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# Biosynthesis of Dichloroimines in the Tropical Marine Sponge *Stylotella aurantium.*

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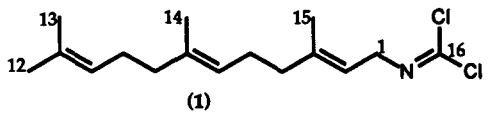
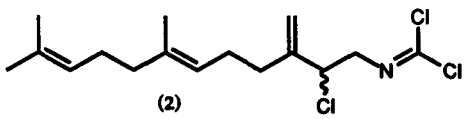
***Abstract:*** **The biosynthetic origin of the dichloroimine carbon in the stylotellanes A and B, (1) and (2), is defined by specific incorporation of sodium [$^{14}$C] cyanide. Sodium [$^{14}$C] thiocyanate is also involved in their biosynthesis. A mechanistic scheme is presented for the formation of these bioactive metabolites.** © 1997 Elsevier Science Ltd.

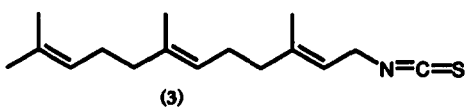
Marine sponges of the order Axinellida, Halichondrida and Haplosclerida often contain bioactive terpenes with isocyanide, isothiocyanate, and formamide functionality; the rarer isocyanate and thiocyanate substituents are also known.[1] These unique metabolites have been novel targets for study with $^{14}$C- and $^{13}$C-labelled precursors to determine the biosynthetic origin of the non-terpenoid carbon atom.[2,3] The dichloroimine (= carbonimidic dichloride) moiety represents an rare example of a functional group containing both nitrogen and carbon which to our knowledge has only been found in terpene metabolites of the Indo-Pacific sponge *Pseudaxinyssa pitys*.[4-6] During field work at Heron Island, we collected a bright orange, soft-textured sponge, subsequently identified as *Stylotella aurantium*,[7,8] containing dichloroimine metabolites. In this paper we report the results of biosynthetic experiments which shed light on the origin of the dichloroimine carbon.




(1)




(2)

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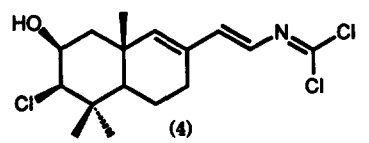


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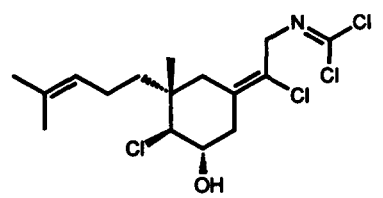
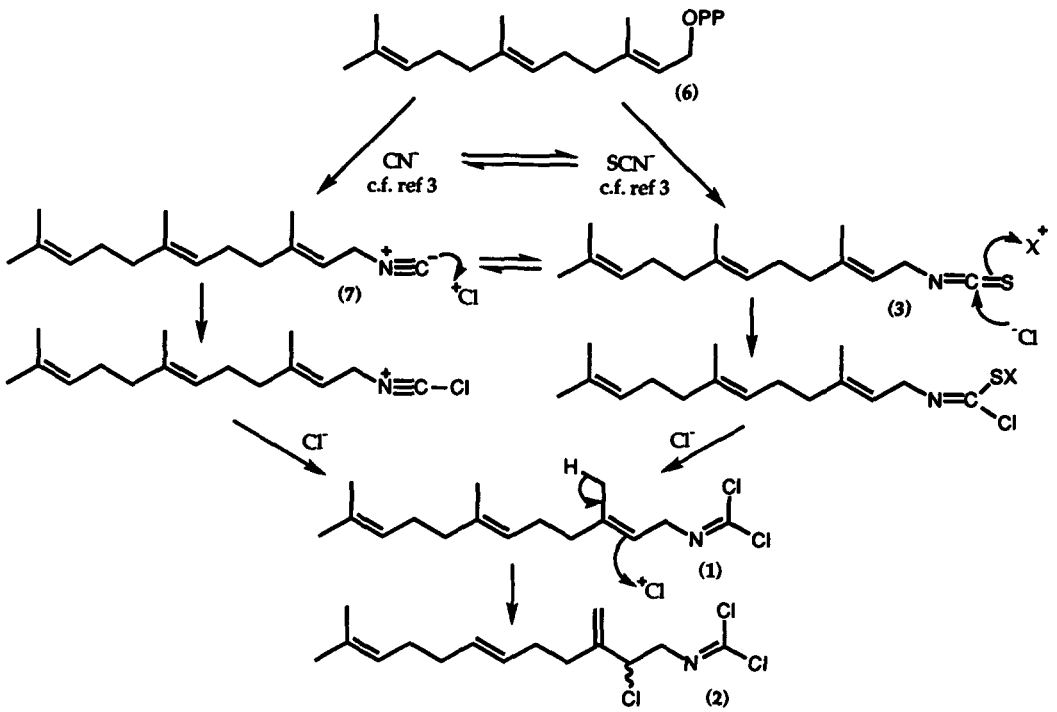



