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New Cdc25B Tyrosine Phosphatase Inhibitors, Nocardiones A and B, Produced by *Nocardia* sp. TP-A0248: Taxonomy, Fermentation, Isolation, Structural Elucidation and Biological Properties

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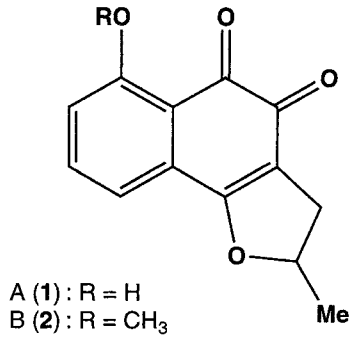
Strain TP-A0248 which produces two new Cdc25B tyrosine phosphatase inhibitors and also possessing antifungal activity, designated nocardiones A (**1**) and B (**2**), was considered to belong to the genus *Nocardia* on the basis of literature comparison of chemotaxonomic properties. The nocardiones were isolated by solvent extraction of fermentation broth of *Nocardia* sp. TP-A0248 and purified by the conventional column chromatography. Spectroscopic studies led to determination that **1** and **2** belong to a class compound of naphtho[*1,2*-b]furan-4,5-diones. Compound **1** inhibited the activity of Cdc25B, PTP1B and FAP-1 protein tyrosine phosphatases at a concentration of 10 μM. It also showed moderate *in vitro* antifungal and cytotoxic activity.

Recent studies have demonstrated that protein tyrosine phosphatases (PTPase) and dual specific phosphatases (DSPase) are key enzymes in the signal transduction pathway for a wide range of cellular processes[1]. Cdc25 is known to be DSPase involved in both cell cycle regulation and response to the stimulation of growth factors[2]. Three Cdc25 homologs (Cdc25A, B and C) from human cells have been cloned by complementation of a temperature-sensitive Cdc25 mutant of *Schizosaccharomyces pombe*. Among them Cdc25B was found to dephosphorylate human $P34^{cdc2}$ kinase on Thr-14 and Tyr-15[3]. Cdc25B is the most highly expressed, and its elevated expression is observed particularly in some cancer cells[4~6]. In addition, it is known that most antitumor drugs developed previously showed cell cycle-inhibition activity on tumor cells. Based upon these observations, compounds which inhibit the function of Cdc25 are considered to show inhibitory effects on the growth of tumor cells. Therefore, we have used Cdc25B phosphatase as a target for the screening of cell cycle inhibitors from microbial metabolites to develop antitumor agents which inhibit specific targets in the cell cycle control.

During the screening of microbial products for Cdc25B phosphatase inhibitors, we found that a strain TP-A0248 produced the two new antifungal antibiotics, hereafter termed nocardiones A (**1**) and B (**2**). As shown in Fig. 1, NMR and MS spectroscopic analyses suggested that these components belong to a class of a tricyclic polyketide *ortho*-quinone antibiotics. In this paper, we describe the taxonomic characteristics of the producing strain, isolation, fermentation, structural elucidation of **1** and **2** and biological properties of **1**.

Fig. 1. Structure of nocardiones A (**1**) and B (**2**).



Materials and Methods

Microorganism

The producing strain TP-A0248 was isolated from a soil sample collected in Kosugi-machi, Toyama Prefecture, Japan and has been deposited in the National Institute of Bioscience and Human-Technology, Agency on Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan under accesion number FERM-BP 6372.

