ARTICLE

OBC
www.rsc.org/obc

Biosynthetic pathways to dichloroimines; precursor incorporation studies on terpene metabolites in the tropical marine sponge *Stylotella aurantium*† ‡

Jamie S. Simpson,§ Andreas Brust and Mary J. Garson
Department of Chemistry, School of Molecular and Microbial Sciences, The University of Queensland, Brisbane QLD 4072, Australia. E-mail: m.garson@mailbox.uq.edu.au

Received 9th December 2003, Accepted 30th January 2004
First published as an Advance Article on the web 26th February 2004

The biosynthetic origin of the dichloroimine functional group in the marine sponge terpene metabolites stylotellanes A (**3**) and B (**4**) was probed by the use of [^{14}C]-labelled precursor experiments. Incubation of the sponge *Stylotella aurantium* with [^{14}C]-labelled cyanide or thiocyanate resulted in radioactive terpenes in which the radiolabel was shown by hydrolytic chemical degradation to be associated specifically with the dichloroimine carbons. Additionally, label from both precursors was incorporated into farnesyl isothiocyanate (**2**). A time course experiment with [^{14}C]-cyanide revealed that the specific activity for farnesyl isothiocyanate decreases over time, but increases for stylotellane B (**4**), consistent with the rapid formation of farnesyl isothiocyanate (**2**) from inorganic precursors followed by a slower conversion to stylotellane B (**4**). The advanced precursors farnesyl isothiocyanate (**2**) and farnesyl isocyanide (**5**) were supplied to *S. aurantium*, and shown to be incorporated efficiently into stylotellane A (**3**) and B (**4**). Feeding of [^{14}C]-farnesyl isothiocyanate (**2**) resulted in a higher incorporation of label than with [^{14}C]-farnesyl isocyanide (**5**). Farnesyl isocyanide was incorporated into farnesyl isothiocyanate in agreement with labelling studies in other marine sponges. Both farnesyl isocyanide and isothiocyanate were further incorporated into axinyssamide A (**11**) as well as the cyclized dichloroimines (**12**)–(**14**), (**16**) that represent more advanced biosynthetic products of this pathway. These results identify the likely biosynthetic pathway leading to the major metabolites of *S. aurantium*.

Introduction

In the marine world, the biological transformations of farnesyl pyrophosphate (**1**) lead to an extraordinary and diverse suite of sesquiterpene products, many of which are nitrogen-functionalised. Sesquiterpenes containing isocyanide and isothiocyanate groups are frequently isolated from sponges and from the molluscs that feed on these sponges.[1–3] Previous research from this group has shown that the inorganic precursors cyanide and thiocyanate can be utilised by marine sponges for isocyanide and isothiocyanate biosynthesis.[2] Thus, sodium [^{14}C]-cyanide is incorporated into the isocyanide groups of diisocyanoadociane,[4] axisonitrile-3,[5] and 9-isocyanopupukeanane,[6] and into the isothiocyanate groups of axisothiocyanate-3[5] and 9-isothiocyanatopupukeanane.[6] [^{14}C]-Labelled thiocyanate has also been demonstrated as a precursor to the isothiocyanate or the isocyanide functionality in these metabolites.[5–7] The Scheuer group has confirmed the role of cyanide in the biosynthesis of diterpene isocyanides, and established by NMR analysis the intact incorporation of [^{13}C, ^{15}N]-cyanide into a sesquiterpene isocyanide.[8]

In contrast to the abundance and diversity of sesquiterpene isocyanides and isothiocyanates, sesquiterpene metabolites containing a dichloroimine (=carbonimidic dichloride) group are rare. Since the first report of their isolation by the Faulkner research group in 1977,[9] a small number of sesquiterpenes with this uncommon functionality have been reported, either from marine sponges (*Pseudaxinyssa pitys*,[9–11] *Axinyssa sp.*,[12] *Ulosa spongia*,[13] and *Stylotella aurantium*[14–16]) or from nudibranchs (*Phyllidia pustulosa*,[12] and *Reticulidia fungia*[17]). Faulkner *et al.* speculated that the biosynthetic origin of the dichloroimine functional group might be linked to that of the corresponding isocyanide,[9] or isothiocyanate.[10] This insight came from recognising that marine sponges are capable of introducing chlorine substituents into terpene skeletons, and hence that this ability to introduce chlorine might be extended to nitrogenous functionality. In both the sponges *P. pitys*[10] and *S. aurantium*,[14] farnesyl isothiocyanate (**2**) is a co-metabolite of dichloroimine metabolites such as the stylotellanes A (**3**) and B (**4**) whose structures clearly suggest that they originate from farnesyl-derived precursors. Scheme 1 shows plausible mechanisms by which the stylotellane metabolites might originate from either farnesyl isothiocyanate (**2**) or from farnesyl isocyanide (**5**) (which has not to date been reported as a natural product). The scheme thus suggests that the inorganic precursors cyanide and thiocyanate might act as precursors to the dichloroimine functional group. This biosynthetic scenario has been explored by our group, initially by incorporation of [^{14}C]-cyanide or thiocyanate into *S. aurantium*.[14] and then by testing the proposed intermediacy of sesquiterpene isocyanides and isothiocyanates in dichloroimine biosynthesis,[18] In this paper, we present full details of these findings, as well as the results of additional experiments that enable us to describe in some detail the overall biosynthetic processes leading to the stylotellane metabolites. We also report on the biosynthesis of cyclic dichloromines in *S. aurantium*.

Results and discussion

Inorganic experiments

We first explored the precursor status of inorganic cyanide and thiocyanate in *S. aurantium*.[14] The incorporation of inorganic cyanide and thiocyanate was performed on healthy specimens collected at Heron Island on the Great Barrier Reef, according to our well-established protocols.[4,5,14,19] The terpene metabolites

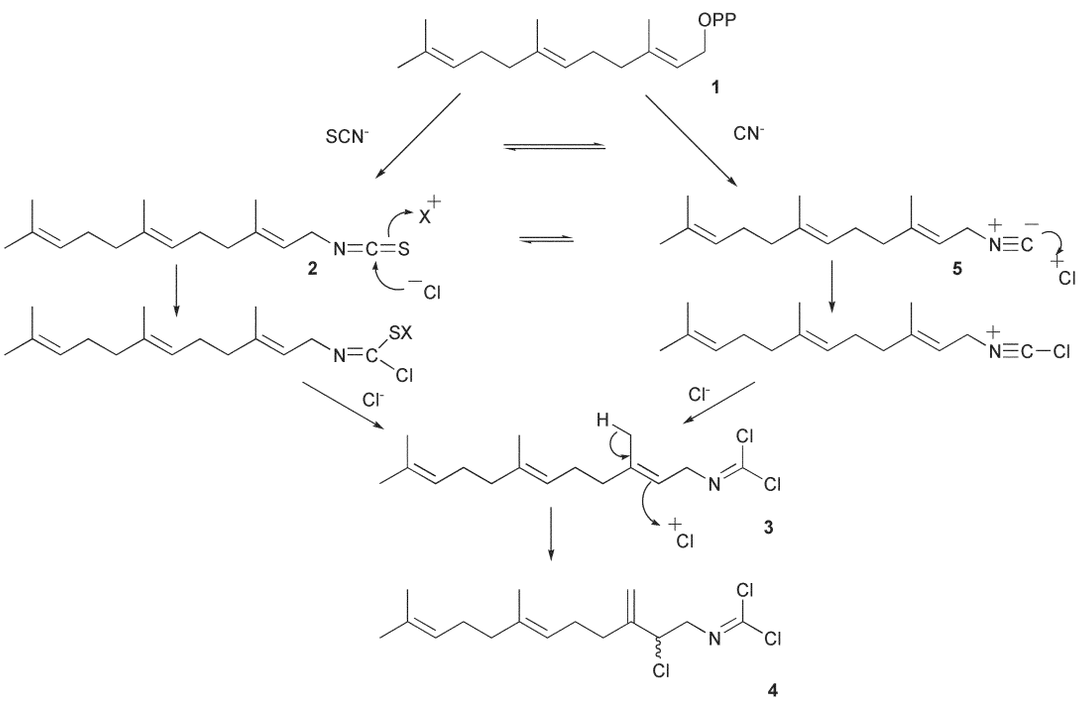
† Dedicated to D. John Faulkner (1942–2002) whose research first characterised dichloroimine metabolites from marine sponges.
‡ Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR spectra for compounds **5**, **9** and **10**. See http://www.rsc.org/suppdata/ob/b3/b315895k/
§ *Current address*: Research School of Chemistry, Institute of Advanced Studies, The Australian National University, Canberra ACT 0200, Australia.

