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Halichonines A, B, and C, novel sesquiterpene alkaloids from the marine sponge *Halichondria okadai* Kadota†

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Novel sesquiterpene alkaloids, halichonines A (1), B (2), and C (3), were identified from the marine sponge *Halichondria okadai* Kadota. By spectroscopic analyses and synthesis, their structures were revealed to include a 6,6-bicyclic ring system and two prenylated amine moieties. In addition, 2 induced apoptosis in HL60 human leukemia cells.

Marine sponges take seawater into their bodies, gather food by filtration, and coexist with symbiotic microorganisms.[1] They are widely known to be a rich source of biologically active and structurally unique secondary metabolites. For example, okadaic acid,[2] halichondrins,[3] and halichlorine[4] have already been isolated from the marine sponge *Halichondria okadai* Kadota, a species abundant in the tidal zone in the Pacific Ocean. These isolated compounds have good potential for clinical or biological use. In fact, a synthetic analogue of halichondrin B, eribulin mesylate (Halaven™), was recently approved for the treatment of patients with metastatic breast cancer in European Union, the United States, Japan, and many other countries.[5] In our continuing search for biologically active substances from marine organisms,[6] we isolated novel sesquiterpene alkaloids, halichonines A (**1**), B (**2**), and C (**3**) (Fig. 1), from *H. okadai* Kadota. We describe here the isolation, structure determination, and biological activities of **1**, **2**, and **3**.

The marine sponge *H. okadai* Kadota (80 kg), collected from the mediolittoral zone in Mie Prefecture, Japan, was immersed in MeOH for 1 week at room temperature. Concentrated extract was partitioned with EtOAc and water, and the EtOAc layer was chromatographed on TSK G3000S polystyrene gel (EtOH/water). The fraction eluted with 40% aqueous EtOH was partitioned with 1.2 N HCl and EtOAc. The water layer was subsequently adjusted to pH 10 with NaOHaq, and then extracted with EtOAc. The extract was then chromatographed on silica gel ($CHCl_3$/MeOH) using Dragendorff's reagent test-guided fractionation. Final purification was achieved by preparative thin layer chromatography ($CHCl_3$/MeOH: 9/1), which gave halichonines A (**1**) (17.1 mg), B (**2**) (2.0 mg) and C (**3**) (1.8 mg), as pale yellow amorphous powders.

The molecular formula of **2** [$[\alpha]_D^{28}$ +13.8 (*c* 0.2, $CHCl_3$)] was determined to be $C_{29}H_{52}N_2$ by HR-ESIMS data (*m*/*z* 429.4220 $[M + H]^+$, calcd for $C_{29}H_{53}N_2$, 429.4209). The NMR spectral data of **2** in CD_3OD are shown in Table 1. A detailed analysis of the COSY and HMQC spectra of **2** allowed us to elucidate the following six partial structures: C1–C3, C5–C7, C9–C11, C12–C13, C15–C18, and C19–C20. The connectivity of these fragments was elucidated based on the HMBC techniques. HMBC crosspeaks of H3, H5, H22 and H23 to quaternary carbon C4 (δ_C 32.5), H3, H22 and H23 to C5 (δ_C 50.4), and H22 and H23 to C3 (δ_C 42.2) established the connection among C3, C4, C5, C22, and C23. HMBC correlations from H5 and H24 to quaternary carbon C10 (δ_C 36.1) and C9 (δ_C 51.7), and of H24 to C1 (δ_C 39.2) constructed the connection of C1, C5, C9, and C24 to C10. HMBC correlations from H25 to C7 (δ_C 121.7), C8 (δ_C 135.4) and C9, and of H11 to C8 indicated the connection of C7, C9 and C25 to C8. These observations

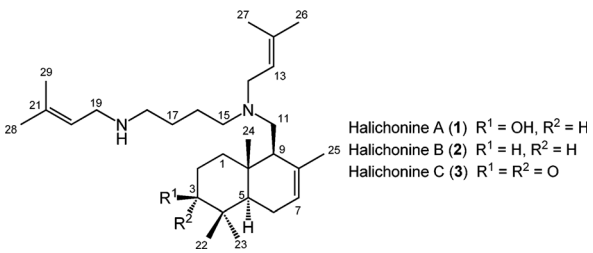

Fig. 1 Structures of halichonines A (**1**), B (**2**), and C (**3**).

