Two New Ring-Contracted Congeners of Rhizopodin Illustrate Significance of the Ring Moiety of Macrolide Toxins on the Actin Disassembly-Mediated Cytotoxicity

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Two new cytotoxic dilactones, bisisorhizopodin (1) and isorhizopodin (2), together with known divalent actin depolymerizer rhizopodin (3), were isolated from the culture broth of a myxobacterium Myxococcus stipitatus. Spectroscopic analyses established that 1 and 2 are doubly and singly acyl-migrated isomers of 3, respectively, and comparison of their cytotoxicity revealed gradual decrease in the activity as the size of the ring contracted. Because the side chains of macrolide toxins uniformly block the contact between the actin protomers, the present result demonstrates substantial contribution of structurally diverse rings to the affinity of macrolide toxins for its target protein.

Key words rhizopodin; Myxobacteria; actin; cytotoxicity

Myxobacteria are a distinctive group of bacteria that alternate their life form in response to the nutritional status: when eutrophic they take a motile and predatory lifestyle, but once eating up available food, they aggregate together and form fruiting bodies to withstand the food-depleted condition as dormant myxospores. Their secondary metabolites are highly unique and often accompanied by potent bioactivity, which make these organisms as one of the best resources for drug discovery from nature. In fact, ixabepilone, derived from epothilone B produced by Sorangium cellulosum, has been approved in 2007 for the treatment of metastatic breast cancer in the U.S.A.

Myxobacteria has been accepted as mesophilic soil bacteria, but attempts to discover new species from other environments identified halophilic, thermophilic, psychrophilic, or anaerobic lineages, revealing rather a broader habitat range for this group. And more importantly, two of the halophiles were found to produce unprecedented metabolites with a β-methoxyacyl group such as haliangicins and miram enamides.

As part of our program to discover new bioactive metabolites from underexploited bacterial resources, we have collected myxobacteria from hotspring environments and screened their fermentation products for cytotoxicity and antimicrobial activity. While most of the cytotoxic extracts contained myxothiazole and exhibited broad antimycotic activity, three were found to selectively inhibit the growth of Saccharomyces cerevisiae. Intrigued by this unique activity, one of the hit strains coded YA1-5B-2, identified as Myxococcus stipitatus based on 16S ribosomal DNA (rDNA) sequence analysis, was chosen for further study. The active principles in its fermentation extract were pursued under the guidance of cytotoxicity against P388 murine leukemia, which resulted in the isolation of the known rhizopodin (3) and its two new congeners (Fig. 1). 3 is a fungicidal and cytotoxic C2-symmetric dilactone discovered from the same organism, and exerts potent bioactivity through depolymerization of actin. Actin is the most common cytoskeletal protein that undertakes motility function in eukaryotic cells, and small molecules that perturb its assembly-disassembly equilibrium are useful in dissecting the molecular mechanism of migration, vesicle and organelle movement, cell division, and establishment of cell shapes. To gain further information on the precise mechanism of actin depolymerization by 3, structure and activity of the new congeners were determined.

Results and Discussion

The producer strain, M. stipitatus YA1-5B-2, was isolated from sandy deposits in a sedimentation basin downstream of an outdoor hot spring bath in Toyama, Japan. It was shake-cultured in V22 medium for 8 d at 32°C, and the culture broth was extracted with n-BuOH. The concentrated extract was partitioned between 60% aqueous MeOH and dichloromethane, and the latter was further separated into 90% MeOH and n-hexane soluble fractions. The 90% MeOH layer, which exhibited potent cytotoxicity, was successively fractionated by octadecl dimethylsilyl-modified silica (ODS)-flash chromatography, gel-filtration on Sephadex LH-20, and ODS-HPLC to yield bisisorhizopodin (1; 0.4 mg), isorhizopodin (2; 0.6 mg), and the known 3 (0.3 mg).

The molecular formulae of 1–3, determined by high resolution-electrospray ionization-time-of-flight-mass spectra (HRESI-TOF-MS) measurements, all coincided with C76H128N4O22. In addition, their 1H-NMR spectra were quite similar with each other (Fig. 2), suggesting that the overall molecular composition is identical but difference exists in the substitution pattern. The signal duplication of an olefinic (6.72 and 7.12, H29) and formyl protons (8.30 and 8.06, H30) were again attributed to the fast cis–trans isomerization of the formamide termini, and, as is the case with 3, the abundance ratio of the cis versus trans isomers was estimated to 2:1 according to the integration.

