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Five New Antifouling Sesquiterpenes from Two Marine Sponges of the Genus *Axinyssa* and the Nudibranch ***Phyllidia pustulosa***[#]

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Abstract: Three new sesquiterpene carbonimide dichlorides have been isolated from a marine sponge of the genus *Axinyssa*, and a guaiane-type sesquiterpene peroxide from another *Axinyssa* sponge. A new isocyanosesquiterpene alcohol was obtained from the nudibranch *Phyllidia pustulosa*. Their structures were determined on the basis of 2D NMR data. These compounds showed potent antifouling activities against larvae of the barnacle *Balanus amphitrite*.

Keywords: Biologically active compounds; Marine metabolites; Sponges; Terpenes and terpenoids

Introduction

In general, marine sessile invertebrates possess chemical defense systems against predators, larval settlement by other fouling organisms, and pathogens. Their secondary metabolites might, therefore, be potential nontoxic antifouling agents.[1] In fact, a variety of compounds that inhibit larval settlement of barnacles were isolated from marine sponges.[2,3] Furthermore, nudibranchs are well known to sequester secondary metabolites from their sponge diets to protect their soft bodies from predators, and these chemical defenses could be developing into an antifouling strategy.[4,5]

[#] This work was performed at the Fusetani Biofouling Project, Exploratory Research for Advanced Technology (ERATO), Research Development Corporation of Japan (JRDC; present name: Japan Science and Technology Corporation), c/o Niigata Engineering Co., Ltd., Isogo-ku, Yokohama, Japan. But the Project has already been terminated. The correspondings should be sent to H. H. (Fax: +81-48-468-4779; E-mail: hirota@cytologic.riken.go.jp) or N. F. (Fax: +81-3-5684-0622; E-mail: anobu@hongo.ecc.u-tokyo.ac.jp)

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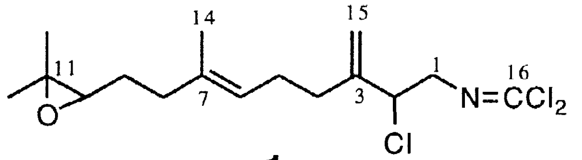
During our search for antifouling substances from Japanese marine invertebrates,[3,5,6] we found that the extracts of two marine sponges of the genus *Axinyssa* and the nudibranch ***Phyllidia pustulosa*** strongly inhibited larval settlement of the barnacle ***Balanus amphitrite.*** Bioassay-guided isolation afforded three new sesquiterpene carbonimide dichlorides named axinyssimides A-C (**1**-**3**) and a guaiane-type sesquiterpene peroxide (**4**) from *Axinyssa* spp., and a new isocyanosesquiterpene alcohol (**5**) from *P. pustulosa*. In this paper we describe isolation and structure elucidation of these antifouling substances.



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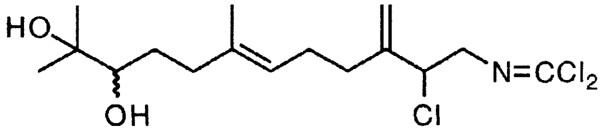

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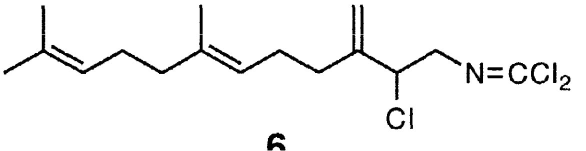

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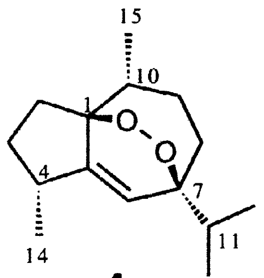

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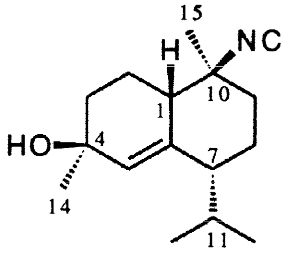

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Results and Discussion

Two marine sponges of the genus *Axinyssa* were collected off Hachijo-jima Island, and their lipophilic parts showed the antifouling activity against larvae of the barnacle *Balanus amphitrite* in our screening system.

