Renieramycin J, a Highly Cytotoxic Tetrahydroisoquinoline Alkaloid, from a Marine Sponge *Neopetrosia* sp.[1]

Naoya Oku,[†] Shigeki Matsunaga,[†] Rob W. M. van Soest,[‡] and Nobuhiro Fusetani*,[†]

Laboratory of Aquatic Natural Products Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan, and Institute for Systematics and Ecology, University of Amsterdam, 1090 GT Amsterdam, The Netherlands

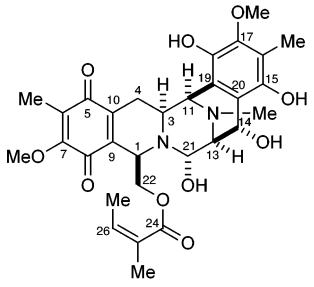
Received March 7, 2003

Renieramycin J (**1**), a new tetrahydroisoquinoline alkaloid, has been isolated from a marine sponge *Neopetrosia* sp. as a potent cytotoxin that induced morphological changes in 3Y1 cells. Such changes are characteristic of RNA and/or protein synthesis inhibitors. The structure of **1** including the absolute stereochemistry was determined by spectroscopic and chemical methods.

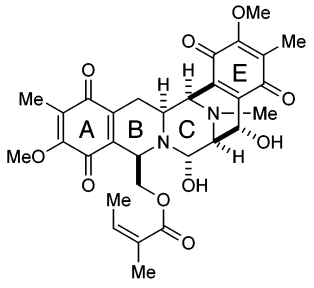
Tetrahydroisoquinoline antibiotics include more than 50 natural products that show antitumor, antimicrobial, and other biological activities.[2] The recent success of ecteinascidin 743 in human clinical trials has shed light on this class of natural products.[3] Since the discovery of renieramycins A−D from a Mexican marine sponge *Reniera* sp. by Frinke and Faulkner,[4] more than 20 tetrahydroisoquinolines have been isolated from marine sponges, tunicates, and a nudibranch.[5−14] In our continuing search for drug leads from Japanese marine invertebrates, we found that the organic extract of a violaceous sponge *Neopetrosia* sp. collected in southern Japan induced morphological changes in 3Y1 cells similar to those induced by RNA or protein synthesis inhibitors. Bioassay-guided fractionation of the extract led to the isolation of renieramycin J (**1**).

The EtOH extract of the frozen sponge (800 g) was partitioned between 60% aqueous MeOH and CH_2Cl_2, and the latter layer was further partitioned between 90% aqueous MeOH and *n*-hexane. The 90% aqueous MeOH layer was then partitioned into a mixture of EtOAc/*n*-heptane/MeOH/H_2O (7:4:4:3), and the lower phase was chromatographed on Sephadex LH-20 followed by HPLC with ODS and phenylhexyl columns to afford **1** as a brownish solid (16.3 mg, 2.0×10^{-3} % wet wt). The ^{1}H NMR spectrum of the freshly prepared **1** contained ca. 10% of a minor set of resonances, which increased gradually during measurements of NMR spectra, suggesting **1** to be susceptible to air oxidation.

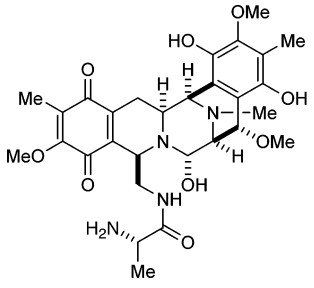
^{1}H NMR and HMQC data of **1** revealed the presence of two *O*-methyls (δ_H 3.97 and 3.69 s; δ_C 61.3 and 60.9), one *N*-methyl (δ_H 2.71 s; δ_C 42.4), and two each of aromatic (δ_H 2.14 and 1.85 s; δ_C 9.5 and 8.6) and olefinic methyls [δ_H 1.65 (dq, J = 7.3, 1.6 Hz) and 1.30 (quintet, J = 1.5 Hz); δ_C 20.4 and 15.2], which were diagnostic of renieramycins.[15] This was supported by a UV absorption maximum at 273 nm. The FAB mass spectrum of **1** exhibited the pseudomolecular ion peak at *m*/*z* 569 corresponding to the formula of $C_{30}H_{37}N_2O_9$, which was designated as the $(M + H)^+$ ion of the dehydration product of the bishydroquinone form. Similar dehydration and reduction reactions were observed in ecteinascidins[11] and safracins[16] during FABMS measurements. Although interpretation of the COSY spectrum was not straightforward due to the presence of long-range couplings (H-1/H-4a, H-1/H-4b, and H-11/H-13) and the absence of a coupling between vicinal



