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Two Sesquiterpene Isocyanides and a Sesquiterpene Thiocyanate from the Marine Sponge *Acanthella* cf. *cavernosa* and the Nudibranch *Phyllidia ocellata*[1]

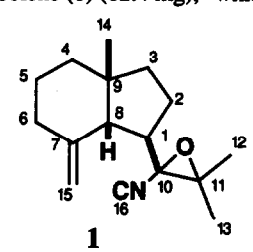
Nobuhiro Fusetani,* Heather J. Wolstenholme, Katsumi Shinoda, Naoki Asai, and Shigeki Matsunaga
Laboratory of Marine Biochemistry, Faculty of Agriculture

Hiroyuki Onuki and Hiroshi Hirota
Department of Chemistry, Faculty of Science
The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Abstract: Three new antifungal sesquiterpenoids, cavernoisonitrile (**1**), cavernothiocyanate (**2**), and 10α-isocyano-4-amorphene (**3**), have been isolated from the marine sponge *Acanthella* cf. *cavernosa*, and their structures were elucidated on the basis of spectroscopic data. The nudibranch *Phyllidia ocellata* also contained **2** and **3**.

Marine sponges of the genera *Acanthella* and *Axinella* and nudibranch molluscs of the genus *Phyllidia* which prey on these sponges often contain sesquiterpene isocyanides which possess antimicrobial, cytotoxic, and ichthyotoxic activities.[2,3] In the course of our study on bioactive substances from Japanese marine invertebrates, the marine sponge *Acanthella* cf. *cavernosa* collected off Hachijo-jima Island showed strong antifungal activity. Bioassay-guided isolation afforded several sesquitepene isocyanides including two new compounds and a new sesquiterpene thiocyanate. The nudibranch mollusc *Phyllidia ocellata* which likely preyed on this sponge also gave rise to sesquiterpenes, which are identical with the sponge metabolites. This paper deals with isolation and structure elucidation of three new metabolites.

The Et_2O soluble portion of the EtOH extract of the frozen sponge (1.0 kg wet weight) was fractionated by the Kupchan procedure. The hexane fraction, which exhibited strong antifungal activity against *Mortierella ramannianus*, was subjected to silica gel flash chromatography (hexane/Et_2O), followed by gel filtration on Sephadex LH-20 (hexane/CH_2Cl_2/MeOH, 2:1:1). Combined active fractions were purified by repeated HPLC on silica gel to yield three new compounds **1** (3.8 mg), **2** (4.0 mg), and **3** (75.6 mg), together with three known isocyano sesquiterpenes, viz, axisonitrile-3 (**4**) (93.1 mg),[4] axisonitrile-2 (**5**) (7.7 mg),[5] and 7-isocyano-7,8-dihydro-α-bisabolene (**6**) (62.4 mg),[6] which were identified by comparison of spectral data with those reported.



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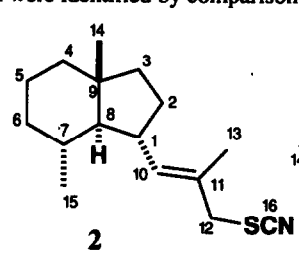

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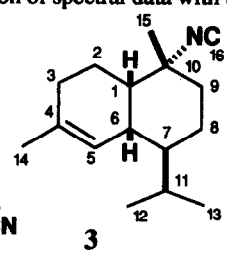

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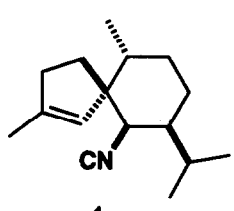

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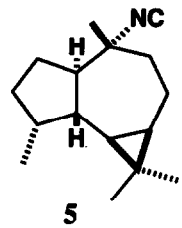

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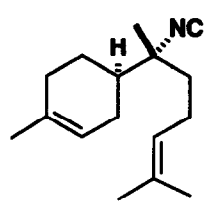

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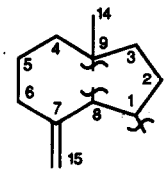


Partial Structure A

Two individuals of the nudibranch *P. ocellata* were collected at the same place, where the sponge was procured (-15m), and immediately steeped in EtOH. The EtOH solution was decanted, concentrated, and extracted with CH_2Cl_2; the extract was fractionated by flash chromatography on silica gel followed by normal phase HPLC to yield **2** (0.5mg), **3** (2.0mg), **4** (3.7mg), and **6** (1.7mg).