In-depth analysis of the 1H, heteronuclear-single quantum coherence (HSQC), and correlation spectroscopy (COSY)
spectra of 1 revealed five fragments C2–C11, C15–C16, C18–
C20, C36–(C21)–C23, and C37–(C25)–C29, which are all present in 3 (Fig. 3). Substitution of a carboxyl group at the
head (C2) and an N-methyl vinylformamide at the tail (C29)
of this fragment sequence, oxazole and isopropylidene inter-
ruptions, and connection between C20 and C21 were also con-
firmed by heteronuclear multiple-bond correlation spectros-
copy (HMBC) correlations relevant to these units, retracing
the same monomeric polyketide–peptide chain in 1. However,
remarkable shiftings (0.1 ppm or more) of proton resonances
were observed in the head (H3–H4) and middle (H15–H19)
regions along this backbone. Especially, the acyloxy methine
proton (H18) was upfield-shifted from 5.32 to 3.60 ppm while
the proximate carbinol proton (H16) downfield-shifted from
3.92 to 5.32 ppm, suggesting migration of both the lactone
linkages from C16 to C18. Although a decisive evidence was
not obtained from 1 due to its scarcity, the other congener 2
endorsed this assignment by exhibiting an HMBC correlation
across the lactone linkage in the same partial structure (vide
infra). Therefore, the structure of 2 proved to be a single acyl migration analog of 3.

To further ensure the structure assignments made as above,
a chromatographic comparison of deacylation products from
1–3 was conducted. Compounds 1–3 (each 35 µg) were treated
with 0.5 N NaOMe (35 µL) and the reaction products were sub-
jected to LC-MS analysis. Neither of the analytes contained
the expected methyl ester monomer 4: instead, an intense
negative ion peak at \( m/z \) 751 was commonly detected at \( t_R \)
20.8 min, supporting the production of carboxylate monomer
5 from 1–3. The precedence of saponification over transesteri-
fication is attributed to the generation of sodium hydroxide
during the micro-scale reaction. Although a rigorous charac-
terization of each product was not possible by NMR due to
the scarcity of 1–3, their identity is quite reasonable in the
light of polyketide biosynthesis, which is stereochemically
controlled.

The occurrence of desymmetrized isomers similar to 2 was
reported for sponge-derived actin-depolymerizing dilactones swinholide A and bistheonellide A. Because the acidic treatment of swinholide A produced singly and doubly lactone bridge-transposed derivatives, potential production of these isomers during separation on acidic silica gel was proposed.

In this work, all purification steps were made under neutral conditions and the yields of the new congeners were higher than the parent compounds, which shows a clear contrast to the abundance of isoswinholide A and isobistheonellide A (less than 1% of the major metabolites).

The cytotoxicity of compounds 1–3 was evaluated as 50% growth inhibition (GI50) 27.2±8.1, 17.0±4.6, and 5.2±2.1 μM, respectively, showing decrease in the activity as the macrolactone ring contracted in size.

Compound 3 incorporates multiple traits that are found in actin depolymerizing cytotoxins: oxazole rings from the tris-oxazole class toxins, N-methyl vinylformamide tails from the tris-oxazoles and the aplyronin class, and dimeric conformation from the swinholide/bistheonellide class and luminolide. In fact, a crystal of actin/3 complex showed binding with two equimolar of G-actin (actin monomer) with virtually an identical mode of interaction with other related toxins at the vinylformamide tails.

In contrast to the clear-cut similarity in the role of the tails

**Fig. 2.** Comparison of 1H-NMR Spectra of Bisisorhizopodin 1 (Top), Isorhizopodin 2 (Middle), and Rhizopodin 3 (Bottom)

Insets magnify spectral regions with major differences.

