Biosynthesis of Isocyanoterpenes in Sponges

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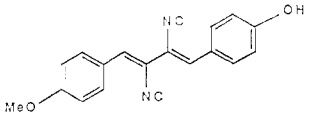
A marine sponge from Hawaii, *Ciocalypta* sp., incorporated [^{14}C]cyanide (1.8%) into 2-isocyanopupukeanane (**8**). Doubly labeled [^{13}C,^{15}N]cyanide was incorporated intact (1.46%) into 9-isocyanoneopupukeanane (**10**). Another sponge, *Acanthella* sp., from Guam incorporated [^{14}C]cyanide (1.5%) into kalihinol-F (**11**). Labeled **11** was transformed into the corresponding triamine and hence the trisbenzylurea, which after purification was devoid of radioactivity, thereby proving that cyanide serves as specific precursor for the isocyano function.

Secondary metabolites containing the isocyano function are rare in nature. The known terrestrial isocyano metabolites bear an obvious or veiled resemblance to amino acids: xanthocillin (**1**), the hazimycin factors (**2**), and the homothallins (**3**) to tyrosine; indolylacryloisocyanide (**4**) to typtophan; and the mannitol diester **5** to valine.[1] In all of these cases the isocyano group is in the position of the original amine, thus suggesting that only the isocyano carbon originates from another source. Achenbach and



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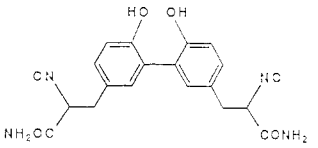

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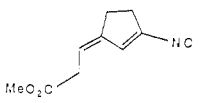

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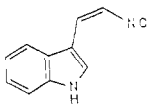

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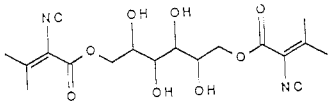

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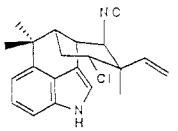

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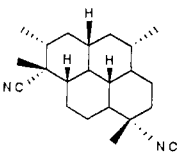
Grisebach[2] failed to detect incorporation of [*Me*-^{14}C]-methionine or [^{14}C]formate into xanthocillin but were able to show that the nitrogen of tyrosine is at least partly retained in the isocyano groups of xanthocillin. In contrast, Puar et al.[3] demonstrated incorporation of [*Me*-^{14}C]- and [*Me*-^{13}C]methionine (1.5%) into the isocyano carbon of hazimycin (**3**). Surprisingly, though, while both [3-^{14}C]- and [3-^{13}C]tyrosine were well incorporated (15% and 8%, respectively), DL-[*N-methyl*-^{13}C]tryosine was not incorporated. Attempts by Herbert and Mann[4] to trace the origin of the isocyano group in the xanthocillins (**1**) have failed. However, Moore and co-workers[5] successfully incorporated cyanide, glycine, serine, methionine, and formate into hapalindole A (**6**), a constituent of the cultured terrestrial cyanophyte *Hapalosiphon fontinalis*.

Marine sponges[6a,b] and blue-green algae[7] are also sources of isonitriles, which usually co-occur with isothiocyanates, formamides, and terpene hydrocarbons, occasionally with isocyanates, ureas, thioureas, dichlorocarbimines, and free amines—all presumably derived from an isocyano precursor.[8] The marine isonitriles bear no resemblance to known amino acids, thereby suggesting different biosynthetic pathways. We,[9] and others,[10] showed that formamide and isothiocyanate are elaborated from isocyanide. Herbert and Mann[4] proposed that marine isonitriles might originate from cyanide ions, which was proven by Garson,[11] who obtained a 1.8% (0.9% per isonitrile) incorporation of [^{14}C]cyanide into diisocyanoadociane (**7**). Garson traced the origin of the isocyano carbon but not of the nitrogen. These results also do not reveal whether incorporation takes place by nonenzymic exchange or substitution reaction on a natural substrate or whether cyanide is concentrated from sea water or biosynthesized from yet another precursor. We therefore examined incorporation of cyanide in two sponges: *Ciocalypta* sp. from Hawaii and *Acanthella* sp. from Guam.



