Isocyanide Terpene Metabolites of *Phyllidiella pustulosa*, a Nudibranch from the South China Sea

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A series of isocyanides, compounds **4**−**11**, including diterpenes never before found in Phyllidiid nudibranchs, have been isolated from a Chinese population of the nudibranch *Phyllidiella pustulosa*. Three new sesquiterpenes (**8**, **10**, and **11**), with eudesmane, guaiane, and bisabolane skeletons, respectively, have been characterized by spectral methods and chemical comparison with known related molecules. The absolute stereochemistry of the major metabolite, the isocyanide **8**, an enantiomer of the known sponge metabolite stylotelline (**12**), has also been determined.

Phyllidiid nudibranchs are chemically characterized by the presence in the lipophilic extracts of highly toxic isocyanide sesquiterpenes.[1,2] A series of these compounds exhibiting different carbon frameworks, such as bisabolane,[3−5] amorphane,[6−8] and pupukeanane[4,6,9] skeletons, have been so far reported from different species from geographically distinct sites. The dietary origin of phyllidiid isocyanides has been strongly supported by several comparative chemical investigations involving the isolation of isocyanide metabolites from both nudibranchs and their sponge preys.[3−5,7,10] In addition, a biosynthetic study conducted on the nudibranch *Phyllidiella pustulosa* and the sponge *Acanthella cavernosa* clearly demonstrated the transfer of the secondary sponge metabolites to the mollusk.[11] The presence of these highly toxic sesquiterpenes in the mucus that the mollusks secrete when attacked by predators strongly supports the involvement of such molecules in the defensive mechanisms displayed by nudibranchs.[1] In addition, isocyanides and related compounds show potent antifouling activity against larvae of the barnacle *Balanus amphitrite*,[12] and bioassay-guided analysis of extracts of different phyllidiid species has led to the finding of many active isocyanosesquiterpenes.

In this work, chemical constituents of a Chinese population of the nudibranch *P. pustulosa* (previously reported as *Phyllidia*) (Cuvier, 1804) have been investigated. Previous studies conducted on individuals of the same species from Japanese,[3,7,12,13] Philippine,[5] Fiji,[10] and Australian[11] coasts have led to the finding of several isonitrile sesquiterpenes (i.e., **1**−**3**, Figure 1), displaying different carbon skeletons.

We add here the isolation of a series of nitrogenous terpenes (Figure 2), including both diterpenes **4**−**7** and sesquiterpenes **8**−**11**, which were detected in both the external and the internal parts of the animal, even though they turned out to be concentrated in the mantle. Diterpenes **4**−**7** have been previously isolated from sponges, whereas sesquiterpene **9** has been reported from different collections of *Phyllidia* spp. The structures of novel sesquiterpenes **8**, **10**, and **11**, exhibiting three different carbon frameworks, were determined by both spectroscopic methods and chemical correlation with related known molecules. The main compound **8** is the enantiomer of the known sponge metabolite stylotellin (**12**).[14] It is interesting to note that this is the first finding of diterpenes in nudibranchs belonging to the family Phyllidiidae.

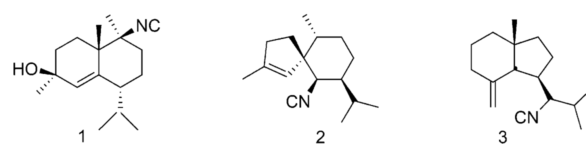

Figure 1. Selected isocyanide sesquiterpenes previously reported from *P. pustulosa*.

Results and Discussion

P. pustulosa (18 specimens) was collected off Hainan Island in the South China Sea in January 2002, at a depth of 20 m. The sample was immediately frozen and then transferred to the ICB laboratories, where it was kept at −20 °C until the extraction. *P. pustulosa* individuals were carefully dissected into mantle and internal organs and then separately extracted by acetone using ultrasound. The diethyl ether-soluble fractions from the acetone extract of both anatomical samples were analyzed by TLC chromatography and found to display qualitatively similar secondary metabolite patterns. In particular a series of components at R_f 0.1−0.5 (light petroleum ether/diethyl ether, 95:5) and at R_f 0.2−0.3 (light petroleum ether/diethyl ether, 1:1) were detected to be more concentrated in the mantle of the mollusk.

