JOURNAL OF NATURAL PRODUCTS

Note

Cite This: J. Nat. Prod. XXXX, XXX, XXX−XXX

pubs.acs.org/jnp

Miuramides A and B, Trisoxazole Macrolides from a *Mycale* sp. Marine Sponge That Induce a Protrusion Phenotype in Cultured Mammalian Cells

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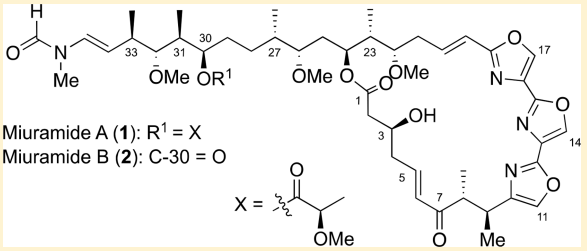
Supporting Information

ABSTRACT: Morphology-guided cell-based screening of the extract of a *Mycale* sp. marine sponge led to the isolation of two trisoxazole macrolides, miuramides A (**1**) and B (**2**), which induced characteristic morphological changes in 3Y1 cells. The structure of **1** including absolute configuration was elucidated by a combination of the analysis of spectroscopic data, derivatization, and degradation. Both compounds exhibit potent cytotoxicity against 3Y1 cells.



Historically, potent and effective cytotoxic natural products were discovered through cell-based assays using cultured cancer cells.[1] With the advancement and dissemination of time-lapse microscopy, cell-based phenotypic screening has been considered as a method of choice among the drug discovery community.[2,3] Phenotypes of cultured cells after administration of biologically active compounds represent a summation of the effects of the compounds toward each molecular target within the cell, permitting us to detect compounds with specific biological activities.[2] During the course of our phenotypic screening, we found that the extract of a *Mycale* sp. sponge, collected off Miura Peninsula, induced characteristic morphological changes in 3Y1 cells immediately after the treatment (Figure 1). Similar morphological changes were observed when actin-binding compounds were applied.[4] Bioactivity-guided fractionation of the extract resulted in the isolation of two trisoxazole macrolides, named miuramides A (**1**) and B (**2**). Here, we describe the isolation, structure elucidation, and biological activity of the miuramides.

The MeOH extract of the *Mycale* sp. sponge was partitioned between $CHCl_3$ and H_2O. The organic layer was further partitioned between *n*-hexane and MeOH−H_2O (9:1), and the latter fraction was subjected to ODS flash column chromatography followed by purification with RP-HPLC to afford miuramides A (**1**, 3.2 mg) and B (**2**, 0.8 mg) as peaks inducing protrusion of the cell membrane.

The molecular formula of miuramide A (**1**) was determined to be $C_{50}H_{72}N_4O_{15}$ by HRESIMS. The presence of three highly deshielded singlet protons (δ 8.07, 8.04, 7.62), five methoxy protons, and a pair of formyl (δ 8.24, 8.01) and *N*-methyl protons (δ 2.97, 3.00) each in a 2:1 ratio in the ^{1}H NMR

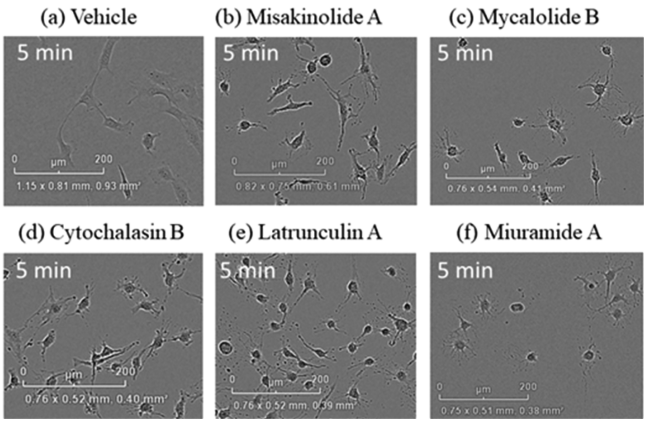


Figure 1. Morphological changes in 3Y1 cells induced by actin depolymerizers. (a, vehicle; b, misakinolide A (0.05 μM); c, mycalolide B (2.7 μM); d, cytochalasin B (41 μM); e, latrunculin A (1.9 μM); f, miuramide A (4.2 μM)).

