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Advanced precursors in marine biosynthetic study. Part 3: The biosynthesis of dichloroimines in the tropical marine sponge *Stylotella aurantium*

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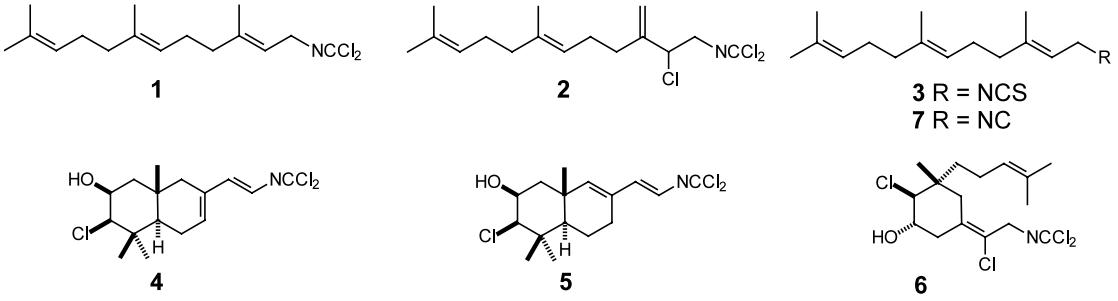
Abstract—The biosynthetic origins of the dichloroimine group in the stylotellanes A and B **1**, **2** have been investigated by incorporation of [^{14}C]-labeled farnesyl isocyanide **7** and farnesyl isothiocyanate **3** into the sponge *Stylotella aurantium*.

Marine sponges show an elaborate range of novel, functionalised, bioactive terpenes possessing isocyanide, isothiocyanate, and thiocyanate functionality.[1] Using ^{14}C-labeled precursors, we have shown that the isocyanide,[2–4] isothiocyanate,[3–5] and thiocyanate[4] functionalities present in some metabolites of tropical sponges originate from cyanide and thiocyanate. The sponge *Stylotella aurantium* contains the rare dichloroimine (=carbonimidic dichloride) functionality. The metabolites isolated from Great Barrier Reef specimens of this sponge include stylotellanes A and B, **1** and **2**, farnesyl isothiocyanate **3**, the bicyclic reticulidin B **4** and isoreticulidin B **5**, and the substituted cyclohexanol **6**.[5,6] We have previously shown that both cyanide and thiocyanate act as precursors to the dichloroimine functional groups in **1** and **2**, and have speculated that their biosynthesis involves farnesyl isocyanide **7** and farnesyl isothiocyanate **3** as likely intermediates.[5] In this paper, we report the results of advanced precursor experiments to test these biosynthetic scenarios.

Our experiments required the synthesis of [^{14}C]-labeled precursors **3** and **7**, which were carried out in a similar manner to the syntheses of [^{14}C]-labeled diisothiocyanatoadociane and 9-isocyanopupukeanane that we reported recently.[7,8] Farnesyl amine **8**, available from farnesol using modified Gabriel conditions,[9,10] was used as a starting material for the radiochemical synthesis (see Scheme 1). The ^{14}C label was incorporated by formylation of amine **8** with [^{14}C]-formic-acetic anhydride[11] to give the [^{14}C]-formamide **9**. Dehydration of the formamide **9** with tosyl chloride in dry pyridine gave [^{14}C]-farnesyl isocyanide **7**. A portion of this isocyanide **7** was converted to the [^{14}C]-farnesyl isothiocyanate **3** by treatment with sulfur at 80°C. All radiochemical products were purified by flash chromatography or by preparative TLC. In particular, we ensured by careful chromatography that the sample of isothiocyanate **3** used in the biological experiments contained no residual [^{14}C]-isocyanide **7**.[8]



