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GRISEOVIRIDIN AND CYCLIC HYDROXAMATES FOUND IN A SCREENING PROGRAM FOR WNT SIGNAL INHIBITOR

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Abstract – A screening study using a luciferase assay to identify natural products which inhibit Wnt signaling was carried out. Bioassay-guided fractionation of the culture broth and mycelia of actinomycetes CKK748 and CKK784, led to the isolation of griseoviridin (**1**), and four cyclic hydroxamates (**4-7**) as active compounds. **1**, **4**, and **5** inhibited TCF/β-catenin transcriptional activity with IC_{50} values of 8.2-14 μM.

The Wnt signaling pathway plays key roles in cell morphology, motility, proliferation, and differentiation. It is also reported to be involved in diabetes and Alzheimer's disease.[1] Furthermore, Wnt/β-catenin signaling can lead to the formation of tumors when aberrantly activated. Wnt target genes have recently been found to be highly expressed in colon cancers, and some are likely to contribute to the formation of cancers.[2] Small molecules which inhibit Wnt signaling may be therefore useful molecular tools for biological research and lead compounds for therapeutic drugs.

During a search for bioactive natural products from various unexplored natural resources,[3] we conducted a screening program targeting the inhibition of Wnt signaling. A number of plant extracts were examined using a cell-based luciferase assay system to evaluate the inhibition of TCF/β-catenin transcriptional activity (SuperTOP-Flash activity),[4,5] which was measured using the cell line STF/293 (a 293 human embryonic kidney cell line stably transfected with SuperTOP-Flash). With this assay system, we have isolated a series of naphthalene glycoside derivatives from an extract of *Eleutherine plamifolia* (Iridaceae).[6,7] We have also investigated bioactive metabolites of actinomycete strains, and isolated a rare phenazine,[8] its glycosides,[9] nonactin derivatives,[10] azaquinone-phenylhydrazone,[11] and naphthopyridazone alkaloid[12] from *Streptomyces* sp. We recently examined the active constituents of

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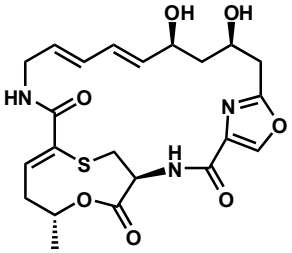
This paper is dedicated to Professor Ei-ichi Negishi for his 77 birthday

fermented broth and mycelia of the actinomycete strains CKK748 and CKK784 leading to the isolation of macrocyclic compounds, griseoviridin (**1**) and four cyclic hydroxamate compounds (**4**-**7**), as Wnt signaling inhibitors. Here, we describe the activity-guided isolation and identification of active compounds.

By using a cell-based luciferase assay system to evaluate the inhibition of TCF/β-catenin's transcriptional activity with the STF/293 cell line, we examined extracts of actinomycetes isolated and cultivated by our group and found that the methanol extracts of fermented broth and mycelia of strains CKK748 and CKK784 on a small scale exhibited marked activity (98% and 96% inhibition at 10 μg/mL with high [>90%] cell viability, respectively). Activity-guided fractionation of the culture broth and mycelia of actinomycete CKK748 led to the isolation of griseoviridin[13] (**1**) as the active compound, together with cyclo(4*R*-hydroxy-L-Leu-D-Pro)[14] (**2**) and cyclo(4*R*-hydroxy-L-Phe-D-Pro)[14] (**3**). From the culture broth and mycelia of actinomycete CKK784, activity-guided separation was carried out by silica gel, ODS column chromatography, and reversed phase HPLC, to afford four cyclic hydroxamate compounds; nocardamine[15] (**4**), dehydroxynocardamine[16] (**5**), desmethylenylnocardamine[16] (**6**), and bisucaberine[17] (**7**), as the active components in the supernatant, together with *N*-formylantimycic acid methy ester[18] (**8**) from mycelia.



