Ciliatamides A−C, Bioactive Lipopeptides from the Deep-Sea Sponge *Aaptos ciliata*[#]

Yoichi Nakao,*[,†,‡] Shizuka Kawatsu,[†] Chikane Okamoto,[†] Masaaki Okamoto,[†] Yoshitsugu Matsumoto,[†] Shigeki Matsunaga,[†] Rob. W. M. van Soest,[§] and Nobuhiro Fusetani*[,⊥]

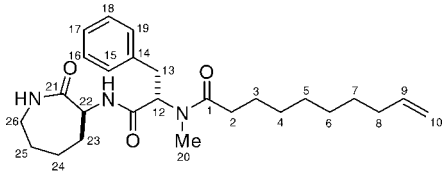
Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan, Institute for Systematics and Ecology, University of Amsterdam, 1090 GT Amsterdam, The Netherlands, and Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1, Minato-cho, Hakodate, 041-8611, Japan

Received January 15, 2008

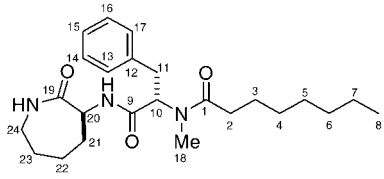
Three lipopeptides, ciliatamides A−C (**1**–**3**), were isolated from the deep-sea sponge *Aaptos ciliata*, and their structures were elucidated on the basis of spectroscopic and chemical methods. Ciliatamides A (**1**) and B (**2**) were found to be antileishmanial, while **2** also exhibited marginal cytotoxicity to HeLa cells.

Leishmaniasis is a disease caused by an obligate intracellular parasite of the genus *Leishmania* and includes several types of disease ranging from self-healing ulcers to fatal visceral leishmaniasis.[1] Pentavalent antimonials are the most common drugs for the treatment of leishmaniasis,[2] but have cardiotoxic effects. Therefore, alternative treatments are urgently required.

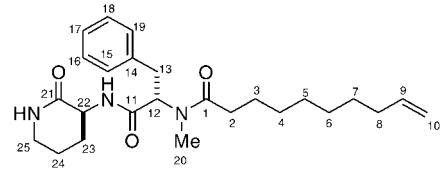
In the course of a drug discovery project from Japanese marine invertebrates, we have reported the isolation of renieramycin A from the sponge *Neopetrosia* sp. as a potential antileishmanial agent.[3] Subsequently, we found significant antileishmanial activity in the organic extract of the deep-sea sponge *Aaptos ciliata.* Fractionation of the extract yielded three new lipopeptides, named ciliatamides A−C (**1**−**3**). Herein, we report the isolation, structure elucidation, and biological activity of these compounds.



1



2



3

The $CHCl_3$-soluble portion of the sponge extract was fractionated by a modified Kupchan procedure[4] to yield an active $CHCl_3$ layer.

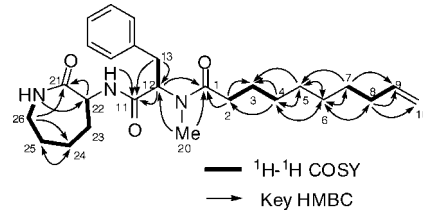


Figure 1. Key COSY and HMBC correlations for ciliatamide A (**1**).

This layer was further separated by ODS flash chromatography, silica gel column chromatography, and repetitive reversed-phase HPLC to yield ciliatamides A (**1**) (1.7×10^{-4} % yield based on wet weight) and B (**2**) (4.4×10^{-4} %), as yellowish oils. Another collection of the same sponge was similarly processed to afford ciliatamide C (**3**) (1.8×10^{-3} %).

Ciliatamide A (**1**) gave a molecular formula of $C_{26}H_{39}N_3O_3$ as established by HRFABMS. The ^{1}H NMR spectrum revealed the presence of two amide NH groups (δ 7.74 and 7.66), a phenyl group [δ 7.22 (2H), 7.21 (2H), and 7.17], and a terminal vinyl group [δ 5.79 and 4.88 (2H)], which was supported by ^{13}C NMR signals for three amide carbonyl carbons (δ 177.1, 177.0, and 171.0), six sp^2 carbons [δ 138.5, 130.0 (2C), 129.5 (2C), and 127.5], and two olefinic carbons (δ 140.0 and 114.6). Analysis of the HSQC spectrum identified a terminal vinyl group, an *N*-methyl, 12 sp^3 methylenes, 2 sp^3 methines, and a monosubstituted phenyl group. Interpretation of the COSY spectrum led to connectivities H-2−H-4, H-7−H-9, H-12−H-13, and H-22−H-26, suggesting the presence of a fatty acid as well as phenylalanine (Phe) and lysine (Lys) residues.

