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Biosynthetic pathways to isocyanides and isothiocyanates; precursor incorporation studies on terpene metabolites in the tropical marine sponges *Amphimedon terpenensis* and *Axinyssa* n.sp.†‡

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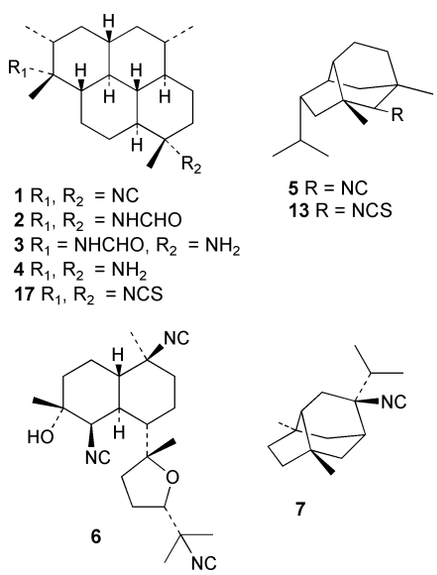
The biosynthetic origins of the isocyanide and isothiocyanate functional groups in the marine sponge metabolites diisocyanoadociane (**1**), 9-isocyanopupukeanane (**10**) and 9-isothiocyanatopupukeanane (**11**) are probed by the use of [^{14}C]-labelled precursor experiments. Incubation of the sponge *Amphimedon terpenensis* with [^{14}C]-labelled thiocyanate resulted in radioactive diisocyanoadociane (**1**) in which the radiolabel is specifically associated with the isocyanide carbons. As expected, cyanide and thiocyanate were confirmed as precursors to the pupukeananes **10** and **11** in the sponge *Axinyssa* n.sp.; additionally these precursors labelled 2-thiocyanatoneopupukeanane (**12**) in this sponge. To probe whether isocyanide-isothiocyanate interconversions take place at the secondary metabolite level, the advanced precursor bisisothiocyanate **17** was supplied to *A. terpenensis*, but did not result in significant labelling in the natural product isocyanide **1**. In contrast, in the sponge *Axinyssa* n.sp., feeding of [^{14}C]-9-isocyanopupukeanane (**10**) resulted in isolation of radiolabelled 9-isothiocyanatopupukeanane **11**, while the feeding of [^{14}C]-**11** resulted in labelled isocyanide **10**. These results show conclusively that isocyanides and isothiocyanates are interconverted in the sponge *Axinyssa* n.sp., and confirm the central role that thiocyanate occupies in the terpene metabolism of this sponge.

Introduction

Marine invertebrates produce an abundance of unique and bioactive compounds, notable among which are the terpene isocyanides.[1,2] Following the first reports of their isolation in the mid-1970's,[3] interest in the biosynthesis of these metabolites, particularly in the origin of the isocyanide group,[3b,4] has led to a number of experimental studies on them.[5,6] Research from our group was the first to show that the isocyanide groups of diisocyanoadociane (**1**) are derived from inorganic cyanide through incorporation of [^{14}C]-cyanide into the sponge *Amphimedon terpenensis*. The radioactivity in the resulting samples of the diterpene **1** was proven to be specifically associated with the isocyanide carbons through stepwise hydrolytic degradation to the corresponding formamide **2**, and then to amines **3** and **4**.[5] Subsequent studies by Karuso and Scheuer showed that [^{14}C]-cyanide is a precursor for 2-isocyanopupukeanane (**5**) in *Ciocalypta* sp. and the diterpene kalihinol F (**6**) in *Acanthella* sp. They also confirmed that the carbon and nitrogen atoms of the isocyanide moiety in 9-isocyanoneopupukeanane (**7**) are derived intact from [^{13}C, ^{15}N]-cyanide.[6b]

Many terpene isocyanides are isolated with the corresponding isothiocyanate substituted metabolites, for example the spiroaxane axisonitrile-3 (**8**) together with axisothiocyanate-3 (**9**)[7] and the tricyclic 9-isocyanopupukeanane (**10**) with 9-isothiocyanatopupukeanane (**11**).[8–10] The related thiocyanates (such as **12**), have been isolated less frequently from marine organisms.[9,11] Incorporation studies with Australian specimens of *Acanthella cavernosa* established that thiocyanate ion was a precursor to the isothiocyanate moiety in axisothiocyanate-3 (**9**), but more surprisingly, that this precursor also labelled the isocyanide group of axisonitrile-3 (**8**).[12] Furthermore, cyanide was a precursor to both the isocyanide and isothiocyanate moieties in this sponge. These results suggested that marine sponges might interconvert cyanide and thiocyanate at the inorganic level (Scheme 1; reactions a and b). The conversion of cyanide to thiocyanate by rhodanese is a well-studied irreversible reaction in microorganisms, plants and higher animals,[13] while peroxidases are known to catalyse the oxidation of thiocyanate to cyanide and sulfate.[13b] Alternatively, marine isocyanides and isothiocyanates might be interconverted at



1 R_1, R_2 = NC
2 R_1, R_2 = NHCHO
3 R_1 = NHCHO, R_2 = NH_2
4 R_1, R_2 = NH_2
17 R_1, R_2 = NCS

5 R = NC
13 R = NCS

6

7

† Dedicated to the memory of Paul J. Scheuer (1915–2003) who was a pioneer of marine isocyanide chemistry.
‡ Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR spectra for compounds **14**–**18**. See http://www.rsc.org/suppdata/ob/b3/b315894b/
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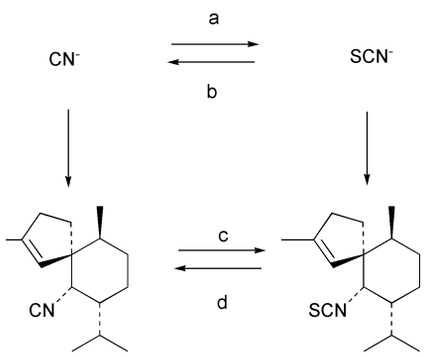
Table 1 Incorporation of inorganic precursors into *Amphimedon terpenensis* and *Axinyssa* n.sp

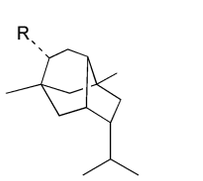
Compound	Precursor, amount	Incorporation period (days)	Molar specific activity (μCi mmol^{-1})	Incorporation (%)
1	SCN^-, 25 μCi	12	0.48	0.049
1	SCN^-, 50 μCi	19	0.92	0.14
10	CN^-, 100 μCi	16	0.006	0.0009
10	CN^-, 100 μCi	27	0.001	0.00005
10	SCN^-, 12.5 μCi	3	0.002	0.0075
10	SCN^-, 25 μCi	16	0.003	0.0015
11	CN^-, 100 μCi	16	13.9	0.3
11	SCN^-, 12.5 μCi	3	6.4	1.14
11	SCN^-, 25 μCi	16	2.6	0.35
12	CN^-, 100 μCi	16	1.23	0.079[a]
12	CN^-, 100 μCi	27	0.007	0.00009
12	SCN^-, 12.5 μCi	3	0.36	0.047
12	SCN^-, 25 μCi	16	0.15	0.029[a]

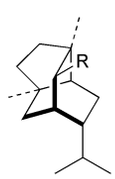
[a] These values have been recalculated since our earlier publication.[16]



Scheme 1 Proposed biosynthetic pathways for *A. cavernosa*.

the secondary metabolite level, as suggested for the spiroaxane series of metabolites in Scheme 1 (reactions c and d). Using novel experimental techniques that left the sponge specimens under study in their natural habitat, the Scheuer group found that 2-isocyanopupukeanane (**5**) was a precursor to the corresponding isothiocyanate **13** in *Hymeniacidon* sp. (= *Ciocalypta* sp.), but they could not detect the reverse isothiocyanate to isocyanide conversion. These experiments used [^{13}C]-labelled precursors and were analysed by GC-MS, using the $[M + 1]^+$ ion to measure the incorporation of [^{13}C]-label.[14]





10 : R = NC
11 : R = NCS
14 : R = NHCHO
15 : R = NHCSN(Et)$_2$
18 : R = NH$_2$

12 : R = SCN
16 : R = SH

In this paper we provide full details of sensitive [^{14}C]-labelling experiments that compare isocyanide–isothiocyanate interconversions in two marine sponges, firstly in *A. terpenensis* which is characterised by isocyanides such as **1**,[15] and in which isothiocyanates are minor metabolites, and then in *Axinyssa* n.sp. which contains the 9-isocyanopupukeanane (**10**) and 9-isothiocyanatopupukeanane (**11**) pair of metabolites, together with thiocyanate **12**.[9,16] As we show below, the two sponges provide contrasting results in these advanced precursor incorporations. Further, our earlier work with *Axinyssa* n.sp. had indicated that while this sponge converts both cyanide and thiocyanate into the isothiocyanate **11**, the isocyanide **10** from both thiocyanate and cyanide feedings appeared not to be labelled.[17] This unconvincing biosynthetic result was most unexpected given the significant incorporations into the diterpene and sesquiterpene isocyanides described above. We therefore report additional precursor incorporation data that enable us to properly describe isocyanide/isothiocyanate biosynthesis in *Axinyssa* n.sp.

