Novel Mammalian Cell Cycle Inhibitors, Tryprostatins A, B and Other Diketopiperazines Produced by *Aspergillus fumigatus*

I. Taxonomy, Fermentation, Isolation and Biological Properties

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Two novel diketopiperazines named tryprostatins A (**1**) and B (**2**) and a new natural product belonging to the diketopiperazine series, designated as demethoxyfumitremorgin C (**3**), together with four known diketopiperazines, fumitremorgin C (**4**), 12,13-dihydroxyfumitremorgin C (**5**), fumitremorgin B (**6**) and verruculogen (**7**), were isolated from the fermentation broth of *Aspergillus fumigatus* BM939 by the combined use of solvent extraction, silica gel column chromatography, preparative TLC and repeated-preparative HPLC. The diketopiperazines showed an inhibitory activity on the cell cycle progression of mouse tsFT210 cells in the M phase with the MIC values of 16.4 μM (**1**), 4.4 μM (**2**), 0.45 μM (**3**), 4.1 μM (**4**), 60.8 μM (**5**), 26.1 μM (**6**) and 12.2 μM (**7**), respectively.

Cell cycle is a strictly regulated process by which the cells complete the division into two daughter cells. It is believed that a universal control mechanism common to all eukaryotic cells exists and cellular factors play crucial roles in the cell cycle regulation[1~4]. The maturation promoting factor (MPF), for instance, is known to be a common M-phase promoting factor for all eukaryotic cells and functions as a universal trigger for mitosis[2~4]. The active MPF functions as a complex of cdc2 kinase and cyclin B and the regulation of MPF activity is associated with a series of phosphorylation/dephosphorylation reaction of cdc2 kinase[3,4]. Development of specific inhibitors is a successful approach to elucidate the biological function of a particular cellular factor and there are many examples applying inhibitors to elucidate the regulatory mechanism of the cell cycle[5~12]. Therefore, new and specific inhibitors of the cell cycle should be useful tools for the investigation of the cell cycle mechanism and also good candidates for cancer chemotherapy[11].

From the above viewpoint, the screening for new mammalian cell cycle inhibitors has been started in our laboratory[5,13,14]. In the course of the screening, we found that a fungal strain BM939 isolated from a sea sediment produced new inhibitors of mammalian cell cycle and we have preliminarily reported two novel diketopiperazines named tryprostatins A (**1**) and B (**2**)[15]. In a continuation of the work, we have recently isolated a new natural product belonging to the diketopiperazine series, designated as demethoxyfumitremorgin C[16] (**3**), together with known diketopiperazines, fumitremorgin C[17,18] (**4**), 12,13-dihydroxyfumitremorgin C[19] (**5**), fumitremorgin B[17,20,21] (**6**) and verruculogen[22] (**7**).

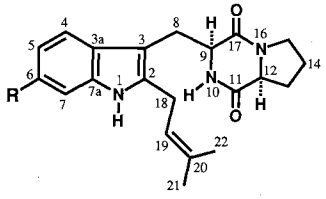
In this paper, we report the taxonomy and fermentation of the producing strain and the isolation and biological properties of the diketopiperazines including tryprostatins in detail.

Materials and Methods

Producing Organism and Taxonomic Studies

The producing fungal strain BM939 was isolated from a sea sediment sample collected in the sea bottom (760 meters deep) at the mouth of Oi river, Sizuoka prefecture, Japan. The strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, under the accession number FERM P-15067. Taxonomic studies of the fungal strain BM939 were done according to the method of KLICH and PITT[23]. The color guide of KORNERUP and WANSCHER[24] was used in determining and standardizing colors. The media, CYA (yeast extract 0.5%, sucrose 3.0%, K_2HPO_4 0.1%, $NaNO_3$ 0.3%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, $ZnSO_4 \cdot 7H_2O$ 0.001%, $CuSO_4 \cdot 5H_2O$ 0.0005%, agar 1.5%), CY20S (CYA medium with sucrose increased to 20%) and MEA (malt extract 2.0%, peptone 0.1%, glucose 2.0%, agar 2.0%), were used for the identification of the fungus. Light microscope (LM; Leitz Orthoplan) and scanning electron microscope (SEM; Hitachi S-2400) were used for morphological observation. For scanning electron microscopy, the isolate sporulating on CYA was fixed and dehydrated[25]. After critical-point drying with a Hitachi HCP-2, the material was coated with Pt-Pd (*ca.* 200 Å thick) in an Eiko ion coater (IB-3).

Fig. 1. Structures of tryprostatins A (**1**) and B (**2**) and other diketopiperazines **3**~**7**.



