# Cupolamide A: A Cytotoxic Cyclic Heptapeptide from Two Samples of the Sponge *Theonella cupola*

Lea S. Bonnington, Junichi Tanaka, and Tatsuo Higa*

*Department of Chemistry, Biology, and Marine Science, University of the Ryukyus, Nishihara, Okinawa 903-01*

Junji Kimura and Yuki Yoshimura

*Department of Chemistry, College of Science and Engineering, Aoyama Gakuin University, 6-16-1 Chitosedai, Setagaya, Tokyo, 157*

Yoichi Nakao, Wesley Y. Yoshida, and Paul J. Scheuer*

*Department of Chemistry, University of Hawai'i at Manoa, 2545 The Mall, Honolulu, Hawaii 96822-2275*

*Received June 9, 1997*[⊗]

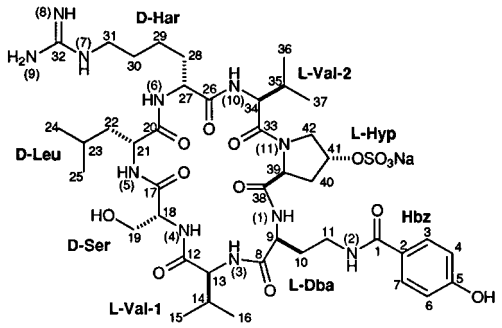
A new cyclic heptapeptide cupolamide A (**1**) was isolated from two samples of the sponge *Theonella cupola* collected in Indonesia and Okinawa. It contains two L-Val, one D-Leu, and one D-Ser, and three uncommon amino acid residues, D-homoarginine (Har), *trans*-4-hydroxy-L-proline (Hyp), and L-2,4-diaminobutanoic acid (Dba). The structure elucidation of **1** was performed by NMR spectroscopic techniques and the stereochemistry was determined by Marfey's method. Cupolamide A (**1**) was active against P388 murine leukemia cells with an $IC_{50} = 7.5$ $\mu$g/mL.

## Introduction

The relatively rare sponge genus *Theonella* has given rise to a remarkably diverse range of secondary metabolites. Among these are macrolides, e.g. the swinholides,[1,2] antiviral alkaloids such as the onnamides,[3,4] and a wealth of peptides.[5] In addition, our Hawaii group recently reported a halogenated nucleoside, kumusine, from an unidentified Indonesian *Theonella* species.[6] Continuing work with the methanolic extract of the freeze-dried sponge led our Hawaii/Tokyo group to the isolation of a new cytotoxic heptapeptide initially named kumamide. Our Okinawa group had also isolated a new heptapeptide, cupolamide A, in addition to kumusine and other peptides from *Theonella cupola* collected in Okinawa.[7] Occurrence of the same distinctive classes of metabolites in the two samples suggested that both groups were working with the same sponge. *T. cupola*. We named the new peptide cupolamide A (**1**) and now describe its isolation and structure elucidation. Three of the amino acid residues, 4-hydroxyproline (Hyp), homoarginine (Har), 2,4-diaminobutanoic acid (Dba), are of uncommon occurrence, as is amide formation of the $\gamma$-amino group of Dba with *p*-hydroxybenzoic acid.

## Result and Discussion

The Hawaii specimen was collected in Manado, Indonesia, in 1992.[6] A freeze-dried sample (503 g) was extracted with MeOH and after solvent removal the residue was partitioned between $H_2O$ and $CHCl_3$. The aqueous layer was extracted with *n*-BuOH, and the BuOH extract, which exhibited inhibition of serine proteases (thrombin and trypsin), was subjected to ODS flash chromatogaphy (aqueous MeOH gradient). An active fraction, which also showed inhibition against thrombin, was separated on an ODS column (MeCN/$H_2O$ 35:65), furnishing cupolamide A (**1**) as an amorphous solid (7 mg, $1.4 \times 10^{-3}$% based on dry weight).




**1**

On the other hand, a sample (600 g wet wt) of the sponge collected in Yonaguni, Okinawa, in 1994 was extracted with acetone. After solvent removal the aqueous suspension was extracted with EtOAc. The aqueous layer was concentrated to dryness and the residue was washed with MeOH. The MeOH extract, which exhibited cytotoxicity against P388 ($IC_{50}$ 10 $\mu$g/mL) was chromatographed on RP-18 silica ($H_2O$/MeOH) followed by Sephadex LH-20 (MeOH/$CH_2Cl_2$) and finally by HPLC (RP-18, $H_2O$/MeCN) to give **1** (101 mg, $1.7 \times 10^{-2}$% based on wet wt) as an amorphous solid.