Results and discussion

Precursor incorporation studies were performed using our standard protocols[5,12] on healthy species of *A. terpenensis* or of *Axinyssa* n.sp. collected at Heron Island or at Lizard Island on the Great Barrier Reef. The specimens were equilibrated in aquaria after collection, and then maintained at ambient temperature and with aeration in seawater in small jars. After addition of the precursor, the radioactivity content in the seawater was monitored to confirm precursor uptake during incubation. After *ca.* 12 h under these static conditions, the sponges were either maintained in aquaria under flowing fresh seawater conditions for an interval, typically 10–20 days depending on the health of the specimens, or returned underwater for longer incorporation periods. Sponges were then frozen for subsequent workup and isolation of terpene metabolites. In all experiments the isolated products were rigorously purified to constant radioactivity.

Inorganic precursor experiments with *A. terpenensis* and *Axinyssa* n.sp

The precursor status of cyanide[5] and of thiocyanate[15] in diisocyanoadociane (**1**) biosynthesis has previously been probed in *A. terpenensis*. In our original experiment using thiocyanate, a sponge sample from Lizard Island was treated with 50 μCi [^{14}C]-thiocyanate giving radiolabelled diisocyanoadociane (6240 dpm mg^{-1}; 0.14%) after 19 days incubation (Table 1).[15] Subsequently, a sponge sample collected at Heron Island was supplied with sodium [^{14}C]-thiocyanate (25 μCi), and an incorporation of 0.049% (3270 dpm mg^{-1}; 0.48 μCi mmol^{-1}) was obtained after 12 days incorporation. As with cyanide,[5] incorporations levels into **1** appear to increase as the incubation time is extended. The specificity of labelling in the diisocyanoadociane sample isolated from the Lizard Island sponge was investigated using the degradation sequence developed earlier.[5,15] Glacial acetic acid treatment gave bisformamide **2** and resulted in the retention of 95.1% of the radioactivity. The removal of a single formyl group was accomplished using 2.5 M sodium hydroxide to give monoamide **3**, which retained

Table 2 Degradation of diisocyanoadociane (**1**) isolated from [^{14}C]-thiocyanate incorporation experiments with *Amphimedon terpenensis*

Compound	Mol. spec. activity (μCi mmol^{-1})	Relative activity (%)
1	0.918	100.0
4	0.025	2.4
1	0.143	100.0
2	0.136	95.1
3	0.071	49.7
4	0.006	4.2

Table 3 Conversion of labeled metabolites **10**, **11**, and **12** from [^{14}C]-cyanide incorporation experiments with *Axinyssa* n.sp

Compound	Mol. spec. activity (μCi mmol^{-1})	Relative activity (%)
10	0.0063	100
14	0.0063	100
11	13.9	100
15	14.8	106[a]
12	1.23	100.0
16	0.001	0.1

[a] The radiochemical yield shows that the degradation product was of slightly higher radiochemical purity than the starting material.

Table 4 Conversion of labeled metabolites **10**, **11**, and **12** from [^{14}C]-thiocyanate incorporation experiments with *Axinyssa* n.sp

Compound	Mol. spec. activity (μCi mmol^{-1})	Relative activity (%)
10	0.0032	100
14	0.0030	94
11	2.6	100
15	2.7	104[a]
12	0.150	100.0
16	0.0005	0.3

[a] See footnote to Table 3.

49.7% of the radioactivity. The second formyl group was removed by refluxing with 6 M HCl to give bisamine **4**, with only 4.2% of the radioactivity remaining, while direct hydrolysis of the isolated **1** to **4** with 6 M HCl also gave unlabelled material (Table 2).

The roles of cyanide and thiocyanate in the biosynthesis of the *Axinyssa* metabolites **10**–**12** next required investigation. These experiments were conducted at Heron Island, and have in part been the subject of a preliminary communication.[17] Firstly, sponge material was incubated with [^{14}C]-cyanide for 16 days, yielding 9-isocyanopupukeanane (**10**) with low radioactivity (60 dpm mg^{-1} above background; 0.006 μCi mmol^{-1}; 0.0009% incorporation; 33.9 mg isolated), 9-isothiocyanatopupukeanane (**11**) with much higher ^{14}C content (116270 dpm mg^{-1}; 13.9 μCi mmol^{-1}; 0.3%; 5.7 mg), and 2-thiocyanatoneopupukeanane (**12**) (10270 dpm mg^{-1}; 1.23 μCi mmol^{-1} 0.079%; 17.0 mg) (Table 1). A second incorporation experiment of 27 days duration resulted in 9-isocyanopupukeanane (**10**) (10 dpm mg^{-1} above background; 5×10^{-5}%; 10.0 mg isolated) and 2-thiocyanatoneopupukeanane (**12**) (58 dpm mg^{-1}; 9×10^{-5}%; 3.6 mg). Incorporation of [^{14}C]-thiocyanate for 16 days yielded 9-isocyanopupukeanane (**10**), again with low radioactivity (30 dpm mg^{-1} above background; 0.0015%; 27.9 mg isolated), 9-isothiocyanatopupukeanane (**11**) with much higher ^{14}C content (22000 dpm mg^{-1}; 0.35%; 8.8 mg isolated), and 2-thiocyanatoneopupukeanane (**12**) (1255 dpm mg^{-1}; 0.029%; 12.9 mg).

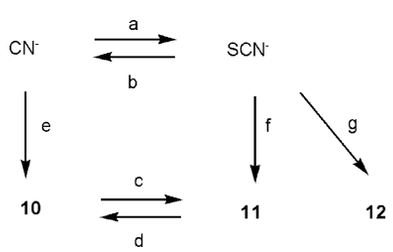
We speculated that the low incorporation of cyanide and thiocyanate into the isocyanide was a result of the extended incorporation period, and therefore tested this using thiocyanate in an incorporation experiment of 3 days duration. 9-Isocyanopupukeanane (**10**) (19 dpm mg^{-1} above background; 0.0075%; 11.1 mg isolated), 9-isothiocyanatopupukeanane (**11**) (53500 dpm mg^{-1}; 1.14%; 5.9 mg), and 2-thiocyanatoneopupukeanane (**12**) (3036 dpm mg^{-1}; 0.047%; 4.3 mg) were isolated, thus the low incorporation into **10** was not related to the length of the incubation period. Comparing the various incorporation experiments, over time the specific activity of the isothiocyanate **11** and thiocyanate **12** metabolites decreases.

Conversion of the isolated samples of isocyanide **10** into the corresponding formamide **14** occurred with significant retention of radioactivity (Tables 3 and 4) which supported genuine labelling. Because of the low ^{14}C content of the samples, further degradation could not be undertaken. By analogy with the results from *A. terpenensis* in Australia,[5] and from Hawaiian sponges,[6b] we infer that the label is associated with the isocyanide carbon atom. The radioactive isothiocyanate products **11** from the cyanide and thiocyanate (16 day) incubations were refluxed with diethylamine, yielding samples of *N*,*N*-diethylthiourea **15** that retained the radioactivity content (Tables 3 and 4). The isothiocyanate **11** and thiourea **15** resisted hydrolytic degradation, preventing experimental confirmation of the site of labelling; again we inferred by analogy with earlier research that the [^{14}C]-label was associated with the isothiocyanate functionality. This assumption was subsequently supported by advanced precursor experimentation as explained below. The thiocyanate metabolite **12** was labelled in all experiments; it was possible to reduce samples of thiocyanate **12** by brief treatment with $LiAlH_4$ to give the thiol **16**.[11] Reaction of thiocyanate **12** from the [^{14}C]-cyanide incubation gave thiol **16** which was found to be unlabelled (Table 3). Similarly, the thiocyanate **12** isolated from incorporation of

[^{14}C]-thiocyanate ion gave unlabelled thiol **16** on reduction (Table 4).