Interpretation of HMBC data could assign the two amino acid moieties as *N*-methylphenylalanine (MePhe) and Lys (Figure 1). The connection of the acyl group and MePhe through the *N*-methyl amide was established by HMBC correlations (Me-20/C-1 and C-12), while CO (MePhe) and NH-22 (Lys) were connected by a HMBC cross-peak (NH-22/C-11). The identification of deca-9-enoic acid was straightforward from HMBC cross-peaks (H-8/C-10 and C-6, H-7/C-6 and C-5, H-6/C-8, C-7, C-5, and C-4, H-5/C-7, C-6, C-4, and C-3, H-4/C-6 and C-5, and H-2/C-1). Judging from the degrees of unsaturation, **1** must contain a ring, which was realized by HMBC correlations between NH-26/C-22 and H-26/C-21, thereby permitting the construction of an ϵ-caprolactam moiety. Comparison of the ^{1}H NMR data of **1** with those of acetyl Lys and α-acetoamide-ϵ-caprolactam prepared from Lys and α-amino-ϵ-caprolactam, respectively, substantiated this assignment (Figure 2). Marfey's analysis[5] of the acid hydrolysates of **1** disclosed the L-configuration of both Lys and MePhe. Thus, the structure of ciliatamide A (**1**) was assigned as shown.

[#] Dedicated to Dr. G. Robert Pettit of Arizona State University for his pioneering work on bioactive natural products.

* Authors to whom correspondence should be addressed. (Y.N.) Tel/Fax: +81-3-5286-2568. E-mail: ayocha@waseda.jp. (N.F.) Tel/Fax: +81-138-40-8884. E-mail: anobu@fish.hokudai.ac.jp.

[†] The University of Tokyo.

[‡] Present address: School of Advanced Science and Engineering, Waseda University, Shinjuku-ku, Tokyo 169-8555, Japan.

[§] University of Amsterdam.

[⊥] Hokkaido University.

10.1021/np8000317 CCC: $40.75 © 2008 American Chemical Society and American Society of Pharmacognosy
Published on Web 02/08/2008

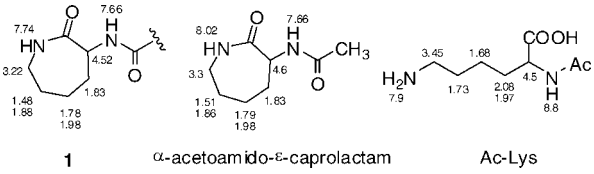


Figure 2. ^{1}H NMR chemical shifts of **1**, α-acetoamido-ϵ-caprolactam, and Ac-Lys.

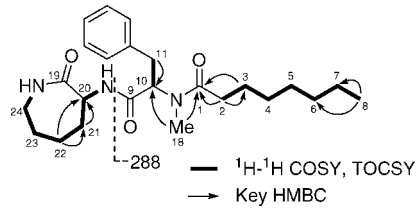


Figure 3. Key correlations and MS fragmentation (*m*/*z*) for ciliatamide B (**2**).

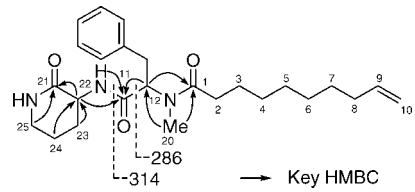


Figure 4. Key HMBC correlations and MS fragmentation (*m*/*z*) of ciliatamide C (**3**).

