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

STRUCTURE ELUCIDATION

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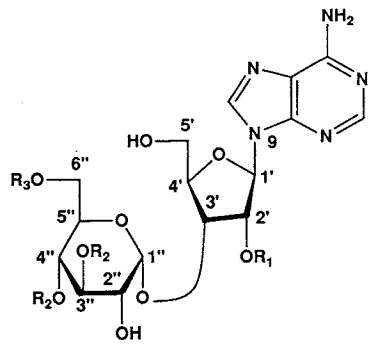
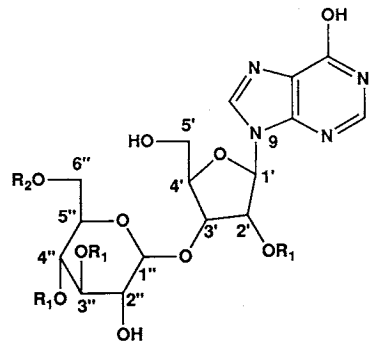
Adenophostins A (**1**, $C_{16}H_{26}N_5O_{18}P_3$) and B (**2**, $C_{18}H_{28}N_5O_{19}P_3$), potent agonists of inositol-1,4,5-trisphosphate ($InsP_3$) receptor, were isolated from the culture broths of *Penicillium brevicompactum* SANK 11991 and SANK 12177. Hydrolysis of **2** with aq NaOH gave **1**. Oxidation of **2** with $NaNO_2$ gave the hypoxanthine derivative (**3**). Treatment of **1** or **2** with alkaline phosphatase gave **4** and **5**. Treatment of **4** with α-glucosidase gave adenosine. Thus, their structures were deduced to be adenosine nucleotides by NMR, MS and the enzymatic degradation. The inhibitory constants (*Ki* value) of **1**, **2**, **3** and $InsP_3$ itself for binding to the $InsP_3$ receptor were 0.18 nM, 0.18 nM, 0.29 nM and 15 nM, respectively.

Mobilization of intracellular Ca^{2+} is a common response to activation of a variety of surface receptors. In many cases the second messenger involved in this signal transduction process is inositol-1,4,5-trisphosphate ($InsP_3$). $InsP_3$, formed in response to receptor-mediated enhancement of inositolphospholipid turnover, releases Ca^{2+} from intracellular stores. There is substantial evidence that the pathway is involved in the control of smooth muscle contractility, secretion, neuronal excitability, the activation of inflammatory cells, and cell proliferation. The therapeutic potential of drugs which selectively modulate this signal system is enormous[1].

Fig. 1. Structures of adenophostins and its derivatives.



	R_1 (2′)	R_2 (3″,4″)	R_3 (6″)
Adenophostin A (**1**)	PO_3H_2	PO_3H_2	H
B (**2**)	PO_3H_2	PO_3H_2	$COCH_3$
4	H	H	H
5	H	PO_3H_2	H



	R_1	R_2
3	PO_3H_2	$COCH_3$

Table 1. ^{1}H and ^{13}C NMR spectral data of adenophostins A (**1**) and B (**2**).

Group	1		2		J=Hz	J_{H-P}=Hz
	CMR[a]	PMR[b]	CMR[a]	PMR[b]		
Ribose						
1′	87.9*	6.30	87.7*	6.38	$J_{1',2'}=6.4$	
2′	75.8*	5.29	75.9*	5.29	$J_{2',3'}=2.9$	9.5
3′	74.4*	4.64	74.5*	4.62	$J_{3',4'}=5.3$	
4′	85.1	4.47	84.9	4.45	$J_{4',5'a}=3.0$; $J_{4',5'b}=3.4$	
5′a	60.7	3.91	61.5	3.88	$J_{5'a,5'b}=12.5$	
b		3.81		3.83		
Glucose						
1″	98.5	5.34	98.5	5.30	$J_{1'',2''}=3.3$	
2″	72.3*	3.83	71.0*	3.80	$J_{2'',3''}=10.3$	
3″	77.7*	4.51	77.6*	4.51	$J_{3'',4''}=9.5$	9.5
4″	73.1*	4.09	73.4*	4.15	$J_{4'',5''}=9.5$	9.5
5″	71.3*	3.83	69.8*	4.05	$J_{5'',6''a}=7.3$; $J_{5'',6''b}=0$	
6″a	61.8	3.83	63.8	4.50	$J_{6''a,6''b}=11.0$	
b		3.77		4.27		

[a] Chemical shifts are given in ppm using Dioxane at 67.0 ppm, as internal reference in D_2O.
[b] Chemical shifts are given in ppm using H_2O signal at 4.7 ppm, as internal reference in D_2O.
* Signals were split with C-P coupling.

