RKS-1778, a New Mammalian Cell-Cycle Inhibitor and a Key Intermediate of the [11]Cytochalasin Group

Hideaki Kakeya,[†] Masayuki Morishita,[†,‡] Chizuko Onozawa,[†] Ron Usami,[‡] Koki Horikoshi,[‡] Ken-ichi Kimura,[§] Makoto Yoshihama,[§] and Hiroyuki Osada*,[†]

Antibiotics Laboratory, The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako-shi, Saitama 351-01, Japan, Department of Engineering, Toyo University, Nakanodai 2100, Kujirai, Kawagoe, Saitama 350, Japan, and Research Institute of Life Science, Snow Brand Milk Products Co., Ltd., Ishibashi-machi, Shimotsuga-gun, Tochigi 329-05, Japan

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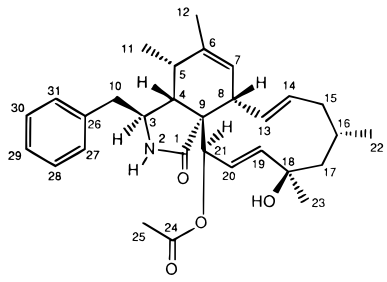
In the course of screening for the mammalian cell-cycle inhibitors, we have isolated a new [11]cytochalasin, RKS-1778 (**1**), and epoxycytochalasin H (**2**) from a fungus, *Phoma* sp. SNF-1778. The structure of **1** was determined to be 21-acetoxy-18-hydroxy-10-phenyl-5,6,16,18-tetramethyl[11]cytochalasa-6,13,19-trien-1-one, on the basis of spectroscopic methods, including ^{1}H- and ^{13}C-2D NMR techniques. RKS-1778 (**1**) may be a precursor of **2** and the key direct product of a proposed biosynthetic intramolecular Diels-Alder reaction. Both **1** and **2** completely arrested the cell-cycle progression of tsFT210 cells in the M phase at concentrations of 2.1 and 2.0 μM, respectively.

In eukaryotic cells, actin, microtubles, and intermediate filaments interact to form the cytoskeletal network involved in determination of cell architecture, motility, and mitosis.[1−3] Development of a specific inhibitor of mitosis is a useful approach to investigate the mechanism of the cell cycle. Furthermore, a mitosis inhibitor, for example, Taxol,[4] may be a useful candidate for cancer chemotherapy. We have established a convenient and practical bioassay method using tsFT210 cells, a mouse carcinoma temperature-sensitive (ts) *cdc2* mutant cell line, FM3A, as an indicator for the screening of new mammalian cell-cycle inhibitors.[5] In the course of screening, we found acetophthalidin;[6] tryprostatins A and B;[7,8] spirotryprostatins A and B;[9] and cyclotryprostatins A, B, C, and D[10] to be active. In continuation of our research, we isolated a new cell-cycle inhibitor, RKS-1778 (**1**), and epoxycytochalasin H (**2**) from a fungus, *Phoma* sp. SNF-1778. These results and experiments with metyrapone, a cytochrome *P*-450 inhibitor, provide a possible biosynthetic pathway for **2**. We describe herein the isolation, structure determination, and biological activity of **1** and **2**.



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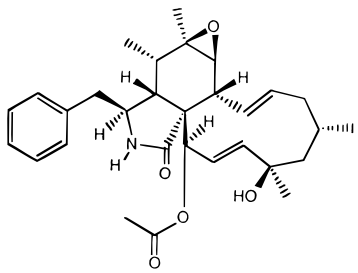

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Results and Discussion

Bioassay-guided isolation of the fermentation broth of *Phoma* sp. SNF-1778 afforded a new [11]cytochalasin, RKS-1778 (**1**), and the known [11]cytochalasin, epoxycytochalasin H (**2**), as the cell-cycle inhibitors that arrest at the M phase. The molecular formula of **1** was determined to be $C_{30}H_{39}NO_4$ by HRFABMS (*m/z* 478.2920 $(M + H)^+$, calcd 478.2958), indicating the index of hydrogen deficiency was 12. The IR spectrum of **1** showed absorptions at 3425, 1745, and 1695 cm^{-1}, indicating the functional groups of a hydroxyl, an ester, and a γ-lactam, respectively.