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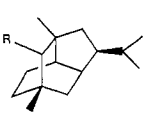
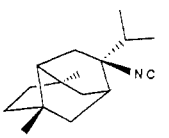


8 R = NC

9 R = NHCHO



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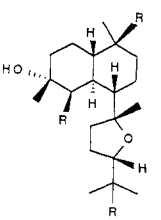

11 R = NC

12 R = NHCONHCH$_2$Ph

Ciocalypta sp. contains predominantly one compound, a monofunctional sesquiterpene, 2-isocyanopupukeanane (**8**),[12] while *Acanthella* sp. contains eight major highly

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Table I. Results of Cyanide Incorporation into Sponges *Ciocalypta* Sp. *Acanthella* Sp.

precursor	amount fed, μCi	incubation time, weeks	total recovered, μCi	specific incorporation, %
Ciocalypta				
[^{14}C]cyanide	100	3	2.42	1.8
[^{13}C,^{15}N]cyanide		9		1.46
Acanthella				
[^{14}C]cyanide	100	5	7.61	1.5

functionalized diterpenes, the kalihinols.[13a-c]

Labeled precursor was encapsulated in lipid vesicles by agitating its aqueous solution in a test tube coated with a lipid film.[14a,b] The resulting milky mixture was injected into a sponge in situ.[15] The sponges were incubated for 3–9 weeks, collected, frozen on dry ice, and transported to the laboratory. The frozen sponges were blended thrice with ethanol; the filtrate was reduced to half its volume and partitioned with hexane and then chloroform.

The hexane extract from *Ciocalypta* sp. that had been incubated with sodium [^{14}C]cyanide was evaporated and subjected to gel filtration on BioBeads SX-8 in toluene. A pale green band following yellow carotenes contained all sesquiterpenoids (TLC). Fractions containing **8** were chromatographed on silica (BondElut; hexane/ethyl acetate, 99:1), yielding a colorless, usually crystalline product, which was further purified by normal-phase HPLC (Karusosorb[16] 5 μm, 1 × 25 cm; hexane/ethanol, 99.95:0.05), followed by reversed-phase HPLC (Adsorbosphere HS 5 μm, 0.46 × 25 cm; acetonitrile/water, 85:15). This product was purified to constant activity by recrystallization from hexane or aqueous acetonitrile. After incubation for 18 days *Ciocalypta* yielded **8** with a specific activity of 1.26 μCi/mmol (1.8% incorporation, see Table I). As a final check of radiochemical purity, **8** was converted to its formamide (**9**) with aqueous acetic acid and purified by HPLC. Due to the extremely hindered nature of the isonitrile, it was impossible to hydrolyze **8** to the amine.

To ascertain whether the observed incorporation was the result of an enzymic process or not, 20 mg of **8** was dissolved in ethanol (1 mL) and 0.2 μm filtered sea water (9 mL). Sodium [^{14}C]cyanide (4.0 μCi) was added, and the mixture was stirred for 4 weeks. Similarly, a 25-mg mixture of crude *Ciocalypta* hydrocarbons (mostly amorphane but also containing some 2-isothiocyanopupukeanane and other isothiocyanates and isocyanates) was stirred with 12.6 μCi of [^{14}C]cyanide. Both reactions were worked up similarly to crude sponge extracts (omitting the gel filtration step). The hexane partition from the hydrocarbon experiment was diluted with **8** (20 mg) and subsequently purified to zero activity. The hexane extract of the pupukeanane experiment was purified to a residual specific activity of 80 dpm/mg above background, which is insignificant when compared to the 12 000 dpm/mg obtained from a typical sponge incubation. We thus showed that cyanide incorporation is an enzymic process.