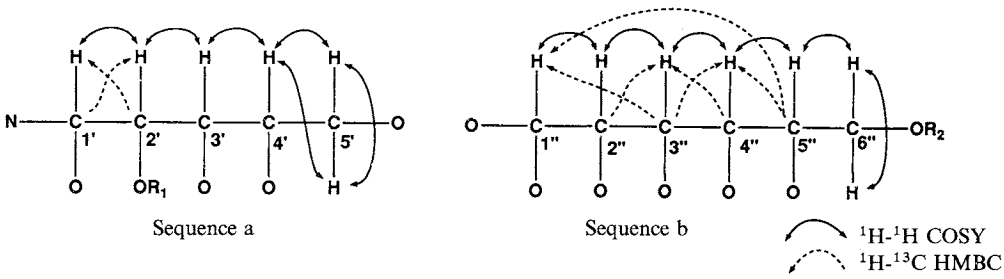
In the course of our screening program for $InsP_3$ receptor agonists or antagonists, we discovered the new microbial products with potent agonistic activities, adenophostins A and B, from the fermentation broth of *Penicillium brevicompactum* SANK 11991 and SANK 12177. In the preceding paper[2], we described the taxonomy of the producing organisms and the fermentation, isolation, physico-chemical and biological properties of adenophostins A and B. In this paper, we report the structural elucidation of adenophostins A (**1**) and B (**2**).

The molecular formulae of adenophostin A (**1**, $C_{16}H_{26}N_5O_{18}P_3$, $M+H^+$ *m/z* found; 670.0513, calcd; 670.0564), and B (**2**, $C_{18}H_{28}N_5O_{19}P_3$, $M+H^+$ *m/z* found; 712.0729, calcd; 712.0669) were determined by elemental analysis and HRFAB-MS spectra. The UV spectra of **1** and **2** showed maxima at 256 nm in acidic and at 260 nm in alkaline solution, which indicated the presence of an *N*-9 substituted adenine moiety. The signals at 8.38(s) and 8.27(s) ppm in the ^{1}H NMR and 154.0(s), 150.0(d), 148.9(s), 142.5(d) and 119.0(s) in the ^{13}C NMR and the fragment ion at *m/z* 136 in the FAB-MS of **1** were assigned to the adenine structure. This partial structure was confirmed by deamination of **2** with $NaNO_2$ in acetic acid which gave the hypoxanthine (inosine) derivative **3** with a UV absorption maximum at 248 nm. This result indicated that the amino function at *N*-6 in the adenine skeleton was unsubstituted. The ^{1}H and ^{13}C NMR data of **1** and **2** are summarized in Table 1.

The ^{1}H NMR spectrum of **2** was similar to that of **1** but exhibited an additional acetyl signal at 2.11 ppm. Hydrolysis of **2** with aqueous NaOH solution gave a deacetylated compound, which was identical with adenophostin A. The position of the acetyl group in **2** was unambiguously determined by comparing the ^{1}H NMR spectra of **1** and **2**. The 6″-CH_2 signal in **2** was observed 0.5~0.7 ppm further down-field than that in **1**. The structure elucidation is mainly based on the NMR spectra of **2**, whose signals are better separated than those of **1**. The ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMBC spectra of **2** clearly revealed the presence of a C_5 unit of sequence (a) and a C_6 unit of sequence (b) as shown in Scheme 1.

Treatment of **2** with an excess of alkaline phosphatase (28 mg of **2** and 40 units, *Escherichia coli* A-19,

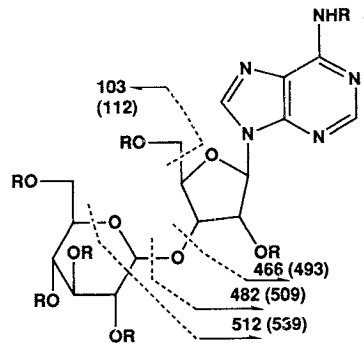
Scheme 1. Partial structures of adenophostins.