Ciliatamide B (**2**) was assigned a molecular formula of $C_{24}H_{37}N_3O_3$, smaller than **1** by C_2H_2, based on the HRFABMS and NMR data. In the ^{1}H NMR spectrum of **2**, there were two sets of signals that were also seen in the ^{1}H NMR spectra of **1** in CD_3OH. Presumably, these signal sets were due to 4:1 mixtures of two conformers around the *N*-methyl amide bond between MePhe residues and fatty acids. The NMR spectra of **2** were closely comparable with those of **1**, except for signals of the terminal vinyl group that were missing in **2**. In fact, octanoic acid was identified by 2D NMR data (Figure 3), instead of deca-9-enoic acid in **1**. Although the presence of an ϵ-caprolactam moiety was not secured by NMR data, the ESIMS fragment ion peak at *m*/*z* 288 was consistent with its presence (Figure 3). Again, the absolute configuration at C-10 and C-20 was determined to be both *S* as in the case of **1** by the Marfey's analysis, furnishing the structure of **2** as shown.

Ciliatamide C (**3**) showed a molecular formula of $C_{25}H_{37}N_3O_3$, as established by HRFABMS. The ^{1}H NMR and ^{13}C NMR spectra also indicated 4:1 mixture of two conformers and were similar to those of ciliatamides A (**1**) and B (**2**). ESIMS fragment ion peaks at *m*/*z* 314 and 286 indicated the presence of a cyclized Orn moiety, instead of an ϵ-caprolactam functionality. The existence of cyclized Orn was confirmed by HMBC data (Figure 4), leading to the planar structure of **3** as shown. Marfey analysis was used to assign L-Orn and L-MePhe.

Ciliatamides A−C (**1**−**3**) were evaluated for antileishmanial activity in a fluorometric microplate assay using *L. major*/egfp promastigotes.[6] As a result, ciliatamides A (**1**) and B (**2**) showed 50% and 45.5% growth inhibition at 10 μg/mL, respectively, while C (**3**) was not active. Since the organic extract of *A. ciliata* showed 86% inhibition at 10 μg/mL, more potent minor components may be contained in the sponge. Ciliatamides A (**1**), B (**2**), and C (**3**) also inhibited growth of HeLa cells with IC_{50} values of 50, 4.5, and 50 μg/mL, respectively.

Although a wide variety of bioactive compounds have been reported from marine sources, there are only a limited number of compounds with antileishmanial activity. Examples are peroxides related to plakortin,[7] alkaloids related to the manzamines,[8] and renieramycin A, whose isolation was guided by a bioassay using *L. amazoensis*/egfp promastigotes.[3] From marine organisms, compounds including ϵ-caprolactam are rare. Bengamides isolated from a marine sponge by Adamczeski et al. are one such example and showed anthelminthic activity.[9]

Experimental Section

General Experimental Procedures. Optical rotations were determined on a JASCO DIP-1000 digital polarimeter in MeOH. UV spectra were recorded on a Shimadzu BioSpec-1600 spectrophotometer. NMR spectra were recorded on a JEOL A600 NMR spectrometer. ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peaks, δ_H 3.31 and δ_C 49.0 for CD_3OD and CD_3OH and δ_H 1.94 and δ_C 118.69 for CD_3CN. FABMS were measured on a JEOL JMS-700T tandem mass spectrometer. ESI mass spectra were measured on a JEOL JMS-T100LC mass spectrometer.

Animal Material. The sponge samples were collected by dredging at a depth of 150 m off Oshima-Shinsone, 28°52′40″, 129°33′19″ Kagoshima prefecture, Japan, in 2001, immediately frozen, and kept frozen at −20 °C until used. Both sponges were identified as *Aaptos ciliata* Wilson 1925. The voucher specimens were deposited at the Zoological Museum, University of Amsterdam (ZMAPOR19858 and ZMAPOR19861).