The precursor experiments described above establish the precursor status of inorganic cyanide and thiocyanate in the biosynthesis of terpene isocyanides, isothiocyanates and thiocyanates in *A. terpenensis* and *Axinyssa* n.sp. The labeling of thiocyanate **12** by cyanide supports the interconversion of cyanide and thiocyanate as it is difficult to envisage the incorporation of cyanide into a terpene, followed by insertion of sulfur to give an organic thiocyanate. The alternative cyanation of a thiol suggested by Pham *et al.*[11] is less likely given the incorporation of both cyanide and thiocyanate. Sulfur insertion into an isocyanide to give an isothiocyanate (by an enzyme functionally equivalent to rhodanese)[13,18] followed by isomerization remains a possibility, however, the isomerization equilibrium usually favours an isothiocyanate over a thiocyanate.[19]

The high incorporation into the isothiocyanate metabolite **11** is in striking contrast to the low incorporation into the major isocyanide metabolite **10**. These contrasting incorporation levels can be partly explained by the quantities of metabolite isolated. 9-Isocyanopupukeanane (**10**) is the major metabolite of *Axinyssa* n.sp., and the background levels of this metabolite in the sponge at the start of an incorporation experiment dilute the radioactivity content of the isolated product. However, in our earlier work with *A. cavernosa*,[12] we had observed higher levels of incorporation from [^{14}C]-cyanide and [^{14}C]-thiocyanate into the spiroaxane metabolite axisonitrile-3 (**8**) compared to axisothiocyanate-3 (**9**), despite the status of axisonitrile-3 as a major metabolite in this sponge. Precursor dilution during biosynthesis by unlabelled metabolite cannot adequately explain the *Axinyssa* results. We therefore propose that in *Axinyssa* n.sp., the major biosynthetic route (Scheme 2 reactions a, f and d) is from cyanide to thiocyanate, followed by incorporation into the thiocyanate **12** or isothiocyanate **11** and subsequent conversion of the isothiocyanate into the isocyanide **10**. If these steps occur at a slow rate, then isocyanide **10** would be labelled at a lower level than the isothiocyanate **11**. The alternative direct utilisation of cyanide for isocyanides (Scheme 2 reaction e) then conversion to isothiocyanate **11** (reaction c), may occur, but does not adequately account for the significant labelling found in the thiocyanate **12**, or the low level of labelling of the isocyanide **10**. We reasoned that incorporation of [^{14}C]-labelled samples of **10** and **11** into *Axinyssa* n.sp. would enable us to evaluate isocyanide–isothiocyanate interconversions at the secondary metabolite level, and hence to explore the status of the individual biosynthetic steps in Scheme 2.

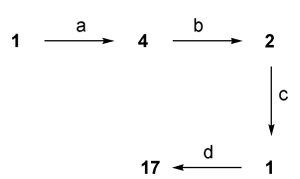


Scheme 2 Interconversions in *Axinyssa* n.sp.

The terpene chemistry of *A. terpenensis* is dominated by isocyanides over isothiocyanates; bisisothiocyanate **17** has not been isolated from this sponge, however two isomeric monoisothiocyanates have been isolated from the related sponge *Cymbastela hooperi*.[20] A precursor incorporation study with **17** was undertaken to complement the experiments in *Axinyssa* n.sp. by enabling us to evaluate whether isothiocyanates are precursors to isocyanides in *A. terpenensis*.

Synthesis of advanced precursors

To test these biosynthetic ideas, we required syntheses of [^{14}C]-labelled samples of the proposed cycloamphilectane precursor **17** and of the pupukeanane metabolites **10** and **11**. We chose to follow the synthetic approach of Hagadone *et al.*,[14] who used the amine derived from the natural isocyanide as starting material for their labelled synthesis. Owing to the ease of isolation of diisocyanoadociane (**1**), we first explored synthesis of **17** (Scheme 3). Thus diisocyanoadociane (**1**) was converted to the bisamine **4** by acid hydrolysis, then conversion to the formamide **2** was explored. Standard methods for the formation of amide bonds such as DCC coupling gave poor yields, but use of formic pivalic anhydride gave acceptable yields of **2**. [^{14}C]-Formic acid is available commercially as its sodium salt, however trial reactions using unlabelled sodium formate gave low yields of formamide **2**, in contrast to reactions using formic acid. Sodium [^{14}C]-formate (400 μCi) was therefore equilibrated with unlabelled formic acid before reaction with pivaloyl chloride, then the amine **4** was added to this [^{14}C]-labelled mixed anhydride. The reaction was then forced to completion by the addition of excess unlabelled mixed anhydride. Workup of the reaction gave **2** in excellent chemical yield, but only in 30% radiochemical yield which likely reflects the effectiveness of the formate equilibration step. The formamide product was immediately dehydrated with toluenesulfonyl chloride in dry pyridine to give [^{14}C]$_2$-diisocyanoadociane (**1**) in 57% radiochemical yield after purification by silica chromatography and repeated recrystallisation from hexane. A portion of the [^{14}C]$_2$-diisocyanoadociane (**1**) sample was degraded by acid hydrolysis to the formamide **2** which retained 94.5% radioactivity, then using 2.5 M NaOH to the monoamide **3** which retained 35.1% of the [^{14}C]-label. Thus both isocyanide groups of the diterpene **1** were labelled, but the equatorial, and more nucleophilic, amino substituent of the parent bisamine **4** was preferentially labelled over the axial amino group by our procedure. Treatment of the remaining [^{14}C]$_2$-diisocyano-adociane (**1**) with sulfur at 120 °C then gave [^{14}C]$_2$-bis-isothiocyanatoadociane (**17**) in 40% yield (with 33% retention of radioactivity) after preparative TLC. This material was used in incorporation studies at Lizard Island, as reported in our preliminary communication.[15] A second batch of [^{14}C]$_2$-bisisothiocyanatoadociane was prepared subsequently for incorporation study at Heron Island. Starting from amine **4** and sodium [^{14}C]-formate (250 μCi), the formamide **2** was prepared in 12.1% radiochemical yield, then dehydrated (TsCl/Pyr) to the diisocyanide **1** (59% radiochemical yield) and treated with sulfur at 120 °C to give [^{14}C]$_2$-bisisothiocyanatodociane **17** in 97% radiochemical yield after preparative TLC.



Scheme 3 Synthesis of [^{14}C]$_2$-bisisothiocyanate **17**. (a) 6 M HCl, reflux, 68%; (b) [^{14}C]-formic pivalic anhydride, Et_3N, CH_2Cl_2, 88%; (c) *p*-TsCl in pyridine, 60%; (d) Sulfur, 120 °C, 56%.

In synthesizing the samples of [^{14}C]$_2$-bisisothiocyanatoadociane (**17**) from the diisocyano analog **1**, we recognized that there is a risk of contamination from unreacted [^{14}C]$_2$-diisocyanoadociane (**1**), which would label the biosynthetic product of the incorporation experiment, and possibly lead to a misleading result. To avoid this, a cold carrier dilution and separation protocol was used. The sample of [^{14}C]$_2$-**17** (11.7 mg; 6.7 μCi) for use at Heron Island was mixed with unlabelled diisocyanide **1** (11 mg), and the mixture separated by silica chromatography resulting in the bisisothiocyanate **17** (9.5 mg; containing 5.98 μCi) and diisocyanoadociane (**1**) (11.0 mg; containing 0.01 μCi). Purification of the recovered isocyanide sample by normal phase HPLC and recrystallisation from hexane gave diisocyanoadociane (9.5 mg) with a very low level

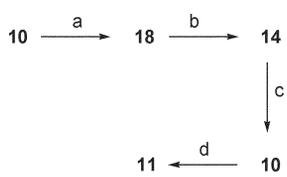
Table 5 Advanced precursor incorporations with *Amphimedon terpenensis* and degradation of diisocyanoadociane (**1**)

Compound	Precursor	Molar specific activity (μCi mmol^{-1})	Incorporation (%)	Relative activity (%)
1	[^{14}C]-**17**[a]	0.059	0.02	100.0
2		0.057	—	97[b,c]
1	[^{14}C]-**17**[a]	0.011	0.002	100.0
4		0.001	—	9.1[d]
1	[^{14}C]-**17**[e]	<0.0059	<0.0002	

[a] Incorporation of 11.5 μCi. [b] Samples of **2** are hygroscopic which slightly lowers the measured specific activity. [c] Insufficient material for further degradation. [d] The amine **4** is difficult to purify, resulting in residual activity, which is unlikely to be genuine labelling. [e] Incorporation of 5.3 μCi.

of radioactivity (<70 dpm mg^{-1}; 0.0003 μCi). In a second cycle of this purification, the radiolabelled **17** was again mixed with unlabelled **1** (10.0 mg), and recovered by preparative TLC, yielding 9.2 mg of **17** (5.3 μCi) and 9.4 mg of **1** (0.001 μCi). This procedure removed any unreacted [^{14}C]$_2$-diisocyanide from the synthetic bisisothiocyanate product and confirmed the radiochemical purity of the recovered bisisothiocyanate **17** for use in incorporations.

