Tetrahedron 67 (2011) 5651–5655

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Nitrogenous sesquiterpenes from the Thai marine sponge *Halichondria* sp.

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ARTICLE INFO

Article history:
Received 11 January 2011
Received in revised form 6 May 2011
Accepted 23 May 2011
Available online 30 May 2011

Keywords:
Halichondria
Marine sponge
Nitrogenous sesquiterpene
(−)-Axisonitrile-3
(+)-Axamide-3
(−)-Halichamine
Cytotoxic activity

ABSTRACT

Five nitrogenous sesquiterpenes having an isonitrile [(−)-axisonitrile-3], a formamide [(+)-axamide-3, axamide-2 and (3*S**,5*R**,6*R**,9*R**)-3-formamido-1(10)-cadinene], and an amine [(−)-halichamine] functionality were isolated from the Thai marine sponge *Halichondria* sp., together with two steroids, ergosterol and ergosterol peroxide. (−)-Axisonitrile-3 was isolated from the natural source for the first time, while (+)-axamide-3 and (−)-halichamine were new metabolites. The structures of these compounds were elucidated on the basis of their spectroscopic data and by chemical transformations. All sesquiterpenes were tested for their cytotoxic activity against six cancer cell lines (HeLa, HuCCA-1, A549, MOLT-3, HepG2, MDA-MB231). Only (−)-axisonitrile-3 showed strong activity to the HepG2 cell line with an IC_{50} value of 1.3 μM.

1. Introduction

Marine sponges of the genus *Halichondria* have been reported to contain several types of compounds, particularly nitrogenous terpenoids,[1–4] alkaloids,[5,6] steroids,[7] macrolides,[8,9] lipids,[10] and peptides.[11] Some of these natural products exhibit biological activities, such as antifungal,[10,11] cytotoxic,[9,10] and inhibition of L-type Ca^{2+} channel[12] activities. However, there are no reports on the metabolites of this genus from material originating from Thailand.

In our continuing project to search for cytotoxic constituents from Thai marine organisms,[13] the methanolic extract of *Halichondria* sp. (CRI328) collected off PP Island was found to exhibit moderate cytotoxic activity against HeLa, HuCCA-1, A549, MOLT-3, HepG2, and MDA-MB231 cancer cell lines. Chemical investigation of the EtOAc-soluble fraction from the methanolic extract led to the isolation of five nitrogenous sesquiterpenes **1**–**5**, of which compounds **1**, **2**, and **5** were new, together with two steroids **6**, **7** (Fig. 1). The present work describes the isolation and structural elucidation of these compounds, as well as an evaluation of the cytotoxicity of compounds **1**–**5**.

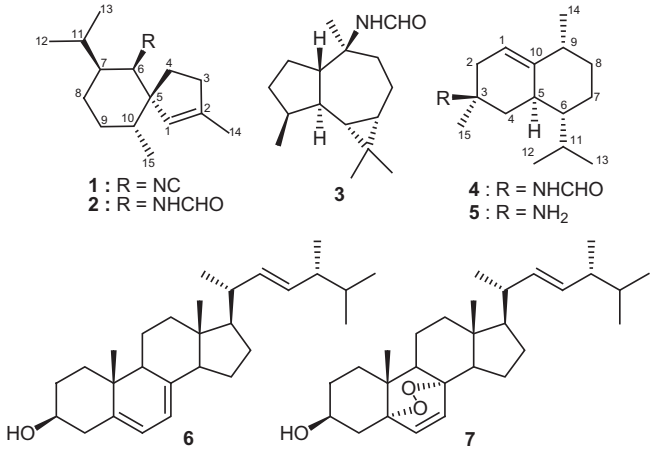

Fig. 1. Structures of isolated compounds **1**–**7**.