Taxonomy

The characteristics of strain TP-A0248 were determined during cultivation on various media recommended by SHIRLING and GOTTLIEB[7], WAKSMAN[8], and ARAI[9]. The color names and hue numbers were assigned by Manual of Color (Japanese Color Enterprise Co., Ltd. 1987). Temperature range for growth was determined using a temperature gradient incubator TN-2148 (Advantec Toyo Co.,). The carbon utilization was determined according to the procedure of PRIDHAM and GOTTLIEB[10]. Cell wall composition was analyzed by the method of LECHEVALIER *et al.*[11] using TLC as described by STANECK *et al.*[12]. The whole-cell sugar pattern was analyzed according to the method of BECKER *et al.*[13]. Mycolic acid was prepared and detected by TLC according to the method of MINNIKIN *et al.*[14]. The acyl type of muramic acid was analyzed by the glycolate test as described by UCHIDA *et al.*[15]. Menaquinones were extracted and purified by the method of COLLINS *et al.*[16], and then isoprene units were analyzed by HPLC using a Shim-pack ODS column (Shimadzu) with MeOH/*iso*-PrOH (3 : 2) as a mobile phase. Phospholipid and fatty acid compositions were determined by the method of LECHEVALIER *et al.*[17] and MINNIKIN *et al.*[18], respectively.

Experimental

Melting points were determined on a YANACO MP J-3 apparatus and uncorrected. UV and IR spectra were recorded on a BECKMAN DU 640 and a SHIMADZU FT IR-300 spectrometer, respectively. The MS spectra were measured on a JEOL JMS-HX110A spectrometer using 3-nitrobenzyl alcohol as a matrix. All NMR experiments were performed on a JEOL JMN-LA400 NMR spectrometer in $CDCl_3$ with TMS as an internal standard. Optical rotations were measured on a HORIBA SEPA-300 polarimeter.

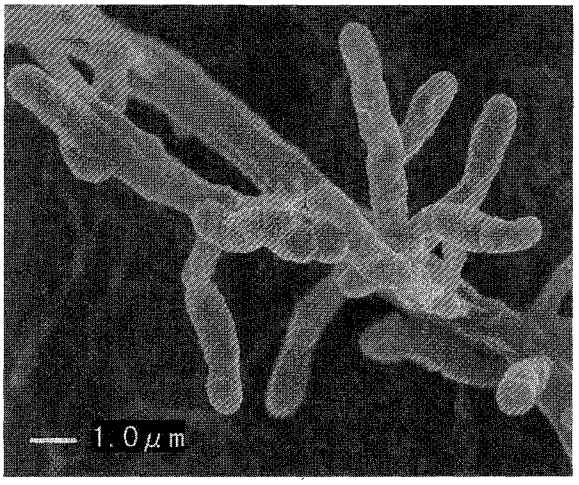
Biological Activities

The respective inhibitory activity against Cdc25B, PTP1B and FAP-1 protein tyrosine phosphatases was determined as follows: for Cdc25B phosphatase, the test samples were diluted with 10 mM HEPES buffer (pH 8.0) containing 50 mM NaCl and 5 mM DTT (50 μl), and then 200 mM *p*-nitrophenylphosphate hexahydrate as a substrate (25 μl) and Cdc25B protein tyrosine phosphatase (200 mU/ml, 25 μl) were added. After incubation at 37°C for 60 minutes, the inhibitory activity was colorimetrically determined at 415 nm using a microplate reader. The effect of phosphatase inhibitor was determined by comparing the absorbance of enzyme reaction mixture with phosphatase inhibitor. For the other enzyme assay, GST-PTP1B catalytic domain fusion protein (Upstate Biotechnology) and 25 mM imidazole (pH 8.0) containing 50 mM NaCl, 2.5 mM EDTA and 5 mM DTT, and FAP-1 catalytic domain protein (Bio Signal) and 10 mM HEPES buffer (pH 8.0) containing 50 mM NaCl, 0.1 mM $CaCO_3$, 5 mM DTT and 0.1% BSA were used, respectively.

The MIC value as *in vitro* antifungal activity was determined by a serial 2-fold agar dilution method using Sabouraud's agar (Nissui Seiyaku) with a 36-hours incubation at 30°C.