**Fig. 3.** Structural Fragments (Bold Lines) Deduced by COSY and Key HMBC Correlations (Arrows) Supporting the Structures of 1 and 2
| Position | $\delta_C$ | $\delta_H$ mult. \((J\text{ in Hz})\) | HMBC | Position | $\delta_C$ | $\delta_H$ mult. \((J\text{ in Hz})\) | HMBC | Position | $\delta_C$ | $\delta_H$ mult. \((J\text{ in Hz})\) | HMBC |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 172.3 | | | | 171.8 | 171.8 | | | | | |
| 2 | 43.1 | 2.41 d (6.6) | 1, 3, 4 | 43.1 | 2.41 d (6.6) | 1, 3, 4 | 1' | 173.3 | 173.3 | | |
| 3 | 66.5 | 3.97 m | | 66.0 | 3.95 m | | 3' | 66.7 | 66.7 | 4.06 m | |
| 4 | 43.3 | 1.74 m | 1.52 m | 43.3 | 1.75 m | 3, 5 | 4' | 43.4 | 43.4 | 1.82 m | 3', 5' |
| 5 | 81.0 | 3.79 m | | 80.9 | 3.79 m | 31 | 5' | 81.0 | 81.0 | 3.82 m | 7', 31' |
| 6 | 131.8 | 5.37 dd (15.0, 8.3) | 8 | 132.0 | 5.37 dd (6.8, 15.0) | 8 | 6' | 132.0 | 132.0 | 5.40 dd (8.1, 15.1) | 8' |
| 7 | 134.9 | 6.22 dd (15.0, 10.5) | 5, 6 | 134.7 | 6.22 dd (10.2, 15.1) | 5, 9 | 7' | 134.9 | 134.9 | 6.18 m | 5' |
| 8 | 133.0 | 6.12 dd (15.0, 10.6) | | 132.9 | 6.12 dd (10.2, 15.8) | | 8' | 133.1 | 133.1 | 6.08 dd (10.3, 15.6) | |
| 9 | 131.2 | 5.68 dt (14.9, 7.3) | 7 | 131.1 | 5.66 m | 7, 11 | 9' | 131.1 | 131.1 | 5.66 m | 7', 11' |
| 10 | 38.5 | 2.55 m | 8, 11, 12 | 38.5 | 2.55 m | 8 | 10' | 38.2 | 38.2 | 2.62 m | 8', 11', 12' |
| 11 | 76.2 | 4.16 t (6.4) | 13, 32 | 76.4 | 4.16 t (6.6) | 9, 13, 32 | 11' | 76.3 | 76.3 | 4.22 t (6.6) | 13', 32' |
| 12 | 140.8 | | | 141.0 | | | | | | 139.9 | |
| 13 | 138.0 | 7.68 s | 12, 14 | 137.9 | 7.67 s | 12, 14 | 13' | 137.7 | 137.7 | 7.68 s | 14' |
| 14 | 164.1 | | | 164.2 | | | | | | 166.2 | |
| 15 | 29.5 | 3.14 dd (15.4, 2.1) | 14 | 29.5 | 3.13 m | 14, 16 | 15' | 31.7 | 31.7 | 2.96 m | 14' |
| 16 | 76.2 | 5.32 dd (10.7, 2.0) | | 76.6 | 5.32 m | 14, 34, 1' | 16' | 74.0 | 74.0 | 3.92 dd (10.3, 2.5) | 16' |
| 17 | 42.1 | | | 42.0 | | | | | | 42.2 | |
| 18 | 72.5 | 3.60 m | | 72.4 | 3.59 m | 33 | 18' | 76.2 | 76.2 | 5.29 m | 1' |
| 19 | 33.2 | 1.51 m | 1.39 m | 33.2 | 1.51 m | 20 | 19' | 31.6 | 31.6 | 1.61 m | 19' |
| 20 | 83.3 | 3.33 m | | 83.3 | 3.33 m | 20' | 82.7 | 82.7 | 3.03 m | 20' |
| 21 | 35.5 | 1.80 m | | 35.5 | 1.80 m | 21' | 35.1 | 35.1 | 1.78 m | 21' |
| 22 | 26.0 | 1.77 m | 1.27 m | 25.8 | 1.80 m | 22' | 25.7 | 25.7 | 1.78 m | 22' |
| 23 | 41.6 | 2.57 m | 21, 22, 24 | 41.6 | 2.58 m | 22, 24 | 23' | 41.6 | 41.6 | 2.58 m | 21', 22', 24' |
| 24 | 215.9 | | | 215.9 | | | | | | 215.9 | |
| 25 | 50.0 | 2.80 m | 37 | 49.8 | 2.79 m | 24, 26, 27, 37 | 25' | 49.8 | 49.8 | 2.79 m | 24, 26, 27, 37 |
| 26 | 83.5 | 3.46 m | 25, 37, 38 | 83.7 | 3.46 m | | 26' | 83.7 | 83.7 | 3.46 m | 26' |
| 27 | 30.9 | 2.50 m | 29, 28 | 30.9 | 2.50 m | 29, 28 | 27' | 30.9 | 30.9 | 2.50 m | 26, 28 |
| 28 | 107.0 | 5.18 m | | 106.8 | 5.18 m | 29 | 28' | 106.8 | 106.8 | 5.18 m | 29 |
| 29 | 131.7 | 6.72 d (13.8) | 30, 39 | 131.6 | 6.71 d (13.9) | 27, 30, 39 | 29' | 131.6 | 131.6 | 6.71 d (13.9) | 27, 30, 39 |
| 29Z | 126.9 | 7.12 d (14.5) | | 126.9 | 7.12 d (14.6) | 27, 39Z | 29Z' | 126.9 | 126.9 | 7.12 d (14.6) | 27, 39Z |

