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Antimalarial Isocyano and Isothiocyanato Sesquiterpenes with Tri- and Bicyclic Skeletons from the Nudibranch *Phyllidia ocellata*

Andrew M. White,[†] Gregory K. Pierens,[‡] Tina Skinner-Adams,[§] Katherine T. Andrews,[§] Paul V. Bernhardt,[†] Elizabeth H. Krenske,[†] Ernesto Mollo,[⊥] and Mary J. Garson*[,†]

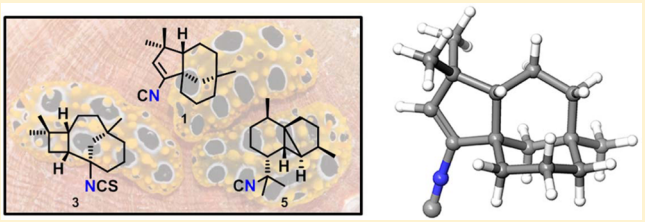
[†]School of Chemistry and Molecular Biosciences and [‡]Centre for Advanced Imaging, The University of Queensland, Brisbane, QLD 4072, Australia

[§]Eskitis Institute for Drug Discovery, Griffith University, Brisbane, QLD 4111, Australia

[⊥]Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, Pozzuoli (Na), 80078, Italy

Supporting Information

ABSTRACT: Five new isocyano/isothiocyanato sesquiterpenes (**1**−**5**) with tri- or bicyclic carbon skeletons have been characterized from Australian specimens of the nudibranch *Phyllidia ocellata*. Spectroscopic analyses at 900 MHz were informed by DFT calculations. The 1*S*, 5*S*, 8*R* configuration of 2-isocyanoclovene (**1**) was determined by X-ray crystallographic analysis of formamide **6**. A biosynthetic pathway to clovanes **1** and **2** from epicaryolane precursors is proposed. Isocyanides **1**, **2**, and **4** showed activity against *Plasmodium falciparum* (IC_{50} 0.26−0.30 μM), while isothiocyanate **3** and formamide **6** had IC_{50} values of >10 μM.



Terpene isonitriles and isothiocyanates are characteristic metabolites of phyllidid nudibranchs and their dietary sponges[1,2] and are documented to possess antimalarial,[3,4] antifouling,[5] and other biological activities.[6] An ongoing and broad-scale investigation into the chemical defensive properties of brightly colored nudibranchs led us to examine the chemistry of phyllidid nudibranchs collected from southeast Queensland, including specimens of *Phyllidia ocellata*. Previous reports on the chemistry of this species have described a range of sesquiterpene isonitriles with bicyclic skeletons.[7] Herein, we report the isolation and characterization of five new sesquiterpene metabolites, comprising three tricyclic isonitriles, one tricyclic isothiocyanate, and one bicyclic isonitrile, from specimens of *P. ocellata* collected by scuba at Mudjimba Island (Mooloolaba, Australia).

Sixteen individuals were extracted with acetone, and the diethyl ether-soluble material was fractionated by silica flash chromatography and normal-phase HPLC to give nine metabolites, five (**1**−**5**) of which were new (Figure 1), together with axisonitrile-2,[8] axisonitrile-3,[9] 1-isocyanoaromadendrane,[10] and halichonadin C.[11] The major component of the extract, 2-isocyanoclovene (**1**), was isolated by NP-HPLC (1% EtOAc/hexanes) as a colorless oil. A molecular formula of $C_{16}H_{23}N$ was established by HRMS (ESI *m*/*z* 230.1906 $[M + H]^+$), implying six degrees of unsaturation. Inspection of the ^{13}C NMR (Table 1) spectra, together with DEPT data, provided 16 signals including three methyls, six methylenes, one methine, and three nonprotonated carbons in the aliphatic region. There were also two alkene signals at δ_C 132.2 (q) and 138.0 (d), respectively, in addition to an isocyano carbon at δ_C 164.7 (t, $^1J_{NC}$ = 5.6 Hz) that was detected only when a recycle time of 12 s and a 90° pulse angle were used for 1D spectral acquisition. For comparison, a diene isonitrile isolated from the fungus *Trichoderma hamatum*[12] has ^{13}C NMR shifts at δ_C 132.6 (q, alkene) and 169.4 (vinyl NC), while a synthetic sample of axisonitrile-4 from *Axinella cannabina*[13] has signals at δ_C 134.3 and 162.0, matching substituted alkene and vinyl NC carbons, respectively. The ^1H NMR spectrum of **1** ($CDCl_3$) showed three methyl singlets at δ_H 0.91, 1.00, and 1.09, an olefinic singlet at δ_H 5.64 (H-3), and a distinctive AB system (δ_H 1.27 (ddd, *J* = 12.4, 2.5, 2.5 Hz) and 1.18 (d, *J* = 12.4 Hz)) for H_2-12.

