Pergamon

Tetrahedron 54 (1998) 13467–13474

TETRAHEDRON

Antimalarial Activity of Kalihinol A and New Relative Diterpenoids from the Okinawan Sponge, *Acanthella* sp.

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Received 28 August 1998

Abstract: Three new kalihinane diterpenoids Δ^9-kalihinol Y (**1**), 10-epikalihinol I (**2**) and 5,10-bisisothiocyanatokalihinol G (**3**) were isolated in the present study along with previously detected kalihinane diterpenoids kalihinol A (**4**), kalihinene (**5**) and 6-hydroxykalihinene (**6**) from the Okinawan marine sponge, *Acanthella* sp. Kalihinol A (**4**) was noted to possess remarkable *in vitro* antimalarial activity. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: antimalarial compounds; marine metabolites; natural products; terpenes and terpenoids

Various natural products have recently been isolated from marine invertebrates and many of which, from marine sponges, are of considerable interest for their unique structural features and biological activity.[1] Over thirty kalihinane diterpenoids, having isonitrile, isothionitrile and/or formamido functionalities have been obtained from marine sponges.[2-14] Members of this series of compounds have antimicrobial,[2-4] antifungal,[2-4,6,8] cytotoxic,[6] anthelmintic[5,7] and antifouling[11-14] properties. The antimalarial activity of axisonitrile-3 containing isonitrile group, isolated from the marine sponge *Acanthella klethra*, has been demonstrated,[15] which prompted the isolation of various antimalarial compounds containing isonitrile or isothionitrile groups have been isolated from marine sponges.[16-21] In the present study on biological active compounds of Okinawan marine invertebrates,[22] a new isocyano diterpenoid Δ^9-kalihinol Y (**1**) and two new isothiocyano diterpenoids 10-epikalihinol I (**2**) and 5,10-bisisothiocyanatokalihinol G (**3**) were isolated along with the previously detected kalihinol A[2,4] (**4**), kalihinene[6] (**5**) and 6-hydroxykalihinene[10] (**6**). This paper describes the isolation, structural elucidation and antimalarial activity of these compounds.

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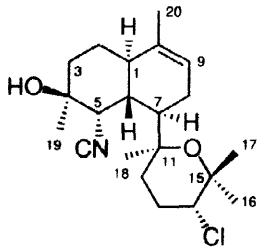
PII: S0040-4020(98)00818-7

Sponge specimens (wet weight 1.4 kg), obtained from the coral reef of Ishigaki Island, Okinawa, Japan, in September 1995, were immersed in MeOH and then acetone. The MeOH and acetone extracts were combined and partitioned between AcOEt and H_2O. The AcOEt soluble portion (14.8 g) was repeatedly chromatographed on a silica gel column to give isocyano diterpenoid Δ^9-kalihinol Y (**1**) (12 mg), isothiocyano diterpenoids 10-epikalihinol I (**2**) (14 mg), 5,10-bisisothiocyanatokalihinol G (**3**) (11 mg), along with the previous isolated kalihinol A[2,4] (**4**) (85 mg), kalihinene[6] (**5**) (31 mg) and 6-hydroxykalihinene[10] (**6**) (6.7 mg).



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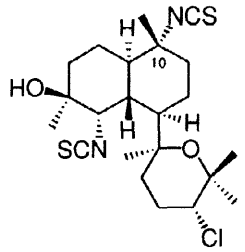

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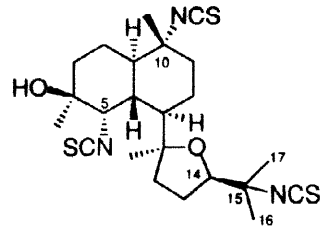
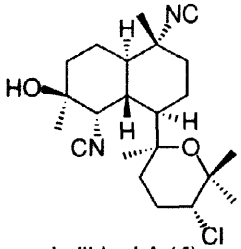
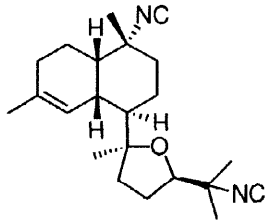