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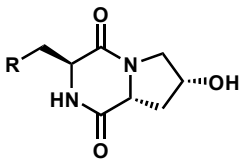
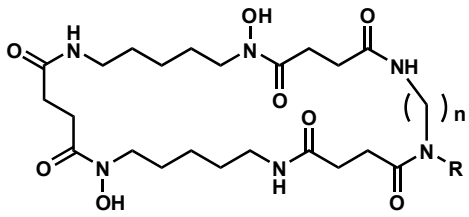
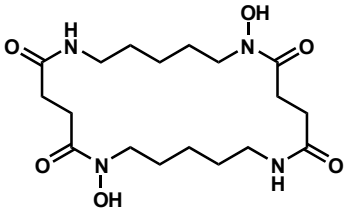


2: R = CHMe$_2$
3: R = Ph



4: R = OH, n = 5
5: R = H, n = 5
6: R = OH, n = 4



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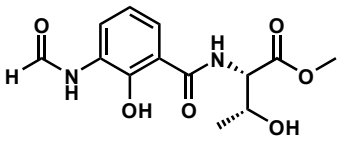

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Each of these compounds was identified on the basis of the NMR data and optical rotation data with reference to the literature. The effects of these compounds on Wnt signaling are not known.

The inhibitory activity of the isolated compounds was evaluated using the luciferase reporter gene assay. Wnt signaling activates gene transcription by forming a complex between DNA-binding proteins of the TCF/LEF family and β-catenin, and in this condition SuperTOP-Flash, a β-catenin-responsive reporter

plasmid with multiple TCF-binding sites (CCTTTGATC), is activated. A selective Wnt inhibitor would inhibit SuperTOP-Flash activity, but would not show any inhibition of transcription in SuperFOP-Flash-transfected cells because SuperFOP-Flash has eight mutated TCF-binding sites (CCTTTGGCC).

The results regarding the inhibition of TCF/β-catenin transcriptional activity (SuperTOP-Flash activity and SuperFOP-Flash activity) are shown in Figure 1 along with the cell viability since a decrease in cell number may contribute to the false positive inhibition of TCF/β-catenin transcriptional activity. Compounds **1**, **4**, and **5** exhibited inhibition of SuperTOP-Flash activity with IC_{50} values of 13, 14, and 8.2 μM, with high viability [>80%], and a small or moderate decrease in SuperFOP-Flash activity, while **6** did not show dose-dependent inhibition. **7** decreased the SuperTOP-Flash activity with an IC_{50} of 1.1 μM. However, **7** (0.4-2 μM) also decreased SuperFOP-Flash activity, implying that it did not selectively inhibit Wnt signaling at 0.4-2 μM. **8** (2-50 μM) did not show any inhibition of SuperTOP-Flash activity (data not shown).

We also examined the cytotoxicity of these compounds (**1** and **4**-**7**) against human colorectal cancer cells, DLD1, HCT116, and SW480 cells, in which Wnt signal pathway is known to be activated,[19] together with 293T cells as a non-cancer cell line. **1** showed 48-62 % viability against DLD1, versus more than 60% viability against HCT 116, SW480, and 293T, at 6.3-50 μM. From these results, **1** was weakly toxic to DLD1, but not HCT 116, SW480, or 293T.

Compounds **4**-**6** did not show cytotoxicity against DLD1, HCT116, SW480, or 293T cells (viability was more than 60% at 0.8-20 μM). **7** was toxic to 293T cells, with an IC_{50} of 14 μM, but not to DLD1, HCT116, and SW480. Although the compounds (**1** and **4**-**7**) inhibited TCF/β-catenin transcriptional activity, they did not show significant cytotoxicity against colorectal cancer cell lines. It was reported that a cyclic siderophore, nocardamine (**4**), did not show cytotoxicity against human breast cancer and malignant melanoma cell lines, but inhibited the formation of colonies by these cells.[20]

In our search for bioactive natural compounds in Actinomycetes, we isolated five macrocyclic compounds as Wnt inhibitors. Although griseoviridin (**1**) and cyclic hydroxamate compounds (**4**-**7**) have been known as a broad spectrum antibiotic and siderophore, respectively, their effect on Wnt signaling has not been reported. In this paper we found that **1**, **4**, and **5** inhibit Wnt signal activity. Thus, these compounds may warrant further study as potential Wnt signaling inhibitors.