(5)

The crude sponge extract, which weakly inhibited the growth of a $P_{388}$ mouse leukaemia cell line, contained terpenes by TLC and NMR. The DCM-solubles of the crude extract were processed by normal phase flash column chromatography (hexanes/EtOAc 95:5) and NPHPLC (hexanes/EtOAc 99.8:0.2), to give two major sesquiterpenes, named by us as stylotellanes A (1) and B (2), together with farnesyl isothiocyanate (3). The structures of the the new metabolite (1)[9] and the known metabolites (2)[10] and (3) were confirmed by 2D-NMR studies, including geHMBC, and by comparison with literature data.[4-6] Small amounts of two unstable

cyclic metabolites were also isolated; complete characterisation proved impossible, however comparison with published data suggested the structures (4) and (5) shown.[4,5]

Earlier research from this group on the sponge *Amphimedon terpenensis* has shown that marine isocyanides are derived by functionalization of a terpene precursor using inorganic cyanide[11] while our recent work with *Acanthella cavernosa* has shown the interconvertibility of cyanide and thiocyanate for the biosynthesis of sesquiterpene isocyanides and isothiocyanates in this axinellid sponge.[3] The coocurrence of an isothiocyanate together with dichloroimines in *S. aurantium* suggests the involvement of cyanide/thiocyanate in the biosynthesis of the dichloroimine group, since isocyanide dihalides can be synthesized by addition of chlorine to isocyanides, [4,12] or by chlorination of isothiocyanates.[12] We therefore tested the possibility that the stylotellanes might be biosynthesized from farnesyl pyrophosphate (6) via the terpenes (3) or (7). Plausible biosynthetic mechanisms invoking a chloroperoxidase[13] enzyme are shown in **Scheme 1.**



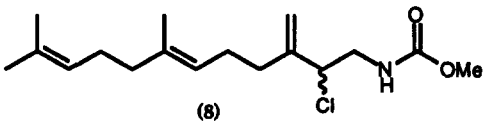

**Scheme 1.**

We supplied 50 μCi potassium [$^{14}$C]cyanide to a specimen of *S. aurantium* according to our established protocols.[3,10,14] After 9 days aquarium incubation, the sponge sample was frozen and stylotellanes A and B were isolated and rigorously purified by HPLC to constant specific radioactivity. The samples of (1) and (2) were significantly radioactive, as shown in **Table 1**, consistent with the use of cyanide for the biosynthesis of the dichloroimine group. The percentage incorporation levels measured were low as a result of loss of volatile metabolites during the purification process combined with the chemical instability of the dichloroimine group. To test the specificity of incorporation, terpene (2) was degraded to the methyl carbamate (8) and the amine (9) using 0.1N phosphoric acid in 95% methanol.[4,15] The carbamate product (8) was radioactive, whereas the

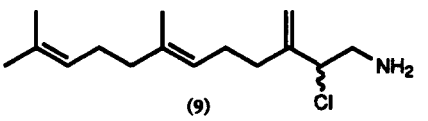
**Table 1.** Molar Specific Activities of *S. aurantium* Metabolites and Degradation Products

| Precursor | Cmpd | Molar Specific Activity (μCi/mMole) | Incorporation (%) | Radioactivity (%) |
|---|---|---|---|---|
| Na[$^{14}$C]CN[a] | (1) | 1.136 | 0.004 | - |
| Na[$^{14}$C]CN[a] | (2) | 1.472 | 0.033 | |
| Na[$^{14}$C]CN[a] | (2) | 0.332[b] | - | 100.0 |
| Na[$^{14}$C]CN[a] | (8) | 0.326 | - | 98.2 |
| Na[$^{14}$C]CN[a] | (9) | 0.004 | - | 1.2 |
| Na[$^{14}$C]SCN[c] | (1) | 0.354 | 0.00034 | - |
| Na[$^{14}$C]SCN[c] | (2) | 0.224 | 0.00056 | - |

[a] Incorporation of 50 μCi; [b] After dilution with unlabelled metabolite; [c] Incorporation of 13 μCi




(8)




(9)

amine (9) was devoid of radioactivity, therefore the [$^{14}$C] label was exclusively associated with the imine carbon, as required by the above biosynthetic scheme. Incorporation of sodium [$^{14}$C] thiocyanate into a second piece of sponge also gave radioactive metabolites (**Table 1**),[3,14] however there was insufficient material for chemical degradation. Likely the [$^{14}$C] label was again associated with the imine carbon. In each experiment, the isolated farnesyl isothiocyanate (3) was also radioactive.[3] Ongoing work is directed towards incorporation of labelled (3) and (7); interestingly, farnesyl isocyanide (7) has not yet been isolated from *S. aurantium*.