DOI: 10.1039/b315895k

Table 1 Incorporation of inorganic precursors [^{14}C]-cyanide and [^{14}C]-thiocyanate into *S. aurantium*

Metabolite	Precursor, amount	Incorporation period (days)	Molar specific activity (μCi mmol^{-1})	Incorporation (%)
2	CN^-, 100 μCi	10	696	1.98
2	CN^-, 100 μCi	28	117	0.43
2	SCN^-, 25 μCi	10	69.8	0.78
2	SCN^-, 12.5 μCi	14	0.71	0.01
3	CN^-, 50 μCi	9	1.29[a]	0.021[a]
3	CN^-, 100 μCi	10	4.74	0.015
3	SCN^-, 25 μCi	10	0.26	0.087
3	SCN^-, 12.5 μCi	14	0.35	0.002[a]
4	CN^-, 50 μCi	9	1.47	0.03
4	CN^-, 100 μCi	10	3.33	0.08
4	CN^-, 100 μCi	28	26.4	0.50
4	SCN^-, 25 μCi	10	0.27	0.015
4	SCN^-, 12.5 μCi	14	0.22	0.004[a]

[a] These values have been revised since our earlier publication.[14]

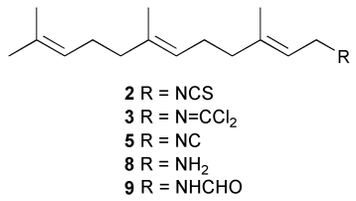


Scheme 1 Biosynthesis of the stylotellanes.

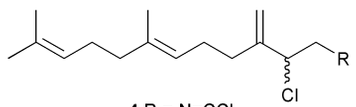
were isolated using flash chromatography followed by silica HPLC. In all experiments the isolated products were rigorously purified to constant specific radioactivity.

The incorporation of [^{14}C]-cyanide into the metabolites stylotellane A (**3**) and stylotellane B (**4**)[14] was compared with the utilization of this precursor for biosynthesis of farnesyl isothiocyanate (**2**). After a 9 day incorporation, the flash chromatography fraction containing the stylotellanes was subjected to normal phase HPLC (0.2% EtOAc in hexane) to give stylotellanes A (**3**) (9360 dpm mg^{-1}; 1.29 μCi mmol^{-1}) and B (**4**) (9630 dpm mg^{-1}; 1.47 μCi mmol^{-1}). The isolated farnesyl isothiocyanate (**2**) was significantly radioactive, but insufficient amounts prevented purification to constant specific activity. A second experiment, of 10 days duration, gave stylotellanes A (**3**) (34550 dpm mg^{-1}), B (**4**) (21780 dpm mg^{-1}) and farnesyl isothiocyanate (**2**) (5821300 dpm mg^{-1}; 696 μCi mmol^{-1}) (see Table 1). An additional experiment was then performed to investigate how the incorporation levels and specific activities changed over time. A piece of sponge was incubated with precursor, then placed on an underwater grid for 28 days to prolong the incorporation period, as previous specimens were not healthy beyond 10 days in an aquarium. Extraction gave stylotellane B (**4**) (172560 dpm mg^{-1}) and farnesyl isothiocyanate (**2**) (979870 dpm mg^{-1}) (see Table 1). In this experiment, stylotellane A (**3**) could not be cleanly separated from stylotellane B (**4**) (1 : 1 ratio), but the mixture of the two metabolites was found to be highly radioactive (716250 dpm mg^{-1}), indicating that stylotellane A was labelled. A comparison of the incorporation results from these three experiments reveals an interesting trend. The specific activity and incorporation levels decrease for farnesyl isothiocyanate (**2**) and increase for stylotellane B (**4**) over time, consistent with the rapid formation of isothiocyanate **2** from the precursor, while the dichloroimine **4** is formed more slowly. This is consistent with the isothiocyanate being converted to the dichloroimine over the longer incorporation period.

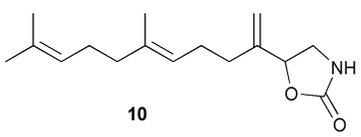
To demonstrate the specificity of labelling, a sample of the labelled stylotellane B (**4**) was diluted with unlabelled material and purified to constant specific activity by HPLC. The diluted material was then hydrolysed (H_3PO_4; 0.03 M in MeOH) to give the carbamate **6** and amine **7**. Purification of the carbamate **6** gave material with the same specific activity as the diluted



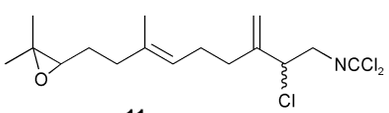
2 R = NCS
3 R = N=CCl_2
5 R = NC
8 R = NH_2
9 R = NHCHO



4 R = N=CCl_2
6 R = NHCOOMe
7 R = NH_2



10



11

stylotellane B (**4**), while the purified amine **7** showed negligible radioactivity (Table 2). Cyanide is therefore specifically incorporated into the dichloroimine functional group, similar to previously reported incorporations into isocyanides.[4,8]

Similar experiments were conducted using [^{14}C]-thiocyanate as precursor. After a 14 day incorporation, isolation of metabolites gave stylotellanes A (**3**) (2580 dpm mg^{-1}), B (**4**) (1480 dpm mg^{-1}) and farnesyl isothiocyanate (**2**; 5950 dpm mg^{-1}). A second experiment (10 day incorporation) gave stylotellane A (**3**) (1860 dpm mg^{-1}), stylotellane B (**4**) (1800 dpm mg^{-1}) and farnesyl isothiocyanate (**2**) (583 600 dpm mg^{-1}) (see Table 1). The lack of sufficient radiolabelled metabolite prevented chemical degradation to establish the sites of labelling, but considering earlier results it is likely that the [^{14}C]-label from thiocyanate was incorporated specifically into the dichloroimine carbon atom.