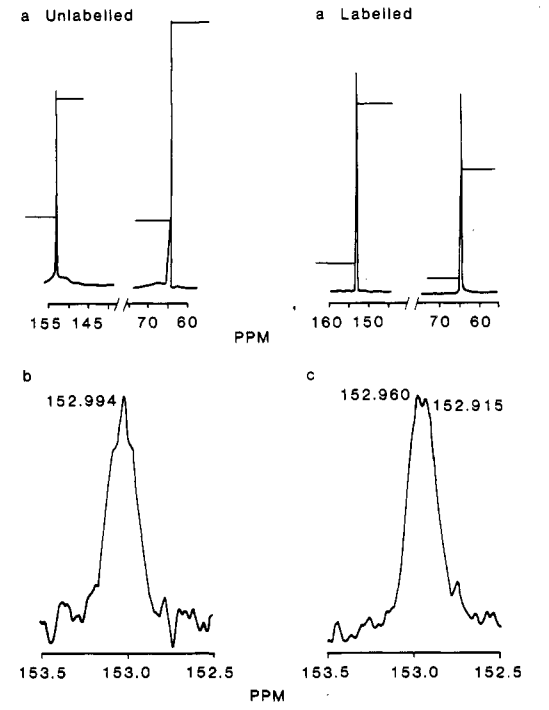

Figure 1. (a) ^{13}C NMR resonances of unlabeled and labeled C (65 ppm)–NC (153 ppm); (b) enlarged signal of unlabeled isocyanocarbon; (c) isocyanocarbon difference ^{13}C NMR spectrum of ^{13}C^{15}N-labeled minus unlabeled compound.

An incorporation level of 1.8% (based on total radioactivity fed) from **8** suggested that incorporation of stable isotopes should be possible. Thus 500 mg of potassium [^{13}C,^{15}N]cyanide was fed to one specimen of *Ciocalypta* in five injections over a period of 9 weeks. The usual workup surprisingly revealed that this sponge did not contain **8** but a rather complex mixture of metabolites of which the major substituent was **10** (83 mg), isolated as a colorless oil.[17,18] Further collections of sponges from the same area and extensive chromatography produced a 30-mg sample of unlabeled **10**. ^{13}C NMR spectra of labeled and unlabeled samples under identical conditions revealed that the isocyano carbon was enhanced by 156% (Figure 1), which corresponds to an enrichment of 1.46% (natural abundance of ^{13}C = 1.10%). Subtraction of the unlabeled spectrum from the labeled gave good cancellation of all signals except that of the isocyano carbon, which remained as a doublet, $^{1}J_{^{15}N^{13}C}$ = 5.9 Hz, 0.037 ppm upfield of the unlabeled resonance (Figure 1), thereby proving that cyanide furnishes both nitrogen and carbon for the isocyano group.

The chloroform extract from the Guam *Acanthella* sp. that had been injected with sodium [^{14}C]cyanide (100 μCi) was subjected to gel filtration on Sephadex LH-20. The first pale yellow fractions eluted after the orange pigments contained the kalihinols. Fractions containing kalihinol-F (TLC) were combined and chromatographed on silica. The fraction that eluted with ethyl acetate/dichloromethane

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(15) Because of the delicate nature of the sponge, it was not possible to transfer and maintain it on plastic plates. Removal of the sponge with substrate was difficult and met with only limited success. Incorporation levels were found to be higher by injection rather than allowing the sponge to filter the vesicles from sea water in a small aquarium.

(16) Prepared by P.K. from commercial 5-μm silica gel (Sigma).

(17) We normally experiment with *Ciocalypta* sp. from Pupukea on the north shore of O'ahu. This site is inaccessible for diving from October through April because of high surf. We located an alternate source of *Ciocalypta* sp. off Ala Moana reef on the south shore of O'ahu. Surprisingly, we discovered after the first incubation experiment that its profile of secondary metabolites differed from that of the north shore *Ciocalypta* sp.

