COMMUNICATIONS TO THE EDITOR

Spirotryprostatin B, a Novel Mammalian Cell Cycle Inhibitor Produced by *Aspergillus fumigatus*

Sir:

In previous papers[1~3], we reported three new natural diketopiperazines, tryprostatins A, B and demethoxyfumitremorgin C together with fumitremorgin C, 12,13-dihydroxyfumitremorgin C, fumitremorgin B and verruculogen, as a new group of M-phase inhibitors of the mammalian cell cycle, which were isolated from the secondary metabolites of a fungus *Aspergillus fumigatus*. In order to obtain larger amounts of those compounds to examine their biological activities and mechanism of action in detail, we carried out a large scale fermentation of the producing strain and from the fermentation broth, larger quantity of the former four compounds were prepared. In the course of the preparation, we have now isolated a novel compound named spirotryprostatin B (**1**) which inhibited the cell cycle progression of tsFT210 cells at the G2/M phase. In this communication, we report the isolation, structure and biological activities of **1**.

The producing strain was cultured in a 600-liter jar fermenter containing 400 liters of fermentation medium (glucose 3%, soluble starch 2%, soybean meal 2%, K_2HPO_4 0.5% and $MgSO_4 \cdot 7H_2O$ 0.05%, adjusted to pH 6.5 before sterilization) containing 0.05% of CA-123 and KM-68 antifoam, respectively. The fermentation was carried out for 66 hours at 28°C under the following condition; 350 rpm stirring speed and 200 liters/minute aeration rate.

The whole broth was filtered to separate to a broth supernatant (370 liters) and a mycelial cake. The latter was extracted with 90% aqueous acetone which was evaporated *in vacuo* to remove acetone. Both the broth supernatant and the mycelium extract (60 liters) were extracted respectively with EtOAc. The EtOAc solution obtained was combined and concentrated *in vacuo* to afford an oily extract (1.2 liters) which was further

Fig. 1. Structure of spirotryprostatin B (**1**).

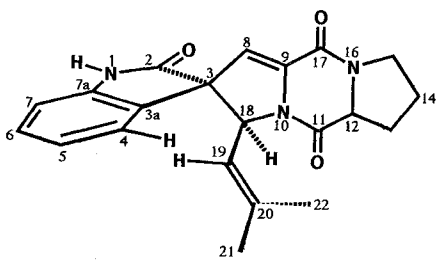

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Fig. 2. Isolation procedure for spirotryprostatin B (**1**).

EtOAc Extract (1.2 liters)
| *n*-Hexane
Insoluble Part
| $CHCl_3$
Soluble Part
| *n*-Hexane
Insoluble Part
| Partition between $CHCl_3$ & H_2O
$CHCl_3$ Extract (66 g)
| Silica gel Column Chromtg. *n*-Hexane - $CHCl_3$ - MeOH
n-Hexane - $CHCl_3$ (1 : 9) and $CHCl_3$ Eluent (800 mg)
| MeOH
Soluble Part
| HPLC { CAPCELL PAK C-18 (2×25 cm); MeOH-H_2O (60 : 40), 10 ml/minute; 210 nm
Active Fraction (Rt=32minutes) (32 mg)
| HPLC { CAPCELL PAK C-8 (2×25 cm); MeOH-H_2O (40 : 60), 10 ml/minute; 210 nm
Spirotryprostatin B (**1**) (Rt=71 minutes, 11 mg)

Fig. 3. UV spectrum of spirotryprostatin B (**1**) in MeOH solution.

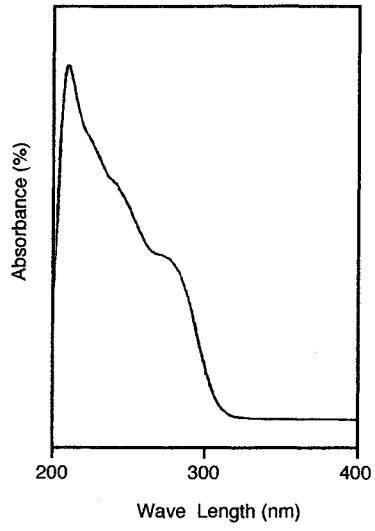


Fig. 4. IR spectrum of spirotryprostatin B (**1**) on a KBr disk.