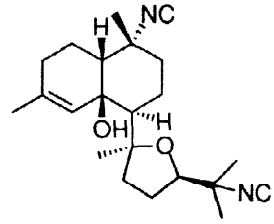
5,10-bisisothiocyanatokalihinol G (**3**)



kalihinol A (**4**)



kalihinene (**5**)



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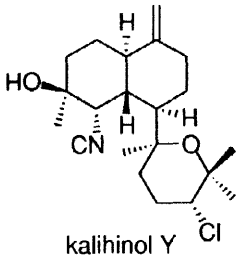
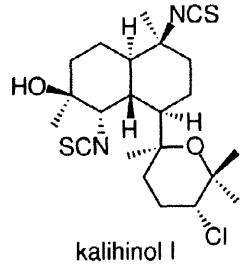
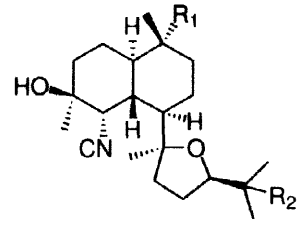


kalihinol Y



kalihinol I



kalihinol G : R_1 = -NC, R_2 = -NCS
kalihinol H : R_1 = -NCS, R_2 = -NC

Δ^9-Kalihinol Y (**1**) was found to have the molecular formula $C_{21}H_{32}NO_2Cl$ based on high resolution mass measurement. The IR spectrum of **1** showed absorptions at 3449 cm^{-1} due to the hydroxyl group and IR absorption at 2160 cm^{-1} and a singlet at δ_C 156.4, a broad triplet (J = 5 Hz) at δ_C 63.7 in ^{13}C NMR spectrum indicated the presence of one isocyano group (Table 1). A trisubstituted double bond was shown to be present by the NMR spectrum [δ_H 5.32 (1H, dd like), 1.63 (3H, s); δ_C 134.4 (C), 120.5 (CH), 20.7 (CH_3)]. The NMR spectra of **1** were closely related to those of reported kalihinol Y[4] except for signals of the trisubstituted double bond instead of the *exo*-methylene group in kalihinol Y, suggesting Δ^9-kalihinol Y to

have the structure of **1**. The plane structure of **1** was confirmed by COSY, HMQC and HMBC spectra. In the ^{1}H NMR spectrum, H-1 appeared as a broad triplet (J = 10.5 Hz) at δ_H 2.18, indicating an axial configuration. NOESY correlation was indicated between H-1 and H-7 (δ_H 1.78). The decalin ring system of **1** is thus shown to be *trans*-fused and H-7 to have the axial configuration. The equatorial orientation of Me-19 was confirmed by its chemical shift (δ_C 28.7). The equatorial orientation of H-5 was determined based on NOESY correlations (H-5/H-6 and H-5/H-18). The axial orientation of Me-18 was confirmed by its chemical shift (δ_C 18.0). The coupling constants of H-14 (J = 12, 4.4 Hz) indicated axial orientation of H-14. The relative configuration of Δ^9-kalihinol Y was thus concluded to be **1**.

Table 1 NMR data for Δ^9-kalihinol Y (**1**), 10-epikalihinol I (**2**) and 5,10-bisisothiocyanatokalihinol G (**3**)