spectrum was reminiscent of a trisoxazole macrolide.[5] Interpretation of the COSY spectrum enabled us to assign four spin systems: **a** (C-2 to C-6), **b** (8-Me to C-9), **c** (C-19 to C-35), and **d** (C-37 to C-38) (Figure 2). Units **a** and **b** were connected through C-7 (δ 202.4) on the basis of HMBC cross-peaks from H-6 and H-8 to C-7. Units **a** and **c** were connected through C-1 (δ 171.9) on the basis of HMBC cross-peaks from H-2 and H-24 to C-1. Units **c** and **d** were connected on the

Received: January 31, 2018

DOI: 10.1021/acs.jnatprod.8b00101
J. Nat. Prod. XXXX, XXX, XXX−XXX

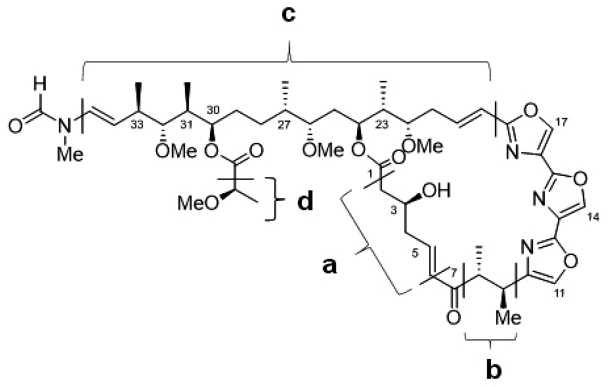
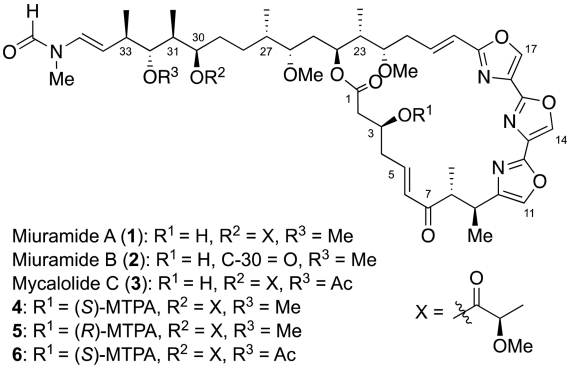


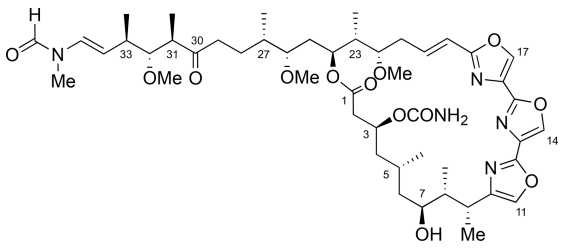
Figure 2. Structure of miuramide A (**1**) with partial structures indicated.

basis of HMBC cross-peaks from H-30 and H-37 to C-36 (δ 172.5). The HMBC data and $^1J_{CH}$ value of the three aromatic singlets indicated the trisoxazole moiety,[6] which was inserted between units **b** and **c**. C-35 was connected to the *N*-methylformylamino group on the basis of HMBC cross-peaks from the *N*-Me protons to C-35 and the formyl carbon (δ 162.1). The locations of the five *O*-methyl groups were determined on the basis of the HMBC correlations from the *O*-methyl protons to the corresponding oxymethine carbons. The configurations of the three olefins in **1** were determined to be all *E* on the basis of large vicinal coupling constants: $^3J_{H5-H6}$ (15.9 Hz), $^3J_{H19-H20}$ (15.9 Hz), and $^3J_{H34-H35}$ (14.3 Hz). Therefore, **1** has the planar structure similar to mycalolide C (**3**), whose 32-acetoxy group was replaced by a methoxy group.[7,8]

