JOURNAL OF NATURAL PRODUCTS

Note

pubs.acs.org/jnp

Naphthoquinone-like Polyketide Isolated from *Streptomyces* sp. RI-77 and Its Predicted Biosynthetic Pathway

Miho Izumikawa,[†] Ryutaro Satou,[‡] Keiichiro Motohashi,[†] Aya Nagai,[†] Yasuo Ohnishi,[‡] Motoki Takagi,*[,†] and Kazuo Shin-ya*[,§]

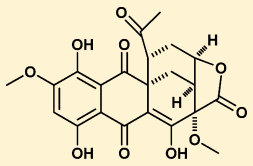
[†]Biomedicinal Information Research Center (BIRC), Japan Biological Informatics Consortium (JBIC), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan

[‡]Department of Biotechnology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

[§]Biomedicinal Information Research Center (BIRC), National Institute of Advanced Industrial Science and Technology (AIST), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan

Supporting Information

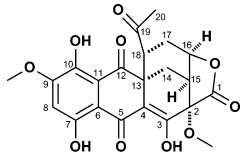
ABSTRACT: A novel naphthoquinone-like polyketide, JBIR-85 (**1**), with a unique skeleton and antioxidative activity was isolated from a culture of *Streptomyces* sp. RI-77. The planar structure of **1** was established on the basis of extensive NMR and MS analyses. The structure of **1** including the absolute configuration was established via X-ray crystallographic analysis. Since **1** exhibits a unique skeleton, we performed feeding experiments to reconfirm the structure and predict the biosynthetic pathway.



JBIR-85 (**1**)

Members of the class Actinobacteria have been extensively studied because of their ability to produce pharmaceutically useful compounds. In order to construct a natural product library for bioactivity screening, we isolated a diverse variety of actinomycetes from marine organisms, including marine sponges and tunicates, and mangrove soils, in addition to the conventionally utilized terrestrial soils. As a result, we discovered novel compounds JBIR-23 and JBIR-24,[1,2] which are cytotoxic compounds, JBIR-68,[3] which is an anti-influenza compound, and JBIR-88,[4] which is an antimicrobial compound. Further chemical screening with LC-MS has resulted in the isolation of a novel antioxidative naphthoquinone-like polyketide, designated JBIR-85 (**1**), from a culture of *Streptomyces* sp. RI-77 obtained from a soil sample collected in Okinawa Prefecture, Japan. This paper describes the fermentation, isolation, structural elucidation, and biosynthesis of **1**.

Streptomyces sp. RI-77 was cultured in a production medium, and the mycelial cake extracted with acetone and concentrated in vacuo. The concentrate was partitioned between EtOAc and H_2O, and the EtOAc layer concentrated in vacuo and subjected to sequential normal-phase MPLC and gel filtration chromatography. The pure form of **1** was finally isolated as a yellow solid using reversed-phase HPLC.



JBIR-85 (**1**)

The HRESIMS of **1** revealed a molecular formula of $C_{22}H_{20}O_{10}$. In accordance with a highly conjugated aromatic framework, **1** absorbed UV radiation at 243 and 419 nm. The IR spectrum of **1** revealed the presence of a γ-lactone group with an absorption at 1781 cm^{-1}. The ^1H and ^{13}C NMR spectroscopic data for **1** are shown in Table 1. The structure of **1** was further elucidated by a series of 2D NMR analyses including HSQC, constant-time HMBC,[5] and double-quantum-filtered (DQF)-COSY spectra (Figure 1).

