ADENOPHOSTINS A AND B: POTENT AGONISTS OF INOSITOL-1,4,5-TRISPHOSPHATE RECEPTOR PRODUCED BY *Penicillium brevicompactum*

TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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New inositol-1,4,5-trisphosphate ($InsP_3$) agonists, adenophostins A(**1**) and B(**2**), were isolated from the culture broth of *Penicillium brevicompactum* SANK 11991 and SANK 12177. Its structures were related to adenine nucleotides. The agonistic activity of adenophostins A or B for binding to the $InsP_3$ receptor was higher than $InsP_3$ itself.

It is now well known that calcium ions play an important role in many cellular processes, including neural activity, muscle contraction, various secretion reactions and cellular growth and differentiation. Accordingly, the increase of intracellular calcium concentration is important to the operation of these processes, and compounds with the ability to control this release clearly have great potential for use in therapy. Inositol-1,4,5-trisphosphate ($InsP_3$) is a second messenger in a wide variety of cell types, and has an important role on the release of calcium ions from internal stores to the cytosol[1].

In our search for activities inhibiting the binding of [^{3}H]-$InsP_3$ to rat cerebellar membranes, we found adenophostins A and B, which were isolated from the cultured broth of *Penicillium brevicompactum* SANK 11991 and SANK 12177. Adenophostins A and B are potent $InsP_3$ receptor agonists, which bind to the $InsP_3$ receptor, and induce Ca^{2+} release from $InsP_3$ sensitive calcium stores. In this paper, we report the taxonomic studies of strain SANK 11991 and SANK 12177, the fermentation of the producing organism and the isolation, physico-chemical and biological properties of adenophostins A and B. Structures and biological activity for $InsP_3$ receptor of adenophostins in detail will be published elsewhere.

Materials and Methods

Taxonomic Studies

The adenophostins-producing strains were identified by using technique as described by PITT[2]. The colors are indicated herein according to KORNERUP and WANSCHER[3].

Inhibitory Activity on [^{3}H]-$InsP_3$ Binding to $InsP_3$ Receptor

The $InsP_3$ receptor was purified form rat cerebellum using heparin-agarose and concanavalinA-Sepharose[4]. Binding of [^{3}H]-$InsP_3$ to the purified $InsP_3$ receptor was determined by using poly-ethyleneglycol (PEG) precipitation method[5]. Samples were incubated in 250 μl of 1 μg of the purified receptor, 50 mM of Tris-HCl (pH 8.0), 1 mM of EDTA, 1 mM of 2-mercaptoethanol and 10 nM of [^{3}H]-$InsP_3$ at 4°C. After 5 minutes, the sample was mixed with 5 μl of 5% (w/v) γ-grobulin and 250 μl of 30% PEG

6000. After an additional 5 minutes, the samples were centrifuged for 5 minutes at 12,000 rpm at 4°C to precipitate the $InsP_3$ receptor. The supernatant was removed and the radioactivity in pellet was then determined. Nonspecific binding was measured in the presence of 1 μM of cold $InsP_3$.

HPLC Analysis

HPLC analysis was performed on a YMC-pak, 6 × 150 mm, ODS, AQ-312. The column was eluted with gradient from 0.05 M phosphate buffer (pH 6.8) to 4% CH_3CN - 0.05 M phosphate buffer solution over a period of 20 minutes, at a flow rate of 1.5 ml/minute. The effluent was monitored using a photodiode array throughout the wavelengths from 220 nm to 350 nm.

Fermentation

A seed culture was produced by inoculating with a loopful of the slant culture of a strain SANK 11991 in a 2-liter Erlenmeyer flask containing 600 ml of GPMY medium containing glycerol 5%, fresh potato 5%, yeast extract 0.5% and malt extract 0.5%, adjusted to pH 6.0 before sterilization. The culture vessel was incubated at 26°C and shaken at 220 rpm on a rotary shaker for 6 days. Six hundred ml of the resultant seed culture were transferred into the 60-liter jar fermentor containing 30 liters of the same medium. A second seed cultivation was carried out for 2 days at 26°C under an aeration rate of 30 liters/minute, and the agitation speed 165 rpm, which was automatically controlled to maintain the dissolved oxygen concentration at 5 ppm. Three hundred liters of the same GPMY medium were placed in 600-liter stainless tank. 6 liters of the second seed culture were transferred into tank, and the fermentation was carried out for 5 days at 26°C, under aeration rate 300 liters/minute, an internal pressure of 1.0 kg/cm^2, and the agitation speed 82.5 rpm.

Isolation and Purification

Isolation procedure was guided by inhibition of [^{3}H]-$InsP_3$ binding assay and was monitored by HPLC.

In a preliminary extraction test, both fractions of cultured filtrate and acetone extracts of mycelia possessed an activity in the binding assay. Thus, isolation procedure in large scale was refined.