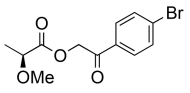


The absolute configurations in **1** were studied by a combination of modified Mosher's method,[9] chemical degradation, and chiral-phase HPLC. The absolute configuration of C-3 was determined by the modified Mosher's method. The $\Delta\delta$ values observed between the (*S*)- and (*R*)-MTPA esters of **1** (**4** and **5**, respectively) indicated that the absolute configuration of C-3 was *S* (Table S1). ^{1}H NMR data including chemical shifts and coupling constants within the macrolide ring of **1** were almost superimposable on those of **3** (Table S2). With the identical C-3 absolute configuration, it was considered that the absolute configurations of the macrolide portions of **1** and **3** were identical. As expected, the (*S*)-MTPA esters of **1** and **3** (**4** and **6**, respectively) displayed almost identical ^{1}H NMR data for the signals within the macrolide portion (Table S3).

In order to determine the absolute configurations of the C-25−C-35 portion, we took advantage of the similarity of **1** to kabiramide C (**7**), which also possesses the C-32-methoxy group.[10,11] Miuramide A (**1**) was oxidized with RuO_4 followed by reduction with borane dimethyl sulfide complex and alkaline hydrolysis to afford **8**. A corresponding fragment was prepared from kabiramide C (**7**) through reduction with $NaBH_4$, acetylation of the resulting C-30-alcohol, oxidation with RuO_4, and reduction of the resulting dicarboxylic acid to afford **9** and **10**, which were epimeric at C-30.



Kabiramide C (**7**)



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The relative configurations at C-30 in **9** and **10** were assigned by determining the $J_{H30, H31}$ value with selective decoupling experiments. The *syn*-relationship between H-30 and H-31 in **9** was deduced from the coupling constant of 1.7 Hz, whereas the *anti*-relationship between H-30 and H-31 in **10** from the coupling constant of 6.6 Hz. The ^{1}H NMR spectrum of **8** was superimposable on that of **9** (Figure 3), indicating that **8** and **9** were identical including total relative configuration. Because the (22*S*, 23*R*, 24*S*)-absolute configurations were established for miuramide A (**1**), the absolute configurations of the remaining stereogenic centers in **8** and **9** were identical.

O-Methyllactic acid was liberated from **1** by alkaline hydrolysis and converted to the *p*-bromophenacyl ester (**11**). LC-MS analyses of **11** and authentic standards, which were prepared from (*S*)-lactic acid and racemic lactic acid through *O*-methylation followed by esterification, showed the 37*R*-absolute configuration (Figure S20) Thus, the absolute configuration of **1** was determined to be 3*S*, 8*R*, 9*S*, 22*S*, 23*R*, 24*S*, 26*S*, 27*S*, 30*R*, 31*R*, 32*R*, 33*R*, and 37*R*.

The molecular formula of **2** was determined to be $C_{46}H_{64}N_4O_{13}$ by HRESIMS, which is less than that of **1** by $C_4H_8O_2$. The obvious difference of miuramide B (**2**) and miuramide A (**1**) was the absence of the C-30-oxymethine and the *O*-methyl lactate and the presence of a C-30 ketone carbonyl (δ 214.0) in the former. This replacement was confirmed by the 2D NMR data including HMBC correlations from H-31, 31-Me, and H-29 to C-30. Therefore, miuramide B is a hybrid of mycalolide C (C-1 to C-9) and kabiramide C (C-19 to C-35). Even though the absolute configurations of **2** were not elucidated due to the paucity of the sample, it is likely that **1** and **2** share the same absolute configurations because of a shared biogenesis.