2. Results and discussion

The MeOH extract of the marine sponge *Halichondria* sp. was partitioned between EtOAc and H_2O. The constituents of the EtOAc portion were separated by a combination of Sephadex LH-20, silica gel as well as RP-8 HPLC chromatography, to afford seven

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0040-4020/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2011.05.094

compounds (**1**–**7**). Compounds **3** and **4** were identified as axamide-2 and (3S^*,5R^*,6R^*,9R^*)-3-formamido-1(10)-cadinene, respectively, by comparison of the physical and spectroscopic data with the literature values.[14–16] The ^{1}H and ^{13}C NMR spectroscopic data of compounds **6** and **7** were in agreement with those of ergosterol and ergosterol peroxide, respectively.[17,18]

Compound **1** was isolated as a colorless solid. Its molecular formula was determined to be $C_{16}H_{25}N$ by high resolution electrospray ionization (HRESI) mass spectrometric analysis. Inspection of the IR spectrum and ^{13}C NMR spectrum indicated the presence of an isonitrile group from the strong absorption band at 2130 cm^{-1}, together with the characteristic signal of the isonitrile carbon at δ_C 155.8 ppm and the carbon attached to this group at δ_C 64.5 ppm. These appeared as small splitting triplets due to the ^{13}C–^{15}N spin–spin couplings.[19,20] The ^{13}C NMR spectrum displayed 16 carbon signals, one of which was assigned to the isonitrile group; therefore this compound was suggested to be a sesquiterpene. The partial connectivities of C-3 to C-4, C-6 to C-10, C-7 to C-11, C-11 to C-12 and C-13, and C-10 to C-15 were deduced by ^{1}H–^{1}H COSY and HSQC analysis as shown in Fig. 2. HMBC spectral data (Table 1) indicated that compound **1** was a spiro[4,5] dec-1-ene (spiroaxane sesquiterpene) having the isonitrile group on C-6, the isopropyl group on C-7, and two methyl groups on C-2 and C-10. This compound showed the same gross structure as (+)-axisonitrile-3[19,21–29] and (−)-10-*epi*-axisonitrile-3,[19,30] previously isolated from different marine sponges. The ^{1}H and ^{13}C NMR spectroscopic data (Table 1) were coincident with those of (+)-axisonitrile-3, suggesting the same arrangement of the protons and carbons.[28] The relative stereochemistry was confirmed by the NOESY spectrum, in which the significant correlations were observed between H_{ax}-8 and H-10; H-1 and H_{ax}-9; H-1 and H-7; and H-6 and H-13 as illustrated in Fig. 3. This compound displayed an optical rotation value of $[\alpha]_D^{26}$ −79.0 (*c* 1.93, $CHCl_3$), opposite to the reports for (+)-axisonitrile-3 [lit.[21] $[\alpha]_D$ +68.4 (*c* 1.0, $CHCl_3$), lit.[28] $[\alpha]_D$ +43.8 (*c* 0.006, $CHCl_3$)]. On the basis of this evidence, compound **1** was a mirror image of (+)-axisonitrile-3 and concluded to be (−)-axisonitrile-3. The structure of (−)-axisonitrile-3 was initially reported in 1978 as a synthetic compound,[31] $[\alpha]_D^{25}$ −71.0 (*c* 0.35, $CHCl_3$) from (+)-dihydrocarvone, and the absolute stereochemistry was determined to be 5*S*,6*R*,7*S*,10*R*. On the other hand, the absolute stereochemistry for (+)-axisonitrile-3, isolated from many sources of marine sponges, was determined by comparison of the specific rotations with those of synthetic (−)-axisonitrile-3. Recently, the total synthesis of (+)-axisonitrile-3 was carried out,[32] providing further confirmation of the absolute stereochemistry. The physical and spectroscopic data were given in this present work.

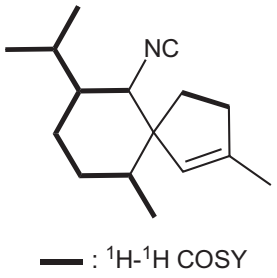

Fig. 2. ^{1}H–^{1}H COSY of (−)-axisonitrile-3 (**1**).