The molecular formula, $C_{42}H_{67}N_{11}O_{14}SNa$, of cupolamide A (**1**) was determined by HR-FABMS (*m/z* 1004.4467, $\Delta$ −1.4 mmu). Salient spectral features—IR bands at 3343 and 1660 $cm^{-1}$, amide ($\delta_H$ 7−8.5, $\delta_C$ 170−173) and α-proton ($\delta_H$ 4−5) NMR signals—revealed the peptide nature of cupolamide A. The presence of a sodium sulfate function was indicated by a FABMS

[⊗] Abstract published in *Advance ACS Abstracts,* October 1, 1997.

(1) Carmely, S.; Kashman, Y. *Tetrahedron Lett.* **1985**, *26*, 511−514.
(2) Kobayashi, M.; Tanaka, J.; Katori, T.; Matsuura, M.; Yamashita, M.; Kitagawa, I. *Chem. Pharm. Bull.* **1990**, *38*, 2409−2418.
(3) Sakemi, S.; Ichiba, T.; Kohmoto, S.; Saucy, G.; Higa, T. *J. Am. Chem. Soc.* **1988**, *110*, 4851−4853.
(4) Matsunaga, S.; Fusetani, N.; Nakao, Y. *Tetrahedron* **1992**, *48*, 8369−8376.
(5) Fusetani, N.; Matsunaga, S. *Chem. Rev.* **1993**, *93*, 1793−1806.
(6) Ichiba, T.; Nakao, Y.; Scheuer, P. J.; Sata, N. U.; Kelly-Borges, M. *Tetrahedron Lett.* **1995**, *36*, 3977−3980.
(7) At a seminar at the University of Hawai'i on April 4, 1997, one of the authors (T.H.) revealed the structure of cupolamide A, which appeared closely related to kumamide. Careful comparison of spectral data revealed their identity. According to T. Ichiba, collector of the Indonesian sponge, the two specimens possess identical morphology.