The mantle ether extract (150 mg) was chromatographed on a Sephadex LH-20 column eluting with chloroform/methanol, 1:1. Two fractions, A (16 mg) and B (93 mg), contained terpenoids, and a third was a glyceride mixture (24 mg). Mixture A was submitted to reverse-phase HPLC purification to afford two pure known diterpene isonitriles, kalihinene **4**[15] (1.7 mg) and amphilectene **5**[16] (1.1 mg), previously found in sponges. Mixture B was purified by a silica gel column eluted with a light petroleum ether/diethyl ether gradient, giving kalihinol-A **6** (2.6 mg), reported from sponges of the genus *Acanthella*,[17−19] and a more polar fraction. After *n*-phase HPLC on this polar fraction, kalihinol-E (**7**, 0.9 mg), previously found in *Acanthella* species,[18,20] was isolated along with a less polar mixture (49 mg) of sesquiterpenes. This fraction was subjected to a

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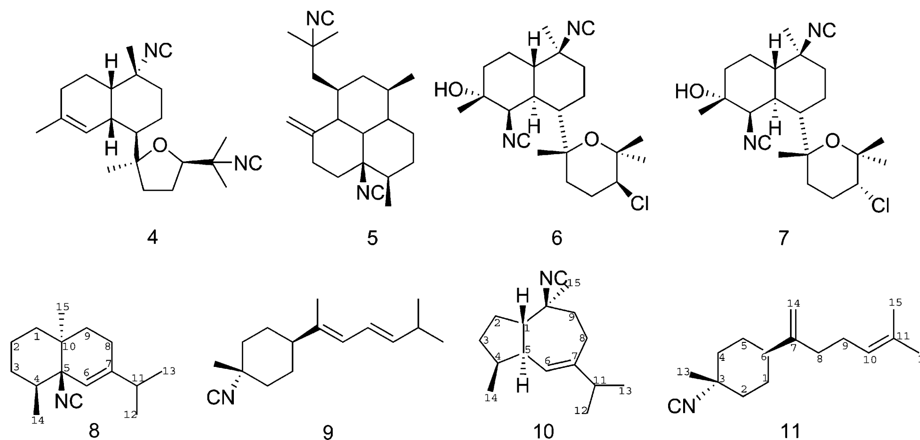

Figure 2. Diterpenes and sesquiterpenes isolated in this work from *P. pustulosa*.

silica gel chromatographic column (light petroleum ether/diethyl ether gradient), giving the new compound **8** (20 mg), main metabolite of the mantle extract, 3-isocyanotheonellin **9** (3 mg), already reported from *Phyllidia* spp.,[3-5] and a fraction (8 mg) that afforded by *n*-phase HPLC purification two minor novel sesquiterpenes, **10** (0.6 mg) and **11** (0.7 mg).

The digestive gland ether extract (236 mg) was purified in an analogous fashion, to give, along with sesquiterpene **8** (40 mg), which was also isolated as main metabolite from the mantle extract, another abundant compound, the known ergosta-4,22-dien-3-one (44 mg).[21] The remaining terpene-containing minor fractions, qualitatively similar to those obtained from mantle extract, were not processed.

The known terpenes were identified by comparison of spectral data (NMR, MS, and $[\alpha]_D$) with those reported in the literature, while the novel metabolites **8**, **10**, and **11**, all of which have the molecular formula $C_{16}H_{25}N$, indicating five degrees of unsaturation, were characterized using spectroscopic techniques.