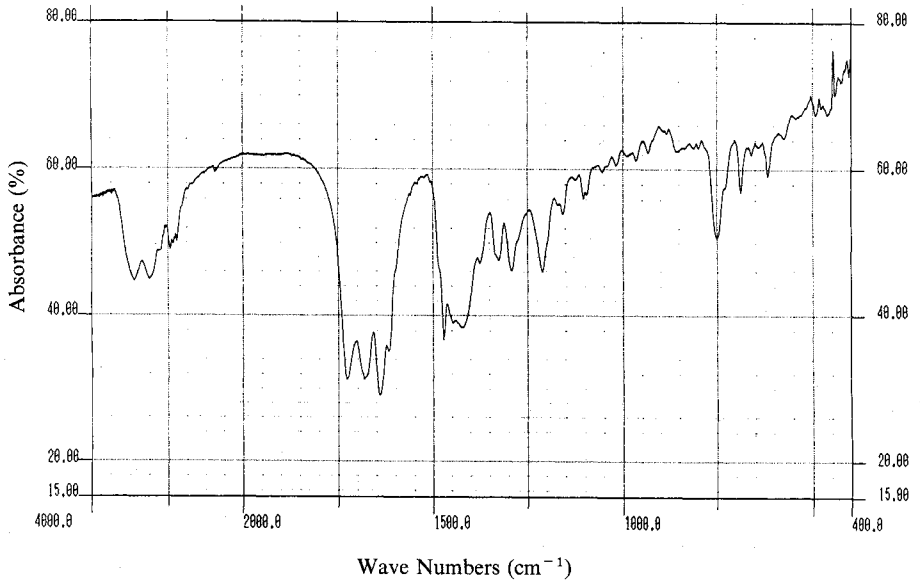


Fig. 5. 500 MHz ^{1}H NMR spectrum of spirotryprostatin B (**1**) in chloroform-*d*.

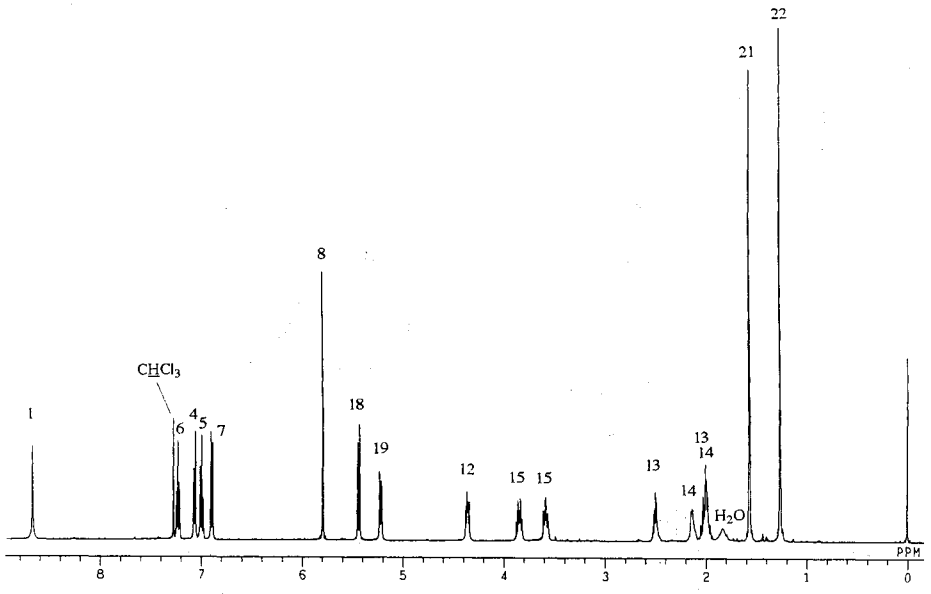
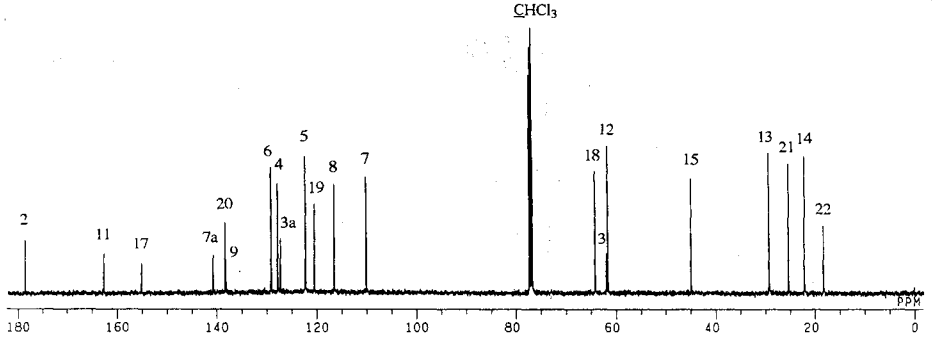