Miuramides A (**1**) and B (**2**) showed cytotoxicity against 3Y1 cells with IC_{50} values of 7 nM for both. As shown in Figure 1, protrusions in the cell membrane were induced by miuramide A. This morphology was similar to those observed by treatment with other actin polymerization inhibitors such as mycalolide B, misakinolide A, cytochalasin B, and latrunculin A, although the phenotypes after prolonged times were different (Figure S21).[12,13] Actin polymerization inhibitors bind to G-actin in

DOI: 10.1021/acs.jnatprod.8b00101
J. Nat. Prod. XXXX, XXX, XXX−XXX

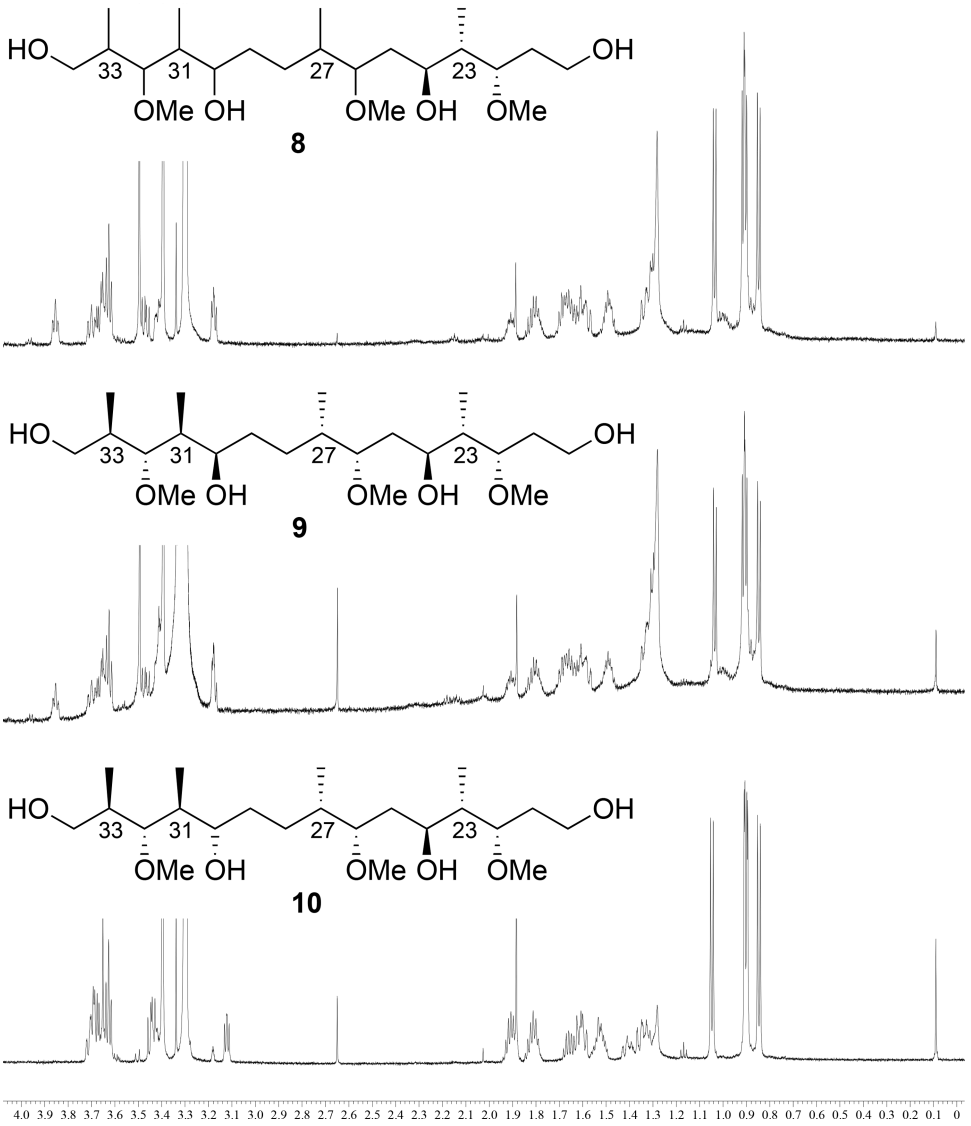

Figure 3. Comparison of the ^{1}H NMR spectra of C-20−C-34 fragments **8**, **9**, and **10**.