	Δ^9-kalihinol Y (**1**)		10-epikalihinol I (**2**)		5,10-bisisothiocyanatokalihinol G (**3**)	
	^{13}C NMR[a]	^{1}H NMR[b]	^{13}C NMR[a]	^{1}H NMR[b]	^{13}C NMR[a]	^{1}H NMR[b]
1	37.1 (CH)	2.18 (br t, 10.5)	43.6 (CH)	1.64	43.3 (CH)	1.68
2	25.6 (CH_2)	1.23	22.1 (CH_2)	1.52	22.0 (CH_2)	1.57
		1.90		1.76		1.78
3	33.9 (CH_2)	1.56	33.3 (CH_2)	1.60	33.2 (CH_2)	1.64
		1.90		1.74		1.75
4	71.0 (C)	-	71.0 (C)	-	71.5 (C)	-
5	63.7* (CH)	4.31	66.0 (CH)	4.68 (br t, 1.9)	65.3 (CH)	4.44 (br t ,1.9)
6	38.7 (CH)	2.01	38.2 (CH)	2.06	38.0 (CH)	2.06
7	45.4 (CH)	1.78	49.1 (CH)	1.58	46.8 (CH)	1.73
8	26.8 (CH_2)	1.64	22.6 (CH_2)	0.98 (qd, 13.5, 3.4)	24.8 (CH_2)	1.19
						1.69
9	120.5 (CH)	5.32 (dd like)	40.1 (CH_2)	1.80 (dt, 3.7, 13.3)	40.2 (CH_2)	1.87
				1.97 (dt,13.3, 3.5)		1.98
10	134.6 (C)	-	63.8 (C)	-	63.8 (C)	-
11	76.8 (C)	-	77.0 (C)	-	87.3 (C)	-
12	37.7 (CH_2)	1.51	38.1 (CH_2)	1.59	38.1 (CH_2)	1.64
		1.64		1.60		1.75
13	27.4 (CH_2)	2.00	27.4 (CH_2)	2.03	25.1 (CH_2)	1.82
		2.11		2.08		2.05
14	64.7 (CH)	3.76 (dd,12, 4.4)	64.2 (CH)	3.76 (dd,12, 4.8)	83.5 (CH)	3.94 (dd, 9.1, 4.2)
15	76.8 (C)	-	76.1 (C)	-	63.6 (C)	-
16	22.9 (CH_3)	1.37	23.0 (CH_3)	1.35	25.5 (CH_3)	1.36
17	30.9 (CH_3)	1.39	30.9 (CH_3)	1.36	24.5 (CH_3)	1.38
18	18.0 (CH_3)	1.20	19.4 (CH_3)	1.19	17.8 (CH_3)	1.05
19	28.7 (CH_3)	1.43	29.3 (CH_3)	1.35	29.1 (CH_3)	1.39
20	20.7 (CH_3)	1.63	20.9 (CH_3)	1.31	20.9 (CH_3)	1.34
5-NC	156.4 (C)					
5-NCS			130.7 (C)		130.5 (C)	
10-NCS			131.1 (C)		131.1** (C)	
15-NCS					131.2** (C)	

[a] 100MHz, $CDCl_3$., [b] 400MHz, $CDCl_3$. *signal appears as a broad triplet (J = 5Hz), **interchangeable

10-Epikalihinol I (**2**) was found to have the molecular formula $C_{22}H_{33}N_2O_2S_2Cl$ based on high resolution mass measurement. The IR spectrum of **2** showed absorptions at 3518 cm^{-1} due to the hydroxyl group. Two isothiocyano groups were recognized by IR absorption at 2179 cm^{-1}, UV absorption (λ_{max} 246 nm) and ^{13}C NMR spectrum (δ_C 130.7 and 131.1). The plane structure of **2** was clarified from COSY, HMQC and HMBC spectra. The NMR spectra of **2** were closely related to those of reported kalihinol I[7] except for chemical shifts of Me-20 (δ_C 20.9) and C-10 (δ_C 63.8), suggesting compound **2** to be 10-epikalihinol I. The relative stereochemistry of **2** was confirmed from NOESY correlations and chemical shifts of methyl groups in ^{13}C NMR.

5,10-Bisisothiocyanatokalihinol G (**3**) was found to have the molecular formula $C_{23}H_{33}N_3O_2S_3$ based on high resolution mass measurement. The IR spectrum of **3** indicated absorptions at 3501 cm^{-1} due to the hydroxyl group. Three isothiocyano groups were recognized by IR absorption at 2109 cm^{-1}, UV absorption (λ_{max} 246 nm) and ^{13}C NMR spectrum (δ_C 130.5, 131.1 and 131.2). The plane structure of **3** was determined by COSY, HMQC and HMBC spectra. The NMR spectra of **3** were closely related to those of previously noted for kalihinol G.[4] The relative stereochemistry of **3** was confirmed by NOESY correlations and chemical shifts of methyl groups in ^{13}C NMR.