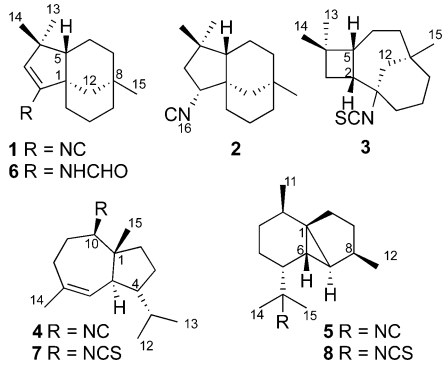


Figure 1. Structures of compounds **1**−**8**.

Detailed inspection of gCOSY, HSQC, H2BC, and HMBC spectra elucidated the planar structure of **1** (Figure 2). The

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Table 1. ^{1}H and ^{13}C NMR Data of 1−3 (in $CDCl_3$)[a,b]

	1		2		3	
no.	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)
1	49.2, C		44.9, C		66.2, C	
2	132.3, C		62.0, CH	3.59, m	41.5, CH	2.61, ddd (11.2, 8.2, 8.2)
3	138.0, CH	5.64, s	45.0, CH_2	1.85, m	36.4, CH_2	1.85, m
				1.82, m		1.79, m
4	45.0, C		39.0, C		33.6, C	
5	49.8, C	1.64, m	50.5, CH	1.33, m	50.0, CH	1.86, m
6	21.1, CH_2	1.62, m	22.3, CH_2	1.57, m	23.1, CH_2	1.71, m
		1.41, m		1.27, m		1.47, m
7	33.9, CH_2	1.36, m	33.3, CH_2	1.36, m	36.6, CH_2	1.54, m
		1.33, m		1.27, m		1.19, m
8	29.9, C		29.4, C		33.1, C	
9	38.7, CH_2	1.35, m	39.5, CH_2	1.34, m	40.2, CH_2	1.34, m
		1.10, ddd (13.4, 13.4, 5.1)		1.10, ddd (13.3, 13.3, 5.0)		1.12, ddd (12.9, 12.9, 3.8)
10	19.9, CH_2	1.68, m	20.7, CH_2	1.68, m	19.9, CH_2	1.58, m
		1.63, m		1.58, m		1.41, m
11	35.7, CH_2	1.61, m	37.9, CH_2	1.54, m	42.1, CH_2	1.87, m
		1.31, m		1.28, m		1.67, ddd (12.9, 12.9, 4.1)
12	40.5, CH_2	1.27, ddd (12.4, 2.5, 2.5)	37.9, CH_2	1.39, m	42.9, CH_2	2.31, ddd (13.8, 2.1, 2.1)
		1.18, d (12.4)		0.97, m		1.23, br d (13.8)
13	24.1, CH_3	1.00, s	25.6, CH_3	0.99, s	24.4, CH_3	0.93, s
14	31.5, CH_3	1.09, s	31.6, CH_3	0.96, s	28.7, CH_3	1.17, s
15	32.9, CH_3	0.91, s	33.0, CH_3	0.94, s	35.0, CH_3	0.95, s
16	164.7, NC		155.6, NC		128.9, NCS	

[a]Recorded at 900 and 225 MHz for ^{1}H and ^{13}C NMR, respectively. [b]Chemical shifts (ppm) referenced to $CHCl_3$ (δ_H 7.26, δ_C 77.16).

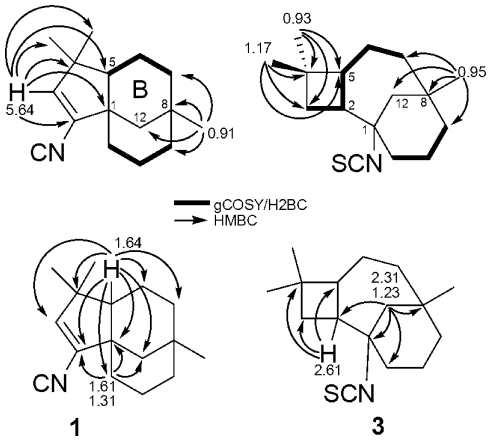

Figure 2. Selected 2D NMR correlations of 1 and 3.

gCOSY spectrum linked H-5 at δ_H 1.64 to H_2-6 and H_2-7 in ring B, and a second fragment of H_2-9 to H_2-11 was established in ring C. H-12a at δ_H 1.27 showed W couplings to H-9 at δ_H 1.35 and H-11 at δ_H 1.31, whereas the alkene proton did not show any long-range COSY interactions, in agreement with the lack of couplings apparent in the ^{1}H NMR spectrum. The HMBC data revealed correlations from H-3 to C-1, C-2, C-4, and C-5 (Figure 2), as well as from Me-13 and Me-14 to C-3, C-4, and C-5 (not shown in Figure 2), which established the cyclopentene ring. HMBC correlations from Me-15 to C-7, C-8, C-9, and C-12 joined together rings B and C, while correlations from H-5 to C-1, C-2, C-3, C-4, C-6, C-7, C-11, and C-12, as well as from H_2-11 to C-1, C-2, and C-12, completed construction of the tricyclo [6.3.1.$0^{1,5}$] dodecane ring system. The methylation pattern matched the clovane skeleton[14] rather than the recently reported penicibilaene skeleton.[15] Oxygenated clovane derivatives have previously been isolated from plants,[16] but to our knowledge the only reported example of oxygenated metabolites with the clovane skeleton from marine sources are from the gorgonian coral *Rumphella antipathies*.[17] Metabolite **1** represents the first