Cavernoisonitrile (**1**)[7] had a molecular formula of $C_{16}H_{23}NO$ (*m/z* 245.1756, Δ 2.2 mmu) as determined by high-resolution EI mass spectroscopy. The ^1H and ^{13}C NMR spectra revealed the presence of three methyls, five methylenes, two methines, and three quaternary sp^3 carbons, in addition to an exocyclic methylene (δ 111.3 t and 146.3 s) and an isocyanide (δ 160.2 br s). Therefore, cavernoisonitrile is a rare oxygenated sesquiterpene isocyanide with three rings. Careful interpretation of the COSY spectrum with concomitant analysis of the CH-COSY spectrum gave rise to partial structure A. Connectivities of C4/C5/C6 and C8/C1/C2/C3 were easily deduced by tracing COSY cross-peaks. Long-range couplings between H_215 and H6α, between H15a and H6β, and between H15b and H8 placed the exomethylene group between C6 and C8. 14-CH_3 was correlated with H3β and H4α through long-range couplings, thereby accommodating this methyl group between C3 and C4. Coupling constants between H4α and H5β (13.4 Hz) and between H5β and H6α (13.6 Hz) implied that these protons were axial protons in a six-membered ring, thereby connecting C8 and C9.

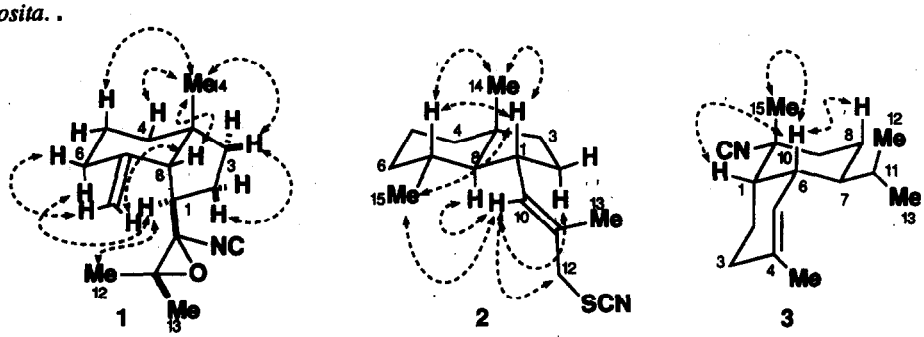
Table.1 ^1H NMR Data of Compounds **1** and **2**

	1			**2**	
	^1H ($CDCl_3$)	^{13}C ($CDCl_3$)		^1H (C_6D_6)	^{13}C ($CDCl_3$)
1	2.27(m)	43.6 d	1	2.42(dddd, 10.9, 10.4, 9.5, 5.9)	39.7 d
2 α	1.99(m)	24.2 t	2 α	1.33(dddd, 13.5, 10.5, 5.9, 1.6)	29.7 t
β	2.05(m)		β	1.99(dddd, 13.5, 10.4, 9.5, 8.5)	
3 α	1.66(m)	40.1 t	3 α	1.24(ddd,10.5,10.5,9.5)	39.8 t
β	1.59(m)		β	1.41(m)	
4 α	1.37(ddd, 13.4, 13.4, 3.8)	33.2 t	4 α	1.10(td, 12.6, 6.5)	39.4 t
β	1.27(m)		β	1.64(m)	
5 α	1.60(m)	23.4 t	5 α	1.52(m)	22.2 t
β	1.47(m)		β	1.50(m)	
6 α	1.92(m)	30.7 t	6 α	0.85(m)	36.8 t
β	2.17(m)		β	1.60(m)	
7		146.3 s	7	1.37(m)	32.3 d
8	2.29(d, 10.2)	56.9 d	8	0.64(t,10.9)	60.7 d
9		43.8 s	9		43.0 s
10		76.4 s	10	5.25(dd,9.5,1.4)	141.8 d
11		65.1 s	11		124.5 s
12	1.21(s)	20.1 q	12	2.67(d,12.4)	44.5 t
				2.80(d,12.4)	
13	1.48(s)	21.3 q	13	1.43(d,1.4)	14.9 q
14	1.02(s)	24.5 q	14	0.75(s)	18.5 q
15 a	4.79(dd, 2.1, 2.1)	111.3 t	15	0.89(d,6.0)	21.2 q
b	4.75(dd, 2.1, 2.1)				
16	-NC	160.2 br s	16	-SCN	112.5 s