Cytotoxic activity against HeLa human cervical carcinoma and SBC-5 human non-small cell lung carcinoma cells was determined as follows: cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum at 37°C overnight in a humidified CO_2 incubator. The exponentially growing cells were harvested and suspended in the culture medium at 1×10^3 cells/100 μl/well. The cells obtained were plated into 96-well tissue culture plates with the test materials (100 μl) and then incubated for 72 hours. The cytotoxic activity was colorimetrically determined at 540 nm with a microplate reader after staining viable cells with crystal violet solution. The IC_{50} value was calculated using Probit's method.

Fig. 2. The scanning electron micrograph of strain TP-A0248.



Results

Taxonomy of Producing Strain TP-A0248

A scanning electron micrograph of spores of strain TP-A0248 is shown in Fig. 2. The spores were oval in shape, 0.5~0.6×1.0~1.2 μm in size, with a smooth surface. The cultural characteristics and physiological properties of strain TP-A0248 are summarized in Tables 1 and 2. This strain grew relatively well on various media. Aerial mass color was beige white to pale beige. Vegetative growth was light orange to pale grayish-brown. Melanoid pigment formation and nitrate reduction gave a positive reaction. D-Glucose and D-fructose were utilized for growth by strain TP-A0248. Whirl, sclerotic granules, sporangia, and flagellated spores were not observed.

Whole-cell hydrolysate of strain TP-A0248 demonstrated the presence of *meso*-diaminopimelic acid, arabinose and galactose. Therefore, the cell wall type was IV and sugar pattern was type A, according to the classification of LECHEVARIER *et al.*[19]. Analysis of whole-cell phospholipids revealed the presence of phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylinositol, belonging to type-PII. This strain had mycolic acid. MK-8(H_4) 86.8% and MK-8(H_2) 13.1% were detected as the major menaquinones. The glycolyl type of muramic acid was observed in the cell wall. Fatty acids consisted of hexadecanoic acid (16:0) 20%, 14-methylhexadecanoic acid (*iso*-16) 16%, and heptadecanoic acid (17:0) 15% as well as other minor components.

Based on literature comparison of chemotaxonomic properties, strain TP-A0248 is considered to belong to the genus *Nocardia*. However, it should be noted that under a light microscope this strain did not form a typical fragmentation of substrate mycelium nomally observed in this genus[20].

Fermentation

A loopful of strain TP-A0248 was inoculated into 500-ml K-1 flask containing 100-ml of a seed medium composed of soluble starch 1.0%, glucose 0.5%, NZ-case (Humco Scheffied Chemical) 0.3%, yeast extract (Difco) 0.2%, tryptone (Difco) 0.5%, K_2HPO_4 0.4%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0 before sterilization). After incubation at 30°C for 4 days on a rotary shaker at 200 rpm, five mililiter aliquots of this seed culture were transfered into 500-ml K-1 flasks each containing 100 ml of a producing medium consisting of glucose 0.5%, glycerol 2.0%, soluble starch 2.0%, Pharmamedia (Trader's Protein) 1.5%, yeast extract 0.3%, and Diaion HP-20 (Mitsubishi Chemical) 1.0% (pH 7.0 before sterilization). The fermentation was carried out at 30°C for 6 days on the same rotary shaker. Progress of the fermentation was monitored by a paper-disk diffusion method and by bioautography using *Candida albicans* 3147 as the test organism and chromatography using Silica gel 60 F_{254} TLC plate (Merck No.5175) developed with chloroform or chloroform/ethyl acetate (10:1). When Diaion HP-20 resin was not present in the production medium, strain TP-A0248 showed no productivity of **1** and **2** at any detectable level.