Light microscopic inspection of sponge tissue revealed the absence of microbial symbionts other than bacteria, therefore the stylotellanes are likely synthesised within the sponge cells.[16] *S. aurantium* is a conspicuous orange sponge which is soft-textured, yet rarely fouled. Preliminary ecological studies indicate that *S. aurantium* may strongly inhibit the settlement of ascidian larvae.[17] Samples of stylotellanes A and B were submitted for biological testing, but gave only weak $P_{388}$ activity associated with stylotellane B.

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7. Sponge samples were collected using SCUBA at Heron Island, Great Barrier Reef (-10-14 m) in September 1996 and March 1997. A voucher sample (registry number QM G307133) is lodged at The Queensland Museum, Brisbane.
8. The documented natural products chemistry of *S. aurantium* is: (a) a sesquiterpene isocyanide; Paris, M.; Fontaine, C.; Laurent, D.; La Barre, S.; Guittet, E. *Tetrahedron Lett.*, **1987**, *28*, 1409; (b) ring-A rearranged sterols: Bohlin, L.; Gehrken, H.K.; Scheuer, P.J.; Djerassi, C. *Steroids*, **1980**, *35*, 295; (c) cyclopeptides: Pettit, G.R.; Cichacz, Z.; Barkoczy, J.; Dorsaz, A.C.; Herald, D.L.; Williams, M.D.; Doubek, D.L.; Schmidt, J.M.; Lamy, P.T; Brune, D. *J. Nat. Prod.*, **1993**, *56*, 260; Pettit, G.R.; Srirangam, J.K.; Herald, D.L.; Erickson, K.L.; Doubek, D.L.; Schmidt, J.M.; Tackett, L.P.; Bakus, G.J. *J. Org. Chem.*, **1992**, *57*, 7217; Pettit, G.R.; Srirangam, J.K.; Herald, D.L.; Xu, J.; Boyd, M.R.; Cichacz, Z.; Kamano, Y.; Schmidt, J.M.; Erickson, K.L. *J. Org. Chem.*, **1995**, *60*, 8257; (d) chlorinated guanidines: Kinnel, R.B.; Gehrken, H.P.; Scheuer, P.J. *J. Am. Chem. Soc.*, **1993**, *115*, 3376; Kato, T.; Shizuri, Y.; Izumida, H.; Yokoyama, A.; Endo, M. *Tetrahedron Lett.*, **1995**, *36*, 2133.
9. *Stylotellane A* (**1**) (Found $[M\text{-}Cl]^{+\cdot}$ 266.1666. $C_{16}H_{25}NCl$ requires 266.1666). E.i. mass spectrum: 305, 303, 301, 268, 266, 230, 189, 161, 137, 121, 107, 69 (100%). C.i. mass spectrum: 306 (1%), 304 (7%), 302 (10%), 268 (13%), 266 (44%), 230 (12%), 205 (60%), 161 (15%), 149 (43%), 137 (100%), 121 (61%), 109, (59%) and 95 (70%). $\upsilon_{max}$ 2940, 1660, 1645 and 870 $cm^{-1}$. $^1H$ n.m.r. (500 MHz, $CDCl_3$) $\delta$ 5.28 (1H, t, 7 Hz, H-2), 5.09 (1H, m, H-6), 5.08 (1H, m, H-10), 4.10 (2H, d, 7Hz, H-1), 2.10-1.97 (8H, m, H-4, H-5, H-8, H-9), 1.69 (3H, s, H-15), 1.65 (3H, s, H-12), 1.59 (3H, s, H-13) and 1.58 (3H, s, H-14). $^{13}C$ n.m.r. (125 MHz, $CDCl_3$) 138.7 (C-3), 135.4 (C-7), 131.3 (C-11), 124,5 (C-16), 124.3 (C-10), 123.7 (C-6), 118.3 (C-2), 53.0 (C-1), 39.7 (C-8), 39.1 (C-4), 26.7 (C-9), 26.3 (C-5), 25.7 (C-12), 17.7 (C-13), 16.5 (C-15) and 16.0 (C-14) ppm.
10. *Stylotellane B* (**2**) had $^{13}C$ 126.8 ppm (C-16) and $[\alpha]_D$ = -29° ($CHCl_3$, 1.0); values not reported in ref 4.
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14. *S. aurantium* (w. wt. 24 g) was placed in an aquarium containing 200 mL aerated seawater at ambient temperature (20-23°). Sodium [$^{14}C$] cyanide (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 9 days, then frozen for subsequent radiochemical analysis. Extraction with DCM:MeOH gave a crude extract (110 mg), which was fractionated by stepwise gradient silica flash chromatography using hexanes/EtOAc as eluant and individual terpenes isolated by silica HPLC (μ-partisil, 0.2% EtOAc/hexanes). The radioactivity content was monitored at each stage of the purification sequence. A sodium [$^{14}C$] thiocyanate (13 μCi; 9 days incorporation) experiment, used a 12 g piece of sponge.
15. Satisfactory analytical and spectroscopic data were obtained for (**8**) and (**9**).
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