The remaining portion including a third ring had a composition of C_5H_6NO, comprising two methyls (δ_H 1.21 s and 1.48 s; δ_C 20.1 and 21.3), an isocyanide, and two non-protonated oxygenated carbons (δ 65.1 and 76.4). The signal at 76.4 ppm appeared as an 1:1:1 triplet when broad-band decoupled, indicating that the isocyano group was on this carbon. The chemical shift values of the two non-protonated carbons and the presence of one ring demanded an epoxide. Because the two methyl proton signals were not broadened by coupling with the isocyano nitrogen, they had to be on the carbon resonating at 65.1 ppm. In the HMBC spectrum, both methyl proton signals gave cross peaks with each other and with the two oxygenated carbons. A cross peak between H1 and the carbon at 76.4 ppm (C10) revealed connectivity between C1 and C10, thus constructing an axane carbon skeleton.[8,9]

Stereochemistry of **1** was inferred from the NOESY data in $CDCl_3/C_6D_6$ (5:1), which gave a better separation of the H1 and H8 signals. The NOESY cross-peaks observed between 14-CH_3 and H8 indicated a *cis* -ring fusion, while a cross-peak between H1 and the axial proton on C6 revealed that H1 was *trans* to H8 (Scheme 1). A coupling constant of 10.3 Hz between H8 and H1 and the absence of a NOESY cross peak between them were consistent with their *trans* relationship. Thus, cavernoisonitrile (**1**) had the same relative stereochemistry as axisonitriles 1 and 4.[8,9]

An HREI mass spectrum showed the molecular formula of cavernothiocyanate (**2**)[10] as $C_{16}H_{25}NS$ (*m/z* 263.1661, Δ 4.7 mmu). In addition to a trisubstituted double bond (δ_H 5.25 dd; δ_C 141.8 d, 124.5 s), the presence of a thiocyanate group was readily inferred from the ^{13}C NMR (112.5ppm) and IR data (2150 cm^{-1});[11] thus **2** must be bicyclic. With the HMQC data in hand, interpretation of the COSY spectrum was straightforward, allowing us to establish an axane-type structure. Several long-range couplings observed in the COSY spectrum helped the assignment, viz. 14-CH_3/H4α, 14-CH_3/H3α, 13-CH_3/H12ab and H4α/H6α. Vicinal coupling constants of 10-12Hz observed for axial protons in the C4-C8 portion indicated that these carbons were in a six-membered ring. The thiocyanate group was placed on C12 by means of HMBC cross-peaks between $H_2$12 and C16. Relative stereochemistry was determined by the NOESY data (Scheme 1) and vicinal coupling constants. The two rings were *trans*-fused and both C1 and C7 substituents were *anti* to the C9 methyl. Therefore **2** had the same relative stereochemisty as oppositol,[15] isolated from the red alga, *Laurencia subopposita*. .