EXPERIMENTAL

Microbial strain and Fermentation. CKK748 was separated on humic acid-vitamin (HV) agar,[21] a medium for the selective isolation of actinomycetes, from a sea water sample collected in Kujukuri-Hama in Chiba, Japan in October 2009, as described in the literature.[22] Briefly, the sea water sample was

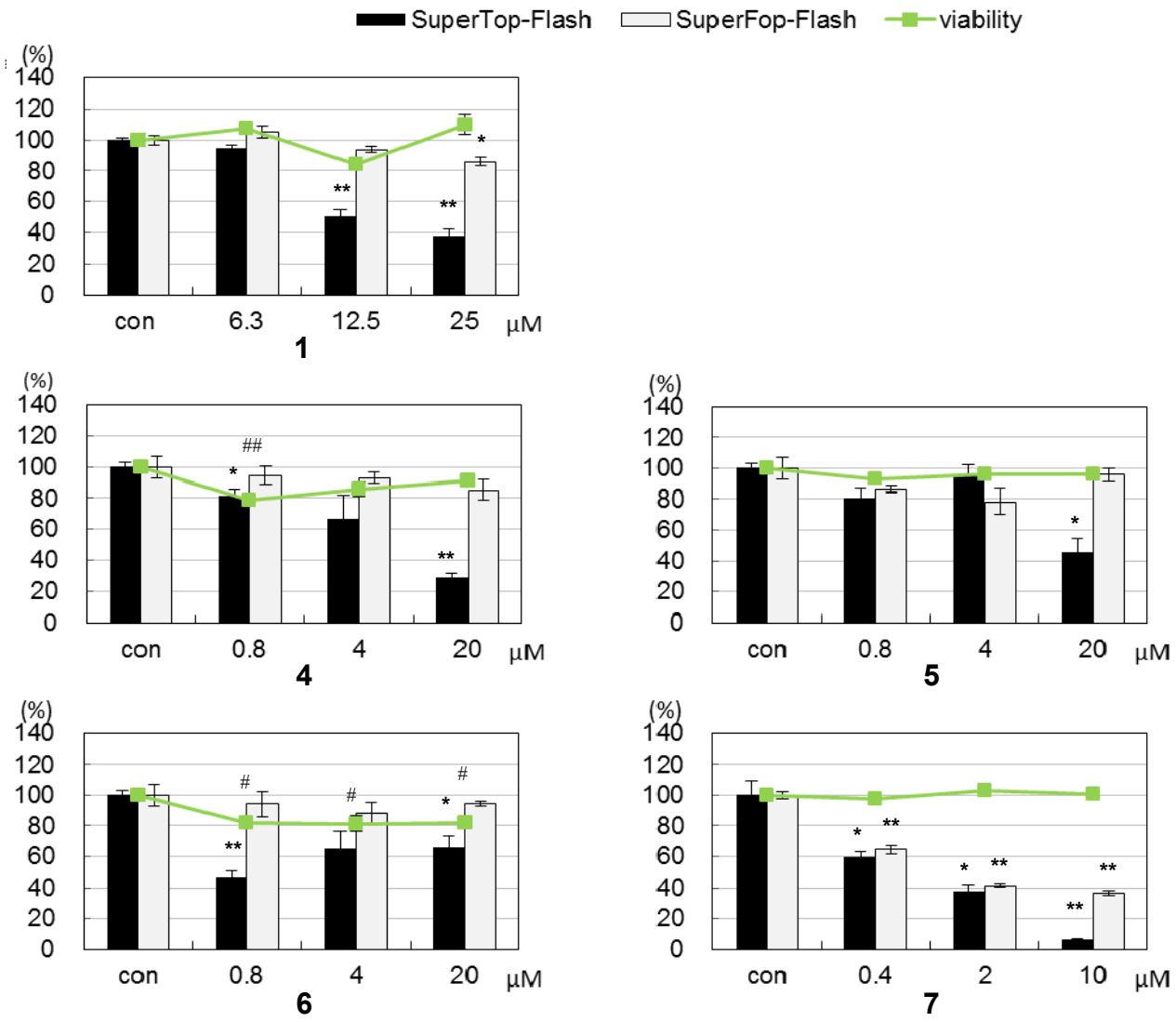

Figure 1. Effects of **1** and **4-7** on SuperTOP-Flash and SuperFOP-Flash activity in STF/293 and 293T cells determined by the luciferase assay, and viability in STF/293 cells determined by the FMCA method. The data are shown as the mean ± SE. The significance of differences was determined with Student's 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 a positive control, with 28.8% TopFlash activity and 93.9% viability at 27.7 μM.

filtrated using a nitrocellulose membrane filter (pore size 0.22μM, GSWP, Millipore), and the filter was overlaid on a plate of HV agar. After three or four days' incubation, the filter was removed from the agar and the plate was reincubated until growth was observed. CKK784 was separated on humic acid-vitamin (HV) agar, from a soil sample collected at Chiba City, Japan in August 2010, as described previously.[23] Spores of these strains kept in a freezer in a 15% glycerol solution, and grown on solid

Waksman medium containing agar for 2-3 days were transferred to a flask (500 mL) and inoculated into 100 mL of a Waksman medium consisting of glucose (2 g/100 mL), meat extract (0.5 g/100 mL), peptone (0.5 g/100 mL), dried yeast (0.3 g/100 mL), NaCl (0.5 g/100 mL), and $CaCO_3$ (0.3 g/100 