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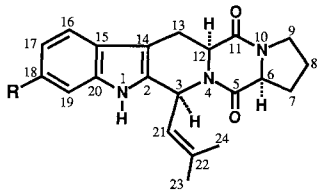
2 R=H



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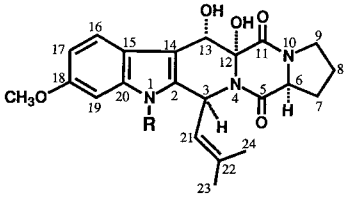
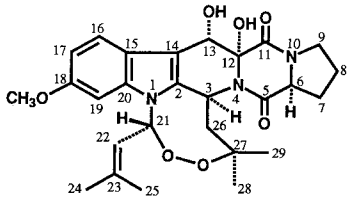


5 R=H
6 R=Isoprenyl



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Fermentation of the Fungal Strain BM939

The strain BM939 on a potato dextrose agar slant was inoculated into a 500-ml cylindrical flask containing 70 ml of the seed medium consisting of glucose 3%, soluble starch 2%, soybean meal 2%, K_2HPO_4 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05% (adjusted to pH 6.5 prior to sterilization) and cultured at 28°C for 48 hours on a rotary shaker at 300 rpm. The seed culture was transferred into 18 liters of the production medium having the same composition of the seed medium with 0.05% of antifoam reagents, CA-123 and KM-68, in a 30-liter jar fermenter (Komatsugawa Chemical and Industrial Machine Co.). The fermentation was carried out at 28°C for 72 hours with an agitation rate of 350 rpm and an aeration rate of 7 liters/minute.

Conditions for Isolation of the Diketopiperazines

Analytical TLC and preparative TLC were done on pre-coated silica gel 60 F_{254} plates (0.25 or 0.5 mm thick, 20 × 20 cm, Merck) and the spots were detected under UV lights (254 and 365 nm) or by the use of 10% aqueous sulfuric acid reagent. Silica gel 60 (230~400 mesh, Merck) was used for column chromatography. Analytical HPLC was carried out on a reversed phase column (CAPCELL PAK C_{18}, 4.6 × 250 mm, Shiseido Co., Japan) by the use of a HPLC equipment with a Hitachi L-6000 pump and a Waters 991J photodiode array detector system. Preparative HPLC was performed on a HPLC system equipped with a Hitachi L-6000 pump, a SSC UV detector and a CAPCELL PAK C_{18} column (20 × 250 mm, Shiseido).

Bioassay for Cell Cycle Inhibitory Activity

A mouse tsFT210 cell line, which is a temperature-sensitive $p34^{cdc2}$ mutant isolated from the mouse mammary carcinoma cell line FM3A[26,27], was used for the bioassay. The cells were routinely maintained at 32°C in RPMI-1640 medium supplemented with 5% calf serum (HyClone Inc., Logan, UT, U.S.A.) in the presence of 30 μg/ml of penicillin and 42 μg/ml of streptomycin under a humidified atmosphere of 5% CO_2 and 95% air.

For randomly cultured assay, each 5 μl of sample solutions was added into each well of the 24 multi-well plate containing the cells at the density of 2×10^5 cells/ml in 0.5 ml of fresh medium and the cells were asynchronously cultured at 32°C for 17 hours.

In the synchronously cultured assay, the cells at the density of 2×10^5 cells/ml in the 24 multi-well plate were pre-incubated at 39.4°C (nonpermissive temperature) for 17 hours to obtain G2-arrested cells. Each 5 μl of sample solutions was added into each well and the cells were incubated at 32°C for 4 hours.

After incubation, both the synchronously cultured or asynchronously cultured cells were treated in the same way as described below. The cells in each well of the 24 multi-well plate were respectively transferred into each of 1.5 ml Eppendorf centrifuge tubes and harvested by centrifugation at 3000 rpm for 3 minutes at 4°C. The cells were washed once with cold phosphate-buffered saline and harvested again by centrifugation under the same condition. Then, 150 μl of propidium iodide in water solution (propidium iodide 50 μg/ml, sodium citrate 0.1% and Nonidet P-40 0.2%) was added into each of tubes and the cells were stained at 4°C for 30 minutes. The cells were then subjected to a flow cytometric analysis and the distribution within the cell cycle was calculated using a computing program, CytoLogic (Coulter).

Staurosporine and reveromycin A isolated in our laboratory were dissolved in methanol (MeOH) at a concentration of 43 μM and 146 μM, respectively, and used as a positive control for assays. The samples tested were

dissolved in water-MeOH and added into the cell culture at a final MeOH concentration of less than 1%.

