Morphology Reversion Activity of Phosmidosine and Phosmidosine B, a Newly Isolated Derivative, on *src* Transformed NRK Cells

NOBUYASU MATSUURA†, RIE ONOSE and HIROYUKI OSADA*

The Institute of Physical and Chemical Research (RIKEN)
Wako, Saitama 351-01, Japan

(Received for publication November 6, 1995)

An antifungal antibiotic, phosmidosine (1) was previously isolated (J. Antibiotics 44: 375~381, 1991). Phosmidosine derivatives, phosmidosines B (2) and C (3) were newly isolated as detransforming compounds from the fermentation broth of *Streptomyces* sp. strain RK-16 which is a producer strain of phosmidosine (1). The structures of 2 and 3 were established by spectroscopic methods, including UV, HRFAB-MS, and NMR. 1 and 2 showed the inhibitory activity of the cell cycle progression and the morphological reversion activity on src^{ts}-NRK cells. On the other hand, 3 had no activity. These results indicate that the prolyl group in phosmidosine derivatives plays an important role in the inhibitory activity against the cell cycle progression and the morphological reversion activity on src^{ts}-NRK cells.

It is known that products of oncogenes and tumor suppressor genes are involved in the mammalian cell cycle regulation[1,2]. Nowadays it becomes common understanding that disorder of the regulation system of the cell cycle induces or enhances tumorigenesis. Low molecular compounds which inhibit the cell cycle might be good candidates for tumor chemotherapy. Therefore, we have conducted a screening program for microbial metabolites which inhibit the cell cycle progression of the src^{ts}-NRK cells (NRK cells transformed by a temperature sensitive Rous sarcoma virus)[3]. During the screening, a cyclic lipopeptide, enamidonin[4] and a polyketide, reveromycines A~D[5,6] were isolated.

In this paper, we report that the purification and structural determination of new members of phosmidosine (1), phosmidosines B (2) and C (3) (Fig. 1). Although 1 was discovered as an antifungal antibiotic[7], new biological activities of 1 and 2 on src^{ts}-NRK cells are reported below.

Materials and Methods

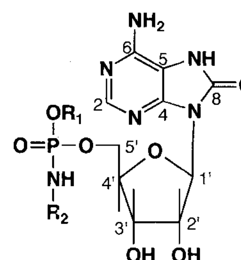
Instrumental Analyses

Melting points and UV spectra were measured on a Yanagimoto melting point apparatus and a Hitachi 220A spectrophotometer, respectively. NMR spectra were recorded on JEOL JNM-α-400 and GSX-500 spectrometers. Chemical shifts in ^1H and ^{13}C NMR were respectively reported relative to residual DHO (δ 4.65) and dioxane (δ 67.6) in D_2O. High resolution fast atom bombardment mass spectra (HRFAB-MS) and optical rotations were obtained on a JEOL JMS DSX-300 and a Jasco J-720 spectropolarimeter.

Fermentation and Isolation

For the seed culture, spores of the strain *Streptomyces* sp. RK-16 were inoculated in a 500-ml flask containing 70 ml of medium composed of glucose 2%, soluble starch 1%, meat extract 0.1%, dried yeast 0.4%, soybean flour 2.5%, NaCl 0.2%, and K_2HPO_4 0.005%, adjust to pH 7.0 before sterilization. It was cultivated for 48 hours at 28°C on a rotary shaker. For large scale fermentation, the culture of the ten flasks after 48 hours cultivation

Fig. 1. Structures of phosmidosine (1), phosmidosines B (2) and C (3).



	R_1	R_2
phosmidosine (1)	Me	prolyl (5''–NH, 4'', 3'', 2''–C(=O), 1'')
phosmidosine B (2)	H	prolyl (NH, C(=O))
phosmidosine C (3)	Me	H

† Present address: Toyama Prefectural University, Biotechnology Research Center, Kosugi-machi, Toyama 939-03.

was inoculated into a 600-liter fermentation tank containing 400 liters of the same medium, which was cultured for 88 hours at 28°C.