The ethanol extract of the darker and smaller specimen (53 g, wet weight) was partitioned between $CHCl_3$ and water. The $CHCl_3$ layer was first partitioned between hexane and 90 % MeOH, and then the 90 % MeOH layer was partitioned between CH_2Cl_2 and 60 % MeOH. The hexane and CH_2Cl_2 layers showed potent antifouling activity. The hexane layer was fractionated on silica gel with a hexane/EtOAc system. The active fractions eluted with hexane/EtOAc (97:3) and hexane/EtOAc (95:5) were separately purified by normal-phase HPLC with hexane/EtOAc systems to afford axinyssimide A (**1**; yield: 1.2 mg, 2.3×10^{-3} %)

and axinyssimide B (**2**; 0.6 mg, $1.1\text{x}10^{-3}$ %). The CH_2Cl_2 layer was also separated on silica gel with a $CHCl_3$-MeOH system. The active fraction eluted with $CHCl_3$ was purified by normal-phase HPLC with hexane/EtOAc (8:2) to afford axinyssimide C (**3**; 0.3 mg, $5.7\text{x}10^{-4}$ %).

Axinyssimide A (**1**) exhibited a strong IR absorption at 1655 cm^{-1} and a low intensity ^{13}C signal at δ 127.0, and its EI mass spectrum revealed a $[C_{16}H_{24}Cl_2NO]^+$ cluster as the highest *m/z* peaks. These signals were reminiscent of carbonimide dichloride derivatives.[7,8] Comparison of our data with those in the literature,[7a] *e.g.* compound **6** obtained from the marine sponge *Pseudaxinyssa pitys*, suggested that the $[C_{16}H_{24}Cl_2NO]^+$ cluster ion was an $[M\text{-}Cl]^+$ ion. Thus, the molecular formula of **1** was $C_{16}H_{24}Cl_3NO$, which contains one more oxygen atom than **6**. Interpretation of $^1H\text{-}^1H$ COSY and HMBC spectra of **1** (Table 1) together with the comparison with those of **6** demonstrated that the oxygen atom of **1** formed an oxirane ring, thus the structure of axinyssimide A (**1**) was a 10,11-epoxy derivative of **6**, that is, 2-chloro-10,11-epoxy-3-methylene-7,11-dimethyl-6-dodecenyl-dichloromethylenamine. This was supported by $^1J_{CH}$ of C-10 (167 Hz) and C-2 (157 Hz). The stereochemistry of **1** remains to be elucidated.

Axinyssimide B (**2**) also exhibited a strong IR absorption at 1650 cm^{-1}, a low intensity ^{13}C signal at δ 127.0, and the same $[C_{16}H_{24}Cl_2NO]^+$ cluster as the highest ion peaks in EIMS.

Table 1. NMR Data for Axinyssimides A-C (**1**-**3**)