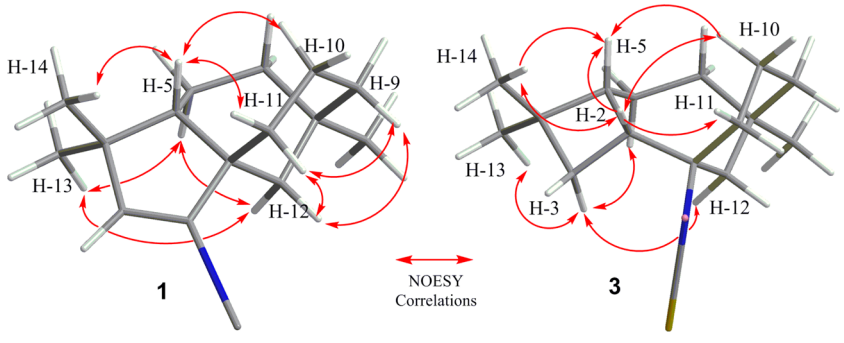

Figure 3. Key NOESY correlations observed for 1 and 3.

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example of a nitrogen-functionalized clovene derivative in the natural products literature.

Based on NOESY correlations from H-5 to Me-14 and H-11b at δ 1.31 (Figure 3), diastereomer **1** was selected for molecular modeling and DFT calculations. The C-5 epimer would be expected to show an NOE between H-5 and H-12. A Monte Carlo conformational search of **1** with the Merck Molecular Force Field (MMFF) provided two low-energy conformers (<3 kcal/mol of the global minimum) with ring B in a boat (80%) or chair (20%) conformation. The conformations were further optimized by DFT calculations at the B3LYP/6-31G(d) level in the gas phase using Gaussian software.[18] The structures were further refined using B3LYP/6-311++G(2d,p) with chloroform solvent (IEFPCM), and the energies obtained were used to calculate the Boltzmann population. The calculated Boltzmann-weighted ^{1}H NMR and ^{13}C NMR chemical shifts supported the chosen relative configuration, with a maximum variation of 0.15 ppm for H-3 and a mean absolute error (MAE) of 0.05 and 1.8 ppm for the ^{1}H and ^{13}C chemical shift values, respectively.[19]

Next, 2-isocyanoclovene was treated with glacial AcOH, and the formamide product **6** crystallized from 3% 2-propanol/hexanes; X-ray analysis confirmed the predicted relative configuration of **6**. Anomalous dispersion effects further established the absolute configuration of **6**, and by implication of **1**, as 1*S*, 5*S*, 8*R* (Figure 4). The structure comprised two molecules (each with a *cisoid* formamide rotamer) in the asymmetric unit; in one of these, ring B was disordered between a boat (63%) and a chair (37%) conformation, whereas the other molecule was 100% boat conformation. The preference for the boat conformer stems from alleviation of repulsion between the *syn* methylene protons attached to C-6 and C-10. In the chair conformer, there is a severe H···H nonbonding contact between the protons attached to C-6 and C-10 (~1.70 Å).[18] In solution in $CDCl_3$, the formamide group of **6** is present as a 1:1 mixture of the *cis* and *trans* rotamers, whereas in the solid state a single rotamer is obtained because of the strong intermolecular hydrogen bonds involving the formamide NH and carbonyl groups.

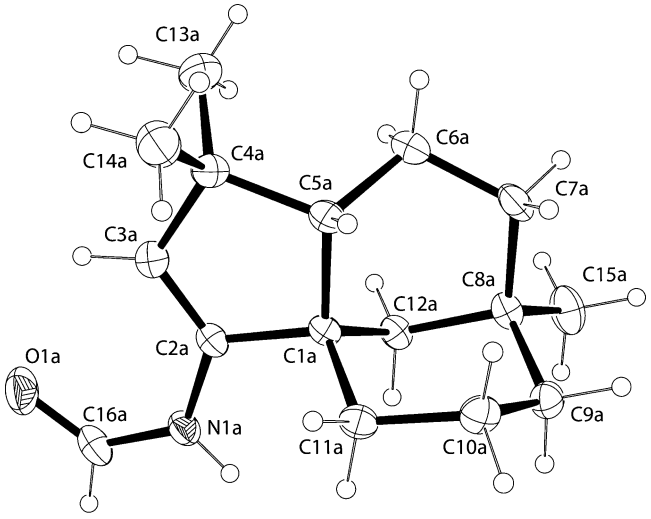

Figure 4. X-ray crystallographic structure of **6** (showing 30% probability ellipsoids).

