Cytotoxic Nitrogenous Terpenoids from Two South China Sea Nudibranchs *Phyllidiella pustulosa*, *Phyllidia coelestis*, and Their Sponge-Prey *Acanthella cavernosa*

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Abstract: A detailed chemical investigation of two South China Sea nudibranchs *Phyllidiella pustulosa* and *Phyllidia coelestis*, as well as their possible sponge-prey *Acanthella cavernosa*, led to the isolation of one new nitrogenous cadinane-type sesquiterpenoid xidaoisocyanate A (1), one new naturally occurring nitrogen-containing kalihinane-type diterpenoid bisformamidokalihinol A (16), along with 17 known nitrogenous terpenoids (2–15, 17–19). The structures of all the isolates were elucidated by detailed spectroscopic analysis and by the comparison of their spectroscopic data with those reported in the literature. In addition, the absolute stereochemistry of the previously reported axiriabiline A (5) was determined by X-ray diffraction (XRD) analysis. In a bioassay, the bisabolane-type sesquiterpenoids 8, 10, and 11 exhibited cytotoxicity against several human cancer cell lines.

Keywords: nitrogenous terpenoids; South China Sea; sponge; nudibranch; cytotoxicity

1. Introduction

Sea slugs of the genus *Phyllidiella* and *Phyllidia* are prolific in the South China Sea. They are well known for their ability to ingest toxic nitrogenous sesquiterpenoids from their diets, and use either these metabolites themselves or their biosynthetically transformed derivatives as a weapon for chemical defense [1–7]. An intriguing ecological study showed that when sea slugs are under attack, they release a lot of mucus containing these nitrogenous metabolites to poison their enemies [8]. The dietary origin of nitrogenous sesquiterpenoids has been supported by chemical investigations involving the isolation of such metabolites from both nudibranchs and their sponge-preys [9–13].

Marine sponges of the genus *Acanthella* are well known as a rich source of diverse diterpenoids and sesquiterpenoids containing nitrogenous functional groups, including cyano, isocyano, isothiocyano, and formamido functionalities [14–18]. Many of these secondary metabolites merit further investigation due to their various biological activities ranging from cytotoxic [15], antimalarial [19,20], and antimicrobial [21,22] to antifouling properties [14,23–27]. Some of them, with novel structures and...
promising biological activities, have attracted much attention from chemists seeking to perform their total synthesis in parallel with intensive biological studies towards new drug leads \[28–31\].

In our previous chemical investigation on South China Sea (Hainan) nudibranchs and sponges, nitrogenous terpenoids were isolated and structurally characterized \[1,17,18,32–34\]. In the course of our continuing project on searching for chemically fascinating and biologically active secondary metabolites from Hainan marine molluscs, as well as the chemical ecology study between nudibranchs and their sponge-preys, we made different collections of two nudibranchs, Phyllidiella pustulosa and Phyllidia coelestis, as well as their sponge-prey Acanthella cavernosa, from the same location (Xidao Island, Hainan Province, China), with the aim of accumulating their nitrogenous metabolites for further study of their bioactivities, as well as studying the dietary relationship between P. pustulos, P. coelestis, and their sponge-prey A. cavernosa.

2. Results

Chemical investigation of the collected two nudibranchs, P. pustulosa and P. coelestis, as well as one sponge, A. cavernosa, led to the isolation of one new cadinane-type sesquiterpenoid (1), one naturally occurring kalihinane-type diterpenoid (16), along with 14 known sesquiterpenoids (2–15) and three known diterpenoids (17–19) (Figure 1). All the compounds contain nitrogen atoms in different functional groups, such as isocyanate, isothiocyanate, and formamide. Herein, we describe the isolation, structure elucidation, and cytotoxic activity of these compounds, as well as their possible biosynthetic origin influenced by the prey-predator relationship.

Figure 1. Structures of compounds 1–20.

2.1. Phyllidiella pustulosa

The Et$_2$O soluble portion of the acetone extract of the mollusc P. pustulosa was subjected to silica gel chromatography (petroleum ether/ether gradient). Guided by NMR analysis, the selected terpene-containing fractions were subsequently purified on repeated column chromatography (silica gel, Sephadex LH-20, reversed phase-C18 and RP-HPLC) to afford one new cadinane-type sesquiterpenoid (1), along with nine known metabolites (2, 3, 6–8, 12–14, 17) (Figure 1). The known compounds were identified as two cadinane-type sesquiterpenoids: halichon G (2) \[35\] and 10-isothiocyanato-4-cadinene (3) \[13,28,36–38\], one eudesmane-type sesquiterpenoid: 11-formamido-7\'H-eudesm-5-ene (6) \[39,40\], two bisabolane-type sesquiterpenoids: $\Delta^7,14$-3-isocyanotheonellin (7) \[1,41\] and 3-isocyanotheonellin...
(8) [1], two aromadendrane-type sesquiterpenoids: 1-isothiocyanatoaromadendrane (12) [42] and axamide-2 (13) [43,44], one mixture of pupukeanane-type sesquiterpenoids: 9-thiocyanatopupukeanane isomers (14) [6], and one kalihinane-type diterpenoid: kalihinol A (17) [45].