Assay for cdc2 Kinase Activity

The cdc2 kinase activity was measured based on the method of ARION and MEIJER[28] using the tsFT210 cells. The cell-extract was prepared, as described below, from the tsFT210 cells synchronized in the M-phase. The cells (5×10^6) were synchronized at the late G2 phase by incubation at 39.4°C for 17 hours and released to enter into M-phase by incubation at 32°C for 2 hours. Then, the cells were washed twice with cold phosphate-buffered saline, suspended in 1 ml of homogenizing buffer (50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 0.1% Triton X-100, 5 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM DTT), and disrupted by sonication. A clear supernatant which contained the total protein at a concentration of 1~2 mg/ml was obtained by centrifugation at 1500 rpm for 10 minutes at 4°C.

The 40 μl of Sepharose beads linked to $p13^{suc1}$ were added to 200 μl of the tsFT210 cell lysate, and the mixture was incubated for 30 minutes at 4°C to co-precipitate cdc2 kinase. The supernatant was removed by centrifugation at 5000 rpm for 3 minutes at 4°C, and the beads were washed twice with the homogenizing buffer. All of the precipitated beads which containing cdc2 kinase were used for the following phosphorylation assay. The reaction mixture consisted of the beads, 1 mg/ml histone H1, 3.7×10^4 Bq[γ-^{32}P]ATP and various concentrations of a sample in 25 μl. After 30 minutes incubation at 32°C, the reaction was stopped by adding SDS-PAGE sample buffer[29] and boiling for 3 minutes. After the gel electrophoresis, the kinase activity was visualized by autoradiography and quantified by a laser densitometer (Molecular Dynamics Co. Ltd., Sunnyvale, CA, U.S.A.).

Measurement of Protein Kinase Activities

The cyclic AMP dependent protein kinase (PKA) and Ca^{2+}/phospholipid dependent protein kinase (PKC) were prepared from the HeLa cell lysate according to the procedure reported[30]. Histone H2B and histone H1 were used as substrates for PKA and PKC, respectively, in the kinase assay. Autophosphorylation of epidermal growth factor (EGF) receptor was measured by the method in the literature[31].

Antimicrobial Activity

Antimicrobial activity was determined by the conventional paper disk method using ADVANTEC TOYO paper disks (i.d. 8 mm, thin, Toyo Roshi Kaisha, Ltd., Japan). Nutrient agar medium, potato sucrose agar medium and glucose-peptone-yeast extract agar medium were used for bacteria, fungi and *Chlorella vulgaris*, respectively. Antimicrobial activity was observed after 24 hours at 37°C for bacteria, 48 hours at 28°C for fungi and 120 hours at 24°C under light for *Chlorella vulgaris*.

Results

Taxonomy

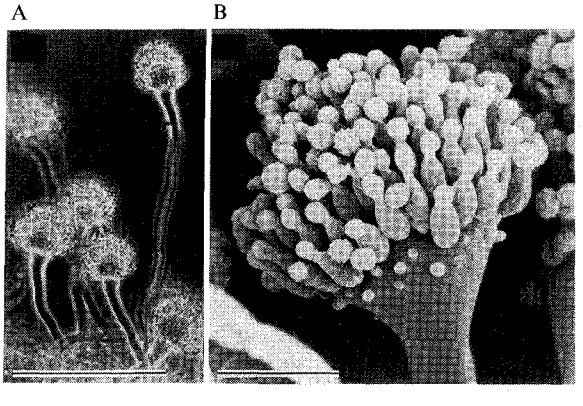
Cultural and morphological characteristics of the producing strain BM939 (FERM P-15067) are shown below (Fig. 2).

Colonies on CYA at 25°C after 7 days reaching 45~50 mm in diameter, dark green (25F4-6) in the center to opaline green (25B-C 4-6); mycelium white; exudate absent; reverse light yellow; soluble pigment absent; texture velutinous, plane to slightly radially furrowed. Colonies on CY20S at 25°C after 7 days reaching *ca.* 55 mm in diameter, dark green (25F4-6); mycelium inconspicuous, white; reverse bright to brownish yellow; texture velutinous, plane; others same on CYA. Colonies on MEA at 25°C after 7 days reaching *ca.* 60 mm in diameter, dark green (27F5-6) in the center to dull green (27D3-4); mycelium inconspicuous; reverse dull yellow; texture velutinous, plane; others same on CYA. Colonies on CYA at 37°C after 7 days reaching more than 70 mm in diameter, greenish grey (25-27F1) to grey; mycelium inconspicuous; reverse yellowish white; texture velutinous, plane to slightly radially furrowed; others same on CYA. Conidial heads columnar. Stipes (conidiophores) on CYA smooth-walled, 150~500 × 5~10 μm, expanding gradually into pyriform vesicles (15~25 μm in diameter). Aspergilla uniseriate. Phialides on CYA over upper two-thirds of the vesicle, obpyriform, straight or slightly curving at the edge of a vesicle, 5~8 × 2.5~3 μm. Conidia on CYA globose to subglobose, finely roughened under LM, spinose under SEM, 2.5~3.5 μm in diameter.

