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Bioorganic & Medicinal Chemistry 14 (2006) 6954–6961
Bioorganic & Medicinal Chemistry

Cytotoxic labdane alkaloids from an ascidian *Lissoclinum* sp.: Isolation, structure elucidation, and structure–activity relationship

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Received 9 May 2006; revised 16 June 2006; accepted 17 June 2006
Available online 18 July 2006

Abstract—Four labdane alkaloids, haterumaimides N–Q (**1**–**4**), were isolated from an ascidian *Lissoclinum* sp. and their structures were elucidated by chemical and spectral analyses. Investigation of the structure–activity relationships of haterumaimides J–K, N–Q, and 14 related compounds suggested that the presence of hydroxyl groups at C-6, C-7, C-12, and C-18, a chlorine atom at C-2, and an imido NH in ring **C** should be essential for cytotoxicity against P388 cells.

1. Introduction

It has been amply demonstrated that ascidians are prolific producers of novel bioactive secondary metabolites.[1–3] A significant number of ascidian-derived compounds have entered into preclinical and clinical trials as antitumor agents.[4] Examples include didemnin B (went through phase II clinical trials but was withdrawn by NCI because it proved to be too toxic to use as a drug),[5] aplidine (currently under phase II clinical trials in Europe and Canada),[6] and ecteinascidin 743 (passed through phase II trials for the treatment of sarcoma and is enlisted for phase III in Europe).[7] The biomedical potential of the ascidian metabolites has resulted in the focused interest in these primitive chordates. As part of our ongoing chemical and biological studies on Okinawan marine organisms,[8,9] we investigated an ascidian *Lissoclinum* sp. collected off the coast of Hateruma Island. A lipophilic extract of the ascidian showed that it could inhibit the division of fertilized sea urchin eggs. In our recent report, we described the isolation and structure elucidation of haterumaimides A–I (**5**–**13**)[10,11] together with dichlorolissoclimide (**16**)[12] and chlorolissoclimide (**17**)[13] from the toxic extract of the ascidian. Further bioassay-guided fractionation of the same extract led to the isolation of four new cytotoxic labdane alkaloids, haterumaimides N–Q (**1**–**4**)[14], and haterumaimides J (**14**) and K (**15**).[15] Haterumaimides L (**21**) and M (**22**), and 3β-hydroxychlorolissoclimide (**23**) were recently reported from the molluscs *Pleurobranchus albiguttatus* and *Pleurobranchus forskalii* by Schmitz's group.[16] Labdane alkaloids of this type have attracted considerable interest because of their potential use as protein synthesis inhibitors,[17] as antitumor drugs,[18] and due to their unusual structural features.[19–21] In this report, we describe the isolation, structure elucidation, and biological activities of haterumaimides N–Q (**1**–**4**), J (**14**), and K (**15**), and the structure–activity relationships (SARs) of haterumaimides A–K (**5**–**15**), N–Q (**1**–**4**), dichlorolissoclimide (**16**), chlorolissoclimide (**17**), and three synthetic derivatives of **17** (**18**–**20**).

Keywords: Diterpene; Labdane alkaloid; Haterumaimide; Cytotoxicity; Structure–activity relationship.
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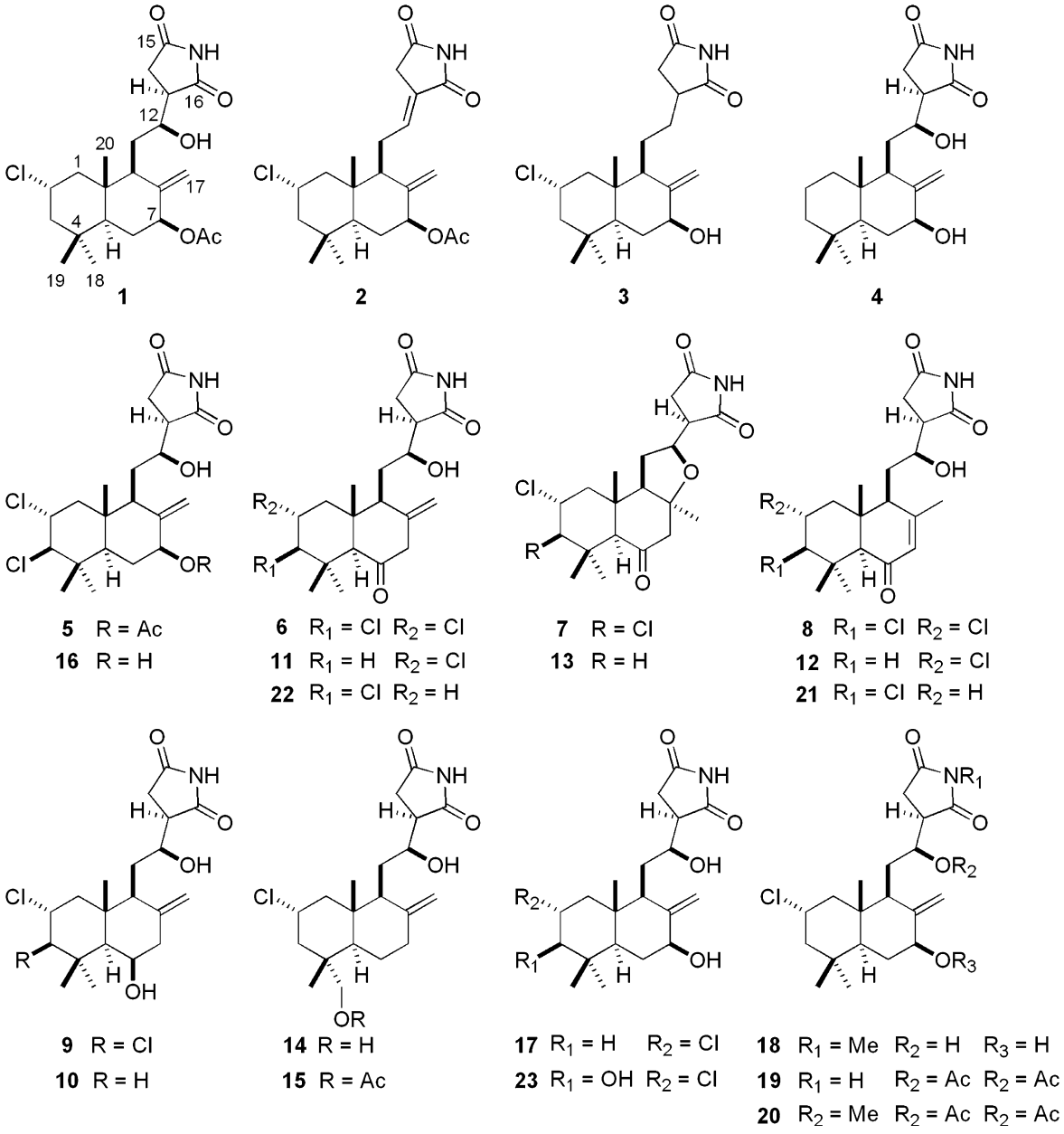
doi:10.1016/j.bmc.2006.06.043