In the HRESIMS spectrum of **8** a pseudomolecular ion $(M + Na)^+$ was observed at m/z 254.1889 along with a diagnostic peak at m/z 205 due to the loss of HCN from the molecular ion. The presence of an isocyanide group was confirmed by both IR absorption at 2139 cm^{-1} and ^{13}C NMR signals at δ 154.3 (−NC) and 68.7 (C-5). Analysis of the ^1H NMR spectrum indicated a trisubstituted double bond [δ 5.41 (1H, bs, H-6)] and four methyl groups all resonating at δ 1.00−1.02 that suggested the presence of a bicyclic sesquiterpene skeleton, based on the unsaturation degrees of the molecular formula. In the ^{13}C NMR spectrum, in addition to sp^2 carbons resonating at δ 121.5 (C-6) and 143.9 (C-7), 12 sp^3 signals (five CH_2, four CH_3, two CH, and one C) were present between δ 39.5 and 16.3. These data were consistent with an eudesmane framework bearing a tertiary −NC function, as indicated in formula **8**. All proton and carbon resonances were assigned as reported in Table 1 by careful analysis of 2D-NMR experiments. However, the spectral data for **8**, which were also recorded in acetone, were identical with those reported for stylotelline (**12**), a eudesmane isocyanide isolated from the sponge *Stylotella* sp.,[14] except for the optical rotation value ($[\alpha]_D$ **8**: +23° ($CHCl_3$, *c* 2); $[\alpha]_D$ **12**: −47° ($CHCl_3$, *c* 1.7)), indicating that **8** was *ent*-stylotelline. The absolute stereochemistry of stylotellin had been determined by chemical conversion of **12** into the corresponding hydrocarbon (+)-δ-selinene (**13**) (Figure 3), which was identified by comparison with an authentic sample.[14] The absolute stereochemistry of compound **8** was also established by transformation to the corresponding known diene (−)-δ-selinene

Table 1. NMR Data[a] of Compound **8**

C	δ_C[b]	m[c]	δ_H[d]	m, *J* (Hz)	HMBC correlations[e]
1	35.9	CH_2	1.68	m	C-2, C-9, C-10
			1.34	m	
2	20.7	CH_2	1.50	m	C-1
3	30.6	CH_2	1.50	m	
			1.37	m	
4	39.5	CH	1.78	m	
5	68.7	C			
6	121.5	CH	5.41	bs	C-8, C-10, C-11
7	143.9	C			
8	22.9	CH_2	2.12	m	C-6, C-7, C-11
			1.99	m	
9	28.6	CH_2	2.09	m	C-1, C-5, C-7, C-8, C-10
			1.23	m	
10	35.1	C			
11	34.5	CH	2.19	m	C-6, C-7, C-12, C-13
12	21.4	CH_3	1.00	d (6.9)	C-7, C-11, C-13
13	21.2	CH_3	1.00	d (6.9)	C-7, C-11, C-12
14	16.3	CH_3	1.00	d (6.9)	C-3, C-4, C-5
15	23.9	CH_3	1.02	s	C-1, C-9, C-10
16	154.3	NC			

[a] Bruker 300, 400, and 500 MHz; $CDCl_3$; chemical shifts (ppm) referred to $CHCl_3$ (δ 7.26) for proton and to $CDCl_3$ (δ 77.0) for carbon. [b] Assignments by HMQC and HMBC experiments. [c] By DEPT sequence. [d] Assignments by $^1H-^1H$ COSY experiment. [e] J = 10 Hz.

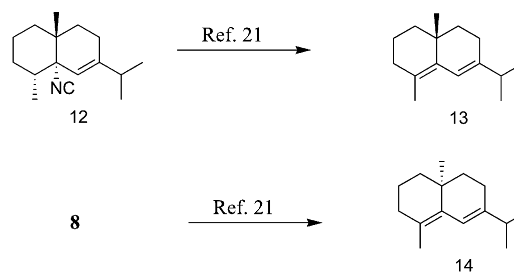

Figure 3. Chemical conversion of *ent*-stylotelline (**8**) into (−)-δ-selinene (**14**).

(**14**), by acid elimination of the tertiary isocyanate group (Figure 3), as for stylotellin (**12**).[14,22]

The HRESIMS spectrum of compound **10** displayed a pseudo-molecular peak at m/z 254.1883 $(M + Na)^+$, together with an intense peak at m/z 205, formed by loss of HCN from the molecular ion. Analysis of ^1H and ^{13}C NMR spectra indicated the presence of a trisubstituted double bond [δ_C 123.8 (C-6) and 147.8 (C-7); δ_H 5.45 (1H, bs, H-6)], which again implied, from the required degrees of unsaturation, a bicyclic sesquiterpene framework. ^1H NMR showed three methyl doublets at δ 0.88 (d, J = 6.9 Hz, H_3-14), 0.97 (d, J = 6.8 Hz, H_3-13), and 0.98 (d, J = 6.9 Hz, H_3-12), along with a methyl singlet at δ 1.40 (H_3-15), which

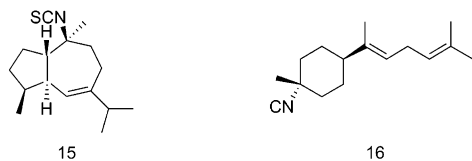

Figure 4. Sponge nitrogen sesquiterpenes structurally correlated with *Phyllidiella* metabolites **10** and **11**.