Atom		**1**		**2**		**3**	
No.	H	C	HMBC	H	C	H	C
1	3.82 (2H)	59.5 (t)	2, 3, 16	3.82 (2H)	59.5 (t)	3.82 (2H)	59.5 (t)
2	4.60	62.4 (d)	1, 3, 4, 15	4.60	62.4 (d)	4.59	62.4 (d)
3		145.8 (s)			145.8 (s)		145.7 (s)
4	2.10	31.6 (t)	3, 5, 6, 15	2.07	31.7 (t)	2.09	31.6 (t)
	2.25		3, 5, 15	2.22		2.24	
5	2.22 (2H)	26.2 (t)	4	2.21 (2H)	26.2 (t)	2.21 (2H)	26.2 (t)
6	5.17	123.9 (d)	8, 14	5.18	124.1 (d)	5.20	124.8 (d)
7		135.2 (s)			135.5 (s)		134.4 (s)
8	2.08	36.3 (t)	6, 7, 9, 14	2.05	36.4 (t)	2.09	37.1 (t)
	2.16		6, 7, 9, 14	2.28		2.30	
9	1.62 (2H)	27.4 (t)	10, 11	1.44	29.7 (t)	1.66	31.2 (t)
				1.70		1.95	
10	2.68	64.1 (d)	8, 9, 11, 12	3.47	78.4 (d)	3.78	73.6 (d)
11		58.3 (s)			76.2 (s)		72.8 (s)
12	1.28 (3H)	24.9 (q)	10, 11, 13	1.58 (3H)	29.2 (q)	1.28 (3H)	26.5 (q)
13	1.25 (3H)	18.8 (q)	10, 11, 12	1.53 (3H)	27.2 (q)	1.27 (3H)	25.2 (q)
14	1.63 (3H)	16.1 (q)	6, 7, 8	1.62 (3H)	16.1 (q)	1.61 (3H)	16.0 (q)
15	5.04	114.5 (t)	2, 4	5.05	114.5 (t)	5.03	114.5 (t)
	5.18		2, 3, 4	5.19		5.19	
16		127.0 (s)			127.0 (s)		127.0 (s)

Interpretation of the ^1H-^1H COSY and HMBC spectra of **2** (Table 1) led to the same carbon connectivity as in **1**. But the IR spectrum of **2** showed the presence of OH at 3425 cm^{-1}, and the ^{13}C chemical shifts of C-10 and C-11 were quite different; that is, δ 78.4 and 76.2 for C-10 and C-11, respectively, in **2**, while δ 64.1 and 58.3 for those of **1**. Thus, axinyssimide B (**2**) was deduced to be a 10,11-dihydroxy derivative of **6**.

Axinyssimide C (**3**) had almost the same physico-chemical data as those of axinyssimide B (**2**). In fact, **3** was consistent with a 10,11-dihydroxy derivative of **6**, that is, **2** and **3** were diastereomers of each other.

The stereochemical elucidation of **2** and **3** was hampered not only by limited samples, but also the very poor stereochemical information from their NOESY spectra.

The carbonimide dichloride (= dichloroimine) moiety represents a rare example of a functional group in natural products. The first marine natural products of this class including compound **6** were reported from the marine sponge *Pseudaxinyssa pitys* in 1977.[7] Recently, farnesyl dichloromethylenamine and **6** were isolated for the second example as stylotellanes A and B, respectively, from the marine sponge *Stylotella aurantium*, and biosynthetic experiments were also reported using sodium [^{14}C]cyanide/thiocyanate, suggesting that these compounds were biosynthesized via farnesyl isocyanide or isothiocyanate.[8] Compounds **1**-**3** might be also derived through the same biosynthetic pathway. They showed fairly strong antifouling activity against larvae of the barnacle (*vide infra*). Compound **6** was not found to possess any biological activity,[7a] while stylotellane B was reported to show only weak cytotoxicity against P388, even though preliminary ecological studies indicated that *S. aurantium* might strongly inhibit the settlement of ascidian larvae.[8]

The ethanol extract of the brighter and larger specimen of *Axinyssa* sp. (528 g, wet weight) was partitioned between $CHCl_3$ and water. The $CHCl_3$ layer was partitioned between hexane and 90 % MeOH. The hexane layer was fractionated twice on silica gel by hexane/EtOAc systems. Part of the most active fraction eluted with hexane/EtOAc (97:3) from the second column was repeatedly separated by normal-phase HPLC with hexane/EtOAc systems to afford pure epidioxy compound (**4**; 10.0 mg, 5.3×10^{-3} %). Even though almost the same isolation procedure as for the former sponge was used, no trace of axinyssimides A-C (**1**-**3**) or their derivatives was detected.