10-Epikalihinol I (**2**), 5,10-bisisothiocyanatokahihinol G (**3**), kalihinol A (**4**), kalihinene (**5**) and 6-hydroxykalihinene (**6**) expressed cytotoxic activity toward the malaria parasite *Plasmodium falciparum* (Table 2). Additional examination of these compounds for their effects on the FM3A cells has led to the establishment of an experimental selectivity index (SI) by which observed antiplasmodial activity can be assessed as a specific ally or general by toxic. Kalihinol A (**4**) was found to have particularly potent (EC_{50} 1.2 x 10^{-9} M) and selective (SI 317) *in vitro* antimalarial activity.

Table 2 Cytotoxicity of Compounds **2-6** to FM3A and *Plasmodium falciparum* (Antimalarial Activity)

compounds	*P. falciparum* EC_{50} (M)	FM3A EC_{50} (M)	SI[b]
10-epikalihinol I (**2**)	>1.8x10^{-6} (62%)[a]	-	-
5,10-bisisothiocyanatokalihinol G (**3**)	2.6x10^{-6}	7.0x10^{-7}	-
kalihinol A (**4**)	1.2x10^{-9}	3.8x10^{-7}	317
kalihinene (**5**)	1.0x10^{-8}	3.7x10^{-8}	4
6-hydroxykalihinene (**6**)	8.0x10^{-8}	1.2x10^{-6}	15
mefloquine[c]	3.2x10^{-8}	2.9x10^{-6}	90

[a] Growth percentage at concentration indicated.
[b] Selectivity index (SI) defined as ratio of FM3A cells cytotoxicity to *P. falciparum*
[c] Antimalarial standard.

The absolute configuration of kalihinane diterpenoids has not yet been reported. A total synthesis of kalihinol A is now underway to clarify its absolute configuration.

Experimental

Melting points were measured on a Yazawa BY-2 micro melting point apparatus without correction. Optical rotation was measured with a JASCO DIP-360 automatic polarimeter. Infrared (IR) spectra were recorded with a Perkin-Elmer FT-IR 1710 spectrometer. Ultraviolet (UV) spectra were recorded with a JASCO V-550 spectrophotometer. ^1H and ^{13}C NMR spectra were recorded with a Bruker AM-400 or Bruker AM-500. Chemical shifts were expressed on a δ (ppm) scale with tetramethylsilane (TMS) as the internal standard (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad). Electron impact mass spectra (EIMS), fast atom bombardment mass spectra (FABMS) and high resolution electron impact mass spectra (HREIMS) were obtained with a Hitachi M-80 or VG Auto Spec spectrometer.

Animal Material, Extraction and Isolation. Specimens of orange marine sponge, *Acanthella* sp. were collected by scuba diving at depths of 5-8 m off the coral reef of Ishigaki Island (Okinawa, Japan) in September 1995, and kept frozen until extraction. Wet specimens (1.4 kg) were immersed successively in MeOH (6 L, 3 L x 5) and then acetone (6 L x 2). The MeOH extracts (100 g) and acetone extracts (2.32 g) were combined and partitioned between H_2O (450 mL) and AcOEt (500 mL, 250 mL x 10).

The AcOEt-soluble portion (14.8 g) was chromatographed on a silica gel column to give three fractions: fraction 1 (4.62 g) eluted with hexane-AcOEt (5:1), fraction 2 (3.28 g) eluted with hexane-AcOEt (1:1) and fraction 3 (106 g) eluted with AcOEt and MeOH.