2. Results and discussion

2.1. Isolation, structure elucidation, and relative stereochemistries

The encrusting gray ascidian *Lissoclinum* sp. collected off the coast of Hateruma Island, Okinawa, was extracted with acetone. The acetone extract was first partitioned between H_2O and EtOAc. The EtOAc extract was suspended in aqueous MeOH and the suspension was successively partitioned with hexanes, $CHCl_3$, and 1-BuOH. Only the $CHCl_3$ extract inhibited the division of fertilized sea urchin eggs. Bioassay-directed fractionation of $CHCl_3$ extract by a series of chromatographic processes, including silica gel and ODS column chromatography, and silica gel and ODS HPLC, led to the isolation of haterumaimides A–I (**5**–**13**), dichlorolissoclimide (**16**), and chlorolissoclimide (**17**). Further HPLC separation of the minor toxic fractions yielded haterumaimides N (**1**, 13.9×10^{-4} % of wet weight), O (**2**, 1.1×10^{-4} %), P (**3**, 1.0×10^{-4} %), Q (**4**, 2.5×10^{-4} %), J (**14**, 7.5×10^{-4} %), and K (**15**, 3.2×10^{-4} %).

Haterumaimide N (**1**) had a molecular formula of $C_{22}H_{32}ClNO_5$, as deduced from HRFABMS [*m*/*z* 426.2058 $(M+H)^+$, Δ +1.1 mmu and *m*/*z* 428.2062 $(M+H+2)^+$; intensity ratio (3:1)]. Thus, the molecular formula requires seven degrees of unsaturation. The IR spectrum of **1** contained characteristic absorption bands at ν_{max} 3505 (OH), 3400 (NH), 1720 (C=O), 1705 (C=O), 1700 (C=O), and 1605 (C=O), and the UV spectrum showed one absorption maxima at λ_{max} ($\log\varepsilon$) 215 nm (3.6). The detailed analysis of ^1H and ^{13}C NMR data (Tables 1 and 2) of **1** indicated the presence of three methyl groups, an exomethylene, two oxymethines, a chloromethine, ester, and imide (or amide) carbonyls. Therefore, haterumaimide N must be tricyclic

Table 1. [1]H NMR[a] data for haterumaimides N–Q (**1**–**4**)

Position	**1**[b] δ (mult, *J*/Hz)	**2**[c] δ (mult, *J*/Hz)	**3**[c] δ (mult, *J*/Hz)	**4**[c] δ (mult, *J*/Hz)
1β	2.20 (ddd, 13.0, 3.5, 1.5)	2.20 (ddd, 12.5, 3.5, 1.5)	2.18 (ddd, 12.5, 4.0, 1.5)	1.60 (ddd, 13.5, 5.5, 3.0)
1α	1.25 (dd, 13.0, 12.5)	1.38 (t, 12.5)	1.35 (dd, 13.0, 12.5)	0.91 (dd, 13.5, 4.0)
2	4.13 (tt, 12.5, 3.5)	4.15 (tt, 12.5, 3.5)	4.36 (tt, 12.5, 4.0)	1.50 (m)
				1.42 (m)
3β	2.05 (ddd, 12.5, 3.5, 1.5)	2.05 (ddd, 12.5, 3.6, 1.5)	1.95 (ddd, 13.0, 4.0, 1.5)	1.36 (ddd, 13.0, 5.5, 3.5)
3α	1.23 (t, 12.5)	1.25 (t, 12.5)	1.50 (dd, 13.0, 12.5)	1.11 (m)
5	1.52 (dd, 12.0, 5.0)	1.55 (dd, 12.5, 4.0)	1.21 (dd, 12.5, 3.5)	1.12 (dd, 12.0, 3.5)
6β	1.98 (dt, 13.0, 5.0)	1.34 (m)	1.88 (ddd, 12.5, 6.0, 3.4)	1.87 (ddd, 12.0, 5.5, 3.5)
6α	1.35 (dt, 13.0, 12.0)	1.24 (m)	1.08 (q, 12.0)	1.13 (m)
7	5.10 (dd, 12.0, 5.0)	5.15 (dd, 12.5, 5.0)	3.76 (ddd, 12.5, 5.8, 5.0)	3.75 (ddd, 11.0, 5.5, 4.5)
9	1.74 (dd, 10.0, 6.0)	1.95 (dd, 9.5, 6.5)	1.63 (m)	1.44 (dd, 10.0, 5.5)
11a	1.80 (ddd, 14.0, 8.0, 6.0)	2.39 (m)	1.50 (m)	1.58 (m)
11b	1.58 (ddd, 14.0, 10.0, 7.5)	2.32 (ddd, 13.0, 9.5, 7.0)	1.35 (m)	1.40 (m)
12	4.28 (dt, 7.5, 1.5)	6.65 (tt, 6.5, 2.5)	1.50 (m)	3.99 (dddd, 9.0, 6.5, 5.0, 2.0)
			1.45 (m)	
13	2.88 (ddd, 9.0, 5.0, 1.5)		2.80 (dddd, 9.0, 8.5, 4.5, 1.5)	2.80 (ddd, 8.5, 5.0, 2.0)
14a	2.82 (dd, 17.5, 5.0)	3.28 (d, 2.5)	2.74 (dd, 17.5, 9.0)	2.52 (dd, 17.5, 5.0)
14b	2.66 (dd, 17.5, 9.0)	3.28 (d, 2.5)	2.36 (dd, 17.5, 4.5)	2.46 (dd, 17.5, 8.5)
17a	5.50 (br s)	5.10 (br s)	5.20 (d, 1.5)	5.18 (br s)
17b	4.83 (br s)	4.55 (br s)	4.70 (d, 1.5)	4.76 (br s)
18	0.94 (s)	0.98 (s)	0.92 (s)	0.85 (s)
19	0.82 (s)	0.86 (s)	0.81 (s)	0.75 (s)
20	0.73 (s)	0.78 (s)	0.65 (s)	0.57 (s)
22	2.10 (s)	2.12 (s)		
NH	8.45 (s)	11.0 (s)	11.04 (s)	10.99 (s)
OH-7			4.92 (d, 5.0)	4.91 (d, 4.5)
OH-12				4.92 (d, 5.0)