 Compound 1, namely xidaoisocyanate A, was obtained as a colorless oil, $\alpha_{D}^{20} = -3.6$ (c 0.1, MeOH). Its molecular formula, $C_{16}H_{25}NO$, was established by HREIMS ($m/z$ 247.1927, [M]+, calcd. 247.1936), indicating five degrees of unsaturation (Figures S1 and S2). The diagnostic $^1$H and $^{13}$C NMR resonances, as well as coupling constants of the connected protons (Table 1, Figures S3 and S4), indicated the presence of one trisubstituted double bond ($\delta^1H$ 5.58, s, $\delta^{13}C$ 130.4, CH; $\delta^{13}C$ 136.5, qC) and four methyl groups ($\delta^1H$ 0.97 (3H, d, Me-12); 0.90 (3H, d, Me-13); 1.40 (3H, s, Me-14); 1.42 (3H, t, Me-15)). The typical $^{13}$C NMR signal of sp$^3$ quaternary carbon ($\delta^{13}C$ 63.3, qC), bearing in mind the odd molecular weight of 1, suggested the presence of an isocyano group ($-NC$ group). The above functionalities account for three out of the five degrees of unsaturation, suggesting a bicyclic ring system in 1. The above structural features were reminiscent of the co-occurring molecule 2, as well as a previously reported axinisothiocyanate J (20) [46] which was isolated from the sponge Axinyssa sp.

### Table 1. $^1$H and $^{13}$C NMR data of 1 and 16, and their model compounds 20 and 17, respectively, recorded in CDCl$_3$ $^a$.

| No. | $\delta^1H$ (Mult if $J$ in Hz) | $\delta^{13}C$ (Char) | $\delta^{13}C$ (Char) | No. | $\delta^1H$ (Mult if $J$ in Hz) | $\delta^{13}C$ (Char) | $\delta^{13}C$ (Char) |
|-----|--------------------------------|------------------------|------------------------|-----|--------------------------------|------------------------|------------------------|
| 1   | 1.96 m                         | 45.9 CH                 | 46.9 CH                 | 1   | 1.37 m                         | 42.6 CH                 | 43.6 CH                 |
| 2a  | 1.69 m                         | 21.6 CH$_2$             | 21.6 CH$_2$             | 2a  | 1.47 m                         | 21.7 CH$_2$             | 21.6 CH$_2$             |
| 2b  | 1.94 m                         |                         | 2b                      | 1.61 m                        |                         |
| 3a  | 1.53 m                         | 36.3 CH$_2$             | 36.2 CH$_2$             | 3   | 1.51 m, 2H                     | 33.7 CH$_2$             | 32.6 CH$_2$             |
| 3b  | 1.96 m                         |                         |                         | 3b  | 1.61 m                         |                        |
| 4   | -                              | 69.5 qC                 | 69.3 qC                 | 4   | -                              | 71.6 qC                 | 70.5 qC                 |
| 5   | 5.58 s                         | 130.4 CH                | 130.2 CH                | 5   | 4.18 (d, 10.4)                 | 59.8 CH                 | 63.7 CH                 |
| 6   | -                              | 136.5 qC                | 137.2 qC                | 6   | 2.35 m                         | 36.6 CH                 | 36.0 CH                 |
| 7   | 1.64 m                         | 47.3 CH                 | 47.4 CH                 | 7   | 1.57 m                         | 45.8 CH                 | 48.4 CH                 |
| 8a  | 1.49 m                         | 21.8 CH$_2$             | 22.5 CH$_2$             | 8a  | 1.62 m                         | 23.1 CH$_2$             | 21.9 CH$_2$             |
| 8b  | 1.69 m                         |                         |                         | 8b  | 1.02 m                         |                         |
| 9a  | 1.51 m                         | 39.4 CH$_2$             | 40.4 CH$_2$             | 9a  | 1.72 m                         | 40.7 CH$_2$             | 39.7 CH$_2$             |
| 9b  | 2.01 (d, 10.0)                 |                         |                         | 9b  | 1.55 m                         |                         |
| 10  | -                              | 63.3 qC                 | 66.0 qC                 | 10  | -                              | 55.0 qC                 | 59.0 qC                 |
| 11  | 2.14 m                         | 26.8 CH                 | 26.8 CH                 | 11  | -                              | 79.0 qC                 | 76.8 qC                 |
| 12  | 0.97 (d, 6.8)                  | 22.1 CH$_3$             | 22.1 CH                 | 12  | 1.48 m                         | 38.1 CH$_2$             | 38.0 CH$_2$             |
|     | 1.57 m                         |                         |                         | 12b | 1.57 m                         |                         |
| 13  | 0.90 (d, 6.8)                  | 17.5 CH$_3$             | 17.5 CH                 | 13  | 1.99 m                         | 27.7 CH$_2$             | 27.4 CH$_2$             |
|     | 2.06 m                         |                         |                         | 13b | 2.06 m                         |                         |
| 14  | 1.40 s                         | 27.1 CH$_3$             | 26.7 CH$_3$             | 14  | 3.68 (dd, 1.2, 4.4)            | 64.4 CH                 | 64.1 CH                 |
| 15  | 1.42 (t, 1.8)                  | 28.9 CH$_3$             | 28.2 CH$_3$             | 15  | -                              | 76.7 qC                 | 76.0 qC                 |
| NC (1) and NCS (20) | n.d. $^b$ | n.d. $^b$ | | 16 | 1.37 s                         | 23.5 CH$_3$             | 22.8 CH$_3$             |
| 17  | 1.31 s                         | 31.4 CH$_3$             | 30.5 CH$_3$             |
| 18  | 1.27 s                         | 19.7 CH$_3$             | 19.2 CH$_3$             |
| 19  | 1.19 s                         | 18.8 CH$_3$             | 29.0 CH$_3$             |
| 20  | 1.18 s                         | 29.0 CH$_3$             | 20.7 CH$_3$             |
| CHO-1 or NC | 8.25 (d, 12.0) | 163.7 CH | 157.0 qC | CHO-2 or NC | 8.10 (d, 11.4) | 167.6 CH | 153.0 qC |

$^a$ Assignments were deduced by the analysis of 1D and 2D NMR spectra. $^b$ n.d. means not detected.
Detailed comparison of the NMR data revealed that 1 should possess the same cadinane ring system as 20. The only significant difference of these two compounds was the presence of an isocyano group at C-10 in 1 instead of the isothiocyanato group (−NCS group) in 20. According to this, the 13C NMR data of C-1, C-9, and C-10 in 1 were upfield shifted (δC 45.9, CH, Δδ = −1.0 ppm; δC 39.4, CH2, Δδ = −1.0 ppm; δC 63.3, qC, Δδ = −2.7 ppm), respectively, compared with those in 20. Further 2D NMR spectra, including COSY, HSQC, and HMBC (Figures S5–S7), allowed the unambiguous determination of the planar structure of compound 1 (Figure 2).

