New Antifouling Kalihipyrans from the Marine Sponge *Acanthella cavernosa*

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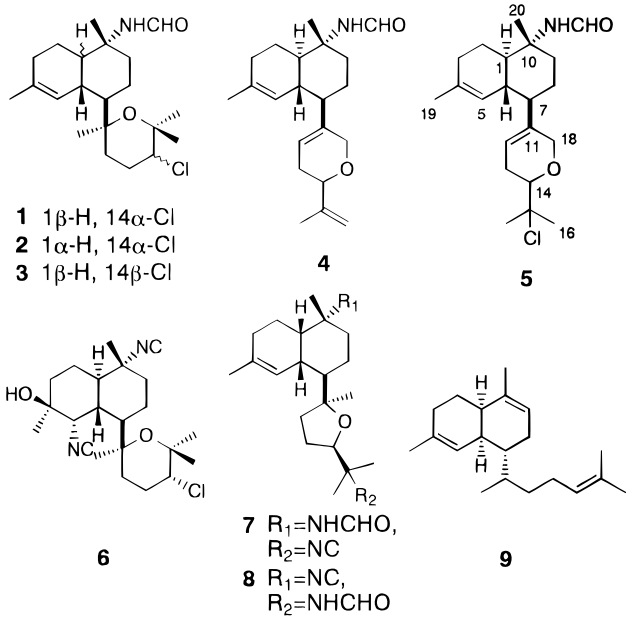
Two new diterpene formamides were isolated from the marine sponge *Acanthella cavernosa* collected off Yakushima Island, along with seven known diterpenes. Their structures proved to be kalihipyran derivatives on the basis of spectral data. All nine compounds showed potent antifouling activity against larvae of the barnacle *Balanus amphitrite.*

Fouling in the marine environment is an economic burden. Sessile marine organisms, such as mussels and barnacles, often cause serious problems by settling on ships' hulls, on cooling systems for power plants, and on aquaculture cages. Organotin compounds, which are at present the most effective antifouling agents, are under criticism because of environmental concerns. Therefore, nontoxic alternates are urgently needed.[2−4]

Sessile marine organisms have developed chemical defenses by producing and exuding secondary metabolites against fouling organisms. These secondary metabolites are presumably potential antifoulants, and active antifouling compounds have, in fact, been obtained from marine organisms (*e.g.*, bromopyrroles,[5] pukalides,[6] renillafoulins,[7] sulphated phenolic acids,[8] and 2,5,6-tribromo-1-methylgramine[9] were isolated from a sponge, a gorgonian, a sea pansy, a seagrass, and a bryozoan, respectively). In our search for natural antifouling substances, we have isolated isocyanoditerpenes, formamidoditerpenes, isocyanosesquiterpenes, and formamidosesquiterpenes from the marine sponge *Acanthella cavernosa* collected off Yakushima and Hachijo-jima Islands.[10,11] Several isocyanosesquiterpenes were also isolated from nudibranchs of the family Phyllidiidae.[12] Bromotyrosine derivatives were isolated from the sponge *Pseudoceratina purpurea*.[13,14] An oroidin dimer and a spermidine derivative from sponges also showed antifouling activity.[15,16] Further study of the Yakushima collection of *A. cavernosa*, which contained the biosynthetically interesting, previously described kalihinenes X−Z (**1**−**3**), kalihinol A (**6**), and 10-formamidokalihinene (**7**),[10] afforded the new antifouling kalihipyrans A (**4**) and B (**5**), together with the known 15-formamidokalihinene (**8**)[17] and biflora-4,9,15-triene (**9**).[11] In this paper we describe the isolation and structure elucidation of these antifouling substances.