The experiments described above establish the precursor status of inorganic cyanide and thiocyanate in the biosynthesis of terpene dichloroimines in *S. aurantium*. We next explored the proposed advanced precursor roles of farnesyl isothiocyanate (**2**) and farnesyl isocyanide (**5**) in the biosynthesis of stylotellanes by incorporation of these compounds in [^{14}C]-labelled form.

Preparation of advanced precursors

Our preparation of labelled precursors followed the syntheses of [^{14}C]-labelled diisothiocyanatoadociane and 9-isocyanopupukeanane that we reported recently.[7,19,20] Farnesyl amine (**8**), available from farnesol in two steps using modified Gabriel conditions,[21] was used as a starting material for the radiochemical synthesis. The [^{14}C]-label was incorporated by formylation of amine **8** with [^{14}C]-formic acetic anhydride to give the [^{14}C]-formamide **9**. Dehydration of the formamide **9** with tosyl chloride in dry pyridine gave [^{14}C]-farnesyl isocyanide (**5**). The isocyanide proved to be unstable during workup, which may explain the fact that this metabolite has not been reported from the sponge.[10] This instability does not exclude the possibility that isocyanide **5** is an intermediate in dichloroimine biosynthesis. A portion of isocyanide **5** was converted to [^{14}C]-farnesyl isothiocyanate (**2**) by treatment with sulfur at 80 °C.

Table 2 Degradation of stylotellane B (**4**) from [^{14}C]-cyanide precursor studies in *S. aurantium*

Cpd.	Mol. spec. activity (μCi $mmol^{-1}$)	Relative activity (%)
4	0.332[a]	100.0
6	0.326	98.2
7	0.004	1.2

[a] After dilution with unlabelled material and purification by HPLC.

The synthetic product had identical spectroscopic data to the natural compound isolated from *S. aurantium*. All synthetic steps involved careful purification to constant specific activity.

Advanced precursor incorporation

[^{14}C]-Farnesyl isothiocyanate (**2**) (13.0 mg, 3.56 μCi) was incubated with a small specimen of *S. aurantium* according to our standard protocols.[7,19,20] The uptake of precursor was essentially complete during the first hour of incubation. After a 14 day incorporation period, the sponge was extracted, yielding stylotellane A (**3**) (7308 dpm mg^{-1}) and stylotellane B (**4**) (2364 dpm mg^{-1}). Similarly [^{14}C]-farnesyl isocyanide (**5**) (38.0 mg, 11.80 μCi) was fed to a specimen of *S. aurantium* in a second incorporation experiment and gave stylotellane A (**3**) (4292 dpm mg^{-1}) and stylotellane B (**4**) (722 dpm mg^{-1}). Farnesyl isothiocyanate (**2**) was also recovered, and was found to be radioactive (5078 dpm mg^{-1}). Comparing the two parallel 14 day incubations, the incorporation of [^{14}C]-farnesyl isothiocyanate (**2**) gave higher specific activities into both stylotellane metabolites than did [^{14}C]-farnesyl isocyanide (**5**), likely because of the instability of farnesyl isocyanide.[10] In a third incorporation experiment, [^{14}C]-farnesyl isocyanide (**5**) (38.0 mg, 11.80 μCi) was supplied to the sponge for 28 days, yielding after work up stylotellane A (**3**) (3654 dpm mg^{-1}), stylotellane B (**4**) (539 dpm mg^{-1}) and farnesyl isothiocyanate (**2**) (5546 dpm mg^{-1}). Finally, [^{14}C]-formamide **9** (14.2 mg, 4.19 μCi) was supplied to a fourth specimen of *S. aurantium* for 7 days. In this experiment, as anticipated from the earlier labelling work of Iengo *et al.*,[22] the isolated samples of the stylotellane metabolites showed no significant radioactivity. Specific activities and percentage incorporation levels for all incorporation experiments are shown in Table 3.

In these incorporation experiments, the radiolabel is unlikely to have been incorporated into the sesquiterpene portion of the natural products. The precursors had been specifically labelled by the synthetic procedure, while the possibility of metabolic degradation and reincorporation of the label *via* general metabolism is remote given the high incorporation yields obtained. In particular, the complete lack of incorporation of formamide **9** suggests that general metabolic degradation and reincorporation does not occur. To confirm the expected sites of radiolabelling, the samples of stylotellane B (**4**) isolated from incorporation of [^{14}C]-**2** and [^{14}C]-**5** were degraded with 0.03 M phosphoric acid in methanol to the cyclic carbamate **10** and the amine **7**. In both experiments, the carbamate product **10** retained the radioactivity (90–96%), whereas the amine **7** was devoid of radioactivity (Table 4). These data are in accordance with specific labelling of the dichloroimine carbon atom. These experiments therefore establish the precursor role of farnesyl isothiocyanate and isocyanide in the biosynthesis of the stylotellanes.

Some additional dichloroimine metabolites were isolated and purified from the advanced precursor experiments with farnesyl intermediates. Axinyssamide A (**11**) (818 dpm mg^{-1}), reticulidin B (**12**) (945 dpm mg^{-1}) and isoreticulidin B (**13**) (504 dpm mg^{-1}) were isolated following incorporation of [^{14}C]-farnesyl isothiocyanate (**2**). The 14 day [^{14}C]-farnesyl isocyanide (**5**) incorporation yielded axinyssamide A (**11**) (1132 dpm mg^{-1}), reticulidin B (**12**) (1078 dpm mg^{-1}) and isoreticulidin B (**13**)

Table 3 Advanced precursor incorporations with *S. aurantium*

Compound	Precursor	Mol. spec. act. (μCi mmol^{-1})	Incorporation (%)
3	[^{14}C]-**2**[a,b]	1.00	0.23
4		0.36	0.27
11		0.13	0.08
12		0.15	0.03
13		0.08	0.06
2	[^{14}C]-**5**[b,c]	0.61	0.03
3		0.58	0.04
4		0.11	0.13
11		0.18	0.02
12		0.17	0.02
13		0.17	0.02
2	[^{14}C]-**5**[c,d]	0.66	0.01
3		0.50	0.02
4		0.08	0.02
11		0.12	0.02
12		0.14	0.02
13		0.14	0.02
3	[^{14}C]-**9**[e,f]	$<10^{-5}$	—
4		$<10^{-5}$	—

[a] 3.6 μCi. [b] 14 day incorporation. [c] 11.8 μCi. [d] 28 day incorporation. [e] 7 day incorporation. [f] 4.2 μCi.