Fraction 1 was subjected to chromatography on silica gel with hexane-AcOEt (10:1) to give fraction 1-1 (297 mg), fraction 1-2 (4.00 g), fraction 1-3 (212 mg), fraction 1-4 (677 mg) and fraction 1-5 (517 mg). Fraction 1-4 was subjected to chromatography on ODS with MeOH-H_2O (15:1) to give fraction 1-4-1 (121 mg), fraction 1-4-2 (57.5 mg), fraction 1-4-3 (8.5 mg) and fraction 1-4-4 (274 mg). On fraction 1-5, chromatography on ODS was conducted with MeOH-H_2O (8:1) to give fraction 1-5-1 (20.0 mg), fraction 1-5-2 (164 mg), fraction 1-5-3 (21.1 mg), fraction 1-5-4 (33.8 mg) and fraction 1-5-5 (204 mg). Fractions 1-4-1 and 1-5-1 were combined and subjected to repeated chromatography on silica gel with $CHCl_3$-Et_2O (30:1), hexane-Et_2O (2:1) and to repeated flash chromatography on ODS with MeOH- H_2O (6:1) to give kalihinene[6] (**5**) (34.0 mg). Fraction 1-5-2 was subjected to flash chromatography on silica gel with hexane-acetone (8:1) and crystallized from AcOEt-hexane to give crude crystalline Δ^9-kalihinol Y (**1**) (43.2 mg). The crude crystals were recrystallized from EtOH to give colorless needles of Δ^9-kalihinol Y (**1**) (12.0 mg). Fraction 1-5-3 was subjected to flash chromatography on ODS with MeOH- H_2O (8:1) to give 6-hydroxykalihinene[10] (**6**) (6.7 mg).

Fraction 2 was subjected to chromatography on silica gel with hexane-AcOEt (5:1) to give fraction 2-1 (77.7 mg), fraction 2-2 (2.11 g) and fraction 2-3 (633 mg). Fraction 2-1 underwent repeated chromatography on silica gel with $CHCl_3$-Et_2O (30:1) and hexane-acetone (8:1) to give crude crystalline 10-epikalihinol I (**2**) (20.5 mg). The crude crystals were recrystallized from EtOH to give colorless needles of 10-epikalihinol I (**2**) (14.0 mg). Fraction 2-3 was subjected to chromatography on silica gel with $CHCl_3$-Et_2O (9:1) to give fraction 2-3-1 (57.8 mg), fraction 2-3-2 (12.0 mg) and fraction 2-3-3 (350 mg). On fraction 2-3-1, repeated flash chromatography on silica gel was carried out with hexane-acetone (5:1) and on ODS with MeOH-H_2O (9:1) to give 5,10-bisisothiocyanatokahihinol G (**3**) (11.0 mg). Fraction 2-3-2 was diluted with EtOH and filtered through active carbon to give crude crystalline kalihinol A. The crude crystals were recrystallized from EtOH to give colorless needles of kalihinol A[2,4] (**4**) (85.0 mg).

Δ^9-Kalihinol Y (1): colorless needle: mp 188-190 °C; $[\alpha]_D$ +38.4° (*c* 0.24, $CHCl_3$); IR (KBr) 3449, 2160 cm^{-1}; ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) see Table 1; HMBC correlation (H/C) 1/6, 2/3, 5/1, 5/3, 5/4, 5/6, 5/NC(5), 7/11, 7/18, 9/1, 9/7, 9/8, 9/20, 12/18, 14/13, 14/15, 14/16, 14/17, 16/15, 17/14, 17/15, 17/16, 18/7, 18/11, 18/12, 19/3, 19/4, 19/5, 20/1, 20/9, 20/10; NOESY correlation (H/H) 1/7, 5/6, 5/18, 5/19; EIMS m/z 365(M^+); HREIMS: Calcd for $C_{21}H_{32}ClNO_2$ (M^+) 365.2122: Found: 365.2118.

10-Epikalihinol I (2): colorless pillar: mp 209-210 °C; $[\alpha]_D$ -52.4° (*c* 0.3, $CHCl_3$); IR (KBr) 3518, 2179, 2137 cm^{-1}; UV (MeOH) λ_{max} 246 nm (ε 1161); ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) see Table 1; HMBC correlation (H/C) 5/1, 5/3, 5/4, 5/6, 5/19, 5/NCS(5), 6/1, 6/7, 9/1, 9/7, 9/8, 9/20, 13/11, 13/12, 13/14, 13/15, 14/15, 14/16, 14/17, 16/15, 17/15, 18/11, 18/12, 19/3, 19/4, 19/5, 20/1, 20/9, 20/10; NOESY correlation (H/H) 1/7, 5/6, 5/18, 5/19, 8ax/20, 9eq/20; FABMS m/z 456(M^+); EIMS m/z 438(M^+-H_2O); HREIMS: Calcd for $C_{22}H_{31}ClN_2OS_2$ (M^+-H_2O) 438.1566: Found: 438.1563.