Scheme 1: NOESY correlation of **1**,**2** and **3**

Compound **3**[16] had a molecular formula of $C_{16}H_{25}N$ (*m/z* 231.1963, Δ 2.2 mmu), which was determined by HREIMS. The presense of a trisubstituted double bond (δ 123.8 d and 134.6 s) and an isonitrile (δ 157.1, 1:1:1 triplet, J = 4.6 Hz) indicated **3** to be bicyclic. Despite severely overlapping signals between 1.92 and 2.05 ppm in $CDCl_3$, a gross structure could be deduced by interpretation of the COSY and HMBC spectra. The assignment

of the C6-C10 portion was straightforward because the ^1H signals were well separated. The shape of the C10 carbon signal at δ 61.1 (1:1:1, triplet, J=5.0 Hz after ^1H decoupling) and the 15-methyl proton signal (broad in $CDCl_3$; 1:1:1 triplet in C_6D_6), indicated that the isonitrile and a methyl group were on C10. The 15-methyl proton signal was correlated with C9, C10, and C1 in the HMBC spectrum, thereby allowing us to place C10 between C9 and C1. The H5 olefinic proton, which was coupled to H6 by 4.9 Hz, gave HMBC cross-peaks with C1, C7, C6, C14, and C3, leaving only the C2 signal unassigned. The placement of C2 between C1 and C3 was deduced by the COSY spectrum in C_6D_6, where only H1 and one of the C2 methylene protons overlapped.

The relative stereochemistry was determined by the NOESY spectrum in C_6D_6 as shown in Scheme 1. H6 and H7 were both axial, since they are coupled by 11.9 Hz as determined by ^1H-^1H decoupling experiment. NOESY cross-peaks, 15-CH_3/H6, 15-CH_3/H8ax, indicated that 15-CH_3 was also axial. Moreover, H6 signal (br m) became a broad doublet (J=11.9 Hz) upon irradiation of H5 at δ 5.36, therefore H1 was *syn* to H6.

Interestingly, only *Phyllidia ocellata* contained cavernothiocyanate among *Phyllidia* nudibranchs including *P. bourguini* [17] and *P. pustlosa* [18] which were collected at the same place, although several sesquiterpene isocyanides were common metabolites.

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7. **1**: $[\alpha]_D$ +27° (*c* 0.17, MeOH); IR (KBr) 2937, 2119, 1645, 1458, 1385, 901 cm^{-1}; HREIMS *m/z* 245.1756 (calcd for $C_{16}H_{23}NO$, 245.1778).
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10. **2**: $[\alpha]_D$ -37.8° (*c* 0.037, $CHCl_3$); IR (film) 2940, 2150, 1453, 1375, 1225 cm^{-1}; HREIMS *m/z* 263.1661 (calcd for $C_{16}H_{25}NS$, 263.1708).
11. The differentiation between thiocyanate and isothiocyanate groups could be accomplished by the ^{13}C chemical shift values; thiocyanates[12-14], 111.8-114.2 ppm; isothiocyanates[13,19], 126.0-131.4 ppm.
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16. **3**: $[\alpha]_D$ +101° (*c* 0.52, MeOH); IR (KBr) 2951, 2127, 1466, 1385, 1132, 893 cm^{-1}; HREIMS *m/z* 231.1963 (calcd for $C_{16}H_{25}N$, 231.1985); ^1H NMR (C_6D_6) δ 0.62 (3H, d, 6.9, H12), 0.67 (m, H8), 0.72 (3H, d, 6.9, H13), 1.03 (m, H8), 1.11 (m, H7), 1.14 (3H, 1:1:1, t, J_{HN}=2 Hz, H15), 1.47 (m, H9), 1.50 (m, H1), 1.53 (3H, s, H14), 1.54 (m, H2), 1.61 (ddd, 13.7, 13.7, 4.3, H9), 1.72 (m, H6), 1.79 (m, H3), 1.84 (dqq, 3.2, 6.9, 6.9, H11), 2.07 (m, H2), 5.36 (d, 4.9, H5); ^{13}C NMR(C_6D_6) δ 15.1 (q, C12), 19.9 (t, C8), 20.6 (t, C2), 21.4 (q, C13), 23.5 (q, C14), 26.5 (d, C11), 26.8 (q, C15), 31.1 (t, C3), 34.4 (t, C9), 35.0 (d, C6), 43.5 (d, C7), 43.5 (d, C1), 60.7 (br s, C10), 123.8 (d, C5), 134.6 (s, C4), 157.1 (br s, -NC).
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