Table 4 Chemical conversions of labelled metabolites from advanced precursor studies with *S. aurantium*

Cpd.	Prec.	Mol. spec. act. (μCi mmol^{-1})	Inc. (%)
4	[^{14}C]-**2**	0.36	100.0
7		$<10^{-5}$	0.0
10		0.32	90.0[a]
4	[^{14}C]-**5**	0.062[b,c]	100.0
7		$<10^{-5}$	0.0
10		0.060[b]	96.7[a,b]

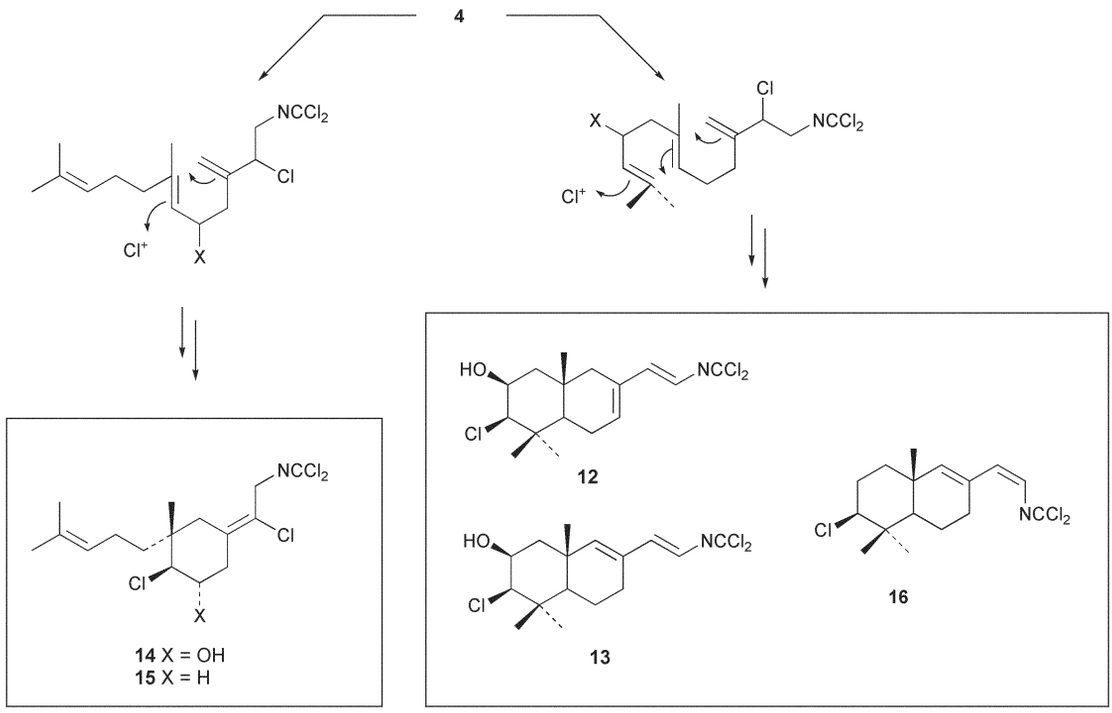
[a] The small amount of carbamate **10** available after purification meant the specific activity was lower than desired, however it is unlikely this level of labelling would be retained after purification of an unlabelled compound. [b] These values have been corrected since our earlier publication.[18] [c] The labelled sample (0.11 μCi mmol^{-1}) was diluted with unlabelled material prior to degradation.

(1052 dpm mg^{-1}), while the 28 day [^{14}C]-farnesyl isocyanide incorporation yielded axinyssamide A (**11**) (755 dpm mg^{-1}), reticulidin B (**12**) (656 dpm mg^{-1}) and isoreticulidin B (**13**) (883 dpm mg^{-1}). In the [^{14}C]-farnesyl isothiocyanate incorporations, the specific activity and incorporation levels are higher for the linear stylotellane metabolites **3** and **4** compared to the bicyclic metabolites **12** and **13**. In both the 14 and 28 day farnesyl isocyanide incorporations, the specific activities are highest for farnesyl isothiocyanate (**2**) and stylotellane A (**3**), then the bicyclic metabolites **12** and **13**, and are least for stylotellane B (**4**).

Scheme 2 shows mechanistic proposals for the formation of the suite of cyclic dichloroimine metabolites that have been isolated from *S. aurantium*. These metabolites divide naturally into two groups: (a) monocyclic dichloroimines exemplified by cyclohexanol **14**[9] and by compound **15** which we isolated recently;[23] (b) bicyclic dichloroimines with the reticulidin skeleton, *e.g.* **12**, **13**, **16**.[10,11,17] Both groups of metabolites can in principle originate from stylotellane B. Thus, attack of Cl^+ on the central double bond of stylotellane B (**4**) and cyclisation leads to metabolites **14** or **15** whereas attack at the terminal double bond, followed by cyclisation provides the carbon skeleton of the bicyclic compounds **12**, **13**, **16**. Allylic hydroxylation of stylotellane B prior to chlorine-induced cyclisation is an attractive route to **12**–**14**, although hydroxylation could also occur after cyclisation (*e.g.* **15** ⟶ **14** or **16** ⟶ **13**). The proposed role for stylotellane B in the biosynthesis of these cyclic dichloroimine metabolites could be tested by incorporation studies using labelled stylotellane B, however our attempts to synthesise this metabolite in labelled form have not yet succeeded. A preliminary experiment has been conducted using biosynthetically-generated [^{14}C]-stylotellane B (**4**) recovered from the incorporation of [^{14}C]-farnesyl isocyanide. A sample of stylotellane B (**4**) (0.049 μCi, 100 000 dpm) was supplied to *S. aurantium* for 15 days, and the isolated metabolites carefully purified by HPLC. The HPLC fraction containing an inseparable mixture of the cyclic metabolites **15** and **16**[23] had radioactivity 53.2 dpm mg^{-1} above background (0.05% incorporation), whereas the individually isolated samples of reticulidin B (**12**) and isoreticulidin B (**13**) were not radioactive. Given this very low utilisation of precursor, we reisolated stylotellane B (**4**) from this sponge sample, and after HPLC purification, this metabolite was radioactive (316 dpm mg^{-1} above background) which confirms precursor uptake by the sponge. Additional experiments using stylotellane B samples of higher radioactivity content are needed to better explore its proposed precursor role.

Conclusions

The observed incorporation of radioactivity into the dichloroimine metabolites from inorganic precursors NC^- and NCS^- as well as from farnesyl precursors **2** and **5** enables us to understand in more detail the biosynthetic paths leading to dichloroimine secondary metabolites. Firstly, the incorporation of radioactivity from [^{14}C]-cyanide as well as [^{14}C]-thiocyanate into the carbon atom of farnesyl isothiocyanate and into the dichloroimine metabolites supports the origin of the farnesyl isothiocyanate (**2**) or farnesyl isocyanide (**5**) from farnesyl pyrophosphate (**1**) and inorganic thiocyanate or cyanide. Our results next show that farnesyl isothiocyanate (**2**) and farnesyl isocyanide (**5**) can be utilised by the sponge *S. aurantium* as advanced precursors in dichloroimine biosynthesis. Further we have also demonstrated the R–NC to R–NCS interconversion at the secondary metabolite level (**5** ⟶ **2**). The fact that farnesyl isocyanide (**5**) has not been detected in *S. aurantium*, combined with its instability, prevented assessment of the reverse R–NCS to R–NC transformation, which has been demonstrated in some sponges (*Axinyssa* n.sp.[19] and *Ciocalypta* sp.[24]), but does not appear to be an efficient transformation in others such as



Scheme 2 Proposed biosynthesis of cyclic dichloroimines.