[a] Recorded at 500 MHz.
[b] Recorded in $CDCl_3$ (δ_H 7.24).
[c] Recorded in DMSO-d_6 (δ_H 2.49).

Table 2. [13]C NMR[a] data for haterumaimides N–Q (**1**–**4**), J (**14**), and K (**15**)

Compound	**1**[b] δ	**2**[c] δ	**3**[c] δ	**4**[c] δ	**14**[c] δ	**15**[b] δ
1	49.1	49.2	48.7	38.0	48.4	49.0
2	55.0	54.8	56.8	18.9	57.3	54.7
3	51.8	51.6	51.7	41.5	45.9	46.2
4	35.9	35.9	35.6	33.1	40.3	39.1
5	51.9	51.7	50.8	52.2	46.0	48.4
6	29.8	29.6	33.0	33.5	22.9	23.6
7	74.5	73.9	71.7	72.0	37.0	37.5
8	144.6	143.9	149.8	151.1	147.6	147.1
9	51.6	53.9	53.0	49.8	51.4	53.5
10	41.5	41.2	41.2	38.7	41.3	41.7
11	29.0	24.7	28.8	29.5	30.0	29.3
12	68.7	138.9	20.2	68.8	68.9	69.2
13	46.8	126.7	40.7	45.3	45.5	46.8
14	29.4	33.0	35.0	28.9	29.0	29.4
15	179.2	173.2	181.6	181.1	181.1	178.8
16	176.8	168.9	178.2	178.8	178.8	176.4
17	106.1	106.4	104.2	103.6	107.7	109.0
18	33.1	33.1	32.9	33.3	69.3	71.7
19	22.0	22.0	21.7	21.5	17.9	18.0
20	14.8	14.8	14.5	14.2	15.0	15.4
21	170.2	169.8				171.0
22	21.2	21.1				21.0

[a] Recorded at 125 MHz.
[b] Recorded in $CDCl_3$ (δ_C 77.0).
[c] Recorded in DMSO-d_6 (δ_C 39.5).

to account for the seven sites of unsaturation required by the molecular formula. The presence of a unique succinimide moiety in **1** was deduced from the HMBC correlations of NH proton signal at δ_H 8.45 to δ_C 179.2 (s), 176.8 (s), 46.8 (d), and 29.4 (t). Thus, the ^1H and ^{13}C NMR data suggested that the remaining two rings are carbocyclic. Further detailed analysis of DQF-COSY (Fig. 1) and HOHAHA spectral data allowed us to elucidate three partial structures, C1–C3, C5–C7, and C9–C14. The connectivity of these partial structures was established from the HMBC correlations (Fig. 1) of H_3-18/C-3, H_3-19/C-3, H_3-18/C-4, H_3-19/C-4, H_2-3/C-4, H-5/C-4, H_3-18/C-5, H_3-19/C-5, H_2-7/C-8, H-9/C-8, H_2-17/C-7, H_2-17/C-8, H_2-17/C-9, H-9/C-10, H_2-1/C-10, H_3-20/C-10, H_3-20/C-1, H_3-20/C-9, H_3-20/C-5, H-5/C-10, H-13/C-16, NH/C-16, H_2-14/C-15, and NH/C-15 to give the entire carbon framework of **1**, leaving the chlorine atom at C-2, a hydroxyl group (or an acetoxyl group) at C-12, and an exomethylene double bond between C-8 and C-17. The downfield proton chemical shift of H-7 (δ_H 5.10) compared to that of H-12 (δ_H 4.28) indicated that the acetoxyl group residing at C-7 and the hydroxyl group is at C-12. The position of acetoxyl group resides at C-7 was further supported by a strong HMBC correlation between δ_H 5.10 (s) and δ_C 170.2 (s). Therefore, the planar structure of haterumaimide N (**1**) was elucidated to be a monochlorinated labdane alkaloid with a succinimide moiety, as shown in **1**. The relative stereochemistry of the decalin part of **1** was determined to be 2*S**, 5*S**, 7*S**, 9*R**, and 10*S** from the NOESY correlations (Fig. 2) of H-2/H_3-20, H-2/H_3-19, H_3-19/H_3-20, H-1β/H_3-20, H-3α/H-1α, H-3α/H_3-18, H-5/H-3α, H-5/H-1α, H-5/H-7, H-5/H-9, H-5/H-6α, H-6β/H_3-20, H-6β/H_3-19, H-7/H-9, and H-9/H-1α,

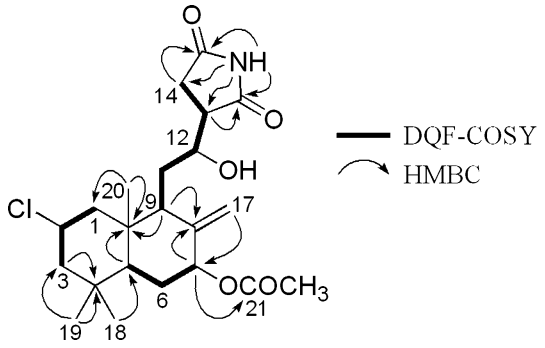


Figure 1. Planar structure of haterumaimide N (**1**) based on DQF-COSY and selected HMBC correlations.