$^1H-^{13}C$ long-range couplings from the singlet aromatic proton H-8 (δ_H 6.77) to aromatic carbons C-6 (δ_C 105.4), C-7 (δ_C 159.4), C-9 (δ_C 157.2), and C-10 (δ_C 147.3) were observed in the HMBC spectrum of **1**. Two phenolic hydroxy protons, 7-OH (δ_H 12.22) and 10-OH (δ_H 12.01), are hydrogen-bonded to the carbonyl carbons in the *peri* position C-5 (δ_C 188.2) and C-12 (δ_C 201.4), respectively. In addition, 7-OH and 10-OH were long-range coupled to C-6, C-7, and aromatic carbon C-8 (δ_C 107.6), and C-9, C-10, and aromatic quaternary carbon C-11 (δ_C 111.7), respectively, thereby demonstrating the existence of the naphthoquinone-like structure shown in Figure 1. The $^1H-^{13}C$ long-range couplings from methoxy proton 9-*O*Me (δ_H 4.00) to C-9 and from the aromatic proton H-8 to a carbonyl carbon C-5 (δ_C 188.2) proposed assignments for these signals. A sequence from methylene protons H_2-14 (δ_H 2.66 and 1.88) to methine proton H-18 (δ_H 3.05) through methine proton H-15 (δ_H 3.23), oxymethine proton H-16 (δ_H 4.93), and methylene protons H_2-17 (δ_H 2.33 and 1.61) was observed in the DQF-COSY spectrum. The $^1H-^{13}C$ long-range couplings from H_2-14 to methine carbon C-18 (δ_C 54.8) and from H-15, H-18, and H_2-17 to quaternary carbon C-13 (δ_C 48.4) confirmed the cyclohexane ring moiety. In addition, the

Received: August 4, 2011
Published: December 6, 2011

dx.doi.org/10.1021/np200651e | *J. Nat. Prod.* 2011, 74, 2588–2591

Table 1. ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR Spectroscopic Data for JBIR-85 (1) in $CDCl_3$

position	δ_C	δ_H (multiplicity, J in Hz)
1	169.9	
2	77.6	
3	169.3	
4	109.0	
5	188.2	
6	105.4	
7	159.4	
8	107.6	6.77, s
9	157.2	
10	147.3	
11	111.7	
12	201.4	
13	48.4	
14	19.5	2.66, dd (14.4, 4.1)
		1.88, dd (14.4, 3.5)
15	36.2	3.23, ddd (7.6, 4.1, 3.5)
16	71.4	4.93, ddd (8.6, 8.4, 7.6)
17	29.5	2.33, ddd (15.2, 8.4, 2.1)
		1.61, ddd (15.2, 8.6, 5.8)
18	54.8	3.05, dd (5.8, 2.1)
19	208.4	
20	31.6	1.90, s
2-OMe	53.2	3.32, s
9-OMe	56.6	4.00, s
3-OH		14.84, s
7-OH		12.22, s
10-OH		12.01, s

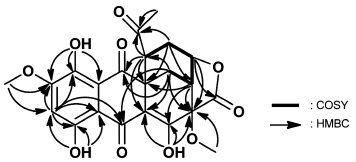


Figure 1. Key correlations observed in the 2D NMR spectra of **1** (bold lines show $^{1}H-^{1}H$ DQF-COSY results, and arrows show HMBC results).

long-range couplings from singlet methyl protons H_3-20 (δ_H 1.90) and H-18 to carbonyl carbon C-19 (δ_C 208.4) revealed that an acetyl group was bonded to C-18.

$^{1}H-^{13}C$ long-range couplings from phenolic hydroxy proton 3-OH (δ_H 14.84) to oxygenated quaternary carbon C-2 (δ_C 77.6) and olefinic quaternary carbons C-3 (δ_C 169.3) and C-4 (δ_C 109.0) revealed that this hydroxy group was attached to C-3. Additional $^{1}H-^{13}C$ long-range couplings from H-15 to oxygenated quaternary carbon C-2 and olefinic quaternary carbon C-3, and from H_2-14 to C-2 and an olefinic quaternary carbon C-4, showed that these carbons compose a cyclohex-2-ene-1,2-dioxy moiety. Long-range coupling between methoxy protons 2-*O*Me (δ_H 3.32) and C-2 revealed the bond between the methoxy group and C-2, while $^{1}H-^{13}C$ long-range coupling from the oxymethine proton H-16 to ester carbonyl carbon C-1 (δ_C 169.9) indicated the presence of a γ-lactone moiety.

$^{1}H-^{13}C$ long-range couplings from the methylene protons H_2-14 and the methine proton H-18 to the carbonyl carbon C-12 clarified that the carbonyl functional group was connected to C-13. In addition, the hydrogen bond between the carbonyl carbon in the *peri* position and 10-OH (vide supra) suggested the connectivity between C-11 and C-12. Thus, these results established a unique naphthoquinone-like chromophore (Figure 1).