mL), and the mixture cultured at 28 °C for 3-5 days on a reciprocating shaker. Then the seed culture (20 mL) was transferred into a flask (3 L) containing 700 mL of the Waksman medium and cultured at 28 °C for 4-7 days on the reciprocating shaker.

Extraction and Isolation.

The culture broth (22.4 L in total) of CKK748 was harvested and centrifuged (6,000 rpm, 15 min.) to separate the mycelia and supernatant. The mycelium was homogenized in MeOH. The MeOH extract was filtrated and concentrated in vacuo and then partitioned with EtOAc (250 mL x 3) to give the EtOAc-soluble fraction (1.2 g). The supernatant was concentrated under reduced pressure to approximately 500 mL and partitioned between EtOAc (500 mL x 3) to give the EtOAc-soluble fraction (5.6 g). The combined EtOAc-soluble fractions of the supernatant and mycelia were subjected to silica gel column chromatography (φ43 x 340 mm) eluted stepwise ($CHCl_3$:MeOH = 1:0, 98:2, 95:5, 93:7, 90:10, 85:15, 80:20, 70:30, 50:50, 0:1) to give ten fractions, 1A to 1J. Fraction 1F (284 mg) eluted with $CHCl_3$:MeOH = 90:10-85:15 was separated by preparative HPLC (CAPCELL PACK C18 MG-2, 10 x 250 mm; eluent, 50% MeOH; flow rate, 1.0 mL/min; UV detection at 254 nm) to give griseoviridin (**1**, 56 mg, t_R 33 min) and a crude fraction (14 mg, t_R 18-22 min). The crude fraction was further purified by preparative HPLC (CAPCELL PACK C18 MG-2, 10 x 250 mm; eluent, 46% MeOH; flow rate, 1.0 mL/min; RI detection) to give cyclo(4*R*-hydroxy-L-Leu-D-Pro) (**2**, 5.6 mg, t_R 23.1 min) and cyclo(4*R*-hydroxy-L-Phe-D-Pro) (**3**, 3.0 mg, t_R 25.5 min).