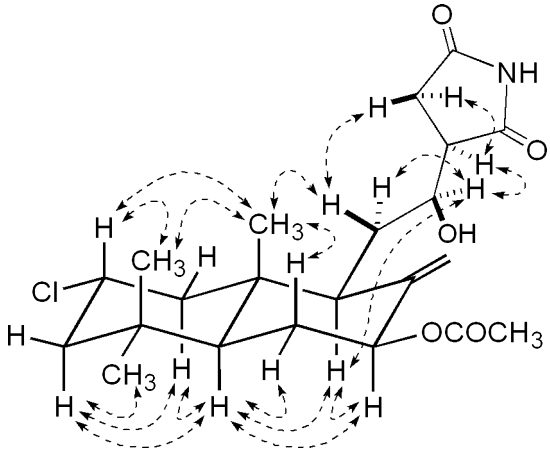


Figure 2. Selected NOEs of haterumaimide N (**1**).

together with large vicinal coupling constants of H-2 (tt, $J = 12.0$, 3.5 Hz), H-5 (dd, $J = 12.0$, 5.0 Hz), and H-7 (dd, $J = 12.0$, 5.0 Hz). It was more challenging to assign the relative configurations at C-12 and C-13 because of the rotational freedom enjoyed by the succinimide moiety in contrast to the bulk of the molecule. However, a careful examination of the NOESY correlations of H-12/H-13, H-12/H-9, H-11a/H-12, H-13/H-14a, and H-11b/H-14b together with vicinal coupling constants of 1.5 Hz for H-13/H-12, 5.50 Hz for H-13/H-14a, and 9.0 Hz for H-13/H-14b, and comparison with related compounds isolated from the same organisms suggested the 12*S** and 13*R** relative configurations.[10–14] Base-catalyzed hydrolysis of **1** furnished chlorolissoclimide (**17**). The ^{1}H and ^{13}C NMR data and $[\alpha]_D$ of the hydrolysate of **1** were identical to those of **17**, which confirmed the structure of haterumaimide N (**1**).

The molecular formula of haterumaimide O (**2**) was deduced to be $C_{22}H_{30}ClNO_5$ based on HRFABMS [*m*/*z* 408.1914 $(M+H)^+$, Δ −2.7 mmu]. The molecular formula of **2** suggested an additional degree of unsaturation compared to that of haterumaimide N (**1**). The ^{1}H and ^{13}C NMR data (Tables 1 and 2) were similar to those of **1** except for the two carbon signals resonating at δ_C 138.9 (d) and 126.7 (s), and a proton chemical shift at δ_H 6.65 (tt, $J = 7.5$, 2.5 Hz). The HMBC correlations of δ_H 6.65 (H-12) to δ_C 126.7 (s, C-13), 24.7 (t, C-11), and 33.0 (t, C-14) clearly demonstrated the presence of a double bond between C-12 and C-13 in **2**. The double bond was tentatively assigned an *E* geometry as suggested by the relatively low field chemical shift (δ_H 6.65)[22] of H-12 due to deshielding of the carbonyl (C-16) and the lack of NOEs between H-12 and H_2-14. A detailed analysis of the 1D and 2D NMR data finally disclosed the planar structure of haterumaimide O. Since the NOEs observed for the decalin part of **2** resembled those described above for **1**, both compounds had to possess an identical stereochemistry for the decalin moiety. We could not conclude that **2** is a natural product, since it is possible that **2** may be derived from **1** by dehydration during isolation.

Haterumaimide P (**3**) was isolated as a minor constituent and had a molecular formula of $C_{22}H_{30}ClNO_3$, as determined by HRFABMS [*m*/*z* 368.1982 $(M+H)^+$, Δ −1.0 mmu]. The ^{1}H and ^{13}C NMR data (Tables 1 and 2) were similar to those of chlorolissoclimide (**17**). In contrast to **17**, compound **3** contained a methylene instead of an oxymethine. Extensive analysis of the 1D and 2D NMR data led to the planar structure of **3**. Although the relative stereochemistry of the decalin part of **3** was determined in the same manner as described above for **1**, the relative stereochemistry at C-13 was not assigned.

The molecular formula of haterumaimide Q (**4**) was deduced to be $C_{22}H_{31}NO_4$ based on HRFABMS [*m*/*z* 372.2159 $(M+Na)^+$, Δ +0.8 mmu] and ^{13}C NMR data. Despite the absence of a chlorine atom in the molecular formula, the ^{1}H, ^{13}C NMR, and IR spectra of **4** suggested that it might belong to the same class of labdane alkaloids. The ^{1}H and ^{13}C NMR data resembled those of chlorolissoclimide (**17**). The only difference between the two compounds was the presence of a methylene in **4** instead of the chlorinated methine at C-2 in **17**, indicating that **4** is the dechloro analogue of **17**. The planar structure and the relative stereostructure of **4** were determined in the same manner as described above for **1**.

The structures of haterumaimides J (**14**) and K (**15**) were determined in the same way as for **1**, as described in the earlier communication.[15] Base treatment (NaOMe/MeOH) of **15** gave **14** with identical ^{1}H and ^{13}C NMR data, $[\alpha]_D$, and HPLC retention time.

2.2. Structure–activity relationships

So far we have isolated a total of 17 labdane alkaloids (**1**–**17**) with a unique succinimide moiety, including two known compounds.[10,11,15] Six of these are dichlorinated (**5**, **6**, **7**, **8**, **9**, and **16**), 10 are monochlorinated (**1**, **2**, **3**, **10**, **11**, **12**, **13**, **14**, **15**, and **17**), and one is a dechloro analogue (**4**). Beyond the basic skeletal structure and the chlorine substituent(s), this class of alkaloids possesses a wide variety of functionalities. Due to their intrinsic structural variety and impressive biological activities, we were interested in establishing the structure–activity relationships of natural haterumaimides A–K and N–Q. We also prepared three synthetic derivatives of **17** (**18**, **19**, and **20**) to examine the effects of the hydroxyl group at C-12 and the imide NH in ring **C** on toxicity. *N*-Methyl

compound **18** was obtained by the reaction between **17** and CH_2N_2. Compounds **17** and **18** were treated with Ac_2O/pyridine to give **19** and **20**, respectively. The cytotoxicity of compounds **1**–**20** was evaluated against mouse lymphocytic leukemia (P388) cells. The toxicity of **1**–**20** against fertilized sea urchin eggs was also evaluated. The results are shown in Table 3.