(18) The major isocyanosesquiterpene of the Ala Moana *Ciocalypta* sp. possesses a new tricyclic skeleton. *J. Org. Chem.*, following paper in this issue.

(1:9) was subjected to silica gel HPLC followed by reverse-phase HPLC to yield pure kalihinol-F (11). Constant activity was attained by recrystallization from aqueous methanol. After an incubation of 5 weeks the sponge yielded 11 (9.1 mg) with a specific activity of 4.79 μCi/mmol (1.5% incorporation, see Table I). Hydrolysis of 11 with 6 N hydrochloric acid yielded the triamine, which was purified as the tris(benzylurea) 12 and found to be essentially devoid of radioactivity. This established that all the radioactivity was associated with the isonitrile carbons.

We have confirmed that cyanide is incorporated into sponge sesqui- and diterpenoids and that cyanide is incorporated intact. Hence the biosynthesis of isonitriles in sponges differs fundamentally from that in microorganisms. Experiments to ascertain the metabolic origin of the cyanide ions and the biosynthesis of the terpene skeleton are currently under way in our laboratory.

Experimental Section

All solvents were reagent grade and distilled from appropriate drying agents prior to use. Extractions were performed in the usual manner with the solvents and wash solutions indicated.

Analytical thin-layer chromatography (TLC) separations were carried out on Merck silica gel 60 F-254 (0.2 mm) precoated aluminum plates. Once developed with the solvents indicated, plates were visualized by spraying with 5% vanillin in sulfuric acid followed by gentle heating. Column chromatography was performed on Analytichem International BondElut disposable columns (3 mL). High-performance liquid chromatography (HPLC) of radioactive compounds was performed on homemade columns, with a Waters Associates 6000A solvent delivery system. For preparative separations Sigma HPLC sorbent (5 μm) was packed into a Valco (25 × 1 cm) column (n = 50 000 plates/m). Semipreparative chromatography was performed on Spherisorb (5 μm) silica gel packed into a Valco (25 × 0.46 cm) column (n = 100 000 plates/m) or Adsorbosphere HS (5 μm) reverse-phase column (25 × 0.46 cm) purchased from Alltech. Gradient separations were performed with two Waters 6000A pumps with a Waters Solvent Programmer. Gel filtration was performed on BioRad BioBeads (SX-8) packed into a glass (100 × 1 cm) column or Pharmacia Sephadex LH-20 also packed in a glass column (150 × 2 cm). The solvent (toluene for BioBeads and chloroform/methanol, 1:1, for Sephadex) was delivered from a Mariotte resevoir at 2–4 mL/h. Fractions were collected with an Isco Model 1200 Pup auto fraction collector.

Melting points (mp) were taken on a Kofler-type hot-stage microscope apparatus and are uncorrected. Infrared absorption spectra (IR) were recorded with a Nicolet MX-S FTIR spectrometer in chloroform solution. Ultraviolet–visible absorption spectra (UV) were recorded on a Hewlett-Packard 8452A spectrometer in 1-cm quartz cells in methanol solution. Nuclear magnetic resonance spectra (NMR) were taken in designated solvents on a Nicolet NT-300 instrument. Carbon-13 spectra were exponential signal enhanced prior to Fourier transform and subtracted in double precision mode. Electron impact mass spectroscopy (EIMS) was performed on a MAT 311 instrument. Fast atom bombardment (FAB) mass spectrometry was performed in monothioglycerol matrix on a VG 70 SE mass spectrometer.

