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Advanced Precursors in Marine Biosynthetic Study: The Biosynthesis of Diisocyanoadociane in *Amphimedon terpenensis*.

Jamie S. Simpson and Mary J. Garson*

Department of Chemistry, The University of Queensland, Brisbane QLD 4072, Australia.

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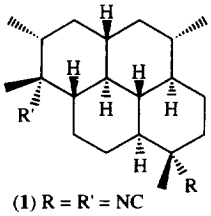
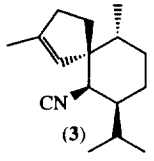
Abstract: The biosynthetic origin of the isocyanide groups in diisocyanoadociane (**1**) is defined by incorporation of the advanced precursor [$^{14}C_2$]-diisothiocyanatoadociane (**2**) into *Amphimedon terpenensis*. [^{14}C]-Thiocyanate is also incorporated specifically into the isocyanide functional groups.

Keywords: Biosynthesis; sponges; terpenes and terpenoids; isocyanides; isothiocyanates.

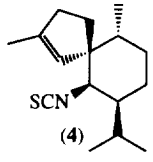
Terpene isocyanides are among the most distinctive metabolites of marine sponges.[1] Research from this group has shown that diisocyanoadociane (**1**) from *Amphimedon terpenensis* is derived by functionalization of a terpene precursor using inorganic cyanide.[2] Recently we have demonstrated the use of both cyanide and thiocyanate by *Acanthella cavernosa* in the biosynthesis of axisonitrile-3 (**3**) and axisothiocyanate-3 (**4**),[3] by *Stylotella aurantium* in the biosynthesis of the dichloroimines (**5**) and (**6**),[4] and by *Axinyssa* n.sp. for the biosynthesis of 2-thiocyanatoneopupukeanane (**7**) and 9-isothiocyanatopupukeanane (**8**).[5] Our results further suggested that the two inorganic precursors may be interconverted by the sponges. An alternative biosynthetic process is that the -NC/-NCS interconversion occurs at the secondary metabolite level, eg. conversion of an isocyanide metabolite such as (**3**) to the isothiocyanate analogue (**4**) or vice versa.



(**1**) R = R' = NC
(**2**) R = R' = NCS
(**9**) R = R' = NHCHO
(**10**) R = NH_2, R' = NHCHO
(**11**) R = R' = NH_2



(**3**)



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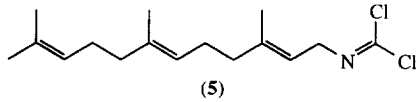
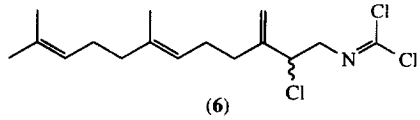
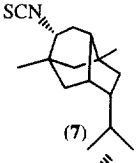


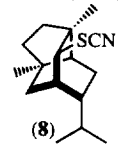
(**5**)



(**6**)



(**7**)



(**8**)

Advanced precursors were first used in sponge biosynthetic study in *Axinella cannabina*[6] to investigate isocyanide biosynthesis, but gave ambiguous results. In pioneering work using [^{13}C]-labelled precursors, Hagadone *et al.* found evidence to suggest that terpene isocyanides were the biosynthetic precursors of the corresponding isothiocyanates,[7] however the low sensitivity of detection of ^{13}C by mass spectrometry complicated interpretation of their results. We now demonstrate the role of isothiocyanate advanced precursors in isocyanide biosynthesis in the sponge *A. terpenensis*, using the experimentally more sensitive approach of [^{14}C]-labelling.

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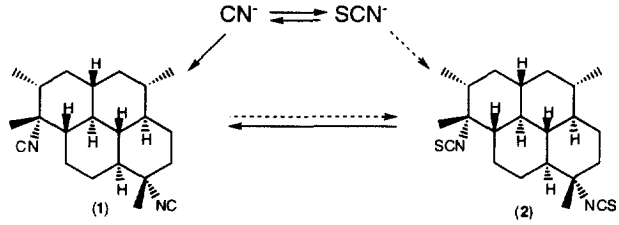
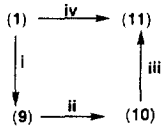
Amphimedon terpenensis and the closely-related *Cymbastela hooperi*[8] contain a rich variety of diterpene metabolites, including isonitriles, isothiocyanates and isocyanates.[9,10] Samples of *A. terpenensis* collected at Lizard Island on the Great Barrier Reef contained diterpenes metabolites by GC-MS, TLC and NMR; the hexane-solubles of the crude extract were processed by normal phase flash column chromatography (5% EtOAc/hexanes) and NPHPLC (0.25% EtOAc/hexanes), to give diisocyanoadociane (**1**).[9a] The GC-MS profile of the diterpene fractions showed other peaks corresponding to minor isocyanides and isothiocyanates.