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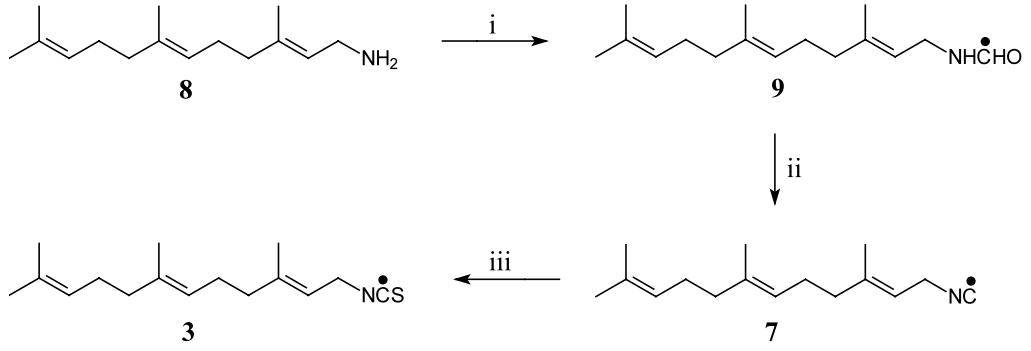
Precursor incorporation experiments were performed at Heron Island Research Station according to our usual protocol.[2–5,7,8] [^{14}C]-Farnesyl isothiocyanate **3** (38.0 mg, 3.6 μCi) was dissolved in 1 mL acetone and added to a glass beaker containing a small piece of *S. aurantium* in aerated sea-water, kept at ambient temperature and light levels.[12] After overnight incubation followed by a 14 day incorporation period, extraction of the sponges and purification to constant specific activity gave samples of stylotellanes A and B, **1** and **2**, which were significantly radioactive (see Table 1). Similarly [^{14}C]-farnesyl isocyanide **7** (13.0 mg, 11.8 μCi) was fed to each of two small pieces of *S. aurantium* in a second incorporation experiment.[13] The two pieces of sponge were then incubated for 14 and 28 days, respectively. The samples of stylotellanes A and B, **1** and **2**, recovered from these incubations were significantly radioactive, as was the isolated farnesyl isothiocyanate **3** (see Table 1). Finally, a sample of [^{14}C]-formamide **9** (14.2 mg, 4.2 μCi) was supplied to a specimen of *S. aurantium* for 7 days.[14] In this third experiment the isolated samples of stylotellane A and B, **1** and **2**, showed no significant radioactivity (see Table 1).

To establish the sites of radiolabeling, the sample of stylotellane B **2** isolated from incorporation of **3** or **7** was degraded to the cyclic carbamate **10** and the amine **11** using 0.1N phosphoric acid in 90% methanol (Scheme 2). In each case, the carbamate product retained almost full radioactivity (90–95%), whereas the

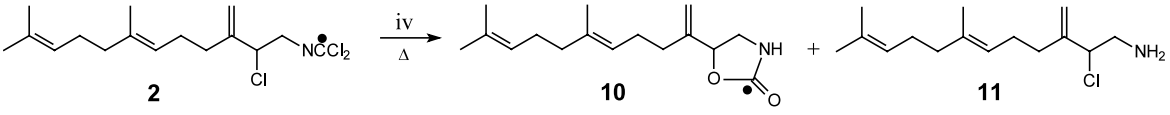


Scheme 1. Synthesis of [^{14}C]-advanced precursors. (● = ^{14}C label) (i) [^{14}C]-formic acetic anhydride, dichloromethane, then silica flash chromatography (hexane to CH_2Cl_2 gradient); yield 97%; (ii) *p*-TsCl in pyridine, silica flash chromatography (hexane); yield 70%, (iii) sulfur, 80°C, then silica chromatography (hexane); yield 24%.

