New Biologically Active Marine Sesquiterpenoid and Steroid from the Okinawan Sponge of the Genus *Axinyssa*

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A new bisabolane-type sesquiterpenoid, (*E*)-3-isocyanobisabolane-7,10-diene (1), and a new epidioxyergostane-type steroid, 9(11)-dehydroaxinysterol (2), were isolated from the Okinawan sponge of the genus *Axinyssa*. Their structures were elucidated based on the results of spectroscopic analysis and chemical conversion. Epidioxysterol 2 was found to show significant growth inhibitory effects against human cancer cell lines.

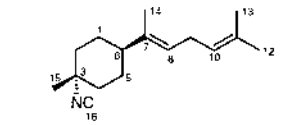
Key words marine sponge; genus *Axinyssa*; bisabolane sesquiterpenoid; 5α,8α-epidioxysterol

During the course of our investigations of biologically active chemical substances from the Okinawan invertebrates,[2—4)] a bisabolane-type sesquiterpenoid (*E*)-3-isocyanobisabolane-7,10-diene (**1**) and an ergostane-type steroid 9(11)-dehydroaxinysterol (**2**) were newly isolated from a sponge of the genus *Axinyssa* along with known compounds such as axinysterol (**3**).[5)] This paper describes the structural elucidation of these compounds, the lethal bioassay for brine shrimp[6)] of **1**, and the growth inhibitory activity against human cancer cell lines[7)] of **2**.

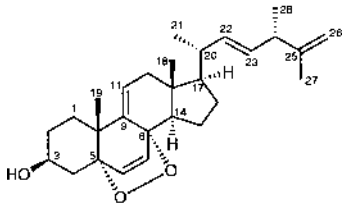
The isolation and purification were carried out as described in the Experimental section.

The molecular formula of **1** was found to be $C_{16}H_{25}N$ by high-resolution electron impact MS (HR-EI-MS). All the carbons in **1** appeared in the ^{13}C-NMR spectrum (Table 1). The distortionless enhancement by polarization transfer (DEPT) spectrum indicated four methyls, five sp^3 methylenes, one sp^3 methine, two sp^2 methines, one sp^3 quaternary carbon, and three sp^2 quaternary carbons. Two of the quaternary carbon signals at δ 56.3 (J_{NC}=5.5 Hz) and 156.1 (J_{NC}=5.5 Hz) ppm in C_6D_6 were both observed as a triplet due to the coupling with ^{14}N.[8)] These findings together with the IR absorption at 2130 cm^{-1} strongly suggest the presence of an isonitrile group (–NC) in **1**. The ^{1}H-NMR analysis showed the presence of three olefinic methyls at δ 1.45 (s), 1.61 (s), and 1.71 (s) ppm, one methyl group connected to quaternary carbon at δ 1.00 (t, J=2.0 Hz) ppm, and two olefinic protons at δ 5.14 (t, J=7.1 Hz) and 5.27 (t, J=7.1 Hz) ppm (Table 1). All the connections for each carbon–proton direct bonding were made from the analysis of ^{13}C–^{1}H correlation spectroscopy (^{13}C–^{1}H COSY). Partial structures of **1**, as shown in Fig. 1, were deduced from ^{1}H–^{1}H correlation spectroscopy (^{1}H–^{1}H COSY).

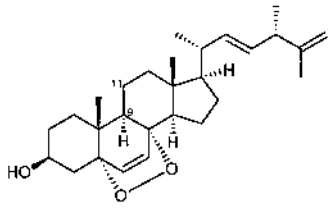
The gross structure of **1** was established by the following heteronuclear multiple bond correlation (HMBC) analysis (Fig. 1). ^{1}H–^{13}C long-range correlations observed from H-2 (CH_2) and H-4 (CH_2) to C-3, and from H-15 (CH_3) to C-2, C-3 and C-4, indicated the carbon–carbon connections around C-3. Correlations from H-8 (CH) to C-6, from H-9



(*E*)-3-isocyanobisabolane-7,10-diene (**1**)