Fig. 6. 125 MHz ^{13}C NMR spectrum of spirotryprostatin B (**1**) in chloroform-*d*.



purified as shown in Fig. 2 to give an active extract (66 g). This extract was then separated by a combination of column chromatography (silica gel 60, Merck) and repeated HPLC (CAPCELL PAK C-18 and CAPCELL PAK C-8, Shiseido) to give 11 mg of **1** (Fig. 2).

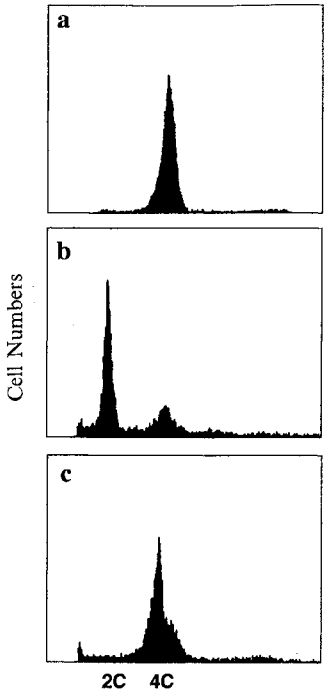
The separation procedure for **1** was monitored by the inhibitory activity on the cell cycle progression of mouse tsFT210 cells which have a temperature-sensitive $p34^{cdc2}$ and the cells were growing normally at 32°C, but arrested in the G2 phase at 39°C[4,5]. The bioassay was carried out by the synchronously cultured assay as we have previously reported[2].

Spirotryprostatin B (**1**) was obtained as a slightly yellow-colored crystalline solid having melting point 137~138°C and showed $[\alpha]_D^{22}$ −162.1° (*c* 0.92, $CHCl_3$). Its molecular formula was determined to be $C_{21}H_{21}N_3O_3$ by HR-EI-MS measurement (Found *m/z* 363.1612 (M^+), Calcd for $C_{21}H_{21}N_3O_3$ 363.1609). In the UV spectrum, **1** showed an unique absorption curve with absorption maxima at 212 (ε 36660), 227 (sh, 28820), 242 (sh, 24830), 272 (sh, 17350) and 286 nm (sh, 14810) and the IR spectrum of **1** showed the absorption at 3440, 3240 (N–H), 1730 (γ-lactam C=O), 1680, 1655 (amide C=O) and 1640 cm^{-1} (C=C) in the functional group region. The UV and IR spectra of **1** are shown in Figs. 3 and 4, respectively. The amide carbonyl absorption at 1680 and 1655 cm^{-1} together with the absence of the amide II band near 1550 cm^{-1} in the IR spectrum suggested the presence of a diketopiperazine system[6~8] in **1**. The carbonyl absorption at 1730 cm^{-1} could be assigned, after structural elucidation by NMR studies, to the carbonyl of the γ-lactam moiety[9] which fused to a benzene ring in **1**.