	R_1	R_2
1	PO_3H_2	H
2	PO_3H_2	$COCH_3$

Takara Shuzo Co.) in 1 M Tris-HCl buffer (pH 8.0) at 37°C for 2 days gave the phosphorus free product **4**. On the other hand, with an equivalent amount of the enzyme (56 mg of **1** and 56 units of the enzyme) and at 25°C for 3 days, **1** gave the diphosphate derivative **5**. The position of the newly liberated hydroxyl group in **5** was deduced to be at 2′ (sequence a) from the up-field shift of the ^{1}H NMR signals (5.29 ppm at 2′-H in **1** to 4.91 ppm in **5**).

Fig. 2. Mass fragmentations of TMS (TMS-d_9) derivatives of **4**.



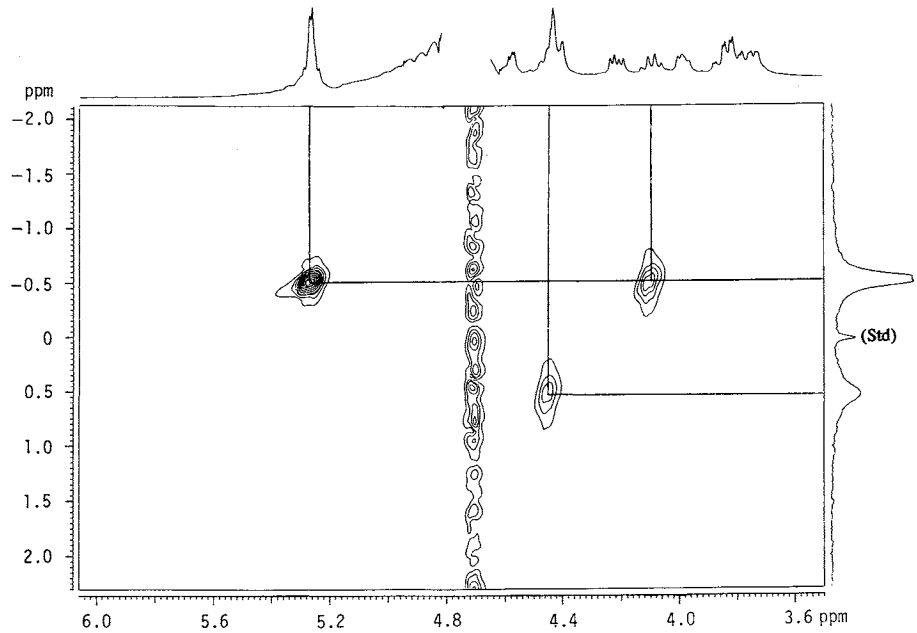
R=TMS (TMS-d_9)

After treatment of **4** with anhydrous 10% HCl in MeOH at 90°C overnight in a sealed tube, followed by trimethylsilylation, adenine, ribose and glucose were detected in the hydrolysate by GC-MS analysis. The sequences a and b were assigned to those of ribose and glucose, respectively. In addition, treatment of **4** with α-glucosidase (maltase, Sigma) in citrate buffer (pH 6.2) at 37°C gave adenosine. From these results, it was concluded that adenophostins were α-glucosyl adenosine derivatives containing three phosphate groups.

The site of the glucosyl linkage at the ribose of **2** was deduced to be the 3′ position by the following evidence. First, the well known down-field shift[3] of ^{13}C NMR signals upon glucosidation was observed in **2**: the C-3′ signal, which appeared at 70.2 ppm in 2′-AMP, was shifted to 74.5 ppm in **2**. Secondly, an NOE was observed between 3′-H of ribose and 1″-H of glucose in **2**. Thirdly, in the MS/MS (EI positive) spectra of trimethylsilyl (TMS) and deuterium trimethylsilyl (TMS-d_9) derivatives of **4**, molecular ion peaks with the seven TMS groups appeared at *m/z* 923 and at *m/z* 986, respectively. The fragment ions at 466 (493), 482 (509) and 512 (539) in the TMS (TMS-d_9) derivatives were attributed to the cleavage of the glucose moiety (Fig. 2). A fragment ion at *m/z* 103 (112), corresponding to 5′-hydroxymethyl was observed in each fragment at 466 (493), 482 (509) and 512 (539), by MS/MS spectra of TMS (TMS-d_9) derivatives, respectively.