The structure and absolute configuration of **1** were confirmed using X-ray crystallographic analysis. X-ray crystallography of a platelet crystal of **1** obtained from crystallization from MeOH confirmed the proposed absolute structure of the unique fused tricyclic ring system with a Flack parameter[6] of x = 0.09(9) (Figure 2). Thus, the absolute configuration of **1** was

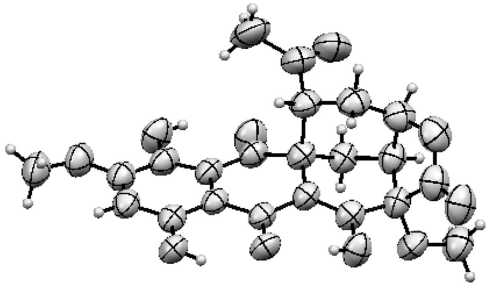


Figure 2. Molecular structure of **1** obtained by X-ray crystallographic analysis.

assigned as 15*S*, 2*S*, 13*S*, 18*R*, and 16*R*. The structure of **1** consists of a characteristic naphthoquinone-like chromophore with a tricyclic skeleton. Feng et al.[7] recently reported the only compound (UT-X26/F129) with an analogous chromophore and tricyclic substructure, and this was biosynthesized using a heterologous expression system with the environmental DNA harboring type II polyketide synthase. In comparison with UT-X26/F129, **1** has a methoxy group at C-2 instead of a hydroxy group and additional methoxy and hydroxy groups at C-9 and C-10, respectively. Compound **1** is the first example of this structure type for which the producing microorganism can be identified.

Since **1** consists of a unique polyketide, the biosynthesis is intriguing. Feng et al.[7] elucidated this biosynthetic pathway for the only known related structure, UT-X26/F129, via gene annotation. We sought to examine the biosynthesis of JBIR-85 by feeding experiments utilizing [1-^{13}C]acetate and [1,2-^{13}C]-acetate. The intact incorporation of ^{13}C-labeled acetate into the polyketide backbone of **1** was indicated by the high abundance of ^{13}C (Table 2). The labeling pattern of [1-^{13}C]acetate-derived **1** provided reasonable evidence that **1** is biosynthesized from a decaketide precursor by polyketide synthases (Figure 3). In contrast to the [1-^{13}C]acetate feeding experiment, the C-1 and C-18 (the closed square and triangle, respectively, in Figure 3) signals in the ^{13}C NMR spectrum of [1,2-^{13}C]acetate-labeled **1** were observed as uncoupled (singlet) signals. Thus, we predict that a bond between C-1 and C-18 in a linear tetracyclic intermediate is cleaved via a Baeyer–Villiger-type reaction, which is similar to the mechanism in the biosynthetic pathway of mithramycin.[8] The resultant linear tricyclic intermediate is then sequentially cyclized through two different possible pathways to generate the fused-pentacyclic ring system shown in Figure 3.

Compound **1** exhibited DPPH radical scavenging activity with an IC_{50} value of 165 μM, which is weak compared with that of α-tocopherol (IC_{50} = 28 μM). In addition, **1** exhibited no cytotoxic activity against several cancer cell lines and no antibacterial activity against *Micrococcus luteus* or *Escherichia coli*.

dx.doi.org/10.1021/np200651e | *J. Nat. Prod.* 2011, 74, 2588–2591

Table 2. ^{13}C (150 MHz) NMR Spectroscopic Data for JBIR-85 (1) and Enrichment Ratios of the Labeled Precursors

position	δ_C	[1-^{13}C]acetate enrichment ratio[a]	[1,2-^{13}C]acetate enrichment ratio[a]	$^1J_{C,C}$ (Hz)
1	170.1	7.1	6.6	s[b]
2	77.9	1.0	3.2	51
3	169.6	6.0	6.5	51
4	109.3	0.8	6.5	55
5	188.4	7.4	9.1	55
6	105.7	0.9	3.4	65
7	159.6	5.8	4.8	65
8	107.8	0.9	6.6	72
9	157.5	6.7	5.2	72
10	147.6	0.9	5.5	70
11	112.0	5.1	6.4	70
12	201.7	0.9	9.2	43
13	48.7	5.5	6.1	43
14	19.8	1.0	6.8	34
15	36.4	5.3	6.3	34
16	71.1	0.8	6.2	37
17	29.8	5.4	9.3	37
18	55.1	1.0	6.4	s[b]
19	208.6	5.7	10.6	41
20	31.9	1.4	6.8	41
2-OMe	53.9	1.0	1.0	s[b]
9-OMe	56.8	0.9	1.4	s[b]