Table 2. NMR Data[a] of Compound **10**

C	δ_C[b]	m[c]	δ_H[d]	m, *J* (Hz)	HMBC correlations[e]
1	50.0	CH	2.31	m	C-10
2	25.0	CH_2	1.95	m	
			1.69	m	
3	3.6	CH_2	1.65	m	
			1.41	m	
4	37.8	CH	2.21	m	
5	42.4	CH	2.28	m	C-8
6	123.8	CH	5.45	bs	
7	147.8	C			C-6
8	24.7	CH_2	2.26	m	C-10
9	41.6	CH_2	1.90	m	C-7
				m	
10	62.6	C			
11	37.1	CH	2.21	m	C-12, C-13
12	21.6	CH_3	0.98	d (6.9)	C-7, C-11, C-13
13	21.2	CH_3	0.97	d (6.8)	C-7, C-11, C-12
14	15.3	CH_3	0.88	d (6.9)	C-3, C-4, C-5
15	20.4	CH_3	1.40	bs	C-1, C-9, C-10
16	nd	NC			

[a] Bruker 300, 400, and 500 MHz; $CDCl_3$; chemical shifts (ppm) referred to C*H*Cl_3 (δ 7.26) for proton and to *C*DCl_3 (δ 77.0) for carbon. [b] Assignments by HMQC and HMBC experiments. [c] By DEPT sequence. [d] Assignments by $^1H-^1H$ COSY experiment. [e] $J = 10$ Hz.

was linked to a tertiary carbon (δ 62.6, C-10) bearing an isonitrile function, the carbon signal of which was not detected. Comparison of NMR values of compound **10** with literature data for different sesquiterpene skeletons led to suggest a *trans*-fused guaiane system as reported in Figure 2, exhibiting a trisubstituted double bond and a tertiary isocyanate group. In particular, the ^{13}C NMR resonances of **10** correlated strongly with those of isothiocyanate **15** (Figure 4), reported from the sponge *Trachyopsis aplysinoides*,[23] suggesting that **10** was the corresponding isocyanide derivative. Almost all the NMR values were assigned by $^1H-^1H$ COSY, HSQC, and HMBC correlations as reported in Table 2. Unfortunately, the sample degraded before recording NOE experiments, but the strong similarities of both carbon and proton values with those of isothiocyanate **15** suggested that **10** had the same relative stereochemistry. The absolute stereochemistry of **15** has not yet been defined.

The HRESIMS spectrum of compound **11** was similar to those of the compounds described above, displaying both peaks at *m*/*z* 254 and 205. The presence of an isocyanide functionality was also indicated by a weak IR band at 2130 cm^{-1}. The ^1H NMR spectrum contained two broad 3H singlets at δ 1.61 (3H, bs, H_3-15) and 1.69 (3H, bs, H_3-12), attributed to two vinyl methyls, and a 3H singlet at δ 1.44 (bs, H_3-13), assigned to a methyl linked to a tertiary carbon bearing the −NC function. Two methine broad singlets at δ 4.76 (H-14a) and 4.79 (H-14b) and a triplet at δ 5.11 (1H, H-10), assigned to an exomethylene and an olefinic proton, respectively, were also observed. Analysis of the ^{13}C NMR spectrum confirmed the presence of two double bonds, one of which was disubstituted [δ 107.8 (CH_2) and 152.3 (C)] and the other trisubstituted [δ 123.9 (CH) and 131.5 (C)]. The remaining resonances were observed between 56.8 and

