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Madurahydroxylactone, an Inhibitor of *Staphylococcus aureus* FtsZ from *Nonomuraea* sp. AN100570

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FtsZ, a bacterial cell-division protein, is an attractive antibacterial target. In the screening for an inhibitor of *Staphylococcus aureus* FtsZ, madurahydroxylactone (**1**) and its related derivatives 2–5 were isolated from *Nonomuraea* sp. AN100570. Compound 1 inhibited *S. aureus* FtsZ with an IC_{50} of 53.4 μM and showed potent antibacterial activity against *S. aureus* and MRSA with an MIC of 1 μg/ml, whereas 2–5 were weak or inactive. Importantly, 1 induced cell elongation in the cell division phenotype assay, whereas 2–5 did not. It indicates that 1 exhibits its potent antibacterial activity via inhibition of FtsZ, and the hydroxyl group and hydroxylactone ring of 1 are critical for the activity. Thus, madurahydroxylactone is a new type of inhibitor of FtsZ.

Keywords: Madurahydroxylactone, FtsZ, inhibitor, *Nonomuraea*, antibacterial

Multidrug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) have become a significant global health concern [1–3]. One approach to combat antibiotic resistance is to identify new drugs that function through novel mechanisms of action [4]. FtsZ, a bacterial cell-division protein, is an attractive target for antibacterial drug discovery, since the target is essential for the viability of bacteria and well conserved throughout bacterial species [5–7]. During bacterial cell division, FtsZ undergoes GTP-dependent polymerization to form the Z ring at the mid-cell. So far, few compounds have been reported to inhibit FtsZ and subsequently inhibit bacterial cell division [8–11]. The compounds berberine [12], sanguinarine [13], and viriditoxin [10, 14] were reported as FtsZ inhibitors of natural origin. As a synthetic inhibitor, PC190723 was reported to be a potent inhibitor of *S. aureus* FtsZ in both in vitro and in vivo mice infection models, which demonstrated FtsZ as an antibacterial target [8, 15]. Microorganisms have been a rich source for new antibiotics. Until now, only viriditoxin is reported as a FtsZ inhibitor of microbial origin [10]. Thus, more unique FtsZ inhibitors are needed from microorganisms.

In the course of screening for an inhibitor of *S. aureus* FtsZ from microbial sources, a benzo[a]naphthacenequinone-type metabolite, madurahydroxylactone (**1**), and four related derivatives 2–5 were isolated from the fermentation broth of an actinomycetal strain, AN100570 (Fig. 1). Madurahydroxylactone was previously isolated as a potent antibacterial from *Nonomuria rubra* (formerly *Actinomadura rubra*) [16]. Its antibacterial mechanism, however, has not been reported yet. In this paper, we report that 1 showed its potent antibacterial activity by inhibiting FtsZ. Additionally, the NMR assignments of 1–5 were reported in this study for the first time.

The actinomycetal strain AN100570 was isolated from soil collected near Gongju city, Chungcheongnam-do, Korea. The strain was identified as *Nonomuraea* sp. based on the 16S rRNA sequence. Culture was carried out in a liquid

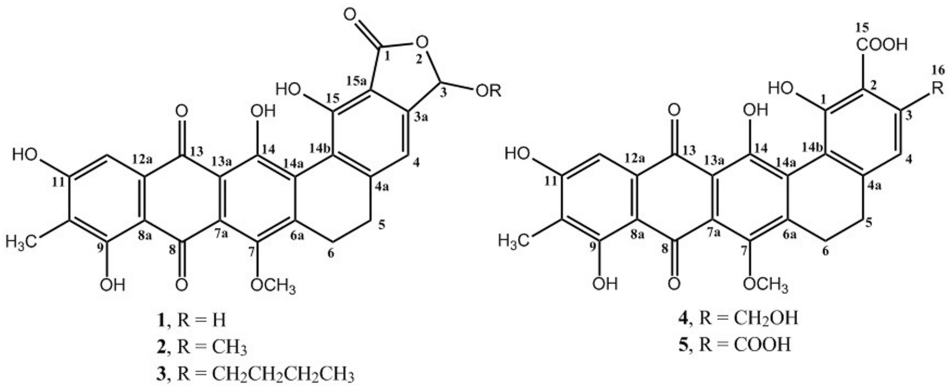

Fig. 1. Chemical structures of madurahydroxylactone (1), methyl acetal of madurahydroxylactone (2), butylmaduramycin (3), BE-39589 B-1 (4), and BE-39589 C-1(5) isolated from *Nonomuraea* sp. AN100570.