The culture broth (400 liters) was filtered and applied on a column of the cation exchange resin IRC-50 (H^+ form). After washing with water, the active component was eluted with 0.5 N NH_4OH (260 liters). The eluted solution was immediately neutralized with 3 N HCl and applied on a column of Diaion HP-20. After washing with water, the active component was eluted with 70% aqueous methanol and concentrated *in vacuo* to a small volume, then lyophilized to give a crude powder (60 g). A part of the powder (2 g) was dissolved in a small volume of water and separated into 100 fractions by a column chromatography of Sephadex LH-20 (35 × 550 mm) equilibrated with water. Active fractions 30 ~ 40 and 61 ~ 70 were respectively combined and lyophilized to yield 15 mg white powder and 220 mg brown powder. Fractions 30 ~ 40 were further purified a column of Sephadex LH-20 (15 × 550 mm) with affording 10 mg of phosmidosine B (**2**) as a white powder. Fractions 61 ~ 70 were further purified by preparative HPLC (column: Pegasil ODS 20 × 250 mm, Senshu Scientific Co. Ltd., Japan, temperature: 40°C, solvent: 7% MeOH containing 0.1% TFA, flow rate: 9.0 ml/minute, detector: 260 nm) affording 3 mg of phosmidosine (**1**) as a white powder. As the UV profile of **3** was indistinguishable from that of **1** and **2**, **3** was also purified from the elute (fractions 51 ~ 60) of the Sephadex LH-20 column.

Cell Cycle Analysis of *src*ts-NRK Cells

*Src*ts-NRK cells were cultured at permissive temperature (32°C) or at nonpermissive temperature (39°C) in EAGLE's minimal essential medium (MEM, Nissui pharmaceutical, Japan) supplemented with 10% calf serum (CS, Hyclone, Utah, U.S.A.). The effect of **1**, **2** and **3** on the cell cycle of *src*ts-NRK cells was investigated by using a flow cytometer (Epics Profile II, Coulter). *Src*ts-NRK cells were plated at 1×10^5 cells/ml in a dish and incubated for 17 hours at 39°C for arresting the cell cycle at G1 phase. The cells were simultaneously treated with various concentrations of the compounds and shifted to 32°C for releasing from the G1 arrest. After incubation for 17 hours at 32°C, the cells were harvested and treated with 50 μg/ml propidium iodide (Sigma, St. Louis, U.S.A.), 0.1% sodium citrate and 0.2% Tween 20 at 4°C for 30 minutes. DNA histograms were obtained by using a flow cytometer equipped with an argon-ion laser at 488 nm.

Morphological Reversion Activity on *src*ts-NRK Cells

*Src*ts-NRK cells maintained at 32° were seeded into a 96-well microtiter plate (2×10^4 cells/200 μl/well: Sumitomo Bakelite, Tokyo, Japan) and cultured for 4 hours at 32°C in 5% CO_2 atmosphere. Various concentrations of the compounds were added to *src*ts-NRK cells and the morphological reversion activity on *src*ts-NRK cells was measured under a microscope after 17 hours incubation at 32°C. The activity were represented as the rate of normal flat cells in total cells.

Results

Isolation

Isolation procedure of phosmidosine (**1**), phosmidosines B (**2**) and C (**3**) was summarized in Fig. 2. After Sephadex LH-20 column chromatography, fractions 61 ~ 70 strongly inhibited the cell cycle progression of *src*ts-NRK cells and fractions 30 ~ 40 weakly inhibited it. The further purification of fractions 61 ~ 70 by preparative HPLC indicated that the active principle of the fractions was phosmidosine (**1**), which had been isolated as an antifungal antibiotic. Fractions 30 ~ 40 contained phosmidosine B (**2**), which was later determined as desmethylphosmidosine. Furthermore, the other fractions (51 ~ 60) which contained phosmidosine C (**3**) were investigated for the purpose of discussing the structure-activity relationships.

Structural Determination

The physico-chemical properties of phosmidosine (**1**), phosmidosines B (**2**) and C (**3**) are summarized in Table 1. The structure of **1** was previously determined by analyses of ^{1}H and ^{13}C NMR and FAB-MS.

The molecular formula of **2**, $C_{15}H_{22}N_7O_8P$ was determined by HRFAB-MS which gave MH^+ *m/z* 460.1301 (Calcd for $C_{15}H_{23}N_7O_8P$, MH^+ 460.1345).

Fig. 2. Isolation and purification of phosmidosine (**1**), phosmidosines B (**2**) and C (**3**).