The ^1H and ^{13}C NMR spectra of **1** are given in Figs. 5 and 6, respectively, which were exactly assigned by means of 2D NMR techniques including pulse field-gradient heteronuclear multiple-bond correlation (PFG-HMBC) spectroscopy. Analyses of the ^1H and ^{13}C NMR spectra with the aid of DEPT, ^1H-^1H COSY and PFG heteronuclear multiple-quantum coherence (PFG-HMQC) spectra, coupled with the difference NOE experiments under the respective irradiation at 1-H and 7-H, enabled us to elucidate all of the proton spin system in **1**. The connectivity between those spin systems and the final planar structure of **1** were determined by detailed analysis of its PFG-HMBC spectrum. Then, the relative stereochemistry at C_3 and C_{18} in **1** could be determined as shown in Fig. 1 on the basis of NOE's observed between 4-H and 19-H. Studies on the relative stereochemistry at C_{12} and the absolute configuration of **1** are being undertaken currently.

The molecule of **1** has an unique structural skeleton with a spiro ring system composed from a γ-lactam fused to a benzene ring and a pentacyclic enamine fused to a diketopiperazine moiety, which are derived from a tryptophan unit, a proline residue and an isoprenyl group. The tryptophan unit in **1** has been modified by dihydrogenation at C_2/C_3, oxidation at C_2 and dehydrogenation at C_8/C_9 positions. Although naturally occuring spiro compounds belonging to the diketopiperazine series derived from tryptophan and proline residues are known[6~8,10], none has a spiro ring skeleton similar to that of **1**. Tryptoquivalines have a spiro ring system composed from two heteropentacyclic rings[11], but their spiro rings are quite different from those in **1**. The present result provides spirotryprostatin (**1**), a novel natural diketopiperazine derivative having an unique spiro ring skeleton, as a new inhibitor of the mammalian cell cycle.

Fig. 7. Effect of **1** on the cell cycle progression of tsFT210 cells.



The tsFT210 cells were synchronized in the G2 phase 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 maintained at 39.4°C up to 4 hours (a) or allowed to progress into mitosis at 32°C for 4 hours in the absence (b) or in the presence of spirotryprostatin B (12.5 μg/ml) (c).

Spirotryprostatin B (**1**) completely inhibited the cell cycle progression of tsFT210 cells in the G2/M phase at final concentrations over 12.5 μg/ml as shown in Fig. 7. In addition, **1** also showed cytotoxic activity on the growth of human chronic myelogenous leukemia K562 cells and human promyelocytic leukemia HL-60 cells with the MIC values of 35 μg/ml and 10 μg/ml, respectively. Further studies on the biological activities of **1** and the mechanism of its action are being undertaken currently.

Details of the structural and biological studies on spirotryprostatin B will be reported elsewhere.

Acknowledgments

The authors thank Dr. TOMIO YAMASHITA and KIYOSHI ISONO of Kaken Pharmaceutical Co. Ltd., Japan, for a jar fermentation of *A. fumigatus* and helpful advise, respectively. This research was supported in part by a Grant from the Ministry of Education, Science and Culture, Japan and a Grant for "Biodesign Research Program" from RIKEN (C.-B. C. & H. O.).

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(Received March 13, 1996)

References

1) 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
2) CUI, C.-B.; H. KAKEYA, G. OKADA, R. ONOSE & H. OSADA: Novel mammalian cell cycle inhibitors, tryprostatins A, B and other diketopiperazines produced by *Aspergillus fumigatus*. I. Taxonomy, fermentation, isolation and biological properties. J. Antibiotics 49: 527～533, 1996
3) 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
4) 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
5) 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* gene product. Cell 63: 313～324, 1990
6) STEYN, P. S.: The structures of five diketopiperazines from *Aspergillus ustus*. Tetrahedron 29: 107～120, 1973
7) STEYN, P. S.: Austamide, a new toxic metabolite from *Aspergillus ustus*. Tetrahedron Lett. 1971: 3331～3334, 1971
8) BIRCH, A. J. & J. J. WRIGHT: Studies in relation to biosynthesis-XLII. The structural elucidation and some aspects of the biosynthesis of the brevianamide-A and -E. Tetrahedron 26: 2329～2344, 1970
9) RAO, C. N. R.: Chemical Applications of Infrared Spectroscopy. pp. 258～261, Academic Press Inc., New York, 1963
10) WILLIAMS, R. M.; E. K. H. COFFMAN & T. GLINKA: Remarkable, enantiodivergent biogenesis of brevianamide A and B. J. Am. Chem. Soc. 111: 3064～3065, 1989
11) 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