The radioactivity of labeled compounds was measured with a Beckman LS-230 liquid scintillation counter with 3a70B counting cocktail purchased from Research Products International. [^{14}C]Toluene was used as external standard and unlabeled isocyanoterpenes as background standard. [^{14}C]Toluene was also used as internal standard to calculate quench correction. For measurement of specific activity (±5%), the sample was dissolved in hexane, and an aliquot was dispersed in the scintillation cocktail and counted against an equal quantity of unlabeled sample. Accurate background readings over long periods of time were obtained prior to counting samples of low specific activity. All radioisotope labeled compounds were purchased from Research Products International.

Fist-sized (40–100 g wet weight) specimens of *Ciocalypta* sp. for radiolabeled experiments were found at Pupukea on the north shore of O'ahu, Hawaii, in caves at −3 m. One larger specimen (~250 g) from Ala Moana on the south shore of O'ahu was used for the stable isotope experiment. Specimens of *Acanthella* sp. growing at −25 m on the "sponge mound" in Apra Harbor, Guam were used in the radiolabeling experiments.

Radioactive precursors were encapsulated in lipid vesicles before direct injection into the sponge. Lipid vesicles were produced from L-α-phosphatidylcholine, L-α-dioleylphosphatidylcholine, DL-α-dipalmitidylphosphatidic acid and cholesterol (12:2:1:4) by dissolving the phospholipids and sterol in chloroform and evaporating a film onto the inside of a chromic acid cleaned test tube a 40 °C. Introduction of the labeled compound dissolved in a small volume of buffered water and agitation with a vortex mixer resulted in a fine suspension of lipid vesicles, which was diluted with 0.2 μm of filtered sea water and injected directly into marked sponges. After 3–9 weeks the sponge was collected and immediately frozen on dry ice. Frozen sponges were blended in ethanol three times, and the combined filtrates were reduced to half their initial volume by rotary evaporation. The aqueous ethanol was partitioned three times with hexane. The aqueous layer was then diluted with an equal volume of water and partitioned three times with chloroform. The aqueous, chloroform, hexane, and dried solid residue were counted separately to obtain total radioactivity recovered.

***Ciocalypta* sp.** The hexane extract (which contained >50% of 8) was subjected to gel filtration (BioBeads 8 × 8, eluted with toluene). Fractions containing 8 (TLC; hexane/ethanol, 1000:1) were combined and chromatographed on silica gel (BondElut; ethyl acetate/hexane, 99:1). The entire eluate was subjected to preparative HPLC (hexane/ethanol, 99.95:0.05) to yield pure 8 as a cyrstalline solid. Further purification was achieved by reversed-phase HPLC (acetonitrile/water, 85:15), and constant specific activity achieved by recrystallization from aqueous acetonitrile followed by recrystallization from hexane, mp 81 °C. A final check of radiochemical purity was achieved by derivatization of 8 to its formamide.

2-Formamidopupukeanane (9). 2-Isocyanopupukeanane (0.010 g) was dissolved in glacial acetic acid (0.2 mL) and water (0.1 mL). Silica gel (0.1 g) was added, and the reaction mixture was stirred for 30 min and then filtered. The filtrate was evaporated under a stream of dry nitrogen, and the residue was purified by reversed-phase HPLC (methanol/water, 80:20) to yield pure 9. This product was recrystallized from aqueous methanol to give colorless crystals (0.010 g; 93%): mp 185 °C (lit.[12] mp 170–174 °C); ^{1}H NMR ($CDCl_3$) δ 8.82 (b s) NHCHO, 7.92 (dd, J = 11.8, 0.6 Hz) NHCHO, 5.7 (b s) NH, 5.3 (b s) NH, 3.62 (d, J = 10.3 Hz) CHNHCHO, 2.76 (d, J = 11.2 Hz) CHNHCHO, 1.2–2.2 (m, 12 H), 0.93 (s) Me, 0.85 (d, J = 6.5 Hz) 2 Me, 0.79 (s) Me; EIMS m/z 249 (38, $M^{\bullet+}$).