culture medium containing 1% soluble starch, 2% glucose, 2.5% soybean meal, 0.1% beef extract, 0.4% yeast extract, 0.2% NaCl, 0.025% K_2HPO_4, and 0.2% $CaCO_3$ (adjusted to pH 7.2 before sterilization). For the production of the active compound, 5 ml of the seed culture was transferred into 500-ml Erlenmeyer flasks (35 flasks) containing 100 ml of the same medium, and then cultivated for 6 days at 28°C. The fermented whole medium (9 L) was centrifuged at 6,000 rpm for 10 min, and the resultant supernatant was successively extracted twice with an equal volume of ethyl acetate (EtOAc) and *n*-butanol (BuOH). The EtOAc extract was subjected to Silica gel TLC (No. 1.05715.0001; Merck, Germany) and developed with $CHCl_3$-methanol (MeOH)-water (150:30:1). The active band (14 mg) with an R_f of 0.13 was further purified by RP-18 TLC (Merck No 1.15389.0001), and developed with acetone-water (60:40) to give 1 (7.5 mg) with an R_f of 0.43 as a red powder. The BuOH extract was subjected to Silica gel TLC developed with $CHCl_3$-MeOH-water (150:50:1) to give three bands, I, II, and III, with R_f of 0.43, 0.14, and 0.04, respectively. Band I (22.2 mg) was further purified by RP-18 TLC developed with acetone-water (60:40) to give 2 (9.4 mg) and 3 (8.4 mg), with R_f of 0.29 and 0.14, respectively, as red powders. Bands II (14.9 mg) and III (26.7 mg) were further purified by RP-18 TLC developed with acetone-water (60:40) to give 4 (6.1 mg) and 5 (6.7 mg), with R_f of 0.56 and 0.54, respectively, as red powders.

The chemical structures of 1–5 were determined by MS and NMR analyses (Supplemental material). Independent NMR and MS analyses of 1–5 led to identification of madurahydroxylactone [16, 17], methyl acetal of madurahydroxylactone [18], butylmaduramycin [19], BE-39589 B-1, and BE-39589 C-1 (Masahisa *et al*. 1998. Jpn. Kokai Tokkyo Koho. JP 10017527 A), respectively (Fig. 1). Since 2, a methyl acetal of 1, was not detected in the EtOAc extracts and also in the isolation procedure using acetonitrile instead of methanol, it is an artifact produced during the isolation procedure. Compound 2 has been reported as a semisynthetic derivative of madurahydroxylactone [18]. Butylmaduramycin, BE-39589 B-1, and BE-39589 C-1 have been isolated as antibacterials from *Actinomadura rubra* [19] and a *Microtetraspora* strain, respectively. To our best knowledge, the ^1H and ^{13}C NMR assignments of 1–5 are reported in this study for the first time (Supplemental material).

Compound 1: $C_{26}H_{18}O_{10}$; a red powder; UV: 235 (4.49), 289 (4.39), 330 (sh, 3.94), 473 (4.02); ESI-MS: 491.1 $[M+H]^+$, 489.1 $[M-H]^-$; ^1H-NMR (500 MHz, DMSO-d_6): δ 2.02 (3H, s, 10-CH_3), δ 2.77 (4H, brs, H-5 and H-6), δ 3.78 (3H, s, 7-OCH_3), δ 6.54 (1H, brs, H-3), δ 7.10 (1H, s, H-4), δ 7.22 (1H, s, H-12), δ 13.54 (1H, s, 9-OH); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 8.1 (10-CH_3), δ 22.3 (C-6), δ 29.7 (C-5), δ 61.0 (7-OCH_3), δ 96.4 (C-3), δ 106.4 (C-12), δ 109.6 (C-8a), δ 112.4 (C-15a), δ 113.3 (C-4), δ 113.8 (C-7a or 13a), δ 118.4 (C-10), δ 120.0 (C-14b), δ 122.2 (C-7a or 13a), δ 129.2 (C-14a), δ 130.6 (C-12a), δ 146.6 (C-14 or C-6a), δ 149.6 (C-6a or C-14), δ 149.7 (C-4a), δ 150.5 (C-7), δ 153.6 (C-3a), δ 156.5 (C-15), δ 162.1 (C-11), δ 162.5 (C-9), δ 167.1 (C-1), δ 185.5 (C-13), δ 187.6 (C-8).