different ways, with respect to the binding site and the orientation of the macrocyclic portion.[14] The ability to sever F-actin is also different among inhibitors.[13] The different phenotypes observed after long-term treatment may reflect the modes of actin binding by the inhibitors.[2]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Jasco DIP-1000 polarimeter. UV spectra were measured on a Shimadzu BioSpec-1600 spectrophotometer. NMR spectra were measured on a JEOL alpha 600 NMR spectrometer and referenced to the solvent peak: δ_H 7.24 and δ_C 77.0 for $CDCl_3$ containing 0.08% pyridine-d_5. ESI mass spectra were recorded on a JEOL JMS-T100LC mass spectrometer. LC-MS experiments were performed on a Shimadzu LC-20AD solvent delivery system and interfaced to a Bruker amaZon SL mass spectrometer. The results of the MTT assay were recorded with a Molecular Devices SPECTRA Max M2. Time-lapse imaging of cells was conducted on the Essen Bioscience IncuCyteZoom. Mycalolide C and kabiramide C were isolated as previously reported.[8,10]

Animal Material. The *Mycale* sp. sponge was collected by snorkeling at a depth of ca. 1 m at an entrance of Aburatsubo Bay, Miura, Kanagawa, Japan, on May 11, 2017 (35°9′36″ N, 139°36′42″ E). The sponge is soft, yellowish-green in life, pale yellowish-green in ethanol. The surface of the sponge is hispid because of the protruding bundles of megascleres. Spicules are composed of subtylostyles (mycalostyles) as megasclere, palmate anisochelae in two size classes and one type of sigma as microscleres. Subtylostyles are straight, smooth, and fushiform with sharp tips, 280−300 μm in length and 2−3 μm in thickness. Palmate anisochelae are abundant. Larger anisochelae are 22−25 μm in length. Smaller anisochelae are 12−15 μm in length. Sigmas are C or S shaped, 17−25 μm in length. Up to now, 14 species of the genus *Mycale* have been reported from Japanese waters.[15] Our specimen is distinct from the 14 known species by its spicule composition and dimensions. The specimen used for the identification (SMBL-Po050) is deposited at Sugashima Marine Biological Laboratory, Graduate School of Science, Nagoya University.

Extraction and Isolation. The sponge (850 g) was homogenized in MeOH and extracted with MeOH and MeOH/$CHCl_3$ (1:1). The extracts were combined, concentrated in vacuo, and partitioned between H_2O and $CHCl_3$. The $CHCl_3$ layer was further partitioned between *n*-hexane and MeOH/H_2O (9:1). The aqueous MeOH fraction was subjected to ODS flash column chromatography with stepwise elution of 20% MeOH, 60% MeOH, 80% MeOH, MeOH, and $CHCl_3$/MeOH (1:1). The 80% MeOH fraction and the MeOH fraction were first purified by RP-HPLC on a COSMOSIL 5C_{18}-AR-II column with gradient elution from 63% MeOH to 80% MeOH to afford two fractions. Each fraction was further purified by RP-HPLC

DOI: 10.1021/acs.jnatprod.8b00101
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 1. ^{1}H (600 MHz) and ^{13}C NMR Data (150 MHz) of Miuramide A (1) and Miuramide B (2) in $CDCl_3$[a,b]