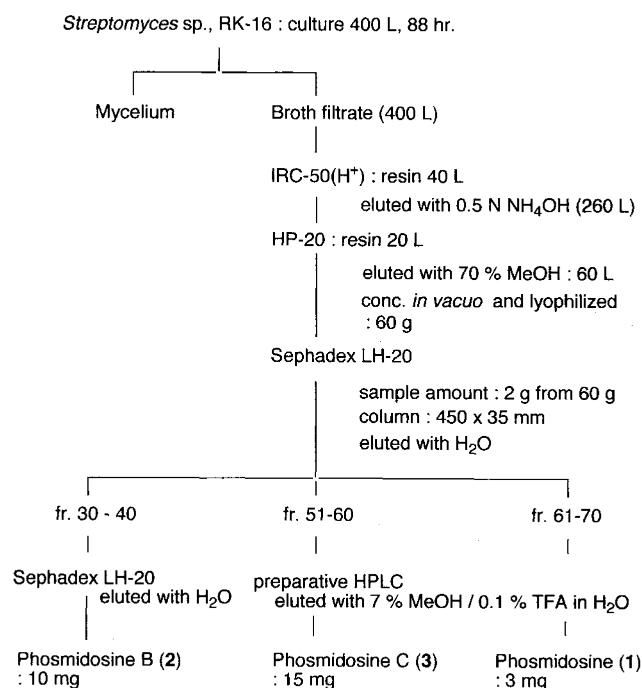


Table 1. Physico-chemical properties of phosmidosine (1) phosmidosines B (2) and C (3).

	1	2	3
mp.	230℃ (dec.)	230℃ (dec.)	230℃ (dec.)
Molecular formular	$C_{16}H_{24}N_7O_8P$	$C_{15}H_{22}N_7O_8P$	$C_{11}H_{17}N_6O_7P$
M.W.	473	459	376
HRFAB-MS (m/z)			
Calcd	474.1502 (MH^+)	460.1345 (MH^+)	377.0975 (MH^+)
Found	474.1493 (MH^+)	460.1301 (MH^+)	377.0992 (MH^+)
UV (nm, H_2O) (log ε)	267 (3.93)	267(3.90)	267(3.93)
$[\alpha]_D^{20}$ (C: 0.1, H_2O)	$-29°$	$-11°$	$-11°$

Table 2. ^1H NMR spectral data of phosmidosine (1) phosmidosines B (2) and C (3).

1	2	3
1.93 (3H, m, 4")	1.99 (3H, m, 4")	
2.14 (1H, m, 3")	2.20 (1H, m, 3")	
3.31 (2H, m, 5")	3.36 (2H, m, 5")	
3.51 (3H, d, J = 11 Hz, P-OMe)		3.63 (3H, d, J = 11 Hz, P-OMe)
4.07 (1H, m, 2")	4.10 (1H, m, 2")	
4.15 (1H, m, 5')	4.14 (1H, m, 5")	4.22 (2H, m, 5')
4.19 (1H, m, 4')	4.18 (1H, m, 4")	4.25 (1H, m, 4')
4.58 (1H, t, J = 5 Hz, 3')	4.53 (1H, t, J = 5 Hz, 3')	4.62 (1H, t, J = 5 Hz, 3')
5.15 (1H, t, J = 5 Hz, 2')	5.12 (1H, t, J = 5 Hz, 2')	5.15 (1H, t, J = 5 Hz, 2')
5.85 (1H, t, J = 5 Hz, 1')	5.86 (1H, d, J = 5 Hz, 1')	5.87 (1H, d, J = 5 Hz, C-1')
8.30 (1H, s, H-2 of base)	8.12 (1H, s, H-2 of base)	8.30 (1H, s, H-2 of base)

^1H NMR (400 MHz, D_2O) δ ppm.

The molecular formula is consistent with ^1H and ^{13}C NMR data shown in Tables 2 and 3. The UV spectrum of **2** showed the same characteristics as **1**. In the ^1H NMR spectrum of **2** (Table 2), the signal (δ 3.51, d, J= 11 Hz) corresponding to the methoxy group coupled with phosphorus of **1** disappeared, and other signals were the same as **1**. In the ^{13}C NMR spectrum, the signal (δ 55.4) assigned to the methoxy carbon connected with phosphorus also disappeared. Based on these results, the structure of **2** was determined to be desmethylphosmidosine (Fig. 1).