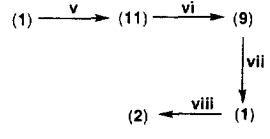


Scheme 1 Biosynthesis of diisocyanoadociane (see ref. 13)

To test the role of inorganic thiocyanate in diisocyanoadociane biosynthesis, we supplied 50 μCi sodium [^{14}C] thiocyanate to a specimen of *A. terpenensis* according to our established protocols.[2-5,11] After 19 days aquarium incubation, the sponge sample was frozen and diisocyanoadociane (**1**) was isolated and rigorously purified by HPLC and recrystallisation to constant specific radioactivity. The diisocyanide (**1**) was significantly radioactive, as shown in **Table 1** (Expt. 1), consistent with the use of thiocyanate for the biosynthesis of the isocyano groups (**Scheme 1**). To test the specificity of incorporation, terpene (**1**) was hydrolysed first to the bisformamide (**9**), then to monoamide (**10**) using acidic conditions, and finally to diamine (**11**) (**Scheme 2a**).[2] The diamine product (**11**) was not radioactive[12] (**Table 1**, Expt. 1) therefore the [^{14}C]-label was exclusively associated with the isocyanide carbons. Direct hydrolysis of the isolated diisocyanoadociane (**1**) to the diamine (**11**) using 6N HCl also gave unlabelled material (**Table 1**, Expt. 1).



Scheme 2a Degradation of (**1**)



Scheme 2b Synthesis of [$^{14}C_2$]-(**2**)

i. g. Acetic Acid, then silica/DCM-EtOAc/MeOH 1:1,100%; ii. 2.5M NaOH, reflux, then C18/MeOH:H_2O-MeOH, 97%;
iii. 6N HCl, reflux, then C18/MeOH:H_2O-MeOH, 33%; iv. 6N HCl, reflux, then C18/MeOH:H_2O-MeOH, 30.5%
v. 6N HCl, reflux, 68%; vi. [^{14}C]-Formic-pivalic anhydride, Et_3N, THF, 100%; vii. *p*-TsCl in pyridine, 29%; viii. Sulphur, 120°C, 40%

Next we tested the role of organic isothiocyanates in diisocyanoadociane biogenesis by synthesis and incorporation of [$^{14}C_2$]-diisothiocyanatoadociane (**2**).[13] A sample of diisocyanoadociane (**1**), isolated from the sponge, was converted to the diamine (**11**) by acid hydrolysis, then treated with (i) [^{14}C]-Formic-pivalic anhydride in THF; (ii) *p*-TsCl in pyridine; (iii) Sulphur at 120°C to give (**2**) (**Scheme 2b**).[14] The intermediate [$^{14}C_2$]-diisocyanoadociane was degraded (**Table 1**, Expt. 2) by hydrolysis first to bisformamide (**9**) then to diamine (**11**), thereby confirming exclusive labelling in both isocyano groups. The labelled diisothiocyanate (**2**) was dissolved in 1 mL of acetone and divided equally between two glass beakers, each containing a small piece of *A. terpenensis* in aerated sea- water kept at ambient temperature and light levels.[11] After 19 days aquarium incubation, the two sponge samples were frozen and diisocyanide (**1**) was isolated and rigorously purified by

Table 1. Molar Specific Activities of Diisocyanoadociane and Degradation Products

Expt	Description	Cmpd	Molar Specific Activity (μCi/mMole)[a]	Incorporation (%)	Radioactivity (%)
1	Feeding of Na[^{14}C]SCN[b]	(**1**)	0.918 (1)[c]	0.141	100.0
		(**11**)	0.025	-	2.3
		(**1**)	0.143 (1)[c]	-	100.0
		(**9**)	0.136	-	95.7[d]
		(**10**)	0.071	-	49.8
		(**11**)	0.006	-	4.2
2	[^{14}C]-(**2**)	(**1**)	0.065 (4)	-	100.0
		(**9**)	0.061	-	94.5[d]
		(**10**)	0.022	-	35.1
		(**11**)	0.001	-	1.7
3	Feeding of [^{14}C]-(**2**)[e]	(**1**)	0.059 (1)	0.019	100.0
		(**9**)	0.051	-	95.0[d,f]
4	Feeding of [^{14}C]-(**2**)[e]	(**1**)	0.011 (2)	0.002	100.0
		(**11**)	0.001	-	9.1[g]

