Phosphatoquinones A and B, Novel Tyrosine Phosphatase Inhibitors Produced by *Streptomyces* sp.

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Phosphatoquinones A and B were isolated from the cultured broth of *Streptomyces* sp. TA-0363 and their structures were elucidated by spectroscopic analyses. Phosphatoquinones A and B inhibited the protein tyrosine phosphatase activity prepared from human Ball-1 cells with IC_{50} of 28 μM and 2.9 μM, respectively.

Protein tyrosine phosphatases (PTPase) and tyrosine kinases are involved in the regulation of the signal transduction in mammalian cells. In fact, recent findings indicate that a transmembrane PTPase, CD45[1,2] is required for induction of early tyrosine phosphorylation of intracellular proteins $p59^{fyn}$ or $p56^{lck}$ in T-cell activation[3,4]. On the other hand, a protein-serine/threonine kinase, $p34^{cdc2}$, which controls the cell cycle transition from G2 to M phase, is activated by CDC25-catalyzed dephosphorylation of the tyrosine residue in its ATP-binding site[5].

Therefore, we have screened PTPase inhibitors from microbial metabolites by an assay *in vitro* using a CD45-enriched membrane fraction of Ball-1 cells[6]. As a result, we found that a strain TA-0363 produced new PTPase inhibitors named phosphatoquinones A (**1**) and B (**2**). Herein, the isolation, structure elucidation and biological activities of (**1**) and (**2**) are described.

Taxonomy

The vegetative mycelium was well developed. The aerial mycelium was moderately short with simple branches and formed flexuous spore chains with 10~20 spores per chain. The spores were oval in shape (0.7~0.9×0.9~1.1 μm) with a warty surface. No sporangia, motile spores or fragmentation of mycelium were observed. The whole-cell hydrolysate contained LL-diaminopimelic acid. The predominant menaquinones were MK-9(H_6) and MK-9(H_8).

The cultural and physiological characteristics of strain TA-0363 are shown in Table 1 and 2, respectively. Based on these characteristics described above, strain TA-0363 belongs to the genus *Streptomyces*. Strain TA-0363 has been deposited in the National Institute of Biosciense and Human-Technology, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-16331.

Fig. 1. Structures of phosphatoquinone A (**1**) and B (**2**).

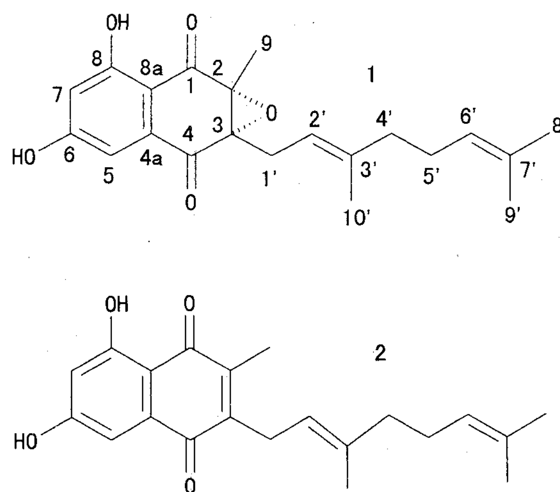


Table 1. Cultural characteristics of strain TA-0363.

Medium	Growth	Aerial mycelium	Reverse side	Soluble pigment
Yeast extract - malt extract agar (ISP No. 2)	Moderate	Poor, light yellow	Dark brown	Brown
Oatmeal agar (ISP No. 3)	Moderate	Poor, white	Pale orange	Yellow
Inorganic salts - starch agar (ISP No. 4)	Poor	Poor, white	Dark brown	None
Glycerol - asparagine agar (ISP No. 5)	Moderate	None	Dark brown	Purple
Peptone - yeast extract iron agar (ISP No. 6)	No growth			
Tyrosine agar (ISP No. 7)	Moderate	None	Dark brown	Brown
Sucrose - nitrate agar	Poor	Poor, white	Colorless	None
Nutrient agar	Moderate	None	Light orange	None
Glucose - asparagine agar	Poor	None	Light orange	None

Table 2. Physiological characteristics of strain TA-0363.