![Figure 2. 1H-1H COSY, key HMBC and NOESY correlations of compounds 1 and 16.](image)

The relative configuration of 1 was deduced by NOESY spectra (Figure 2 and Figure S8). The NOE correlation between H-5 (δH 5.58, s) and H-11 (δH 2.14, m) indicated the Z-geometry of Δ5,6. The correlations of H-1 (δH 1.96, m) with Me-15 (δH 1.42, t) and H-7 (δH 1.64, m) indicated that these protons were on the same side of the molecule and were tentatively assigned to be α-oriented. Furthermore, the obvious NOE correlation between Me-15 and H-2b (1.94, m), and between Me-14 (1.40, s) and H-2a (1.69, m) suggested the α-orientation of Me-14. Therefore, the structure of compound 1 was determined as shown in Figure 1, which was further confirmed by its similar NMR data to those of axinisothiocyanate J (20) based on a biogenetic consideration [46]. In fact, compound 1 was identified as a C-10 epimer of a known isocyanosesquiterpene alcohol, which was first isolated from the nudibranch Phyllidia pustulosa [12].

2.2. Phyllidia coelestis

The abovementioned usual workup of the Et2O-soluble portion of the acetone extract of the animals of P. coelestis yielded six pure compounds: 6, 8–11, and 14 (Figure 1). The known compounds were identified as one eudesmane-type sesquiterpenoid: 6 [39,40], four bisabolane-type sesquiterpenoids: 8 [1], theonellin formamide (9) [33], theonellin isothiocyanate (10) [33], and 7-isocyaano-7,8-dihydro-α-bisabolene (11) [42], and one mixture of pupukeanane-type sesquiterpenoids: 14 [6] by direct comparison of its NMR data and specific rotation with those reported in the literature.

2.3. Acanthella cavernosa

The frozen A. cavernosa animals were cut into pieces and exhaustively extracted by acetone. The Et2O-soluble portion of the acetone extract was repeatedly chromatographed to yield pure compounds 4, 5, 15, 16, 18, and 19 (Figure 1). The known compounds were readily identified as one cadinane-type sesquiterpenoid: 10-formamido-4-cadinene (4) [24], one eudesmane-type sesquiterpenoid: arixiabiline A (5) [32], one spiroaxane-type sesquiterpenoid: axamide-3 (15) [27], along with two kalihinane-type diterpenoids: 10β-formamido-5β-isothiocyanatokalihinol-A (18) [14] and 10β-formamido-5-isocyanatokalihinol-A (19) [14] by comparing their NMR spectroscopic data and specific optical rotation with those reported in the literature.
Compound 16 was isolated as an optically active colorless oil, $[\alpha]_D^{20} +19$ (c 0.1, CHCl$_3$). Its molecular formula was determined as C$_{22}$H$_{37}$N$_2$O$_4$Cl by HRESIMS ($m/z$ 429.2522 [M+H]$^+$, calcd. 429.2515), indicating five degrees of unsaturation (Figure S9). The IR spectrum (Figure S10) of 16 showed absorptions at $\nu_{\text{max}}$ 1665 cm$^{-1}$ and 3440 cm$^{-1}$, indicating the presence of the amide carbonyl and hydroxy groups, respectively. The $^{13}$C NMR and DEPT spectra of 16 displayed 22 carbon signals, including five sp$^3$ methyls, six sp$^3$ methylenes, five sp$^3$ methines, four sp$^3$ quaternary carbons, and two sp$^2$ methines. The spectroscopic data (Table 1, Figures S11 and S12) showed highly similarity to those of co-occurring related known compounds 18 and 19, indicating that 16 is also a kalihinane-type diterpenoid. In fact, they differed from each other only by the substitution at C-5 position of the kalihinane ring. Bearing in mind the two additional protons present in its molecular formula in comparison to 19, a –NHCHO group ($\delta_H$ 8.10 s, $\delta_C$ 167.6, CH) should be attached to the C-5 of compound 16. Intriguingly, resonances for both formamides were observed as a plethora of signals between $\delta_H$ 8.0 and 8.3. These included eight signals arising from the four isomeric arrangements possible for the two formamides at C-5 and C-10 [47]. Detailed analysis of the 1D and 2D NMR spectra, including $^1$H–$^1$H COSY, HSQC, and HMBC (Figures S13–S15), allowed the establishment of the planar structure of 16 (Figure 2), the same as a known compound named bisformamidokalihinol A, which was obtained from the hydrolysis of kalihinol A with acetic acid [48].

The relative configuration of 16 was also determined to be the same as co-occurring compounds 17–19 by careful interpretation of its NOESY spectrum with the clear NOE correlations of H-1/H-7, H-5/H-6/H$_3$-20, and H$_2$-19/NHCHO at C-5 (Figure 2 and Figure S16). Since the absolute configuration of 17 has been previously determined by total synthesis [29], from a biogenetic point of view, the absolute configuration of compound 16 was tentatively assigned as 15,4R,5R,6S,7S,10S,11R,14S.