9(11)-dehydroaxinysterol (**2**)



axinysterol (**3**)

Table 1. NMR Data of **1** in C_6D_6 (δ ppm)[a)]

No.	^{13}C	^{1}H	No.	^{13}C	^{1}H
1	26.7	0.98 (dtd, 4.2, 11.4, 13.9), 1.35 (m)	9	27.4	2.78 (2H, t, 7.1)
2	38.4	1.49 (m), 1.54 (m)	10	123.7	5.27 (t, 7.1)
3	56.3 (t, 5.5)[b)]		11	131.3	
4	38.4	1.49 (m), 1.54 (m)	12	25.8	1.71 (3H, s)
5	26.7	0.98 (dtd, 4.2, 11.4, 13.9), 1.35 (m)	13	17.7	1.61 (3H, s)
6	44.7	1.57 (m)	14	14.6	1.45 (3H, s)
7	137.8		15	24.8	1.00 (3H, t, 2.0)
8	122.6	5.14 (t, 7.1)	16	156.1 (t, 5.5)[b)]	

a) ^{13}C; 125 MHz. ^{1}H; 500 MHz, J in Hz. Assignments of the ^{13}C and ^{1}H signals were based on HMQC. *b*) J_{NC} in Hz. Coupled to ^{14}N observed as 1 : 1 : 1.[6)]

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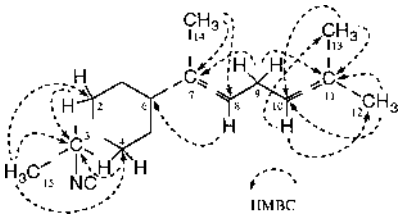

Fig. 1. Partial Structures and Important HMBC for **1**

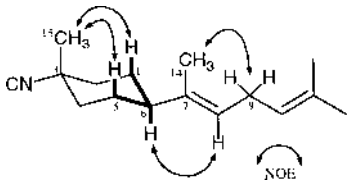

Fig. 2. Important NOE Correlations and *trans*-Diaxial Relationship between H-1, H-5, and H-6

(CH_2) to C-7, and from H-14 (CH_3) to C-7 and C-8 demonstrated the connections around C-7. Finally, correlations from H-9 (CH_2) to C-11, from H-10 (CH) to C-12 and C-13, and from H-12 (CH_3) and H-13 (CH_3) to C-10 and C-11, indicated the terminal structure of the side chain in **1**. These spectroscopic findings together with the unsaturation number (U=5) disclosed that compound **1** had the structure of a bisabolane-type sesquiterpenoid with an isonitrile moiety at C-3.[8—10] This gross structure of **1** indicated that the molecule of **1** has a plane of symmetry across the C-3 and C-6 positions on the cyclohexane ring, and thus **1** is achiral and optically inactive; $[\alpha]_D^{25}$ 0.0° ($CHCl_3$). However, the stereochemistry of the olefin moiety and the relative configuration between C-3 and C-6 must be determined.

Stereochemistry of the trisubstituted olefin between C-7 and C-8 was confirmed to be an *E* configuration by nuclear Overhauser effect (NOE) analysis, as shown in Fig. 2. The relative configuration between C-3 and C-6 was revealed by the combined analysis of NOESY and *J* values observed in ^{1}H-NMR spectrum. As shown in Fig. 2, NOE correlations between the methyl protons (H-15) at C-3 and one of the methylene protons at both C-1 (H-1a) and C-5 (H-5a) [δ 0.98 (dtd, J=4.2, 11.4, 13.9 Hz)] indicated that H-15, H-1a, and H-5a were directed to the same side of the cyclohexane ring. The ^{1}H–^{1}H large coupling constant (J=11.4 Hz) of the triplet signal of H-1a and H-5a demonstrated these protons to be axial and to couple with the adjacent axial protons (H-2, H-6 for H-1 and H-4, H-6 for H-5). These findings indicated H-6 to be axially directed to the opposite side of H-15, H-1a, and H-5a, and thus the side chain group at C-6 to be equatorially directed, as shown in Fig. 2. The structure of **1** was thus concluded to be (*E*)-3-isocyanobisabolane-7,10-diene.