Isolation and Purification Procedure

Nocardione complex was isolated from fermentation broth by a solvent-extraction procedure. The harvested broth (4.5 liters, pH 6.5) was filtered and the inhibitory activity was found in both mycelial cake and broth filtrate. The mycelial cake was extracted twice with methanol. The extracts were combined and evaporated *in vacuo* to give an aqueous concentrates which was extracted with ethyl acetate at pH 4. The broth filtrate was also extracted twice with ethyl acetate. The two resulting extracts were combined and concentrated *in vacuo* to give a crude oily residue (4.83 g). The crude complex was applied to a silica gel column eluting with chloroform - methanol (10:1). The active fractions obtained were combined and concentrated *in vacuo* to give a reddish brown residue (160 mg). The crude residue containing **1** and **2** was further purified by preparative reverse-phase HPLC with Inertsil ODS-3

Table 1. Cultural characteristics of strain TP-A0248.

Medium	Growth	Aerial mycelium	Soluble pigment
Yeast extract-starch agar	Light orange [65]	Light orange [62]	Absent
Tyrosine agar (Waksman med. No.42)	Dark reddish-brown [102]	Pale reddish-yellow [130]	Dark brown [103]
Glycerol-asparagine agar (ISP med. No.5)	Dark grayish-brown [121]	Light gray [397]	Grayish-brown [112]
Inorganic salts-starch agar (ISP med. No.4)	White [388]	White [388]	Absent
Tyrosine agar (ISP med. No.7)	Grayish-brown [118]	Pale beige [86]	Yellowish-brown [97]
Oat meal agar (ISP med. No.3)	Light orange [64]	Beige white [392]	Absent
Yeast extract-malt extract agar (ISP med. No.2)	Vivid orange [71]	Yellowish-pink [14]	Soft yellow [147]
SCM agar	Pale yellowish-pink [7]	White [388]	Absent
Bennet' agar (Waksman med. No.30)	Soft orange [83]	Pale beige [86]	Absent

Figure in parentheses shows color code number in New Color Dictionary (1987)

Table 2. Physiological characteristics of strain TP-A0248.

Tempereture range for growth (YS agar)		17～45℃
Optical temperature		23～40℃
Formation of melanoid pigment (ISP No.7 med.)		Positive
Liquefaction of gelatin		Negative
Coagulation of milk (30℃)		Negative
Peptonization of milk (30℃)		Negative
Hydrolysis of starch (ISP No.4 med.)		Negative
Reduction of nitrate (ISP No.8 med.)		Positive
Decomposition of cellulose		Negative
Optimum pH for growth		6～8
Growth in NaCl (YS agar)		～4 %
Utilization of carbon sources	Positive	D-Glucose, D-Fructose, Glycerol
	Negative	L-Arabinose, D-Xylose, Sucrose, Inositol, Raffinose, Raffinose, D-Mannitol,

Table 3. Physico-chemical properties of nocardiones A (**1**) and B (**2**).

	Nocardione A (**1**)	Nocardione B (**2**)
Appearance	Reddish brown powder	Reddish brown powder
Melting point	115~120°C	79~81°C
$[\alpha]_D^{26}$	-85.4 (c=1.0, $CHCl_3$)	-29.8 (c=0.32, $CHCl_3$)
HR-EIMS found:	230.0570 $(M)^+$	244.0737 $(M)^+$
calcd:	230.0579 (for $C_{13}H_{10}O_4$)	244.0735 (for $C_{14}H_{13}O_4$)
Molecular formula	$C_{13}H_{10}O_4$	$C_{14}H_{13}O_4$
UV λ_{max} nm (ε)		
in MeOH	203 (16,400), 237 (15,100), 259 (18,000), 416 (4,900)	201 (20,300), 230 (15,900), 258 (13,200), 395 (3,700)
in 0.01N HCl-MeOH (1:9)	237 (16,000), 259 (18,300), 415 (5,000)	233 (13,600), 258 (13,500), 396 (4,000)
in 0.01N NaOH-MeOH (1:9)	205 (15,500), 238 (16,300), 259 (14,400), 474 (4,900)	207 (8,300), 231 (11,900), 259 (11,300), 396 (3,000)
IR ν_{max} (cm^{-1})	3400, 1640, 1620, 1580, 1450, 1410, 1320, 1270, 1030	1650, 1620, 1580, 1465, 1300, 1270, 1030
Solubility		
soluble in	$CHCl_3$, acetone	$CHCl_3$, acetone
insoluble in	*n*-hexane, water	*n*-hexane, water
TLC (Rf)[a]	0.25	0.08
	0.42[b]	0.11[b]
HPLC (Rt)[c]	5.9 min	6.3 min