The planar structure of **4** was elucidated mainly from the ^1H-^1H COSY and HMBC spectra (see Table 2) to be 1,7-epidioxy-5-guaiene. Faulkner and coworkers[9] reported the synthesis of two 1,7-epidioxy-5-guaienes from natural 1(5),6-guaiadiene without stereochemical determination. Comparison of ^1H NMR chemical shifts of **4** with those in the literature[9] disclosed that **4** was identical with one of them (peroxide **8b** in the literature[9]). In the NOESY spectrum of **4** (see Table 2), both secondary methyls (4-Me and 10-Me) showed correlation peaks with the same methylene proton of C-3. This indicated these two methyl groups had the same orientation. Furthermore, these two methyl groups showed NOE correlation with each other, implying that the 1,7-epidioxy moiety was oriented on the opposite side of these methyl groups. Thus, the compound **4** was deduced to be (1*S**,4*R**,7*S**,10*R**)-1,7-epidioxy-5-guaiene.

Table 2. NMR Data for Compound **4**

Atom No.	H	C	HMBC	NOE
1		91.4 (s)		
2	α 2.09 (ddd)	36.4 (t)	3, 4, 5, 10	2β, 3α, 15
	β 1.80 (m)		1, 3	2α, 3α, 3β
3	α 1.26 (m)	31.7 (t)	4, 14	2α, 2β, 3β, 14, 15
	β 1.92 (m)		1, 4, 5	2β, 3α, 4
4	2.84 (m)	36.8 (d)	3, 5, 6, 14	3β, 14
5		150.3 (s)		
6	5.96 (br s)	122.8 (d)	1, 4, 7, 8, 11	11, 12 and/or 13, 14
7		83.3 (s)		
8	α 1.73 (m)	31.0 (t)	6, 7, 9, 10	8β
	β 1.66 (m)		9, 10, 11	8α, 9α, 9β
9	α 1.00 (m)	30.3 (t)	1, 7, 8, 10	8β, 9β, 15
	β 1.78 (m)		8, 10, 15	8β, 9α, 10
10	2.02 (m)	39.1 (d)	1, 9	9β, 15
11	1.81 (m)	35.4 (d)	12, 13	6, 12, 13
12	0.93 (3H, d)	16.8 (q)	7, 11, 13	(6), 11
13	0.94 (3H, d)	17.0 (q)	7, 11, 12	(6), 11
14	1.11 (3H, d)	19.1 (q)	3, 4, 5	3α, 4, 6, 15
15	0.89 (3H, d)	18.0 (q)	1, 9, 10	2α, 3α, 9α, 10, 14

Interestingly, this was the third discovery of an epidioxy derivative in our search for antifouling substances. 1,7-Epidioxy-5-cadinene had been isolated with very weak activity from the nudibranch *Phyllidia krempfi*,[5] and four steroid peroxides which elongated the swimming period of the larvae of the barnacles were obtained from the marine sponge *Acanthella cavernosa*.[3k]

One specimen of the nudibranch *Phyllidia pustulosa* was obtained from Katsuura on the Kii Peninsula in western Japan. The EtOH extract was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 layer was fractionated twice on silica gel with hexane/ether systems. The antifouling hexane/ether (9:1) fraction of the second column was separated twice by normal-phase HPLC with hexane/EtOAc (9:1) and hexane/ether (3:1), respectively, to afford a pure antifouling compound (**5**; 0.9 mg).