[a]Enrichment ratios are relative to the 2-*O*Me signal as 1.0. [b]Signal was a singlet since the carbon atom did not couple with others.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation was measured using a Horiba SEPA-300 polarimeter. UV and IR spectra were measured using a Beckman Coulter DU730 UV/vis spectrophotometer and a Horiba FT-720 spectrophotometer, respectively. NMR spectra were collected using a Varian NMR System 500 or 600 NB CL; samples were dissolved in $CDCl_3$ (δ_C 77.0, δ_H 7.25 ppm) with the residual solvent peak serving as the internal standard. HRESIMS data were recorded using a Waters LCT-Premier XE mass spectrometer. Normal-phase MPLC was conducted on a Purif-Pack SI-30 column (Shoko Scientific). Analytical reversed-phase HPLC was conducted using a CAPCELL PAK C_{18} MGII column (4.6 i.d. × 150 mm; Shiseido) in conjunction with a Waters 2996 photodiode array detector and a Waters 3100 mass detector. Preparative reversed-phase HPLC was conducted using a CAPCELL PAK C_{18} MGII column (20 i.d. × 150 mm, Shiseido) in conjunction with a Waters 2996 photodiode array detector and a Waters 3100 mass detector. All other reagents and solvents were of the highest grade available.

Fermentation. *Streptomyces* sp. RI-77 was isolated from a soil sample collected in Shuri, Okinawa Island, Japan, and cultivated in 50 mL test tubes that contained 15 mL of a seed medium consisting of 1.0% starch (Kosokagaku), 1.0% Polypepton (Nihon Pharmaceutical), 1.0% molasses (Dai-Nippon Meiji Sugar), and 1.0% meat extract (Extract Ehlrich, Wako Pure Chemical Industry) (pH 7.2). The test tubes were shaken on a reciprocal shaker (320 rpm) at 27 °C for two days. Aliquots (2.0 mL) of the broth were then transferred to 500 mL baffled Erlenmeyer flasks containing 100 mL of a production medium consisting of 2.0% starch, 0.25% glycerin, 0.5% Pharmamedia (Traders Protein), 1.0% Diaion HP20 (Mitsubishi), 5 mg of $CuSO_4$, 5 mg of $MnCl_2$, and 5 mg of $ZnSO_4$ (pH 7.2) and cultured on a rotary shaker (150 rpm) at 27 °C for five days.