Compound **14** was the most cytotoxic against P388 cells, with IC_{50} value of 0.23 ng/mL, followed by **15** at 0.45 ng/mL. Thus, acetylation of the hydroxyl group at C-18 of **14** has no significant effect on toxicity. In a comparison of **14** and **15** with **17** and its acetyl congener **1**, the cytotoxicity increases three fold when the hydroxyl group at C-7 in **17** and the acetoxyl group at C-7 in **1** are replaced with those at C-18 in **14** and **15**, respectively. Compounds **9** and **10** showed pronounced toxicity with IC_{50} values of 4.1 and 5.5 ng/mL. Oxidation of a C-6 hydroxyl group in **9** or **10** to a keto group in **6** or **11** significantly reduced the toxicity, which suggested that the relative hydrophilicity of this part of the molecule contributes to the activity. The conversion of an exomethylene double bond in **6** or **11** to a trisubstituted double bond in **8** or **12** does not markedly change the cytotoxic activity. The effect of the hydroxyl group at C-12 and the imide NH in ring **C** on the cytotoxicity is more pronounced, since compounds **2**, **3**, **7**, **13**, **18**, **19**, and **20** show remarkably low cytotoxicity compared to **17**. There is a two fold decrease in cytotoxicity with the acetyl group at C-7 in **5** or **1**, compared to the free alcohol **16** or **17**. Comparison of the cytotoxicity of dichloro and monochloro analogues clearly showed that the chlorine atom at C-3 does not significantly contribute to the cytotoxicity. The chlorine atom at C-2 has a pronounced effect on the cytotoxicity, since the dechloro congener **4** shows about a 30-fold decrease in toxicity compared with the monochloro analogue **17**. A similar trend in the structure–activity relationship for **1**–**20** was observed in the assay with fertilized sea urchin eggs (Table 3).

Table 3. Bioactivities of haterumaimides (A–K), (N–Q) and compounds **16**, **17**, **18**, **19**, and **20**

Haterumaimides	IC_{50} (μg/mL) against P388	Inhibition (%) of the division of fertilized sea urchin eggs at 3 ppm
A (**5**)	3.5×10^{-3}	100
B (**6**)	2.1	100
C (**7**)	>10	10
D (**8**)	0.9	100
E (**9**)	4.1×10^{-3}	100
F (**10**)	5.5×10^{-3}	100
G (**11**)	>10	100
H (**12**)	2.7	100
I (**13**)	>10	50
J (**14**)	2.3×10^{-4}	100
K (**15**)	4.5×10^{-4}	100
N (**1**)	3.4×10^{-3}	100
O (**2**)	1.3	80
P (**3**)	1.2	100
Q (**4**)	5.0×10^{-2}	100
16	1.0×10^{-3}[a]	100
17	1.7×10^{-3}[b]	100
18	1.4	10
19	2.4	0
20	0.14	50

[a] Data taken from Ref. 12.
[b] Data taken from Ref. 13.

2.3. Conclusion

In this report, we have described the isolation, structure elucidation, and biological activities of haterumaimides N–Q (**1**–**4**), detailed experimental procedures for haterumaimides J (**14**) and K (**15**), and structure–activity relationships of haterumaimides A–K (**5**–**15**), N–Q (**1**–**4**), dichlorolissoclimide (**16**), chlorolissoclimide (**17**), and three synthetic derivatives of **17** (**18**, **19**, and **20**). Metabolites **1**–**17** with chlorine and a succinimide moiety are rare in nature. Based on the SAR results, it appears that several structural features of haterumaimides, such as the presence of hydroxyl groups at C-6, C-7, C-12, and C-18, a chlorine atom at C-2, and an imido NH in ring **C**, are very important for the cytotoxicity. The hydrophilic OH and $(CO)_2NH$ groups in haterumaimides might increase cell membrane permeability to the molecules and/or enhance the stereo-electronic interaction between the molecules and a target molecule. Further chemical and biological studies on these unique metabolites are in progress in our laboratory.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a JASCO DIP-1000 polarimeter. IR and UV spectra were measured using a JASCO FT/IR-300 spectrometer and a JASCO UVDEC 610 spectrophotometer, respectively. HRFABMS were determined on a JEOL JMS-LG2000 mass spectrometer. The ^1H, ^{13}C, and 2D NMR spectra were recorded on a JEOL α-500 spectrometer, and ^1H and ^{13}C chemical shifts were referenced to the solvent peaks (δ_H 2.49 and δ_C 39.5 in DMSO-d_6 and δ_H 7.24 and δ_C 77.0 in $CDCl_3$). Column chromatography was performed on Kieselgel 60 (70–230 mesh, Merck) and Cosmosil 75C18-OPN (Nacalai Tesque). HPLC was performed using a COSMOSIL Si60 HPLC (5SL, 10 × 250 mm) and a COSMOSIL ODS HPLC column (C18, 10 × 250 mm). Analytical TLC was performed using Kieselgel 60 F_{254} DC-fertigplatten (Merck). All solvents used were of reagent grade.