The molecular formula of **5** was established as $C_{16}H_{25}NO$ by HREIMS. The presence of hydroxyl and isocyano groups was implied from prominent IR absorptions at 3320 and 2120 cm^{-1} and a small ^{13}C signal at δ 153.3 together with a triplet signal at δ 61.8. Extensive

NMR experiments including ^{1}H-^{1}H COSY and HMBC (see Table 3) disclosed that **5** had a cadinane-type planar structure with 4-hydroxy and 10-isocyano functionalities. Stereochemistry of **5** was assigned by NOESY spectrum. The 1β-H (δ 2.34) showed NOE correlation peaks with 7β-H (1.79) and 9β-H (1.95); these hydrogen atoms were located at 1,3-diaxial positions of each other. NOE correlation was observed among 8α(axial)-H (1.11), 9α-H (2.07), and 10-Me protons, implying α-orientation of the 10-Me group. Two tertiary methyl protons (4-Me and 10-Me) showed correlation peaks with the same 2α-H and 3α-H. Thus, stereochemistry of the four chiral centers was deduced as 1β-H, 4α-Me, 7β-H, and 10α-Me, that is, the structure of **5** was confirmed as (1*S**,4*S**,7*R**,10*S**)-10-isocyano-5-cadinen-4-ol. There was a report of 10-isothiocyanato-5-amorphen-4-ol isolated from the marine sponge *Axinyssa fenestratus* without complete elucidation of relative stereochemistry, where the equatorial stereochemistry of both *tert*-Me's was mentioned.[10] As ^{13}C NMR data were also fairly different from those of **5**, the stereostructure of the reported compound[10] would be different from that of **5**.

We have already obtained several cadinane- and amorphane-type isocyanides from various nudibranchs as antifoulants,[5] but this was the first case to afford an antifouling isocyanide with a hydroxy moiety.

Table 3. NMR Data for Compound **5**

Atom No.		H	C	HMBC	NOE
1		2.34 (t-like)	45.8 (d)	2, 5, 6, 10, 15	3β, 7, 9β
2	α-ax	1.75 (m)	20.2 (t)		2β, 14, 15
	β-eq	2.08 (m)			2α, 3α, 3β
3	α-eq	1.59 (m)	29.8 (t)		2β, 3β, 14, 15
	β-ax	1.93 (m)		1, 4	1, 2β, 3α
4			80.4 (s)		
5		5.52 (br s)	125.3 (d)	1, 3, 7, 14	11, 12, 14
6			140.7 (s)		
7		1.79 (m)	47.7 (d)		1, 13
8	α-ax	1.11 (m)	21.8 (t)		8β, 9α, 12, 15
	β-eq	1.71 (m)			8α, 12, 13
9	α-eq	2.07 (m)	41.3 (t)	8, 10	8α, 9β, 15
	β-ax	1.95 (m)			1, 9α
10			61.8 (s)		
11		2.10 (m)	26.5 (d)		5
12		0.83 (3H, d)	17.4 (q)	7, 11, 13	5, 8α, 8β, 13
13		0.96 (3H, d)	22.0 (q)	7, 11, 12	7, 8β, 12
14		1.31 (3H, s)	24.2 (q)	3, 4, 5	2α, 3α, 5
15		1.24 (3H, br s)	21.5 (q)	1, 9, 10	2α, 3α, 8α, 9α
16			153.3 (s)		

Antifouling Activity against Barnacle Larvae.

These sesquiterpenes (**1**-**5**) showed antifouling activity against *cypris* larvae of the barnacle *Balanus amphitrite*; **1**: EC_{50} 1.2 μg/mL; **2**: 70 % inhibition at the concentration of 0.5 μg/mL; **3**: more than 90 % inhibition at the concentration of 0.5 μg/mL; **4**: EC_{50} 23 μg/mL; **5**: EC_{50} 0.17 μg/mL. The isolated amounts of **2** and **3** were too small to obtain EC_{50} values. At these EC_{50} concentrations for **1**, **4**, and **5** or the mentioned concentrations for **2** and **3**, the lethality was less than 5 %. It should be noted that an EC_{50} value of $CuSO_4$ was 0.15 μg/mL and the lethality at this concentration was more than 5 %.[6a]

Conclusion

Three new sesquiterpene carbonimide dichlorides **1**-**3** have been isolated from a marine sponge of the genus *Axinyssa*, while a guaiane-type sesquiterpene peroxide **4** from another *Axinyssa* sponge. This was the third report to obtain compounds with a rare carbonimide dichloride moiety from marine invertebrates, and the oxygenated functionalities would play an important role for their antifouling activity.