**Table 1.** Comparison of the NMR Spectroscopic Data (500 MHz, CD3OD) for Rhizopodins 1–3

- **Bisisorhizopodin (1)**
- **Isorhizopodin (2)**
- **Rhizopodin (3)**
that physically disrupt the contact between actin monomers, ring portions of the macrolides are structurally diverse and occupy different sites on the protein surface. Nevertheless, their roles seem to be identical, because both tris-oxazoles and dimeric macrolides competed with two actin-capping proteins of the gelsolin superfamily in competition-binding experiments. Examination of the actin-bound crystals corroborated superposition of their binding interfaces, and taking results of kinetic analyses into consideration, a gelsolin-mimetic mode of binding was proposed for these toxins. More specifically, the ring portions work as adapters to attach the closest recognized relative in the DDBJ database using a fruiting body was picked by a flame-sterilized disposable syringe needle transferred on Bennetts-2 (Bn-2) agar, and repeatedly transferred until pure. The isolate, coded YA1-5B-2, was sequenced by the method described previously. For a gene sequence by the method described previously.

**Experimental**

**General Procedures** UV, IR, and NMR spectra were recorded on a Hitachi U-3210, a Perkin-Elmer Spectrum 100 FT-IR spectrometer, and a 500 MHz Bruker AVANCE II instrument, respectively. HR-ESI-TOF-MS and LC-MS analyses were conducted with an Agilent 1200 HPLC-DAD system coupled to a Bruker micrOTOF mass spectrometer. The optical rotations were measured on a JASCO P-1030 polarimeter.

**Microorganism** Myxococcus stipitatus strain YA1-5B-2 was isolated from sandy deposits in a sedimentation basin from an outdoor hot spring bath in Ogawa-onsen Hotspring (36°52′39.77"N, 137°38′02.28"E), Toyama, Japan, on April, 2011. A spartalul of the specimen was placed on a sterilized filter paper layer on medium D solidified with agar. The plate was incubated for several weeks at room temperature (ca. 25°C) until fruiting bodies of myxobacteria appeared on the surface of the inculum or filter paper. The upper part of a fruiting body was picked by a flame-sterilized disposable syringe needle transferred on Bennetts-2 (Bn-2) agar, and repeatedly transferred until pure. The isolate, coded YA1-5B-2, was subjected to phylogenetic analysis based on 16S rRNA gene sequence by the method described previously. For a 1425 bp nucleotide sequence thus amplified was searched the closest recognized relative in the DDBJ database using BLAST algorithm, which retrieved Myxococcus stipitatus (GenBank accession No. AJ233922) with 99% identity.

**Fermentation** The producer strain grown on Bn-2 agar was seeded with agar substratum in V-22 liquid medium (100 mL in a K-flask) using a 10 µL disposable inoculation loop. The 30 flasks were cultured on a rotary shaker operated at 200 rpm at 30°C for 8 d.