[a] Silica gel TLC (Merck Art 5715): ($CHCl_3$), [b] ($CHCl_3$-EtOAc=10:1)
[c] HPLC conditions: Column: Inertsil ODS-2 (250 x 4.6 mm, i.d.), Mobile phase: CH_3CN-0.05% TFA (60:40), Flow rate: 0.8 ml/min, Detection: UV-230 nm.

column (250×10 mm, i.d., GL Sciences) with CH_3CN/0.05 % TFA (60 : 40) for **1** and (40 : 60) for **2** at flow rates of 4 ml/minute. The compounds were detected at 230 nm. The eluted peaks showing inhibitory activity were collected and after evaporation lyophilized to yield **1** (8 mg) and **2** (0.3 mg). Separation by analytical HPLC using an Inertsil ODS-2 column (250×4.6 mm, i.d.) and elution with CH_3CN/0.05% TFA (60 : 40) for **1** and (40 : 60) for **2** at the flow rate of 0.8 ml/minute gave the following retention time; A: 5.9 min utes B: 6.3 minutes.

Physico-chemical Properties

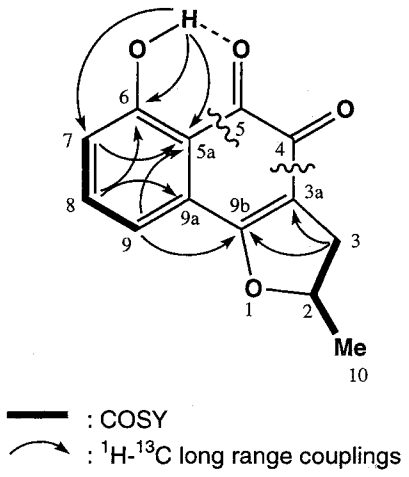
The physico-chemical properties of **1** and **2** are summarized in Table 3. **1** and **2** were both obtained as reddish-brown powders with melting point of 117~119°C and 79~80°C, respectively. They were soluble in chloroform and ethyl acetate, but insoluble in *n*-hexane and water, and optically active with $[\alpha]_D^{26}$ value of −85.4 (c=1.0, $CHCl_3$) for **1** and −29.8 (c=0.32, $CHCl_3$) for **2**. Molecular formulae of **1** and **2** were determined as $C_{13}H_{10}O_4$ and $C_{14}H_{13}O_4$, respectively, based on the molecular ion peaks observed at *m/z* 230 and *m/z* 244 in EI-MS and ^1H and ^{13}C NMR spectra. In the UV spectrum of **1**, the absorption maximum at 416 nm was shifted to 474 nm in alkaline-methanolic solution, showing the presence of a phenolic hydroxyl function, whereas no similar shift was observed in the spectrum of **2**. In the IR spectra, both compounds showed two absorption bands between 1620 and 1650 cm^{-1} due to the streching of the carbonyl group and **1** exhibited a band at 3400 cm^{-1} due to the hydroxyl group.