Sponge specimens were extracted with EtOH, and the extract was partitioned between Et_2O and H_2O. The Et_2O layer was then partitioned between hexane and MeOH/H_2O (9:1). Two antifouling fractions were obtained from the hexane-soluble layer by Si gel column chromatography. The less polar fraction afforded biflora-4,9,15-triene (**9**), which was readily identified from spectral data.[11] The more polar fraction was separated by Si gel column chromatography followed by ODS HPLC to yield eight active compounds (**1**−**8**). The



structures of the formamidoditerpenes kalihinenes X−Z (**1**−**3**) were elucidated from spectral data.[10] Kalihinol A (**6**), 10-formamidokalihinene (**7**), and 15-formamidokalihinene (**8**) were identified by comparison with data reported in the literature.[17,18]

Most ^{1}H- and ^{13}C-NMR signals for **4** and **5** were doubled due to the presence of a formamide group, as in the cases of **1**−**3**. This function was supported by IR absorption (**4**; 1664 cm^{-1}, **5**; 1660 cm^{-1}). The formamide group of **4** resonated at δ_H 5.62 (NH), 8.25 (d, CHO), and δ_C 162.7 (CHO) for the s-*trans* isomer and at δ_H 5.09 (NH), 8.05 (br s, CHO), and δ_C 160.4 (CHO) for the s-*cis* isomer; these assignments were secured by COSY and HMQC data. Compound **5** exhibited the corresponding signals at δ_H 5.53 (NH), 8.26 (d, CHO), and δ_C 162.6 (CHO) for the s-*trans* isomer and at δ_H 5.08 (NH), 8.06 (br s, CHO), and δ_C 160.3 (CHO) for the s-*cis* isomer.

The molecular formula of **5** was established as $C_{21}H_{32}ClNO_2$ by HRFABMS. The ^{1}H- and ^{13}C-NMR spectra revealed four methyls, six methylenes, four sp^3 methines, two sp^3 quaternary carbons, and two trisubstituted olefins in addition to a formamide group, which accounted for the elements of $C_{21}H_{32}NO$; one oxygen and one chlorine atom were missing from the molecular formula. COSY correlations led to a partial structure $CH_2(8)-CH_2(9)$ and a methylcyclohexene ring, which was supported by HMBC cross peaks between Me-19 and C-3, 4, and 5. HMBC correlations from Me-20 to

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C-1, 9, and 10 indicated that the quaternary C-10 was connected to C-1, 9, and 20, while HMBC cross peaks from H-5 and H-9 to C-7 led to the connectivities from C-7 to C-6 and C-8. No correlation to the formamide group in the s-*trans* isomer was observed, although the formyl proton (δ 8.06) in the s-*cis* isomer was coupled to C-10 (δ 57.0) in the HMBC spectrum. Thus, the same decalin system containing a formamide group as in **2** was established. A unit attached to C-7 comprised the elements of $C_8H_{12}ClO$. A partial structure, $CH_2(18)-C(11)=CH(12)-CH_2(13)-CH(14)$, was obtained by COSY correlations together with an allylic coupling between an olefinic proton (H-12) and an oxymethylene (H-18) and a homoallylic coupling between methylene protons (H-13) and an oxymethylene (H-18). Furthermore, HMBC correlations (H-12/C-13, H-12/C-14, H-12/C-18, H-18/C-11, and H-18/C-12) supported the connectivities from C-18 to C-14, which, in turn, was connected to the oxymethylene C-18 as revealed by HMBC correlations H-14/C-18 and H-18/C-14. The HMBC spectrum also revealed the connectivities between two methyl groups Me-16 and Me-17 and the oxymethine C-14 as well as the quaternary C-15; a chlorine atom was placed at C-15. Thus, a dihydropyran system resulted. Finally, an HMBC cross peak from H-12 to C-7 connected C-7 in the decalin to C-11 in the dihydropyran, thereby completing the planar structure of **5**.

In the ^{1}H-NMR spectrum, H-5 appeared as a broad singlet, indicating that the dihedral angle between H-5 and H-6 was about 90°. Thus, the decalin ring system of **5** is *trans*-fused as in the case of **2**, whose ^{13}C-NMR chemical shifts for the decalin ring system are almost superimposable on those of **5** except for C-7 and C-8. The axial orientation of Me-20 was confirmed by its chemical shift (δ_C 18.9). The coupling constants of H-14 (J = 10.6, 3.1 Hz) indicated its axial configuration. NOESY correlations (H-5/H-12, H-5/H-14, H-5/H-18, H-6/H-12, and H-6/H-18) supported the axial orientation of H-7. Thus, the relative stereochemistry of C-7 was assumed to be the same as that of the known kalihinane diterpenes whose structures were determined by X-ray diffraction.