Temperature range for growth (°C)	12 ~ 37°C
Optimum temperature (°C)	28 ~ 30°C
Formation of melanoid pigment	−
Hydrolysis of starch	+
Liquefaction of gelatin	+
Coagulation of milk	−
Peptonization of milk	+
Utilization of carbon sourse	
L-Arabinose	±
D-Fructose	±
D-Glucose	+
Inositol	−
D-Mannitol	−
Raffinose	+
Sucrose	−
D-Xylose	+

+, Positive; ±, doubtful; −, negative.

Fermentation

Streptomyces sp. TA-0363 cultured on a mature slant culture was inoculated to a 500-ml Erlenmeyer flask containing 100 ml of the first seed medium consisting of glucose 0.5%, soluble starch 2%, yeast extract 0.2%, pharmamedia 1%, $CaCO_3$ 0.3% (pH 7.0). The flask was incubated at 28°C on a shaker at 200 rpm for 4 days. This seed culture was transferred into a flask containing the second seed medium same as the first seed medium and cultured for 3 days. This seed culture was transferred into a 50-liter jar fermenter containing 30 liters of the production medium consisting of glucose 1%, dextrin 2.5%, oatmeal 2%, dried yeast 0.2%, fishmeal 0.5%, pharmamedia 1%, beet molasses 0.5%, allophocyte 0.5%, $CaCO_3$ 0.3% (pH 7.0). The fermentation was carried out at 28°C for 3 days with an aeration rate of 30 liters per minute and agitation of 200 rpm.

Isolation

The cultured broth (60 liters) was centrifuged to separate the supernatant and the mycelia, which was extracted with acetone (20 liters). The supernatant was applied on a column of Diaion HP-20 (3 liters). After washing the column with water, the adsorbed material was eluted with acetone. This acetone fraction was combined with the mycelial extract and concentrated *in vacuo* to remove acetone. The obtained residue was extracted with EtOAc to afford an EtOAc extract (120 g). This extract was separated by column chromatography on silica gel and compounds were eluted stepwise with ethyl acetate - *n*-hexane. Active fractions were then applied on a Sephadex LH-20 (Pharmacia) column and eluted with *n*-hexane - chloroform - methanol (5 : 5 : 1). The fractions showing activity were combined and further purified by preparative HPLC on an ODS column (YMC-Pack ODS-AM, 20 i.d. × 250 mm) with eluent CH_3CN - H_2O (90 : 10) to give a brown oil of **1** (577.5 mg) and a reddish-brown amorphous powder of **2** (245.1 mg).

Physico-chemical Properties and Structure Elucidation

Physico-chemical properties of phosphatoquinone A

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

	1	**2**
Appearance	Brown oil	Reddish-brown amorphous
$[\alpha]_D$ (*c* 0.1, 25°C, $CHCl_3$)	−284	4
Molecular formula	$C_{21}H_{24}O_4$	$C_{21}H_{24}O_5$
HRFAB-MS		
Calcd for $(M-H)^-$	339.1596	355.1545
Found.	339.1597	355.1562
UV λ_{max} nm (ε) (MeOH)	205 (31150), 250 (15900), 272 (sh), 295 (8500), 365 (7600)	217 (34800), 270 (15000), 288 (11900), 425 (4170)
IR ν_{max} (KBr) cm^{-1}	3369, 2926, 1698, 1637, 1386, 1321, 1161	3413, 2927, 1633, 1455, 1335, 1158

Table 4. ^{13}C and ^1H NMR data of **1** and **2** in $CDCl_3$ and $CDCl_3$-CD_3OD (1 : 1), respectively.