Synthesis of the required radiolabelled pupukeanane metabolites was next undertaken. Amine **18**, available by 6 M HCl hydrolysis of the sponge-derived isocyanide **10**, was used as starting material (see Scheme 4). The [^{14}C]-label was incorporated by formylation of amine **18** with 750 μCi of [^{14}C]-formic pivalic anhydride to give the [^{14}C]-formamide **14** with the retention of 19% of the radioactivity. Dehydration of the formamide with toluenesulfonyl chloride in dry pyridine gave [^{14}C]-9-isocyanopupukeanane (**10**) in 93% radiochemical yield. A portion of this isocyanide was converted to the isothiocyanate **11** by treatment with sulfur at 120 °C in 45% radiochemical yield. The sample of [^{14}C]-**11** was mixed with unlabelled **10**, the mixture separated by preparative TLC and the two individual compounds purified. Two cycles of this purification protocol resulted in unlabelled **10** (<0.0001 μCi), confirming the radiochemical purity of the recovered [^{14}C]-9-isothiocyanatopupukeanane (**11**) for use in incorporations.



Scheme 4 Synthesis of [^{14}C]-isocyanide **10** and [^{14}C]-isothiocyanate **11**. (a) 6 M HCl, reflux, 66%; (b) [^{14}C]-formic pivalic anhydride, Et_3N, CH_2Cl_2, 59%; (c) *p*-TsCl in pyridine, 84%; (d) sulfur, 120 °C, 33%.

Advanced precursor incorporation

Precursor incorporation experiments were performed in the field according to our usual protocol.[5,12] First, at Lizard Island, [^{14}C]$_2$-bisisothiocyanate **17** was dissolved in acetone and provided equally to two small pieces of *A. terpenensis* in aerated seawater. After 19 days incorporation, the diisocyanoadociane isolated from each specimen was found to contain a low level of radioactivity (Table 5). One sample of **1** was degraded to the formamide **2**, which retained 97% of the radioactivity, but lack of material prevented further degradation to amine products, while the other sample was hydrolysed directly to the bisamine **4** that contained 9.1% of the activity of the diisocyanide. The higher than expected ^{14}C content of this sample of **4** was attributed to side products of the hydrolysis reaction, and the difficulty of purification. At first sight, these results were consistent with a low conversion of precursor **17** to diisocyanide **1** by the sponge.[15] On reflection, interpretation of the data from these initial advanced precursor studies was complicated by concerns about the purity of the precursor supplied, given that it had been prepared from radioactive **1**. Any carry-through of unreacted radiolabelled diisocyanide during purification of the bisisothiocyanate precursor would interfere with the incorporation results. Subsequently, an incorporation experiment was conducted on *A. terpenensis* at Heron Island using a sample of bisisothiocyanate **17** that had been carefully treated with unlabelled **1** as described above to remove any traces of residual [^{14}C]$_2$-**1**. The diisocyanoadociane isolated from this biosynthetic experiment had a radioactivity content of 4 dpm mg^{-1} above background, and was essentially non radioactive. Thus there was no incorporation of bisisothiocyanate **17** into diisocyanoadociane **1** in this experiment. Although a negative biosynthetic result does not necessarily disprove a biosynthetic conversion, our data suggest that this precursor may not be part of the biosynthetic pathway to **1** in this sponge. The bisisothiocyanate has not been reported from *A. terpenensis* or the closely-related *C. hooperi*,[20] so it is possible that the lack of incorporation occurs because the enzymes responsible for the pathway to diisocyanide **1** do not recognize bisisothiocyanate **17** as a substrate.

We next explored advanced precursor studies with *Axinyssa* n.sp.[16] Prior to these experiments, the radiochemical purity of the precursors used was defined by the cold carrier dilution method described above. [^{14}C]-9-Isocyanopupukeanane (**10**) was dissolved in methanol and incubated with a small piece of *Axinyssa* n.sp. overnight, followed by a 21 day incorporation period. Extraction of the sponge, followed by purification to constant specific activity gave a sample of 9-isothiocyanatopupukeanane (**11**) which was significantly radioactive (Table 6). Fractions containing 9-isocyanopupukeanane (**10**) were highly radioactive, but were not purified further. Confirmation of the radiochemical purity of the isolated sample of 9-isothiocyanatopupukeanane (**11**) was obtained by preparation of the thiourea **15** (Et_2NH reflux), which retained the radioactivity after purification. In a second experiment, [^{14}C]-9-isothiocyanatopupukeanane (**11**) was dissolved in acetone and supplied to a specimen of *Axinyssa* n.sp. in a similar incorporation. The sample of 9-isocyanopupukeanane (**10**) isolated from this incorporation experiment was significantly radioactive (see Table 6). Fractions containing 9-isothiocyanatopupukeanane (**11**) were highly radioactive, but were not purified further. Confirmation of the radiochemical purity of the isolated 9-isocyanopupukeanane (**10**) was achieved by hydration (g. AcOH) to give formamide **14** which retained the radioactivity after purification. In these two incorporation experiments, the specificity of incorporation was not tested, and is not in question as the precursors supplied were specifically labelled at the isocyanide or isothiocyanate carbons. The high incorporation levels obtained do not suggest that metabolic degradation and re-utilization of labelled breakdown products via general metabolism had occurred.

These results show the conversion of isocyanides to isothiocyanates and also of isothiocyanates to isocyanides in *Axinyssa* n.sp. Although the incorporation percentages are similar for the isocyanide to isothiocyanate conversion (0.16%) and the reverse isothiocyanate to isocyanide conversion

Table 6 Advanced precursor incorporations with *Axinyssa* n.sp

Compound	Precursor	Molar specific activity (μCi mmol^{-1})	Incorporation (%)	Relative activity (%)
11	[^{14}C]-**10**	4.6	0.16[a]	100
15		4.1	—	89[b]
10	[^{14}C]-**11**	0.14	0.12[a]	100
14		0.14	—	100

[a] These values were inadvertently transposed in our earlier publication.[20] [b] The small amount and difficulty in purification meant the specific activity was lower than desired, however it is unlikely that this level of labeling would be retained after purification of an unlabelled compound.

(0.12%), the endogenous levels of these metabolites (and the consequent dilution) mean that the rate of transfer of isocyanide (major metabolite) to isothiocyanate (minor metabolite) is significantly faster than the reverse conversion. The possibility that these conversions occur without the action of enzymes was considered. The procedures used for the synthesis of the radiolabelled metabolites provide limited evidence in support of the enzyme-mediated nature of these conversions. In the cold carrier dilution and separation experiments, labelled isothiocyanate is mixed with unlabelled isocyanide, and the isolated isocyanide was found to be non radioactive. Any chemical conversion would result in labelling of the isolated isocyanide, unless the conversion was extremely slow at room temperature. Additionally the lack of utilization of bisisothiocyanate **17** by *A. terpenensis*, suggests that isothiocyanates are not converted chemically to isocyanides under incorporation conditions within sponge tissue. Although this evidence is not conclusive, it suggests that the non-enzymatic conversion of isothiocyanates to isocyanides either does not occur, or occurs only at a very slow rate under normal conditions.

Summary

The issue of whether the isocyanide and isothiocyanate secondary metabolites can be interconverted by marine sponges was first addressed by Scheuer *et al.* using [^{13}C]-labelled precursor incorporation.[14] Some mass spectrometric evidence was obtained for the conversion of the tricyclic metabolite 2-isocyanopupukeanane into 2-isothiocyanatopupukeanane by the Hawaiian sponge *Ciocalypta* sp., however the reverse transformation (isothiocyanate⟶isocyanide) was not detected using this methodology. Our data, using sensitive [^{14}C]-labelling experiments, do not provide convincing evidence for an isothiocyanate to isocyanide conversion in the Great Barrier Reef sponge *A. terpenensis*. The incorporation values obtained are low and variable, possibly because the isothiocyanate precursor supplied is not a natural metabolite of this sponge. In contrast, we have obtained definitive evidence for isocyanide–isothiocyanate interconversions by advanced precursor experiments with *Axinyssa* n.sp. In this sponge, the efficiency of incorporation is similar in either direction. Incorporation of the inorganic precursors cyanide or thiocyanate into the *Axinyssa* metabolites **10** and **11** gives contrasting data for the two metabolites. High incorporations of these two precursors were achieved with **11**, whereas the isocyanide **10** was poorly labelled. These combined experimental results suggest to us that the major pathway operating in *Axinyssa* n.sp. is cyanide⟶thiocyanate⟶isothiocyanate **11**⟶isocyanide **10**, which implies that there may be a major biosynthetic role for thiocyanate ion in this sponge. The labelling of thiocyanate metabolite **12** by cyanide ion *via* thiocyanate ion (Scheme 2 reactions a and g) in this sponge is an additional piece of biosynthetic information which adds weight to the proposed pathway as it also confirms a key role for thiocyanate ion.[17] The biochemical origin of the thiocyanate or cyanide ion used for marine terpene biosynthesis in these sponges remains elusive. Finally we note that it is possible that the organic interconversions detected in *Axinyssa* n.sp. represent an equilibrium within the sponge that allows adjustment of the concentration of the metabolites to suit the environmental needs of the sponge.[2,21] Additional biochemical roles for cyanide, thiocyanate and their organic counterparts are under investigation in our laboratory.[22]

Experimental

General experimental

Thin layer chromatography (TLC) was carried out using Kieselgel 60 F_{254} silica containing a UV fluorescence indicator coated on aluminium plates and visualised under UV light (254 nm). Further visualisation was achieved with vanillin spray (50 mg vanillin, in 25% sulfuric acid in ethanol (50 mL)) followed by heating. Flash chromatography was carried out using Kieselgel 60 230–400 mesh silica. Silica PTLC was carried out on Merck 05717 2 mm glass backed plates with a UV fluorescence indicator (254 nm) and visualised under UV light. Bands were scraped off and the silica placed in a column, then the compounds were eluted with CH_2Cl_2 and EtOAc. High performance liquid chromatography (HPLC) was carried out on Waters μPorasil (10 μm) columns (300 mm × 7.8 mm) with isocratic elution using hexane/ethyl acetate mixtures; compounds were detected by refractive index detection.