The divergence in the absolute stereochemistry of (−) and (+)-axisonitrile-3 could be explained by the proposed isomerization of the isopropyl group and methyl group involving the hydride shifts[35] in the intermediates in the proposed biosynthesis of these compounds.[21]

Compound **2** was obtained as a colorless oil and the molecular formula was determined to be $C_{16}H_{27}NO$ by HRESI mass spectrometric analysis. The IR spectrum suggested the presence of a formamide group from the absorption bands at 3308 and 1656 cm^{-1}. The ^{1}H and ^{13}C NMR spectra exhibited the typical signals of a compound containing the formamide group from the equilibrium of two geometrical isomers,[16,20] approximately 2:1 of cis:trans, as deduced from two formamide protons at δ_H 8.26 (br s, cis) and 8.04 (d, *J*=11.8 Hz, trans). The NMR spectral data indicated that this compound possessed the same core structure (spiro[4,5] dec-1-ene) as compound **1**, except for the presence of the formamide instead of the isonitrile group. The assignments for each isomer (Table 2) were based on the results of ^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY analyses. ^{1}H–^{1}H COSY and HSQC were used to determine the proton signals. The HMBC provided further confirmation from the significant correlations as illustrated in Fig. 4. In the NOESY spectrum, correlations were found between (i) NH proton of cis-form (δ_H 5.82,) and H-8$_{ax}$ (δ_H 0.97), H_{ax}-10 (δ_H 1.41); (ii) NH proton of trans-form (δ_H 6.22) and H_{ax}-10 (δ_H 1.41); (iii) H_{eq}-6 of cis-form (δ_H 4.22) and H-4 (δ_H 1.83, 1.65); (iv) H_{eq}-6 of trans-form (δ_H 3.28) and H-4 (δ_H 1.73, 1.65); and (v) H-7 of both forms (δ_H 1.28) and H-1 (δ_H 5.26, 5.27). The chemical shifts and the relative stereochemistry were identical to those of (−)-axamide-3, previously isolated from *Axinella cannabina*[21] and *Acanthella cavernosa*.[20] However, the optical rotation value $[\alpha]_D^{27}$ +17.5 (*c* 3.05, $CHCl_3$) was different from the reports for (−)-axamide-3 [lit.[20] $[\alpha]_D$ −15.0 (*c* 0.06, $CHCl_3$), lit.[21] $[\alpha]_D$ −6.86 (*c* 1.0, $CHCl_3$)]. Therefore, the structure of this compound was an antipode of (−)-axamide-3 and elucidated to be (+)-axamide-3. This compound showed the same absolute stereochemistry (5*S*,6*R*,7*S*,10*R*) as that of (−)-axisonitrile-3 (**1**). Furthermore, (+)-axamide-3 (**2**) was prepared by hydration

Table 1
NMR spectroscopic data of (−)-axisonitrile-3 (**1**) recorded in $CDCl_3$ (600 MHz for ^{1}H data and 150 MHz for ^{13}C data)

No.	δ_C	δ_H	HMBC (H→C)
1	123.6	5.14 (1H, br d, *J*=1.4 Hz)	2, 4, 5, 14
2	144.8		
3	35.8	2.18–2.30 (2H, m)	1, 2, 5, 14
4	34.9	1.91–2.01 (2H, m)	1, 2, 3, 5, 6, 10
5	57.1		
6	64.5	3.59 (1H$_{eq}$, br s)	1, 5, 7, 8, 10, 11, NC
7	43.8	1.12–1.19 (1H$_{ax}$, m)	8, 9, 11
8	24.9	1.74–1.84 (1H$_{eq}$, m)	6, 7, 9
		1.34 (1H$_{ax}$, td, *J*=13.2, 4.1 Hz)	6, 7, 9, 10, 11
9	31.2	1.52 (1H$_{eq}$, dt, *J*=13.6, 3.7 Hz)	5, 7, 8, 10, 15
		1.07 (1H$_{ax}$, td, *J*=13.4, 4.1 Hz)	5, 7, 8, 10, 15
10	34.3	1.74–1.87 (1H$_{ax}$, m)	5, 6, 8, 9
11	29.7	1.55–1.63 (1H, m)	6, 7, 8, 12, 13
12	20.7	0.94 (3H, d, *J*=6.7 Hz)	7, 11, 13
13	20.3	0.91 (3H, d, *J*=6.6 Hz)	7, 11, 12
14	16.9	1.74 (3H, br d, *J*=0.7 Hz)	1, 2, 3
15	16.0	0.77 (3H, d, *J*=6.7 Hz)	5, 9, 10
NC	155.8		

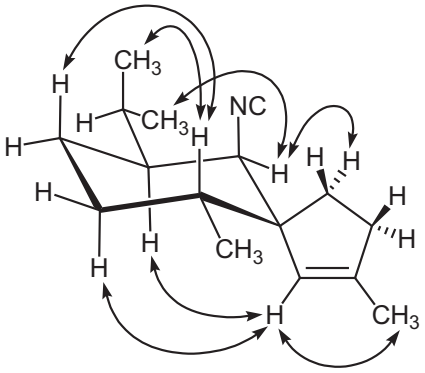

Fig. 3. Significant NOESY correlations of (−)-axisonitrile-3 (**1**).

