6-dione (9), 1.2 mg (0.01% of dry weight).

PMR spectrum (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 6.17 (1H, s), 5.22 (1H, dd, J = 6.9, 15.0), 5.17 (1H, dd, J = 7.5, 15.0), 2.70 (1H, dd, J = 3.9, 15.6), 2.47–2.55 (2H, m), 1.168 (3H, s, Me-19), 1.025 (3H, d, J = 6.6, Me-21), 0.914 (3H, d, J = 6.9, Me-28), 0.841 (3H, d, J = 6.9, Me-26 or -27), 0.818 (3H, d, J = 6.6, Me-27 or -26).

^{13}C NMR spectrum (75.5 MHz, $CDCl_3$, δ, ppm): 12.1, 17.5, 17.6, 19.6, 19.9, 20.8, 20.9, 23.9, 28.3, 33.1, 34.0, 34.2, 35.5, 39.0, 39.8, 40.1, 42.4, 42.8, 46.8, 51.0, 55.8, 56.6, 125.4, 132.2, 135.3, 161.1, 199.5, 202.4.

Mass spectrum (EI, 70 eV, *m*/*z*, I_{rel}, %): 410 (100) $[M]^+$, 367 (22), 312 (66), 283 (61), 253 (28), 243 (24), 229 (16), 189 (18), 175 (20), 147 (22), 137 (61), 124 (24), 109 (41), 95 (36), 83 (72), 69 (100), 55 (100).

Reductive hydrogenation was carried out as follows. Each ketosteroid (**7**, **8**, and **9**; 0.9, 0.8, and 1.2 mg, respectively) dissolved in EtOH (200 μL) was hydrogenated under H_2 over Adams catalyst for 24 h at room temperature with continuous stirring on a magnetic stirrer to afford (24*S*)-24-methyl-5β-cholestan-3α-ol (1.0 and 0.9 mg from **7** and **8**, respectively) and (24*S*)-24-methyl-5α-cholestan-3β,6β-diol (1.7 mg from **9**).

(24*S*)-24-Methyl-5β-cholestan-3α-ol, 1.0 and 0.9 mg, $[\alpha]_D^{25}$ +27.8° (*c* 0.09, $CHCl_3$).

PMR spectrum (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 3.62 (1H, m), 0.919 (3H, s, Me-19), 0.902 (3H, d, J = 6.6, Me-21), 0.855 (3H, d, J = 6.9, Me-26), 0.784 (3H, d, J = 6.9, Me-27), 0.775 (3H, d, J = 6.6, Me-28), 0.639 (3H, s, Me-18).

(24*S*)-24-Methyl-5α-cholestan-3β,6β-diol, 1.7 mg. PMR spectrum ($CDCl_3$) agreed with that published [23].

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