Six hundred eighty liters of cultured broth were mixed with 20 kg of Celite 545 filter aid, and the mixture was filtered to separate the filtrate and mycelial cake. The mycelial cake was extracted twice with acetone, each time with 400 liters. The acetone extract was concentrated *in vacuo* to remove acetone. This aqueous solution (400 liters) was passed through 60 liters of Diaion HP-20 column. In this column, lipophilic substances were removed. The effluent was adjusted to pH 3.0 with HCl, and was applied on a column containing 60 liters of active carbon. After washing with 300 liters of water and then active principles were eluted with 300 liters of 1 : 1 mixture of acetone and 0.2 N NH_4OH solution, and concentrated *in vacuo* to afford 10 liters of a condensed solution. Meanwhile, 660 liters of the filtrate as mentioned above were passed through a column containing 60 liters of Diaion HP-20 as above mentioned manner. The collected effluent was adsorbed on a carbon column, and eluted with 50% acetone - 0.1 N NH_4OH solution and concentrated *in vacuo*. The condensed solution, combined with the 10 liters of condensed solution previously obtained from mycelia, were applied on column containing 6 liters of DEAE Sephadex A-25, which had been previously equilibrated with 0.05 M phosphate buffer (pH 6.8). The column was then eluted with stepwise from 0.1 M to 0.5 M NaCl - 0.05 M phosphate buffer. The active fractions, mainly 0.3 M to 0.5 M NaCl solution, were pooled and desalted with carbon column and lyophilized to crude powder. This process was repeated twice. 1.69 g of crude powder containing adenophostins were obtained. Finally, adenophostins A and B were separated by preparative HPLC. The crude powder was dissolved in 1.7 ml of water, and 100 μl of the solution was injected into YMC column (ODS, AQ-type, 10 × 250 mm), and eluted with a gradient of 0.05 M phosphate buffer (pH 6.8) and 4% CH_3CN solution at a flow rate of 5 ml/minute. The fractions containing adenophostins A or B were desalted on carbon column and lyophilized, separately. Adenophostins A (30 mg) and B (100 mg) were obtained both as white powders.

Results and Discussion

Taxonomic Characteristics

The mycological properties of adenophostin-producing strains are as follows: SANK 11991 was

isolated from a soil sample collected in Yubari-shi, Hokkaido, Japan.

Colony on CYA is 16 mm in diameter (25°C, 7 days). Thick and dense hyphal mat is formed, and the surface is downy. The colony is slightly umbonate at the center, and radially sulcate. Grayish green (26E3) conidia are formed at the center. Hyaline to golden yellow exudate is produced. No soluble pigments are produced. The reverse side of the colony is yellowish brown (5D5), and radially and concentrically sulcate.

Colony on MEA is 16 mm in diameter (25°C, 7 days). Thick and dense hyphal mat is formed and the surface is velutinous to downy, plane. Grayish green to dull green (26E3) conidia are formed on almost the whole surface of the colonies. The reverse side of the colony is grayish yellow (4B4).

Colony on G25N is 7 mm in diameter (25°C, 7 days). Thin and dense hyphal mat is formed and the surface is velutinous. Grayish green (26D5) conidia are formed on almost the whole surface of colonies, however, the sporulation area is not so large. Conidia germinate at 5°C to form micro-colonies. At 37°C, no germination is observed on either CYA or MEA medium.

Penicilli are terverticillate, partly biverticillate. The conidiophore wall is smooth or very finely roughened. Metulae are cylindrical, $8\sim12\,\mu$m long. Phialides are ampulliform, $6\sim12\,\mu$m. Conidia are spherical to subspherical, $2\sim4$ (6) μm, with smooth to finely roughened walls, borne on phialides in divergent and disordered chains.

SANK 12177 was isolated from a soil sample collected in Fukue-shi, Nagasaki Prefecture, Japan. The colony on CYA is 36 mm in diameter (25°C, 7 days). Thick and dense hyphal mat is formed and the surface is velvety. Downy hyphae develop at the center. The colony is slightly umbonate at the center, and radially sulcate. Conidia are formed on the whole surface of the colony, and dull green (25E3) in color. Greenish yellow (1A8) exudate is formed. Soluble pigment of the same color is excreted into the medium. The reverse side presents grayish yellow (4C6), and radially and concentrically sulcate.

Colony on MEA is 33 mm in diameter (25°C, 7 days). The surface is velutinous and plane. Conidia are dull green (25D3) in color, and are formed on almost the whole surface of the colonies. The reverse side presents dark yellow (4C8).

Colonies on G25N is 26 mm in diameter (25°C, 7 days). Thin and dense hyphal mat is formed and the surface is velutinous, umbonate at the center. Dull green (25E3) conidia are formed on almost the whole surface. Conidia germinate at 5°C to form microcolonies. At 37°C, no germination is observed on either CYA or MEA.