Table 2
NMR spectroscopic data of (+)-axamide-3 (**2**) recorded in $CDCl_3$ (600 MHz for ^1H data and 150 MHz for ^{13}C data)

No.	cis		trans	
	δ_C	δ_H[a]	δ_C	δ_H[a]
1	125.1	5.26 (1H, br s)	125.2	5.27 (1H, br s)
2	143.5		143.7	
3	36.0	2.07–2.32 (2H, m)	36.1	2.07–2.32 (2H, m)
4	33.6	1.77–1.89 (1H, m)	34.5	1.58–1.78 (2H, m
		1.58–1.68 (1H, m)		
5	58.1		57.9	
6	54.0	4.22 ($1H_{eq}$, br d, J=10.9 Hz)	59.6	3.28 ($1H_{eq}$, dd, J=11.2, 2.2 Hz)
7	44.2	1.25–1.33 ($1H_{ax}$, m)	44.0	1.25–1.33 ($1H_{ax}$, m)
8	25.3	1.77–1.89 ($1H_{eq}$, m)	24.9	1.77–1.89 ($1H_{eq}$, m)
		0.92–1.06 ($1H_{ax}$, m)		0.92–1.06 ($1H_{ax}$, m)
9	31.6	1.47–1.55 ($1H_{eq}$, m)	31.3	1.47–1.55 ($1H_{eq}$, m)
		1.09–1.21 ($1H_{ax}$, m)		1.09–1.21 ($1H_{ax}$, m)
10	35.5	1.36–1.46 ($1H_{ax}$, m)	35.8	1.36–1.46 ($1H_{ax}$, m)
11	29.5	1.28–1.34 (1H, m)	28.8	1.37–1.44 (1H, m)
12	21.2	0.92 (3H, d, J=6.0 Hz)	20.6	0.90 (3H, d, J=6.6 Hz)
13	20.9	0.87 (3H, d, J=6.0 Hz)	20.5	0.85 (3H, d, J=6.6 Hz)
14	16.9	1.74 (3H, s)	16.9	1.76 (3H, s)
15	16.2	0.76 (3H, d, J=6.6 Hz)	16.1	0.76 (3H, d, J=6.6 Hz)
NH		5.82 (1H, d, J=10.3 Hz)		6.22 (1H, t, J=11.0 Hz)
CHO	161.1	8.26 (1H, s)	164.5	8.04 (1H, d, J=11.7 Hz)

[a] ^1H NMR signals were assigned from ^1H–^1H COSY and HSQC.

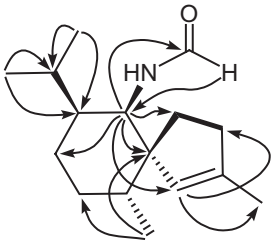

Fig. 4. Significant HMBC correlations of (+)-axamide-3 (**2**).

of (−)-axisonitrile-3 (**1**) (see Experimental section). The spectroscopic data and the optical rotation value $[\alpha]_D^{27}$ +16.8 (*c* 2.52, $CHCl_3$) of the semi-synthetic (+)-axamide-3 (**2**) were identical to those of the natural product, providing strong evidence of the structure.