3.2. Collection, extraction, and purification

The encrusting gray ascidian was collected by hand from the coast of Hateruma Island, Okinawa, in June 1999, and identified as *Lissoclinum* species. The identified sponge was kept frozen until use. A voucher specimen was deposited at the University of the Ryukyus (Specimen no. URKU-31). The animal specimen (1.0 kg, wet weight) was first extracted with acetone and then concentrated in vacuo to give an acetone extract (5.7 g). The acetone extract was partitioned between H_2O

(200 mL) and EtOAc (3 × 200 mL). The EtOAc extract completely inhibited the first cleavage of fertilized sea urchin eggs at 10 ppm. The bioactive EtOAc extract (3.4 g) was suspended in aqueous MeOH (1:1, 500 mL). The suspension was partitioned with hexanes (300 mL × 2), $CHCl_3$ (300 mL × 2), and *n*-BuOH (200 mL × 2), respectively, to give non-polar fatty hexane extract (0.5 g), a lipophilic $CHCl_3$ extract (2.5 g), and polar *n*-BuOH extract (0.2 g). All three extracts were tested for toxicity against fertilized sea urchin eggs. The lipophilic $CHCl_3$ extract completely inhibited the first cleavage of fertilized sea urchin eggs at 5 ppm, while the remaining two extracts were inactive. The active $CHCl_3$ extract was first subjected to a column chromatography on silica gel (300 g) using hexanes with increasing proportions of EtOAc [hexanes (500 mL)→hexanes/EtOAc (5:1, 400 mL→1:1, 400 mL→1:3, 400 mL) and EtOAc with increasing proportions of MeOH [EtOAc (400 mL)→EtOAc/MeOH (9:1, 400 mL)] as eluents to give nine fractions. All nine fractions were tested for toxicity against fertilized sea urchin eggs at 5 ppm. Only fraction 5 eluted with hexanes/EtOAc (1:1) and fraction 6 eluted with hexanes/EtOAc (1:3) completely inhibited cell division in fertilized sea urchin eggs. The active fifth fraction (0.4 g) was further chromatographed on ODS (150 g) using 35% H_2O in MeOH (250 mL) and finally washed with MeOH (250 mL) to give two fractions. The active polar fraction (0.3 g) was subjected to further separation by HPLC on Si60 using hexanes/CH_2Cl_2/EtOAc/MeOH (12:4:3:1) to give 12 fractions. The 4th fraction was purified by reversed-phase HPLC on ODS using MeOH/H_2O/CH_3CN (6.5:2.7:0.8) to afford haterumaimide N (**1**, 13.9 mg) and haterumaimide K (**15**, 3.2 mg). The 10th fraction was subjected to reversed-phase HPLC on ODS using MeOH/H_2O/CH_3CN (11:7:2) to yield haterumaimide O (**2**, 1.1 mg). The 6th fraction was purified by repeated reversed-phase HPLC on ODS using MeOH/H_2O/CH_3CN (6.5:2.7:0.8) and MeOH/H_2O/CH_3CN (6:3:1) as a solvent system to give haterumaimide P (**3**, 1.0 mg). The 12th fraction was further purified by reversed-phase HPLC on ODS using MeOH/H_2O/CH_3CN (5.4:3.6:1.0) to furnish haterumaimide J (**14**, 7.5 mg). The active sixth fraction of aforementioned column chromatography on silica gel (0.4 g) was chromatographed on ODS (150 g) using 30% H_2O in MeOH (250 mL) and MeOH (250 mL) to give two fractions. Both fractions were tested for toxicity against fertilized sea urchin eggs, and the polar fraction was found to be active at 5 ppm. The active polar fraction (0.3 g) was subjected to further fractionation by HPLC on Si60 using hexanes/CH_2Cl_2/EtOAc/MeOH (10:3:6:1) to give five factions. The fifth fraction was purified by normal-phase HPLC on Si60 using hexanes/CH_2Cl_2/EtOAc/MeOH (12:3:4:1) to afford haterumaimide Q (**4**, 2.5 mg).

3.2.1. Haterumaimide N (1). Colorless oil; $[\alpha]_D^{32}$ +59.7 (*c* 0.79, MeOH); UV (MeOH) λ_{max} (log ε) 215 nm (3.6); FT/IR (film) ν_{max} 3505, 3400, 2900, 1720, 1705, 1700, 1605, 1370, 1240, 1180, and 1050 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$) data are listed in Tables 1 and 2; HRFABMAS [*m*/*z* $(M+H)^+$ 426.2058 (calcd for $C_{22}H_{32}{}^{35}ClNO_5$, 426.2047)].

3.2.2. Haterumaimide O (2). Colorless oil; $[\alpha]_D^{28}$ +16.0 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε) 235 nm (3.9); FT/IR (film) ν_{max} 3300, 2920, 2840, 1720, 1715, 1710, 1660, 1380, 1280, 1120, and 1040 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6) data are listed in Tables 1 and 2; HRFABMS [*m*/*z* $(M+H)^+$ 408.1914 (calcd for $C_{22}H_{31}{}^{35}ClNO_4$, 408.1941)].

3.2.3. Haterumaimide P (3). Colorless oil; $[\alpha]_D^{29}$ +68.8 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 210 nm (3.6); FT/IR (film) ν_{max} 3400, 2910, 1705, 1700, 1600, 1380, 1200, and 1080 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6) data are listed in Tables 1 and 2; HRFABMAS [*m*/*z* $(M+H)^+$ 368.1982 (calcd for $C_{20}H_{31}{}^{35}ClNO_3$, 368.1992)].

3.2.4. Haterumaimide Q (4). Colorless oil; $[\alpha]_D^{28}$ +36.0 (*c* 0.19, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 204 nm (3.6); FT/IR (film) ν_{max} 3405, 2930, 1720, 1710, 1600, 1350, and 1165 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6) data are listed in Tables 1 and 2; HRFABMAS [*m*/*z* $(M+Na)^+$ 372.2159 (calcd for $C_{22}H_{31}NaNO_4$, 372.2151)].

3.2.5. Haterumaimide J (14). Colorless oil; $[\alpha]_D^{29}$ +68.0 (*c* 0.92, MeOH); UV (MeOH) λ_{max} (log ε) 210 nm (3.6); FT/IR (film) ν_{max} 3400, 2910, 1720, 1710, 1220, and 1050 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6) data were described in the earlier paper;[11] HRFABMAS [*m*/*z* $(M+H)^+$ 384.1925 (calcd for $C_{20}H_{31}{}^{35}ClNO_4$, 384.1942)].

3.2.6. Haterumaimide K (15). Colorless oil; $[\alpha]_D^{29}$ +59.6 (*c* 0.19, MeOH); UV (MeOH) λ_{max} (log ε) 210 nm (3.6); FT/IR (film) ν_{max} 3405, 2902, 1720, 1710, 1250, and 1040 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6) data were described in the earlier paper;[11] HRFABMAS [*m*/*z* $(M+H)^+$ 426.2032 (calcd for $C_{22}H_{33}{}^{35}ClNO_5$, 426.2047)].