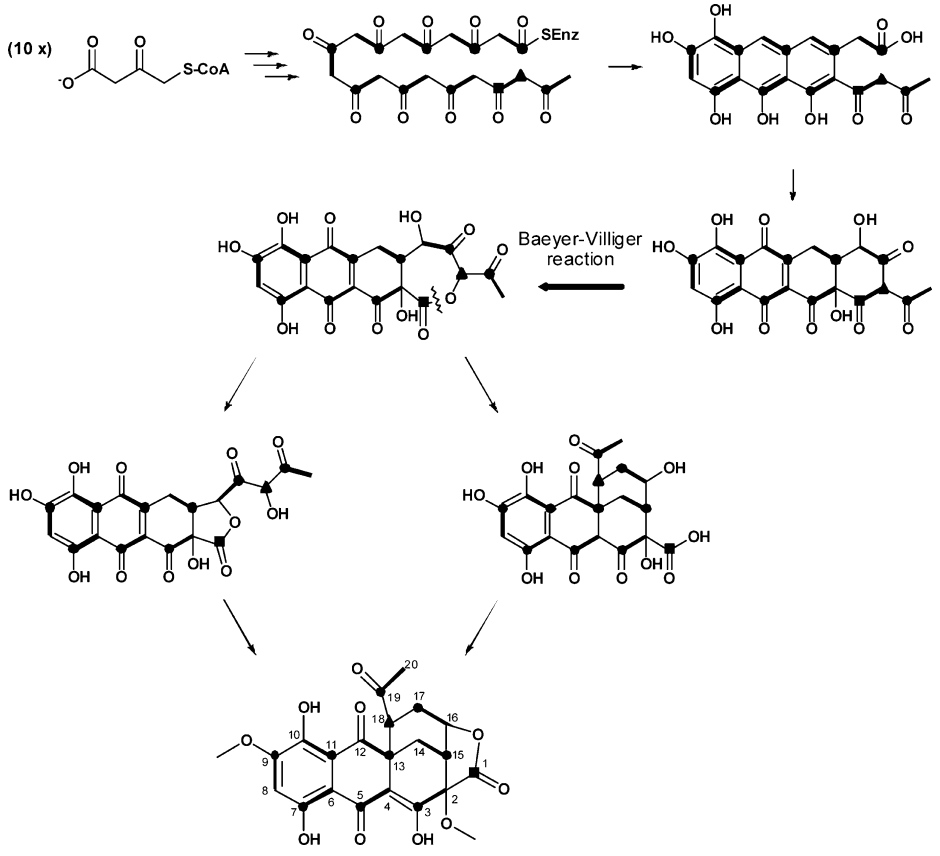


Figure 3. Labeling pattern and proposed biosynthetic pathway of **1**. Solid circles indicate the carbons enriched by [1-^{13}C]acetate, and thick bars indicate $^{13}C-^{13}C$ couplings observed by the incorporation of [1,2-^{13}C]acetate. Solid squares indicate that C-1 is labeled by [1-^{13}C]acetate, but has no coupling with others. Solid triangles indicate that C-18 is labeled by [1,2-^{13}C]acetate but not by [1-^{13}C]acetate and has no coupling with others.

dx.doi.org/10.1021/np200651e | *J. Nat. Prod.* 2011, 74, 2588–2591

Isolation. The fermentation broth (2 L) was centrifuged, the mycelial cake was extracted with Me_2CO (0.5 L) and filtered, and the filtrate was concentrated in vacuo. The aqueous concentrate was extracted with EtOAc (100 mL × 3). After being dried over Na_2SO_4, the organic layer was evaporated to dryness. The residue (810 mg) was subjected to normal-phase MPLC using an *n*-hexane−EtOAc stepwise solvent system (0, 10, 20, 25% EtOAc) and $CHCl_3$−MeOH (0, 2, 5, 10% MeOH), successively. The 2% MeOH-eluted fraction (56 mg) was subjected to a Sephadex LH-20 gel filtration column (15 × 550 mm; GE Healthcare) using $CHCl_3$−MeOH (50:50) as the eluent. Finally, the obtained fraction was further purified by preparative reversed-phase HPLC using a CAPCELL PAK C_{18} MGII column developed with 55% MeOH−H_2O containing 0.1% formic acid (flow rate: 10 mL/min) to yield JBIR-85 (**1**, 13.4 mg, t_R = 17.4 min).

JBIR-85 (1): yellow solid; $[\alpha]^{24}_D$ +72.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 243 (4.14), 419 (4.16) nm; IR (KBr) ν_{max} 3420, 1781, 1700 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), see Table 1; HRESIMS *m*/*z* 443.0977 $[M - H]^-$ (calcd for $C_{22}H_{19}O_{10}$, 443.0978).

X-ray Crystal Structure. A yellow platelet crystal of **1** was crystallized from MeOH. Crystal data for **1** were collected using a Rigaku VariMax with a RAPID system: $C_{22}H_{20}O_{10}$, crystal size 0.12 × 0.05 × 0.04 mm, space group $P2_12_12$ (#18), a = 24.6881(4) Å, b = 7.0022(1) Å, c = 13.9254(2) Å, V = 2407.31(7) $Å^3$, Z = 4, D_{calcd} = 1.460 g/cm^3, Cu Kα (λ = 1.54187 Å) radiation, 4403 unique reflections (R_{int} = 0.0591), final R_1 = 0.01110 (wR_2 = 0.3420) for 28 557 reflections with $I > 2.00\sigma(I)$. The structure was solved using direct methods, refined with full-matrix least-squares on F^2, and expanded using Fourier techniques. The non-hydrogen atoms were refined anisotropically, and the hydrogen atoms were refined using the Riding model. The absolute configuration was determined on the basis of a Flack parameter of 0.09(9) using 1860 Friedel pairs.[6]

DPPH Radical Scavenging Activity. A 384-well plate was used for the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay.[9,10] Compound **1** and α-tocopherol, which acted as a positive control, were dissolved in MeOH. In the well plate, 10 μL of 400 μM DPPH dissolved in EtOH and 10 μL of the samples were mixed. After 30 min of incubation at room temperature, the absorbance was measured at 525 nm.