Compound **5** was isolated as a colorless oil. The molecular formula was determined to be $C_{15}H_{27}N$ by HRESI mass spectrometric analysis. The ^1H and ^{13}C NMR spectral data were closely related to those of compound **4**. However, the NMR spectra displayed only one set of proton and carbon signals, different from those of compound **4**, which showed two sets of signals due to the presence of the formamide group. Thus, this compound was suggested to be a functional group analogue of compound **4**. The formamide group in compound **4** was replaced by the amine group, which was supported by the absorption band at 3366 cm^{-1} in the IR spectrum. The ^{13}C NMR spectral data indicated that this compound possessed four methyls, four methylenes, five methines, and two quaternary carbons. The structure assignment was confirmed by the detailed analysis of ^1H–^1H COSY, HSQC, HMBC, and NOESY experiments. ^1H–^1H COSY and HSQC spectra revealed the connectivities of C-1 to C-2, C-4 to C-9, C-6 to C-11, C-11 to C-12, C-11 to C-13, and C-9 to C-14 as shown in Fig. 5. HMBC correlations (Table 3) provided further confirmation of the structure as related to compound **4**. The relative stereochemistry was deduced by the NOESY spectrum (Fig. 6), in which cross peak correlations were found between H_{ax}-5 and H_{ax}-7, H-14, H-15; and H_{ax}-7 and H-14, indicating the same relative stereochemistry as that of compound **4**. In addition, compound **4** was hydrolyzed to the analogue amine **5** with 10% NaOH (see Experimental section) to compare the physical and spectroscopic

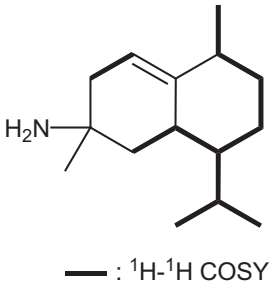

Fig. 5. ^1H–^1H COSY of (−)-halichamine (**5**).

Table 3
NMR spectroscopic data of (−)-halichamine (**5**) recorded in DMSO-d_6 (600 MHz for ^1H data and 150 MHz for ^{13}C data)

No.	δ_C	δ_H	HMBC (H→C)
1	117.7	5.28 (1H, dt, J=5.9, 1.8 Hz)	2, 3, 5, 9
2	40.0	1.74 ($1H_{eq}$, ddt, J=16.6, 5.9, 2.5 Hz)	1, 3, 4, 10, 15
		1.86–1.92 ($1H_{ax}$, m)	1, 3, 10, 15
3	48.5		
4	42.6	1.63 ($1H_{eq}$, ddd, J=12.5, 6.0, 2.5 Hz)	2, 3, 5, 10, 15
		9.95–1.01 ($1H_{ax}$, m)	—
5	33.9	1.95–2.04 ($1H_{ax}$, m)	1, 4, 6, 10
6	49.6	0.88–0.94 ($1H_{ax}$, m)	—
7	17.9	1.37 ($1H_{eq}$, dq, J=13.4, 3.4 Hz)	5, 6, 8, 9, 11
		1.28 ($1H_{ax}$, qd, J=13.0, 3.4 Hz)	5, 6, 8, 9, 11
8	31.9	1.51–1.57 ($1H_{eq}$, m)	6, 7, 9, 10, 14
		1.43 ($1H_{ax}$, tt, J=13.4, 4.1 Hz)	6, 7, 9, 10, 14
9	36.8	2.36–2.46 ($1H_{eq}$, m)	1, 5, 7, 8, 10, 14
10	142.7		
11	26.1	1.85–1.92 (1H, m)	5, 6, 7, 12, 13
12	14.7	0.76 (3H, d, J=6.9 Hz)	6, 11, 13
13	21.3	0.86 (3H, d, J=6.9 Hz)	6, 11, 12
14	19.7	0.97 (3H, d, J=7.2 Hz)	8,9,10
15	25.2	0.91 (3H, s)	2, 3, 4

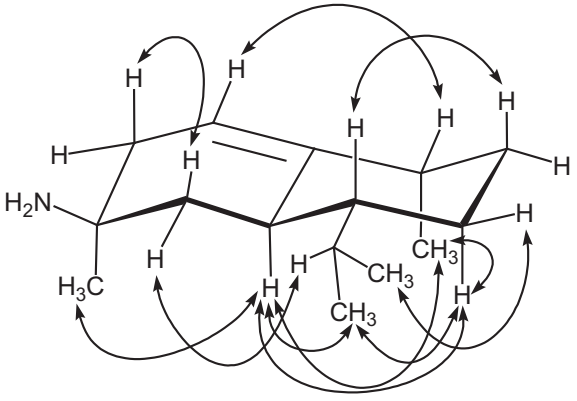

Fig. 6. Significant NOESY correlations of (−)-halichamine (**5**).

data, which were identical to each other. Consequently, the structure of this compound was elucidated as shown, namely (−)-halichamine.