It is worth noting that compound 5 was previously isolated from the Hainan sponge Axinysa variabilis, and its absolute configuration was determined by a combination of ROESY experiment and time dependent density functional theory-electronic circular dichroism (TDDFT-ECD) calculation [32]. In this work, we obtained a single crystal of 5, and X-ray diffraction (XRD) analysis on a suitable crystal of 5 by employing Ga Kα radiation ($\lambda = 1.34139$ Å) with small Flack parameter 0.02 (16) allowed not only the unambiguous definition of the planar structure as illustrated in Figure 3, but also the revision of its absolute configuration from 4S,5R,10S to 4R,5S,10S.

![Figure 3. Perspective Oak Ridge Thermal Ellipsoid Plot (ORTEP) drawing of the X-ray structure of 5.](image-url)
a preliminary structure-activity relationship could be addressed, that is, the bisbolane skeleton might be good for activity, while regarding the inactive compounds 7 and 9, the terminal olefin or the formamide group might be harmful for activity. More diverse bisabolenes should be discovered and tested for cytotoxicity to support our proposal.

Table 2. Cytotoxicity of compounds 1–19 against four human cancer cell lines.

| Compounds | A549 IC_50 (µM) | HT-29 IC_50 (µM) | SNU-398 IC_50 (µM) | Capan-1 IC_50 (µM) |
|-----------|----------------|-----------------|-----------------|-----------------|
| 8         | 8.60 ± 6.36    | 3.35 ± 3.12     | 0.50 ± 0.46     | 1.98 ± 1.76     |
| 10        | >50            | >50             | 2.15 ± 0.93     | >50             |
| 11        | >50            | >50             | 0.50 ± 0.35     | >50             |
| VCR       | 10.13 nM       | 0.23 nM         | 0.04 nM         | 0.30 nM         |

*Compounds 1–7, 9, 12–19 were considered to be inactive with IC_50 values of more than 50 µM; VCR: vincristine.

3. Discussion

In recent years, several marine molluscs were found by our group to contain the same or similar secondary metabolites as those in marine corals or sponges, which was further proved to be due to the predator–prey relationship between these animals. For example, isoquinolonequinones were discovered from both the nudibranch *Jorunna funebris* and its sponge-prey *Xestospongia* sp. [49,50], while cladiellane-type diterpenoids were isolated from both the nudibranch *Tritoniopsis elegans* and its soft coral prey *Cladiella krempfi* [51]. In this study, similar results were observed by the chemical investigation of the three title animals. As shown in Figure 4, by comparison of the typical nitrogenous terpenoids in the two nudibranchs *P. pustulosa* and *P. coelestis* with those in the sponge *A. cavernosa*, four common structural skeletons were observed in both *P. pustulosa* and *A. cavernosa*, including cadinane, eudesmane, aromadendrane, and kalihinane, whereas one common eudesmane skeleton was found in all three animals. In addition, our previous chemical investigation of the marine sponge *A. variabilis* from the same water area in the South China Sea revealed the main secondary metabolites as bisabolene sesquiterpenoids [52], which was the common skeleton found in both *P. pustulosa* and *P. coelestis* (Figure 4). Therefore, on the basis of these research observations, we hold the belief that the two nudibranchs *P. pustulosa* and *P. coelestis* feed on the sponges *A. cavernosa* and *A. variabilis* and accumulate the useful dietary metabolites from the sponges, especially those toxic isocyanide derivatives, to be employed as their own chemical defensive agents for surviving in the harsh marine living environment. More intriguingly, it is obvious that one nudibranch can feed on various sponges to obtain diverse isocyanide metabolites, so as to use them as specially appointed chemical weapons on particular occasions.

In summary, the chemical investigation of the two nudibranchs *P. pustulosa* and *P. coelestis*, as well as the sponge *A. cavernosa*, led to the isolation and determination of 19 nitrogenous terpenoids with high chemical diversity. In fact, a total of seven different chemical skeletons were observed: four cadinane-type sesquiterpenoids (1–4), two eudesmane-type sesquiterpenoids (5–6), five bisabolene-type sesquiterpenoids (7–11), two aromadendrane-type sesquiterpenoids (12 and 13), one pupukeanane-type sesquiterpenoid (14), one spiroaxane-type sesquiterpenoid (15), and four kalihinane-type diterpenoids (16–19). Their structures including relative stereochemistry were elucidated by comprehensive NMR analyses. The absolute configuration of two new metabolites (1 and 16) were tentatively assigned based on the biogenetic consideration, whereas that of the known compound 5 was revised by the XRD analysis. In bioassay, the bisabolane-type sesquiterpenoids 8, 10, and 11 displayed considerable cytotoxicity against several cancer cell lines, which is worth further pharmacological study. Further chemical ecological research on the basis of the predator–prey relationship to prove our hypothesis would be interesting to be conducted in the future.
4. Materials and Methods

4.1. General Experimental Procedures

Optical rotations were measured in CHCl$_3$ on a Perkin-Elmer 241MC polarimeter (PerkinElmer Inc., Waltham, MA, USA). IR spectra were recorded on a Nicolet 6700 spectrometer (Thermo Scientific, Waltham, MA, USA) with KBr pellets; peaks are reported in cm$^{-1}$. 1D and 2D NMR spectra were measured on a Bruker DRX-400 or Bruker DRX-500 spectrometer (Bruker Biospin AG, Fallanden, Germany), using the residual CHCl$_3$ signal ($\delta_H$ 7.26 ppm) as an internal standard for $^1$H NMR and CDCl$_3$ ($\delta_C$ 77.00 ppm) for $^{13}$C NMR. Chemical shifts are expressed in $\delta$ (ppm) and coupling constants ($J$) in Hz. $^1$H and $^{13}$C NMR assignments were supported by $^1$H–$^1$H COSY, HSQC, HMBC, and NOESY experiments. EIMS and HREIMS spectra were recorded on a Finnigan-MAT-95 mass spectrometer (FinniganMAT, San Jose, CA, USA). HRESIMS spectra were recorded on an Agilent G6250 Q-TOF (Agilent, Santa Clara, CA, USA). Reversed-phase (RP) HPLC purification was carried out on an Agilent 1260 series liquid chromatography equipped with a DAD G1315D detector at 210 and 254 nm and with a semi-preparative ODS-HG-5 column (5 µm, 250 × 9.4 mm). Commercial silica gel (Qingdao Haiyang Chemical Group Co., Ltd., Qingdao, China, 200–300 and 300–400 mesh) was used for column chromatography, and precoated silica gel plates (Yan Tai Zi Fu Chemical Group Co., Yantai, China, G60 F-254) were used for analytical Thin-layer chromatography (TLC). Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde H$_2$SO$_4$ reagent. All the chemicals were obtained from commercial sources. All solvents used for column chromatography (CC) were of analytical grade, and solvents used for HPLC were of HPLC grade.
4.2. Biological Material, Extraction, and Isolation