According to the above characteristics, the fungal strain BM939 was identified as *Aspergillus fumigatus*.

Fig. 2. Aspergilla of *Aspergillus fumigatus* BM939, on CYA, 7 days, 25°C.

A. phase contrast (bar = 100 μm). B. SEM (bar = 10 μm).



Fermentation and Isolation

In the course of the fermentation of strain BM939, the production of active components was monitored by the inhibitory activity of both broth supernatant and mycelial extract on the cell cycle progression of the tsFT210 cells. The broth supernatant and mycelial extract for determination of the activity were obtained by centrifugation of the whole broth (50 ml) at 3500 rpm for 20 minutes followed by extraction of the mycelia with 80% aqueous acetone. The inhibitory activity reached the maximum at 48~72 hours fermentation.

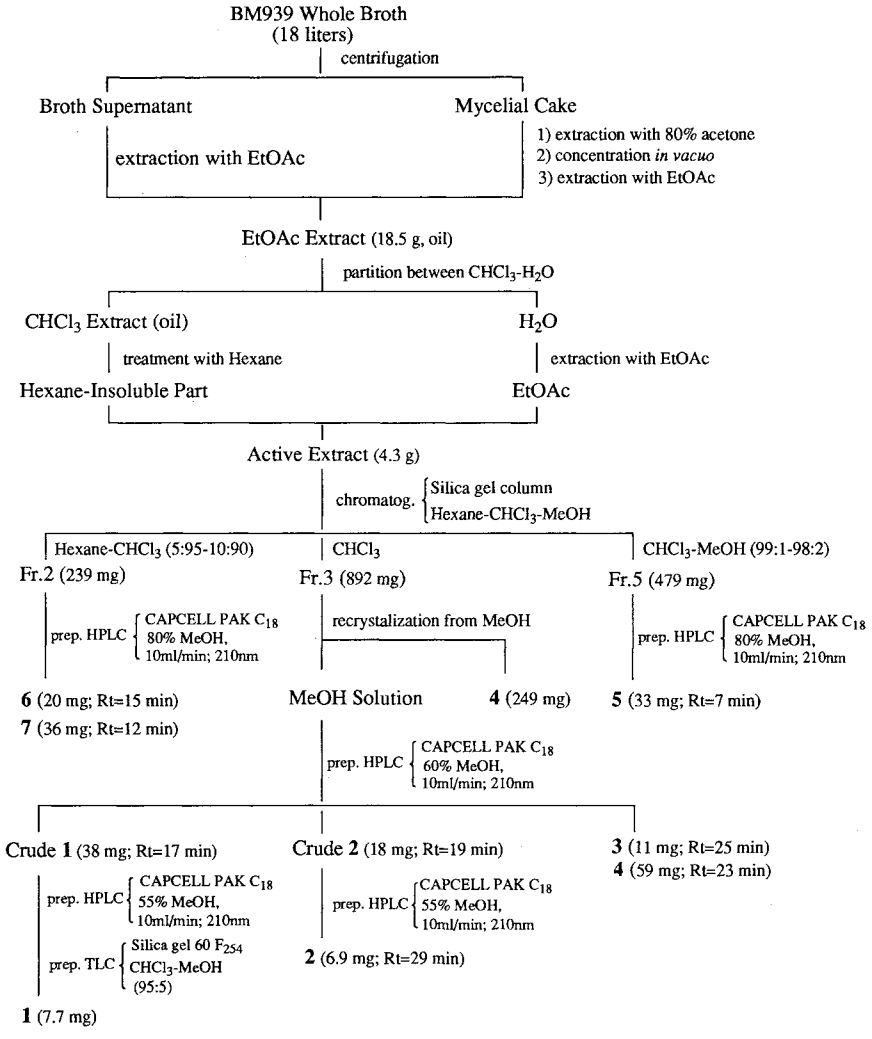
The diketopiperazines were isolated from the whole broth of the fermentation. The isolation procedure are outlined in Fig. 3. As the broth supernatant and the mycelial extract showed an inhibitory activity on the cell cycle progression of tsFT210 cells, they were extracted respectively with the same volume of EtOAc and the following separation procedure was monitored by the inhibitory activity. The EtOAc solutions obtained were combined and concentrated *in vacuo* to afford an oily extract (18.5 g) which was further purified to give an active extract (4.3 g). This extract was then separated by a combination of column chromatography, repeated HPLC and preparative TLC to obtain **1** (7.7 mg), **2** (6.9 mg), **3** (11 mg), **4** (308 mg), **5** (33 mg), **6** (20 mg) and **7** (36 mg), respectively (Fig. 3).