The sequiterpenes **1**–**5** were evaluated for their cytotoxic activity against six tumor cell lines, including HeLa (human cervical adenocarcinoma), HuCCA-1 (human cholangiocarcinoma), A549 (human non-small-cell lung carcinoma), MOLT-3 (mouse T-lymphoblastic leukemia), HepG2 (human hepatocellular carcinoma), and MDA-MB231 (human hormone-independent breast cancer). The preliminary screening of the sponge methanolic extract of *Halichondria* sp. exhibited moderate cytotoxic activity against all

cancer cell lines. However, for cytotoxic testing of pure compounds (Table 4), only the (−)-axisonitrile-3 selectively exhibited against HepG2 cell line with IC_{50} valued of 1.3 μM. Compound **4** [(3*S**,5*R**,6*R**,9*R**)-3-formamido-1(10)-cadinene] showed moderate cytotoxic activity against HeLa, MOLT-3, and HepG2 cell lines with IC_{50} valued of 32.1, 33.4, and 16.0 μM, respectively, while compound **5** [(−)-halichamine] also showed moderate cytotoxic activity against HuCCA-1, MOLT-3, HepG2, and MDA-MB231 cell lines with IC_{50} valued of 20.3, 34.6, 19.9, and 22.6 μM, respectively, as shown in Table 4. It seemed that the isonitrile group in sesquiterpenes is the important functionality for potent cytotoxic activity. The diterpene isonitriles, kalihinene, isokalihinol, and kalihinol D are known to exhibit cytotoxic activity.[29,34] (+)-Axisonitrile-3 has also been reported to exhibit cytotoxicity against A549 cell line (IC_{50} 10.6 μM),[29] while (−)-axisonitrile-3 (**1**) was inactive (Table 4).

Table 4
Cytotoxic activity of sesquiterpenes **1**–**5**

Compound	IC_{50} (μM)					
	HeLa	HuCCA-1	A549	MOLT-3	HepG2	MDA-MB231
1	>100	>100	>100	75.2	1.3	>100
2	—[a]	>100	>100	63.9	>100	>100
3	96.2	>100	>100	57.0	66.2	94.2
4	32.1	84.2	>100	33.4	16.0	78.2
5	—[a]	20.3	45.2	34.6	19.9	22.6
Doxorubicin	0.074	0.791	0.478	—[a]	0.442	0.405

[a] Not determined.

3. Conclusions

The present work reported the isolation and identification of five nitrogenous sesquiterpenes **1**–**5** and two steroids **6**, **7** from Thai marine sponge *Halichondria* sp. (−)-Axisonitrile-3 (**1**) and (+)-axamide-3 (**2**) were antipodes of the previously isolated compounds obtained from the different marine sponges. It is noteworthy that the opposite stereochemistry has been found from the natural source. Axamide-2 (**3**) having an aromadendrane skeleton has previously been isolated from the marine sponge *Axinella cannabina*[14] and the marine nuribranch *Hexabranchus sanguineus*.[15] This skeleton type has also been isolated from the *Halichondria* sp.[3,4] found in Okinawa. The presence of the cadinene skeleton, (3*S**,5*R**,6*R**,9*R**)-3-formamido-1(10)-cadinene (**4**) and (−)-halichamine (**5**), was anticipated to be the constituent of our material due to the fact that this skeleton was previously found in the related species, *Halichondria panicea*.[1] Ergosterol (**6**) and ergosterol peroxide (**7**) are commonly found in natural sources. From the cytotoxic activity results, only (−)-axisonitrile-3 (**1**) displayed promising activity to the HepG2 cell line as compared to doxorubicin. This study has confirmed the typical presence of nitrogenous sesquiterpenes metabolites in *Halichondria* sponges and validates the pattern of chemical contituents reported in the literature for several species belonging to this genus.[1–4]

4. Experimental

4.1. General experimental procedures

^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 using a Bruker AVANCE 600 spectrometer (600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR). HRMS values were performed on a Bruker Micro TOF-LC mass spectrometer. Optical rotations were measured on a JASCO P-1020 polarimeter using a cylindrical glass cell (10 mm i.d.×10 mm). FTIR spectra were recorded on a universal attenuated total reflectance attached to a Perkin–Elmer Spectrum One spectrometer. For column chromatography, silica gel (70–230 mesh, Merck) and Sephadex LH-20 (Amersham Biosciences) were used. HPLC was performed using a system comprised of Thermo Separation Products (San Jose, CA) instruments (P4000 pump, UV6000LP for analysis, UV2000 for preparative). A reverse phase column (SunFire Prep C8 250×19 mm, 10 μm, Waters) was used for preparative HPLC.