Amphimedon terpenensis.[7,19] As anticipated,[22] the formamide **9** was not utilised for dichloroimine biosynthesis. The slightly higher incorporation rates in the experiments with farnesyl isothiocyanate (**5**), an isolated metabolite, compared with activities from the farnesyl isocyanide (**5**) experiments may possibly indicate that farnesyl isothiocyanate (**2**) may be the key intermediate in the biosynthetic pathway to dichloroimine metabolites; unfortunately this interpretation is complicated by the instability of farnesyl isocyanide. The proposed reaction steps from isothiocyanate **2** to dichloroimine metabolites involve oxidative chlorination of the –NCS moiety giving stylotellane A (**3**), and then further chlorination of isothiocyanate **2** yielding stylotellane B (**4**). Stylotellane B (**4**) is proposed to undergo Cl^+-induced cyclisation to give the monocyclic products **14**, **15** or the bicyclic products **12**, **13**, **16**, however this could not be verified by precursor incorporation study.

With this biosynthetic scheme in mind, we are now able to look more purposefully into the enzymatic "tool box" of the sponge, and to pose additional questions that will have to be solved in the future. Firstly, in sponges a sulfur transferring enzyme, *e.g.* rhodanese,[25] has not yet been described, although our results infer that enzymes such as these may be present in *S. aurantium*. Secondly, the possible role of halogenating[26] enzymes in relation to the proposed introduction of chloride in electrophilic form should be probed. Finally, the role that enzymes may play in the various Cl^+ induced cyclisations (Scheme 2) that generate the extraordinary suite of dichloroimine metabolites found in *S. aurantium* clearly warrants further attention.

Experimental

General experimental

General experimental procedures have been reported elsewhere.[19]

Radiochemical measurements

Scintillation counting was carried out on a Wallac 1410 liquid scintillation counter using Wallac Optiphase Hisafe aqueous organic scintillation liquid. Each chemical sample was counted in triplicate. Other organic samples were counted in triplicate in either benzene or methanol, using 1% of the available material in each scintillation vial. Samples were dissolved in benzene (10 mL), and 0.1 mL aliquots transferred directly into scintillation vials, then diluted with 3.9 mL of scintillant. Samples of low activity were then recounted using 30% of the available material in each scintillation vial. All samples were counted until the number of counts recorded reached a 2σ confidence limit. Sample radioactivity values were corrected for background radiation by subtraction of an averaged value obtained by counting in triplicate blank samples containing the same volume of solvent.

Animal material

Sponge samples were collected by SCUBA at Coral Gardens and Coral Spawning dive sites, Heron Island (23°27′S, 151°55′E), Great Barrier Reef (−14–16 m). A voucher sample (registry number QM G312575) was identified as *Stylotella aurantium* and is lodged at The Queensland Museum, Brisbane.

Isolation of metabolites

In a typical procedure frozen sponge sample (50 g wet wt.) was extracted exhaustively with $CHCl_3$: MeOH (1 : 1), and the concentrated extract partitioned into CH_2Cl_2 to give after drying ($MgSO_4$), an orange coloured extract (3–5% of sponge wet wt.) which was subjected to flash chromatography on silica using gradient elution with hexane, gradually changing to dichloromethane, and finally to EtOAc, to give four main fractions. The first fraction (R_f 0.75–0.8 in hexane : CH_2Cl_2, 2 : 1) was further purified by silica HPLC (hexane : CH_2Cl_2, 8 : 1) to give farnesyl isothiocyanate (**2**) (1.6 mg),[10,27] and a mixture of stylotellanes A and B which were further separated by HPLC (hexane 0.5% EtOAc) to yield stylotellane A (**3**) (2.5 mg) and stylotellane B (**4**) (8.7 mg).[9,14] The second fraction with R_f 0.35–0.70 was purified by silica HPLC using hexane : CH_2Cl_2 (6 : 1) and gave farnesyl isothiocyanate (**2**) (0.8 mg) together with a mixture of the dichloroimines **15** and **16** (4.7 mg).[11,23] The third fraction (R_f 0.2) was recrystallised from hexane to give reticulidin B (**12**) (12 mg).[17] The fourth fraction (R_f 0.1) was separated by preparative TLC (hexane : CH_2Cl_2 (1 : 3)) followed

by silica HPLC (hexane : CH_2Cl_2 (5 : 1)) to give isoreticulidin B (**13**) (10 mg),[11] and axinyssamide A (**11**) (7 mg).[12]

Incorporation experiments with inorganic precursors

Incorporation of [^{14}C]-cyanide. A specimen of *S. aurantium* (24 g wet wt.) was placed in an aquarium containing 200 mL aerated seawater at ambient temperature. [^{14}C]-NaCN (50 μCi) was added and the sponge allowed to assimilate radioactivity for 12 h overnight. Water samples were taken at regular intervals and these confirmed that the precursor was taken up during this period. The sponge was then kept in running seawater in a 10 L aquarium at ambient temperature for 9 days, and then frozen. The frozen sample was extracted as described above and the metabolites purified to constant specific radioactivity. Weights and recoveries were as follows: stylotellane A (**3**) (2.5 mg, 0.01% chemical yield; 9360 dpm mg^{-1}, 0.021% radiochemical incorporation) and stylotellane B (**4**) (3.8 mg, 0.016%; 9630 dpm mg^{-1}, 0.03%).

A second piece of sponge (65 g wet wt.) was allowed to metabolise the precursor [^{14}C]-NaCN (100 μCi) for 10 days, then extracted to give stylotellane A (**3**) (0.9 mg, 0.0014%; 34 550 dpm mg^{-1}, 0.015%), stylotellane B (**4**) (8.1 mg, 0.012%; 21 780 dpm mg^{-1}, 0.08%) and farnesyl isothiocyanate (**2**) (0.7 mg, 0.0011%; 5 821 300 dpm mg^{-1}, 1.98%).

A third piece of sponge (50 g wet wt.) was allowed to metabolise the precursor [^{14}C]-NaCN (100 μCi) overnight then the sponge sample was placed on an underwater grid at –16 m for 28 days, then extracted to give stylotellane B (**4**) (6.3 mg, 0.013%; 172 560 dpm mg^{-1}, 0.5%) and farnesyl isothiocyanate (**2**) (0.9 mg, 0.0018%; 979 870 dpm mg^{-1}, 0.43%).

Incorporation of [^{14}C]-thiocyanate. The incorporation of [^{14}C]-NaSCN (12.5 μCi) was performed in the same way on a sponge of wet wt. 12 g for 14 days and gave stylotellane A (**3**) (0.3 mg, 0.0025%; 2580 dpm mg^{-1}, 0.0028%), stylotellane B (**4**) (0.8 mg, 0.0067%; 1480 dpm mg^{-1}, 0.0041%), and farnesyl isothiocyanate (**2**) (0.5 mg, 0.0042%; 5950 dpm mg^{-1}, 0.01%). A second piece of sponge (50 g wet wt.) was treated with the precursor [^{14}C]-NaSCN (25 μCi) for 10 days and gave stylotellane A (**3**) (1.9 mg, 0.038%; 1860 dpm mg^{-1}, 0.087%), stylotellane B (**4**) (13.9 mg, 0.0278%; 1800 dpm mg^{-1}, 0.015%) and farnesyl isothiocyanate (**2**) (0.7 mg, 0.0014%; 583 600 dpm mg^{-1}, 0.78%).