The biological activity of **1** was examined to estimate the lethal concentration (LC_{50}) for brine shrimp,[6] and compound **1** showed strong activity at LC_{50} 0.1 μg/ml. The other bioactivities for **1** are currently under investigation.

The molecular formula of compound **2**, $C_{28}H_{40}O_3$, was determined by HR-EI-MS analysis. IR, ^{1}H-, and ^{13}C-NMR spectra of **2** were quite similar to those of axinysterol (**3**),[5] which had the molecular formula $C_{28}H_{42}O_3$, suggesting **2** to

Table 2. NMR Data of **2** and **3** in $CDCl_3$ (δ ppm)

No.	**2**[a]		**3**
	^{13}C (125 MHz)	^{1}H (500 MHz, J in Hz)	^{13}C (100 MHz)
1	32.6	1.68 (m), 2.06 (m)	34.8
2	30.6	1.57 (m), 1.92 (m)	30.2
3	66.3	4.00 (tt, 5.1, 11.4)	66.6
4	36.1	1.92 (dd, 11.4, 13.7), 2.10 (m)	37.1
5	82.7		82.3
6	135.5	6.28 (d, 8.5)	135.4[b]
7	130.7	6.59 (d, 8.5)	130.8
8	78.3		79.5
9	142.6		51.2 (CH)
10	38.0		37.1
11	119.7	5.42 (dd, 1.9, 6.0)	20.7 (CH_2)[c]
12	41.2	2.08 (m), 2.26 (dd, 6.0, 17.0)	39.5
13	43.6		44.7
14	48.2	1.83 (dd, 8.2, 12.3)	51.8
15	20.9	1.57 (m), 1.68 (m)	23.5[c]
16	28.6	1.38 (m), 1.78 (m)	28.7[c]
17	55.8	1.37 (m)	56.3
18	12.9	0.73 (3H, s)	13.0
19	25.5	1.09 (3H, s)	18.3
20	39.7	2.05 (m)	39.6
21	20.5	1.00 (3H, d, 6.6)	20.69[d]
22	135.2	5.23 (dd, 7.8, 15.3)	135.5[b]
23	132.0	5.29 (dd, 6.7, 15.3)	132.0
24	43.6	2.71 (quint, 6.7)	43.7
25	149.7		149.8
26	108.9	4.69 (m), 4.70 (m)	109.0
27	20.6	1.67 (3H, s)	20.74[d]
28	18.8	1.08 (3H, d, 6.7)	18.9

a) Assignments of the ^{13}C and ^{1}H signals were based on ^{13}C–^{1}H COSY. *b*, *c*, *d*) Values with the same subscript may be interchanged.

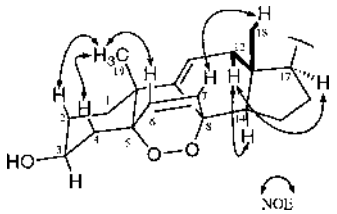

Fig. 3. Important NOE Correlations and Long-Range Coupling (Bold)

be a dehydro-congener of **3**. Differences between **2** and **3** were found in the NMR spectra, due to the presence of a new trisubstituted olefin at δ 5.42 (dd, J=1.9, 6.0 Hz) ppm in the ^{1}H-NMR, and at δ 119.7 (CH) and 142.6 (C) ppm in the ^{13}C-NMR spectra of **2** (Table 2).

