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Thiocyanate Biosynthesis in the Tropical Marine Sponge *Axinyssa* n.sp.

Jamie S. Simpson and Mary J. Garson*

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

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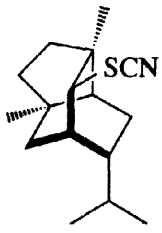
Abstract: The biosynthetic origin of the thiocyanate carbon in 2-thiocyanatoneopupukeanane (**1**) is defined by incorporation of sodium [^{14}C] cyanide and [^{14}C] thiocyanate into *Axinyssa* n.sp. The specificity of incorporation is demonstrated by reduction of (**1**) to the thiol (**7**).

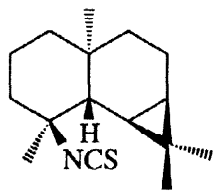
Keywords: Biosynthesis; sponges; terpenes and terpenoids; thiocyanates.

Thiocyanates have been documented as metabolites in several genera of marine sponges,[1-3] as well as in nudibranchs[1c,2b,4] and ascidians.[5] The origin of the thiocyanato group has been the subject of much biosynthetic speculation.[6] Scheuer *et al.* suggested the cyanation of a thiol,[2a] which appears to be a reasonable pathway to the amino acid-derived psammaplin thiocyanate.[3] In contrast, in those sponges in which thiocyanates co-occur with isonitriles or with isothiocyanates, the involvement of the ambident[7] thiocyanate ion has been invoked.[1a,1b,8]

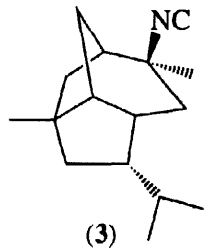
Earlier research from this group has shown that marine isocyanides are derived by functionalization of a terpene precursor using inorganic cyanide.[9,10] More recently our group has demonstrated the use of both cyanide and thiocyanate by *Acanthella cavernosa* in the biosynthesis of isocyanides and isothiocyanates,[11] and by *Stylotella aurantium* in the biosynthesis of dichloroimines,[12] suggesting that these inorganic precursors may be interconverted by the sponges. Thus it seemed plausible to us that cyanide might also be a precursor to the thiocyanato group in marine terpenes. In this paper we report on biosynthetic experiments with the sponge *Axinyssa* n.sp. which provide evidence for the origin of the thiocyanate functionality.



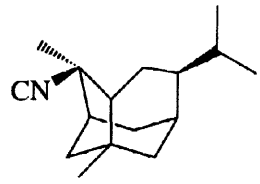
(**1**)



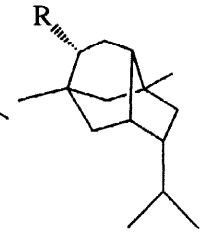
(**2**)



(**3**)



(**4**)



(**5**) R = NC
(**6**) R = NCS

Sponges of the genus *Axinyssa* have been reported to contain a wide variety of sesquiterpene metabolites. including isonitriles, isothiocyanates and thiocyanates.[1] We recently described the terpene chemistry of *Axinyssa* n.sp. collected at Heron Island on the Great Barrier Reef.[1c,13] The sponge contained sesquiterpenes metabolites by GC-MS, TLC and NMR, and 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 the known 2-thiocyanatoneopupukeanane (**1**),[1b,4] together with epipolasin-A (**2**), 9-isocyanoallopupukeanane (**3**), 2-isocyanotrachyopsane (**4**), and the 9-pupukeanane isocyanide/isothiocyanate pair (**5/6**), of which the

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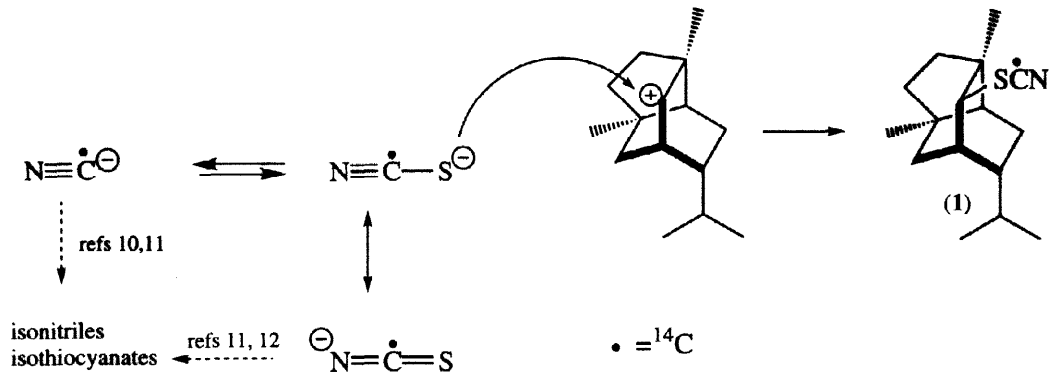
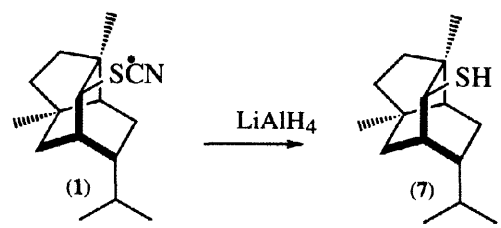
isothiocyanato analogue was previously unknown.[1c] The GC-MS profile of the sesquiterpene fraction showed a number of other peaks including isonitriles and isothiocyanates.