Extraction and Isolation. The frozen sample (700 g, wet weight) was homogenized and extracted with MeOH (2 L × 3), EtOH (2 L × 1), and acetone (2 L × 1). Fractionation of the extract was carried out by monitoring cytotoxicity against HeLa cells and antileishmanial activity to obtain less toxic antileishmainal compounds. The extracts were combined, evaporated in vacuo, and partitioned between H_2O and $CHCl_3$. The organic layer was further partitioned between 90% MeOH and *n*-hexane. The 90% MeOH layer was diluted with H_2O to 60% MeOH and extracted with $CHCl_3$. The $CHCl_3$ layer was subjected to ODS flash chromatography [50 and 70% MeOH, 70 and 85% MeCN, 100% MeOH, and $CHCl_3$−MeOH−H_2O (7:3:0.5)] to yield six fractions. The active fraction eluted with 70% MeCN was separated by silica gel column chromatography with $CHCl_3$−MeOH−H_2O (8:2:0.1, 7:3:0.5, and 6:4:1). The active fraction eluted with $CHCl_3$−MeOH−H_2O (8:2:0.1) was fractionated by HPLC [Inertsil ODS-3 (250 × 20 mm, i.d.); flow rate, 8 mL/min; detection, UV 220 nm; solvent, 45% 1-PrOH] to give 10 fractions. The active antileishmanial and abundant fraction was purified by HPLC [Inertsil ODS-3 (GL Sciences, 250 × 20 mm, i.d.); flow rate, 8 mL/min; detection, UV 220 nm; solvent, 60% MeCN] to obtain 3.1 mg of ciliatamide B (**2**). The three other active fractions were combined and purified by HPLC [Inertsil ODS-3 (250 × 20 mm, i.d.); flow rate, 8 mL/min; detection, UV 220 nm; solvent, 60% MeCN] to yield 15 fractions. Among these, the most abundant active fraction was purified by HPLC [Cosmosil 5C_{18} AR-II (Nacalai, 250 × 20 mm, i.d.); flow rate, 8 mL/min; detection, UV 220 nm; solvent, 60% MeCN] to yield 1.2 mg of ciliatamide A (**1**).

The sponge from which ciliatamide C was obtained had been kept as a different batch and was identified to be the same *A. ciliata*. A frozen specimen (220 g, wet weight) was homogenized and extracted with MeOH and $CHCl_3$. Fractionation of the extracts was carried out following the cytotoxicity against P388 murine leukemia cells. The combined extracts were partitioned between $CHCl_3$ and water, and the evaporated $CHCl_3$ layer was dissolved in 90% MeOH and extracted with *n*-hexane. The obtained 90% MeOH layer was evaporated and separated by ODS flash column chromatography with an aqueous MeOH system to afford two cytotoxic fractions eluted with 70% MeOH and 80% MeOH, respectively. These were combined, followed by countercurrent partition chromatography (CPC) separation with EtOAc−heptane−MeOH−H_2O (7:4:4:3, upper phase as the mobile phase and lower phase as stationary phase) to afford 57 fractions (Nos. 1–57). Fractions No. 7 to 14 were combined, concentrated, and then separated by ODS HPLC on Inertsil ODS-3 with a linear gradient of 30–90% MeOH. One of the obtained fractions was further separated by purification by reversed-phase HPLC (Phenomenex Luna 5 μm phenylhexyl with 30% MeCN) to give 4.0 mg of ciliatamide C (**3**).

Ciliatamide A (1): yellow oil; $[\alpha]_D^{20}$ +40 (*c* 0.05, MeOH); UV (MeOH) λ_{max} 206 (ϵ 29 000) nm; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m*/*z* 464.2892 $(M + Na)^+$ (calcd for $C_{26}H_{39}N_3O_3Na$, Δ +0.3 mmu).

Table 1. NMR Spectroscopic Data for Ciliatamides A−C (**1**–**3**)