4.2.1. Biological Material

The molluscs and sponges were collected using scuba at Xidao Island, Hainan Province, China, in March 2014, at a depth of −15 to −20 m, and identified by Professor Xiu-Bao Li from Hainan University. The voucher sample is deposited at the Shanghai Institute of Materia Medica, CAS.

4.2.2. Extraction and Isolation of 1–19

The lyophilized bodies of *P. pustulosa* (24 specimens, 11.1 g, dry weight) were carefully dissected into internal organs and mantle that were separately extracted by acetone using ultrasound. Filtration of the two homogenates gave an aqueous-Me₂CO filtrate that was concentrated in vacuo to give a gummy residue. The residue was suspended in H₂O and extracted sequentially with diethyl ether and n-BuOH. The mantle ether extract (431.3 mg) was subjected to a silica gel column eluting with light petroleum ether/diethyl ether gradient to yield 11 fractions (A–K), including pure compounds 3 (5.3 mg), 12 (2.6 mg), and 13 (1.0 mg). A less polar fraction E was chromatographed over Sephadex LH-20 eluting with CHCl₃/MeOH (2:1), followed by silica gel CC (PE/CHCl₃, 100:1 to 50:1) to afford 7 (2.0 mg), 8 (2.2 mg), and 14 (2.6 mg). A middle polar fraction I was separated by a column of Sephadex LH-20 eluting with CHCl₃/MeOH (1:1), followed by ODS CC (MeOH/H₂O, 60:40) to afford 1 (1.5 mg) and 2 (1.0 mg). Fraction J was chromatographed over Sephadex LH-20 eluting with CHCl₃/MeOH (1:1), followed by silica gel CC (PE/CHCl₃, 6:4), and was further purified by ODS CC (MeOH/H₂O, 50:50) to yield 5 (2.0 mg) and 17 (3.1 mg). The digestive gland ether extract (60.0 mg) was purified by a silica gel column eluting with light petroleum ether/diethyl ether gradient, followed by a similar procedure as above, to give compounds 3 (1.3 mg), 5 (0.5 mg), 7 (1.3 mg), 8 (1.8 mg), 14 (0.9 mg), and 17 (1.9 mg).

The lyophilized bodies of *P. coelestis* (seven specimens, 25.5 g, dry weight) were extracted by acetone using ultrasound. The extracts of both internal organs and mantle were combined due to the similar TLC results, to give 700 mg extract. An approach similar to the abovementioned fractional extraction was applied to give a total of seven fractions (A–G). Compounds 8 (5.2 mg) and 9 (3.4 mg) were obtained directly from fractions B and G after purification by HPLC, respectively. Fraction B was chromatographed over Sephadex LH-20 eluting with PE/CHCl₃/MeOH (2:1:1), followed by HPLC purification to give compounds 10 (1.5 mg) and 11 (1.2 mg). Fraction P was treated by the same procedure as above to give compound 6 (1.7 mg).

The frozen *A. cavernosa* sponges (55 g, dry weight) were cut into pieces and extracted exhaustively with acetone at room temperature (6 × 2.0 L). The organic extract was evaporated to give a brown residue, which was then partitioned between H₂O and Et₂O. The upper layer was concentrated under reduced pressure to give a red residue (1.0 g). The resultant residue was separated into six fractions (A–F) by gradient silica gel column chromatography. The resulting fractions were then fractionated into sub-fractions by Sephadex LH-20. The sub-fraction E4 of fraction E gave compounds 4 (3.1 mg), 6 (4.1 mg), and 15 (2.7 mg).

*Xidaoisocyanate A* (1), colorless oil, [α]D²⁰ = −3.6 (c 0.1, MeOH); for 1H and 13C NMR spectroscopic data, see Table 1; HREIMS: m/z calcld for C₁₆H₂₃NO [M]+: 247.1936; found: 247.1927.

*Bisformamidokalihinol A* (16), colorless oil, [α]D²⁰ +19 (c 0.1, CHCl₃); for 1H and 13C NMR spectroscopic data, see Table 1; HREIMS: m/z calcld for C₂₂H₃₈N₂O₂Cl [M+H]+: 429.2515; found: 429.2522.