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Table 1 NMR data for halichonine B (**2**) in CD_3OD

Position		δ_C[a] (mult.)	δ_H[b] (mult., *J* in Hz)	HMBC[c] ($^1H \rightarrow {}^{13}C$)
1	a	39.2 (t)	1.01 (m)	C2
	b		2.03 (m)	
2	a	18.5 (t)	1.39 (m)	C1, 10
	b		1.55 (m)	
3	a	42.2 (t)	1.16 (m)	C4, 22
	b		1.38 (m)	C5, 22, 23
4		32.5 (s)		
5		50.4 (d)	1.18 (m)	C4, 6, 9, 10, 24
6		23.5 (t)	1.95 (m, 2H)	
7		121.7 (d)	5.34 (s)	C25
8		135.4 (s)		
9		51.7 (d)	1.84 (m)	
10		36.1 (s)		
11		53.6 (t)	2.31 (m, 2H)	C8, 12, 15
12	a	51.0 (t)	2.86 (dd, 7.8, 13.9)	C11, 13, 14, 15
	b		3.11 (dd, 5.7, 13.9)	C11, 13, 14, 15
13		121.5 (d)	5.22 (m)	C26, 27
14		134.0 (s)		
15	a	53.0 (t)	2.22 (m)	C11, 12, 17
	b		2.52 (m)	C11, 12, 16
16		24.3 (t)	1.43 (m, 2H)	C17
17		26.2 (t)	1.45 (m, 2H)	C15
18		48.0 (t)	2.55 (m, 2H)	C17, 19
19		45.8 (t)	3.18 (d, 6.5, 2H)	C18, 20, 21
20		119.0 (d)	5.22 (m)	C28, 29
21		137.4 (s)		
22		21.1 (q)	0.88 (s, 3H)	C3, 4, 5, 23
23		32.6 (q)	0.85 (s, 3H)	C3, 4, 5, 22
24		12.8 (q)	0.74 (s, 3H)	C1, 5, 9, 10
25		21.6 (q)	1.74 (s, 3H)	C9, 7, 8
26		24.7 (q)	1.70 (s, 3H)	C13, 14, 27
27		16.7 (q)	1.63 (s, 3H)	C13, 14, 26
28		24.6 (q)	1.74 (s, 3H)	C20, 21, 29
29		16.7 (q)	1.68 (s, 3H)	C20, 21, 28

[a] Recorded at 100 MHz. [b] Recorded at 600 MHz. [c] Recorded at 400 MHz.

indicated that **2** possessed a 6,6-bicyclic ring system. Meanwhile, the existence of two prenyl moieties was demonstrated based on HMBC correlations at H26/C13, H26/C14, H27/C13, H27/C14, H28/C20, H28/C21, H29/C20, and H29/C21. In addition, HMBC correlations at H18/C19 and H19/C18 suggested that C18 was attached through nitrogen to a C19–C29 prenyl group. This elucidation was also supported by the analyses of ^1H NMR and COSY spectra of **2** in DMSO-d_6 (NH:δ_H 1.59). Similarly, HMBC correlations at H11/C12, H11/C15, H12/C11, H12/C15, H15/C11, and H15/C12 indicated that C11, C12 and C15 were connected by an amine bond. Thus, the planar structure of **2** was determined to be as shown in Fig. 2. The relative configuration of the 6,6-bicyclic rings was determined by the NOESY spectrum (ESI†, Fig. S19B). The NOE correlations at H1a/H3b, H3b/H5, H1b/H2b, H2a/H3a, and H1b/H24 revealed that ring A had a chair conformation. Furthermore, the NOE correlations at H1a/H9 and H5/H9 indicated that ring B had a half-chair conformation. In addition, **2** was successfully obtained as a crystal in the HCl-salt form. The relative stereostructure of **2** was confirmed by X-ray crystallographic analysis (Fig. 3 and S20 in ESI†),[7] and it completely coincided with those determined by the spectroscopic analysis described above.