	1[a]		**2**[b]		**3**[b]	
#	δ_H[c]	δ_C	δ_H[c]	δ_C	δ_H[c]	δ_C
1		177.0		174.8		175.1
2	2.24 (t, 7.6)	34.0	2.16 (m)	33.8	2.18 (dd)	34.1
3	1.37 (m)	25.9	1.38 (m)	25.6	1.33 (m)	27.0
4a	1.15 (m)	29.8	1.11 (m)	29.6	1.12 (m)	29.7
4b			1.18 (m)			
5a	1.27 (m)	30.2	1.11 (m)	29.6	1.22 (m)	29.7
5b			1.18 (m)			
6a	1.23 (m)	29.8	1.19 (m)	32.4	1.27 (m)	30.4
6b			1.34 (m)			
7	1.34 (m)	29.7	1.27 (m)	23.2	1.34 (m)	29.7
8	2.02 (dt, 6.5, 6.9)	34.5	0.85 (t, 7.3)	14.3	2.04 (dt)	34.8
9	5.79 (ddt, 6.5, 10.4, 17.2)	140.0		170.1	5.84 (ddt, 6.6, 10.2, 16.8)	140.6
10a	4.88	114.6	5.35 (dd, 5.6, 10.3), [4.66][d]	58.0	4.93 (d, 10.2)	115.1
10b					5.01 (d, 16.8)	
11a		171.0	2.88 (dd, 10.3, 14.7)	34.3		171.6
11b			3.22 (dd, 5.6, 14.7)			
12	5.41 (dd, 5.4, 10.7)	59.3		139.2	5.40 (dd, 6.0, 6.0), [4.61 (dd)][d]	58.5
13a	2.96 (dd, 10.7, 14.4)	35.0	7.2 (m)	129.8	2.88	34.8
13b	3.26 (dd, 5.4, 14.4)				3.28 (dd, 16.5, 6.0), [3.35 (dd)][d]	
14		138.5	7.2 (m)	129.0		139.0
15	7.21 (d, 6.9)	130.0	7.2 (m)	127.1	7.21	129.0
16	7.22 (dd, 6.9, 7.2)	129.2	7.2 (m)	129.0	7.25	131.0
17	7.17 (t, 7.2)	127.5	7.2 (m)	129.8	7.17	127.0
18	7.22 (dd, 6.9, 7.2)	129.2	2.82 (s), [2.76 (s)][d]	31.9	7.25	131.0
19	7.21 (d, 6.9)	130.0		175.5	7.21	129.0
20	2.90 (s), [2.91 (s)][d]	32.6	4.38 (ddd, 1.8, 6.4, 11.4), [4.43][d]	52.9	2.87 (s), [2.82 (s)][d]	32.3
21a		177.1	1.24 (m)	29.6		171.5
21b			1.75 (m)			
22a	4.52 (dd, 7.6, 10.3)	53.5	1.69 (m)	28.6	4.24 (dt), [4.27 (dt)][d]	51.1
22b			1.9 (m)			
23a	1.83 (m)	29.6	1.84 (m)	32.2	1.61	28.8
23b					2.13	
24a	1.78 (m)	29.0	3.17 (m)	42.0	1.84	22.5
24b	1.98 (m)					
25a	1.48 (m)	32.0			3.22 (br)	42.3
25b	1.88 (brd, 13.7)					
26	3.22 (bdd, 5.5, 6.2)	42.5				
NH-20			7.14			
NH-22	7.66 (d, 6.9)				6.73 (brd, 6.7), [6.85 (d)][d]	
NH-24			6.6 (brs)			
NH-25					6.13 (br), [6.15 (br)][d]	
NH-26	7.74 (bt, 5.5)					

[a] In CD_3OH. [b] In CD_3CN. [c] mult., J in Hz. [d] Signals for the minor conformer.

Ciliatamide B (2): yellow oil; $[\alpha]_D^{20}$ +55 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 206 (ϵ 15 000) nm; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m*/*z* 416.2926 $(M + H)^+$ (calcd for $C_{24}H_{38}N_3O_3$, Δ +1.3 mmu).

Ciliatamide C (3): yellow oil; $[\alpha]_D^{26}$ +74 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 203 (ϵ 13 600) nm; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m*/*z* 428.2934 $(M + H)^+$ (calcd for $C_{25}H_{37}O_3N_3$, Δ +2.1 mmu).

Amino Acid Analysis of 1–3. A solution of each of **1**−**3** (0.1 mg) in 6 M hydrochloric acid (1 mL) was stirred at 100 °C in a sealed tube overnight. The reaction mixture was concentrated to dryness under nitrogen at 80 °C for 2 h. After being reacted with 0.1 M $NaHCO_3$ (100 μL) and L-FDAA (fluoro dinitrophenyl-L-alaninamide) in acetone (50 μL) at 80 °C for 5 min, each product was treated with a solution of 0.2 M HCl (50 μL) and 50% MeCN containing 0.1% TFA (50 μL). D- and L-amino acid standards were treated separately with FDAA in the same manner. The FDAA derivatives were analyzed by HPLC [Cosmosil 5C_{18}-MS (Nacalai, 250 × 4.6 mm, i.d.); flow rate, 1 mL/min; detection, UV 340 nm; solvent, 40% MeCN + 0.05% TFA]. The stereochemistry of MePhe and Lys in **1** and **2** was determined to be L. The stereochemistry of MePhe and Orn in **3** was analyzed under the same conditions [Cosmosil 5C_{18}-MS (Nacalai, 250 × 4.6 mm, i.d.); flow rate, 1 mL/min; detection, UV 340 nm; solvent, 40% MeCN + 0.05% TFA] to show both L forms.