S0022-3263(97)01028-1 CCC: $14.00 

**Table 1. NMR Data for Cupolamide A (1) in DMSO-$d_6$**

| | # | $^{13}$C | $^{1}$H | HMBC | | # | $^{13}$C | $^{1}$H | HMBC |
|---|---|---|---|---|---|---|---|---|---|
| **Hbz** | 1 | 166.1 s | | | **Leu** | 22a | 39.5 t | 1.63 ddd 5, 9, 14 | C: 20, 21, 23−25 |
| | 2 | 125.2 s | | | | 22b | | 1.48 m | C: 20, 21, 23−25 |
| | 3 | 129.0 d | 7.72 d9 | C: 1, 5 | | 23 | 24.3 d | 1.78 m | C: 22, 24, 25 |
| | 4 | 114.7 d | 6.77 d9 | C: 2, 5 | | 24 | 22.8 q | 0.93 d7 | C: 22, 23, 25 |
| | 5 | 160.0 s | | | | 25 | 21.4 q | 0.88 d7 | C: 22, 23, 24 |
| | 6 | 114.7 d | 6.77 d9 | C: 2, 5 | | NH-5 | | 8.47 d4 | C: 17, 21, 22 |
| | 7 | 129.0 d | 7.72 d9 | C: 1, 5 | **Har** | 26 | 170.4 s | | |
| **Dba** | 8 | 171.0 s | | | | 27 | 52.1 d | 4.19 m | C: 26, 29, 30 |
| | 9 | 60.6 d | 3.60 m | C: 8, 10, 11, 38 | | 28a | 30.2 t | 1.90 m | |
| | 10a | 28.8 t | 2.42 m | C: 8 | | 28b | | 1.47 m | C: 26, 30 |
| | 10b | | 2.18 m | C: 11 | | 29 | 22.7 t | 1.27 m | C: 27, 28, 30, 31 |
| | 11 | 36.7 t | 3.29 m | C: 8, 9, 10 | | 30 | 27.8 t | 1.45 m | C: 26−28, 31 |
| | NH-1 | | 8.77 d7 | C: 9, 10, 38 | | 31 | 40.4 t | 3.08 m | C: 27−29, 32 |
| | NH-2 | | 8.15 t6 | C: 1, 11 | | 32 | 156.8 s | | |
| **Val-1** | 12 | 170.6 s | | | | NH-6 | | 7.58 d9 | C: 20, 27, 28 |
| | 13 | 60.6 d | 4.05 dd 9, 9 | C: 12, 14, 15 | | NH-7 | | 7.44 dd 5, 6 | |
| | 14 | 31.3 d | 1.93 m | C: 13, 15, 16 | **Val-2** | 33 | 171.3 s | | |
| | 15 | 18.5 q | 0.84 d7 | C: 13, 14, 16 | | 34 | 55.8 d | 4.33 dd 10, 10 | C: 26, 33, 35, 36 |
| | 16 | 19.2 q | 0.82 d7 | C: 13, 14, 15 | | 35 | 30.7 d | 2.21 m | C: 34, 36, 37 |
| | NH-3 | | 7.83 d10 | C: 8, 13 | | 36 | 19.2 q | 0.83 d7 | C: 34, 35, 37 |
| **Ser** | 17 | 172.2 s | | | | 37 | 17.9 q | 0.95 d7 | C: 34, 35, 36 |
| | 18 | 52.8 d | 4.73 ddd 4, 5, 9 | C: 17, 19 | | NH-10 | | 7.11 d10 | C: 26, 34 |
| | 19a | 63.3 t | 4.10 m | C: 18 | **Hyp** | 38 | 171.4 s | | |
| | 19b | | 3.62 m | | | 39 | 59.8 d | 4.25 dd 6, 11 | C: 33, 40 |
| | NH-4 | | 8.05 d9 | C: 12 | | 40a | 35.4 t | 2.57 dd 6, 13 | C: 41, 42 |
| | OH | | 5.36 dd 4, 5 | C: 18 | | 40b | | 1.97 ddd 4, 11, 13 | C: 38 |
| **Leu** | 20 | 172.3 s | | | | 41 | 74.6 d | 4.86 dd 3, 4 | C: 39 |
| | 21 | 54.6 d | 3.94 ddd 4, 4, 9 | C: 20, 22, 23 | | 42a | 54.8 t | 3.95 d12 | C: 40, 41 |
| | | | | | | 42b | | 3.85 dd 3, 12 | |

fragment ion at $m/z$ 902 [$(M + 1)^+ - SO_3Na$] and by the IR band at 1246 cm$^{-1}$ and confirmed by positive sodium rhodizonate test. Analysis of 1D and 2D NMR (COSY, TOCSY, HMQC, HMBC) data delineated eight spin systems which could be ascribed to seven amino acid residues [two Val, Leu, Ser, homoarginine (Har), 4-hydroxyproline (Hyp), 2,4-diaminobutanoic acid (Dba)] and to a *p*-hydroxybenzoyl (Hbz) moiety (Table 1). The amino acid composition which included three uncommon residues (Har, Hyp, Dba) was confirmed by amino acid analysis employing the PTC method. The presence of a guanidino function was shown by a positive Sakaguchi test and by a characteristic $^{13}$C NMR chemical shift ($\delta$ 156.8) for C-32. Its location in Har was demonstrated by HMBC correlation of H-31 to C-32. The connectivity of H-27 through H-31 in Har was ascertained by consecutive COSY correlation. Observation of interresidual HMBC correlation, cross peaks between an amide NH and/or α-proton of each residue and a carbonyl carbon of next residue, led to the cyclic sequence Val1-Dba-Hyp-Val2-Har-Leu-Ser-Val1. The linkage of Hbz with the $\gamma$-amino group of Dba was established by HMBC correlation of H−N-2 to C-1 of Hbz as shown in Figure 1.