	1		**2**	
	$^{13}C^a$	$^1H^b$	$^{13}C^a$	$^1H^b$
C-1	196.0 (s)		188.9 (s)	
C-2	64.9 (s)		144.0 (s)	
C-3	67.2 (s)		144.6 (s)	
C-4	191.3 (s)		184.5 (s)	
C-4a	134.3 (s)		134.2 (s)	
C-5	108.2 (d)	7.02 (1H, d, *J*=2 Hz)	108.8 (d)	7.06 (1H, d, *J*=2 Hz)
C-6	163.2 (s)		164.0 (s)	
C-7	108.8 (d)	6.61 (1H, d, *J*=2 Hz)	107.7 (d)	6.55 (1H, d, *J*=2 Hz)
C-8	164.5 (s)		164.7 (s)	
C-8a	108.9 (s)		109.1 (s)	
C-9	11.5 (q)	1.70 (3H, br s)	12.2 (q)	2.15 (3H, s)
C-1′	25.7 (t)	2.32 (1H, dd, *J*=7, 15 Hz) 3.27 (1H, dd, *J*=7, 15 Hz)	25.7 (t)	3.32 (2H, d, *J*=7 Hz)
C-2′	116.3 (d)	5.04 (1H, dt, *J*=1, 7 Hz)	119.3 (d)	4.98 (1H, t, *J*=1, 7 Hz)
C-3′	139.3 (s)		137.7 (s)	
C-4′	39.7 (t)	1.98 (2H, t, *J*=7 Hz)	39.9 (t)	1.99 (2H, t, *J*=7 Hz)
C-5′	26.4 (t)	2.03 (2H, t, *J*=7 Hz)	26.7 (t)	2.05 (2H, t, *J*=7 Hz)
C-6′	123.9 (d)	5.00 (1H, tt, *J*=1, 5 Hz)	124.2 (d)	5.03 (1H, tt, *J*=1.5, 8 Hz)
C-7′	131.6 (s)		131.7 (s)	
C-8′	25.6 (q)	1.60 (3H, br s)	25.1 (q)	1.64 (3H, br s)
C-9′	16.5 (q)	1.71 (3H, br s)	17.7 (q)	1.77 (3H, s)
C-10′	17.7 (q)	1.54 (3H, br s)	16.4 (q)	1.57 (3H, br s)

[a] 100 MHz, chemical shifts in ppm (multiplicity, coupling constants).
[b] 400 MHz, chemical shifts in ppm (multiplicity, coupling constnats).

(**1**) and B (**2**) are summarized in Table 3. The molecular formula for phosphatoquinone A (**1**) and B (**2**) were established as $C_{21}H_{24}O_4$ and $C_{21}H_{24}O_5$, respectively, by HR-FABMS. The UV spectrum of **1** suggested the presence of a semi-naphthoquinone chromophore in **1** with the absorption maxima at 205, 250, 295, 365 nm like those of SF2415B2[7] which was previously reported as a semi-naphthoquinone type antibiotic. The strong absorption at 1637 and 1698 cm^{-1} in the IR spectrum of **1** also suggested the semi-quinone carbonyl group.

The ^1H NMR spectrum of **1** (Table 4) showed signals due to four singlet methyl groups, one pair of meta coupled aromatic protons, two olefinic protons, and a hydrogen-bonded phenolic proton. The ^{13}C NMR spectrum of **1** (Table 4), analyzed by the DEPT method, indicated the presence of two carbonyl carbons, eight

Fig. 2. Partial structures for **1**.

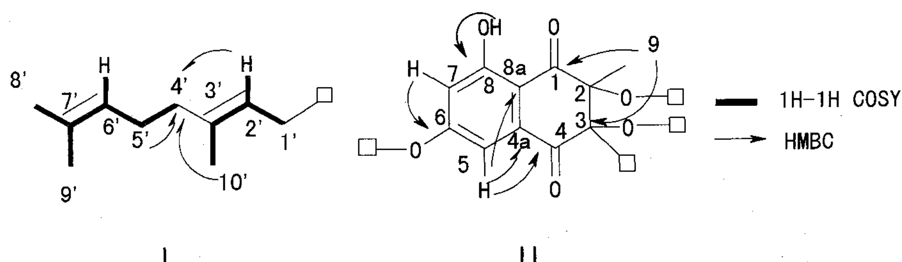
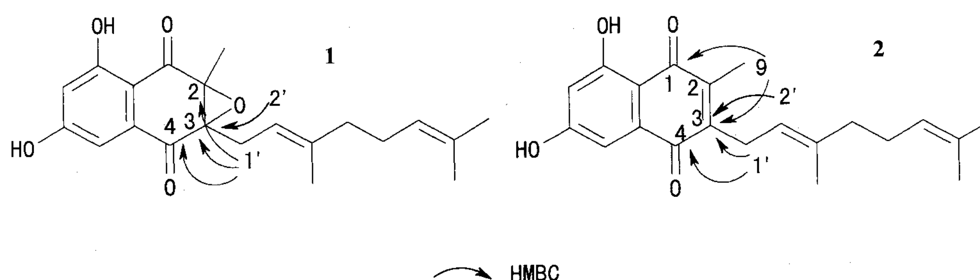


Fig. 3. Planar structures of **1** and **2**.



quaternary carbons, and three methylene carbons.