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX500 instrument at 500 MHz in deuteriochloroform and referenced to residual solvent (δ_H $CHCl_3$ = 7.25 ppm, δ_C $CDCl_3$ = 77 ppm). Electron impact (EI) mass spectra were recorded on a Kratos MS25RFA spectrometer at 70 eV. Electrospray ionisation (ESI) mass spectra were recorded on a P E SCIEX API III triple quadrupole mass spectrometer for solutions in methanol.

The specific activities of the [^{14}C]-radiolabelled precursors were sodium cyanide 40–60 μCi mmol^{-1}, sodium thiocyanate 5–25 μCi mmol^{-1} and sodium formate 40–60 mCi mmol^{-1}, and were purchased from ICN.

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. For diisocyanoadociane samples of low activity, aliquots of recrystallised disocyanoadociane were weighed to the nearest 0.01 mg and transferred directly into scintillation vials, then dissolved with 0.1 mL benzene and diluted with 3.9 mL of scintillant. 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 10 mL solvent and 0.1 mL aliquots transferred for counting in 3.9 mL scintillant. For samples of 10 mg or less, samples were dissolved in 1 mL solvent, and 0.01 mL aliquots transferred for counting in 4.0 mL 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 the same solvent.

Animal material

Specimens of *A. terpenensis* were collected by hand using SCUBA (−10–15 m) at dive sites on the southern side of Heron Island or at North Point, Lizard Island, Great Barrier Reef, Australia. A voucher sample (registry number QM G307133) was identified as *Amphimedon terpenensis* (Order: Haplosclerida, Family: Niphatidae), and is lodged at The Queensland Museum, Brisbane.

Specimens of *Axinyssa* n.sp were collected by hand using SCUBA (−10–15 m) at Coral Spawning dive site at Heron Island on the Great Barrier Reef, Australia. A voucher sample (registry number QM G312575) was identified as *Axinyssa* n.sp. (1939) (Order: Halichondrida, Family: Halichondriidae), and is lodged at The Queensland Museum, Brisbane.

Incorporation experiments with inorganic precursors

[^{14}C]-Thiocyanate and *A. terpenensis*. A piece of *A. terpenensis* (30.5 g wet wt.) was placed in a beaker containing unfiltered, continuously aerated seawater (200 mL) and maintained at ambient temperature by a flowing seawater bath surrounding the beaker. Precursor (NaS^{14}CN, 25 μCi) was then added, and the sponge allowed to assimilate the precursor for a 12 h period overnight. Water samples were taken at regular intervals which revealed that 33% of the precursor was taken up during this period. The sponge was then transferred to a large flowing seawater aquarium for a 12 day incorporation period, then frozen for subsequent workup. The sponge was sliced finely and extracted with CH_2Cl_2 : MeOH (1 : 1, 4 × 100 mL). The extracts were evaporated to an aqueous suspension, which was extracted with CH_2Cl_2 (3 × 60 mL). The CH_2Cl_2 layers were dried and evaporated to give a green residue (300 mg, 0.98% chemical yield; 6140 dpm mg^{-1}, 3.3% radiochemical incorporation) which was chromatographed on silica eluting stepwise with hexane to ethyl acetate. Fractions were combined on the basis of radioactivity and TLC comparison with an authentic sample of diisocyanoadociane (**1**). The fraction containing diisocyanoadociane (**1**) (31.7 mg, 0.1% chemical yield; 5220 dpm mg^{-1}, 0.3% radiochemical incorporation) was further purified by repeated normal phase HPLC (5% EtOAc in hexane), followed by two recrystallisations from hexane to give diisocyanoadociane (**1**) (8.2 mg, 0.027%; 3270 dpm mg^{-1}, 0.049%), identical in all respects with authentic material and literature data.[5,23] A second piece of sponge (45 g wet wt.) was treated with precursor (NaS^{14}CN, 50 μCi) for an incorporation period of 19 days, followed by workup as above, resulting in diisocyanoadociane (**1**) (24.8 mg, 0.055%; 6240 dpm mg^{-1}, 0.14%).

*Hydration of [^{14}C]$_2$-diisocyanoadociane (**1**) to [^{14}C]$_2$-bisformamide **2**.* A small sample of [^{14}C]-diisocyanoadociane (**1**) was diluted with unlabelled material, then recrystallised before use in degradation studies. [^{14}C]$_2$-Diisocyanoadociane (**1**) (26.2 mg, 0.143 μCi mmol^{-1}) was dissolved in glacial acetic acid (1 mL) and allowed to stand at room temperature overnight. The reaction was diluted with water (5 mL), and saturated sodium bicarbonate solution (1 mL), then extracted with CH_2Cl_2 (4 × 10 mL). The CH_2Cl_2 layers were washed with saturated sodium bicarbonate solution (3 × 10 mL) until effervescence ceased. The CH_2Cl_2 layers were dried and evaporated to give a colourless oil (22.2 mg). This residue was purified by silica chromatography (eluting with CH_2Cl_2, then EtOAc stepwise to MeOH) to give [^{14}C]$_2$-bisformamide **2** (19.0 mg, 65% chemical yield; 0.136 μCi mmol^{-1}, 95.1% radiochemical yield), identical with previously reported material.[5]

*Hydrolysis of [^{14}C]$_2$-bisformamide **2** to [^{14}C]-monoamine **3**.* Bisformamide **2** (8.7 mg, 0.024 mmol; 0.136 μCi mmol^{-1}) was dissolved in ethanol (0.75 mL) and 2.5 M sodium hydroxide (0.75 mL) was added. The solution was heated to reflux for three hours, then cooled to room temperature overnight. The reaction mixture was then applied to a reverse phase chromatography cartridge (eluted with H_2O, 30% MeOH/H_2O, MeOH, then CH_2Cl_2) to give monoamine **3** (7.9 mg, 99%; 0.078 μCi mmol^{-1}, 57%). Further reverse phase cartridge chromatography (eluting with H_2O, 30% MeOH/H_2O, MeOH, then CH_2Cl_2) gave monoamine **3** (6.5 mg, 81%; 0.071 μCi mmol^{-1}, 49.7%).[5]

*Hydrolysis of [^{14}C]-monoamine **3** to bisamine **4**.* Monoamine **3** (3.4 mg, 0.071 μCi mmol^{-1}) was dissolved in acetone (2 mL) and added to hydrochloric acid (1.5 mL, 6 M) and heated to reflux for 3 h, then cooled to room temperature and stirred overnight. The reaction mixture was washed with EtOAc (2 mL), the EtOAc layer extracted with hydrochloric acid (1.5 mL, 6 M) and the combined acid layers made basic with sodium hydroxide. The aqueous solution was applied to a reverse phase chromatography cartridge and eluted with H_2O, 20% MeOH/H_2O, MeOH, then CH_2Cl_2 to give bisamine **4** (1.5 mg, 49%; 0.006 μCi mmol^{-1}, 4.2%), identical with previously reported material.[5]

*Hydrolysis of diisocyanoadociane (**1**) to bisamine **4**.* Undiluted diisocyanoadociane (**1**) (8.9 mg, 0.918 μCi mmol^{-1}) was added to hydrochloric acid (6 M, 3 mL) and refluxed for 3 h, then allowed to cool to room temperature overnight. The reaction solution was made basic with sodium hydroxide, then applied to a reverse phase chromatography cartridge (eluted with H_2O, 20% MeOH/H_2O, MeOH, then CH_2Cl_2) to give bisamine **4** (2.6 mg, 31%; 0.025 μCi mmol^{-1}, 2.4%).