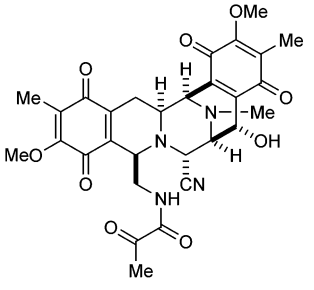
1



2



3



4

* To whom correspondence should be addressed. Tel: 81-3-5841-5299. Fax: 81-3-5841-8166. E-mail: anobu@mail.ecc.u-tokyo.ac.jp.
† The University of Tokyo.
‡ University of Amsterdam.

10.1021/np030092g CCC: $25.00
Published on Web 07/31/2003

Table 1. ^{1}H and ^{13}C NMR Data for Renieramycin J (**1**) in CD_3OD

position	^{13}C	^{1}H, m, *J* (Hz)	HMBC	NOESY
1	54.4.	4.33 ddd 2.4, 2.4, 2.4	9, 10	21
3	52.1	3.42 ddd 2.7, 3.1, 11.2	19	4b, 11
4a	25.7	1.56 ddd 2.3, 11.1, 17.4	3, 9, 10, 11	4b
4b		3.03 dd 2.4, 17.4	5, 9, 10	3, 4a, 11
5	187.0			
6	129.0			
6-CH_3	8.6	1.85 s	5, 6, 7, 7-OCH_3, 8	7-OCH_3
7	157.5			
7-OCH_3	61.3	3.97 s	7	6-CH_3
8	182.4			
9	138.1			
10	142.7			
11	58.4	4.55 brd 2.8	13, 18 19, 20	3, 4b, 12-CH_3
12-CH_3	42.4	2.71 s	11, 13	11, 13
13	63.9	3.82 brs	11, 14, 20, 21	12-CH_3, 14, 21
14	64.3	4.79 s	15, 16, 19, 20, 21	13, 21
15	148.1			
16	120.7			
16-CH_3	9.5	2.14 s	15, 16, 17, 17-OCH_3, 18, 20	17-OCH_3
17	147.5			
17-OCH_3	60.9	3.69 s	17	16-CH_3
18	142.1			
19	113.2			
20	118.7			
21	90.2	4.58 d 2.4	1, 3	1, 13, 14, 22
21-OCH_3				
22	64.4	4.25 brs (2H)	9, 24	21
24	168.6			
25	128.2			
25-CH_3	20.4	1.30 dq 1.5, 1.5	24, 25, 26	26
26	140.0	5.87 qq 1.6, 7.2	24, 25-CH_3, 26-CH_3	25-CH_3, 26-CH_3
26-CH_3	15.2	1.65 dq 7.3, 1.6	24, 25, 26	26

protons (H-13/H-14), the framework of the tricyclic portion (rings B, C, and D) could be determined on the basis of the HMBC data (Table 1). Placement of hydroxyl groups on C-14 and C-21 was assigned from comparison of the ^{1}H and ^{13}C chemical shifts with those of saframycin Mx1 (**3**)[17] and saframycin G (**4**),[18,19] while analysis of COSY and HMBC data led to the assignment and placement of an angelate ester on C-22 (Table 1). Assignment of rings A and E was carried out in the same fashion as those of saframycin Mx1[17] and saframycin E[18] again on the basis of the HMBC data (Table 1), thereby determining the gross structure of **1** as the ring E reduced form of 14-hydroxyrenieramycin E.[5] With the assignment of the major set of signals in hand, interpretation of the NMR data for the minor set of signals was unexceptional, leading to the ring E quinone structure **2** (Table 2).

The relative stereochemistry of **1** was deduced from the analysis of coupling constants and NOESY data. Magnitudes of coupling constants for protons on rings C, D, and E in **1** were almost identical with those reported for saframycins,[17,18,20] suggesting the identical stereochemistry with that of other compounds of this class. This was supported by the NOESY data (Figure 1).