4.2. Animal material

The marine sponge *Halichondria* sp. (Order Halichondrida, Family Halichondriidae) was collected off PP Island in the Andaman Sea, Southern Thailand in April 2005 by scuba diving at a depth of 10–15 m, and identified by Dr. Sumaitt Putchakarn, Institute of Marine Science, and Burapha University, Thailand. A voucher specimen (CRI328) was deposited at the Laboratory of Natural Products, Chulabhorn Research Institute, Bangkok, Thailand.

4.3. Extraction and isolation

A frozen sample (wet wt., 620 g) of sponge *Halichondria* sp. (CRI328) was chopped finely and extracted with MeOH (3×2 L) at room temperature. After removal of the organic solvent by evaporation, the aqueous layer was partitioned with EtOAc (3×200 mL). The EtOAc phase was combined and concentrated to give a dark brown solid (5.85 g). This EtOAc extract was subjected to vacuum–liquid chromatography on silica gel and eluted with a mixture of *n*-hexane and EtOAc (stepwise, 1:1 to pure EtOAc) to obtain seven fractions (A–G). Fraction B (419.9 mg) was applied to a Sephadex LH-20 column using solvent system CH_2Cl_2/acetone (1:1) to provide three fractions (B-1–B-3). Fraction B-2 (395.0 mg) was purified by preparative RP-8 HPLC using 80% aqueous MeOH as solvent system (flow rate 8.0 mL/min, UV 220 nm) to afford compound **1** (21.5 mg). A solution of fraction D was crystallized to yield compound **6** (199.8 mg). Fraction F (901.7 mg) was applied to a Sephadex LH-20 column using solvent system CH_2Cl_2/acetone (1:1) to obtain five fractions (F-1 to F-5). Fraction F-3 (614.1 mg) was further purified by preparative RP-8 HPLC using gradient solvent system 73→92% aqueous MeOH to provide compounds **2** (39.2 mg), **3** (21.4 mg), **4** (45.5 mg), and **7** (9.5 mg). Finally, fraction G (610.6 mg) was applied to a Sephadex LH-20 column using solvent system CH_2Cl_2/MeOH (1:4) to obtain four fractions (G-1–G-5). Fraction G-5 (33.6 mg) was purified by a small silica gel column with CH_2Cl_2/MeOH (49:1) as solvent system to afford compound **5** (2.6 mg).

*4.3.1. (−)-Axisonitrile-3 (**1**).* Colorless solid, $[\alpha]_D^{27}$ −79.0 (*c* 1.93, $CHCl_3$); [(+)-axisonitrile-3, lit.[21] $[\alpha]_D$ +68.4 (*c* 1.0, $CHCl_3$); lit.[28] $[\alpha]_D$ +43.8 (*c* 0.006, $CHCl_3$)]; IR (UATR) ν_{max} 2925, 2130, 1658, 1463 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$): see Table 1; HRESIMS, *m*/*z*: 254.1875 $[M+Na]^+$ (calcd for $C_{16}H_{25}NNa$, 254.1879).

*4.3.2. (+)-Axamide-3 (**2**).* Colorless oil, $[\alpha]_D^{27}$ +17.5 (*c* 3.05, $CHCl_3$); [(−)-axamide-3, lit.[20] $[\alpha]_D$ −15.0 (*c* 0.06, $CHCl_3$); lit.[21] $[\alpha]_D$ −6.86 (*c* 1.0, $CHCl_3$)]; IR (UATR) ν_{max} 3308, 3044, 2927, 1656, 1533, 1462 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$): see Table 2; HRESIMS, *m*/*z*: 250.2161 $[M+H]^+$ (calcd for $C_{16}H_{28}NO$, 250.2165).