	1		2	
position	δ_C, type	δ_H, mult (J in Hz)	δ_C[c], type	δ_H, mult (J in Hz)
1	171.9, C		n.d[d]	
2a	42.5, CH_2	2.57, dd (14.5, 10.5)	42.8, CH_2	2.58, dd (10.7, 14.8)
2b		2.45, m		2.47, m
3	67.5, CH	4.39, m	67.7, CH	4.40[e]
4	40.8, CH_2	2.46, m	41.0, CH_2	2.47, m
5	145.6, CH	7.21, dt (15.9, 7.2)	145.9, CH	7.20, dt (15.9, 7.2)
6	133.2, CH	6.20, d (15.9)	133.4, CH	6.21, d (15.9)
7	202.4, C		202.3, C	
8	44.0, CH	3.96, m	44.2, CH	3.96, m
8-Me	13.2, CH_3	0.90, d (6.6)	13.7, CH_3	0.89, d (6.6)
9	77.5, CH	4.33, d (8.3)	77.7, CH	4.34, d (8.3)
9-OMe	56.8, CH_3	3.16, s	56.9, CH_3	3.16, s
10	139.4, C		n.d.	
11	137.2, CH	7.62, s	137.2, CH	7.62, s
12	155.4, C		155.2, C	
13	131.0, C		131.0, C	
14	137.1, CH	8.07, s	137.4, CH	8.07, s
15	156.4, C		156.3, C	
16	129.9, C		129.8, C	
17	136.8, CH	8.04, s	137.5, CH	8.04, s
18	162.7, C		162.6, C	
19	116.4, CH	6.33, d (15.9)	116.7, CH	6.33, d (15.9)
20	140.4, CH	7.09, m	140.6, CH	7.10, m
21a	34.8, CH_2	2.65, m	35.0, CH_2	2.64, m
21b		2.42, m		2.43, m
22	79.7, CH	3.41, m	80.0, CH	3.42, m
22-OMe	58.2, CH_3	3.32, s	58.4, CH_3	3.33, s
23	40.5, CH	1.84, m	40.9, CH	1.86, m
23-Me	8.9, CH_3	0.88, d (6.6)	9.3, CH_3	0.90, brd (6.6)
24	73.1, CH	5.23, ddd (1.8, 6.9, 9.7)	73.5, CH	5.26, m
25a	31.4, CH_2	1.53, m	32.3, CH_2	1.61, m
25b		1.47, m		1.52, m
26	81.5, CH	2.93, m	81.9, CH	2.98, m
26-OMe	58.1, CH_3	3.27, s	58.2, CH_3	3.30, s
27	34.7, CH	1.71, m	34.7, CH	1.70, m
27-Me	9.9, CH_3	0.83, d (7.2)	15.7, CH_3	0.82, d (6.6)
28a	27.1, CH_2	1.43, m	25.0, CH_2	1.77, m
28b		0.93, m		1.25, m
29	30.7, CH_2	1.55, m	42.6, CH_2	2.49, m
30	74.0, CH	5.28, m	214.0, C	
31	39.7, CH	1.57, m	49.3, CH	2.65, m
31-Me	15.5, CH_3	0.81, d (7.2)	13.3, CH_3	0.89, brd (6.6)
32	86.5, CH	2.73, dd (2.8, 9.5)	87.5, CH	3.28, dd (2.8, 9.9)
32-OMe	61.8, CH_3	3.42, s	61.5, CH_3	3.31, s
33	37.9, CH	2.41, m	37.6, CH	2.35, m
33-Me	19.9, CH_3	1.12 (6.6)	19.6, CH_3	1.13, d (6.6)
34	111.5, CH	5.06, dd (9.0, 14.3)	111.6, CH	5.07, dd (9.2, 14.3)
[34]	[113.3], CH	[5.08, dd (9.2, 14,3)]	[113.4], CH	[5.09, m]
35	128.6, CH	6.43, d (14.3)	129.0, CH	6.43, d (14.3)
[35]	[124.6], CH	[7.09, m]	[125.0], CH	[7.09, m]
35-NMe	27.5, CH_3	2.97, s	27.8, CH_3	3.00, s
[35-NMe]	[33.0], CH_3	[3.00, s]	[33.4], CH_3	[3.04, s]
35-NCHO	162.1, CH	8.24, s	162.3, CH	8.26, s
[35-NCHO]	[160.8], CH	[8.01, s]	[n.d]	[n.d]
36	172.5, C			
37	76.5, CH	3.84, q (6.8)		
37-OMe	57.8, CH_3	3.38, s		
38	18.7, CH_3	1.38, d (6.8)		

[a]Signals for the minor isomer are shown in square brackets. [b]0.08% pyridine-d_5 was added to the solvent. [c]Assigned by HSQC and HMBC data. [d]Not detected. [e]Assigned by COSY data.

on a COSMOSIL 5C_{18}-AR-II column with gradient elution from 50% MeCN to 70% MeCN to afford miuramide A (**1**, 3.2 mg) and miuramide B (**2**, 0.8 mg).