Structure Determination

The ^1H and ^{13}C NMR spectra of **1** showed 8 proton and 13 carbon signals, respectively, as summarized in Table 4. The DEPT spectra indicated the presence of four methines, one methylene, one methyl group and seven quaternary carbons. The connectivity of proton and carbon atoms was confirmed by HMQC data. The proton at 11.95 ppm was exchangeable. The ^1H-^1H COSY spectrum and spin decoupling experiments revealed two separate proton spin systems: H-3/H-2/H-10 and H-7/H-8/H-9. ^1H-^{13}C long range couplings observed in the HMBC spectrum are shown in Fig. 3. The phenolic hydroxyl proton at 11.95

ppm, which is hydrogen bonded to a quinone carbonyl oxygen at the *peri* position, was long range coupled to aromatic carbons C-5a (113.99 ppm), C-6 (164.96 ppm), and C-7 (123.77 ppm). The position of the phenolic hydroxyl group was deduced to be C-6 from the ^{13}C chemical shift. Long range couplings between aromatic protons and carbons were also observed from H-7 to C-5a, H-8 to C-6 and C-9a and H-9 to C-5a. These NMR data led to the identification of a 2,3-disubstituted phenol substructure. Another substructure was determined as follows. Methylene protons H-3 were long range coupled to sp^2 carbons C-3a and C-9b. ^{13}C chemical shifts of C-2 (85.04 ppm) and C-9b (169.58 ppm) indicated their attachment to oxygen atoms. Taking into account the molecular formula, the presence of a dihydrofuran substructure was revealed. The connnectivity of two substructures between C-9a and C-9b was confirmed by the long range coupling from H-9 to C-9b. The remaining two sp^2 carbons C-4 (175.53 ppm) and C-5 (185.93 ppm) were deduced to be carbonyl carbons from their chemical shifts and the molecular formula. Thus, the gross structure of **1** was detemined as shown in Fig. 1.

Fig. 3. NMR analysis of nocardione A (**1**).



In the ^{1}H NMR specrum of **2**, a singlet signal due to a methyl group at 3.98 ppm appeared instead of a phenolic proton. The ^{13}C NMR spectrum of **2** showed a new signal at 56.24 ppm. The long range coupling from the methyl protone to C-6 revealed the methylation of the phenolic residue. Thus by combining with the molecula formula, component **2** was determined to be the *O*-methyl analog of **1**, as shown in Fig. 1. The positions of carbonyl carbons were assigned as shown in Table 4, since the chemical shift of hydrogen bonded-carbonyl carbon C-5 of **1** was shifted upfield from 185.93 ppm to 180.24 ppm in **2** in which the hydrogen bonding was disconnected. The absolute configuration of nocardiones is unknown.

Biological Activity

The antifungal activity of **1** is presented in Table 5. **1** exhibited moderate activity with MIC values at the range

Table 4. ^{13}C- and ^{1}H-NMR data for nocardiones A (**1**) and B (**2**).

Position	Nocardione A ^{13}C		Nocardione A ^{1}H	Nocardione B ^{13}C		Nocardione B ^{1}H
2	85.04	d	5.25 (1H, m)	84.12	d	5.22 (1H, m)
3	34.04	t	2.73 (1H, dd, J =15.6, 7.3Hz) 3.27 (1H, dd, J =15.6, 9.8Hz)	33.51	t	2.71 (1H, dd, J =15.2, 7.3Hz) 3.25 (1H, dd, J =15.2, 9.6Hz)
3a	115.67	s		114.34	s	
4	175.53	s		175.54	s	
5	185.93	s		180.24	s	
5a	113.99	s		114.64	s	
6	164.96	s		161.80	s	
6-OH			11.95 (1H, s)			
6-OCH3				56.24	q	3.98 (3H, s)
7	123.77	d	7.13 (1H, d, J =8.8Hz)	117.26	d	7.27 (1H, d, J =8.3Hz)
8	138.03	d	7.54 (1H, dd, J =8.8, 7.3Hz)	135.79	t	7.58 (1H, t, J =8.3Hz)
9	117.98	d	7.2 (1H, d, J =7.3Hz)	116.70	d	7.17 (1H, d, J =8.5Hz)
9a	128.00	s		129.54	s	
9b	169.58	s		169.29	s	
10	22.45	q	1.57 (3H, d, J =6.4HZ)	21.92	q	1.56 (3H, d, J =6.3HZ)

^{1}H and ^{13}C NMR spectra were recorded at 400MHz and 100MHz, respectively, in $CDCl_3$ solution.
TMS was used as an internal standard.