Conidiophore wall is smooth. Penicilli are terverticillate, partly biverticillate. Metulae are cylindrical, $8.5\sim16.5\,\mu$m long. Phialides are ampulliform, and $6.5\sim13.5\,\mu$m long, Conidia are formed on phialides in chain, spherical to subspherical, $2\sim5$ (6) μm long, with nearly smooth walls.

Based on properties above, both strains were identified as *Penicillium brevicompactum* Dierckx.

Physico-chemical Properties

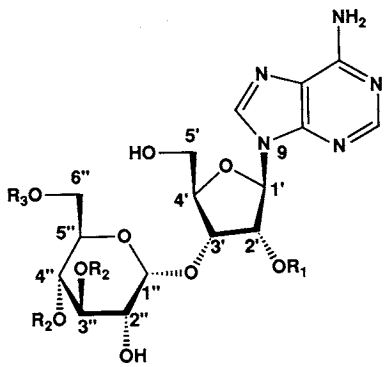
Physico-chemical characteristics of adenophostins A and B are summarized in Table 1. They are white, powdery, acidic, and soluble in water and slightly soluble in DMSO. The molecular formulae were determined by elementally analyses and HRFAB-MS spectra. Their characteristic UV absorptions at 258 nm and ^{1}H and ^{13}C NMR data suggest the presence of adenine and sugar. The structures of adenophostins A and B are shown in Fig. 1. Details of structure elucidation will be published elsewhere.

Table 1. Physico-chemical properties of adenophostins A and B.

	A	B
Molecular formula	$C_{16}H_{26}N_5O_{18}P_3$	$C_{18}H_{28}N_5O_{19}P_3$
Molecular weight	669	711
HRFAB-MS $(M+H)^+$		
Found:	670.0513	712.0729
Calcd:	670.0564	712.0669
Elemental analysis		
Found (%):	C 25.52, H 5.03, N 12.85, P 11.32	C 26.43, H 4.77, N 11.66, P 10.80
Calcd (%):	$C_{16}H_{26}N_5O_{18}P_3 \cdot 2NH_4 \cdot 3H_2O$	$C_{18}H_{28}N_5O_{19}P_3 \cdot 2NH_4 \cdot 4H_2O$
	C 25.37, H 5.05, N 12.94, P 12.26	C 26.45, H 5.18, N 11.99, P 11.32
$[\alpha]_D^{25°}$ (H_2O)	+28.6° (*c* 0.71)	+33.8° (*c* 0.91)
UV λ_{max}: nm	256 (acidic), 258 (neutral), 260 (alkaline)	256 (acidic), 258 (neutral), 260 (alkaline)
IR γ KBr: cm^{-1}	3200, 1690, 1400, 1050, 930	3200, 1690, 1720, 1400, 1050
HPLC[a]: Rt (minutes)	5.56	16.17
TLC[b]: Rf	0.11	0.14

[a] Descrived in text.
[b] Silica gel plate (No. 5715, Merck), *n*-BuOH - Pyridine - AcOH - H_2O (15 : 10 : 2 : 12).

Fig. 1. Structures of adenophostins.



	R_1	R_2	R_3
Adenophostin A (**1**)	PO_3H_2	PO_3H_2	H
B (**2**)	PO_3H_2	PO_3H_2	$COCH_3$

Biological Activities

Inhibitory constants (*Ki* value) on [^{3}H]-$InsP_3$ binding of adenophostins A and B were both 0.18 nM, while that of $InsP_3$ itself was 15 nM. Nucleotide structurally related, commercial available compounds such as AMP, ADP, ATP, GMP, GTP and CTP did not exhibited an activity in our binding assay. It is known only one report except $InsP_3$, that cyclic ADP ribose isolated from sea urchin egg was reported to possess a releasing activity of calcium ions[6,7]. Our compounds having calcium ions releasing activity are the first report occured from fungal metabolites. It may be worthwhile to explore the therapeutical nature of the present compounds or their derivatives.

Adenophostins A and B showed neither any activities of receptor binding assay except $InsP_3$ receptor, such as α1 and β adrenergic, angiotencin II, benzodiazepine, L-type calcium channel (dihydropyridine sensitive), cholecystokinin A and B, dopamine D2, insulin, leukotriene B4 and D4, muscarin, neurokinin, NMDA, serotonin 5-HT1, 5-HT2, and 5-HT3, and thromboxane A2 at concentration of 10 μg/ml, nor any antimicrobial activity against about 30 species of Gram-positive and -negative bacteria and fungi at 1,000 μg/ml using the paper disc agar diffusion method.

Acute toxicity of adenophostin B was conducted in mice, and its LD_0 value is more than 200 mg/kg when given intravenously.

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