Compound 2: $C_{27}H_{20}O_{10}$; a red powder; ESI-MS: 505.5 $[M+H]^+$, 505.3 $[M-H]^-$; ^1H-NMR (500 MHz, DMSO-d_6): δ 2.04 (3H, s, 10-CH_3), δ 2.67 (2H, brs, H-5), δ 2.76 (2H, brs, H-6), δ 3.69 (3H, s, 7-OCH_3), δ 6.26 (1H, s, H-3), δ 6.68 (1H, s, H-4), δ 7.09 (1H, s, H-12), δ 13.70 (1H, s, 9-OH); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 8.0 (10-CH_3), δ 21.9 (C-6), δ 30.1 (C-5), δ 60.6 (7-OCH_3), δ 100.3 (C-3), δ 105.7 (C-12), δ 108.9 (C-4), δ 109.3 (C-8a), δ 112.3 (C-15a), δ 114.8 (C-10), δ 118.8 (C-7a or 13a), δ 123.6 (C-7a or 13a), δ 125.8 (C-14b), δ 134.2 (C-12a), δ 135.7 (C-14a), δ 141.5 (C-6a), δ 146.4 (C-3a), δ 147.2 (C-7), δ

148.9 (C-4a), δ 161.3 (C-15), δ 161.5 (C-9), δ 163.1 (C-11), δ 167.8 (C-1), δ 181.6 (C-13), δ 187.2 (C-8).

Compound 3: $C_{30}H_{26}O_{10}$; a red powder; ESI-MS: 547.2 $[M+H]^+$, 545.1 $[M-H]^-$; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 0.90 (3H, t, J = 7.4, H-4′), δ 1.36 (2H, m, H-3′), δ 1.57 (2H, m, H-2′), δ 1.96 (3H, s, 10-CH_3), δ 2.66 (2H, brs, H-5), δ 2.74 (2H, brs, H-6), δ 3.67 (3H, s, 7-OCH_3), δ 3.69 (2H, t, H-1′), δ 6.31 (1H, s, H-3), δ 6.65 (1H, s, H-4), δ 6.88 (1H, s, H-12); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 8.2 (10-CH_3), δ 13.6 (C-4′), δ 18.6 (C-3′), δ 21.9 (C-6), δ 30.2 (C-5), δ 31.2 (C-2′), δ 60.8 (7-OCH_3), δ 67.9 (C-1′), δ 99.8 (C-3), δ 106.6 (C-12), δ 106.9 (C-8a), δ 108.7 (C-4), δ 112.1 (C-15a), δ 114.4 (C-10), δ 119.3 (C-7a or 13a), δ 124.6 (C-7a or 13a), δ 125.8 (C-14b), δ 134.0 (C-14a), δ 134.3 (C-12a), δ 141.9 (C-6a), δ 146.5 (C-3a), δ 146.9 (C-7), δ 148.8 (C-4a), δ 160.5 (C-9), δ 161.9 (C-11), δ 163.0 (C-15), δ 168.3 (C-1), δ 183.5 (C-13), δ 184.7 (C-8).