A second tricyclic compound, 2-isocyanoclovane, was isolated as a colorless oil by NP-HPLC (3% EtOAc/hexanes) and revealed a molecular formula of $C_{16}H_{25}N$ by HRMS (ESI m/z 254.1876 $[M + Na]^+$), two mass units more than **1**. Together with the absence of alkene signals in the ^{1}H NMR spectrum, these data suggested the dihydro analogue **2**. Assuming the same absolute configurations at C-1, C-5, and C-8 as in **1**, NOESY correlations from H-2 to H-5, Me-14, and H_2-11 supported a 1*S*, 2*R*, 5*S*, 8*R* configuration. The C-2 epimer would have been expected to show NOEs between H-2 and H-3, H-6b, and H_2-12.

The third tricyclic component, 1-isothiocyanatoepicaryolane (**3**),[20] was isolated as a colorless oil by NP-HPLC (1% EtOAc/hexanes). The sample did not ionize under ESIMS conditions, and so (+)-HRESIMS measurement was not feasible; instead, a molecular formula of $C_{16}H_{25}NS$ was established by HREIMS (m/z 263.1706 $[M]^+$). An IR band at 2085 cm^{-1} also supported an isothiocyanate. The ^{13}C NMR (Table 1) spectrum again provided 16 signals including three methyls and an isothiocyanato carbon (δ_C 128.9 (q)), while the ^{1}H NMR spectrum ($CDCl_3$) showed three methyl singlets (δ_H 0.93, 0.95, 1.17) and a distinctive methine signal at δ_H 2.61 (ddd, J = 11.2, 8.2, 8.2 Hz) as well as an AB system (δ_H 2.31 (ddd, J = 13.8. 2.1, 2.1 Hz) and 1.23 (br d, J = 13.8 Hz)). Analysis of 2D NMR spectra elucidated the planar structure of **3** (Figure 2). gCOSY and H2BC data provided fragments C-3 to C-7 and C-9 to C-11, while HMBC correlations from H-2, Me-13, and Me-14 to C-3, C-4, and C-5 established the cyclobutane ring. These fragments were joined by HMBC correlations from Me-15 to C-7, C-8, C-9, and C-12 and from H_2-12 to C-1, C-2, C-8, and C-11. The *cis* ring configuration followed from a $^3J_{H\text{-}2/H\text{-}5}$ of 8.2 Hz[21] and from NOESY correlations from H-2 to H-5, H-10b, H-11a, and H-14. MMFF modeling and DFT calculations were undertaken on the anticipated 1*S*, 2*R*, 5*R*, 8*R* stereoisomer. The theoretical coupling constants calculated using the method of Bally et al.[19] averaged by a Boltzmann distribution of all reasonable conformations matched the experimental values shown in Table 1.[18] Caryolane sesquiterpenes have previously been isolated from plants, fungi,[22] and the marine sponge *Eurypon* sp.,[23] but with hydroxy, diol, or ketone substitution rather than a nitrogen-based functionality. Classic mechanistic studies on the acid-catalyzed cyclization of caryophyllene and caryophyllene oxide, yielding caryolan-1-ol and clov-2-ene among other products, led to the elucidation of the stereochemistry of *trans*-caryophyllene and its *cis* epimer isocaryophyllene.[14]

Two additional isocyanides, each with a known ring system, were isolated. The first, isolated as a colorless oil from NP-HPLC (3% EtOAc/hexanes), showed a molecular formula of $C_{16}H_{25}N$ by HRMS (ESI m/z 254.1883 $[M + Na]^+$) and was identified by ^{1}H and ^{13}C NMR data as the isocyano analogue **4** of the known 4,5-epi-10-isothiocyanatodauc-6-ene (**7**).[24] The second isocyanide, **5**, isolated as a colorless oil from NP-HPLC (3% EtOAc/hexanes), and with a molecular formula of $C_{16}H_{25}N$ by (+)-HRESIMS (m/z 254.1885 $[M + Na]^+$), was identified as 13-isocyanocubebane by comparison of its ^{1}H and ^{13}C NMR data with isothiocyanate **8**.[25] Full details are provided in the Supporting Information. The absolute configurations of **4** and **5** were not pursued owing to the small amounts of material isolated.