5,10-bisisothiocyanatokalihinol G (3): colorless oil: $[\alpha]_D$ -62.7° (*c* 0.8, $CHCl_3$); IR (neat) 3501, 2109 cm^{-1}; UV (MeOH) λ_{max} 246 nm (ε 1660); ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) see Table 1; HMBC correlation (H/C) 5/1, 5/3, 5/4, 5/6, 5/NCS(5), 9/7, 14/16, 14/17, 18/7, 18/11, 18/12, 19/3, 19/4, 19/5, 20/1, 20/9, 20/10; NOESY correlation (H/H) 5/6, 5/18, 5/19, 9eq/20, 14/18; EIMS m/z 479(M^+); HREIMS: Calcd for $C_{23}H_{33}N_3O_2S_3$ (M^+) 479.1735: Found: 479.1731.

Antimalarial Assay. Materials; Mefloquine was a gift from F. Hoffman-La Roche LTD (Basel, Switzerland). Parasites of *P. falciparum* and *P. berghei*; *Plasmodium falciparum* strain FCR-3 (ATCC 30932) was used in our study. *P. falciparum* was maintained *in vitro* at 37°C in RPMI 1640 medium (Gibco, NY) containing human red blood cells (RBCs, type A) at 5% hematocrit in 24-well microplates.[23] The microplates were placed in a CO_2 incubator (5% CO_2, 5% O_2, 90% N_2) at 37°C and the medium was changed daily. Mammalian cells; A wild-type mouse mammary tumor FM3A cell line (subclone F28-7) was supplied by the Japanese Cancer Research Resources Bank (JCRB). FM3A cells were maintained in suspension culture at 37°C in a 5% CO_2 atmosphere in plastic bottles containing ES medium (Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 2% heat-inactivated fetal bovine serum (Gibco, NY).[24] *In vitro* antimalarial activity of *Acanthella* sp.; The following procedures were used for routine assay of antimalarial activity.[25] Various concentrations of compounds in dimethylsulfoxide were prepared. Ten microliters of each solution was added to individual wells of a 24-well plate. Erythrocytes with 0.3% parasitemia were added to each well containing 990 μL of culture medium to give a final hematocrit level of 3%. The plates were incubated at 37°C for 72 h in a CO_2 incubator (5% CO_2, 5% O_2, 90% N_2). To evaluate the antimalarial activity of compounds of *Acanthella* sp., we prepared thin smears from each culture and stained them with Giemsa (E. Merck, Germany). More than 10000 erythrocytes were examined under microscopy. All of the test compounds were assayed in duplicate at each concentration. Drug-free control cultures were run simultaneously. All data points represent the mean of at least two experiments. The fifty percent inhibitory concentration, IC_{50}, was determined by comparison to drug-free controls incubated under the same conditions. Toxicity against mammalian cell line; Cells grew with a doubling time of about 12 h. Prior to exposure to drugs, cell density was adjusted to 5 x 104 cells/mL. A cell suspension of 990 μL was

dispensed to the test plate, and compounds at various concentrations suspended in dimethylsulfoxide (10 μL), were added to individual wells of a 24-well plate. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 48 h. Cell numbers were measured using a blood cell counter CC-108 (Toa Medical Electric Co., Japan). All data points represent the mean of at least two experiments. IC_{50} was determined compared with those in drug-free controls incubated under the same conditions.

Acknowledgments: This work was supported in part by a Grant-in-Aids for Scientific Research on Priority Areas (Grant No. 10166224 and 08281105) from the Ministry of Education, Science, Culture and Sports.

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