Two-dimensional NMR spectra [^{1}H–^{1}H COSY, ^{1}H-detected heteronuclear multiple quantum coherence (HMQC), and HMBC] indicated that this new double bond is located between the C-9 and C-11 positions. The relative configuration of **2** was determined based on the following evidence. NOESY analysis focused on the ring junctures of the steroidal skeleton confirmed that **2** has the same relative configuration as that of **3** (Fig. 3). The NOE correlation between H-14 and H-12α, which showed long-range coupling (W-shape) with the angular methyl group (H-18) in the COSY spectrum, as shown in the bold lines in Fig. 3, exhibited the *trans* configuration between H-14 and H-18. The relative configuration of H-17 was assigned as an α orientation due to the NOE correlation between H-12α and H-17. The-α ori-

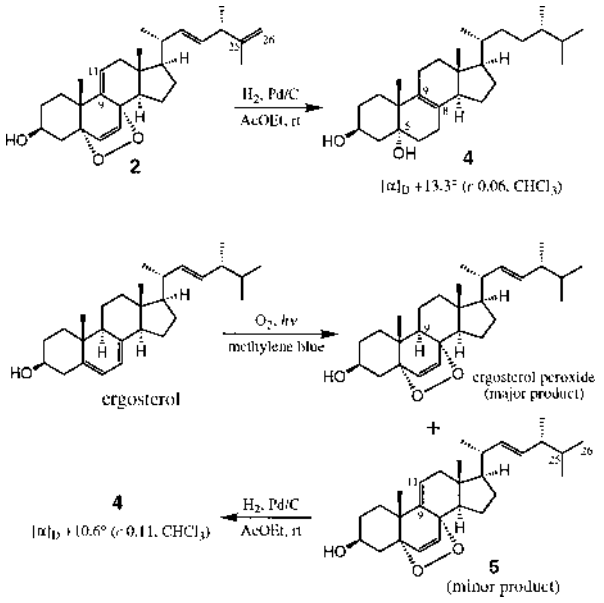

Chart 1

Table 3. Growth Inhibitory Activity (IC_{50}) of **2** against Human Cancer Cell Lines[a)]

Origin of cancer	Cell line	IC_{50} (μg/ml)	Origin of cancer	Cell line	IC_{50} (μg/ml)
Breast	HBC-4	0.85	Colon	HCC2998	0.57
	BSY-1	0.60		KM-12	0.60
	HBC-5	0.96		HT-29	0.57
	MCF-7	0.36		HCT-15	0.75
	MDA-MB-231	1.26		HCT-116	0.48
Lung	NCI-H23	0.54	Ovary	OVCAR-3	0.19
	NCI-H226	0.63		OVCAR-4	0.60
	NCI-H522	0.57		OVCAR-5	0.54
	NCI-H460	0.81		OVCAR-8	0.22
	A549	0.96		SK-OV-3	0.81
	DMS273	0.54	CNS[b)]	U251	0.63
	DMS114	0.48		SF-268	1.02
Stomach	St-4	0.69		SF-295	0.75
	MKN1	0.42		SF-539	0.84
	MKN7	0.48		SNB-75	2.16
	MKN28	0.84		SNB-78	1.17
	MKN45	0.54	Prostate	DU-145	0.54
	MKN74	0.54		PC-3	0.57
Kidney	RXF-631L	0.72	Melanoma	LOX-IMVI	0.60
	ACHN	0.51			

a) Each IC_{50} value was estimated from its GI_{50} value. *b*) CNS, central nervous system.

entation of the epidioxy group was determined by the NOE correlations between H-18 and H-7 (olefinic proton). The relative configuration of the angular methyl group at C-10 was indicated by the NOE correlation between H-6 (olefinic proton) and H-19, and the stereochemistry at C-3 was deduced from the *J* values of H-3 (J=5.1, 11.4 Hz). The stereochemistry of two chiral centers in the side chain was assigned by comparison of the ^{13}C-NMR data from C-20 to C-28 with those in **3** (Table 2). The chemical shift values of nine carbons in **2** were essentially identical to those of **3**.

The following chemical conversions[5)] from **2** to the known diol **4**[11)] were carried out to establish the absolute configuration of **2**, as summarized in Chart 1. Catalytic hydrogenation of **2** in the presence of 10% palladium on carbon in ethyl acetate gave **4** {$[\alpha]_D^{25}$ +13.3°}. The authentic diol **4** {$[\alpha]_D^{25}$ +10.6°} was independently prepared from ergosterol in two steps with photosensitized oxidation,[5,12)] in which the desired epidioxysterol **5** was obtained as a minor product, followed by catalytic hydrogenation. The spectral data of **4** derived from **2** were identical to those of authentic **4**, including the sign of its optical rotation value. These findings together with the spectral analysis confirmed the structure of this new sterol **2** to be 9(11)-dehydroaxinysterol.