*Axtirabiline A* (5), colorless crystal, m.p. 105–107 °C, [α]D²⁰ = −123 (c 0.1, CHCl₃); X-ray crystal data for compound 5: C₁₆H₂₇NO M = 249.38, orthorhombic, a = 11.5594(2) Å, b = 12.0694(2) Å, c = 21.2049(4) Å, α = 90.00°, β = 90.00°, γ = 90.00°, V = 2958.40(9) Å³, T = 170.01 K, space group P2(1)2(1)2(1), Z = 8, 28095 reflections measured, 5616 independent reflections (Rint = 0.0569). The final R₁ values were 0.0416 (I > 2σ(I)). The final wR(F²) values were 0.1051 (I > 2σ(I)). The final R₁ values were 0.0446 (all data). The final wR(F²) values were 0.1081 (all data). The structure was solved by direct methods (SHELXS97).
and refined using full-matrix least-squares difference Fourier techniques. All non-hydrogen atoms were refined anisotropically, and all hydrogen atoms were placed in idealized positions and refined as riding atoms with their related isotropic parameters. Crystallographic data (excluding structure factors) for the structure in this paper have been deposited with the Cambridge Crystallographic Data Center as supplementary publication no. CCDC 1880256. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

4.3. Bioassay Procedures

Cytotoxic Activity

Compounds 1–19 were evaluated for their cytotoxic activity against four human cancer cell lines (A549, HT-29, SNU-398, and Capan-1) using the sulforhodamine B (SRB, Sigma, St. Louis, MO, USA) method. Four cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cytotoxic activity in vitro was indicated in terms of IC\textsubscript{50} (µM), that is, the concentration of a compound that inhibited the proliferation rate of tumor cells by 50% as compared to the untreated control cells. Vincristine was used as a reference drug.

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-3397/17/1/56/s1, Figure S1. LREIMS spectrum of compound 1. Figure S2. HREIMS spectrum of compound 1. Figure S3. H NMR spectrum of compound 1 in CDCl\textsubscript{3}. Figure S4. 13C NMR spectrum of compound 1 in CDCl\textsubscript{3}. Figure S5. HSQC spectrum of compound 1 in CDCl\textsubscript{3}. Figure S6. 1H-1H COSY spectrum of compound 1 in CDCl\textsubscript{3}. Figure S7. HMBC spectrum of compound 1 in CDCl\textsubscript{3}. Figure S8. NOESY spectrum of compound 1 in CDCl\textsubscript{3}. Figure S9. HRESIMS spectrum of compound 16. Figure S10. IR spectrum of compound 16. Figure S11. 1H NMR spectrum of compound 16 in CDCl\textsubscript{3}. Figure S12. 13C NMR spectrum of compound 16 in CDCl\textsubscript{3}. Figure S13. HSQC spectrum of compound 16 in CDCl\textsubscript{3}. Figure S14. 1H-1H COSY spectrum of compound 16 in CDCl\textsubscript{3}. Figure S15. HMBC spectrum of compound 16 in CDCl\textsubscript{3}. Figure S16. NOESY spectrum of compound 16 in CDCl\textsubscript{3}.

Author Contributions: Y.-W.G. and X.-W.L. conceived and designed the experiments; Q.W., W.-T.C., S.-W.L., J.-Y.Y., and X.-J.H. performed the experiments; Q.W., W.-T.C., and Z.-H.M. analyzed the data; L.-G.Y. contributed materials; M.G. and H.W. analyzed the chemical ecology relationship. Y.-W.G., X.-W.L., Q.W., and W.-T.C. wrote the paper. M.G. and H.W. analyzed the chemical ecology relationship.

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References

1. Manzo, E.; Ciavatta, M.L.; Cavagnin, M.; Mollo, E.; Guo, Y.-W.; Cimino, G. Isocyanide terpene metabolites of Phyllidiella pustulosa, a nudibranch from the South China Sea. J. Nat. Prod. 2004, 67, 1701–1704. [CrossRef] [PubMed]

2. Jomoria, T.; Shibutani, T.; Ahmadi, P.; Suzuki, T.; Tanaka, J. A New Isocyanosesquiterpene from the nudibranch Phyllidiella pustulosa. Nat. Prod. Commun. 2015, 10, 1913–1914. [PubMed]

3. Jaisamut, S.; Prabpai, S.; Tancharoen, C.; Yuenyongsawad, S.; Hannongbua, S.; Kongsaeer, P.; Plubrukarn, A. Bridged tricyclic sesquiterpenes from the tubercle nudibranch Phyllidia coelestis Bergh. J. Nat. Prod. 2013, 76, 2158–2161. [CrossRef] [PubMed]

4. White, A.M.; Pierens, G.K.; Skinner-Adams, T.; Andrews, K.T.; Bernhardt, P.V.; Krenske, E.H.; Mollo, E.; Garson, M.J. Antimalarial isocyno and isothiocyanato sesquiterpenes with tri- and bicyclic skeletons from the nudibranch Phyllidia ocellata. J. Nat. Prod. 2015, 78, 1422–1427. [CrossRef]
5. Sim, D.C.; Wayan, M.I.; White, A.M.; Martiningsih, N.W.; Loh, J.J.M.; Cheney, K.L.; Garson, M.J. New sesquiterpenoid isonitriles from three species of Phyllid nudibranchs. *Fitoterapia* 2017, 126, 69–73. [CrossRef] [PubMed]

6. Yasman, Y.; Edrada, R.A.; Wray, V.; Proksch, P. New 9-thiocyanatopupukeanane sesquiterpenes from the nudibranch *Phyllidia varicosa* and its sponge-prey *Axinyssa aculeata*. *J. Nat. Prod.* 2003, 66, 1512–1514. [CrossRef]

7. Dumdei, E.J.; Flowers, A.E.; Garson, M.J.; Moore, C.J. The biosynthesis of sesquiterpene isocyanides and isothiocyanates in the marine sponge *Acanthella cavernosa* (Dendy): Evidence for dietary transfer to the dorid nudibranch *Phyllidia pustulosa*. *Comp. Biochem. Physiol.* 1997, 118, 1385–1392. [CrossRef]

8. Thompson, J.E.; Walker, R.P.; Wratten, S.J.; Faulkner, D.J. A chemical defense mechanism for the nudibranch *Cadlina luteomarginata*. *Tetrahedron* 1982, 38, 1865–1873. [CrossRef]