[^{14}C]-Cyanide and *Axinyssa* n.sp. The incorporation of Na^{14}CN (100 μCi) was performed in the same way using a piece of *Axinyssa* n.sp. (88 g wet wt.) in 200 mL seawater. After a 16 day incorporation, the sponge was extracted with CH_2Cl_2 : MeOH (1 : 1) (4 × 140 mL). The extract was then evaporated to an aqueous residue which was extracted with hexane (3 × 80 mL). The hexane layers were dried and evaporated to give a green residue (906 mg). This residue was fractionated on a silica flash column by stepwise gradient elution using hexane/ethyl acetate mixtures,[9] and fractions combined on the basis of TLC, NMR and radioactivity. Purification by repeated normal phase HPLC (0.25% EtOAc/hexane) gave 9-isocyanopupukeanane (**10**) (33.9 mg, 0.038%; 60 dpm mg^{-1}, 0.0009%) and 2-thiocyanatoneopupukeanane (**12**) (17.0 mg, 0.019%; 10270 dpm mg^{-1}, 0.079%).[9] Repeated normal phase HPLC (hexane) of another fraction gave 9-isothiocyanatopupukeanane (**11**) (5.7 mg, 0.0065%; 116270 dpm mg^{-1}, 0.3%).[9] A second piece of sponge (70 g) was treated identically with Na^{14}CN (100 μCi). The sponge was then transferred to an underwater grid at −14 m for 27 days. The sponge was extracted as above to give 9-isocyanopupukeanane (**10**) (10.1 mg, 0.014%; 10 dpm mg^{-1}, 0.00005%) and 2-thiocyanatoneopupukeanane (**12**) (3.6 mg, 0.0051%; 58 dpm mg^{-1}, 0.00009%). The small amount of **11** isolated in this experiment was not able to be purified to constant specific activity.

*Hydration of 9-isocyanopupukeanane (**10**) to formamide **14**.* 9-Isocyanopupukeanane (**10**) (14.2 mg, 0.006 μCi mmol^{-1}) was dissolved in glacial acetic acid (2 mL) and left to stand overnight. The resulting solution was poured onto saturated sodium bicarbonate solution (20 mL), extracted with CH_2Cl_2 (3 × 20 mL), dried and evaporated to give a colourless oil. Purification by silica chromatography gave formamide **14** (14.5 mg, 95%; 0.006 μCi mmol^{-1}, 100%). δ_H (2 rotamers) 0.74 (3H, s), 0.83 (3H, d, *J* 6.3), 0.84 (3H, d, *J* 6.3), 0.98 (3H, s), 1.01 (1H, m), 1.14 (1H, m), 1.25 and 1.35 (1H, m), 1.26 (1H, m), 1.30 (2H, m), 1.36 (1H, m), 1.46 (1H, m), 1.84 (1H, m), 2.04 (1H, m), 2.20 (1H, m), 3.11 and 3.86 (1H, m), 5.65 and 5.95 (1H, br m), 7.98 and 8.18 (1H, m). δ_C (2 rotamers) 21.5, 21.7, 24.3, 26.8, 26.9, 28.4, 28.5, 30.0, 31.5, 31.7, 33.9, 38.3, 38.8, 43.9, 44.0, 46.8,

47.2, 48.5, 49.4, 49.5, 55.4, 162.2, 167.5. *m/z* (EI) = 249 ($M^{+\bullet}$), 204 (100%), 161, 133, 106, 41. *m/z* (EI) 249.2089 [$M^{+\bullet}$] $C_{16}H_{27}NO$ requires 249.2092.

*Conversion of 9-isothiocyanatopupukeanane (**11**) to diethylthiourea **15**.* 9-Isothiocyanatopupukeanane (**11**) (2.8 mg, 13.9 μCi mmol^{-1}) was refluxed in diethylamine (2.5 mL) overnight, then evaporated. Silica chromatography gave a residue (2.7 mg) which was purified using normal phase HPLC (5% EtOAc in hexane) giving thiourea **15** (1.8 mg, 50.6%; 14.8 μCi mmol^{-1}, 106%; see footnote to Table 3). δ_H 0.80 (3H, s), 0.83 (3H, d, *J* 7.8), 0.84 (3H, d, *J* 7.9), 0.98 (3H, s), 1.09 (1H, m), 1.14 (1H, m), 1.23 (6H, t, *J* 7.0), 1.24 (1H, m), 1.26 (1H, m), 1.28 (1H, m), 1.30 (1H, m), 1.37 (1H, m), 1.45 (1H, m), 1.48 (1H, m), 1.84 (1H, dd, *J* 13 and 11), 2.07 (1H, m), 2.47 (1H, m), 3.65 (4H, q, *J* 7), 4.34 (1H, m), 5.40 (1H, br d, *J* 8). δ_C 12.9, 21.6, 21.8, 24.7, 27.1, 29.2, 30.0, 31.7, 34.2, 38.3, 38.7, 44.1, 45.0, 48.5, 48.7, 49.3, 57.2, 179.9. *m/z* (ESI) 337.3 (M + H^+, 100%). *m/z* (EI) 337.2684 $C_{20}H_{37}N_2S$ requires 337.2677.

*Reduction of 2-thiocyanatoneopupukeanane (**12**) to thiol **16**.* 2-Thiocyanatoneopupukeanane (**12**) (6.8 mg, 0.025 mmol, 1.23 μCi mmol^{-1}) in dry THF (1.2 mL) was added to $LiAlH_4$ (4.9 mg, 0.13 mmol) and stirred at room temperature for 8 minutes, then poured into saturated aqueous ammonium chloride (5 mL) and extracted with EtOAc (3 × 10 mL), dried over anhydrous magnesium sulfate and evaporated to give 6.6 mg of a residue. Purification by silica chromatography followed by normal phase HPLC (0.05% EtOAc in hexane) gave thiol **16** (2.4 mg, 39%; 0.001 μCi mmol^{-1}, 0.1%).[17] δ_H 0.78 (3H, d, *J* 6.5), 0.86 (2H, m), 0.89 (3H, d, *J* 6.5), 0.92 (1H, m), 0.95 (1H, m), 1.00 (3H, s), 1.05 (3H, s), 1.20 (1H, m), 1.25 (1H, m), 1.34 (1H, m), 1.38 (1H, m), 1.42 (1H, m), 1.50 (1H, m), 1.78 (1H, ddd, *J* 3.5, 11.0 and 14.5), 2.10 (1H, ddd, *J* 5.5, 9.5 and 14.5), 2.80 (1H br d, *J* 7). δ_C 20.9, 21.3, 22.1, 26.3, 26.7, 32.2, 32.2, 32.7, 37.9, 39.5, 40.9, 45.7, 44.6, 50.5, 53.3. *m/z* (EI) 238 ($M^{+\bullet}$), 223, 205 (100%), 149, 107, 95, 41.

[^{14}C]-Thiocyanate and *Axinyssa* n.sp. The incorporation of Na^{14}CN (25 μCi) was performed in the same way using *Axinyssa* n.sp. (77 g wet wt.) in 200 mL of seawater. After a 16 day incorporation, the sponge was extracted to give 9-isocyanopupukeanane (**10**) (27.9 mg, 0.036%; 30 dpm mg^{-1}, 0.0015%), 2-thiocyanatoneopupukeanane (**12**) (12.9 mg, 0.017%; 1255 dpm mg^{-1}, 0.029%) and 9-isothiocyanatopupukeanane (**11**) (8.8 mg, 0.011%; 22 000 dpm mg^{-1}, 0.35%). Another piece of sponge (24 g) was treated identically with Na^{14}CN (12.5 μCi). After a 3 day incorporation period, the sponge was worked up to give 9-isocyanopupukeanane (**10**) (11.1 mg, 0.046%; 19 dpm mg^{-1}, 0.0075%), 2-thiocyanatoneopupukeanane (**12**) (4.3 mg, 0.0018%; 3036 dpm mg^{-1}, 0.047%) and 9-isothiocyanato-pupukeanane (**11**) (5.9 mg, 0.024%; 53 500 dpm mg^{-1}, 1.14%).

*Hydration of 9-isocyanopupukeanane (**10**) to formamide **14**.* 9-Isocyanopupukeanane (**10**) (7.4 mg, 0.0032 μCi mmol^{-1}) was treated with acetic acid (2 mL) as before and gave formamide **14** (6.9 mg, 87%; 0.0030 μCi mmol^{-1}, 94%).

*Conversion of 9-isothiocyanatopupukeanane (**13**) to diethylthiourea **15**.* 9-Isothiocyanatopupukeanane (**11**) (4.0 mg, 2.6 μCi mmol^{-1}) was refluxed in diethylamine as before to give thiourea **15** (2.4 mg, 47%; 2.7 μCi mmol^{-1}, 104%; see footnote to Table 3).

*Reduction of 2-thiocyanatoneopupukeanane (**12**) to thiol **16**.* 2-Thiocyanatoneopupukeanane (**12**) (4.1 mg, 0.015 mmol, 0.15 μCi mmol^{-1}) in dry THF (1.2 mL) was added to $LiAlH_4$ (2.9 mg, 0.076 mmol) and stirred at room temperature for 8 minutes, then poured into saturated aqueous ammonium chloride (5 mL) and extracted with EtOAc (3 × 10 mL), dried and evaporated to give 4.8 mg of a residue. Purification by silica chromatography followed by normal phase HPLC (0.05% EtOAc in hexane) gave thiol **16** (1.6 mg, 44%; 0.0005 μCi mmol^{-1}, 0.3%).