Biological Properties

Inhibitory activities of **1**~**7** on the cell cycle progression of tsFT210 cells were determined by both randomly cultured and synchronously cultured methods.

In the randomly cultured assay, compounds **1**, **3** and **4** inhibited the cell cycle progression of tsFT210 cells in the M phase with the MIC values of 44.6 μM (**1**), 9.46 μM (**3**) and 87.1 μM (**4**), respectively, and **2** showed a strong cytotoxicity at the concentrations higher than 71 μM and no effect at the concentrations lower than 35 μM. While the others, **5**~**7**, showed no effect on the cell cycle progression of tsFT210 cells even at a concentration of 100 μg/ml. The compounds **1**, **3** and **4** had IC_{50} values

Fig. 3. Isolation procedure for **1**~**7**.



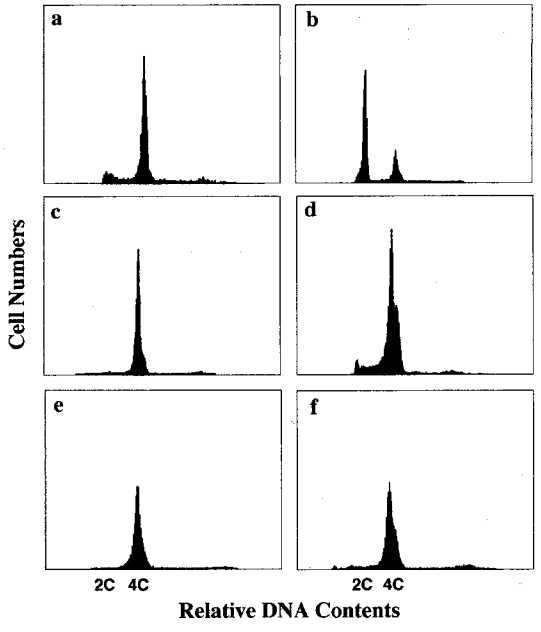
of 78.7 μM (**1**), 13.5 μM (**3**) and 187.3 μM (**4**), respectively, in the randomly cultured assay.

In contrast to the randomly cultured assay, all of the compounds **1**~**7** showed an inhibitory activity on the cell cycle progression of tsFT210 cells at the M phase in the synchronously cultured assay. The MIC and IC_{50} values for **1**~**7** are summarized in Table 1. Compounds **5**~**7** slightly inhibited the cell cycle in the M phase throughout the concentration range from MIC to 100 μg/ml and their exact IC_{50} values could not be determined because of their insolubility in the assay solution. As a typical result, several flow cytometric histograms obtained by the synchronously cultured assay with **1**~**4** are shown in Fig. 4. Morphological observations of the corresponding cells are given in Fig. 5.

Table 1. Inhibitory effects of **1**~**7** on the cell cycle progression of tsFT210 cells.

Compounds	1	2	3	4	5	6	7
MIC (μM)	16.4	4.4	0.45	4.1	60.8	26.1	12.2
IC_{50} (μM)	78.7	18.8	1.78	14.0	>243.0	>209.0	>196.0

Fig. 4. Effects of **1**~**4** on the cell cycle progression of tsFT210 cells.



The tsFT210 cells were synchronized in the G2 phase (a) by incubation at 39.4°C for 17 hours at a density of 2×10^5 cells/ml in RPMI-1640 medium supplemented with 5% calf serum. Then the cells were transferred to 32°C for 4 hours in the absence (b) or in the presence of 50 μg/ml of **1** (c), 12.5 μg/ml of **2** (d), 2.5 μg/ml of **3** (e), and 50 μg/ml of **4** (f), respectively.

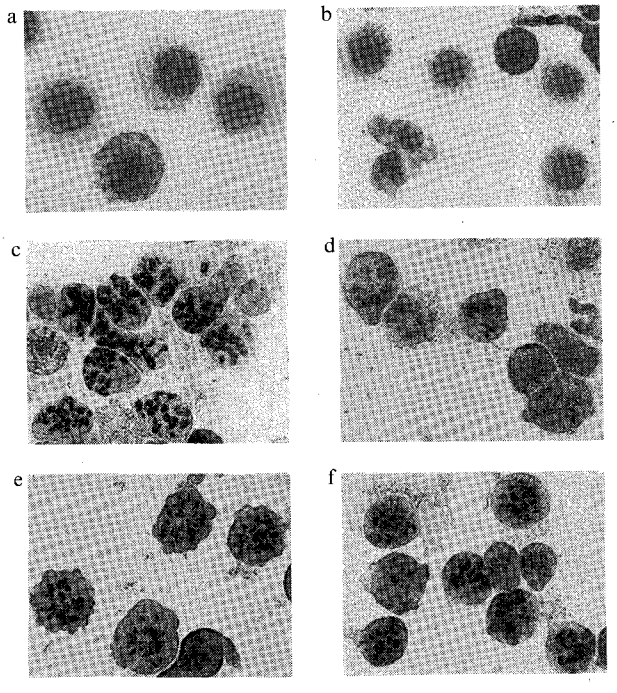
Kinase assays were carried out for **1**~**4**. In the kinase assays, **1**~**4** did not inhibit cdc2 kinase, PKA and PKC activities or the autophosphorylation of EGF receptor even at the concentration of 1000 μg/ml.