The molecular formula of **1** $[[\alpha]_D^{25}$ +35.5 (*c* 0.55, $CHCl_3)]$ was determined to be $C_{29}H_{52}ON_2$by HR-ESIMS data (*m*/*z* 445.4166 $[M + H]^+$, calcd for $C_{29}H_{53}ON_2$, 445.4158). The molecular formula of **3** $[[\alpha]_D^{25}$ +8.42 (*c* 0.19, $CHCl_3)]$ was determined to be $C_{29}H_{50}ON_2$ by EIMS data (*m*/*z* 442). The NMR signals of **1** and

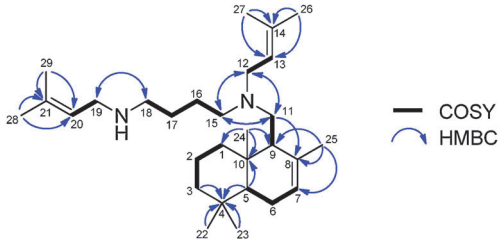


Fig. 2 COSY and selected HMBC correlations of **2**.

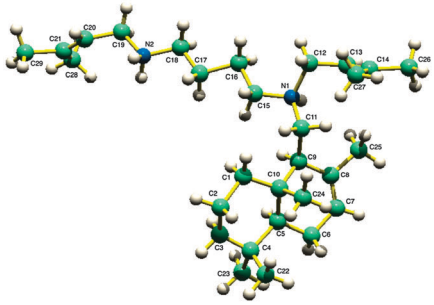
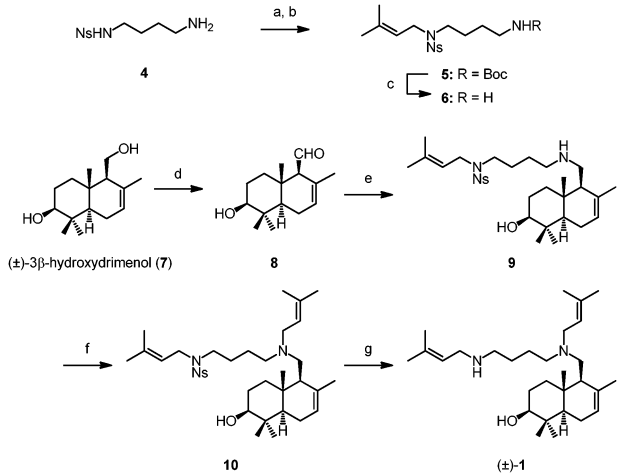


Fig. 3 The crystal structure of HCl complex of **2**. The counter anions of Cl^- and the solvent water molecules are omitted for clarity.

3 were almost identical to those of **2**, except for the signals around the C3 moiety. Therefore, the structures of **1** and **3** were elucidated based on a comparison of their spectroscopic data to those of **2** (ESI, Tables S1 and S3†). A hydroxyl group was revealed to be present at C3 of **1** by COSY and HMQC analyses. The connectivity around C3 was revealed by the HMBC correlations of H3/C5, H3/C22, H2/C3, H22/C3, and H23/C3. Meanwhile, a carbonyl group was revealed to be present at position C3 of **3** based on its ^1H and ^{13}C NMR spectra (δ_C 216.7). From an analysis of ^1H-^1H/^{13}C-^1H COSY spectra, the planar structure of **3** could be determined. Thus, **1–3** were revealed to possess different levels of oxidation at position C3. The NOESY spectrum of **1** demonstrated that the relative stereostructure of the 6,6-bicyclic rings was the same as that of **2**. The NOE correlations at H1a/H3, H3/H5 and H3/H23, and the coupling constants ($J_{2a,3}$ = 4.4 Hz, $J_{2b,3}$ = 11.0 Hz) showed that the hydroxyl group at the C3 position of **1** had an equatorial orientation (ESI†, Fig. S19A). Furthermore, the CD curve of **3** exhibited a negative Cotton effect ($\Delta\varepsilon$ = −0.7) at 294 nm. Application of the octant rule[8] clearly indicated that the axial-oriented methyl group at C10 is located in the upper position (ESI†, Fig. S22). Thus, the absolute configuration of C5, C9, and C10 in **3** was clarified to be 5*R*, 9*R*, and 10*S*. From the viewpoint of the likely similar biosynthesis within the same collected sample, we propose that the configuration of **1–3** may be superimposed on each other. Each halichonine congener included a sesquiterpene skeleton and two prenylated amine moieties as novel and unique structural features. Therefore, they are likely to be synthesized from a farnesol and an ornithine *via* unusual biosynthetic routes. To the best of our knowledge, this is the first report of the isolation of such new type of sesquiterpene alkaloids from marine organisms.