A new isocyanosesquiterpene alcohol **5** was also obtained from the nudibranch *Phyllidia pustulosa*. This was the first case to afford an antifouling isocyanide with a hydroxyl moiety from nudibranchs. This compound **5** possessed almost the same potent antifouling activity as $CuSO_4$ against larvae of the barnacle *Balanus amphitrite* with very weak lethality.

Experimental

General methods:

NMR spectra were recorded on a BRUKER ARX-500 spectrometer in $CDCl_3$ at 500.14 MHz for ^1H and 125.77 MHz for ^{13}C at 300 K. Chemical shifts were referenced to solvent peaks: δ_H 7.24 and δ_C 77.0 for $CDCl_3$. Optical rotations were determined by a JASCO DIP-1000 digital polarimeter. EI mass spectra were measured on a JEOL SX-102A mass spectrometer. IR spectra were recorded on a JASCO IR-700 infrared spectrometer.

Antifouling assay:

Adult barnacles *Balanus amphitrite* attached on bamboo poles of oyster farms were procured from Lake Hamana in Shizuoka, Japan, and maintained in an aquarium at 25 °C by feeding *Artemia salina* nauplii. Broods released I-II stage nauplii upon immersion in seawater after dried for 2 days. Nauplii thus obtained were cultured in 80 % filtered seawater at 25 °C by feeding with the diatom *Chaetoceros gracilis*. The seawater and diet were renewed everyday. Larvae reached the *cypris* stage in 5 days. The cyprids were stored at 5 °C until used.

Test samples were dissolved in ethanol; aliquots of the solution were supplied to wells of 24-well polystyrene tissue culture plates and air-dried. To each well were added 2 mL of 80 % filtered seawater and six one-day-old cyprids. Four wells were used in a set of experiment. The plates were kept in the dark for 48 h at 25 °C, and the numbers of larvae which attached, metamorphosed, died, or did not settle were counted under a microscope.

Axinyssimides A-C (1-3)

A marine sponge was collected at a depth of 15 m off Hachijo-jima Island (300 km south of Tokyo) using scuba and identified as *Axinyssa* sp. (Class Demospongea, Order Halichondrida, Family Halichondriidae) by Dr. Rob van Soest; a voucher specimen (ZMA POR. 11063) was deposited at the Institute for Systematics and Population Biology, University of Amsterdam. The frozen specimen (53 g) was homogenized and extracted with EtOH (4 x 150 mL). The EtOH extract was partitioned between $CHCl_3$ and water (3 x 150 mL). The $CHCl_3$ layers were combined, and dried into residue. The residue was resolved into 90% MeOH (100 mL), and the solution was extracted with hexane (100 mL) three times. The hexane layers were combined and dried to a residue of 340 mg of crude antifouling fraction. To the 90 % MeOH was added water for adjustment to 60 % MeOH, and the 60 % MeOH solution was extracted with CH_2Cl_2 (100 mL) three times. The CH_2Cl_2 layers were combined and dried into 290 mg of another crude antifouling fraction.