The absolute configuration of **1** was determined by the modified Mosher method.[21] To avoid confusion by esterification of phenolic hydroxyl groups, **1** was first air-oxidized[17] to **2**, which was derivatized with (*S*)- or (*R*)-MTPACl. The $\Delta\delta$ values around C-14 indicated 14*S* configuration (Figure 2). Therefore, the absolute stereochemistry of **1** was identical with that of saframycins.[2]

Table 2. ^{1}H and ^{13}C NMR Data for Bisquinone (**2**) in CD_3OD

position	^{13}C	^{1}H, m, *J* (Hz)	HMBC
1	54.1	4.34 ddd 2.4, 2.4, 2.4	
3	51.0	3.24 ddd 2.7, 3.1, 11.5	
4a	26.8	1.17 ddd 2.4, 11.8, 16.6 (ax)	3, 9, 10
4b		2.67 dd 2.6, 16.9 (eq)	5, 9, 10
5	186.9		
6	129.0		
6-CH_3	8.6	1.85 s	5, 6, 7, 7-OCH_3, 8
7	157.5		
7-OCH_3	61.3	3.97 s	7
8	182.4		
9	138.5		
10	142.5		
11	56.2	3.99 d 2.4	3, 13, 18, 19, 20
12-CH_3	42.7	2.47 s	11, 13
13	62.3	3.38 d 1.5	11, 14, 20, 21
14	61.8	4.31m[a]	15, 18, 19, 20, 21
15	188.1		
16	130.5		
16-CH_3	8.7	1.91 s	15, 16, 17, 17-OCH_3, 18
17	156.7		
17-OCH_3	61.4	3.97 s	17
18	184.5		
19	136.1		
20	142.8		
21	90.6	4.31 m[a]	3
22a	64.9	4.23 dd 2.6, 11.4	1, 24
22b		4.29 dd 2.7, 11.3	1, 24
24	168.5		
25	128.1		
25-CH_3	20.7	1.54 dq 1.5, 1.5	22, 24, 25, 26
26	140.3	5.96 qq 1.6, 7.2	25-CH_3
26-CH_3	15.9	1.72 dq 7.3, 1.6	24, 25, 26

[a] Coupling constants were not determined due to overlapped signals.

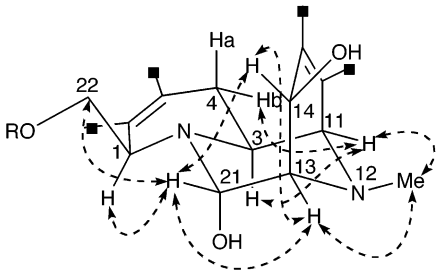


Figure 1. NOESY cross peaks for the CDE ring portion in **1**.

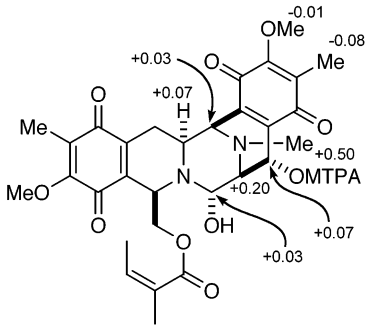


Figure 2. Distribution of $\Delta\delta$ values for the MTPA esters of **2**.

Shrinkage or disappearance of nucleoli in 3Y1 cells was observed by treatment with 86 nM **1** for 6 h. Inhibition of filopodial development was also evident. After exposure to renieramycin J for 12 h, cellular boundaries became obscure and the number of dead cells increased. By day five almost all cells were dead as observed under a light microscope. A similar course of events is observed upon treatment with actinomycin D (an inhibitor of RNA synthesis) or cycloheximide (inhibitor of protein synthesis). Upon exposure to a higher concentration of renieramycin J (1.7 μM), cells became round in 6 h and ca. 90% of them were dead within 24 h.