Synthesis of [^{14}C]-labelled advanced precursors

[16-^{14}C]-*N*-[(3*E*,7*E*)-3,7,11-Trimethyl-2,6,10-dodecatrienyl]-formamide (farnesyl formamide) (9). [^{14}C]-Formic acetic anhydride was generated by a 2 h equilibration of freeze dried [^{14}C]-sodium formate (1 mCi) with 90% aqueous solution of formic acid (0.19 mL, 4.42 mmol), followed by addition of acetic anhydride (0.66 mL, 7.0 mmol) and warming to 60 °C for a further 2 h. To this freshly prepared solution was added farnesyl amine (**8**)[21] (680 mg, 3.1 mmol) in dry CH_2Cl_2 (5 mL). After 1 h, the reaction mixture was diluted with CH_2Cl_2 (30 mL) and poured into saturated $NaHCO_3$solution (20 mL). The organic layer was washed with water (3 × 10 mL) and dried over $MgSO_4$ to give the formamide **9** as a colourless oil, which was further purified by silica flash chromatography (hexane to chloroform). The combined fractions with R_f 0.36 ($CHCl_3$: methanol 100 : 1) yielded the [^{14}C]-formamide **9** (640 mg, 83%, 190 μCi, 19% radiochemical yield); δ_H (400 MHz; $CDCl_3$) 1.59 (6 H, s, 12-H_3, 13-H_3), 1.67 (6 H, s, 14-H_3, 15-H_3), 1.96–2.10 (8 H, m, 4-H_2, 5-H_2, 8-H_2, 9-H_2), 3.89 (2 H, t, *J* 6 Hz, 1-H_2), 5.09 (2 H, m, 2-H, 6-H), 5.19 (1 H, t, *J* 6.4 Hz, H-10), 5.50 (1 H, bs, N*H*), 8.15 (1 H, s, *H*CO); δ_C (125 MHz, $CDCl_3$) 16.0, 16.3, 17.7, 25.7 (4 × q, C-12, C-13, C-14, C-15), 26.2, 26.7 (2 × t, C-4, C-8), 36.0, 39.4 (2 × t, C-5, C-9), 39.7 (t, C-1), 119.3, 123.5, 124.3 (3 × d, C-2, C-6, C-10), 131.4, 135.5, 140.6 (3 × s, C-3, C-7, C-11), 160.9 (1 × d, NH*C*HO); *m*/*z* (EI) 249 ($M^{+\bullet}$, 80%), 204 (90), 189 (30), 180 (40), 161 (70); *m*/*z* (EI) 249.2091 ($M^{+\bullet}$) $C_{16}H_{27}NO$ requires 249.2092.

[16-^{14}C]-(3*E*,7*E*)-1-(Isocyano)-3,7,11-trimethyl-2,6,10-dodecatriene (farnesyl isocyanide) (5). To a solution of formamide **9** (150 mg, 0.6 mmol, 44.5 μCi) in dry pyridine (3 mL) was added tosyl chloride (1.14 g, 6 mmol). The mixture was heated for about 1 h to 115 °C, then poured into hexane (30 mL), then washed with saturated $NaHCO_3$ solution (1 × 10 mL) and water (1 × 20 mL). After drying over $MgSO_4$, and evaporating, repeated purification on silica gel with hexane as eluent yielded farnesyl isocyanide (**5**) as a colourless oil (97 mg, 70%; 30 μCi, 67.6% radiochemical yield); δ_H (500 MHz, $CDCl_3$) 1.59 (6 H, s, 12-H_3, 13-H_3), 1.66, 1.69 (3 H each, 2 s, 14-H_3, 15-H_3), 1.96–2.10 (8 H, m, 4-H_2, 5-H_2, 8-H_2, 9-H_2), 3.98 (2 H, d, *J* 6.5 Hz, 1-H_2), 5.08 (2 H, m, 2-H, 6-H), 5.27 (1 H, t, *J* 6.5 Hz, 10-H); δ_C (125 MHz, $CDCl_3$) 16.0, 16.4, 17.7, 25.8 (4 × q, C-12, C-13, C-14, C-15), 26.0, 26.4 (2 × t, C-4, C-8), 39.1, 39.6 (2 × t, C-5, C-9), 39.4 (t, J_{NC} 6.5 Hz, C-1), 115.7, 123.2, 124.2 (3 × d, C-2, C-6, C-10), 131.5, 131.8, 142.1 (3 × s, C-3, C-7, C-11), 154.4 (bs, N*C*); *m*/*z* (EI) 231 ($M^{+\bullet}$, 40%), 230 (M − 1, 80), 216 (M − CH_3, 25), 204 (10), 190 (20%); *m*/*z* (EI) 231.1983 ($M^{+\bullet}$) $C_{16}H_{25}N$ requires 231.1987.

[16-^{14}C]-(3*E*,7*E*)-1-(Isothiocyanato)-3,7,11-trimethyl-2,6,10-dodecatriene (farnesyl isothiocyanate) (2).[10,27] In a sealed reaction vial farnesyl isocyanide (**5**) (90 mg, 27.8 μCi) and sulfur were heated together for 18 h at 80 °C. The brown crude product was purified by chromatography on silica gel with hexane as eluent to yield farnesyl isothiocyanate (**2**) as a colourless oil (24.2 mg, 23.6%; 6.35 μCi, 22.9% radiochemical yield); δ_H (500 MHz, $CDCl_3$) 1.60 (6 H, s, 12-H_3, 13-H_3), 1.68 (6 H, s, 14-H_3, 15-H_3), 1.98–2.18 (8 H, m, 4-H_2, 5-H_2, 8-H_2, 9-H_2), 4.07 (2 H, d, *J* 6.5 Hz, 1-H_2), 5.08 (2 H, m, 2-H, 6-H), 5.30 (1 H, t, *J* 6.5 Hz, 10-H); δ_C (125 MHz, $CDCl_3$) 15.5, 15.9, 17.2, 25.2 (4 × q, C-12, C-13, C-14, C-15), 25.7, 26.2 (2 × t, C-4, C-8), 38.7 (t, C-1), 39.1, 42.1 (2 × t, C-5, C-9), 116.3, 122.8, 123.7 (3 × d, C-2, C-6, C-10), 130.9, 135.3, 141.7 (3 × s, C-3, C-7, C-11), 135.7 (s, N*C*S); *m*/*z* (EI) 263 ($M^{+\bullet}$, 50%), 248 (M − CH_3, 25), 230 (M − S, 90), 216 (80); 204 (25); *m*/*z* (EI) 263.1717 ($M^{+\bullet}$) $C_{16}H_{25}NS$ requires 263.1721.

Repeat synthesis. In a second set of synthetic experiments formamide **9** (300 mg, 1.2 mmol, 89 μCi) was converted to isocyanide (**5**) (210 mg, 75%; 66 μCi, 74.2% radiochemical yield). Then farnesyl isocyanide (**5**) (90 mg, 28.3 μCi) was converted to farnesyl isothiocyanate (**2**) (26 mg, 25.3%; 6.8 μCi, 24.1% radiochemical yield).