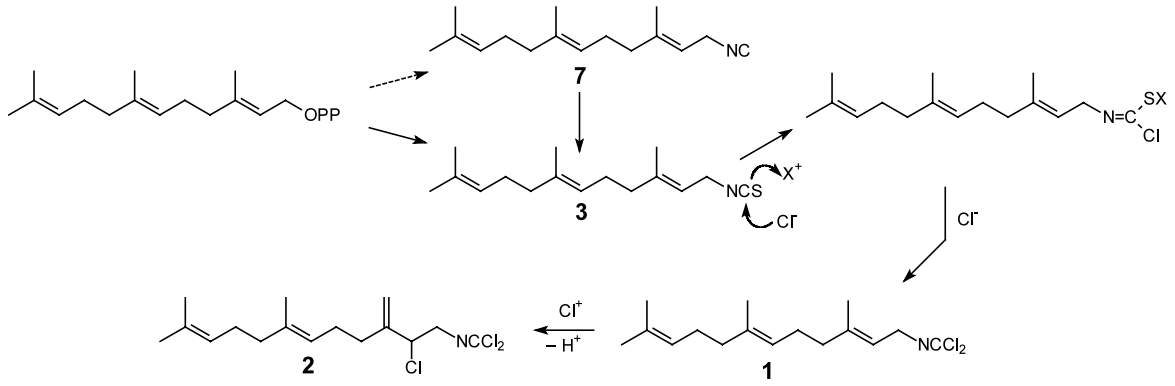
Table 1. Molar specific activities of *S. aurantium* metabolites **1**, **2** and **3** and their degradation products **10** and **11**

Precursor	Compound	Molar specific activity (μCi/mmol)	Incorporation (%)	Radioactivity (%)
[^{14}C]-**3**	**1**	1.00	0.23	–
	2	0.36	0.27	100.0
	10	0.32	–	90.0[a]
	11	$<10^{-5}$	–	0.0
[^{14}C]-**7**[b]	**1**	0.58	0.04	–
	2	0.11	0.13	100.0
	3	0.61	0.03	–
	10	0.59	–	96.0[a]
	11	$<10^{-5}$	–	0.0
[^{14}C]-**7**[c]	**1**	0.50	0.02	–
	2	0.08	0.02	–
	3	0.66	0.01	–
[^{14}C]-**9**	**1**	$<10^{-5}$	–	–
	2	$<10^{-5}$	–	–

[a] The small amount and difficulty in purification meant the specific activity was lower than desired; it is quite unlikely that this level of labeling would be retained after purification if the compound was unlabelled.
[b] 14 day incorporation.
[c] 28 day incorporation.



Scheme 2. Degradation of stylotellane B **2** (iv) 0.1N H_3PO_4/90% methanol, then silica flash chromatography (hexane to EtOAc gradient); yield 16% of **11**, than silica prep. TLC, yield 3% of **10**.



Scheme 3.

amine was devoid of radioactivity. These data are in accordance with specific incorporation into the dichloroimine carbon atom. Therefore no metabolic degradation and reutilization of labeled breakdown products via general metabolism had occurred.

These results clearly show that farnesyl isocyanide **7** and farnesyl isothiocyanate **3** can be utilized by the sponge *S. aurantium* as advanced precursors in dichloroimine biosynthesis. Further we have also demonstrated the –NC to –NCS interconversion at the secondary metabolite level (**7→3**). The low concentrations of farnesyl isocyanate **7** in *S. aurantium*, and additionally its instability, prevented isolation and assessment of the reverse –NCS to –NC transformation. As anticipated, the formamide **9** is not utilized for dichloroimine biosynthesis.[15,16]

The issue of whether the isocyanide–isothiocyanate secondary metabolites can interconvert in marine sponges was first addressed by Scheuer et al. using ^{13}C precursor incorporation and mass spectrometric detection.[16] Evidence was obtained for the conversion of the tricyclic metabolite 2-isocyanopupukeanane into 2-isothiocyanatopupukeanane by *Ciocalypta* sp.; however, the reverse transformation (isothiocyanate→isocyanide) was not detected using this methodology. In contrast, ^{14}C-labeling experiments carried out in our laboratory provided evidence for an isothiocyanate to isocyanide conversion in the Great Barrier Reef sponge *Amphimedon terpenensis*.[7] Unfortunately, the incorporation values were low and variable, possibly because the isothiocyanate precursor supplied was not a natural metabolite of this sponge. Subsequently, we obtained definitive evidence for isocyanide–isothiocyanate interconversions by advanced precursor experiments with *Axinyssa* n.sp.[8] In this sponge, the rate of conversion was similar in either direction.