The growth inhibitory properties of **2** against cancer cells were examined with a disease-oriented panel of 39 human cancer cell lines (HCC panel) in the Japanese Foundation for Cancer Research.[7)] Compound **2** exhibited a strong growth inhibitory effect against some ovarian cancer cells such as OVCAR-3 at IC_{50} 0.19 μg/ml ($\log GI_{50}$ −6.20) and OVCAR-8 at IC_{50} 0.22 μg/ml ($\log GI_{50}$ −6.14), as shown in Table 3, and also indicated significant growth inhibition in 21 human cancer cell lines at less than 0.60 μg/ml. Estimation of its bioactivities is now underway, including *in vivo* bioassay.

Experimental

Optical rotations were measured with a JASCO DIP-370 automatic polarimeter. IR spectra were recorded with a Perkin-Elmer FT-IR 1600 spectrophotometer. NMR spectra were recorded with a Bruker DRX-500 and DPX-400 spectrometer (^{1}H, 500 and 400 MHz; ^{13}C, 125 and 100 MHz) in C_6D_6 for **1** and in $CDCl_3$ for **2**, respectively. Two-dimensional NMR spectra (^{1}H–^{1}H COSY, ^{13}C–^{1}H COSY, HMQC, HMBC, and NOESY) were measured using standard Bruker pulse sequences. Chemical shifts are given on a δ (ppm) scale with either $CHCl_3$ (^{1}H, 7.26 ppm; ^{13}C, 77.0 ppm) for $CDCl_3$ or C_6H_6 (^{1}H, 7.20 ppm; ^{13}C, 128.0 ppm) for C_6D_6 as the internal standard (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad). Mass spectra were recorded with a Micromass Auto Spec spectrometer. Reverse-phase ODS column chromatography was carried out on YMC S-50 gel (ODS-AM120-S50). Normal-phase flash column chromatography was performed on Merck silica gel 60 (230—400 mesh). Medium-pressure liquid chromatography (MPLC) was carried out with a Kusano KHLC-201-43 apparatus using a CIG prepack column (silica gel, CPS-HS-221-05, for normal phase and ODS silica gel, CPO-HS-221-20, for reverse phase). HPLC with a recycling loop was performed on a YMC-Pack SIL-06 column (silica gel, SH-043-5-06, normal phase) and a YMC-Pack ODS-AM column (ODS, SH-343-5AM, reverse phase).

Extraction and Isolation The sponge of the genus *Axinyssa* (order Halichondria, family Desmospongiae) was collected from a coral reef near Ishigaki Island, Okinawa Prefecture, Japan in June 1999, at a depth of 5—10 m. A voucher specimen (No. S-99-3) was deposited at the Tokyo University of Pharmacy and Life Science, Tokyo, Japan. Wet specimens (706 g) were immersed in MeOH (3×2 l). After filtration, MeOH was removed under reduced pressure. The MeOH extract (47.5 g) was partitioned between hexane and H_2O, and the hexane layer was concentrated under reduced pressure to give a hexane-soluble portion (11.4 g). Most of the hexane-soluble portion (11.3 g) was chromatographed on an ODS column (200 g). Stepwise elution with MeOH–H_2O (9 : 1 for five fractions, 19 : 1 for two fractions, 200 ml of each) and MeOH (400 ml) gave eight fractions. The fifth fraction (1.23 g) [eluted with MeOH–H_2O (9 : 1)] containing sesquiterpenoids and the final fraction (1.20 g) containing sterols were obtained. Each fraction was subjected to normal-phase silica gel flash column chromatography, followed by MPLC separation to obtain almost pure **1** and **2**, respectively. Further purification was carried out with recycled HPLC to afford **1** [11.8 mg, using a normal-phase column, hexane–2-propanol (20 : 1)] and **2** [13.4 mg, using a reverse-phase column, MeOH–H_2O (19 : 1)]. Axinysterol (**3**, 339 mg) and some sesquiterpenoids possessing a bisabolane skeleton were also obtained in this separation.