3.3. Methanolysis of 1

To a solution of haterumaimide N (1.1 mg, 2.6 μmol) in MeOH (0.5 mL) was added sodium methoxide (1.5 mg, 27.7 μmol) and the mixture was stirred at room temperature for 4 h. Next, 0.5 mL of water was added to the mixture. The organic solvent was evaporated and the mixture was extracted with ether (3 × 0.5 mL). The combined ether extract was washed with brine, dried ($MgSO_4$), and evaporated. The residue was purified by normal-phase HPLC on Si60 using hexanes/EtOAc/MeOH (6.2:3.3:0.5) to afford the alcohol, chlorolissoclimide (**7**, 0.9 mg, 90%) as a colorless oil: $[\alpha]_D^{29}$ +45.0 (*c* 0.05, MeOH); ^1H NMR (DMSO-d_6, 500 MHz) δ_H 11.01 (1H, s), 5.23 (1H, br s), 4.98 (1H, s), 4.93 (1H, d, *J* = 4.5 Hz), 4.80 (1H, br s), 4.33 (1H, tt, *J* = 12.0, 4.0 Hz), 4.00 (1H, m), 3.78 (1H, m), 2.84 (1H, ddd, *J* = 9.0, 4.5, 1.5 Hz), 2.55 (1H, dd, *J* = 17.5, 4.5 Hz), 2.48 (1H, dd, *J* = 17.5, 9.0 Hz), 2.10 (1H, ddd, *J* = 12.0, 4.0, 1.5 Hz), 1.92 (1H, ddd, *J* = 12.5, 4.0, 1.5 Hz), 1.85 (1H, ddd, *J* = 12.5, 5.5, 2.0 Hz), 1.64 (1H, ddd, *J* = 12.5, 5.5, 2.0 Hz), 1.60 (1H, dd, *J* = 10.5, 9.5 Hz), 1.48 (1H, t, *J* = 12.5 Hz), 1.40 (1H, ddd, *J* = 13.0, 10.5, 6.5 Hz), 1.30 (1H, t, *J* = 12.0 Hz), 1.23

(1H, dd, J = 13.0, 2.0 Hz), 1.10 (1H, q, J = 12.5 Hz), 0.91 (3H, s), 0.81 (3H, s), 0.61 (3H, s); ^{13}C NMR (DMSO-d_6, 125 MHz) δ_C 180.8 (s, C-15), 178.5 (s, C-16), 150.2 (s, C-8), 104.5 (t, C-17), 71.5 (d, C-7), 66.5 (d, C-12), 56.6 (d, C-2), 51.6 (t, C-3), 50.5 (d, C-5), 49.2 (d, C-9), 48.3 (t, C-1), 45.3 (d, C-13), 41.0 (s, C-10), 35.5 (s, C-4), 32.8 (t, C-6), 32.8 (q, C-18), 29.9 (t, C-11), 28.8 (t, C-14), 21.9 (q, C-9), 14.5 (q, C-20).

3.4. Methanolysis of 15

To a solution of haterumaimide K (0.5 mg, 1.2 μmol) in MeOH (0.5 mL) was added sodium methoxide (1.0 mg, 18.5 μmol) and the mixture was stirred at room temperature for 4 h. Next, 0.5 mL of water was added to the mixture. The organic solvent was evaporated and the mixture was extracted with ether (3 × 0.5 mL). The combined ether extract was washed with brine, dried ($MgSO_4$), and evaporated. The residue was purified by reversed-phase HPLC on ODS using MeOH/H_2O/CH_3CN (5.4:3.6:1.0) to afford haterumaimide J (**14**, 0.4 mg, 90%) as a colorless oil. The ^{1}H and ^{13}C NMR data and $[\alpha]_D$ of the hydrolysate of **15** were described previously.[15]

3.5. Methylation of 17

Compound **17** (4.0 mg, 10.41 μmol) was dissolved in MeOH (1.0 mL), and a solution of diazomethane in ether was added until all of **17** had been consumed as determined by TLC. The reaction mixture was concentrated and the crude product was purified by HPLC [COSMOSIL, 5SL, hexanes/EtOAc (1:2.5)] to give compound **18** (2.3 mg, 55%): colorless oil; $[\alpha]_D^{16}$ +70 (*c* 1.0 MeOH); IR ($CHCl_3$) ν_{max} 3612, 3019, 2969, 1699, 1387, 1222 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ_H 5.33 (1H, br s, H_a-17), 4.90 (1H, br s, H_b-17), 4. 34 (1H, m, H-12), 4.14 (1H, m, H-2), 3.98 (1H, dd, J = 11.0, 5.5 Hz, H-7), 2.97 (3H, s, NCH_3), 2.81 (1H, m, H-13), 2.79 (1H, m, H_a-14), 2.63 (1H, dd, J = 18.0, 10.5 Hz, H_b-14), 2.20 (1H, br d, J = 10.5 Hz, H_α-1), 2.08 (1H, m, H_α-6), 2.00 (1H, m, H_α-3), 1.81 (1H, m, H_α-11), 1.60 (1H, m, H_β-11), 1.52 (1H, t, J = 12.5 Hz, H_β-3), 1.24 (1H, m, H_β-6), 1.22 (1H, m, H_β-1), 1.16 (1H, dd, J = 12.5, 1.5 Hz, H-5), 0.96 (3H, s, CH_3-18), 0.84 (3H, s, CH_3-19), 0.72 (3H, s, CH_3-20): ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 178.9 (C-15), 176.7 (s, C-16), 169.9 (s, *C*OCH_3), 149.5 (s, C-8), 105.4 (t, C-17), 73.3 (d, C-7), 68.9 (d, C-12), 55.0 (d, C-2), 52.3 (d, C-5), 51.9 (t, C-3), 51.6 (d, C-9), 49.3 (t, C-1), 45.4 (d, C-13), 41.7 (s, C-10), 35.9 (s, C-4), 33.2 (q, C-18), 33.0 (t, C-6), 29.1 (t, C-11). 28.2 (t, C-14), 24.9 (q, NMe). 22.0 (q, C-19), 14.9 (q, C-20); HRESIMS [*m*/*z* $(M+Na)^+$ 420.1917 (calcd for $C_{21}H_{32}{}^{35}ClNNaO_4$, 420.1910)].