Antimicrobial activities of **1**~**7** were determined under a concentration of 20 μg/disk on the growth of *Escherichia coli* BE1186, *Pseudomonas aeruginosa* L-form, *Staphylococcus aureus* 209P IFO 12732, *Pynicularia oryzae*, *Candida albicans* IFO 594 and *Chlorella vulgaris*. Among the organisms tested, only the growth of *Escherichia coli* BE1186 which is a sensitive strain towards various antibiotics was slightly inhibited by the compounds **1** (17 mm), **2** (12.5 mm), **3** (10.5 mm), **5** (12 mm), **6** (15.5 mm) and **7** (10.5 mm), respectively.

Discussion

Cultural and morphological characteristics of the producing strain BM939 (FERM P-15067) agreed well with the previous descriptions of *Aspergillus fumigatus* Fres.[23,32,33]. We, therefore, identified this strain as *A. fumigatus*. This species has been reported from various

Fig. 5. Morphology of tsFT210 cells treated by **1**~**4**.



The tsFT210 cells were synchronized in the G2 phase (a) by incubation at 39.4°C for 17 hours at a density of 2×10^5 cells/ml in RPMI-1640 medium supplemented with 5% calf serum. Then the cells were transferred to 32°C for 4 hours in the absence (b) or in the presence of 50 μg/ml of **1** (c), 12.5 μg/ml of **2** (d), 2.5 μg/ml of **3** (e), and 50 μg/ml of **4** (f), respectively. The cells were spread on glass slides and visualized by stain with wright modified reagent (Sigma). The nuclei of the cells were observed under a microscope (TMD-2, Nikon, Tokyo, Japan).

substrates in most parts of the world as well as marine habitat[33].

The tsFT210 cells exponentially grew at 32°C with a doubling time of 14 hours and the cell cycle progresses throughout G1, S, G2, and M phases. Therefore, the randomly cultured assay can be used for the screening of all G1-, S-, and G2/M-phase inhibitors. In contrast, when the cells arrested at the late G2 phase by incubation at 39.4°C were transferred to 32°C in the synchronously cultured assay, the cells synchronously entered into mitosis and moved to G1 phase. In our general experience, the G2 synchronously cultured cells were more sensitive to the G2/M-phase inhibitors than the randomly cultured cells (see also the present results). We therefore used the randomly cultured assay to discover that the fungal strain BM939 produces G2/M-phase inhibitors and then, the synchronously cultured assay was the primary assay used in the present study.

Incidentally, we have isolated two novel diketopiperazines named tryprostatins A (**1**) and B (**2**) and a new natural product belonging to the diketopiperazine series, designated as demethoxyfumitremorgin C (**3**), from the fermentation broth of the fungal strain BM939 together with four known diketopiperazines, **4**, **5**, **6**, and **7**, as new inhibitors of the mammalian cell cycle. As described in the following paper[34], the molecules of **1** and **2** are composed from a 2-isoprenyltryptophan moiety and a proline residue, forming a diketopiperazine unit, which are distinguished from the molecules of fumitremorgin series[16~22] in the respect of opening the centered heterocyclic ring at C-N bond between 18 and 10 positions. Only few kinds of natural products structurally related to **1** and **2** such as deoxybrevianamide E had so far been reported[35], and the present result provides the first example of natural product belonging to this novel class as the inhibitor of mammalian cell cycle. Further, demethoxyfumitremorgin C[16] (**3**) was isolated for the first time from the natural source.

The inhibitory effects of **1**~**7** on the cell cycle progression of the tsFT210 cells suggested some structure-activity relationships. As shown in Table 1, the compounds **1**~**4** showed stronger inhibitory activities than those of **5**~**7**. From a view of the structures of **3**~**7** (Fig. 1), lack of substitution at 1, 12 and 13 positions on the penta-heterocyclic ring skeleton in **3**~**7** are a likely requirement for significant inhibition of cell cycle progression in the M phase. On the other hand, it was indicated by comparing the structures (Fig. 1) and inhibitory activities (Table 1) of **3** and **4** that introduction of a methoxy group on the aromatic ring causes a marked decrease in the intensity of the inhibitory activity. The similar result was also observed in the case of **1** and **2**, revealing a potent effect of the methoxy group in **1**~**4** on the inhibition of the cell cycle in the M phase. Also, respective comparison of the inhibitory activities between **1** and **4** and between **2** and **3** suggested that the formation of the centeral heterocyclic ring in **3** and **4** is likely to be an important factor in increasing their inhibitory activities. To our knowledge, this is the first report describing the inhibitory effects of diketopiperazines on the cell cycle progression of the mammalian cells and the present result provides new group of M phase inhibitors of the mammalian cell cycle.

