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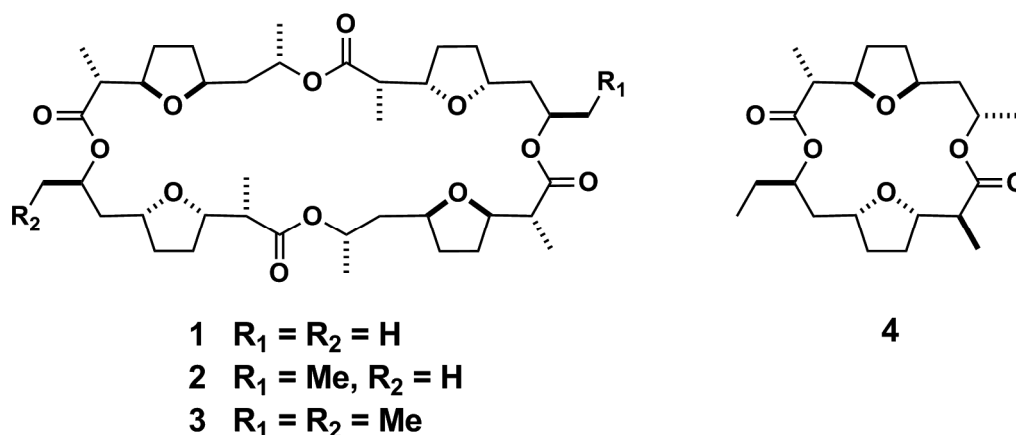
## NONACTIN AND RELATED COMPOUNDS FOUND IN A SCREENING PROGRAM FOR WNT SIGNAL INHIBITORY ACTIVITY

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**Abstract** – A screening study using a luciferase assay to identify natural products with inhibitory activity against Wnt signaling was carried out. Fractionation of the culture extract of an actinomycete, strain CKK179, led to the identification of nonactin (**1**), monactin (**2**), and dinactin (**3**) as active compounds. We examined the effects of dimeric dinactin (**4**), isolated from another actinomycete, *Streptomyces* sp. (YM09-028), on Wnt signaling activity and found that **1** - **4** inhibited TCF/ $\beta$ -catenin transcriptional activity with IC<sub>50</sub> values of 0.6 - 7.4 nM.

Wnt signaling plays a key role in many biological events including cell morphology, motility, proliferation, and differentiation. However, aberrant Wnt/ $\beta$ -catenin signaling can lead to the formation of tumors.<sup>1</sup> Small molecules that inhibit Wnt signaling may therefore suppress cancer cell growth. During a search for bioactive natural products, we recently investigated a screening program targeting Wnt signaling, and examined a number of extracts of plants and microorganisms using a cell-based luciferase assay to evaluate the inhibition of TCF/ $\beta$ -catenin transcriptional activity (TOP activity),<sup>2</sup> which was measured using the cell line STF/293 (a 293 human embryonic kidney cell line stably transfected with SuperTOP-Flash). We recently examined the active compounds from the extract of a cultured actinomycete, strain CKK179, leading to the identification of nonactin (**1**) and its congeners, monactin (**2**) and dinactin (**3**). Their inhibition of TCF/ $\beta$ -catenin transcriptional activity was compared with a related compound, dimeric dinactin (**4**), isolated from another actinomycete, *Streptomyces* sp. (YM09-028),



revealing that these compounds inhibited the TCF/ $\beta$ -catenin transcriptional activity with  $IC_{50}$  values of 0.6 – 7.4 nM. However, it was found that the inhibition of Wnt signaling by **4** was not selective.

By using a cell-based luciferase assay system to evaluate the inhibition of TCF/ $\beta$ -catenin's transcriptional activity with the STF/293 cell line,<sup>2</sup> we examined a series of extracts of cultured actinomycetes in our laboratory,<sup>3</sup> and found that the extract of strain CKK179 showed 88% inhibition with high (> 85%) cell viability at 500  $\mu$ g/mL. Thus, fractionation of this extract was carried out.