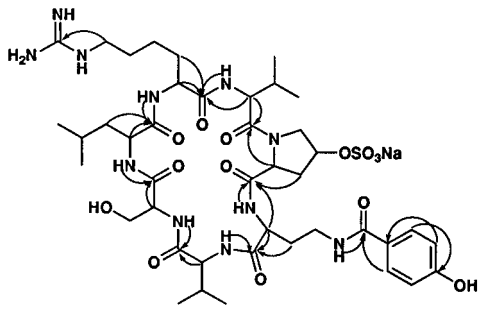



**Figure 1.** Sequential HMBC correlations of **1**.

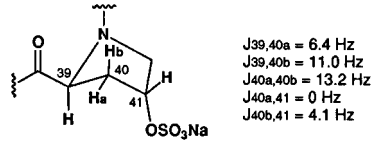



**Figure 2.** Relative stereochemistry of Hyp.

The remaining portion of the molecule, $SO_3Na$, was located as a sulfate ester of the hydroxyl group of Hyp. Other possible positions, either on Ser or Hbz, were excluded by observing chemical shift differences between **1** and its diacetate (**2**). Acetate **2** exhibited large downfield shifts for the methylene protons ($\Delta\delta$ +0.40, +0.58) of Ser and the aromatic protons ($\Delta\delta$ +0.43, +0.17) but were virtually unchanged for the $\gamma$-proton ($\Delta\delta$ −0.02) of Hyp, thereby confirming acetylation of Ser and Hbz in **2** and consequently the location of the sulfate on Hyp. The relative configuration of Hyp was suggested to be *trans* by analysis of coupling constants of the protons in Hyp. A large coupling constant ($J$ = 11.0 Hz) between H-39 ($\delta$ 4.25) and H-40b ($\delta$ 1.97) indicated a *trans* diaxial relationship of the two protons in a preferred half-chair conformation of Hyp. This allows H-40a ($\delta$ 2.57), which shows $J_{39,40a}$ = 6.4 Hz, to be located *cis* to H-39. The couplings $J_{40b,41}$ = 4.1 Hz and $J_{40a,41}$ = 0 Hz revealed that H-41 ($\delta$ 4.86) is *cis* to H-40b and thus *trans* to H-39, as shown in Figure 2.

The stereochemistry of **1** was determined by Marfey analysis,[8] which led to the assignment of the L-configuration for Val, Dba, and Hyp and the D-configuration for Leu, Ser, and Har. Since D-Har and D-Dba were commercially unavailable, racemic mixtures prepared from L-isomers were used as standards in the analysis. L-*trans* stereochemistry for Hyp was ascertained by using the Matsunaga and Fusetani modification[9] of the Marfey analysis.

Of the three uncommon amino acids, *trans*-4-hydroxyproline is a constituent of collagen and occasionally has been found in terrestrial biota.[10] It has also been reported as a constituent of hormothamnin A isolated from the marine cyanophyte *Hormathamnion enteromor-*

(8) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(9) Matsunaga, S.; Fusetani, N. *J. Org. Chem.* **1995**, *60*, 1177−1180.
(10) Mauger, A. B. *J. Nat. Prod.* **1996**, *59*, 1205−1211.

*phoides*[11] and of kahalalides H and J from the marine sacoglossan mollusk *Elysia rufescens*.[12] Homoarginine is a rare constituent of a toxic peptide isolated from a freshwater bluegreen alga.[13] Finally, 2,4-diaminobutanoic acid is known, for example, as a metabolite of *Pseudomonas* spp.[14,15] Its resemblance to GABA may indicate the central nervous system activity of cupolamide A, which has not been assayed. In contrast to the anti-thrombin activity of the crude extract, cupolamide A was active against P388 murine leukemia cells with an $IC_{50}$ of 7.5 $\mu$g/mL but not against thrombin.

## Experimental Section

**General Procedures.** IR spectra were measured as KBr pellets on a FTIR spectrophotometer. Optical rotations were recorded on a digital polarimeter. UV spectra were obtained using a diode array spectrophotometer. NMR spectra were recorded at 500 MHz for $^1$H and 125 MHz for $^{13}$C.

**Material.** The sponge used in Hawaii was collected north of Manado Bay, Indonesia, in October, 1992, at −64 m and identified by Dr. M. Kelly-Borges as *Theonella* sp.[6] The sponge used in Okinawa was collected at −35 m at the Umabana point of Yonaguni, Okinawa, Japan, in September, 1994, and identified by Dr. J. N. A. Hooper as *T. cupola*. A voucher specimen (QMG312708) has been deposited at Queensland Museum, South Brisbane, Australia.