The hexane solubles (340 mg) were subjected to silica gel (Wako gel C-300) column chromatography (3 x 15 cm), which was eluted with hexane, hexane/EtOAc (99:1), hexane/EtOAc (98:2), hexane/EtOAc (97:3), hexane/EtOAc (95:5), hexane/EtOAc (9:1), hexane/EtOAc (8:2), hexane/EtOAc (1:1), and EtOAc, successively (each 1.5 L). The very active hexane/EtOAc (97:3) fraction (31 mg) was separated by HPLC (Shodex SIL-5B, 4.6 x 250 mm; hexane/EtOAc (97:3); flow rate: 1.0 mL/min; detection: refractive index), and an antifouling fraction with T_R 14-15 min was purified again by HPLC (Capcellpak, silica AG 120, 4.6 x 250 mm; hexane/EtOAc (99:1); flow rate: 1.0 mL/min; detection: refractive index) to yield 1.2 mg of pure antifouling compound, axinyssimide A (**1**; 2.3×10^{-3} % yield; T_R 64 min). Another very active hexane/EtOAc (95:5) fraction (12 mg) of the former column chromatography was also separated by HPLC (Shodex SIL-5B, 4.6 x 250 mm; hexane/EtOAc (97:3); flow rate: 1.0 mL/min; detection: refractive index) to afford 0.6 mg of pure antifouling compound, axinyssimide B (**2**; 1.1×10^{-3} %; T_R 32 min).

The CH_2Cl_2 solubles (290 mg) were fractionated by silica gel (Wako gel C-300) column chromatography (2 x 10 cm) with $CHCl_3$-MeOH gradient elution system. The $CHCl_3$ (100%) fraction (3.5 mg) was purified by HPLC (Develosil, silica 60-5, 4.6 x 250 mm; hexane/EtOAc (8:2); flow rate: 1.0 mL/min; detection: refractive index) to obtain 0.3 mg of pure antifouling compound, axinyssimide C (**3**; 5.7×10^{-4} %; T_R 7-8 min).

Axinyssimide A (**1**): $[\alpha]_D$ +1.7° (*c* 0.060, $CHCl_3$); HREIMS: *m/z* 316.1223 (calcd for $C_{16}H_{24}{}^{35}Cl_2NO$ [M-Cl], Δ -1.2 mmu); IR (neat) 1655 cm^{-1}; NMR data, see Table 1.

Axinyssimide B (**2**): $[\alpha]_D$ -22.5° (*c* 0.020, $CHCl_3$); HREIMS: *m/z* 316.1233 (calcd for $C_{16}H_{24}{}^{35}Cl_2NO$ [M-Cl-H_2O], Δ -0.2 mmu); IR (neat) 3425, 1650 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HMBC correlations: H-1/C-2, 3, 16; H-2/C-1, 3, 4, 15; H-9 (δ 1.44)/C-7, 8, 10; H-12(Me)/C-10, 11, 13; H-13(Me)/C-10, 11, 12; H-14(Me)/C-6, 7, 8; H-15 (δ 5.05)/C-4; H-15 (δ 5.19)/C-2, 3.

Axinyssimide C (**3**): $[\alpha]_D$ +7.3° (*c* 0.015, $CHCl_3$); HREIMS: *m/z* 316.1221 (calcd for $C_{16}H_{24}{}^{35}Cl_2NO$ [M-Cl-H_2O], Δ -1.4 mmu); IR (neat) 3390, 1650 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HMBC correlations: H-1/C-2, 3, 16; H-2/C-1, 3, 4, 15; H-12(Me)/C-10, 11, 13; H-13(Me)/C-10, 11, 12; H-14(Me)/C-6, 7, 8; H-15 (δ 5.03)/C-2, 4; H-15 (δ 5.19)/C-2, 3, 4.