Table 3. NMR Data[a] of Compound **11**

C	δ_C[b]	m[c]	δ_H[d]	m, *J* (Hz)	HMBC correlations[e]
1	26.7	CH_2	1.41	m	C-2
			1.78	m	
2	38.5	CH_2	1.82	m	C-1, C-13
			1.89	m	
3	56.8	C			
4	38.5	CH_2	1.82	m	C-5
			1.89	m	
5	26.7	CH_2	1.41	m	C-4
			1.78	m	
6	40.9	CH	1.99	m	C-7, C-14
7	152.3	C			
8	34.7	CH_2	2.03	t (8.38)	C-7, C-9, C-14
9	26.5	CH_2	2.10	m	C-8, C-10, C-11
10	123.9	CH	5.11	t (6.82)	C-9, C-12, C-15
11	131.5	C			
12	25.6	CH_3	1.69	bs	C-10, C-11
13	25.3	CH_3	1.44	bs	C-2, C-4
14	107.8	CH_2	4.76	bs	C-6, C-7
			4.79	bs	
15	17.6	CH_3	1.61	bs	C-10, C-11
16	nd	NC			

[a] Bruker 300, 400, and 500 MHz; $CDCl_3$; chemical shifts (ppm) referred to C*H*Cl_3 (δ 7.26) for proton and to *C*DCl_3 (δ 77.0) for carbon. [b] Assignments by HMQC and HMBC experiments. [c] By DEPT sequence. [d] Assignments by $^1H-^1H$ COSY experiment. [e] $J = 10$ Hz.

17.6 and were attributed to sp^3 carbons. Again, the −NC carbon was not detected. Analysis of $^1H-^1H$ COSY, HSQC, and HMBC experiments led to a bisabolane-type structure **11**, closely related to those of both the co-occurring 3-isocyano-theonellin (**9**) and the isomeric cyanide **16** (Figure 4), recently reported from an *Axinyssa* sponge.[24] In particular, comparison of the proton and carbon chemical shifts of **11** (Table 3) with those of **16** showed that the two compounds differed only in the position of the double bond at C-7.

The relative stereochemistry at C-3 and C-6 was also suggested to be the same, on the basis of the chemical shift values for C-2/C-4 (δ 38.5) and C-1/C-5 (δ 26.7) of **11** with those reported for **9**[3] and **16**.[24] The absolute stereochemistry remained undetermined.

To test the biological properties of these molecules and to establish the probable role as deterrents against predators, feeding experiments with *Carassius auratus* were carried out on all of the isolated compounds, as described in the literature.[25] Isocyanides **5**−**7** were found to be active at a concentration of 50 $\mu g/cm^2$. However, the higher concentration of these dietary isocyanides in the mantle of the mollusk relative to the digestive gland suggests that *P. pustulosa* could efficiently transfer selected chemicals to be used in the defense mechanisms to the external part of the body.

In summary, this study has confirmed that the secondary metabolite pattern of *P. pustulosa* is characterized by isocyanosesquiterpenes, which even though are mainly concentrated in the mantle, are most likely sequestered from dietary sponges, as already reported in the literature,[1] but it has also revealed for the first time the ability of this mollusk to select and accumulate isocyanide diterpenes.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Jasco DIP 370 digital polarimeter. IR spectra were measured on a Biorad FTS 155 FTIR spectrophotometer. 1D- and 2D-NMR spectra were recorded on a Bruker 400 AMX (400.13 MHz) and a Bruker 500 AMX (500.13 MHz) in $CDCl_3$ (δ values are reported referred to C*H*Cl_3 at 7.26 ppm), and ^{13}C NMR were recorded on a 300 AMX Bruker

(75.47 MHz) (δ values are reported to $CDCl_3$, 77.0 ppm). HRESIMS were carried out on a Micromass Q-TOF micro. An HPLC Waters 501 pump with a refractometer detector was used equipped with a Kromasil Silica, 5 μm (250 × 4.60 mm, Phenomenex) direct-phase column and with a Kromasil C-18, 5 μm (250 × 4.60 mm, Phenomenex) reverse-phase column. TLC plates (silica gel 60 F254) were from Merck (Darmstadt, Germany), and silica gel powder (silica gel 60 0.063−0.200 mm) was from Merck (Darmstadt, Germany). Sephadex LH-20 was from Amersham Pharmacia Biotech (Uppsala, Sweden).