A plausible biosynthetic scheme for **1**−**3** from farnesyl diphosphate is shown in Scheme 1. The carbocation intermediate **A**,[26] which can adopt either a boat or chair

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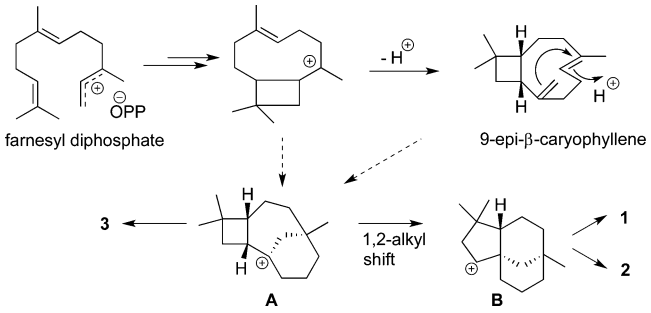
Scheme 1. Proposed Biosynthetic Pathway to 1−3



conformation, leads directly to the epicaryolane derivative **3** after capture by a thiocyanate anion,[1,27] but also provides the clovane skeleton by a 1,2 alkyl shift. Density functional calculations with mPW1PW91/6-31+G(d,p)//B3LYP/6-31+G(d,p)[18] indicated that, relative to the chair conformer of **A**, the energy barriers ($\Delta G^{\ddagger}$) for the 1,2-shift are 11.9 (chair) and 15.1 (boat) kcal/mol; thermodynamically, the 1,2-shift is downhill (−2.6 kcal/mol) for the chair conformer owing to hyperconjugation with the C-1/C-12 bond in **B**.

The new sesquiterpenes **1**−**4** and **6** together with halichonadin C[11] were screened in an in vitro growth inhibition assay against *P. falciparum* malaria parasite lines, using the isocyanides axisonitrile-3[3a] and diisocyanoadociane[3b−d] as reference standards. In preliminary screening against the *P. falciparum* Dd2 (drug resistant) line performed at 10 μM, all six isonitriles showed some antiparasitic activity (98.3−100% growth inhibition), but isothiocyanate **3** and formamide **6** were significantly less effective (<50% inhibition at 10 μM). Further evaluation of isocyanides **1**, **2**, and **4** against the *P. falciparum* Dd2 (drug resistant) line gave 50% growth inhibition (IC_{50}) values of 0.36 (±0.05), 0.83 (±0.25), and 0.87 (±0.20) μM, respectively. IC_{50} values against the chloroquine-sensitive *P. falciparum* 3D7 line were 0.30 (±0.09), 0.29 (±0.07), and 0.26 (±0.072) μM, respectively. When assessed for toxicity using a mammalian cell line (neonatal foreskin fibroblast (NFF) cells), the isocyanides were found to have good parasite-specific selectivity (IC_{50} NFF/IC_{50} *P. falciparum*), with 45−158-fold better activity for *P. falciparum* versus NFF cells.

In conclusion, we isolated five new sesquiterpenes from the nudibranch *P. ocellata*. Two isonitriles contained the rare [6.3.1.$0^{1,5}$] dodecane skeleton with the same methylation pattern as the hydrocarbon clovene. Also isolated was an isothiocyanato-functionalized epicaryolane for which the carbon skeleton may be the precursor to the clovane framework, as well as two isocyanide analogues with the daucene and cubebane skeletons, respectively. Sub-micromolar activity and parasite-specific antimalarial activity were associated with the isocyano-functionalized metabolites.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured in a polarimeter at 589 nm at ambient temperature using a 1 mL quartz cell (10 cm path length) for solutions in $CHCl_3$. IR spectra were recorded on a spectrophotometer for solutions in $CHCl_3$. NMR spectra were recorded at ambient probe temperature on spectrometers operating at 500 or 900 MHz for proton nuclei. All NMR spectra were run in base-filtered $CDCl_3$ and referenced to solvent signals at δ_H 7.26 (^{1}H) and δ_C 77.16 (^{13}C). Positive-ion electrospray mass spectra were determined using ion trap (LRESIMS) or time-of-flight instruments (HRESIMS), each with a standard ESI source. High-resolution electron impact mass spectrometry (HREIMS) was performed on solutions in hexanes on a magnetic sector mass spectrometer, using a direct insertion probe and with high-resolution (8000 resolution) peak matching against perfluorokerosene as reference. Gas chromatography/mass spectrometry (GC/MS) data were recorded on a gas chromatograph−mass spectrometer using a flow rate of 1.5 mL/min; initial oven temperature 100 °C (isothermal for 3 min); ramped 16 °C/min to 270 °C, held for 10 min; injection temperature 250 °C. Thin-layer chromatography (TLC) was carried out on aluminum plates coated with silica gel 60 F_{254} (normal-phase TLC). The plates were visualized under UV (254 and 365 nm) and then treated with a vanillin/concentrated sulfuric acid dip [vanillin (15 g), sulfuric acid (2.5 mL) in ethanol (250 mL)] followed by heating. Analytical reagent-grade solvents were distilled prior to use, while solvents for HPLC were purchased as HPLC grade. Normal-phase flash column chromatography was performed using silica gel 60 (40−63 μm) as the stationary phase and a gradient of hexanes to methanol under a pressure of compressed air for elution with combination of the resulting fractions guided by TLC. Additional chromatography was carried out with normal-phase silica cartridges. Normal-phase HPLC was performed using a 5 μm silica (10 × 250 mm) column with refractive index detection. Separations were performed using isocratic solvent conditions up to 3% EtOAc in hexanes at 2 mL/min using premixed, filtered, and degassed mobile phases.