Synthesis of ^{14}C-labelled advanced precursors

Synthesis of [^{14}C]$_2$-bisisothiocyanatoadociane (17). *Hydrolysis of diisocyanoadociane (**1**) to bisamine **4**.* Diisocyanoadociane (**1**) (500 mg, 1.54 mmol) was hydrolysed with hydrochloric acid (6 M, 3 mL) as described above to give bisamine **4** (319 mg, 68%).

*Formylation of bisamine **4** to give [^{14}C]$_2$-bisformamide **2**.* Sodium formate (400 μCi) was equilibrated with formic acid (17.7 μL, 0.46 mmol) and added to pivaloyl chloride (69.4 μL, 0.56 mmol) in dry THF (3 mL) and triethylamine (92 μL, 0.66 mmol) and cooled to −80 °C. Bisamine **4** (80.4 mg) in dry THF (3 mL) was then added, and the reaction mixture allowed to warm to room temperature. To ensure complete reaction a further amount of pre-prepared pivalic formic anhydride (formic acid (31.9 μL, 0.84 mmol) and pivaloyl chloride (123 μL, 1.0 mmol)) was added at −80 °C, and the reaction again allowed to warm to room temperature. The reaction mixture was diluted with CH_2Cl_2 (10 mL) then washed with sodium hydroxide solution (10 mL, 2.5 M) followed by sulfuric acid (10 mL, 25%), then dried and evaporated. Purification by silica chromatography (eluting with CH_2Cl_2, then EtOAc stepwise to MeOH) gave [^{14}C]-labelled bisformamide **2** (105.3 mg, 110%; 120 μCi, 30%). The >100% recovery presumably results from the hygroscopic nature of **2**; further efforts to purify this material were not worthwhile at this point.

*Dehydration of [^{14}C]$_2$-bisformamide **2** to give [^{14}C]$_2$-diisocyanoadociane (**1**).* The labelled bisformamide **2** (105.3 mg, 0.29 mmol, 120 μCi) was dissolved in dry pyridine (2 mL) and added to toluenesulfonyl chloride (332 mg, 1.75 mmol) and refluxed for 3 h during which time the initially formed purple colour faded to a light brown. The reaction mixture was poured into hexane (10 mL), washed with sodium hydroxide solution (10 mL, 2.5 M) to remove unreacted toluenesulfonyl chloride, followed by sulfuric acid (2 × 10 mL, 25%) to remove pyridine, then dried and evaporated. Purification by silica chromatography (eluting with hexane to CH_2Cl_2 stepwise then EtOAc) followed by recrystallisation from hexane gave diisocyanide **1** (36.0 mg, 38%; 68.6 μCi, 57%).

*Reaction of [^{14}C]$_2$-diisocyanoadociane (**1**) with sulfur to give [^{14}C]$_2$-bisisothiocyanatoadociane (**17**).* The labelled diisocyanide **1** (36.0 mg, 0.11 mmol, 68.6 μCi) was added to sulfur (25 mg, 0.78 mmol) in a reaction vial and heated at 120 °C overnight, after which time TLC analysis indicated no remaining starting material. The reaction mixture was chromatographed on a silica PTLC plate to give [^{14}C]$_2$-bisisothiocyanate **17** (17.2 mg, 40%; 23 μCi, 33 %). δ_H 0.65 (1H, ddd, *J* 10, 10 and 10), 0.74 (1H, m), 0.82 (1H, m), 0.84 (1H, m), 0.85 (3H, d, *J* 6.5), 0.91 (1H m), 0.99 (3H, d, *J* 6.6), 1.01 (1H, m), 1.08 (2H, m), 1.09 (1H, m), 1.10 (1H, m), 1.23 (1H, m), 1.26 (3H, s), 1.36 (3H, s), 1.38 (1H, m), 1.42 (1H, m), 1.50 (1H, m), 1.53 (1H, m), 1.77 (1H, ddd, *J* 13.4, 13.4 and 3.8), 1.87 (1H, dddd, *J* 13.6, 3.6, 3.6 and 3.6), 1.94 (1H, m), 1.98 (1H, m), 2.05 (1H, m). δ_C 16.4, 19.6, 20.4, 23.7, 25.6, 26.1, 26.2, 36.4, 38.2, 40.6, 40.7, 41.6, 42.2, 45.9, 46.6, 47.7, 49.0, 50.2, 64.4, 67.9, 130.0, 130.5. *m/z* (EI) 388 ($M^{+\bullet}$), 345, 330, 271 (100%), 201, 55, 41. *m/z* (EI) 388.2001 [$M^{+\bullet}$] $C_{22}H_{32}N_2S_2$ requires 388.2006. This sample was split and used in two incorporation experiments at Lizard Island.

Degradation of synthetic [^{14}C]$_2$-diisocyanoadociane (1). *Hydration of diisocyanoadociane (**1**) to bisformamide **2**.* A small sample (<0.1 mg) of synthetic [^{14}C]-diisocyanoadociane (**1**) was diluted with unlabelled material, then recrystallised before use in degradation studies. The resulting diisocyanoadociane (**1**) (7.1 mg, 0.022 mmol, 0.065 μCi mmol^{-1}) was treated with glacial acetic acid as before and gave bisformamide **2** (6.9 mg, 88%; 0.061 μCi mmol^{-1}, 94.5%).

*Hydrolysis of [^{14}C]$_2$-bisformamide **2** to [^{14}C]-monoamine **3**.* Bisformamide **2** (6.7 mg, 0.019 mmol; 0.061 μCi mmol^{-1}) was

hydrolysed as before and gave monoamine **3** (4.4 mg, 70%; 0.022 μCi mmol^{-1}, 35.1%).

Repeat synthesis of [^{14}C]$_2$-bisisothiocyanatoadociane (17). Sodium [^{14}C]-formate (250 μCi) was used in reaction with bisamine **4** (62.7 mg, 0.21 mmol) as before to give [^{14}C]-labelled bisformamide **2** (65.4 mg, 88%; 30 μCi, 12.1%). Dehydration with toluenesulfonyl chloride gave diisocyanide **1** (35.0 mg, 60%; 17.7 μCi, 59%). Reaction of the diisocyanide **1** (16.4 mg, 0.506 mmol, 6.9 μCi) with sulfur gave bisisothiocyanate **17** (11.7 mg, 71%; 6.7 μCi, 97%). To remove any traces of unreacted starting material, [^{14}C]-bisisothiocyanate **17**, (11.7 mg, 6.7 μCi) was mixed with unlabelled **1** (11 mg), and the mixture separated by silica chromatography (eluting with hexane to CH_2Cl_2 stepwise) to give [^{14}C]$_2$-bisisothiocyanate **17**, (9.5 mg, 5.98 μCi), and diisocyanoadociane (**1**) (11.0 mg, 0.01 μCi). Further purification of isocyanide **1** by normal phase HPLC (5% EtOAc in hexane) resulted in unlabelled diisocyanoadociane **1**, (9.5 mg, 0.0003 μCi). A repeat of this process used unlabelled isocyanide **1** (10.0 mg) and gave [^{14}C]$_2$-bisisothiocyanate **17** (9.2 mg, 5.3 μCi; 224.7 μCi mmol^{-1}) and diisocyanoadociane (**1**), (9.4 mg, 0.001 μCi). Purification by normal phase HPLC (5% EtOAc in hexane) resulted in unlabelled diisocyanoadociane (**1**), (8.2 mg, 0.00005 μCi).

Synthesis of [^{14}C]-9-isocyanopupukeanane (10) and [^{14}C]-9-isothiocyanatopupukeanane (11). *Hydrolysis of 9-isocyanopupukeanane (**10**) to amine **18**.* 9-Isocyanopupukeanane (**10**) (143 mg, 0.62 mmol) was dissolved in methanol (3 × 1 mL) and added to HCl (6 M, 20 mL) and the solution refluxed for 3 h and then left to stir overnight at room temperature. The resulting solution was then made basic with sodium hydroxide and extracted with ethyl acetate (3 × 40 mL), dried and evaporated to give the amine **18** (89.8 mg, 66%). δ_H 0.81 (3H, d, *J* 6.3), 0.83 (3H d, *J* 6.3), 0.96 (3H, s), 1.00 (1H, m), 1.07 (3H, s), 1.10 (1H, dd, *J* 8.2 and 13.4), 1.28 (1H, m), 1.30 (1H, m), 1.40 (1H, m), 1.45 (1H, m), 1.65 (1H, m), 1.84 (1H, dd, *J* 10.3 and 13.5), 1.90 (1H br d, *J* 12), 2.02 (1H, m), 2.28 (1H, br t, *J* 10.5), 3.05 (1H, br s), 8.34 (1H, br s). δ_C 21.6, 21.7, 24.4, 25.5, 26.3, 29.9, 30.8, 34.4, 38.0, 39.0, 45.7, 48.4, 49.6, 43.5, 55.4. *m/z* (EI) 221 ($M^{+\bullet}$), 204, 178, 161, 133, 119, 107, 91, 56, 43 (100%); *m/z* (EI) 221.2139 ($M^{+\bullet}$), $C_{15}H_{27}N$ requires 221.2144.