**Extraction and Isolation** At the end of the fermentation period was added 1-butanol (50 mL) to each flask, and they were shaken for another 1 h to extract metabolites. The resulting emulsion was centrifuged at 6000 rpm for 10 min, and the upper organic phase was collected by careful decantation and pipettings. The crude extract thus obtained (910 mg) was flash-chromatographed on ODS by a stepwise elution...
with aqueous MeCN (30%, 45%, 60%, 75%, 90%) containing 50 mM NaClO₄ to give 5 fractions. The forth fraction was the second most cytotoxic and found to contain myxalamidine A as the major active principle. The most toxic third fraction was gel-filtered on Sephadex LH-20 with 60% MeCN, yielding the cytotoxicity at the top of eluates. This fraction was finally purified by reversed-phase HPLC (Cosmosil AR-II 10×250 mm, Nacalai Tesque) eluting for the first 5 min with 50% aqueous MeCN then raising the concentration of MeCN linearly to 60% over the next 40 min, yielding bisisorhizopodin (1; 0.4 mg), isorhizopodin (2; 0.6 mg), and rhizopodin (3; 0.3 mg), respectively.

Bisisorhizopodin (1): Amorphous solid; [α]D −79.0 (c=0.033, MeOH). UV (MeOH) nm (logε) 226 (5.05), 232 (5.03), 241 (4.88). IR (attenuated total reflectance (ATR)) ν max cm⁻¹ 3454, 2935, 2824, 1696, 1658, 1601, 1379, 1092, 994. HR-ESI-TOF-MS m/z: 1491.8577 [M+Na]+ (Calcd for C₇₅H₁₂₄N₄NaO₂₂ 1491.8605).

Isorhizopodin (2): Amorphous solid; [α]D −59.1 (c=0.049, MeOH). UV (MeOH) nm (logε): 229 (4.97), 231 (4.96), 240 (4.82). IR (ATR) ν max cm⁻¹ 3454, 2927, 1696, 1657, 1377, 1092, 994. HR-ESI-TOF-MS m/z: 1491.8619 [M+Na]+ (Calcd for C₇₅H₁₂₄N₄NaO₂₂ 1491.8605).

LC-MS Analysis of 1–3 To each 35-µg aliquot of rhizopodins 1–3, precooled on ice in v-bottom vials, was added 50 µL of similarly ice-cooled 0.5% sodium methoxide. The vials were stirred for 3 min at 0°C, after which 51 µL-volume of 0.04% trypan blue in phosphate buffered saline was added to quench the reaction. MeOH was removed under a stream of Ar and the resulting aqueous solutions were extracted four times with 100 µL of EtOAc. The organic extracts were combined and blown to dryness, which was dissolved in 35 µL of MeCN and subjected to LC-MS analysis. 0.3 µL aliquots of the analytes were chromatographed on a Cosmosil AR-II column (2.0×150 mm) under the elution program of 25% MeCN in 0.1% aqueous HCO₂H for 5 min, 1%–50% MeCN concentration ramp to 45%, and the same concentration held for 5 min at a flow rate of 0.2 mL/min. Negative ESI was employed for mass spectral detection and elution of 5 was traced by molecular ion at m/z 751.4 [M+Na]+. Retention times for 5 from 1–3 were all 20.8 min.

Cytotoxicity Testing Cytotoxicity of 1–3 was evaluated using a trypan blue dye-exclusion technique. P388 murine leukemia cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum, 0.1% L-glutamine, 10 µM 2-mercaptoethanol. To each cell suspension prepared at a density of 5×10⁴ cells/mL was added a serially diluted drug solution, and 100 µL aliquots of test cultures were triplicated in a 96-well plate. After incubating the plate at 37°C in a 5% CO₂–95% air atmosphere for 24 h, a 100 µL-volume of 0.04% trypan blue in phosphate buffered saline (PBS) was added to each well, and the number of live cells (nucleus not stained) were counted using microscopic observation. The IC₅₀ values were calculated from triplicated data points taken at each drug concentration.

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Supporting Information 1D- and 2D-NMR spectroscopic data for compounds 1 and 2. This material is available free of charge via the Internet at https://www.jstage.jst.go.jp/browse/cpb.

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