The cytotoxicities of **1** against 3Y1, HeLa, and P388 cells (IC_{50}, nM) were 5.3, 12.3, and 0.53, respectively. The structure−activity relationship study of saframycins has demonstrated the importance of the C-21 substitutent, which should be a hydroxyl or cyano group to exhibit potent activity.[22] The importance of the C-22 appending group was also noted.[23,24] No cytotoxicity data of renieramycins with a C-21 hydroxyl group were reported before this study. The potent cytotoxic activity of renieramycin J (**1**) together with a modest activity of renieramycin G,[6] whose C-21 was oxidized to carbonyl, not only confirms the importance of the C-21 substituent in this class of metabolites but also demonstrates the ability of a C-22-angelate derivative to exhibit potent activity.

Experimental Section

General Experimental Procedures. Optical rotations were obtained on a JASCO DIP-1000 digital polarimeter, and UV spectra on a Shimadzu BioSpec-1600 DNA/protein/enzyme analyzer. NMR spectra were recorded on either a JEOL A500 or A600 NMR spectrometer. ^{1}H and ^{13}C NMR chemical shifts were referenced to solvent peaks, δ_H 3.30 and δ_C 49.0 for CD_3OD. FAB mass spectra were measured on a JEOL JMX SX102/SX102 tandem mass spectrometer.

Animal Material. The violaceous sponge *Neopetrosia* sp. was collected off Kuchinoerabu-jima Island (30°28.31′ N, 130°11.73′ E) at depths of 5−15 m and off Iwo-jima Island (30°48.35′ N, 130°19.07′ E) at a depth of 20 m, the Satsunan Archipelago, in July 2001. They are massively encrusting sponges with upright oscular lobes which may be individual tube-like, height up to 4 cm, or clustered to form ridge-like elevations; oscules 2−5 mm in diameter; color a distinctive blue, with brownish discoloration at the sides and on the basal crust; surface punctate, smooth; consistency firm to hard; skeleton irregularly reticulate, with rounded or squarish meshes formed by one or a few spicules; primary spicule tracts indistinct, "superimposed" on the isotropic structure; no special ectosomal skeleton; spicules oxeas, sharply pointed, straight, of uniform size 225−260 × 11−13 μm. By its structure and firmness this sponge conforms to the newly revived genus *Neopetrosia*, but previously may have been assigned to *Xestospongia* s.l. The voucher is incorporated in the collections of the Zoological Museum of the University of Amsterdam under reg. no. POR. 16999.

Extraction and Isolation. The frozen sponge (800 g) was extracted with EtOH (2.4 L × 5), and the combined extract was concentrated to an aqueous suspension, which was diluted with MeOH to furnish a 60% aqueous MeOH solution (400 mL), which was extracted with CH_2Cl_2 (400 mL × 3). The CH_2Cl_2 layer was concentrated, redissolved in 90% aqueous MeOH (400 mL), and defatted with *n*-hexane (400 mL × 3). The aqueous MeOH layer was concentrated and redissolved in the lower layer (360 mL) of a solvent system EtOAc/*n*-heptane/MeOH/water (7:4:4:3, total 1440 mL) and washed with the upper layer (210 mL × 3) to give 1227.7 mg of the crude active fraction. The fraction was gel-filtered on Sephadex LH-20 (5.1 × 90 cm; mobile phase, MeOH), and the combined active fractions were purified by ODS-HPLC on a Cosmosil ARII column (2 × 25 cm; mobile phase, 38% aqueous MeCN/0.2 M NaCl; UV detection at 210 nm) to yield 40.7 mg of an active fraction, which was finally purified by HPLC with a phenylhexyl column (1 × 25 cm; mobile phase, 38% aqueous MeCN/0.2 M NaCl; UV detection at 210 nm) to afford 16.3 mg (2.0×10^{-3} %, wet wt) of renieramycin J (**1**).

Renieramycin J (1): brownish solid (containing 20−30% of bisquinone **2**); $[\alpha]^{21}_D$ 1.2° (*c* 0.02, MeOH); UV (MeOH) λ_{max} 203 (ϵ 39000), 273 (12000) nm; ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data, see Table 1; HRFABMS (glycerol) *m*/*z* 569.2502 $(M + 2H - OH)^+$ (calcd for $C_{30}H_{37}N_2O_9$, 569.2499).

Preparation of Bisquinone (2). To a solution of **1** (1.7 mg) in MeCN (2.5 mL) was added 2.5 mL of 50 mM sodium phosphate buffer adjusted to pH 7.5, and the mixture was stirred for 3 h in an open atmosphere. The solution was then titrated with 100 mM sodium phosphate buffer to pH below 7, evaporated to dryness, and desalted on a short ODS column to give **2** (1.7 mg).