***Acanthella* sp.** The chloroform partition from the sponge extract was subject to gel filtration (Sephadex LH-20, eluted with methanol/chloroform, 1:1), and the fractions containing kalihinol-F (TLC; hexane/ethyl acetate, 60:40) were combined and subjected to silica gel chromatography (BondElut; dichloromethane to ethyl acetate). The fraction that eluted with ethyl acetate/dichloromethane, 1:9, was purified first by preparative HPLC (hexane/ethyl acetate, 60:40) and secondly by reverse-phase HPLC (methanol/water, 62:38) to yield pure kalihinol-F (11). Constant specific activity was achieved by recrystallization from aqueous methanol, mp 176–178 °C.

Kalihinol-F Tris(benzylurea) (12). Kalihinol-F (11; 0.010 g)[10] was refluxed in hydrochloric acid (6 N; 5 mL) for 3 h under an atomosphere of nitrogen. The pale yellow homogeneous reaction mixture was evaporated in vacuo, and the residue was immediately dissolved in dry benzene (10 mL) containing benzyl isocyanate (0.1 g). The mixture was refluxed under dry nitrogen for 4 h. The cooled reaction mixture was loaded directly onto a silica gel column (BondElut) and flushed with chloroform. Elution with chloroform/ethanol (10:1) yielded the crude tris(urea) 12, which was purified by silica gel HPLC (gradient; 0–10% ethanol/chloroform) followed by reverse-phase HPLC (methanol/water, 9:1). Evaporation of the solvent yielded the tris(urea) 12 as an amorphous white powder (0.013 g; 65%): mp 165 °C; FAB m/z 754.5($M^{\bullet+}$, 100); UV λ_{max} 212 (5000), 260 nm (450); IR ($CHCl_3$) ν_{max} 3400, 2900, 1659, 1561 cm^{-1}; ^{1}H NMR (CD_3COCD_3) δ 7.1–7.3 (15 H, m), 6.66, 6.39, 6.37, 5.78, 5.60, 5.30 (6 NH), 4.0–4.4 (8 H, m), 1.0–2.5 (16 H, m), 1.43, 1.15, 1.11, 1.05, 0.97 (5 Me, s).

Acknowledgment. We thank Dr. Valerie Paul of the University of Guam for invaluable help in the biosynthetic experiments with *Acanthella*, and M. R. Hagadone and N. K. Gulavita for supplying the crude hydrocarbon mixture from *Ciocalypta*. Thanks also to A. Poiner and J. Hill for assistance with the tedious chromatography of **10**. We acknowledge financial support from the National Science Foundation and from the University of Hawaii Sea Grant College Program under Institutional Grant NA81AA-D-0070 from NOAA, Office of Sea Grant, U.S. Department of Commerce.

Registry No. **8**, 73069-50-8; **9**, 893898-14-1; **10**, 119323-93-2; **11**, 93426-91-6; **11** (triamine deriv), 119244-33-6; **12**, 119244-32-5; CN^-, 57-12-5; benzyl isocyanate, 3173-56-6.

Isocyanoneopupukeanane, a New Tricyclic Sesquiterpene from a Sponge[1]

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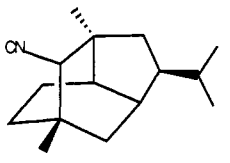
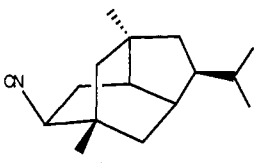
A sponge, *Ciocalypta* sp., from the south shore of O'ahu produces an isocyanosesquiterpene with a new tricyclic symmetrical skeleton. Its structure was determined by extensive NMR measurements, including long-range carbon–hydrogen, NOE, and COSY connectivities.