**Extraction and Isolation. Okinawan Sample.** A wet frozen sample (600 g) was extracted by steeping in acetone. The extract was concentrated and the resulting aqueous suspension was extracted with EtOAc. The aqueous layer was dried under vacuum, and the residue was washed with MeOH. The MeOH-soluble material (5.34 g) which exhibited cytotoxicity ($IC_{50}$ 10 $\mu$g/mL) against P388 was subjected to flash chromatography on RP-18 silica (stepwise gradient of aqueous MeOH). A fraction (198 mg) eluted with $H_2O$/MeOH 1:3 was further separated on Sephadex LH20 ($CH_2Cl_2$/MeOH 1:1) into three fractions. HPLC separation (RP-18, $H_2O$/MeCN 5:2) of the first fraction (114 mg) gave 101 mg of cupolamide A (**1**) as a white amorphous solid, $[\alpha]_D$ −34.28 (*c* 1.94, MeOH); UV (MeOH) $\lambda_{max}$ 253 nm ($\epsilon$ 1.1 × 10$^4$); IR (KBr) 3343, 1660, 1622, 1538, and 1246 cm$^{-1}$; LR-FABMS (NBA matrix) *m*/*z* 1004 ($M^+$), 902, 731, 307, and 289; HR FABMS *m*/*z* 1004.4467 ($\Delta$ −1.4 mmu) ($C_{42}H_{67}N_{11}O_{14}SNa$); $^1$H and $^{13}$C NMR, see Table 1.

**Indonesian Sample.** A freeze-dried sample (503 g) was extracted with MeOH. The MeOH extract was concentrated and the resulting residue was partitioned between $CHCl_3$ and $H_2O$. The aqueous layer was further extracted with *n*-BuOH, and the *n*-BuOH layer which showed activity as a serine protease inhibitor was subjected to ODS flash chromatography using a stepwise gradient system of aqueous MeOH. A part of the most active fraction that eluted with 70% MeOH was triturated with 60% MeCN. The soluble part was separated by ODS HPLC with 35% MeCN to yield 7.0 mg of cupolamide A (**1**): $[\alpha]_D$ −128 (*c* 0.05, MeOH); UV (MeOH) $\lambda_{max}$ 253 nm ($\epsilon$ 1.3 × 10$^4$); HR-FABMS (glycerol matrix): *m*/*z* 902.5071 [$(M + H)^+ - SO_3Na$)] ($C_{42}H_{68}N_{11}O_{11}$), $\Delta$ −2.9 mmu). The $^1$H and $^{13}$C NMR spectra were identical with those of **1** from the Okinawan sample.

**Acetylation of 1.** A mixture of 4.0 mg of **1**, 0.5 mL of acetic anhydride, and 0.5 mL of pyridine was stirred overnight at room temperature. After concentration to dryness under nitrogen, the product mixture was dissolved in MeOH and filtered over a small amount of RP-18 silica in a disposable pipet. The residue of the filtrate was purified by HPLC (RP-18, MeCN/MeOH/$H_2O$ 2:1:2.8) to give 3 mg of diacetate **2** as a white amorphous solid. $^1$H NMR (DMSO-$d_6$) $\delta$ 0.81 (6H, H-15, 37), 0.82 (3H, H-16), 0.88 (3H, H-24), 0.91 (3H, H-36), 0.93 (3H, H-25), 1.23 (1H, H-29b), 1.34 (1H, H-29a), 1.45 (2H, H-22ab), 1.46 (2H, H-30), 1.50 (1H, H-28b), 1.61 (1H, H-28a), 1.75 (1H, H-23), 1.78 (3H, AcO-19), 1.94 (1H, H-40b), 1.95 (1H, H-14), 2.19 (1H, H-35), 2.20 (1H, H-10b), 2.33 (3H, AcO-5), 2.45 (1H, H-10a), 2.59 (1H, H-40a), 3.09 (1H, H-31), 3.30 (2H, H-11), 3.60 (1H, H-9), 3.85 (1H, H-42b), 3.85 (1H, H-27), 3.92 (1H, H-42a), 4.00 (1H, H-13), 4.19 (1H, H-39), 4.20 (1H, H-19b), 4.20 (1H, H-21), 4.33 (1H, H-34), 4.50 (1H, H-19a), 4.84 (1H, H-18), 4.84 (1H, H-41), 7.20 (2H, H-4,6), 7.21 (1H, NH-10), 7.34 (1H, NH-5), 7.39 (1H, NH-7), 7.81 (1H, NH-3), 7.89 (2H, H-3,7), 8.31 (1H, NH-4), 8.37 (1H, NH-6), 8.43 (1H, NH-2), 8.74 (NH-1); LR-FABMS (triethanolamine) *m*/*z* 1088 ($M^+$).