Feeding Experiments. Strain RI-77 was grown at 27 °C in 500 mL Erlenmeyer flasks containing producing medium. Sodium acetate (1-^{13}C, 99%) or sodium acetate ($^{13}C_2$, 99%) was added at a final concentration of 0.1% at 24 h after the inoculation of the seed culture. After 5 days of culture, the fermentation broth (1 L) was centrifuged and the ^{13}C-labeled **1** (3.4 mg for [1-^{13}C]acetate and 5.5 mg for [1,2-^{13}C]acetate) was purified according to the procedure described above. The ^{13}C NMR spectra of nonlabeled and ^{13}C-labeled **1** (2.5 mg of each in 250 μL of $CDCl_3$) were measured under identical conditions (150 MHz, 25 °C, 20 000 scans, acquisition time of 1.0 s, relaxation delay of 3.5 s, pulse width of 45°). Relative enrichments for all carbon atoms of labeled **1** were obtained by comparing the integrals of the ^{13}C signals with those of the natural-abundance standards. The intensities of the signals at 53.9 ppm (2-*O*Me) in the ^{13}C NMR spectra of these compounds from the cultures in either the presence or absence of labeled acetate were aligned. These signals were used as standards for the evaluation of isotopic abundance.

■ ASSOCIATED CONTENT

Supporting Information

NMR and HRESIMS spectra and X-ray crystal structural data of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +81-3-3599-8304. Fax: +81-3-3599-8494. E-mail: motoki-takagi@aist.go.jp (M.T.). Tel: +81-3-3599-8854. Fax: +81-3-3599-8494. E-mail: k-shinya@aist.go.jp (K.S.).

■ ACKNOWLEDGMENTS

This work was supported by a grant from the New Energy and Industrial Technology Department Organization (NEDO). The authors thank the Rigaku Corporation for measuring the X-ray crystal structure of **1**.

■ REFERENCES

(1) Motohashi, K.; Hwang, J. H.; Sekido, Y.; Takagi, M.; Shin-ya, K. *Org. Lett.* **2009**, *11*, 285−288.
(2) Hwang, J. H.; Takagi, M.; Murakami, H.; Sekido, Y.; Shin-ya, K. *Cancer Lett.* **2011**, *300*, 189−196.
(3) Takagi, M.; Motohashi, K.; Nagai, A.; Izumikawa, M.; Tanaka, M.; Fuse, S.; Doi, T.; Iwase, K.; Kawaguchi, A.; Nagata, K.; Takahashi, T.; Shin-ya, K. *Org. Lett.* **2010**, *12*, 4664−4666.
(4) Motohashi, K.; Takagi, M.; Yamamura, H.; Hayakawa, M.; Shin-ya, K. *J. Antibiot.* **2010**, *63*, 545−548.
(5) Furihata, K.; Seto, H. *Tetrahedron Lett.* **1998**, *39*, 7337−7340.
(6) Flack, H. D. *Acta Crystallogr.* **1983**, *A39*, 876−881.
(7) Feng, Z.; Kallifidas, D.; Brady, S. F. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 12629−12634.
(8) Gibson, M.; Nur-e-alam, M.; Lipata, F.; Oliveira, M. A.; Roh, J. *J. Am. Chem. Soc.* **2005**, *127*, 17594−17595.
(9) Izumikawa, M.; Nagai, A.; Doi, T.; Takagi, M.; Shin-ya, K. *J. Antibiot.* **2009**, *62*, 177−180.
(10) Motohashi, K.; Takagi, M.; Shin-ya, K. *J. Nat. Prod.* **2010**, *73*, 226−228.