In the present study, morphological examinations showed that the tsFT210 cells treated by **1**~**4** were trapped mainly in the M phase as shown in Fig. 5. In addition, in the kinase assay, **1**~**4** did not directly inhibit cdc2 kinase activity which is essential to MPF action for the cell cycle to escape G2/M boundary. As **1**~**4** did not inhibit the protein kinase activities of PKA, PKC and EGF receptor, the mode of action should be elucidated in a forthcoming paper. Morphological observations suggest that the effects of **1**~**4** are similar to that of nocodazole or colcemid (data not shown).

Detailed studies on their mechanism of action are currently being undertaken and the structural studies will be reported in a succeeding paper[34].

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References

1) HARTWELL, L. H. & T. A. WEINERT: Checkpoints: Controls that ensure the order of cell cycle events. Science 246: 629~634, 1989
2) O'FARRELL, P. H.; B. A. EDGAR, D. LAKICH & C. F. LEHNER: Directing cell division during development. Science 246: 635~640, 1989
3) NURSE, P.: Universal control mechanism regulating oneset of M-phase. Nature 344: 503~508, 1990
4) NORBURY, C. & P. NURSE: Animal cell cycles and their control. Ann. Rev. Biochem. 61: 441~470, 1992
5) USUI, T.; M. YOSHIDA, K. ABE, H. OSADA, K. ISONO & T. BEPPU: Uncoupled cell cycle without mitosis induced by a protein kinase inhibitor, K-252a. J. Cell Biol. 115: 1275~1282, 1991
6) ABE, K.; M. YOSHIDA, T. USUI, S. HORINOUCHI & T. BEPPU: Highly synchronous culture of fibroblasts from G2 block caused by staurosporine, a potent inhibitor of protein kinases. Exp. Cell Res. 192: 122~127, 1991
7) TH'NG, J. P. H.; X.-W. GUO, R. A. SWANK, H. A. CRISSMAN & E. M. BRADBURY: Inhibition of histone phosphorylation by staurosporine leads to chromosome decomposition. J. Biol. Chem. 269: 9568~9573, 1994
8) YAMASHITA, K.; H. YASUDA, J. PINES, K. YASUMOTO, H. NISHITANI, M. OHTSUBO, T. HUNTER, T. SUGIMURA & T. NISHIMOTO: Okadaic acid, a potent inhibitor of type 1 and type 2a protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. EMBO J. 9: 4331~4338, 1990
9) FELIX, M.-A.; P. COHEN & E. KARSENTI: Cdc2 H1 kinase

is negatively regulated by a type 2A phosphatase in the *Xenopus* early embryonic cell cycle: evidence from the effects of okadaic acid. EMBO J. 9: 675～683, 1990