(1S,4R*,7S*,10R*)-1,7-Epidioxy-5-guaiene (4)*

Another marine sponge was also collected off Hachijo-jima Island and identified as a different *Axinyssa* sp. (Class Demospongea, Order Halichondrida, Family Halichondriidae) by Dr. Rob van Soest; a voucher specimen (ZMA POR. 11511) was deposited at the Institute for Systematics and Population Biology, University of Amsterdam. The frozen specimen (528 g) was homogenized and extracted with EtOH. The EtOH extract was partitioned between $CHCl_3$ and water (3 x 200 mL). The $CHCl_3$ layers were combined, and dried (5.1 g). After the residue was resolved into 90 % MeOH (170 mL), hexane (150 mL) was added for extraction three times. The prominent antifouling active hexane layer (4.5 g) was separated by silica gel (Wako gel C-300) column chromatography (8 x 20 cm), which was eluted with hexane, hexane/EtOAc (99:1), hexane/EtOAc (98:2), hexane/EtOAc (97:3), hexane/EtOAc (95:5), hexane/EtOAc (9:1), hexane/EtOAc (8:2), hexane/EtOAc (1:1), and EtOAc, successively (each 1.5 L). The most active hexane/EtOAc (97:3) fraction (214 mg) was rechromatographed on the same silica gel (Wako gel C-300) column (2.5 x 20 cm) using hexane/EtOAc (98:2) system as the eluant (10 mL/fraction), and the antifouling fractions from Nos. 23 to 36 were collected (138 mg). The 5/14 portion of these fractions was separated by HPLC (Develosil, silica 60-5, 10 x 250 mm; hexane/EtOAc (98:2); flow rate: 3.0 mL/min; detection UV 220 nm and refractive index), and a peak at T_R 13 min (16 mg) was antifouling. This was purified again by HPLC (Develosil, silica 60-5, 4.6 x 250 mm; hexane/EtOAc (98.5:1.5); flow rate: 1.0 mL/min; detection: UV 220 nm and refractive index) to yield 10.0 mg (5.3×10^{-3} %) of pure compound (**4**) at T_R 14 min.

4: $[\alpha]_D$ -49.4° (*c* 0.27, $CHCl_3$); LREIMS: *m/z* 236 (M^+, very weak), 204, 161; HREIMS: *m/z* 204.1874 (calcd for $C_{15}H_{24}$ [M-O_2], Δ -0.4 mmu); IR (neat) 890 cm^{-1}; NMR data, see Table 2.

(1S,4S*,7R*,10S*)-10-Isocyano-5-cadinen-4-ol (5)*

A specimen of the nudibranch *Phyllidia pustulosa* was obtained at a depth of 15 m off Katsuura on the Kii Peninsula (600 km southwest of Tokyo), and was immediately soaked in EtOH. The EtOH extract was partitioned between CH_2Cl_2 and H_2O (3 x 40 mL). The CH_2Cl_2 layers were combined, dried, and then resolved into hexane. The hexane solution was fractionated by silica gel (Wako gel C-300) column chromatography (1.5 x 5 cm), which was eluted with hexane, hexane/ether (99:1), hexane/ether (98:2), hexane/ether (95:5), hexane/ether (9:1), hexane/ether (8:2), and hexane/ether (6:4), successively. The antifouling hexane/ether (95:5) fraction was again separated by silica gel (Wako gel C-300) column chromatography (1.5 x 5 cm). The elution was performed by hexane/EtOAc (96:4), hexane/EtOAc (95:5), hexane/EtOAc (93:7), hexane/EtOAc (9:1), hexane/EtOAc (8:2), and hexane/EtOAc (1:1), successively. The active hexane/EtOAc (9:1) fraction was separated by HPLC (Senshupak silica 1251-N, 4.6 x 250 mm; hexane/EtOAc (9:1); flow rate: 1.0 mL/min; detection: UV 254 nm); a fraction with T_R 19-26 min was obtained. The fraction was again separated by HPLC (Senshupak silica 1251-N, 4.6 x 250 mm; hexane/ether (3:1); flow rate: 1.0 mL/min; detection: UV 220 nm and refractive index) to furnish 0.9 mg of the pure antifouling compound **5** (T_R 12 min).

5: $[\alpha]_D$ +88.8° (*c* 0.025, $CHCl_3$); LREIMS *m/z* 247 (M^+), 229, 203; HREIMS *m/z* 247.1943 (calcd for $C_{16}H_{25}NO$, Δ +0.7 mmu) ; IR (neat) 3320, 2120 cm^{-1}; NMR data, see Table 3.

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