Compound 4: $C_{26}H_{20}O_{10}$; a red powder; ESI-MS: 493.1 $(M+H)^+$; ^{1}H-NMR (800 MHz, DMSO-d_6): δ 2.08 (3H, s, 10-CH_3), δ 2.61 (2H, t, J = 6.6, H-5), δ 2.77 (2H, brs, H-6), δ 3.76 (3H, s, 7-OCH_3), δ 4.67 (2H, s, H-16), δ 6.76 (1H, s, H-4), δ 7.20 (1H, s, H-12); ^{13}C-NMR (200 MHz, DMSO-d_6): δ 8.5 (10-CH_3), δ 22.9 (C-6), δ 29.6 (C-5), δ 61.4 (7-OCH_3), δ 64.1 (C-16), δ 106.4 (C-12), δ 110.1 (C-8a), δ 115.2 (C-4), δ 115.4 (C-2), δ 116.8 (C-7a or 13a), δ 117.3 (C-10), δ 119.9 (C-14b), δ 122.4 (C-7a or 13a), δ 132.9 (C-12a), δ 133.8 (C-14a), δ 143.8 (C-6a), δ 144.1 (C-4a), δ 146.9 (C-3), δ 150.5 (C-7), δ 162.4 (C-11), δ 162.5 (C-9), δ 164.2 (C-1), δ 171.9 (C-15), δ 185.1 (C-13), δ 186.9 (C-8).

Compound 5: $C_{26}H_{18}O_{11}$; a red powder; ESI-MS: m/z 507.0 $(M+H)^+$; ^{1}H-NMR (800 MHz, DMSO-d_6): δ 2.10 (3H, s, 10-CH_3), δ 2.69 (2H, brs, H-5), δ 2.82 (2H, brs, H-6), δ 3.80 (3H, s, 7-OCH_3), δ 7.47 (1H, s, H-4), δ 7.30 (1H, s, H-12); ^{13}C-NMR (200 MHz, DMSO-d_6): δ 8.7 (10-CH_3), δ 22.7 (C-6), δ 29.2 (C-5), δ 61.5 (7-OCH_3), δ 106.6 (C-12), δ 110.2 (C-8a), δ 114.6 (C-7a or 13a), δ 114.9 (C-2), δ 118.3 (C-10), δ 120.1 (C-4), δ 122.6 (C-14b), δ 122.5 (C-7a or 13a), δ 131.5 (C-14a), δ 131.8 (C-12a), δ 135.9 (C-3), δ 143.9 (C-4a), δ 146.5 (C-6a), δ 150.9 (C-7), δ 162.3 (C-1), δ 162.6 (C-11), δ 162.8 (C-9), δ 168.4 (C-16), δ 173.4 (C-15), δ 186.4 (C-8), δ 187.5 (C-13).

For FtsZ GTPase assay, *S. aureus* FtsZ was cloned, expressed, and purified. The *ftsZ* gene was PCR-amplified from the genomic DNA of *S. aureus* RN4220 using oligos ftsZ-F (5′-AAAAAACATATGTTAGAATTTGAACAAGGATTTAATC-3′) and ftsZ-R (5′-AAAAAACTCGAGTTAACGTCTTGT TCTTCTTG-3′). The amplified PCR products were purified, digested with NdeI and XhoI, and cloned into the pET28a vector. The culture of *E. coli* BL21(DE3) transformed with the recombinant expression vector was induced with isopropyl-1-thio-β-D-galactopyranoside (at a final concentration of 0.5 mM). After centrifuging the cell lysate, FtsZ enzyme was purified by nickel-nitrilotriacetic acid column chromatography (Qiagen). The fractions eluted with 200 mM imidazole were pooled and concentrated with Amicon Ultra-15 (Millipore). The concentrated enzyme was dialyzed overnight at 4°C using 100 mM Tris-HCl (pH 8.0) containing 20% (v/v) glycerol, and stored at 4°C until use.