Table 5. Antifungal spectrum of nocardione A (**1**).

Test organism	MIC(μg/ml)
Candida albicans IFO-1060	6.25
C. albicans IFO-1061	6.25
C. albicans ATCC 38247	6.25
C. albicans A-9540	6.25
Cryptococcus neoformans	1.56
Saccharomyces cerevisiae NR5174 S-100	12.5
Kluyveromyces fragilis ATCC 8635	6.25
Aspergillus niger	6.25
Aspergillus fumigatus	3.12
Trichophytone mentagrophytes	3.12

Minimum inhibitory concentration (MIC) was determined by the serial agar dilution method on Sabouraud's agar after incubation for 18 hours against yeast and for 36 hours against fungi at 30°C

of 6.25 to 12.5 μg/ml against yeasts and fungi tested. The cytotoxic activities (IC_{50}) against HeLa and SBC-5 cells were 0.38 and 0.54 μM, respectively. The inhibitory activity (IC_{50}) of **1** against Cdc25B, PTP1B and FAP-1 protein tyrosine phosphatases showed 17, 14 and 89 μM, respectively. In addition, we observed that **1**, at concentrations of less than 6.25 μg/ml, induces cell death with characteristics of apoptosis in U937 human myeloid leukemia cell lines in RPMI 1640 medium.

Discussion

Nocardiones possess a naphtho[*1,2*-b]furan-4,5-dione as a basic skelton. Few natural products which belong to this class have been reported so far. Dunnione and related compounds have been isolated from the leaves of the ornamental herb *Streptocarpus dunnii*[21,22] and the horticultural plant *Calceolaria integrifolia*[23]. The inhibition of topoisomerase II by dunnione has been reported recently[24]. Trypethelones have been isolated from cultures of the mycosymbiont of the tropical cortical lichen *Trypethelium eluteriae* with moderate antibacterial activity against *Bacillus subtilis*[25]. This is the first report to describe the DSPase inhibitory activity of the related compounds.

Sodium *ortho*-vanadate[26] is a well-known and useful inhibitor for investigation of the role of PTPase in the eukaryotic cells. Therefore, its inhibitory activity was examined in our assay system. The respective IC_{50} values of sodium *ortho*-vanadate against Cdc25B and PTP1B phosphatases were 8.6 and 6.2 μM. As microbial secondary metabolites, only a few PTPase or DSPase inhibitors such as danacin[27], dephostatin[28], and RK-682[29] are known. Danacins A1 and B1, benzoquinoid antitumor antibiotics from *Actinosynnena pretiosum*, have been reported to inhibit Cdc25B phosphatase activity in a non-competitive manner at a concentration of 141 μM and 64 μM, respectively. RK-632, which was produced by *Streptomyces* sp. and consisted of a tetronic acid and a saturated fatty acid moiety, has been reported to inhibit the dephosphorylation activity of CD45 and VHR, but it did not inhibit the phosphatase activity of Cdc25B. Dephostatin from the culture broth of *Streptomyces* sp. inhibited PTPase with the IC_{50} value of 7.7 μM. Very recently, KAGAMIZONO *et al.*[30] have reported that phosphatoquinone A and B from *Streptomyces* sp. inhibited PTPase with the IC_{50} of 28 and 2.9 μM, respectively.

Human Cdc25 proteins seem to play important roles in all proliferating cells. Unlike Cdc25B, Cdc25A phosphatase was shown to function at the start of the cell cycle. However, the relationship between inhibition of phosphatase and antitumor activity of these compounds remain to be clarified. Accordingly, we are examining whether they inhibit Cdc25 homologs and arrest the cell cycle or not.

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