The molecular formula of **4** was established as $C_{21}H_{31}NO_2$ by HRFABMS. The *trans*-decalin ring system, including relative stereochemistry, was readily deduced to be the same as that of **5** by comparison of their ^{13}C-NMR signals. The remaining $C_8H_{11}O$ portion was connected to C-7. The connectivities, H-12−H-14, were inferred from the COSY spectrum. Allylic and homoallylic couplings were observed between H-18 and H-12, 13. HMBC correlations from H-18 to C-12 and C-13, together with data mentioned above, constructed the dihydropyran ring. In the HMBC spectrum exomethylene (CH_2-16) and methyl (Me-17) protons were coupled to a quaternary carbon (C-15), which demonstrated that the connectivity of the dihydropyran ring was the same as that of kalihipyran.[19] As a result, the gross structure was completed. The relative stereochemistry was assigned as results of NOESY and ^{13}C-NMR data.

The new kalihipyrans A (**4**) and B (**5**) inhibited larval settlement and metamorphosis of the barnacle *Balanus amphitrite* with IC_{50} of 1.3 and 0.85 μg/mL, respectively. These activities are comparable to those of kalihinene X-Z (IC_{50}: **1**; 0.49, **2**; 0.45, **3**; 1.1 μg/mL), whereas the corresponding isocyano compounds are more active (IC_{50}: **6**; 0.087, **7**; 0.095, **8**; 0.14 μg/mL). Interestingly, the hydrocarbon **9** was moderately active (IC_{50} 4.6 μg/mL). It should be noted that **6** and **7** were more active than $CuSO_4$ (IC_{50} 0.15 μg/mL), and their toxicity was quite low.[20]

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Bruker ARX 500 spectrometer in $CDCl_3$ at 500.14 MHz and 125.77 MHz 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 obtained with a JASCO DIP-1000 digital polarimeter. FABMS were measured on a JEOL JMS-SX 102A mass spectrometer. IR spectra were recorded on a JASCO IR-700 infrared spectrometer.

Sponge Sample. The marine sponge *Acanthella cavernosa* Dendy, 1922 (class Demospongiae, order Halichondrida, family Dictyonellidae) was collected by hand using scuba at depths of 15−20 m off Yakushima Island, 1000 km southwest of Tokyo. A voucher specimen (ZMA POR. 11018) was deposited at the Institute for Systematics and Population Biology, University of Amsterdam.

Extraction and Isolation. The frozen sponge (0.5 kg) was extracted with EtOH (1.5 L × 3) followed by partitioning between Et_2O and H_2O (0.5 L × 3). The Et_2O layer was then partitioned between hexane and MeOH/H_2O (9:1) (0.5 L × 3). The hexane-soluble portion (3.5 g) was separated by Si gel column chromatography (Wako gel C-300) with increasing amounts of Et_2O in hexane, and then EtOAc. The hexane/Et_2O (95:5) eluate was separated by HPLC on Develosil ODS T-5 (4.6 × 250 mm, mobile phase MeOH 100%, flow rate 1.0 mL/min, UV 220 nm) to yield biflora-4,9,15-triene (**9**, 2.6 mg). The EtOAc eluate was fractionated on Si gel with a C_6H_6/EtOAc system. The C_6H_6/EtOAc (8:2) and (7:3) eluates were separated on an ODS column with 90% MeOH. The C_6H_6/EtOAc (8:2) eluate was purified by repeated HPLC on Capcellpak C_{18}AG, Develosil ODS T-5, and HG-5 (10 and 4.6 × 250 mm, mobile phase 75−85% MeOH, flow rate 3.0−1.0 mL/min, RI) to afford kalihinol A (**6**, 1.3 mg). Seven active fractions were obtained by HPLC on Capcellpak C_{18}AG (10 × 250 mm, mobile phase 85% MeOH, flow rate 3.0 mL/min, UV 220 nm) from the C_6H_6/EtOAc (7:3) fraction. Each fraction was purified by ODS HPLC to yield 10-formamidokalihinene (**7**, 7.4 mg), 15-formamidokalihinene (**8**, 6.2 mg), kalihipyran A (**4**, 1.2 mg), kalihipyran B (**5**, 1.2 mg), kalihinene Z (**3**, 1.0 mg), kalihinene X (**1**, 5.4 mg), and kalihinene Y (**2**, 0.7 mg).