Incorporation experiments with advanced precursors

Incorporation of [^{14}C]-farnesyl isothiocyanate (2). A specimen of *S. aurantium* (59 g wet wt.) was supplied with [^{14}C]-farnesyl isothiocyanate (**2**) (3.6 μCi, 69.0 μCi mmol^{-1}) for 14 days, according to our usual protocols,[19] then extracted to give stylotellane A (**3**) (2.5 mg, 0.004%; 7308 dpm mg^{-1}; 0.23%), stylotellane B (**4**) (8.7 mg, 0.014%; 2364 dpm mg^{-1}, 0.26%), axinyssamide A (**11**) (7.7 mg, 0.013%; 818 dpm mg^{-1}, 0.08%), reticulidin B (**12**) (2.1 mg, 0.004%; 945 dpm mg^{-1}, 0.025%), and isoreticulidin B (**13**) (10.0 mg, 0.017%; 504 dpm mg^{-1}, 0.06%).

Incorporation of [^{14}C]-farnesyl isocyanide (5). A specimen of *S. aurantium* (36 g wet wt.) was supplied with [^{14}C]-farnesyl isocyanide (**5**) (11.8 μCi, 71.2 μCi mmol^{-1}) for 14 days, then extracted to give farnesyl isothiocyanate (**2**) (1.6 mg, 0.004%; 5078 dpm mg^{-1}, 0.03%), stylotellane A (**3**) (2.5 mg, 0.007%; 4292 dpm mg^{-1}, 0.035%) stylotellane B (**4**) (4.5 mg, 0.012%; 722 dpm mg^{-1}, 0.009%), axinyssamide A (**11**) (4.5 mg, 0.012%; 1132 dpm mg^{-1}, 0.02%), reticulidin B (**12**) (2.2 mg, 0.006%; 1078

dpm mg^{-1}, 0.009%), and isoreticulidin B (**13**) (4.9 mg, 0.013%; 1052 dpm mg^{-1}, 0.02%).

The 28 day incorporation experiment yielded: farnesyl isothiocyanate (**2**) (1.8 mg, 0.004%; 5546 dpm mg^{-1}, 0.03%), stylotellane A (**3**) (2.8 mg, 0.008%; 3654 dpm mg^{-1}, 0.035%) stylotellane B (**4**) (4.9 mg, 0.013%; 539 dpm mg^{-1}, 0.009%), axinyssamide A (**11**) (4.9 mg, 0.013%; 755 dpm mg^{-1}, 0.02%), reticulidin B (**12**) (2.4 mg, 0.007%; 880 dpm mg^{-1}, 0.009%), and isoreticulidin B (**13**) (5.4 mg, 0.013%; 890 dpm mg^{-1}, 0.02%).

Incorporation of [^{14}C]-farnesyl formamide (9). A specimen of *S. aurantium* (28 g wet wt.) was fed with [^{14}C]-formamide **9** (4.2 μCi, 73.7 μCi $mmol^{-1}$). The sponge extract was processed to give stylotellane A (**3**) (0.2 mg, 0.0007%), stylotellane B (**4**) (2.8 mg, 0.01%) and reticulidin B (**12**) (2.5 mg, 0.008%), all of which were devoid of radioactivity.

Hydrolysis of stylotellane B (4). The isolated stylotellane B (**4**) from the 14 day and 28 day incorporation experiment with isocyanide **5** was combined (9.5 mg, 0.028 mmol; 627 dpm mg^{-1}) and diluted with unlabelled material (5 mg, 0.015 mmol) to result in a final activity of 404 dpm mg^{-1}. The sample was dissolved in 0.03 M phosphoric acid (10 mL, solution in 90% methanol), and refluxed for 6 h. The resulting solution was poured into saturated $NaHCO_3$ solution, then extracted into CH_2Cl_2 (3 × 50 mL), the combined organic extracts washed with water, dried over $MgSO_4$, and concentrated to a syrup. This residue was fractioned on silica gel to give two fractions, one containing methyl carbamate **6** and the cyclic carbamate **10** which were separated by preparative TLC ($CHCl_3$: methanol 50 : 1) to yield the carbamate **10** (1.0 mg, 9%; 498 dpm mg^{-1}, 96%). The second fraction contained the non-radioactive amine **7** (3.5 mg, 30%) (see Table 4).

The isolated stylotellane B (**5**) (8.6 mg, 0.025 mmol; 2364 dpm mg^{-1}) from the incorporation experiment with isothiocyanate (**2**) was treated as above to give non-radioactive amine **7** (1.5 mg, 23%) and the carbamate **10** (1.0 mg, 15%; 2 701 dpm mg^{-1}, 90%) (see Table 4).

[(6*E*)-2-Chloro-7,11-dimethyl-3-methylene-6,10-dodecadienyl]-carbamic acid methyl ester (6).[14] δ_H (400 MHz, $CDCl_3$) 1.59, 1.61 (3 H each, 2 s, 12-H_3, 13-H_3), 1.69 (3 H, s, 14-H_3), 1.98 (2 H, m, 8-H_2), 2.09–2.13 (4 H, m, 5-H_2, 9-H_2), 2.13–2.20 (2 H, m, 4-H_2), 3.40 (2 H, m, 1-H_2), 3.67 (3 H, s, $COOCH_3$), 4.45 (1 H, m, 2-H), 5.05 (1 H, s, -C=CH_{2a}), 5.09–5.11 (2 H, m, 6-H, 10-H), 5.19 (1 H, s, -C=CH_{2b}); δ_C (100 MHz, $CDCl_3$) 16.1, 17.7, 25.7 (3 × q, C-12, C-13, C-14), 26.2, 26.7 (2 × t, C-5, C-9), 31.8, 39.7 (2 × t, C-4, C-8), 46.1 (t, C-1), 52.3 (q, CH_3OOC), 64.2 (d, C-2), 114.4 (t, -C=CH_2), 123.2, 124.3 (2 × d, C-6, C-10), 131.4, 136.0, 145.7 (3 × s, C-3, C-7, C-11), 160.3 ($COOCH_3$); *m*/*z* (EI) 313 ($M^{+\bullet}$, 60%), 294 (20), 277 (60); *m*/*z* (EI) 313.1822 ($M^{+\bullet}$) $C_{17}H_{28}NO_2$ requires 313.1823.

(6*E*)-2-Chloro-7,11-dimethyl-3-methylene-6,10-dodecadienylamine (7).[14] δ_H (400 MHz, $CDCl_3$) 1.60, 1.61 (3 H each, 2 s, 12-H_3, 13-H_3), 1.67 (3 H, s, 14-H_3), 1.88 (2 H, bs, NH_2), 1.95 (2 H, m, 8-H_2), 2.05–2.11 (4 H, m, 5-H_2, 9-H_2), 2.12–2.20 (2 H, m, 4-H_2), 3.03 (2 H, m, 1-H_2), 4.35 (1 H, m, 2-H), 5.01 (1 H, s, -C=CH_{2a}), 5.07–5.11 (2 H, m, 6-H, 10-H), 5.18 (1 H, s, -C=CH_{2b}); δ_C (100 MHz, $CDCl_3$) 16.1, 17.7, 25.7 (3 × q, C-12, C-13, C-14), 26.2, 26.7 (2 × t, C-5, C-9), 31.6, 39.6 (2 × t, C-4, C-8), 45.6 (t, C-1), 64.0 (d, C-2), 115.1 (t, -C=CH_2), 123.1, 124.2 (2 × d, C-6, C-10), 131.5, 136.2, 145.3 (3 × s, C-3, C-7, C-11); *m*/*z* (EI) 255 ($M^{+\bullet}$, 1%), 240 (M − CH_3).