The higher incorporation rates in the experiments with farnesyl isothiocyanate **3**, an isolated metabolite, compared with activities from the farnesyl isocyanide **7** experiments led us to the conclusion that farnesyl isothiocyanate **3** is the major intermediate in the biosynthetic pathway to stylotellane B. The proposed reaction steps involve oxidative chlorination of the –NCS moiety (**3→1**) and further chlorination of **1** to finally yield stylotellane B (**2**) (Scheme 3). The role of farnesyl intermediates and of the stylotellanes **1** and **2** in the formation of cyclic metabolites such as the reticulidins (**4–6**) will next be addressed.

Acknowledgements

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10. All new compounds gave satisfactory ^{1}H and ^{13}C NMR spectroscopic and HR-MS analytical data.
11. [^{14}C]-Formic–acetic anhydride was prepared by dissolving dry sodium-^{14}C-formate (1.0 mCi) in formic acid (190 μL), after 2 h at 25°C acetic anhydride (660 μL) was added and heated for 30 min at 60°C. The resulting solution was then added to amine **8** (680 mg) in dichloromethane (5 mL).

12. Sponge samples were collected on SCUBA at Coral Spawning, Heron Island (23°27′S, 151°55′E), Great Barrier Reef (–14–16 m) in April 2002. A voucher sample (registry number QM G312575) is lodged at The Queensland Museum, Brisbane. A specimen of *S. aurantium* (w. wt. 59 g) was placed in an aquarium containing 200 mL aerated seawater at ambient temperature. [^{14}C]-Farnesyl isothiocyanate **3** (3.6 μCi, 69.0 μCi/mmol) in acetone (1 mL) was added and the sponge allowed to assimilate radioactivity for 12 h overnight. The sponge sample was kept in running seawater in a 10 L aquarium at ambient temperature for 14 days, then frozen for subsequent radiochemical analysis. A chloroform/methanol extract was processed by our previously reported method,[5] followed by repeated silica HPLC (μ-partisil, 0.5% EtOAc/hexane) to give stylotellane A **1** (2.5 mg, 0.004% chemical yield; 7400 dpm/mg, 0.23% incorporation yield) and stylotellane B **2** (8.7 mg, 0.014% chemical yield; 2386 dpm/mg, 0.27% incorporation yield).
13. Two small pieces of *S. aurantium* (each w. wt. approx. 36 g) were each supplied with [^{14}C]-farnesyl isocyanide **7** (11.8 μCi, 71.2 μCi/mmol) and allowed to assimilate radioactivity for 14 or 28 days. The 14 days extract was processed to give stylotellane A **1** (2.52 mg, 0.007% chemical yield; 4277 dpm/mg, 0.04% incorporation yield), stylotellane B **2** (4.52 mg, 0.012% chemical yield; 729 dpm/mg, 0.13% incorporation yield) and farnesyl isothiocyanate **3** (1.6 mg, 0.004% chemical yield, 5140 dpm/mg, 0.03% incorporation yield).
14. A specimen of *S. aurantium* (w. wt. 28 g) was supplied with [^{14}C]-formamide **9** (4.2 μCi, 73.7 μCi/mmol) for 7 days. The sponge extract was processed to give stylotellane A **1** (0.2 mg, 0.0007% chemical yield) and stylotellane B **2** (2.8 mg, 0.01% chemical yield) neither of which were radioactive.
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