10) PAULSON, J. R.; W. A. CIESIELSKI, B. R. SCHRAM & P. W. MESNER: Okadaic acid induces dephosphorylation of histone H1 in metaphase-arrested HeLa cells. J. Cell Science 107: 267～273, 1994
11) ROBERGE, M.; C. TUDAN, S. M. F. HUNG, K. W. HARDER, F. R. JIRIK & H. ANDERSON: Antitumor drug fostriecin inhibits the mitotic entry checkpoint and protein phosphatases 1 and 2A. Cancer Res. 54: 6115～6121, 1994
12) GUO, X. W.; J. P. H. TH'NG, R. A. SWANK, H. J. ANDERSON, C. TUDAN, E. M. BRADBURY & M. ROBERGE: Chromosome condensation induced by fortriecin does not require $p34^{cdc2}$ kinase activity and histone H1 hyperphosphorylation, but is associated with enhanced histone H2A and H3 phosphorylation. EMBO J. 14: 976～985, 1995
13) OSADA, H.: Screening of bioactive microbial products (molecular probes) which regulate mammalian cell functions. Actinomycetol. 8: 120～121, 1994
14) HAMAGUCHI, T.; T. SUDO & H. OSADA: RK-682, a potent inhibitor of tyrosine phosphatase, arrest the mammalian cell cycle progression at G1 phase. FEBS Letters 372: 54～58, 1995
15) CUI, C.-B.; H. KAKEYA, G. OKADA, R. ONOSE, M. UBUKATA, I. TAKAHASHI, K. ISONO & H. OSADA: Tryprostatins A and B, novel mammalian cell cycle inhibitors produced by *Aspergillus fumigatus*. J. Antibiotics 48: 1382～1384, 1995
16) KODATO, S.; M. NAKAGAWA, M. HONGU, T. KAWATA & T. HINO: Total synthesis of (+)-fumitremorgin B, its epimeric isomers, and demethoxy derivatives. Tetrahedron 44: 359～377, 1988
17) HINO, T.; T. KAWATA & M. NAKAGAWA: A synthesis of so-called fumitremorgin C. Tetrahedron 45: 1941～1944, 1989
18) STEYN, P. S. & R. VLEGGAAR: Tremorgenic mycotoxins. Progress in the Chemistry of Organic Natural Products (Founded by L. ZECHMIESTER, Edited by W. HERZ, H. GRISEBACH and G. W. KIRBY). 48: 1～80, 1985
19) ABRAHAM, W.-R. & H.-A. ARFMANN: 12,13-Dihydroxyfumitremorgin C from *Aspergillus fumigatus*. Phytochem. 29: 1025～1026, 1990
20) YAMAZAKI, M.; K. SASAGO & K. MIYAKI: The structure of fumitremorgin B (FTB), a tremorgenic toxin from *Aspergillus fumigatus* Fre. J. Chem. Soc., Chem. Comm. 1974, 408～409
21) YAMAZAKI, M. & H. FUJIMOTO: Crystal structure and absolute configuration of fumitremorgin B, a tremorgenic toxin from *Aspergillus fumigatus* Fre. Tetrahedron Lett. 1975, 27～28
22) FAYOS, J.; D. LOKENSGARD, J. CLARDY, R. J. COLE & J. W. KIRKSEY: Structure of verruculogen, a tremor producing peroxide from *Penicillium verruculosum*. J. Am. Chem. Soc. 96: 6785～6787, 1974
23) KLICH, M. A. & J. I. PITT: A Laboratory Guide to Common *Aspergillus* Species and Their Teleomorphs. Commonwealth Scientific and Industrial Research Organization, Division of Food Processing, North Ryde, Australia, 1988
24) KORNERUP, A. & J. H. WANSCHER: Methuen Handbook of Colour, 3rd ed. Eyre Methuen, London, 1978
25) COLE, G. T. & R. A. SAMSON: Patterns of Development in Conidial Fungi. pp. 127～130, Pitman, London, 1979
26) MINEO, C.; Y. MURAKAMI, Y. ISHIMI, F. HANAOKA & M.-A. YAMADA: Isolation and analysis of a mammalian temperature-sensitive mutant defective in G2 functions. Exp. Cell Res. 167: 53～62, 1986
27) TH'NG, J. P. H.; P. S. WRIGHT, J. HAMAGUCHI, M. G. LEE, C. J. NORBURY, P. NURSE & E. M. BRADBURY: The FT210 cell line is a mouse G2 phase mutant with a temperature-sensitive *CDC2* gen product. Cell 63: 313～324, 1990
28) ARION, D. & L. MEIJER: M-phase-specific protein kinase from mitotic aea urchin eggs: cyclin activation dependent on protein synthesis and phosphorylation but does not require DNA or RNA synthesis. Exp. Cell Res. 183: 361～375, 1989
29) LAEMMLI, U. K: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680～685, 1970
30) INAGKI, M.; Y. GONDA, M. MATSUYAMA, K. NISHIZUKA, Y. NISHI & C. SATO: Intermediate filament reconstitution *in vitro*. The role of phosphorylation on the assembly-disassembly of desmin. J. Biol. Chem. 263: 5970～5978, 1988
31) CARPENTER, G.; L. KING & S. COHEN: Epidermal growth factor stimulates phosphorylation in membrane preparations *in vitro*. Nature 276: 409～410, 1978
32) RAPER, K. B. & D. I. FENNELL: The Genus *Aspergillus*. Williams & Wilkins, Baltimore, 1965
33) DOMSCH, K. H.; W. GAMS & T.-H. ANDERSON: Compendium of Soil Fungi, Vol. 1. pp. 94～98, Academic Press, London, 1980
34) CUI, C.-B.; H. KAKEYA & H. OSADA: Novel mammalian cell cycle inhibitors, tryprostatins A, B and other diketopiperazines produced by *Aspergillus fumigatus*. II. Physico-chemical properties and structures. J. Antibiotics 49: 534～540, 1996
35) STEYN, P. S.: The structures of five diketopiperazines from *Aspergillus ustus*. Tetrahedron 29: 107～120, 1973