*[^{14}C]-Formylation of amine **18** to give [^{14}C]-formamide **14**.* Sodium [^{14}C]-formate (750 μCi) was dissolved in formic acid (28 μL, 0.58 mmol) and stirred for 5 minutes. Pivaloyl chloride (68 μL, 0.55 mmol) was then added and stirred for 25 minutes. Amine **18** (116 mg, 0.53 mmol) in CH_2Cl_2 (1 mL) was then added, followed by triethylamine (363 μL, 2.64 mmol), and the resulting solution stirred for 20 minutes. A further equivalent of pivalic formic anhydride (20 μL formic acid in 65 μL pivaloyl chloride) was then added and stirred for a further 20 minutes. The whole reaction mixture was then poured into saturated aqueous sodium bicarbonate (5 mL) and then extracted with CH_2Cl_2 (3 × 2 mL), dried and evaporated to give a residue (150.3 mg), that was purified by silica chromatography to give [^{14}C]-formamide (**14**) (78.0 mg, 59.3%, 142.6 μCi, 19%).

*Dehydration of [^{14}C]-formamide **14** to give [^{14}C]-9-isocyanopupukeanane (**10**).* [^{14}C]-Formamide **14** (78 mg, 0.31 mmol, 142.6 μCi) was dissolved in pyridine (6 mL) and added to toluenesulfonyl chloride (239 mg, 1.25 mmol) and the solution refluxed for 3 h and then stirred overnight at room temperature. The resulting brown solution was poured into hexane (50 mL) and washed with sodium hydroxide (2.5 M, 2 × 40 mL) then sulfuric acid (25%, 2 × 40 mL), then dried and evaporated to give a brown residue (74.0 mg). The residue was purified by silica chromatography to give [^{14}C]-9-isocyanopupukeanane (**10**) (60.8 mg, 84%, 132 μCi, 93%). A portion of this sample (28.4 mg, 61.5 μCi) was set aside for further reaction. The remaining [^{14}C]-9-isocyanopupukeanane (**10**) was then further purified by silica chromatography to give [^{14}C]-9-isocyanopupukeanane (**10**) (16.3 mg, 40.6 μCi) for biosynthetic use.

*Reaction of [^{14}C]-9-isocyanopupukeanane (**10**) with sulfur to give [^{14}C]-9-isothiocyanatopupukeanane (**11**).* [^{14}C]-9-Isocyanopupukeanane (**10**) (28.4 mg, 0.123 mmol, 61.5 μCi) was placed in a reaction vial and sulfur (25.3 mg, 0.79 mmol) was then added together with toluene (0.5 mL) and the mixture then heated for 24 h at 120 °C after which time TLC analysis showed some remaining starting material. Further sulfur (20 mg, 0.63 mmol) was then added and the reaction heated at 120 °C for a further 24 h. The reaction mixture was chromatographed on a silica PTLC plate to give 9-isothiocyanatopupukeanane (**11**) (18.7 mg, 58%; 27.9 μCi, 45%). Removal of traces of [^{14}C]-9-isocyanopupukeanane (**10**) was accomplished by mixing of [^{14}C]-9-isothiocyanatopupukeanane (**11**) (18.7 mg, 27.9 μCi) with 9-isocyanopupukeanane (**10**) (14 mg) and then separation (silica chromatography) to give 9-isocyanopupukeanane (**10**) which was further purified by repeated normal phase HPLC (0.25% EtOAC in hexane) to give **10** (5.2 mg, 0.0001 μCi), and [^{14}C]-9-isothiocyanatopupukeanane (**11**) (11.1 mg, 27.9 μCi). This process was repeated with a second batch of unlabelled **10** (10.0 mg) which gave 9-isocyanopupukeanane (**10**) (4.4 mg, <0.0001 μCi) after normal phase HPLC, and [^{14}C]-9-isothiocyanatopupukeanane (**11**) (10.7 mg, 25.4 μCi, 622.3 μCi mmol^{-1}) for biosynthetic use.

Incorporation experiments with advanced precursors

Incorporation of [^{14}C]$_2$-bisisothiocyanatoadociane (17) into *A. terpenensis* (Lizard Island). A specimen of *A. terpenensis* (26 g wet wt.) in 400 mL seawater, was incubated with advanced precursor [^{14}C]$_2$-bisisothiocyanatoadociane (**17**) (8.6 mg, 11.5 μCi) in acetone (0.3 mL) for a 12 h period overnight. Water samples taken at regular intervals revealed that 95% of the precursor was taken up during this period. The sponge was then transferred back to the large aquarium for a 19 day incorporation period. Extraction as before gave diisocyanoadociane (**1**) (11.8 mg, 0.045%; 398 dpm mg^{-1}, 0.02%). The fraction containing [^{14}C]-bisisothiocyanate **17** was highly radioactive (6.1 mg, 0.026%; 37420 dpm mg^{-1}, 0.9%). In a parallel incorporation experiment, the sponge (24 g wet wt.) was treated identically with advanced precursor [^{14}C]$_2$-bisisothiocyanatoadociane (**17**) to give, after purification, diisocyanoadociane (**1**) (6.6 mg, 0.028%; 76 dpm mg^{-1}, 0.002%). The fraction containing [^{14}C]-bisisothiocyanate **17** was highly radioactive (3.1 mg, 0.013%; 56910 dpm mg^{-1}, 0.69%).

*Hydration of diisocyanoadociane (**1**) to bisformamide **2**.* Diisocyanoadociane (**1**) (7.1 mg, 0.022 mmol, 0.059 μCi mmol^{-1}) was treated with glacial acetic acid as before to give bisformamide **2** (6.92 mg, 88%; 0.057 μCi mmol^{-1}, 97%).

*Hydrolysis of diisocyanoadociane (**1**) to bisamine **4**.* Diisocyanoadociane (**1**) (2.5 mg, 0.008 mmol, 0.011 μCi mmol^{-1}) was treated with 6 M hydrochloric acid as before to give bisamine **4** (0.9 mg, 40%; 0.001 μCi mmol^{-1}, 9.1%).

Incorporation of [^{14}C]$_2$-bisisothiocyanatoadociane (17) into *A. terpenensis* (Heron Island). The sponge (16.8 g wet wt.) was incubated with advanced precursor [^{14}C]$_2$-bisisothiocyanatoadociane (**17**) (9.2 mg, 5.3 μCi) to give diisocyanoadociane (**1**) (5.4 mg, 0.032%; 3.4 dpm mg^{-1}, <0.0002%). The fraction containing [^{14}C]-bisisothiocyanate **17** was highly radioactive (4.1 mg, 0.024%; 37790 dpm mg^{-1}, 1.3%).

Incorporation of [^{14}C]-9-isocyanopupukeanane (10) into *Axinyssa* n.sp. A piece of sponge (19.2 g wet wt.) in 400 mL seawater was incubated with [^{14}C]-9-isocyanopupukeanane (**10**) (8.1 mg, 20.3 μCi) in methanol (2 × 0.5 mL). After a 21 day incorporation, the sponge was extracted as before to give 9-isothiocyanatopupukeanane (**11**) (2.4 mg, 0.012%; 29440 dpm mg^{-1}, 0.16%).

*Conversion of 9-isothiocyanatopupukeanane (**11**) to diethylthiourea **15**.* 9-Isothiocyanatopupukeanane (**11**) (0.8 mg, 0.003 mmol, 4.6 μCi $mmol^{-1}$) was treated with diethylamine as before to give thiourea (**15**) (0.4 mg, 39%; 4.1 μCi $mmol^{-1}$, 89%).

Incorporation of [^{14}C]-9-isothiocyanatopupukeanane (11) into *Axinyssa* n.sp. A piece of sponge (18.1 g wet wt.) in 400 mL seawater was incubated with [^{14}C]-9-isothiocyanatopupukeanane (**11**) (6.2 mg, 14.6 μCi) in acetone (2 × 0.5 mL). After a 21 day incorporation, the sponge was extracted as above to give 9-isocyanopupukeanane (**10**) (29.8 mg, 0.165%; 1320 dpm mg^{-1}, 0.12%).

*Hydration of 9-isocyanopupukeanane (**10**) to formamide **14**.* 9-Isocyanopupukeanane (**10**) (13.5 mg, 0.058 mmol, 0.14 μCi $mmol^{-1}$) was treated with glacial acetic acid to give formamide **14** (5.5 mg, 38%; 0.14 μCi $mmol^{-1}$, 100%).

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