Finally, the positions of the remaining two phosphate groups were deduced to be C-3″ and C-4″ in

Fig. 3. ^{1}H-$^{31}\{P\}$ Homonuclear spectra of **2** (D_2O, ext. std. for ^{31}P: H_3PO_4; ext. std. for ^{1}H: TMS).



glucose by NMR analysis. The 3″-H and 4″-H in **2** were shifted down-field by substitution with a phosphate group as compared with those of glucose. The C-2″, C-3″, C-4″ and C-5″ signals in the ^{13}C NMR spectra of **1** and **2** were split by C-P long range couplings, and in particular, the C-3″ and C-4″ appeared as doublets of doublets in the coupling pattern of PO-C(3″)-C(4″)-OP (Table 1). Moreover, in the ^{1}H-$^{31}\{P\}$ homonuclear spectra of **2**, the 2′-H at 5.29 ppm in ribose and 3″-H at 4.51 ppm and 4″-H at 4.15 ppm in the glucose moiety showed cross peaks with phosphorus (Fig. 3). These observations indicated that the linkage sites of the three phosphates were at the C-2′, C-3″ and C-4″ positions. Thus, the structures of **1** and **2** were established as shown in Fig. 1. The absolute configuration of adenophostins are now under investigation.

The inhibitory constants (*Ki* value) of **1**, **2**, **3**, **5** and $InsP_3$ for binding to the purified $InsP_3$ receptor were 0.18 nM, 0.18 nM, 0.29 nM, 320 nM and 15 nM, respectively. Adenophostins might therefore fit more tightly to the binding site of the $InsP_3$ receptor than $InsP_3$ itself. Adenophostins induced Ca^{2+} release both from cerebellar microsomes and from intracellular Ca^{2+} stores in permeabilized NG108-15 cells. Adenophostins at concentration as low as 1 nM produced a significant Ca^{2+} release from cerebellar microsomes, and their activities were 100-fold more potent than $InsP_3$[4]. Recently, cyclic ADP ribose having a releasing activity of Ca^{2+} was isolated from sea urchin egg[5]. Its structure is quite different from the structures of the adenophostins. Adenophostins are the first reported agonists from fungal metabolites.

Experimental

General

FAB-MS and MS/MS spectra were obtained on JOEL JMS-HX100 triple analyser tandem mass spectrometer using a 3-nitrobenzyl alcohol matrix on the FAB probe tip and bombarded with 6 kev xenon atoms. FAB-MS/MS spectra were obtained by collisional activation. NMR spectra were measured on

JEOL JNM-GX400 and Bruker AMX360 spectrometers. Chemical shifts are given in ppm using TMS as an external standard. UV spectra were measured on a Shimazu UV-265FW spectrophotometer.

Hydrolysis of **2** with NaOH

2 (18 mg) was hydrolyzed with 0.01 N NaOH (1 ml) at room temperature overnight. The reaction mixture was neutralized with 0.1 N HCl and applied on a column of Sephadex LH-20 (300 ml), and the column was eluted with water. The fraction containing **1** was lyophilized. Twelve mg of **1** was obtained as an amorphous powder, which was identical with the natural product by HPLC analysis, elemental analysis and NMR spectra data.

Deamination of **2** with $NaNO_2$

2 (56 mg) was dissolved in 20% acetic acid (5 ml) and $NaNO_2$ (300 mg) was added. After stirring at room temperature overnight, the reaction mixture was diluted with water (30 ml) and applied on a carbon column (5 ml), and the column was eluted with 0.2 N NH_4OH - MeOH (1 : 1, v/v). The eluate was evaporated and was chromatographed on a Sephadex LH-20 (300 ml) using water as eluent to give 43 mg of **3** as an amorphous powder. FAB-MS: *m/z* 711 $(M-H)^-$. ^1H NMR (D_2O): 8.16 (1H, s), 8.04 (1H, s), 6.14 (1H, d, 6.3), 5.14 (2H, m), 4.47 (1H, m), 4.25~4.40 (3H, m), 4.10 (1H, q, 5.8, 11.7), 4.00 (1H, q, 9.7, 9.3), 3.60~3.90 (4H, m). ^{13}C NMR (D_2O): 176.7 (s), 161.2 (s), 151.3 (s), 148.8 (d), 143.7 (d), 126.9 (s), 100.8 (d), 90.1 (d), 87.0 (d), 79.9 (d), 78.0 (d), 76.8 (d), 75.7 (d), 73.4 (d), 72.0 (d), 66.2 (t), 63.9 (t), 22.9 (q). UV λ_{max} H_2O (ε): 248 nm (11,100).