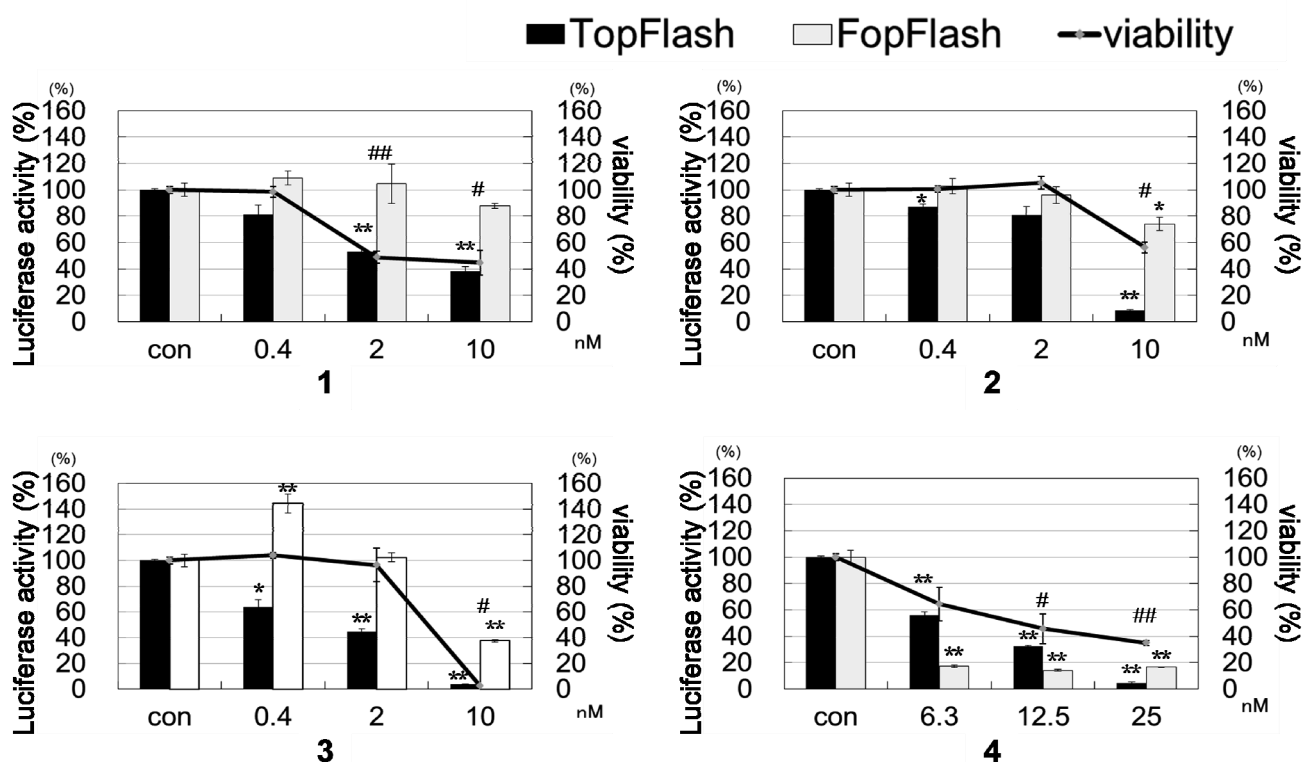
The fermentation broth of CKK179 was centrifuged and the supernatant was extracted with EtOAc. The EtOAc fraction was subjected to chromatography on a silica gel column followed by a Sephadex LH-20 column to give nonactin (**1**), monactin (**2**), and dinactin (**3**),<sup>4</sup> which were identified by ESIMS as well as HPLC using commercially available standards.

Here we examined the inhibitory activity of **1** – **3** using standards along with dimeric dinactin (**4**)<sup>5</sup> by the luciferase reporter gene assay. Compound **4** was originally isolated from another strain of *Streptomyces* sp. (YM09-028) as a cytotoxic compound, and its structural relationship with **1** – **3** caused us to examine its Wnt signaling activity.

Wnt signaling activates gene transcription by forming a complex between DNA-binding proteins of the TCF/LEF family and  $\beta$ -catenin. SuperTOP-Flash, a  $\beta$ -catenin-responsive reporter plasmid with multiple TCF-binding sites (CCTTTGATC), was activated in cells. SuperFOP-Flash has eight mutated TCF-binding sites (CCTTTGGCC), and a selective inhibitor would prevent any enhancement of transcription in SuperFOP-Flash-transfected cells; thus the ratio of TOP/FOP-Flash reporter activity provides a measure of selective inhibition of Wnt signaling.