Detailed analyses of ^{1}H-^{1}H COSY, HMQC and HMBC spectra of **1** led us to postulate the presence of partial structures I and II in Fig. 2. For example, allylic couplings between 2'-H and 10'-H_3 and between 6'-H and 8'-H_3, 9'-H_3 were observed. The trans-relation of Δ^2 and Δ^6 olefin, in partial structure I, was established by comparison of the chemical shifts of three methyl carbons. Namely, the C-9' signal at 16.5 ppm was observed at 9 ppm higher field than the C-8' signal at 25.6 ppm owing to steric shielding effect of the C-5' moiety. The C-10' signal was also observed at 17.7 ppm due to the same effect of the C-1' moiety. Accordingly, the partial structure I was identified as an (E)-3,7-dimethyl-2,6-octadienyl group.

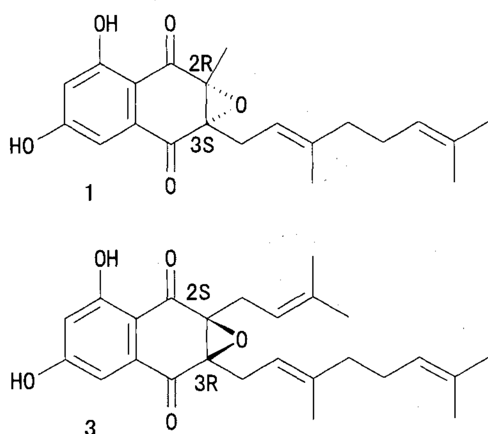
The assignments of the quaternary carbons in the partial structure II were also performed according to the long-range couplings shown by solid line arrows on the partial structure II in Fig. 2, together with the structural information from the UV and IR spectra. For instance, C-1, C-4, C-6 and C-8 were assigned on the basis of the HMBC correlations (9-H_3/C-1; 5-H/C-4; 7-H/C-6; 8-OH/C-8).

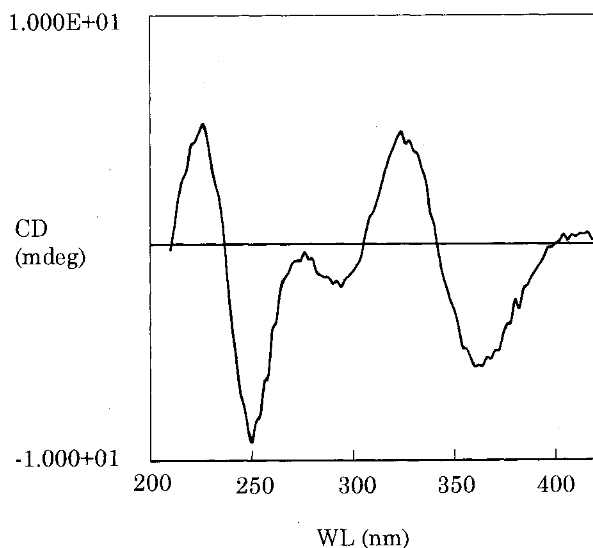
Further inspection of the HMBC spectrum of **1** revealed the connection of the partial structures I and II. Namely, correlation between 1'-H_2 and C-2, C-3 and C-4 and between 2'-H and C-3 indicated that partial structure I attached to C-3 in the partial structure II. Finally, the location of a hydroxyl group at C-6 and epoxy ring formation at C-2 and C-3 were suggested by the degree of unsaturation from the molecular formula. Based on these results, the structure of **1** was inferred to be as shown in Fig. 3 except for the stereochemistry.