Biological Material. Collection of *P. ocellata* was undertaken using scuba at a depth of 12 m. Twenty-one individual specimens were collected in December 2004 from the Mudjimba Island (Old Wooman Island) dive site, located north of Mooloolaba. A voucher specimen (MOOL-12) is held at the School of Chemistry and Molecular Biosciences, University of Queensland. Although considerable individual variation in dorsal pattern occurs in *P. ocellata*, this species can be separated from other members of the genus *Phyllidia* based on its distinctive yellow body with black circles bordered with white.[28] This allowed us an unambiguous identification of all collected specimens. The samples were taken back to the laboratory and stored at −20 °C until extraction.

Extraction and Isolation. Sixteen frozen nudibranchs (15 g) were finely chopped, extracted with acetone (5 × 50 mL), and sonicated (2 min). The extracts were filtered through cotton wool and reduced to an aqueous suspension before partitioning between H_2O (30 mL) and Et_2O (3 × 30 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered through cotton wool, and evaporated under N_2 to yield an orange gum (156 mg). The extract was further separated by NP flash column chromatography with a solvent gradient from hexanes to 100% MeOH prior to GC/MS analysis. The fractions eluting from hexanes/CH_2Cl_2 (4:1) were further purified by NP-HPLC (1% EtOAc in hexanes) to yield 1-isothiocyanatoepicaryolane (**3**), 2-isocyanoclovene (**1**), and axisonitrile-3.[9] The fraction eluting from hexane/CH_2Cl_2 (1:1) was further purified by NP HPLC (3% EtOAc in hexanes) to provide 1-isocyanoaromadendrane,[10] 2-isocyanoclovane (**2**), halichonadin C,[11] axisonitrile-2,[8] 4,5-epi-10-isocyanoisodauc-6-ene (**4**), and 13-isocyanocubebane (**5**). A second extraction of five frozen nudibranchs (5.8 g) was made following the same procedure to yield further material for characterization and biological assays.

*(−)-(1S,5S,8R)-2-Isocyanoclovene (**1**):* colorless oil (9.0 mg); $[\alpha]_D$ −17 (*c* 0.053, $CHCl_3$); IR ($CHCl_3$) ν_{max} 2924, 2866, 2108, 1463 cm^{-1}; ^{1}H NMR and ^{13}C NMR see Table 1; GC/MS *m*/*z* (%) 229 (47), 214 (100), 186 (100); HRESIMS *m*/*z* 230.1906 $[M + H]^+$ (calcd for $C_{16}H_{24}N$, 230.1903).

*(−)-(1S,5S,8R)-2-Isocyanoclovane (**2**):* colorless oil (0.3 mg); $[\alpha]_D$ −42 (*c* 0.06, $CHCl_3$); IR ($CHCl_3$) ν_{max} 2928, 2136, 1456, 1368 cm^{-1}; ^{1}H NMR and ^{13}C NMR see Table 1; GC/MS *m*/*z* (%) 231 (5), 230 (7), 216 (72), 204 (14), 188 (60), 175 (80) 161 (58), 135 (100), 135 (72); HRESIMS *m*/*z* 254.1876 $[M + Na]^+$ (calcd for $C_{16}H_{25}NNa$, 254.1879).

*(−)-(1S,2R,5R,8R)-1-Isothiocyanatoepicaryolane (**3**):* colorless oil (0.2 mg); $[\alpha]_D$ −3 (*c* 0.026, $CHCl_3$); IR ($CHCl_3$) ν_{max} 2931, 2866, 2085, 1462 cm^{-1}; ^{1}H NMR and ^{13}C NMR see Table 1; GC/MS *m*/*z*

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(%) 263 (3), 205 (85), 189 (10), 161 (22), 149 (100); HREIMS m/z 263.1706 $[M]^+$ (calcd for $C_{16}H_{25}NS$, 263.1702).