9. Gulavita, N.K.; de Silva, E.D.; Hagadone, M.R.; Karuso, P.; Scheuer, P.J. Nitrogenous bisabolene sesquiterpenes from the nudibranch *Phyllidia varicosa* and its sponge-prey *Axinyssa aculeata*. *J. Nat. Prod.* 2003, 66, 1512–1514. [CrossRef]

10. Fusetani, N.; Wolstenholme, H.J.; Matsunaga, S. Two new sesquiterpene isonitriles from the nudibranch *Phyllidia pustulosa* isolated from the South China Sea sponge *Acanthella cavernosa*. *Tetrahedron* 2012, 68, 2876–2883. [CrossRef]

11. Wright, A.D. GC-MS and NMR analysis of *Phyllidia pustulosa* and one of its dietary sources, the sponge *Phakellia carduus*. *Comp. Biochem. Physiol.* 2003, 134, 307–313. [CrossRef]

12. Wright, A.D.; McCluskey, A.; Robertson, M.J.; MacGregor, K.A.; Gordon, C.P.; Guenther, J. Anti-malarial, anti-algal, anti-tubercular, anti-bacterial, anti-photosynthetic, and anti-fouling activity of diterpene and diterpene isonitriles from the tropical marine sponge *Phyllidia pustulosa* and *P. varicosa*, and from a Palauan sponge, *Halichondria cf. lendenfeldi*. *J. Org. Chem.* 1991, 56, 3747–3750. [CrossRef]

13. Wright, A.D. GC-MS and NMR analysis of *Phyllidia pustulosa* and one of its dietary sources, the sponge *Phakellia carduus*. *Comp. Biochem. Physiol.* 2003, 134, 307–313. [CrossRef]

14. Wright, A.D.; McCluskey, A.; Robertson, M.J.; MacGregor, K.A.; Gordon, C.P.; Guenther, J. Anti-malarial, anti-algal, anti-tubercular, anti-bacterial, anti-photosynthetic, and anti-fouling activity of diterpene and diterpene isonitriles from the tropical marine sponge *Phyllidia pustulosa* and *P. varicosa*, and from a Palauan sponge, *Halichondria cf. lendenfeldi*. *J. Org. Chem.* 1991, 56, 3747–3750. [CrossRef]

15. Wright, A.D.; McCluskey, A.; Robertson, M.J.; MacGregor, K.A.; Gordon, C.P.; Guenther, J. Anti-malarial, anti-algal, anti-tubercular, anti-bacterial, anti-photosynthetic, and anti-fouling activity of diterpene and diterpene isonitriles from the tropical marine sponge *Phyllidia pustulosa* and *P. varicosa*, and from a Palauan sponge, *Halichondria cf. lendenfeldi*. *J. Org. Chem.* 1991, 56, 3747–3750. [CrossRef]

16. Wright, A.D.; McCluskey, A.; Robertson, M.J.; MacGregor, K.A.; Gordon, C.P.; Guenther, J. Anti-malarial, anti-algal, anti-tubercular, anti-bacterial, anti-photosynthetic, and anti-fouling activity of diterpene and diterpene isonitriles from the tropical marine sponge *Phyllidia pustulosa* and *P. varicosa*, and from a Palauan sponge, *Halichondria cf. lendenfeldi*. *J. Org. Chem.* 1991, 56, 3747–3750. [CrossRef]

17. Wright, A.D.; McCluskey, A.; Robertson, M.J.; MacGregor, K.A.; Gordon, C.P.; Guenther, J. Anti-malarial, anti-algal, anti-tubercular, anti-bacterial, anti-photosynthetic, and anti-fouling activity of diterpene and diterpene isonitriles from the tropical marine sponge *Phyllidia pustulosa* and *P. varicosa*, and from a Palauan sponge, *Halichondria cf. lendenfeldi*. *J. Org. Chem.* 1991, 56, 3747–3750. [CrossRef]
26. Fusetani, N.; Hiroto, H.; Okino, T.; Tomono, Y.; Yoshimura, E. Antifouling activity of isocyanoterpenoids and related compounds isolated from a marine sponge and nudibranchs. J. Nat. Toxins 1996, 5, 249–259.

27. Hirota, H.; Tomono, Y.; Fusetani, N. Terpenoids with antifouling activity against barnacle larvae from the marine sponge Acanthella cavernosa. Tetrahedron 1996, 52, 2359–2368. [CrossRef]

28. Nishikawa, K.; Umezawa, T.; Garson, M.J.; Matsuda, F. Confirmation of the configuration of 10-isothiocyanato-4-cadinene diastereomers through synthesis. J. Nat. Prod. 2012, 75, 2232–2235. [CrossRef]

29. Miyako, A.; Abe, Y.; Sekiya, N.; Mitome, H.; Kawashima, E. Total synthesis of antimalarial diterpenoid (+)-kalihinol A. Chem. Commun. 2012, 48, 901–903. [CrossRef]

30. White, R.D.; Keaney, G.F.; Slown, C.D.; Wood, J.L. Total synthesis of (+/-)-kalihinol C. Org. Lett. 2004, 6, 1123–1126. [CrossRef]

31. White, R.D.; Wood, J.L. Progress toward the total synthesis of kalihinane diterpenes. Org. Lett. 2001, 3, 1825–1827. [CrossRef] [PubMed]

32. Li, X.-W.; Chen, S.-H.; Ye, F.; Mollo, E.; Zhu, W.-L.; Liu, H.-L.; Guo, Y.-W. Axiriabilines A–D, uncommon nitrogenous eudesmane-type sesquiterpenes from the Hainan sponge Axinysa variabilis. Tetrahedron 2017, 73, 5239–5243. [CrossRef]

33. Liu, H.-L.; Xue, D.-Q.; Chen, S.-H.; Li, X.-W.; Guo, Y.-W. New highly oxidized formamidobisabolene-derived sesquiterpenes from a Hainan sponge Axinysa variabilis. Helv. Chim. Acta 2016, 99, 650–653. [CrossRef]