Miuramide A (1): colorless gum; $[\alpha]_D$ −79 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 232 (4.3); ^{1}H and ^{13}C NMR data, Table 1; HRESIMS *m/z* 991.4897 $[M + Na]^+$ (calcd for $C_{50}H_{72}N_4O_{15}Na$, 991.4892).

Miuramide B (1): colorless gum; $[\alpha]_D$ −28 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 231 (4.5); ^{1}H and ^{13}C NMR data, Table 1; HRESIMS *m/z* 903.4411 $[M + Na]^+$ (calcd for $C_{46}H_{64}N_4O_{13}Na$, 903.4368).

Preparation of the MTPA Esters of 1. To a solution of **1** (0.4 mg) in pyridine (100 μL) was added two drops of (*R*)-(−)-MTPACl, and the mixture was left at room temperature for 12 h The reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The organic layer was purified by ODS-HPLC to afford the (*S*)-(−)-MTPA ester (**4**). The (*R*)-(+)-MTPA ester (**5**) of **1** was prepared in the same way using (*S*)-(+)-MTPACl. The ^{1}H NMR data for **4** and **5** in $CDCl_3$ are in Table S1.

Preparation of the MTPA Ester of 3. To a solution of **3** (0.4 mg) in pyridine (100 μL) was added two drops of (*R*)-MTPACl, and the mixture was left at room temperature (rt) for 12 h. The reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The organic layer was purified by ODS-HPLC to afford the (*S*)-MTPA ester (**6**).

Preparation of the C-20−C-34 Fragment 8 from 1. To a solution of 1.5 mg of **1** in CCl_4/MeCN/H_2O (4:4:5, 176 μL) were added $RuCl_3 \cdot nH_2O$ (10 μL, 25 mM in H_2O) and $NaIO_4$ (3 mg), and the mixture was stirred at rt for 1 h. The reaction mixture was diluted with 0.5 N HCl (100 μL) and extracted with EtOAc to give an oily material, which was dissolved in tetrahydrofuran (THF) (300 μL) and then treated with a solution of $BH_3 \cdot Me_2S$ (2.0 M in THF, 30 μL) at 0 °C and left at rt for 12 h. The reaction mixture was quenched with 50% AcOH (50 μL) and concentrated. The residue was dissolved in saturated aqueous $NaHCO_3$ and left at 0 °C for 10 min. The resulting mixture was extracted with EtOAc to give an oily material, which was dissolved in a mixture of MeOH/1 N LiOH in H_2O (1:2, 150 μL) and left at rt for 8 h. To the reaction mixture was added AcOH (10 μL), and the resulting mixture was concentrated and purified by RP-HPLC to afford **8**.

Compound 8: ^{1}H NMR (600 MHz, CD_3OD) δ 0.85 (3H, d, *J* = 7.1 Hz, 23-Me), 0.91, (3H, d, *J* = 6.6 Hz, 27-Me), 0.91 (3H, d, *J* = 6.6 Hz, 31-Me), 1.03 (3H, d, *J* = 7.1 Hz 33-Me), 1.33 (1H, m, H-25), 1.49 (2H, m, H-29), 1.58 (1H, m, H-25), 1.61 (1H, overlapped, H-23), 1.65 (1H, overlapped, H-21), 1.66 (2H, overlapped, H-28), 1.67 (1H, overlapped, H-31), 1.79 (1H, overlapped, H-27), 1.80 (1H, overlapped, H-21), 1.90 (1H, m, H-33), 3.18 (1H, m, H-32), 3.40 (3H, s, 26-OMe), 3.42 (1H, overlapped, H-26), 3.47 (1H, dd, *J* = 10.8, 7.2 Hz, H-34), 3.50 (3H, s, 32-OMe), 3.62 (3H, s, 22-OMe), 3.63 (2H, overlapped, H-20), 3.66 (1H, overlapped, H-22), 3.67 (1H, over-

DOI: 10.1021/acs.jnatprod.8b00101
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lapped, H-34), 3.71 (1H, m, H-24), 3.85 (1H, m, H-30); ESIMS m/z 423 $[M + H]^+$.