(+)-(1S,4R*,5S*,10S*)-4,5-Epi-10-isocyanoisodauc-6-ene (**4**):* colorless oil (0.7 mg); $[\alpha]_D$ +40 (c 0.027, $CHCl_3$); IR ($CHCl_3$) ν_{max} 2955, 2133, 1465, 1387 cm^{-1}; ^1H NMR ($CDCl_3$ 900 MHz) δ 5.20 (1H, br s, H-6), 3.35 (1H, br d, J = 12.0 Hz, H-10), 2.18 (1H, br t, J = 13.9 Hz, H-8), 1.95 (1H, m, H-8), 1.93 (1H, m, H-5), 1.89 (1H, m, H-9), 1.85 (1H, m, H-2), 1.85 (1H, m, H-4), 1.78 (1H, m, H-3), 1.74 (3H, br s, H-14), 1.65 (1H, dd, J = 12.8, 12.0, H-9), 1.59 (1H, m, H-11), 1.43 (1H, m, H-2), 1.40 (1H, m, H-3), 0.87 (3H, d, J = 6.8 Hz, H-13), 0.85 (3H, s, H-15), 0.82 (3H, d, J = 6.8 Hz, H-12); ^{13}C NMR ($CDCl_3$ 225 MHz) δ 154.2 (NC), 137.2 (C-7), 128.7 (C-6), 68.1 (C-10), 51.0 (C-4), 49.1 (C-5), 46.3 (C-1), 40.2 (C-2), 31.5 (C-11), 31.1 (C-8), 28.6 (C-9), 25.5 (C-14), 24.1 (C-3), 22.0 (C-13), 18.9 (C-12), 14.4 (C-15); GC/MS m/z (%) 204 (24), 188 (26), 161 (55), 123 (76), 105 (58), 91 (42), 81 (55); HRESIMS m/z 254.1883 $[M + Na]^+$ (calcd for $C_{16}H_{25}NNa$, 254.1876).

(+)-(1S,2S*,5S*,6S*,7R*,8S*)-13-Isocyanocubebane (**5**):* colorless oil (0.2 mg); $[\alpha]_D$ +26 (c 0.013, $CHCl_3$); IR ($CHCl_3$) ν_{max} 2949, 2869, 2128, 1457, 1369 cm^{-1}; ^1H NMR ($CDCl_3$ 900 MHz) δ 2.28 (1H, m, H-8), 1.93 (1H, m, H-5), 1.86 (1H, m, H-2), 1.73 (2H, m, H-10), 1.72 (1H, m, H-3), 1.59 (1H, m, H-4), 1.55 (1H, m, H-9), 1.44 (3H, br s, H-14), 1.43 (3H, br s, H-15), 1.14 (1H, br t, J = 4.3 Hz, H-7), 1.04 (3H, d, J = 6.9 Hz, H-11), 1.02 (3H, d, J = 6.5 Hz, H-12), 1.00 (1H, m, H-4), 0.92 (1H, m, H-3), 0.89 (1H, br t, J = 3.8 Hz, H-6), 0.72 (1H, m, H-9); ^{13}C NMR ($CDCl_3$ 225 MHz) δ 62.1 (C-13), 44.7 (C-5), 35.7 (C-8), 34.1 (C-3), 32.5 (2C, C-7, C-10), 31.9 (C-2), 31.3 (C-1), 30.1 (C-9), 28.5 (C-14), 27.5 (C-15), 21.4 (C-4), 21.2 (C-11), 19.3 (C-12), 17.2 (C-6); GC/MS m/z (%) 216 (34), 204 (12), 189 (15), 161 (46), 149 (34), 133 (32), 121 (82), 107 (100), 91 (92); HRESIMS m/z 254.1885 $[M + Na]^+$ (calcd for $C_{16}H_{25}NNa$, 254.1876).

*Hydrolysis of 2-isocyanoclovene (**1**) to 2-formamidoclovene (**6**):* 2-Isocyanoclovene (**1**) (5.8 mg) was dissolved in glacial acetic acid (1 mL) and left to stand overnight at room temperature. The solution was diluted with H_2O (2 mL) and taken to neutral pH with sodium bicarbonate (0.5 g). The solution was extracted with CH_2Cl_2 (5 × 10 mL), dried over anhydrous sodium sulfate, and evaporated to give 2-formamidoclovene (**6**) (5.0 mg, 80%). 2-Formamidoclovene (**6**) was recrystallized from hexanes/isopropyl alcohol (3%) at 4 °C to afford a small sample of colorless crystals.