Bisquinone (2): brownish solid; UV (MeOH) λ_{max} 203 (ϵ 27000), 269 (15000) nm; ^{1}H and ^{13}C NMR data, see Table 2; FABMS (glycerol) *m*/*z* 569 $(M + 4H - OH)^+$.

Preparation of MTPA Esters of 2. To a 0.85 mg portion of **2** was added 5 drops of dry pyridine and 10 μL of (*R*)-(−)-MTPACl, and the mixture was stirred for 10 s. After removal of pyridine under vacuum, the reaction mixtures were separated by ODS-HPLC using a linear gradient of aqueous MeCN containing 0.05% TFA (40% to 80% MeCN over 60 min) to furnish 0.2 mg of the (*S*)-MTPA ester and the unreacted bisquinone **2** (0.2 mg). The (*R*)-MTPA ester was prepared in the same way.

(*S*)-MTPA Ester of 2: ^{1}H NMR (CD_3OD) δ 6.59 (1H, qq, J = 1.5, 7.3 Hz, H-26), 5.67 (1H, s, H-14), 4.49 (1H, d, J = 2.7 Hz, H-21), 4.41 (1H, ddd, J = 2.2, 2.7, 3.5 Hz, H-1), 4.31 (1H, dd, J = 2.7, 11.5 Hz, H-22), 4.19 (1H, dd, J = 3.5, 11.5 Hz, H-22), 4.06 (1H, dd, J = 1.2, 3.1 Hz, H-11), 3.98 (3H, s, CH_3-7), 3.97 (3H, s, CH_3-17), 3.32 (overlapped with the solvent peak, H-13), 3.31 (overlapped with the solvent peak, H-3), 2.73 (1H, dd, J = 2.7, 16.9 Hz, H-4), 2.38 (3H, s, *N*CH_3-12), 1.87 (3H, s, CH_3-6), 1.82 (3H, s, CH_3-16), 1.77 (3H, dd, J = 1.5, 7.3 Hz, CH_3-26), 1.60 (3H, dq, J = 1.5, 1.5 Hz, CH_3-25), 1.20 (1H, ddd, J = 2.0, 12.0, 16.0 Hz, H-4).

(*R*)-MTPA Ester of 2: ^{1}H NMR (CD_3OD) δ 6.59 (1H, qq, J = 1.5, 7.3 Hz, H-26), 5.60 (1H, s, H-14), 4.46 (1H, d, J = 2.3 Hz, H-21), 4.40 (1H, ddd, J = 2.3, 2.7, 3.5 Hz, H-1), 4.34 (1H, dd, J = 2.5, 11.4 Hz, H-22), 4.18 (1H, dd, J = 3.1, 11.2 Hz, H-22), 3.98 (3H, s, CH_3-7), 3.98 (3H, s, CH_3-17), 3.97 (overlapped, H-11), 3.24 (1H, ddd, J = 2.3, 3.1, 11.9 Hz, H-3), 3.12 (1H, brs, H-13), 2.70 (1H, dd, J = 2.3, 16.9 Hz, H-4), 1.90 (3H, s, CH_3-16), 1.88 (3H, s, *N*CH_3-12), 1.86 (3H, s, CH_3-6), 1.77 (3H, brd, J = 7.3 Hz, CH_3-26), 1.60 (3H, dq, J = 1.5, 1.5 Hz, CH_3-25), 1.18 (1H, ddd, J = 2.4, 11.8, 16.8 Hz, H-4).

Cell Morphology Assay and Cytotoxicity Tests. The assays were carried out as described in ref 25.

Acknowledgment. This study was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology. N.O. acknowledges a JSPS Research Fellowship for Young Scientists.