34. Wu, Q.; Nay, B.; Yang, M.; Ni, Y.; Wang, H.; Yao, L.; Li, X. Marine sponges of the genus Axinella ambrosia and epi-eudesmane skeletons from the marine sponge Axinella cannabina. J. Nat. Prod. 2002, 65, 851–855. [CrossRef] [PubMed]

35. Prawat, H.; Mahidol, C.; Kaeweitripob, W.; Prachyawarakorn, V.; Tuntiwachwuttikul, P.; Ruchirawat, S. Sesquiterpene isocyanides, isothiocyanates, thiocyanates, and formamides from the Thai sponge Halichondria sp. Tetrahedron 2016, 72, 4222–4229. [CrossRef]

36. Clark, R.J.; Stapleton, B.L.; Garson, M.J. New isocyano and isothiocyanato terpene metabolites from the tropical marine sponge Acanthella cavernosa. Tetrahedron 2000, 56, 3071–3076. [CrossRef]

37. Nishikawa, K.; Nakahara, H.; Shirokura, Y.; Nogata, Y.; Yoshimura, E.; Umezawa, T.; Okino, T.; Matsuda, F. Total synthesis of 10-isocyano-4-cadinene and determination of its absolute configuration. Org. Lett. 2010, 12, 904–907. [CrossRef]

38. Nishikawa, K.; Nakahara, H.; Shirokura, Y.; Nogata, Y.; Yoshimura, E.; Umezawa, T.; Okino, T.; Matsuda, F. Total synthesis of 10-isocyano-4-cadinene and its stereoisomers and evaluations of antifouling activities. J. Org. Chem. 2011, 76, 6558–6573. [CrossRef]

39. Petrichicheva, N.V.; Duque, C.; Dueñas, A.; Zea, S.; Hara, N.; Fujimoto, Y. New nitrogenous eudesmane-type compounds isolated from the Caribbean sponge Axinysa ambrosia. J. Nat. Prod. 2002, 65, 851–855. [CrossRef]

40. Ciminiello, P.; Fattorusso, E.; Magni, S.; Mayol, L. Nitrogenous sesquiterpenes based on allo-aromadendrane and epi-eudesmane skeletons from the marine sponge Axinella cannabina. Can. J. Chem. 1987, 65, 518–522. [CrossRef]

41. Zubia, E.; Ortega, M.J.; Carballo, J.L. Sesquiterpenes from the sponge Axyissa isabela. J. Nat. Prod. 2008, 71, 2004–2010. [CrossRef] [PubMed]

42. Jimaryatno, P.; Stapleton, B.L.; Hooper, J.N.A.; Brecknell, D.J.; Blanchfield, J.T.; Garson, M.J. A comparison of sesquiterpene scaffolds across different populations of the tropical marine sponge Acanthella cavernosa. J. Nat. Prod. 2007, 70, 1725–1730. [CrossRef] [PubMed]

43. Prawat, H.; Mahidol, C.; Wittayalai, S.; Intachote, P.; Kanchanapoom, T.; Ruchirawat, S. Nitrogenous sesquiterpenes from the Thai marine sponge Halichondria sp. Tetrahedron 2011, 67, 5651–5655. [CrossRef]

44. Zhang, W.; Gavagnin, M.; Guo, Y.-W.; Mollo, E.; Ghiselin, M.T.; Cimino, G. Terpenoid metabolites of the nudibranch Hexabranchus sanguineus from the South China Sea. Tetrahedron 2007, 63, 4725–4729. [CrossRef]

45. Okino, T.; Yoshimura, E.; Hiroto, H.; Fusetani, N. Antifouling kalihinenes from the marine sponge Acanthella cavernosa. Tetrahedron Lett. 1995, 36, 8637–8640. [CrossRef]

46. Shimomura, M.; Miyoka, H.; Yamada, Y. Absolute configuration of marine diterpenoid kalihinol A. Tetrahedron Lett. 1999, 40, 8015–8017. [CrossRef]
49. He, W.-F.; Li, Y.; Feng, M.-T.; Gavagnin, M.; Mollo, E.; Mao, S.C.; Guo, Y.-W. New isoquinolinequinone alkaloids from the South China Sea nudibranch 

\textit{Jorunna funebris} and its possible sponge-prey 

\textit{Xestospongia} sp. 

\textit{Fitoterapia} 2014, 96, 109–114. [CrossRef]

50. Huang, R.-Y.; Chen, W.-T.; Kurtán, T.; Mándi, A.; Ding, J.; Li, J.; Li, X.-W.; Guo, Y.-W. Bioactive isoquinolinequinone alkaloids from the South China Sea nudibranch 

\textit{Jorunna funebris} and its sponge-prey 

\textit{Xestospongia} sp. 

\textit{Future Med. Chem.} 2016, 8, 17–27. [CrossRef]

51. Ciavatta, M.L.; Manzo, E.; Mollo, E.; Mattia, C.A.; Tedesco, C.; Irace, C.; Guo, Y.-W.; Li, X.-B.; Cimino, G.; Gavagnin, M. Tritoniopsins A–D, cladiellane-based diterpenes from the South China Sea nudibranch 

\textit{Tritoniopsis elegans} and its prey 

\textit{Cladiella krempfi}. 

\textit{J. Nat. Prod.} 2011, 74, 1902–1907. [CrossRef] [PubMed]

52. Mao, S.-C.; Guo, Y.-W.; van Soest, R.; Cimino, G. New nitrogenous bisabolene-type sesquiterpenes from a Hainan sponge 

\textit{Axinyss} aff. 

\textit{Variabilis}. 

\textit{Helv. Chim. Acta} 2007, 90, 588–593. [CrossRef]

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