Preparation of the C-20-C-34 Fragments 9 and 10 from 7. To a solution of 7 (4.6 mg) in MeOH (500 μL) was added $NaBH_4$ (0.5 mg), and the mixture was stirred at 0 °C for 30 min. The reaction mixture was quenched with 5% AcOH in H_2O (500 μL), and the mixture was concentrated. The residue was dissolved in a 1:1 mixture of acetic anhydride and pyridine (200 μL), and the solution was stirred at rt overnight. After removal of the solvent by lyophilization, the residue was subjected to RP-HPLC to yield dihydrokabiramide C-1 (520 μg) and its C-30 epimer, dihydrokabiramide C-2 (3.0 mg). Dihydrokabiramides C-1 and C-2 were converted to the C-20−C-34 fragments, **9** and **10**, as described above.

Preparation of 11 from 1. To a solution of **1** (100 μg) in CCl_4/MeCN/H_2O (4:4:5, 104 μL) were added $RuCl_3nH_2O$ (10 μL, 25 mM in H_2O) and $NaIO_4$ (2 mg), and the mixture was stirred at rt for 1 h. The reaction mixture was diluted with 0.5 N HCl (100 μL) and extracted with EtOAc to give an oily material, which was dissolved in a mixture of MeOH/1 N LiOH in H_2O (1:2, 150 μL) at rt for 4 h. The reaction mixture was neutralized with AcOH (10 μL), and the resulting mixture was concentrated, redissolved in dimethylformamide (DMF) (200 μL), and treated with *p*-bromophenacyl bromide (0.8 mg) in the presence of KF (0.8 mg) at rt for 40 min. The reaction mixture was diluted with H_2O, extracted with EtOAc, and concentrated to give *p*-bromophenacyl ester **11**.

Synthesis of *p*-Bromophenacyl *O*-Methyl-L-lactate. To a solution of L-lithium lactate (8 mg) in DMF (200 μL) was added *p*-bromophenacyl bromide (1 mg) in the presence of KF (1 mg), and the mixture was stirred at rt for 40 min. The reaction mixture was diluted with H_2O and extracted with EtOAc. The EtOAc layer was concentrated to give *p*-bromophenacyl-L-lactate. To a solution of *p*-bromophenacyl-L-lactate in CH_2Cl_2 (200 μL) were added Me_3OBF_4 (1 mg) and proton sponge (1 mg), and the mixture was stirred at rt for 2 days. The reaction mixture was diluted with H_2O and extracted with EtOAc. The EtOAc layer was concentrated to give *p*-bromophenacyl *O*-methyl-L-lactate. The racemic mixture of *p*-bromophenacyl *O*-methyl lactate was prepared in the same manner.

Cell Culture and MTT Assay. The cytotoxicities of **1** and **2** against 3Y1 cells were evaluated by the MTT assay. 3Y1 cells were cultured in Dulbecco's modified Eagle's medium containing penicillin and streptomycin and 10% fetal bovine serum at 37 °C under an atmosphere of 5% CO_2. After overnight preincubation, **1** and **2** were added to each well of a 96-well microplate containing 200 μL of 3Y1 cell suspension and further incubated for 72 h. Then, MTT saline solution was added to each well, and the plate was further incubated for 3 h. The medium was excluded and formazan dye was dissolved in 150 μL of DMSO.

Phenotypic Screening. 3Y1 cells were cultured as described above. After overnight preincubation, samples were added to each well of a 96-well plate. The 96-well plate was loaded into an IncuCyte live-cell imaging system, and the cells were imaged every 2 h for 72 h.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00101.

1D and 2D NMR spectra for **1**−**6** and **8**−**10**, LC-MS chromatograms of *p*-bromophenacyl esters of *O*-methyl-lactate derivatives, and the results of the morphology-guided cell-based assay of actin depolymerizers (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by JSPS KAKENHI Grant Numbers 25252037, 16H04980, and 17H06403 and JSPS Research Fellowship for Young Researchers 16J07837. We thank Dr. K. Furihata (University of Tokyo) for his assistance in measuring NMR spectra.

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DOI: 10.1021/acs.jnatprod.8b00101
J. Nat. Prod. XXXX, XXX, XXX−XXX