**Amino Acid Analysis.** A solution of **1** (3.3 mg) in 6 N HCl (1 mL) was heated at 110 °C under nitrogen in a sealed tube. After cooling to room temperature, the reaction mixture was concentrated to dryness under vacuum with three consecutive additions of water (5 mL each) to ensure complete elimination of HCl. Part (10 $\mu$g) of this product was treated with a solution of phenyl isothiocyanate (PITC) in MeOH/$H_2O$/$Et_3N$. Amino acid standard mixture, Har, Hyp, and Dba were identically treated separately with PITC. These products were analyzed by HPLC (Wakosil-PTC column) by gradient elution using a commercially available solvent system (A, 6% MeCN; B, 60% MeCN in $AcONH_4$-buffered solution) at a flow rate of A + B at 1 mL/min, varying B from 0 to 70% in 15 min. Elution was monitored by UV at 254 nm. The elution profile of the hydrolysate of **1** showed peaks corresponding to 1 mol equiv of Hyp, Ser, Har, Leu, and Dba at $t_R$ 5.293, 6.496, 8.649, 13.064, and 14.070 min, respectively, and 2 mol equiv of Val at 11.282 min.

**Determination of Absolute Configuration.** The hydrolysate (0.28 mg) of **1** in 40 $\mu$L of $H_2O$, prepared as above, was treated with a solution of $N^\alpha$-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (FDAA, 0.76 mg) in acetone (80 $\mu$L) and 20 $\mu$L of 1 N $NaHCO_3$ at 40 °C for 1 h. L- and D-amino acid standards were treated separately with FDAA in the same manner. For standards of D-Har and D-Dba which were commercially unavailable, racemic mixtures prepared by racemization[16] of L-isomers were used. The FDAA derivatives were analyzed by HPLC (Capcell Pak, UG120) eluting with 0.05 M $H_3PO_4$ in $Et_3N$ (pH adjusted to 3.0)/MeCN (either 7:3 or 8:2) at a flow rate of 2 mL/min. The elution profile was monitored by UV at 340 nm. Retention times of the constituents of **1** matched with L-Val, L-Dba, L-*trans*-Hyp, D-Har, D-Leu, and D-Ser.

**Acknowledgment.** The UH and AGU authors are indebted to Professor Nobuhiro Fusetani of The University of Tokyo for bioassays (enzyme assay and cytotoxicity) and mass spectral measurements and also thank Tatsuya Takahashi of AGU for amino acid analysis. Financial assistance (to UH) by the National Science Foundation, the Sea Grant College Program, and PharmaMar, S. A., is gratefully acknowledged. Y.N. was financially supported by a Japan Society for the Promotion of Science Postdoctoral Fellowships for Research Abroad. The UR authors thank Y. Murakami of Kirin Brewery Co. Ltd. for HR-FABMS and Dr. W. Henderson and W. Jackson of the University of Waikato, New Zealand, for ESMS measurement. They also acknowledge financial support by Grant-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, and Science (No. 08266103).

**Supporting Information Available:** $^1$H and $^{13}$C NMR, COSY, TOCSY, NOESY, HMQC, and HMBC spectra for **1** (7 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JO9710285

(11) Gerwick, W. H.; Jiang, Z. D.; Agarwal, S. K.; Farmer, B. T. *Tetrahedron* **1992**, *48*, 2313−2324.

(12) Goetz, G.; Nakao, Y.; Scheuer, P. J. *J. Nat. Prod.* **1997**, *60*, 562−567.

(13) Namikoshi, M.; Rinehart, K. L.; Sakai, R.; Sivonen, K.; Carmichael, W. W. *J. Org. Chem.* **1990**, *55*, 6135−6139.

(14) Lim, Y.; Suh, J-W.; Kim, S.; Hyun, B.; Kim, C.; Lee, C-H. *J. Antibiot.* **1994**, *47*, 1406−1416.

(15) Jacques, P.; Gwose, I.; Seinsche, D.; Taraz, K.; Budzikiewicz, H.; Schroeder, H.; Ongena, M.; Thonart, P. *Nat. Prod. Lett.* **1993**, *3*, 213−218.

(16) Yamada, S.; Hongo, C.; Yoshioka, R.; Chibata, I. *J. Org. Chem.* **1983**, *48*, 843−846.