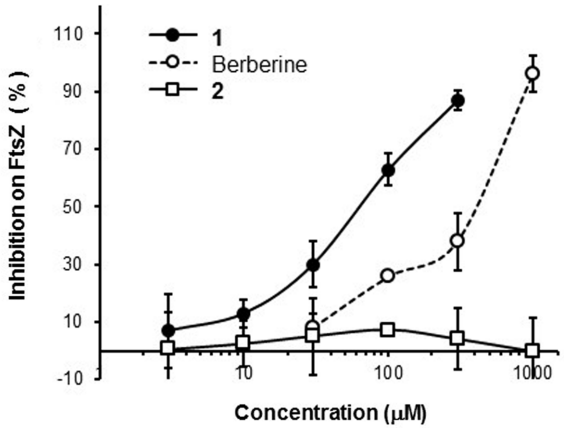

Fig. 2. Inhibitory activity of 1 and related compounds on *S. aureus* FtsZ GTPase.

Reaction containing 10 μM *S. aureus* FtsZ and different doses of 1 (1–300 μM), 2 (1–1,000 μM), and berberine (30–1,000 μM) were initiated by the addition of 1 mM GTP, and incubated at 37°C for 1 h. Each point represents the mean of three independent assays, and the vertical bars show the standard deviation of the mean.

FtsZ GTPase assay was performed according to the method of Domadia *et al.* [12]. Conversion of GTP to GDP by FtsZ was determined by measuring the release of phosphate using malachite green dye in an end-point assay. The reaction contained 10 μM FtsZ and test compounds in the reaction buffer (50 mM MES-NaOH (pH 6.5), 50 mM KCl, and 5 mM $MgCl_2$). The reactions were initiated by the addition of 1 mM GTP, and incubated at 37°C for 1 h; reaction samples (10 μl) were then added to 100 μl of malachite green dye. After 20 min, the absorbance at 620 nm (A_{620}) was measured using an ELISA reader.

Compound 1 inhibited the GTPase activity of *S. aureus* FtsZ with an IC_{50} of 53.4 ± 1.9 μM, which was 5 times higher activity than that (242.7 ± 7.8 μM) of berberine (Sigma) as a positive control (Fig. 2 and Table 1). The inhibitory activity of berberine on *S. aureus* FtsZ in our system is similar to that (272 ± 46.6 μM) in the literature [9]. Compounds 2–5, however, did not inhibit *S. aureus* FtsZ GTPase even at 1,000 μM. This result indicated that the hydroxyl group and the hydroxylactone ring of 1 are critical for inhibition of *S. aureus* FtsZ. The antibacterial

Table 1. Effects of 1–5 on *S. aureus* FtsZ, bacterial growth, and cell elongation.

Compounds	IC_{50} (μM) on saFtsZ[a]	MIC (μg/ml)				Cell elongation activity[d]
		S. aureus RN4220	MRSA 3167[b]	QRSA 3519[c]	*B. subtilis* KCTC1021	
1	53.4 ± 1.9	1	1	1	2	+
2	>1,000	16	16	16	8	-
3	>1,000	16	16	32	4	-
4	>1,000	16	16	32	4	-
5	>1,000	>128	>128	>128	>128	N.T.[e]
Berberine	242.7 ± 7.8	128	128	128	512	+
Ciprofloxacin	>1,000	0.1	2	>128	0.03	-

[a]*S. aureus* FtsZ, [b]Methicillin-resistant *S. aureus* CCARM 3167, [c]Quinolone-resistant *S. aureus* CCARM 3519.
[d]Cell elongation activity was examined in *B. subtilis* KCTC 1021. [e]Not tested.