The results regarding the inhibition of TCF/ $\beta$ -catenin transcriptional activity (TOP-Flash activity) by **1** – **4** are shown in Figure 1 along with those for cell viability since a decrease in cell number may contribute to the inhibition. These compounds inhibited TOP-Flash activity with  $IC_{50}$  values of 7.2, 5.0, 1.3, and 7.4 nM, respectively. The FOP-Flash activity was also examined. Compounds **1** – **3** showed weak

inhibition of FOP-Flash activity at the same concentration as their  $IC_{50}$  values. However, compound **4** caused a greater decrease in FOP-Flash activity and cell viability, implying that it did not selectively inhibited Wnt signaling. Considering the cell viability data (Figure 1), compounds **1** and **2** showed 20% inhibition of TOP-Flash activity at 0.4 nM and 2 nM, respectively, while compound **3** showed 60% inhibition of TOP-Flash activity at 2 nM. Thus, compound **3** showed the most potent inhibition of TOP-Flash activity. The inhibition of TCF/ $\beta$ -catenin transcriptional activity may be caused by  $\beta$ -catenin degradation or inhibition of  $\beta$ -catenin translocation into the nucleus, and further molecular mechanism of these compounds still remained undefined. We also examined the cytotoxicity of these compounds in two colon cancer cell lines, HCT116 and DLD1, since Wnt signal pathways of many human colorectal cancer cells are known to be aberrantly activated, and found that **1** – **4** were cytotoxic with the  $IC_{50}$  values shown in Table 1. Nonactin is known as an ionophore of ammonium and potassium ions and its effect on Wnt signaling pathway has not been reported, while a calcium ionophore A23187 was described to inhibit Wnt/ $\beta$ -catenin pathway through activation of protein kinase C to promote the phosphorylation of  $\beta$ -catenin resulting in proteasome-mediated  $\beta$ -catenin degradation.<sup>6</sup>



**Figure 1.** Effects of **1** - **4** on TOP- and FOP-Flash activity and cell viability. The data are shown as the mean  $\pm$  SE. The significance of difference was determined with Student's t-test (\*:  $p < 0.05$ , \*\*:  $p < 0.01$  vs. control for Luciferase activity, #:  $p < 0.05$ , ##:  $p < 0.01$  vs. control for viability). Quercetin was used as positive control and showed 29% of TopFlash activity and 94% of viability at 27.7  $\mu$ M.

**Table 1.** Cytotoxicity of **1 - 4** against colon cancer cell lines (IC<sub>50</sub> values, nM)

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
HCT116	24.7	30.1	8.9	>50
DLD1	25.9	17.3	10.1	27.4

## EXPERIMENTAL

**Luciferase Assay.** For stable reporter cells, STF/293 cells ( $3 \times 10^4$ ) were split into 96-well plates and 24 h later, treated with compounds combined with 15 mM LiCl, an inhibitor of glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ), which causes the accumulation of non-phosphorylated  $\beta$ -catenin resulting in the increase in TCF/ $\beta$ -catenin transcription.<sup>2</sup> After incubation for 24 h, cells were lysed with CCLR (cell culture lysis reagent; 20  $\mu$ L/well, Promega) and luciferase activity was measured with a Luciferase Assay System (Promega). Assays were performed in triplicate at least. Quercetin was used as a positive control and showed 29% (81% inhibition) of Top-Flash activity and 94% of viability at 27.7  $\mu$ M. Transient transfection was performed using Lipofectamine 2000 (Invitrogen, USA). Briefly,  $1 \times 10^5$  cells (293T) were split into 24-well plates. After 24 h, cells were transfected with 1  $\mu$ g of the luciferase reporter construct (SuperFOP-Flash) and 0.05  $\mu$ g of pRL-CMV (Promega, USA) for normalization. At 3 h posttransfection, compounds were added to a medium containing FBS. Of note, 293T cells were treated with compounds in a FBS-containing medium combined with 15 mM of LiCl. Cells incubated for 24 h were lysed in Passive lysis buffer (Promega, 50  $\mu$ L/well) and luciferase activity was measured with a Dual-Glo Luciferase Assay System (Promega). The assay sample was stored as a 10 mM solution in DMSO, then diluted to the indicated concentrations with medium, the final concentration of DMSO being less than 0.1% (v/v). The data are shown as the mean  $\pm$  SE. The significance of difference was determined with Student's *t*-test (\*:  $p < 0.05$ , \*\*:  $p < 0.01$  vs. control) The IC<sub>50</sub> was estimated from the graphical interpolation.