[a] Number of recrystallisations in brackets; [b] Incorporation of 50 μCi; [c] (**1**) of specific activity 0.918 μCi/mMole was diluted with unlabelled material to give a specific activity of 0.143 μCi/mMole; [d] Samples of (**9**) are hygroscopic which slightly lowers the measured specific activity. [e] Incorporation of 11.5 μCi; [f] Insufficient material for further degradation.; [g] see ref. 12.

repeated HPLC and recrystallisations to constant specific radioactivity. In one sample (Expt. 3), the diisocyanide (**1**) was significantly radioactive, (**Table 1**), consistent with conversion of (**2**) as shown in **Scheme 1**. Terpene (**1**) was hydrolysed to the bisformamide (**9**), which retained the radioactivity, further highlighting the radiochemical purity of the isolated (**1**); lack of material prevented further degradation of this sample. In the duplicate experiment (Expt. 4, **Table 1**), the diisocyanide (**1**) contained less radioactivity, and several passes through HPLC and recrystallisations were required to confirm radiochemical integrity. Degradation of the resulting small sample of diisocyanoadociane (**1**), (77 dpm/mg), to the diamine (**11**) showed that the label was associated with the isocyano carbons. Considering that incorporation via general metabolism is highly unlikely, we attribute the higher than expected ^{14}C content of (**11**) to inseparable side products products from the degradation reaction.[12]

These data show clearly that inorganic thiocyanate, in addition to cyanide,[2] is utilised by *A. terpenensis* for isocyanide biosynthesis, further confirming that cyanide and thiocyanate are used interchangeably in marine sponges for terpene functionalisation.[3-5] Furthermore organic isothiocyanates are utilised by *A. terpenensis* for isocyanide biosynthesis. To date, this is the most convincing labelling result in which advanced biosynthetic precursors are used to investigate secondary metabolism in a marine sponge.[15]

Hagadone *et al.* detected the complementary isocyanide to isothiocyanate conversion in the Hawaiian sponge *Ciocalypta* sp.[7] The sponge *A. terpenensis* is not a suitable candidate to investigate the isocyanide to isothiocyanate transformation, owing to the large amounts of diisocyanoadociane present in the sponge extract; this aspect of isocyanide and isothiocyanate biosynthesis is currently being tested in *Axinyssa* n.sp.[16]

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11. Sponge samples were collected using SCUBA at North Point, Lizard Island, Great Barrier Reef (-14-16 m) in July 1998. A voucher sample (registry number QM G314228) is lodged at The Queensland Museum, Brisbane. *Amphimedon terpenensis* (w. wt. 45 g) was placed in an aquarium containing 400 mL aerated seawater at ambient temperature. Sodium [^{14}C] thiocyanate (50 μCi) was added and the sponge allowed to assimilate radioactivity for 12 h overnight. The sponge was kept in running seawater in a 10 L aquarium at ambient temperature for 19 days, then frozen for subsequent radiochemical analysis. A DCM:MeOH extract was evaporated to give an aqueous suspension which was extracted with DCM to give a crude extract (314 mg), which was fractionated by stepwise gradient silica flash chromatography using hexanes/EtOAc as eluant and diisocyanoadociane isolated by silica HPLC (μ-partisil, 5% EtOAc/hexanes) and recrystallisation to constant specific activity from hexane. [$^{14}C_2$]-Diisothiocyanatoadociane (11.5 μCi, 433 μCi/mmol) in acetone (0.5 mL) was added to each of two samples of *A. terpenensis* (Expt. 3, 26g and Expt. 4, 28 g) in 400 mL of seawater and treated as for the thiocyanate incorporation above, giving crude extracts weighing 204 mg and 188 mg respectively.
12. Diamine (**11**) is difficult to purify rigorously, resulting in some residual activity in these samples.
13. Diisothiocyanatoadociane (**2**) has not yet been isolated from *A. terpenensis*, although related isothiocyanates have been reported.[8,10] GC-MS analysis has not detected (**2**) in our extracts of *A. terpenensis*.
14. Overall chemical yield, 11.6 %; Overall radiochemical yield, 5.75 %.
15. For discussion of advanced precursor studies on sponge sterols, see: (a) Djerassi, C.; Silva, C.J. *Acc. Chem. Res.*, **1991**, *24*, 371-378. (b) Silva, C.J.; Wuensche, L.; Djerassi, C. *Comp. Biochem. Physiol.*, **1991**, *99*, 763-773.
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