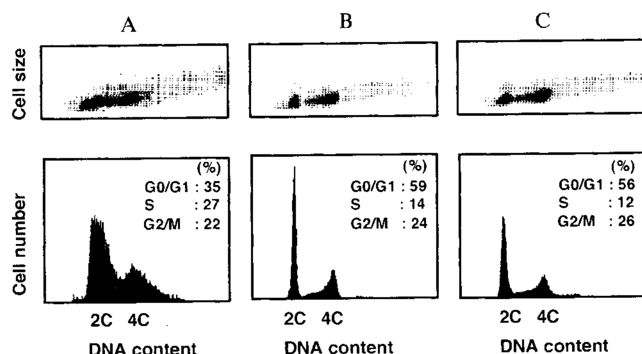
The molecular formula of **3**, $C_{11}H_{17}N_6O_7P$ was determined by HRFAB-MS which gave MH^+ m/z 377.0975 (Calcd for $C_{11}H_{18}N_6O_7P$, MH^+ 377.0992). The physico-chemical properties of **1** and **2** were almost the same. In the ^1H NMR spectrum of **3**, the signals [δ 1.93 (3H, m, H-4″), 2.14 (1H, m, H-3″), 3.31 (2H, m, H-5″), 4.07 (1H, m, H-2″)] corresponding to the prolyl group of **1** disappeared, though the signal (δ 3.63) of methoxy group coupled with phosphorus and other signals assigned in the chromophore were observed. It was con-

Table 3. ^{13}C NMR spectral data of phosmidosine (1) and phosmidosine B (2).

	δ ppm	
C	1	2
2	153.5	153.8
4	149.8[a]	150.0[c]
5	107.1	107.0
6	149.1[a]	149.4[c]
8	155.5	155.5
1'	88.7	88.8
2'	73.0[b]	73.1[d]
3'	64.9	63.6
4'	84.4	84.9
5'	67.7	68.1
1"	179.1	—[e]
2"	72.2[b]	72.5[d]
3"	32.2	32.3
4"	26.2	26.4
5"	48.7	49.1
OMe	55.4	—

100 MHz, D_2O.
[a,b,c,d]: Assignment may be interchangable.
[e]: Not observed.

Fig. 3. The effect of phosmidosine (**1**) and phosmidosine B (**2**) toward the cell cycle progression of *src*^{ts}-NRK cells.

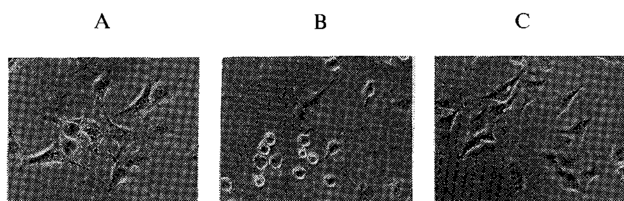


Column A shows the histogram of cells cultured at 32°C for 17 hours after the G 1 arrest. Columns B and C showed the histograms of the cells cultured at 32°C with 4 μg/ml phosmidosine (**1**) and 170 μg/ml phosmidosine B (**2**) after cultured at 39°C for 17 hours.

Fig. 4. Morphology of *src*^{ts}-NRK cells.

Cells cultured at 39°C (nonpermissive temperature) took the normal flat morphology (A). At 32°C (permissive temperature), the cells took the transformed morphology (B). The transformed phenotype was reversed to the normal by the treatment with 17 μg/ml of phosmidosine (**1**) (C).

A B C



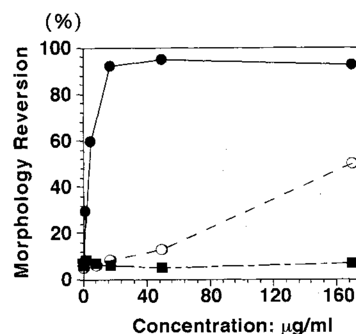
cluded that the structure of **3** is desprolylphosmidosine (Fig. 1).

Biological Activity

The effect of phosmidosine (**1**), phosmidosines B (**2**) and C (**3**) to the cell cycle progression of *src*^{ts}-NRK cells was analyzed using a flow cytometer. In the histogram of the cells cultured at 39°C, the cell cycle progression almost stopped and the band belonging to the S phase-cells disappeared. When the G1 synchronized cells cultured at 39°C were released from the G1 arrest, the broadening of G1 peak was observed (Fig. 3A) When the G1 synchronized cells were simultaneously treated with **1** (4 μg/ml) and shifted to 32°C, the broadening of G1 peak was suppressed. The observation suggests that phosmidosine inhibits the cell cycle progression of *src*^{ts}-NRK cells at G1 Phase (Fig. 3B). Though **2** showed the similar activity as **1** in respect to the cell cycle

Fig. 5. Morphological reversion activity on *src*^{ts}-NRK cells.