Supporting Information Available: The 1D and 2D NMR spectra for **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Bioactive Marine Metabolites. Part 120. Part 119: Oku, N.; Matsunaga, S.; Fusetani, N. *J. Am. Chem. Soc.* **2003**, *125*, 2044−2045.
(2) Scott, J. D.; Williams, R. D. *Chem. Rev.* **2002**, *102*, 1669−1730.
(3) Aune, G. J.; Furuta, T.; Pommier, Y. *Anti-Cancer Drugs* **2002**, *13*, 545−555.
(4) Frincke, J. M.; Faulkner, D. J. *J. Am. Chem. Soc.* **1982**, *104*, 265−269.
(5) He, H.-Y.; Faulkner, D. J. *J. Org. Chem.* **1989**, *54*, 5822−5824.
(6) Davidson, B. S. *Tetrahedron Lett.* **1992**, *33*, 3721−3724.
(7) Parameswaran, P. S.; Nalik, C. G.; Kamat, S. Y. *Ind. J. Chem.* **1998**, *38B*, 1258−1263. For the correction of the structure of renieramycin H, see: Saito, N.; Sakai, H.; Suwanborirux, K.; Pummangura, S.; Kubo, A. *Heterocycles* **2001**, *55*, 21−28.

(8) Fontana, A.; Cavaliere P.; Wahidulla, S.; Naik, C. G.; Cimino, G. *Tetrahedron*, **2000**, *56*, 7305−7308.
(9) Pettit, G.; Knight, J. C.; Collins, J. C.; Herald, D. L.; Pettit, R. K.; Boyd, M. R.; Young, V. G. *J. Nat. Prod.* **2000**, *63*, 793−798.
(10) Wright, A. E.; Forleo, D. A.; Gunawardana, G. P.; Gunasekera, S. P.; Koehn, F. E.; McConnell, O. J. *J. Org. Chem.* **1990**, *55*, 4508−4512.
(11) Rinehart, K. L.; Holt, T. G.; Fregeau, N. L.; Stroh, J. G.; Keifer, P. A.; Sun, F.; Li, L. H.; Martin, D. G. *J. Org. Chem.* **1990**, *55*, 4512−4515.
(12) Sakai, R.; Rinehart, K. L.; Guan, Y.; Wang, A. H.-J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11456−11460.
(13) Sakai, R.; Jares-Erijman, E. A.; Manzanares, I.; Silva Elipe, M. V.; Rinehart, K. L. *J. Am. Chem. Soc.* **1996**, *118*, 9017−9023.
(14) Suwanborirux, K.; Charupant, K.; Amnuoypol, S.; Pummangura, S.; Kubo, A.; Saito, N. *J. Nat. Prod.* **2002**, *65*, 935−937.
(15) Interpretation of the NMR data was conducted with a sample containing ca. 30% of the oxidation product **2**.
(16) Cooper, R.; Unger, S. *J. Antibiot.* **1985**, *38*, 24−30.
(17) Trowitzsch-Kienast, W.; Irschik, H.; Reichenbach, H.; Wray, V.; Hofle, G. *Liebig Ann. Chem.* **1988**, 475−481.
(18) Saito, N.; Harada, S.; Nishida, M.; Inoue, I.; Kubo, A. *Chem. Pharm. Bull.* **1995**, *43*, 777−782.
(19) Renieramycin J (CD_3OD): δ 4.79 s (H-14), 4.58 d (J = 2.5 Hz; H-21); 64.3 (C-14), 90.2 (C-21). Saframycin Mx-1 (CD_3OD): δ 4.82 d (J = 2.8 Hz, H-14), 4.78 s (H-21); 73.0 (C-14), 89.2 (C-21). Saframycin G ($CDCl_3$): δ 4.33 d (J = 0.5 Hz, H-14); 62.1 (C-14).
(20) Lown, J. W.; Joshua, A. V.; Chen, H.-H. *Can. J. Chem.* **1981**, *59*, 2945−2952.
(21) Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092−4096.
(22) Kishi, K.; Yazawa, K.; Takahashi, K.; Mikami, Y.; Arai, T. *J. Antibiot.* **1984**, *37*, 847−852.
(23) Martinez, E. J.; Corey, E. J. *Org. Lett.* **1999**, *1*, 75−77.
(24) Meyers, A. G.; Plowright, A. T. *J. Am. Chem. Soc.* **2001**, *123*, 5114−5115.
(25) Oku, N.; Matsunaga, S.; Wada, S.; Watabe, S.; Fusetani, N. *J. Nat. Prod.* **2000**, *63*, 205−209.

NP030092G