*(−)-(1S,5S,8R)-2-Formamidoclovene (**6**):* white, amorphous solid (5.0 mg); $[\alpha]_D$ −45 (c 0.087, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3297 (br), 2925, 1682, 1544 cm^{-1}; ^1H NMR ($CDCl_3$ 900 MHz; 1:1 mixture of *cis* and *trans* formamide rotamers) δ 8.41 (*trans*, br d, J = 11.4 Hz) and 8.31 (*cis*, br s) (1H, CHO), 6.60 (*trans*) and 6.38 (*cis*) (1H, br s, NH), 5.94 (*trans*) and 5.06 (*cis*) (1H, s, H-3), 1.81 and 1.77 (1H, m, H-10a), 1.66 (1H, m, H-6a), 1.65 and 1.58 (1H, m, H-5), 1.62 and 1.61 (1H, m, H-10b), 1.44 (1H, m, H-6b), 1.40 (1H, m, H-11a), 1.39 (1H, m, H-9a), 1.36 (2H, m, H-7), 1.35 and 1.27 (1H, m, H-12a), 1.34 (1H, m, H-11b), 1.09 and 1.08 (3H, br s, Me-14), 1.06 (1H, m, H-9b), 1.00 and 0.99 (3H, br s, H-13), 0.96 and 0.95 (1H, m, H-12b), 0.89 (3H, br s, H-15); ^{13}C NMR ($CDCl_3$ 225 MHz) δ 162.8 (*trans*) and 159.4 (*cis*) (C-NHCHO), 123.2 (*trans*) and 119.6 (*cis*) (C-3), 137.6, and 140.3 (C-2), 50.0 and 49.3 (C-5), 48.9 and 48.6 (C-1) 45.2 and 44.5 (C-4), 42.4 and 41.8 (C-12), 39.0 and 38.9 (C-9), 37.0 and 36.5 (C-11), 34.6 and 34.4 (C-7), 33.2 and 33.1 (C-15), 32.6 and 32.4 (C-14), 30.4 and 30.2 (C-8), 25.1 and 25.0 (C-13), 21.5 (C-6), 20.5 and 20.3 (C-10); HRESIMS m/z 270.1828 $[M + Na]^+$ (calcd for $C_{16}H_{25}NNaO$, 270.1830).

Crystallographic Data for 2-Formamidoclovene (6). $C_{16}H_{25}NO$, fw = 247.37, monoclinic space group, $P2_1$, unit cell dimensions a = 9.6177(2) Å, b = 12.2824(2) Å, c = 12.3764(2) Å, V = 1458.59(5) Å ^{3}, β = 93.918(2)°, Z = 4, ρ_{calcd} = 1.126 g/cm^3, crystal dimensions 0.2 × 0.2 × 0.2 mm^3, μ = 0.530 mm^{-1}, $F(000)$ = 544. The 15 463 measurements yielded 5213 independent reflections after equivalent data were averaged, and Lorentz and polarization corrections were applied. The final refinement gave R_1 = 0.0275 and wR_2 = 0.1582 [$I > 2\sigma(I)$].

Structure Determination. X-ray diffraction analysis was carried out on an Oxford Diffraction Gemini S Ultra CCD diffractometer using Cu Kα radiation (λ = 1.541 80 Å), and data were collected within the 4.09° < θ < 62.53° scan range. The crystal was preserved at a temperature of 173 K using an Oxford Cryosystems Cryostream Cooler. Data reduction and semiempirical absorption corrections were conducted with the CrysAlisPro software package (version 171.38.28, Agilent). The structure was solved by direct methods and refined by full-matrix least-squares on F^2 with SHELX[29] within the WinGX graphical user interface.[30] ORTEP3 was used to produce the thermal ellipsoid plots,[31] and PLATON[32] was used for other drawings. The absolute structure was established from a statistical analysis of anomalous dispersion effects of 2583 Bijvoet pairs according to the method of Hooft et al.,[33] leading to a probability ($P2$) of the correct enantiomer being 1.00 [Hooft parameter 0.00(6), ν = 15 using Student's t-statistics]. Atomic coordinates, bond lengths and angles, and displacement parameters have been deposited at the Cambridge Crystallographic Data Centre (CCDC number 1039520).

Bioassay Methods and Data. *P. falciparum Growth Inhibition Assay.* In vitro *P. falciparum* growth inhibition assays were carried out using a modified isotopic microtest.[34] Briefly, asynchronous *P. falciparum*-infected erythrocytes (1% parasitemia; 1% hematocrit) were seeded, in triplicate, into wells of 96-well tissue culture plates containing the antimalarial drug control (chloroquine; Sigma-Aldrich, C6628) or test compounds. [^{3}H]-Hypoxanthine (0.5 μCi/well) was then added, and plates were incubated under standard *P. falciparum* culture conditions for 48 h. Cells were harvested onto 1450 MicroBeta filter mats (Wallac), and [^{3}H] incorporation was determined using a 1450 MicroBeta liquid scintillation counter. Percentage growth inhibition compared to vehicle control [DMSO (0.5%)] and background controls was determined. Compounds were initially tested at 10 μM in triplicate wells, in three independent experiments, and only compounds showing >∼50% inhibition in these primary tests were further investigated to determine IC_{50} values. IC_{50} (±SD) values were calculated using log-linear interpolation of inhibition curves[35] for two to three independent experiments, each carried out in triplicate.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details of X-ray crystallographic analysis, modeling and computational studies, antimalarial assays, and copies of 1D and 2D NMR spectra of compounds **1**−**6**. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00354.

■ AUTHOR INFORMATION

Corresponding Author

*Tel (M. J. Garson): +61-7-3365-3605. Fax: +61-7-3365-4273. E-mail: m.garson@uq.edu.au.

Notes

The authors declare no competing financial interest.

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