Scheme 1 Biosynthesis of 2-thiocyanatoneopupukeanane

We supplied 25 μCi sodium [^{14}C] thiocyanate to a specimen of *Axinyssa* n.sp. according to our established protocols.[10-12, 14] After 16 days aquarium incubation, the sponge sample was frozen and thiocyanate (**1**) was isolated and rigorously purified by HPLC to constant specific radioactivity. The thiocyanate (**1**) was significantly radioactive, as shown in **Table 1**, consistent with the use of thiocyanate for the biosynthesis of the thiocyanato group as shown in **Scheme 1**. To test the specificity of incorporation, terpene (**1**) was degraded to the thiol (**7**) using $LiAlH_4$.[2a,15,16] The thiol product (**7**) was not radioactive therefore the [^{14}C] label was exclusively associated with the thiocyanato carbon, as required by the proposed biosynthesis. Incorporation of sodium [^{14}C] cyanide into a second piece of sponge also gave radioactive 2-thiocyanatoneopupukeanane (**1**) (**Table 1**).[14] Degradation to the thiol (**7**) resulted in unlabelled thiol product as before, indicating the label was exclusively associated with the thiocyanato moiety.



These results strongly suggest the conversion of inorganic cyanide into inorganic thiocyanate in the sponge, as it is difficult to envisage the incorporation of cyanide into a terpene, followed by insertion of sulphur to give a thiocyanate. Sulphur insertion to give an isothiocyanate (by an enzyme functionally equivalent to rhodanese[17]) followed by isomerisation remains a possibility, however in chemical reactions at least, the equilibrium usually favours an isothiocyanate over a thiocyanate.[7,18] Our experiments also allowed us to monitor isonitrile/isothiocyanate biosynthesis in this sponge. When the isonitrile/isothiocyanate pair (**5/6**) were isolated, the isothiocyanate (**6**) samples were radioactive (>150,000 dpm/mg), whereas the isonitrile samples from both thiocyanate and cyanide feedings were not significantly labelled (<100 dpm/mg). The specificity of labelling of isothiocyanate (**6**) is currently under investigation. It is extraordinary that, in view of previous experiments with both diterpene isonitriles[10,19] and sesquiterpene isonitriles,[11,19] we have not demonstrated the incorporation of cyanide or of thiocyanate into the major isonitrile component of *Axinyssa* n. sp.

Table 1. Molar Specific Activities of *Axinyssa* n.sp. Metabolites and Degradation Products

Precursor	Cmpd	Molar Specific Activity (μCi/mMole)	Incorporation (%)	Radioactivity (%)
Na[^{14}C]SCN[a]	(1)	0.150	0.0002	100.0
Na[^{14}C]SCN[a]	(7)	<0.001	-	0.3
Na[^{14}C]CN[b]	(1)	1.230	0.002	100.0
Na[^{14}C]CN[b]	(7)	0.001	-	0.1

[a] Incorporation of 25 μCi; [b] Incorporation of 100 μCi

In common with other isonitrile-containing sponges,[20] light and electron microscopic inspection of *Axinyssa* n.sp. revealed the presence of microbial symbionts. The outer layers of sponge tissue were rich in cyanobacteria of a type morphologically similar to *Aphanocapsa feldmanni* while the inner tissue contained high populations of diverse bacterial cell types in addition to sponge cells.[21] Preliminary ecological studies indicate that *Axinyssa* n.sp. may have unusual inhibitory effects on the settlement of ascidian larvae.[22]

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13. Sponge samples were collected using SCUBA at Heron Island, Great Barrier Reef (-14-16 m) in June 1997. A voucher sample (registry number QM G312575) is lodged at The Queensland Museum, Brisbane.
14. *Axinyssa* n.sp. (w. wt. 77 g) was placed in an aquarium containing 200 mL aerated seawater at ambient temperature (20-23°). Sodium [^{14}C] thiocyanate (25 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 16 days, then frozen for subsequent radiochemical analysis. Extraction with DCM:MeOH gave an aqueous suspension which was extracted with hexanes to give a crude extract (1055 mg), which was fractionated by stepwise gradient silica flash chromatography using hexanes/EtOAc as eluant and individual terpenes isolated by silica HPLC (μ-partisil, 0.25% EtOAc/hexanes). The radioactivity content was monitored at each stage of the purification sequence, and terpenes were subjected to repeated hplc until the specific activity was constant. A sodium [^{14}C] cyanide (100 μCi; 16 days incorporation) experiment, used an 88 g piece of sponge, resulting in a crude extract weighing 906 mg, which was analysed as above.
15. *Reduction of thiocyanate (1).* 2-Thiocyanatoneopupukeanane (**1**, 3.90mg, 0.015 mmol) was dissolved in dry THF (2 mL), added to $LiAlH_4$ (2.98 mg, 0.078 mmol) and allowed to stir at room temperature for 8 minutes, then poured into a saturated ammonium chloride solution (15 mL) and extracted with EtOAc (3 x 10 mL). The EtOAc layers were dried over anhydrous magnesium sulphate and evaporated to give 4.2 mg of a clear smelly residue. Purification by silica filtration eluting with hexane was followed by NP-HPLC (0.05% EtOAc/hexanes) to give pure thiol (**7**, 1.78 mg, 0.0077 mmol, 51%).
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