The culture broth (11.2 L in total) of CKK784 was harvested and centrifuged (6,000 rpm, 15 min.) to separate the mycelia and supernatant. The supernatant was concentrated under reduced pressure to approximately 500 mL and partitioned between EtOAc (500 mL x 3) to give the EtOAc-soluble fraction (9.3 g). This fraction was subjected to silica gel column chromatography (φ36 x 360 mm) eluted stepwise ($CHCl_3$:MeOH = 1:0, 95:5, 90:10, 85:15, 80:20, 70:30, 50:50, 0:1) to give seven fractions, 1A to 1G. Fractions 1E and 1F were combined (1140 mg), eluted with $CHCl_3$:MeOH = 85:15-70:30, and separated by ODS column chromatography (φ36 x 360 mm, MeOH:H_2O = 3:7, 5:5, 7:3, 9:1 1:0) to give six fractions, 2A to 2F. Fraction 2C (256 mg) eluted with MeOH:H_2O = 5:5-7:3 was separated by preparative HPLC (Develosil C30 UG-5, 10 x 250 mm; eluent, 50% MeOH; flow rate, 1.0 mL/min; RI detection) to give nocardamine (**4**, 18.8 mg, t_R 33.3 min) and a crude fraction (61.4 mg, t_R 24-30 min). The crude fraction was further purified by preparative HPLC (Develosil ODS MG-5, 10 x 250 mm; eluent, 40% MeOH; flow rate, 1.0 mL/min; RI detection) to give dehydroxynocardamine (**5**, 13.6 mg, t_R 65.0

min), desmethylenylnocardamine (**6**, 12.4 mg, t_R 59.5 min), and bisucaberine (**7**, 1.4 mg, t_R 41.5 min). The culture broth (33.6 L in total) of CKK784 was harvested and centrifuged (6,000 rpm, 15 min.) to separate the mycelia and supernatant. The mycelia were homogenized in MeOH. The MeOH extract was filtrated and concentrated in vacuo and then partitioned with EtOAc (500 mL x 7) to give the EtOAc-soluble fraction (5.9 g). This fraction was subjected to ODS column chromatography (φ40 x 300 mm) eluted stepwise (MeOH: H_2O = 2:8, 4:6, 5:5, 6:4, 7:3, 9:1, and 1:0) to give seven fractions, 3A to 3G. Fraction 3C (100.1 mg) eluted with MeOH: H_2O = 5:5 was separated by preparative HPLC (COSMOSIL 5C18-AR-II, 10 x 250 mm; eluent, 45% MeOH; flow rate, 1.0 mL/min; UV detection at 254 nm) to give *N*-formylantimycic acid methy ester (**8**, 5.7 mg, t_R 39 min).

Luciferase assay.

Stable reporter cells, STF/293 cells (3 x 10^4) were split into 96-well plates and 24 h later treated with compounds and LiCl (final conc. 15 mM). After incubation for 24 h, cells were lysed with CCLR (20 μL/well cell culture lysis reagent; 20 μL/well, Promega) and luciferase activity was measured with a Luciferase 1000 Assay System (Promega). Assays were performed in triplicate at least. Transient transfection was performed using Lipofectamine 2000 (Invitrogen, USA). 293T cells (1×10^5) were split into 24-well plates. After 24 h, cells were transfected with 1 μg of the luciferase reporter construct (SuperFOP-Flash) and 0.05 μg of pRL-CMV (Promega, USA) for normalization. At 3 h posttransfection, compounds were added with 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 μL/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 indicated concentrations with medium. The final concentration of DMSO was less than 0.1% (v/v). The data are shown as the mean ± SE. The significance of difference was determined with Student's *t*-test (*: $p< 0.05$, **: $p< 0.01$ vs. control). The IC_{50} was estimated from graphical interpolation.

Viability Assay. (FMCA assay[24])

293T cells (6 x 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 μg/mL), and after 1 h of incubation, fluorescence was detected. Assays were performed in triplicate at least. The sample was prepared as mentioned above.

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