The ^{1}H and ^{13}C NMR data (Table 4) of **2** were similar to those of **1**. Compared with **1**, the ^{13}C NMR spectrum of **2** revealed low field shifts, at 144.0 ppm and 146.6 ppm of the C-2 and C-3 carbon signals, respectively, due to epoxy ring formation in **1**. HMBC experiments on **2**, as shown in Fig. 3, also supported the existence of naphthoquinone in **2** instead of the semi-quinone in **1**. Thus, the structure of **2** was determined to be as shown in Fig. 3.

Phosphatoquinone A (**1**) exhibits Cotton effects caused by it's α,β-unsaturated ketone moiety. The absolute configuration of the epoxide in **1** could be ascertained by comparison of the CD spectrum of **1** with that of 2,3-epoxy-naphthomevalin (**3**)[8] whose absolute stereochemistry was established by employing the inverse octant rule and by comparison of it's CD spectrum with that of benzoquinone epoxide, (−)-terric acid[9]. The CD spectrum of **1** (Fig. 4) exposes two Cotton effects for n-π^* transitions and is associated with the two individual

Fig. 4. Absolute structure of phosphatoquinone A (**1**) and 2,3-epoxy-naphthomevaline (**3**), and CD spectrum of **1**.





carbonyl chromophores. The n-π^* transition of the C-1 carbonyl could be attributed to the Cotton effect with positive sign at 324 nm, whereas the transition of the C-4 carbonyl refers to the Cotton effect with the negative sign at 362 nm. These signs of the CD spectrum of **1** are opposite to those of **3**, resulting in the determination of the absolute configuration of C-2 and C-3 of **1** to be (2*R*,3*S*) (Fig. 4).

Biological Properties

Phosphatoquinones A and B showed PTPase inhibitory activities with IC_{50} being 28, 2.9 μM, respectively, and they are more potent than a well-known PTPase inhibitor, sodium vanadate. However, they did not inhibit serine/threonine protein phosphatases, PP1 and PP2A.

Discussion

Phosphatoquinones A and B are naphthoquinone derivatives and are structurally similar to SF2415[7], and naphthomevalin[8] which were isolated from the cultured broth of *Streptomyces*. Phosphatoquinone A has the opposite absolute configuration to that of 2,3-epoxy-naphthomevaline and this is the first report to describe the PTPase inhibitory activity of the related compounds. Phosphatoquinones A and B will be useful tools to investigate the biological significance of PTPase in the intercellular signal transduction. Detailed studies on the biological activity of phosphatoquinone A and B are in progress.

Experimental

General

Spectral and physico-chemical data were obtained on the following instruments: UV, JASCO V-520-SR spectrophotometer; IR, PERKIN ELMER 1760 infrared Fourier transform spectrometer; NMR, JEOL GX-400 spectrometer; FABMS, JEOL JMS-SX102 mass spectrometer; Optical rotations, JASCO DIP-360 polarimeter; CD, JASCO J-720 spectropolarimeter.

Taxonomic Study

Cultural and physiological characteristics of the producer strain were determined by the methods of SHIRLING and GOTTLIEB[10]. The carbon utilization was determined by the method of PRIDHAM and GOTTLIEB[11]. The morphological characteristics were observed with a light microscope and a scanning electron microscope. Diaminopimelic acid in the cell wall was analyzed by the method of STANECK and ROBERTS[12]. Menaquinones were analyzed by HPLC as described by TAMAOKA *et al.*[13].

PTPase Assay

For preparation of a membrane fraction, Ball-1 cells were homogenized in hypotonic lysis buffer (25 mM Tris-HCl, 25 mM sucrose, 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM PMSF, pH 7.5), and centrifuged at 300 × *g* for 5 minutes The supernatant containing CD45 was centrifuged at 100,000 × *g* for 60 minutes and solubilized the protein at the concentration of 200 μg/ml using an assay buffer (100 mM sodium acetate, 1 mM EDTA, 0.2% NP-40, pH 6.0). CD45- enriched membrane preparation (20 μg protein) was used for the bioassay. PTPase activity

to phosphotyrosine was measured by colorimetric assay.

Acknowledgments

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