[2′-^{14}C]-5′-[(4*E*)-5,9-Dimethyl-1-methylene-4,8-decadienyl]-oxazolidin-2′-one (10). δ_H (400 MHz, $CDCl_3$) 1.59, 1.60 (3 H each, 2 s, 10-H_3, 11-H_3), 1.67 (3 H, s, 12-H_3), 1.99 (2 H, m, 6-H_2), 2.05–2.12 (4 H, m, 3-H_2, 7-H_2), 2.13–2.20 (2 H, m, 2-H_2), 3.37 (1 H, t, *J* 8 Hz, 5′-H), 3.72 (2 H, t, *J* 8 Hz, 4′-H_2), 5.01 (1 H, s, -C=CH_{2a}), 5.07–5.09 (2 H, m, 4-H, 8-H), 5.17 (1 H, s, -C=CH_{2b}); δ_C (100 MHz, $CDCl_3$) 16.1, 17.7, 25.7 (3 × q, C-10, C-11, C-12), 25.9, 26.6 (2 × t, C-3, C-7), 30.6, 39.6 (2 × t, C-2, C-6), 45.3 (t, C-4′), 78.9 (d, C-5′), 112.2 (t, -C=CH_2), 123.1, 124.2 (2 × d, C-4, C-8), 131.4, 136.2, 145.1 (3 × s, C-1, C-5, C-9), 159.3 (s, C-2′); *m*/*z* (EI) 263 ($M^{+\bullet}$, 50%), 248 (M − CH_3, 50), 234 (M, 20), 220 (80); *m*/*z* (EI) 263.1714 ($M^{+\bullet}$) $C_{16}H_{23}NO_2$ requires 263.1855.

Incorporation of [^{14}C]-stylotellane B (4). Fractions from the chromatography of stylotellane B isolated in the [^{14}C]-farnesyl isocyanide experiment were combined and repurified by HPLC. A specimen of *S. aurantium* (32 g wet wt.) was supplied with this sample of [^{14}C]-stylotellane B (**4**) (17.5 mg; 0.049 μCi, 0.94 μCi $mmol^{-1}$) for 14 days, then extracted to give farnesyl isothiocyanate (**2**) (0.9 mg), stylotellane B (**4**) (2.3 mg; 316 dpm mg^{-1}), a mixture of the cyclic metabolites **15** and **16** (0.9 mg; 53.2 dpm mg^{-1}, 0.05%), reticulidin B (**12**) (1.5 mg), and isoreticulidin B (**13**) (4.0 mg).

Acknowledgements

We thank the Australian Research Council and The University of Queensland for funding. The assistance of Feras Lafi, Emma-Karin Millers, Wimolpun Rungprom and the staff of Heron Island Research Station in performing the research is gratefully acknowledged. We thank Dr John Hooper, The Queensland Museum, for taxonomic identification of samples. This research was performed under permits G96/050, G97/097 and G01/146 issued jointly by GBRMPA and QNPWS.

References

1 C. W. J. Chang, in *Fortschritte der Chemie Organischer Naturstoffe*, eds. W. Herz, H. Falk, G. W. Kirby and R. Moore, Springer-Verlag, New York, 2000, vol 80, pp. 1–186.
2 M. J. Garson, J. S. Simpson, A. E. Flowers and E. J. Dumdei, in *Studies in Natural Products Chemistry*, ed. Atta-ur-Rahman, Elsevier, New York, 2001, vol 21B, pp. 329–372.
3 P. Karuso, in *Bioorganic Marine Chemistry*, ed. P. J. Scheuer, Springer-Verlag, Berlin, 1987, vol 1, pp. 31–60.
4 (*a*) M. J. Garson, *J. Chem. Soc., Chem. Commun.*, 1986, 35–36; (*b*) C. J. R. Fookes, M. J. Garson, J. K. MacLeod, B. W. Skelton and A. H. White, *J. Chem. Soc., Perkin Trans. I*, 1988, 1003–1011.
5 E. J. Dumdei, A. E. Flowers, M. J. Garson and C. J. Moore, *Comp. Biochem. Physiol., A: Physiol.*, 1997, **118**, 1385–1392.
6 J. S. Simpson and M. J. Garson, *Tetrahedron Lett.*, 1998, **39**, 5819–5822.
7 J. S. Simpson and M. J. Garson, *Tetrahedron Lett.*, 1999, **40**, 3909–3912.
8 P. Karuso and P. J. Scheuer, *J. Org. Chem.*, 1989, **54**, 2092–2095.
9 S. J. Wratten and D. J. Faulkner, *J. Am. Chem. Soc.*, 1977, **99**, 7367–7368.
10 S. J. Wratten, D. J. Faulkner, D. Van Engen and J. Clardy, *Tetrahedron Lett.*, 1978, **19**, 1391–1394.
11 S. J. Wratten and D. J. Faulkner, *Tetrahedron Lett.*, 1978, **19**, 1395–1398.
12 H. Hirota, T. Okino, E. Yoshimura and N. Fusetani, *Tetrahedron*, 1998, **54**, 13971–13980.
13 S. Kehraus, G. M. König and A. D. Wright, *J. Nat. Prod.*, 2001, **64**, 939–941.
14 J. S. Simpson, P. Raniga and M. J. Garson, *Tetrahedron Lett.*, 1997, **38**, 7947–4950.
15 (*a*) M. Musman, J. Tanaka and T. Higa, *J. Nat. Prod.*, 2001, **64**, 111–113; (*b*) T. Higa, *Pure Appl. Chem.*, 2001, **73**, 589–593.
16 W. Plangsangkate, B. L. Stapleton and M. J. Garson, *ACGC Chem. Res. Commun.*, 2001, **13**, 23–27.
17 J. Tanaka and T. Higa, *J. Nat. Prod.*, 1999, **62**, 1339–1340.
18 A. Brust and M. J. Garson, *Tetrahedron Lett.*, 2003, **43**, 327–330.
19 J. S. Simpson and M. J. Garson, *Org. Biomol. Chem.*, 2004, **2**, DOI: 10.1039/b315895k, preceding paper.
20 J. S. Simpson and M. J. Garson, *Tetrahedron Lett.*, 2001, **42**, 4267–4269.
21 S. E. Sen and S. L. Roach, *Synthesis*, 1995, 756–758.

22 A. Iengo, C. Santacroce and G. Sodano, *Experientia*, 1979, **35**, 10–11.
23 A. Brust and M. J. Garson, *ACGC Chem. Res. Commun.*, 2004, in press.
24 M. R. Hagadone, P. J. Scheuer and A. Holm, *J. Am. Chem. Soc.*, 1984, **106**, 2447–2448.
25 J. Westley, in *Cyanide in Biology*, eds. B. Vennesland, E. E. Conn, C. J. Knowles, J. Westley and F. Wissing, Academic Press, London, 1981, vol 1, pp. 61–76.
26 K.-H. van Pee and S. Unversucht, *Chemosphere*, 2003, **52**, 299–312.
27 G. Pala, A. Mantegani and G. Coppi, *J. Med. Chem.*, 1969, **12**, 725–726.