Animal Material. *P. pustulosa* (18 specimens) was collected by scuba diving at a depth of 20 m along the coast of Hainan Island, in the South Chinese Sea, during January 2002. Biological material was immediately frozen, then transferred to ICB Naples, and stored at −80 °C until extraction. A voucher specimen is stored for inspection at ICB (HN-15).

Extraction and Isolation. *P. pustulosa* individuals were carefully dissected into mantle and internal organs that were separately extracted by acetone using ultrasound. Filtration of the two homogenates gave an aqueous Me_2CO filtrate that was concentrated in vacuo to give a gummy residue. The residue was suspended in H_2O and extracted sequentially with diethyl ether and *n*-BuOH. The mantle ether extract (150 mg) was subjected to Sephadex LH-20 chromatography eluting with chloroform/methanol, 1:1, to give two fractions, A (16 mg) and B (93 mg), along with a glyceride mixture (24 mg). Mixture A was submitted to reverse-phase HPLC purification (eluent: MeOH/H_2O, 9:1, flow rate 1 mL/min) to afford **4** (1.7 mg, 1.13%) and **5** (1.1 mg, 0.73%). Mixture B was purified on a silica gel column eluted with a light petroleum ether/diethyl ether gradient, giving **6** (2.6 mg, 1.73%), a more polar fraction that afforded by *n*-phase HPLC (eluent: hexane/AcOEt, 74:26, flow rate 1 mL/min) **7** (0.9 mg, 0.6%), and a less polar fraction (49 mg) constituted by a mixture of sesquiterpenes. This mixture was chromatographed on silica gel, eluted with a light petroleum ether/diethyl ether gradient, to obtain **8** (20 mg, 13.3%), along with **9** (3 mg, 2%), and a fraction (8 mg) that afforded by *n*-phase HPLC purification (eluent: hexane/AcOEt, 99:1, flow rate 1 mL/min) **10** (0.6 mg, 0.4%) and **11** (0.7 mg, 0.5%).

The digestive gland ether extract (236 mg) was purified by a Sephadex LH-20 chromatographic column (chloroform/methanol, 1:1) to give *ent*-stylotelline (**8**, 40 mg, 17%) and ergosta-4,22-dien-3-one[20] (44 mg, 19%) as the main metabolites.

Compound 8: oil, R_f 0.5 (petroleum ether/diethyl ether, 95:5); $[\alpha]_D$ +23.0 (*c* 2, $CHCl_3$); IR (KBr) ν_{max} 2139 cm^{-1}; ^1H and ^{13}C NMR data in Table 1; HRESIMS *m/z* 254.1889 [M + Na] (calcd for $C_{16}H_{25}N$+Na 254.1885).

Compound 10: oil, R_f 0.4 (petroleum ether/diethyl ether, 95:5); $[\alpha]_D$ +28.6 (*c* 0.06, $CHCl_3$); IR (KBr) ν_{max} 2957, 2927, 2123, 1706 cm^{-1}; ^1H and ^{13}C NMR data in Table 2; HRESIMS *m/z* 254.1883 [M + Na] (calcd for $C_{16}H_{25}N$+Na 254.1885).

Compound 11: oil, R_f 0.3 (petroleum ether/diethyl ether, 95:5); IR (KBr) ν_{max} 2930, 2862, 2130, 1691 cm^{-1}; ^1H and ^{13}C NMR data in Table 3; HRESIMS *m/z* 254.1890 [M + Na] (calcd for $C_{16}H_{25}N$+Na 254.1885).

Conversion of 8 into 14. Compound **8** was dissolved in 1% HCl/MeOH, 1 h at room temperature, to give (−)-δ-selinene (**14**), $[\alpha]_D$ −105 (*c* 0.2, $CHCl_3$) ($[\alpha]_D$ lit.: −191). ^1H NMR and mass spectra were identical with those reported in the literature.[22]

Biological Assays. Feeding-deterrence tests against goldfish (*Carassius auratus*) were conducted according to literature procedures.[25] Compounds **4**−**11** were assayed at 50 μg/cm^2.

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Note Added after ASAP: In the version posted on September 18, 2004, there was an error in the ^1H NMR data of **8** and a few typographical errors. These have been corrected in the version posted on September 22, 2004.

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