*4.3.3. Synthetic conversion of (−)-axisonitrile-3 (1) into (+)-axamide-3 (**2**).* Acetic acid (2.0 mL) was added to a solution of (−)-axisonitrile-3 (**1**, 20.1 mg) in EtOH (2.0 mL). After stirring at room temperature for 18 h, the reaction was concentrated and purified by a silica gel column using *n*-hexane/EtOAc (4:1) to provide (+)-axamide-3 (**2**, 19.5 mg) as a colorless oil, $[\alpha]_D^{27}$ +16.8 (*c* 2.52, $CHCl_3$) and HRESIMS *m*/*z*: 250.2159 $[M+H]^+$ (calcd for

$C_{16}H_{28}NO$, 250.2165). The NMR spectroscopic data were identical to those of the natural product isolated directly from the sponge.

*4.3.4. Axamide-2 (**3**).* Colorless oil, $[\alpha]_D^{27}$ +22.9 (*c* 1.35, $CHCl_3$); lit.[14] $[\alpha]_D^{20}$ +37.5 (*c* 0.9, $CHCl_3$) and lit.[15] $[\alpha]_D^{20}$ +79.0 (*c* 0.14, $CHCl_3$); IR (UATR) ν_{max} 3292, 3056, 1688, 1537, 1455, 1380 cm^{-1}; HRESIMS, *m*/*z*: 250.2161 $[M+H]^+$ (calcd for $C_{16}H_{28}NO$, 250.2165).

4.3.5. (3S,5R*,6R*,9R*)-3-Formamido-1(10)-cadinene (**4**).* Colorless solid, $[\alpha]_D^{27}$ −47.2 (*c* 0.57, $CHCl_3$); lit.[16] $[\alpha]_D$ −43.6 (*c* 0.55, $CHCl_3$); IR (UATR) ν_{max} 3283, 3048, 1666, 1532, 1542, 1453, 1380 cm^{-1}; HRESIMS, *m*/*z*: 250.2161 $[M+H]^+$ (calcd for $C_{16}H_{28}NO$, 250.2165).

*4.3.6. (−)-Halichamine (**5**).* Colorless oil, $[\alpha]_D^{27}$ −5.9 (*c* 0.15, MeOH); IR (UATR) ν_{max} 3366, 2924, 1736, 1603, 1462 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6): see Table 3; HRESIMS, *m*/*z*: 222.2212 $[M+H]^+$ (calcd for $C_{15}H_{28}N$, 222.2216).

4.3.7. Synthetic conversion of (3S,5R*,6R*,9R*)-3-formamido-1(10)-cadinene (4) into (−)-halichamine (**5**).* NaOH (10%, 5.0 mL) was added to a solution of (3S*,5R*,6R*,9R*)-3-formamido-1(10)-cadinene (**4**, 10.0 mg) in EtOH (15.0 mL), and then heated at 67 °C for 6 h. After removal of EtOH, the aqueous phase was extracted with EtOAc (3×15 mL). The EtOAc extract was evaporated to provide (−)-halichamine (**5**, 6.5 mg) as a colorless oil, $[\alpha]_D^{27}$ −6.1 (*c* 0.17, MeOH) and HRESIMS *m*/*z*: 222.2207 $[M+H]^+$ (calcd for $C_{15}H_{28}N$, 222.2216). The NMR spectroscopic data were identical to those of the natural product.

4.4. Tests for cytotoxic activity in vitro

The cytotoxicity tests for each compound were monitored at several concentration levels against six tumor cell lines; HeLa (human cervical adenocarcinoma), HuCCA-1 (human cholangiocarcinoma), A549 (human non-small-cell lung carcinoma), MOLT-3 (mouse T-lymphoblastic leukemia), HepG2 (human hepatocellular carcinoma), and MDA-MB231 (human hormone-independent breast cancer) and performed using the same method as previously described.[33]

Acknowledgements

The authors thank CRI colleagues from the Laboratory of Immunology, and the Integrated Research Unit for cytotoxicity tests. We also thank Dr. Sumaitt Putchakarn, Head of Marine Biodiversity Research Unit for identification of the animal material.

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