(*E*)-3-Isocyanobisabolane-7,10-diene (**1**): Colorless oil. $[\alpha]_D^{25}$ 0.0° (c=0.75, $CHCl_3$). IR (dry film) ν_{max} cm^{-1}: 2130. ^{1}H- and ^{13}C-NMR, see Table 1. HR-EI-MS *m/z*: 230.1912 (Calcd for $C_{16}H_{25}N$: 230.1909 $[M-H]^+$).

9(11)-Dehydroaxinysterol (**2**): White amorphous solid. $[\alpha]_D^{25}$ +78.9° (c=0.89, $CHCl_3$). IR (dry film) ν_{max} cm^{-1}: 3389, 2962, 1644. ^{1}H- and ^{13}C-NMR, see Table 2. HR-EI-MS *m/z*: 424.2971 (Calcd for $C_{28}H_{40}O_3$: 424.2977 $[M]^+$).

Catalytic Hydrogenation of 2 to 4 A mixture of **2** (0.6 mg) and 10% palladium on charcoal (2 mg) in ethyl acetate (0.5 ml) was vigorously stirred under a hydrogen atmosphere at room temperature for 3.5 h. The mixture was filtered through a short Celite column, rinsed with ethyl acetate, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel flash column chromatography [hexane–ethyl acetate (3 : 1)] and HPLC [hexane–2-propanol (9 : 1)] to obtain diol **4** (0.6 mg, 100% yield) as a white solid. $[\alpha]_D^{25}$ +13.3° (c=0.06, $CHCl_3$).[11] IR (dry film) ν_{max} cm^{-1}: 3406, 3311, 2926. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 0.62 (3H, s, H-18), 0.78 (3H, d, J=6.8 Hz, H-27), 0.79 (3H, d, J=6.8 Hz, H-26), 0.83 (3H, d, J=6.9 Hz, H-28), 0.94 (3H, d, J=6.5 Hz, H-21), 1.13 (3H, s, H-19), 4.12 (1H, tt, J=5.2, 10.6 Hz, H-3). ^{13}C-NMR (100 MHz, $CDCl_3$) δ ppm: 11.0, 15.5, 17.6, 18.9, 20.5, 23.1, 23.3, 23.4, 23.5, 28.7, 29.5, 29.7, 30.7, 31.0, 31.5, 33.7, 36.7, 36.7, 39.1, 40.8, 41.9, 42.1, 51.6, 54.6, 67.2, 74.3, 129.5, 132.8. HR-EI-MS m/z: 398.3534 (Calcd for $C_{28}H_{46}O$: 398.3549 [M−H_2O]$^+$).

Preparation of 4 from Ergosterol The oxidation reaction was carried out according to the procedure described in the literature.[5,12] The mixture of products was separated by silica gel flash column chromatography [hexane–ethyl acetate (4 : 1)], followed by MPLC separation [reverse phase, MeOH–H_2O (9 : 1)] to afford 9(11)-dehydro-5α,8α-epidioxyergosterol (**5**, 25 mg, 12% yield) as a faint yellow solid. A mixture of **5** (4.9 mg) and 10% palladium on charcoal (5 mg) in ethyl acetate (3 ml) was vigorously stirred under a hydrogen atmosphere at room temperature for 3 h. The mixture was filtered through a short Celite column, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel flash column chromatography [hexane–ethyl acetate (1 : 1)], followed by HPLC separation [normal phase, hexane–2-propanol (9 : 1)] to obtain diol **4** (2.8 mg, 59% yield) as a white solid. $[\alpha]_D^{25}$ +10.6° (c=0.11, $CHCl_3$). The spectral data of **4** prepared by this method were identical to those of **4** from natural **2**.

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