●; Phosmidosine, ○; phosmidosine B, ■; phosmidosine C.



inhibition, ED_{50} of **2** was 170 μg/ml higher than that of **1**. **3** had no effect on the cell cycle progression of *src*^{ts}-NRK cells.

When *src*^{ts}-NRK cells were cultured at 32°C, spherical transformed cells were observed. On the other hand, flat normal cells appeared at 39°C in place of spherical cells. **1** and **2** induced morphological reversion of *src*^{ts}-NRK cells from spherical transformed cells to normal flat cells at 32°C (Fig. 4), but **3** didn't show the morphological reversion.

The doses of **1**~**3** required for the morphological reversion of *src*^{ts}-NRK cells were plotted in Fig. 5. Both **1** and **2** showed the cytotoxicity to *src*^{ts}-NRK cells at the concentration of 170 μg/ml or more. The concentration of **1** between the morphological reversion activity and the cytotoxicity is *ca.* 10-fold, but that of **2** is almost the same.

Discussion

Phosmidosine **1** was previously isolated by URAMOTO *et al.* as an antifungal antibiotic[7]. In the course of our screening utilizing mammalian cells, we have identified that **1** had the morphological reversion activity on *src*^{ts}-NRK cells.

The existence of desmethylphosmidosine, phosmidosine B (**2**) and methylprolylamidephosmidosine was predicted on the basis of FAB-MS of *O*-isopropylidine derivatives prepared from the crude phosmidosine isolate[8]. However, those compounds have not been isolated and characterized yet. In this paper, desmethylphosmidosine and desprolylphosmidosine, named phosmidosine B (**2**) and phosmidosine C (**3**), respectively were isolated and characterized for the first time.

Both **1** and **2** showed the inhibitory activity of the cell cycle progression (Fig. 3) and the morphological

reversion activity of src^{ts}-NRK cells (Fig. 4). However, the potency of **1** was superior to **2** (Fig. 5). Moreover **3**, deprolyl derivative of **1**, did not show the inhibitory activity on src^{ts}-NRK cells. According to these observations, it is suggested that prolyl moiety in the phosmidosine molecule is very important for its inhibitory activity and that methyl group enhances the activity.

The mode of action of **1** and **2** should be the next issue to be investigated.

Acknowledgments

We are grateful to Drs. K. Isono and M. Uramoto for helpful discussions and Kaken Pharmaceutical Co. Ltd. for the culture of *Streptomyces* sp. strain RK-16. A part of this work was supported by a Grant from Biodesign Research Program in RIKEN and a Grant from the Ministry of Education, Science, and Culture, Japan.

References

1) Cooper, J. A. & B. Howell: The when and how of Src regulation. Cell. 73: 1051, 1993
2) Weinberg, R. A.: Tumor suppressor genes. Science. 254: 1138~1146, 1991
3) Uehara, Y.; Y. Murakami, Y. Sugimoto & S. Mizuno: Mechanism of reversion of Rous sarcoma virus transformation by herbimycin A: reduction of total phosphotyrosine levels due to reduced kinase activity and increased turnover of $p60^{v\text{-}src1}$. Cancer Res. 49: 780~785, 1989
4) Koshino, S.; H. Koshino, N. Matsuura, K. Kobinata, R. Onose, K. Isono & H. Osada: A new cyclic lipopeptide antibiotic, enamidonin. J. Antibiotics 48: 185~187, 1995
5) Osada, H.; H. Koshino, K. Isono, H. Takahashi & G. Kawanishi: Reveromycin A, a new antibiotic which inhibits the mitogenic activity of epidermal growth factor. J. Antibiotics 44: 259~261, 1991
6) Takahashi, H.; H. Osada, H. Koshino, T. Kudo, S. Amano, S. Shimizu, M. Yoshihama & K. Isono: Reveromycins, new inhibitors of eukaryotic cell growth. I. Producing organism, fermentation, isolation and physicochemical properties. J. Antibiotics 45: 1409~1413, 1992
7) Uramoto, M.; C. J. Kim, K. Shinya, H. Kusakabe & K. Isono: Isolation and characterization of phosmidosine a new antifungal nucleotide antibiotic. J. Antibiotics 44: 375~381, 1991
8) Phillips, D. R.; M. Uramoto, K. Isono & J. M. McCloskey: Structure of the antifungal nucleotide antibiotic phosmidosine. J. Org. Chem. 58: 854~859, 1993