Kalihipyran A (**4**): colorless oil; $[\alpha]^{23}_D$ +38.6° (*c* 0.080, $CHCl_3$); IR (neat) ν max 3320, 1664 cm^{-1}; ^{1}H NMR of s-*trans* isomer ($CDCl_3$, 500.14 MHz) δ 8.25 (1H, d, J = 12.3 Hz, H-21), 5.62 (1H, NH), 5.58 (1H, H-12), 5.26 (1H, br s, H-5), 4.99 (1H, s, H-16), 4.84 (1H, s, H-16), 4.15 (2H, s, H-18), 3.88 (1H, br d, J = 9.4 Hz, H-14), 2.20 (1H, m, H-13), 2.11 (1H, m, H-13), 1.98 (2H, br, H-3), 1.93 (1H, m, H-6), 1.88 (1H, m, H-9), 1.86 (1H, m, H-7), 1.80 (1H, m, H-2), 1.77 (3H, s, Me-17), 1.67 (1H, m, H-8), 1.62 (3H, s, Me-19), 1.59 (1H, m, H-9), 1.48 (1H, m, H-8), 1.28 (1H, m, H-2), 1.25 (3H, s, Me-20), 1.18 (1H, m, H-1); ^{13}C NMR of s-*trans* isomer ($CDCl_3$, 125.77 MHz) δ 162.7 (d, C-21), 145.4 (s, C-15), 138.7 (s, C-11), 134.9 (s, C-4), 122.7 (d, C-5), 120.0 (d, C-12), 110.6 (t,

C-16), 76.9 (d, C-14), 67.0 (t, C-18), 55.3 (s, C-10), 49.0 (d, C-1), 45.2 (d, C-7), 41.9 (t, C-9), 39.1 (d, C-6), 31.0 (t, C-3), 29.7 (t, C-8), 29.6 (t, C-13), 23.3 (q, C-19), 22.7 (t, C-2), 19.0 (q, C-20), 18.9 (q, C-17); HRFABMS (glycerol) m/z $[M + H]^+$ 330.2430; calcd for $C_{21}H_{32}NO_2$ 330.2433.

Kalihipyran B (**5**): colorless oil; $[\alpha]^{23}_D$ +73.4° (*c* 0.035, $CHCl_3$); IR (neat) ν max 1660 cm^{-1}; ^1H NMR of s-*trans* isomer ($CDCl_3$, 500.14 MHz) δ 8.26 (1H, d, J = 12.4 Hz, H-21), 5.58 (1H, H-12), 5.53 (1H, NH), 5.24 (1H, br s, H-5), 4.21 and 4.13 (2H, ABq, J = 15.6 Hz, H_2-18), 3.42 (1H, dd, J = 10.6, 3.1 Hz, H-14), 2.29 (1H, m, H-13), 2.17 (1H, m, H-13), 1.97 (2H, br, H-3), 1.90 (1H, m, H-6), 1.88 (1H, m, H-9), 1.83 (1H, m, H-2), 1.67 (1H, m, H-8), 1.67 (1H, m, H-7), 1.63 (3H, s, Me-19), 1.59 (1H, m, H-9), 1.58 (3H, s, Me-17), 1.57 (3H, s, Me-16), 1.55 (1H, m, H-8), 1.28 (1H, m, H-2), 1.24 (3H, s, Me-20), 1.18 (1H, m, H-1); ^{13}C NMR of s-*trans* isomer ($CDCl_3$, 125.77 MHz) δ 162.6 (d, C-21), 138.4 (s, C-11), 135.2 (s, C-4), 122.6 (d, C-5), 119.7 (d, C-12), 80.9 (d, C-14), 70.6 (s, C-15), 67.8 (t, C-18), 55.2 (s, C-10), 48.9 (d, C-1), 47.4 (d, C-7), 41.8 (t, C-9), 39.0 (d, C-6), 31.0 (t, C-3), 29.4 (t, C-8), 29.1 (q, C-16), 28.3 (q, C-17), 26.0 (t, C-13), 23.3 (q, C-19), 22.7 (t, C-2), 18.9 (q, C-20); HRFABMS (glycerol) m/z $[M + H]^+$ 366.2188; calcd for $C_{21}H_{33}{}^{35}ClNO_2$ 366.2200.

Antifouling Assay. Antifouling activity was determined as described previously.[12]

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