Anal Calcd for $C_{18}H_{27}N_4O_{20}P_3 \cdot 3NH_4 \cdot 3H_2O$: C 26.34, H 5.52, N 11.95, P 11.32.
Found: C 26.42, H 4.97, N 11.83, P 10.86.

Complete Hydrolysis of **2** with Alkaline Phosphatase

2 (28 mg) was dissolved in 1 M Tris-HCl buffer (pH 8.0, 3 ml) and alkaline phosphatase (40 units, *E. coli* A-19, Takara Shuzo) was added. After 2 days at 37°C, the reaction mixture was applied to a column of Diaion CHP-20P (200 ml, Mitsubishi Chemical Industries), and the column was eluted with 20% acetone. The fraction containing **4** was evaporated and chromatographed on Sephadex LH-20 (300 ml) using water as eluent to give 11.9 mg of **4** as an amorphous powder. FAB-MS: *m/z* 430 $(M+H)^+$. ^1H NMR (D_2O): 8.10 (1H, s), 8.00 (1H, s), 5.92 (1H, d, 6.3), 5.01 (1H, d, 3.4), 4.71 (1H, t, 5.3), 4.3~4.4 (2H, m), 3.5~3.8 (6H, m), 3.42 (1H, q, 3.9, 10.2), 3.26 (1H, t, 9.2).

Anal Calcd for $C_{16}H_{23}N_5O_9 \cdot 2H_2O$: C 41.29, H 5.85, N 15.05.
Found: C 40.97, H 5.62, N 14.86, P 0.

Partial Hydrolysis of **1** with Alkaline Phosphatase

1 (56 mg) was dissolved in 1 M Tris-HCl buffer (5 ml) and alkaline phosphatase (56 units) was added. After 3 days at 25°C, the reaction mixture was applied on a carbon column (25 ml). The column was washed with water and eluted with 0.2 N NH_4OH - MeOH (1 : 1, v/v). The eluate was evaporated and lyophilized to give a crude product. A crude product was purified by preparative HPLC (Senshu-pak, ODS, H-5251-AQ, 25×250 mm, 2% CH_3CN - 0.05 M phosphate buffer, 10 ml/minute). The fraction containing **5** was desalted on a carbon column (2 ml) and lyophilized to give 15.9 mg of **5** as a hygroscopic powder. FAB-MS: *m/z* 590 $(M+H)^+$. ^1H NMR (D_2O): 8.31 (1H, s), 8.24 (1H, s), 6.14 (1H, d, 5.2), 5.22 (1H, d, 3.8), 4.91 (1H, t, 5.3), 4.45~4.55 (3H, m), 4.06 (1H, q, 9.2, 9.5), 3.75~3.9 (6H, m), 3.71 (tris).

Anal Calcd for $C_{16}H_{24}N_5O_{15}P_2 \cdot NH_4 \cdot 3H_2O \cdot C_4H_{11}NO_3$(tris): C 30.74, H 5.80, N 12.55, P 7.93.
Found: C 29.58, H 5.57, N 13.52, P 8.30.

Treatment of **4** with α-Glucosidase

4 (1 mg) was dissolved in 20 mM citrate buffer (pH 6.2, 0.3 ml) and 150 μl of α-glucosidase (100 units/ml, maltase, Sigma) was added. After standing 2 days at 37°C, the reaction mixture was analyzed by HPLC with photodiode array detection (YMC, ODS, AQ-312, 6×150 mm, 4% CH_3CN-0.05 M phosphate buffer, 1.5 ml/minute). The retention time (13.41 minutes) and the UV absorption (260 nm) of the product was identical with those of authentic adenosine.

Methanolysis of **4**

4 (1 mg) was hydrolyzed with anhydrous 10% HCl - MeOH (0.5 ml) in a sealed tube at 90°C for

20 hours. The reaction mixture was evaporated to dryness. The residue was dissolved in pyridine (30 μl) and *N*-*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA, 20 μl). After keeping at 60°C for 1 hour, the TMS derivatives were assigned to those of *O*-methyl ribose-3×TMS (Rt: 4.1 minutes), adenine-2×TMS (6.4 minutes), *O*-methyl glucose-4×TMS (7.2 minutes) by gas chromatography (2% OV-1, 3×500 mm, 100~250°C, 10°C/minute temperature programming, carrier gas He: 60 ml/minute). GC-MS analysis of TMS derivatives were also performed under similar conditions to those above.

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