**Assay of Cell Viability (FMCA Assay<sup>7</sup>).** 293T cells ( $6 \times 10^3$ ) were split into 96-well plates and incubated for 24 h. Cells were treated with compounds and incubated for 24 h. They were treated with fluorescein diacetate (Wako) in PBS buffer (10  $\mu$ g/mL), and after 1 h of incubation, fluorescence was detected. Assays were performed in triplicate at least. The sample was prepared as mentioned above. The data are shown as the mean  $\pm$  SE. The significance of difference was determined with Student's *t*-test (#:  $p < 0.05$ , ##:  $p < 0.01$  vs. control). Cytotoxicity against HCT116 and DLD1 cells was also examined as above.

**Fermentation of CKK179.** Spores of strain CKK179 collected in Chiba city, Japan in 2005, and grown on solid Waksman medium, were inoculated into a 500-mL Sakaguchi flask containing 100 mL of liquid medium and cultured for 3 days at 28 °C with reciprocal shaking at 150 rpm to produce a seed culture. A 10-mL sample of the seed culture was transferred into a 3-L flask containing 500 mL of the same medium, and cultured for 5 days at 28 °C with reciprocal shaking at 150 rpm to obtain 4 L of fermentation broth.

**Extraction and Isolation of 1 - 3.** The cultured broth (4 L) was centrifuged at 6000 rpm for 15 minutes to separate the mycelium and supernatant. The supernatant was extracted with EtOAc (300 mL x 3), and the extracts were concentrated *in vacuo* to give a residue (1.42 g). The EtOAc extract (1.42 g) was subjected to silica gel flash column chromatography (40 x 200 mm) and successively eluted with 25 - 100% EtOAc in hexane to give eight fractions (1A–1H). Fraction 1G (7 mg) eluted with 50% EtOAc in hexane was subjected to Sephadex LH-20 column chromatography (10 x 400 mm) eluted with 100% MeOH to give six fractions (2A–2F). Fraction 2C (1.0 mg) was revealed to be a mixture of nonactin derivatives by an <sup>1</sup>H NMR analysis and ESIMS data: *m/z* 759 (M+Na)<sup>+</sup> for **1**, *m/z* 773 (M+Na)<sup>+</sup> for **2**, and *m/z* 787 (M+Na)<sup>+</sup> for **3**. These three compounds were firmly identified by reversed-phase HPLC (capcell pack ACR C-18, 250 x 4.6 mm; flow rate, 1.0 mL/min; eluant, 87% MeOH; detection, UV at 210 nm) to give peaks for **1** (*t<sub>R</sub>* 19.1 min), **2** (*t<sub>R</sub>* 22.9 min), and **3** (*t<sub>R</sub>* 29.6 min), which were identical with the standards obtained from Sigma-Aldrich Japan (Tokyo).

**Fermentation of *Streptomyces* sp. (YM09-028).** *Streptomyces* sp. (YM09-028), collected in Kamaishi city, Japan in 2003, grown on Marine Broth agar plates, was inoculated into three tubes containing 4 mL of liquid medium for 3 days at 30 °C with reciprocal shaking at 130 rpm to produce a seed culture. Aliquots (1 mL) were seeded into 12 Sakaguchi flasks containing 150 mL of the medium and cultured for 3 days at 30 °C with reciprocal shaking at 130 rpm.

**Extraction and Isolation of 4.** The cultured broth (1.8 L) was centrifuged at 3500 rpm for 30 minutes. The supernatant was extracted with MeOH. The extract was concentrated and the aqueous residue was extracted with EtOAc. The EtOAc extract (365 mg) was subjected to silica gel flash column chromatography with 50% EtOAc in hexane to give a crude fraction (62.7 mg) containing **4**. The fraction was purified by ODS flash column chromatography with 80% MeOH-H<sub>2</sub>O to give **4** (16.6 mg), which was identified as dimeric dinactin based on the spectral data.<sup>5</sup>

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