Antileishmanial Assay. Fluorescence signals of *L. major*/egfp promastigotes cultured in 199 medium (NISSUI Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum and 25 mM Hepes buffer (ICN Biomedicals Inc., Aurora, OH) in 96-well plates at 25 °C were measured with a fluorescence microplate reader (Fluoroscan Ascent FL, Dainippon Pharmaceutical Co., Osaka, Japan) with excitation at 485 nm and emission at 538 nm. To each well of 96-well plates containing 100 μL of *L. major*/egfp suspension with 1 × 10^6 cells/mL, 100 μL of test solution (sample dissolved in MeOH) was added, and the plates were incubated in a low-temperature incubator (Yamato Scientific Co., Ltd., Tokyo, Japan) at 25 °C for 72 h. To determine the IC_{50} value of ciliatamides, *L. major*/egfp were cultured at 1 × 10^6 cells/mL with various concentrations of the drugs, and their fluorescence was measured after 72 h incubation. Renieramycin A was used as the positive control (IC_{50} 0.5 μg/mL).

Cytotoxic Assay. HeLa human uterine cervix carcinoma cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum, 2 μg/mL gentamycin, 2 μg/mL antibiotic-antimicotic, and 0.3 M $NaHCO_3$ (adjusted to pH 7.0–7.4 with 2 M HCl) at 37 °C under an atmosphere of 5% CO_2. To each well of 96-well microtiter plates that contained 200 μL of tumor cell suspension containing 1 × 10^4 cells/mL was added 2 μL of test solution (sample dissolved in MeOH), and the plates were incubated in a TABAI BNA-111 CO_2 incubator (Espec Co., Tokyo, Japan) at 37 °C for 72 h. After addition of 50 μL of 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2*H* tetrazolium bromide (MTT) saline solution (1 mg/mL) to each well, the plates were incubated for another 3 h under the same conditions. After the incubation, the cytotoxic activities were judged from absorbance at 550 nm. IC_{50} values were obtained by plotting the percent inhibition in a semilogarithmic scale. Adriamycin was used for the positive control (IC_{50} 2 μg/mL). Cytotoxicity against P388 cells was evaluated by using the MTT assay as described previously.[10]

Acknowledgment. We are indebted to the crew of *R/V Toyoshio-maru* of Hiroshima University for assistance in collection of the samples. This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Supporting Information Available: Tables of HMBC data for **1**−**3**. This information is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Pitzer, K. K.; Werbovetz, K. A.; Brendle, J. J.; Scovill, J. P. *J. Med. Chem.* **1998**, *41*, 4885–4889.
(2) Sharief, A. H.; Khalil, E. A. G.; Theander, T. G.; Kharazmi, A.; Omer, S. A.; Ibrahim, M. E. *Exp. Parasitol.* **2006**, *114*, 247–252.
(3) Nakao, Y.; Shiroiwa, T.; Murayama, S.; Matsunaga, S.; Goto, Y.; Matsumoto, Y.; Fusetani, N. *Mar. Drugs* **2004**, *2*, 55–62.
(4) Kupchan, S. M.; Britton, R. W.; Ziegler, M. F.; Sigel, C. W. *J. Org. Chem.* **1973**, *38*, 178–179.
(5) Merfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591–596.
(6) Goto, Y.; Nakao, Y.; Matsumoto Y.; Kawazu, S.-I.; Matsumoto, Y. Submitted.
(7) Compagnone, R. S.; Piña, I. C.; Rangel, H. R.; Dagger, F.; Suárez, A. I.; Reddy, A. M. V. R.; Faulkner, D. J. *Tetrahedron* **1998**, *54*, 3057–3068.
(8) Rao, K. V.; Santarsiero, B. D.; Mesecar, A. D.; Schinazi, R. F.; Tekwani, B. L.; Hamann, M. T. *J. Nat. Prod.* **2003**, *66*, 823–828.
(9) Adamczeski, M.; Quinoa, E.; Crews, P. *J. Am. Chem. Soc.* **1989**, *111*, 647–654.
(10) Fukuzawa, S.; Matsunaga, S.; Fusetani, N. *J. Org. Chem.* **1995**, *60*, 608–614.

NP8000317