Our biosynthetic experiments toward discovering the origin of the isocyano function in marine sponges[2a,b] have been carried out on *Ciocalypta* sp. from the north shore of O'ahu, HI, where the animal is relatively abundant. More importantly, it contains one major metabolite, 2-isocyanopupukeanane (**1**).[3] High surf makes the north shore of O'ahu inaccessible to diving between October and April. We therefore searched south shore reefs for *Ciocalypta* sp. and found a few specimens on the Ala Moana reef. We injected potassium [$^{13}C^{15}N$]cyanide[2b] into a sponge, *Ciocalypta* sp., only to discover during the isolation that its principal secondary metabolite was not 2-isocyanopupukeanane (**1**) but a new and rearranged tricyclic isocyanosesquiterpene, the structure of which is the subject of this report.

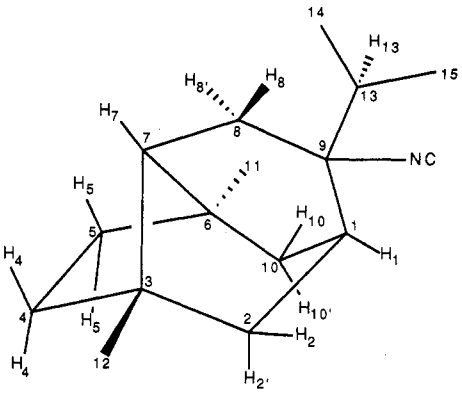
The frozen sponge was blended three times in ethanol. The filtrate was concentrated and partitioned against hexane. The residue of the hexane extract was chromatographed, yielding a complex mixture of which the major constituent was a colorless oil, rather than the anticipated low-melting solid. The highest peak in the EI mass spectrum of the new compound, isocyano*neo*pupukeanane, at m/z 231.1988 corresponded to a molecular formula of $C_{16}H_{25}N$, identical with the composition of **1**. Lack of olefinic ^{13}C or ^1H NMR signals indicated that the compound was a tricyclic saturated sesquiterpene. These compounds possess a few salient spectral features, but there was ample evidence that the compound bore an isocyano function (ν_{max} 2115 cm^{-1}; lack of UV absorption). Secondly, the compound clearly differed from **1** and from the isomeric 9-isocyanopupukeanane (**2**).[4] In contrast to **1** and **2**, in which the isocyano function is linked to a methine, the corresponding carbon (C-9, δ 64.5) in the new compound is quaternary and is only coupled to ^{14}N (t, J ~ 4 Hz). That the new compound furthermore must possess a new skeleton was seen by its five methylenes [δ 41.7 (2×), 39.9, 38.8, 32.1] in contrast to **1** and **2** with three methylenes each. Extensive NMR spectral measurements established the structure of 9-isocyano*neo*pupukeanane (3,6-dimethyl-9-isopropyltricyclo[4.3.$10^{3,7}$]decane) (**3**).



1



2



3

The ^1H decoupled ^{13}C NMR spectrum (Table I) revealed 15 resonances with the signal at 41.7 ppm corresponding to two carbons. Changing solvents from benzene to chloroform, acetone, or cyclohexane did not separate these signals. The fully coupled DEPT spectrum[5] provided the multiplicity and $^1J_{CH}$ coupling constants for all carbons. The signals at 158.3 and 64.5 ppm are 1:1:1 triplets (J ~ 4 Hz), typical of carbons α to an isocyano nitrogen, which show ^{14}N quadrupole couplings due to the low electric field gradient of isocyanides. The presence of 15 skeletal carbons including four methyl groups suggested a sesquiterpene isonitrile, which is typical of this genus of sponge.

The ^1H NMR spectrum revealed four methyls as two singlets and two doublets, plus 13 other protons (from integration), which confirmed the presence of 25 protons. C–H correlated 2D NMR spectroscopy[6] established all C–H connectivities and confirmed that two methylene

(1) A preliminary account was presented at the Gordon Research Conference on Marine Natural Products in Oxnard, CA, 29 February–4 March, 1988.

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(6) Bax, A.; Morris, G. A. *J. Magn. Reson.* **1981**, *42*, 501–505.

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