3.6. Acetylation of 17

Compound **17** (5.0 mg, 13.02 μmol) was dissolved in pyridine (0.250 mL) and Ac_2O (0.100 mL) and the solution was allowed to stand for 30 min. Evaporation of the solvent in vacuo followed by HPLC separation [COSMOSIL, 5SL, hexanes/EtOAc (1:1)] yielded 1.3 mg of **19**: colorless oil; $[\alpha]_D^{16}$ +47 (*c* 0.58 MeOH); IR ($CHCl_3$) ν_{max} 3400, 3027, 2964, 1726, 1372 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ_H 7.89 (1H, br s, NH), 5.43 (1H, m, H-12), 5.20 (1H, br s, H_a-17), 5.18 (1H, br s, H_b-17), 5.05 (1H, dd, J = 9.0, 4.0 Hz, H-7), 4.13 (1H, m, H-2), 3.01 (1H, m, H-13), 2.80 (2H, m, $H_{a,b}$-14), 2.19 (1H, br d, J = 10.5 Hz, H_α-1), 2.12 (3H, s, $COCH_3$), 2.06 (1H, m, H_a-11), 2.02 (1H, m, H_α-6), 2.01 (1H, m, H_α-3), 1.98 (3H, s, $COCH_3$), 1.61 (1H, m, H-9), 1.59 (1H, m, H_b-11), 1.50 (1H, t, J = 13.0 Hz, H_β-3), 1.32 (1H, q, J = 11.5 Hz, H_β-6), 1.20 (1H, m, H_β-1), 1.19 (1H, m, H-5), 0.94 (3H, s, CH_3-18), 0.84 (3H, s, CH_3-19), 0.74 (3H, s, CH_3-20); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 176.5 (s, C-15), 175.2 (s, C-16), 169.9 (s, *C*OCH_3), 169.7 (s, *C*OCH_3), 142.5 (s, C-8), 107.3 (t, C-17), 74.0 (d, C-7), 70.6 (d, C-12), 54.7 (d, C-2), 52.1 (d, C-5), 51.8 (t, C-3), 51.0 (d, C-9), 49.2 (t, C-1), 43.8 (d, C-13), 41.4 (s, C-10), 35.9 (s, C-4), 33.1 (q, C-18), 29.9 (t, C-14), 29.7 (t, C-6), 26.4 (t, C-11), 22.0 (q, C-19), 21.1 (q, CO*C*H_3), 20.8 (q, CO*C*H_3), 14.7 (q, C-20); HRESIMS [*m*/*z* $(M+Na)^+$ 490.1972 (calcd for $C_{24}H_{34}{}^{35}ClNNaO_6$, 490.1964)].

3.7. Methylation of 17 followed by acetylation

Compound **17** (4.0 mg, 10.41 μmol) dissolved in MeOH (1.0 mL) and a solution of diazomethane in ether were added until all of **17** had been consumed as determined by TLC. The reaction mixture was concentrated and dried in vacuo. The crude product was dissolved in pyridine (0.25 mL) and Ac_2O (0.10 mL), and the solution was allowed to stand for 8 h. Evaporation of the solvent in vacuo followed by HPLC separation [COSMOSIL, 5SL, hexanes/EtOAc(1:1)] yielded 2.9 mg of **20**: colorless oil; $[\alpha]_D^{16}$ +38 (*c* 1.0, MeOH); IR ($CHCl_3$) ν_{max} 3019, 2965, 1736, 1705, 1223, 1208 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ_H 5.45 (1H, m, H-12), 5.20 (1H, br s, Ha-17), 5.18 (1H, br s, Hb-17), 5.04 (1H, dd, J = 11.5, 5.0 Hz, H-7), 4.13 (1H, m, H-2), 2.97 (3H, s, NCH_3), 2.94 (1H, m, H-13), 2.73 (2H, m, $H_{a,b}$-14), 2.19 (1H, br d, J = 10.5 Hz, H_α-1), 2.12 (3H, s, $COCH_3$), 2.06 (1H, m, H_a-11), 2.02 (1H, m, H_α-6), 2.01 (1H, m, H_α-3), 1.93 (3H, s, $COCH_3$), 1.61 (1H, m, H-9), 1.57 (1H, m, H_b-11), 1.50 (1H, t, J = 11.5 Hz, H_β-3), 1.32 (1H, q, J = 11.5 Hz, H_β-6), 1.20 (1H, m, H_β-1), 1.19 (1H, m, H-5), 0.94 (3H, s, CH_3-18), 0.84 (3H, s, CH_3-19), 0.74, (3H, s, CH_3-20). ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 176.9 (s, C-15), 175.9 (s, C-16), 169.9 (s, *C*OCH_3), 169.8 (s, *C*OCH_3), 142.6 (s, C-8), 107.3 (t, C-17), 74.4 (d, C-7), 70.7 (d, C-12), 54.8 (d, C-2), 52.1 (d, C-5), 51.8 (t, C-3), 51.1 (d, C-9), 49.3 (t, C-1), 42.6 (d, C-13), 41.4 (s, C-10), 36.0 (s, C-4), 33.1 (q, C-18), 29.8 (t, C-6), 28.9 (t, C-14), 26.1 (t, C-11), 25.0 (q, NMe), 22.0 (q, C-19), 21.1 (q, CO*C*H_3), 20.9 (q, CO*C*H_3), 14.7 (q, C-20); HRESIMS [*m*/*z* $(M+Na)^+$ 504.2129 (calcd for $C_{25}H_{36}{}^{35}ClNNaO_6$, 504.2120)].

Acknowledgments

We thank Professor Tatsuo Higa, University of the Ryukyus, Okinawa, for his invaluable suggestions throughout this study, and Dr. Yuichi Hirose of the same University for identifying the ascidian. This work

was supported in part by Wako Pure Chemical Industries Ltd. and by Grants-in-Aid (Nos. 11558079 and 12045235) from the Ministry of Education, Science, Sports and Culture of Japan.

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