For the confirmation of the structure including the relative configuration at C3, synthesis of **1** was carried out. The synthesis began with *N*-(4-aminobutyl)-2-nitrobenzenesulfonamide (**4**), which was transformed to amine **6** in 42% yield in three steps.[9]



Scheme 1 Total synthesis of (±)-**1**. *Reagents and conditions*: (a) Boc_2O (1.2 equiv.), Et_3N (1.2 equiv), CH_2Cl_2, rt. (b) 1-bromo-3-methyl-2-butene (3.0 equiv.), K_2CO_3 (2.5 equiv.), DMF, rt, 77% yield for 2 steps. (c) TFA, CH_2Cl_2, 0 °C, 54% yield. (d) BAIB (1.2 equiv.), TEMPO (0.1 equiv.), 69% yield. (e) **6** (2.7 equiv.), $NaBH(OAc)_3$ (5.0 equiv.), DCE, rt, 44% yield. (f) 3-methyl-2-butenal (2.5 equiv.), $NaBH(OAc)_3$ (5.0 equiv.), DCE, rt, 32% yield. (g) PhSH (1.3 equiv.), K_2CO_3 (2.3 equiv.), CH_3CN, rt, 14% yield.

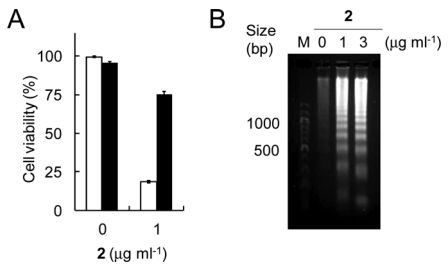


Fig. 4 Induction of apoptosis in HL60 cells by **2**. (A) HL60 cells were preincubated (solid column) or not (open column) with 50 μM Z-VAD-FMK, and then treated with 0 or 1 μg ml^{-1} of **2**. After 24 h, cell viability was determined. Values are the means ± SD of quadruplicate determinations. (B) HL60 cells were incubated with the indicated concentrations of **2** for 24 h. Cellular DNA was then extracted and electrophoresed on an agarose gel.

(±)-3β-Hydroxydrimenol (**7**) was prepared in 5 steps from farnesol as previously reported.[10] Oxidation of **7** with BAIB and a catalytic amount of TEMPO gave aldehyde **8** (78% yield) as a colorless oil.[11] The aldehyde **8** was then subjected to reductive amination with **6**, to give amine **9** (44% yield).[12] Reductive amination of **9** with 3-methyl-2-butenal afforded **10** in 32% yield. The nosyl protecting group of **10** was removed with PhSH and K_2CO_3 to give (±)-**1** and unidentified byproducts. Finally, purification of the mixture using alumina column chromatography (hexane/EtOAc, $CHCl_3$, and $CHCl_3$/MeOH) provided (±)-**1** in 14% yield (Scheme 1). Synthetic **1** was identical to the natural **1** in all respects (^1H and ^{13}C NMR, and HR-ESIMS spectra). Thus, the relative stereostructure of **1** was confirmed.

Next, compounds **1–3** were examined with regard to their growth-inhibitory activities against mammalian cancer cell lines (L1210 and PC13) using the MTT assay. After 72 hours of incubation, each compound showed moderate growth-inhibitory activity toward these cells (ESI†, Table S4). **2** was then subjected to the trypan blue dye exclusion using HL60 human leukemia cells, and showed cytotoxicity (IC_{50} value: 0.60 μg ml^{-1}). In addition, **2**-induced cell death in HL60 cells was suppressed in the presence of Z-VAD-FMK, an irreversible and cell-permeable inhibitor of caspases (Fig. 4A). DNA ladder analysis revealed that **2** induced DNA fragmentation in HL60 cells (Fig. 4B). These results indicated that **2** induced apoptosis in HL60 cells.

In conclusion, we isolated halichonines A (**1**), B (**2**), and C (**3**) from the marine sponge *H. okadai* Kadota. Based on the results of spectroscopic analyses, **1–3** were determined to be novel sesquiterpene alkaloids with a 6,6-bicyclic ring system possessing different levels of oxidation at position C3. In addition, the relative stereostructure of **1** was confirmed by successful synthesis. All three halichonine congeners showed growth-inhibitory activities against mammalian cancer cells, and **2** was revealed to induce apoptosis in HL60 cells. Thus, these halichonines may deserve further studies.

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