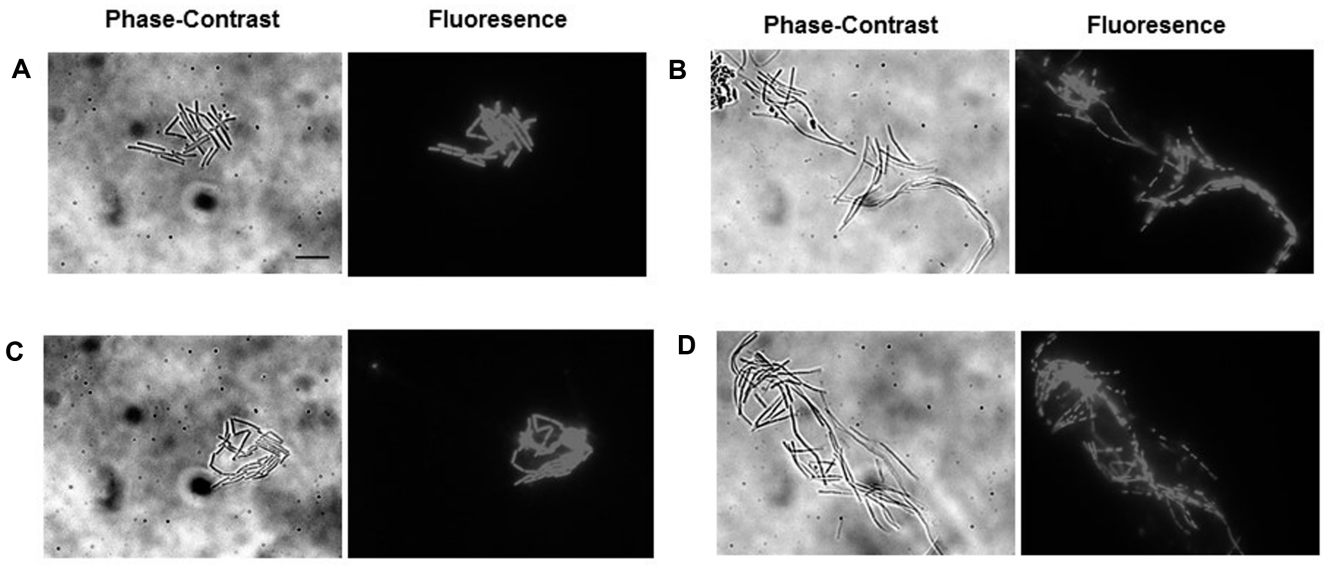

Fig. 3. Effects of 1 and related compounds on cell division and cell morphology of *B. subtilis*.
B. subtilis KCTC 1021 was cultured in the absence (**A**) or presence of 0.5 μg/ml 1 (**B**), 2 μg/ml 2 (**C**), or 128 μg/ml berberine (**D**). The cell samples were collected, stained with DAPI, and observed by phase-contrast light microscopy and fluorescence microscopy. The length of the scale bar is 10 μm.

activities of 1–5 were compared (Table 1). MICs were determined by the broth microdilution method as reported previously [20]. Consistent with its inhibition of *S. aureus* FtsZ, 1 showed potent antibacterial activity against *S. aureus*, including MRSA and quinolone-resistant *S. aureus* (QRSA) with MICs of 1 μg/ml, which was 16–32 times higher activity than that of 2–4. Interestingly, 5 had no antibacterial activity even at 128 μg/ml.

To ascertain whether 1 showed its antibacterial activity via inhibition of FtsZ, a cell morphology assay was employed as reported previously [8, 11]. The disruption of septum formation in dividing *B. subtilis* cells through chemical interference with FtsZ activity leads to an elongation of the cells [5], which is easily visible through microscopy. Mid-log *B. subtilis* KCTC 1021 cells were treated with 1–4 at below MIC for 5 h. Cell morphology was stained with 4′,6-diamidino-2-phenylindole for 30 min and examined using phase-contrast light microscopy or fluorescent microscopy with a Zeiss Axiovert 200 M inverted microscope. Compound 1 significantly increased the cell length of *B. subtilis* (Fig. 3B), as compared with the vehicle (DMSO)-treated cells (Fig. 3A), with a high elongation at 1/4 × MIC (0.5 μg/ml). The formed long cells, called filaments, contained multiple chromosomes. It indicated the inhibition of 1 on cell division. In contrast, 2 did not affect the cell morphology (Fig. 3C). Moreover, 3–5 were inactive (data not shown).

Berberine as a positive control also induced the cell filamentation as expected (Fig. 3D). This result demonstrates that 1 exhibits its potent antibacterial activity by inhibiting FtsZ, whereas 2–4 elicit their weaker antibacterial activity through inhibition of other target(s). Thus, madura-hydroxylactone is a new inhibitor of FtsZ and has the potential for treatment of multidrug-resistant bacteria, including MRSA.

Acknowledgments

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