All the ^{1}H and ^{13}C signals were asssigned as shown in Table 1. In the ^{1}H-NMR spectrum, **1** showed an N−H proton (δ 5.45 br s, 2-H), monosubstituted benzene ring protons (δ 7.14 d, J = 7.0 Hz, 27-H and 31-H; δ 7.31 d, J = 7.0 Hz, δ 7.32 d, J = 7.6 Hz, 28-H and 30-H; and δ 7.25 t, J = 7.6 Hz, 29-H), five olefinic protons (δ 5.36 m, 7-H; δ 5.82 ddd, J = 1.5, 10.4, 15.3 Hz, 13-H; δ 5.23 ddd, J = 4.6, 10.4, 15.3 Hz, 14-H; δ 5.51 dd, J = 2.1, 16.5, 19-H; and δ 5.91 dd, J = 2.8, 16.5, 20-H), five methyl groups (δ 1.13 d, J = 7.4 Hz, 11-H; δ 1.71 s, 12-H; δ 1.03 d, J = 6.4Hz, 22-H; δ 1.34 s, 23-H; and δ 2.21 s, 25-H) along with signals due to several methine and methylene groups. The ^{13}C-NMR spectrum of **1** indicated the presence of an amide carbonyl (δ 175.2 s, C-1), an ester carbonyl (δ 170.1 s, C-24), and five methyl groups (δ 14.0 q, C-11; δ 20.8 q, C-12; δ 26.4 q, C-22; δ 31.5 q, C-23; and δ 20.9 q, C-25) together with ten sp^2 and six sp^3 methines, two sp^2 and two sp^3 quaternary carbons, and three methylene groups. Detailed analyses of the ^{1}H- and ^{13}C-NMR spectra of **1** with the aid of pulse field gradient (PFG) ^{1}H−^{1}H COSY and PFG heteronuclear multiple quantum coherence (PFG−

* To whom correspondence should be addressed. Phone: +81-48-467-9541. FAX: +81-48-462-4669. E-mail: antibiot@postman.riken.go.jp.
[†] Antibiotics Laboratory, The Institute of Physical and Chemical Research (RIKEN).
[‡] Department of Engineering, Toyo University.
[§] Research Institute of Life Science, Snow Brand Milk Products Co., Ltd.
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Table 1. ^{13}C- and ^{1}H-NMR Chemical Shifts (ppm) for RKS-1778 (**1**) in $CDCl_3$[a]

position	^{13}C[b]	^{1}H[c]	(*J*/Hz)	position	^{13}C[b]	^{1}H[c]	(*J*/Hz)
1	175.2 s			16	28.6 d	1.76 m	
2		5.45 br s		17	53.4 t	1.53 m	
3	55.7 d	3.15 m				1.89 m	
4	54.0 d	2.17 dd	4.2, 4.3	18	74.4 s		
5	35.2 d	2.45 m		19	136.9 d	5.51 dd	2.1, 16.5
6	137.7 s			20	126.5 d	5.91 dd	2.8, 16.5
7	127.9 d	5.36 m		21	77.1 d	5.65 dd	2.1, 2.5
8	43.0 d	3.21 m		22	26.4 q	1.03 d	6.4
9	56.0 s			23	31.5 q	1.34 s	
10	46.0 t	2.58 dd	10.1, 13.4	24	170.1 s		
		2.90 dd	4.0, 13.4	25	20.9 q	2.21 s	
11	14.0 q	1.13 d	7.4	26	137.7 s		
12	19.8 q	1.71 s		27, 31	128.9 d	7.14 d	7.0
13	129.1 d	5.82 ddd	1.5, 10.4, 15.3	28, 30	128.9 d	7.31 d	7.0
14	135.4 d	5.23 ddd	4.6, 10.4, 15.3			7.32 d	7.6
15	42.7 t	1.76 m		29	127.0 d	7.25 t	7.6
		2.00 m					

[a] Signal assignments were based on the results of $^{1}H-^{1}H$ COSY, PFG−HMQC, PFG−HMBC, and difference NOE experiments. δ ppm from TMS as an internal standard. [b] 100 MHz. [c] 400 MHz.

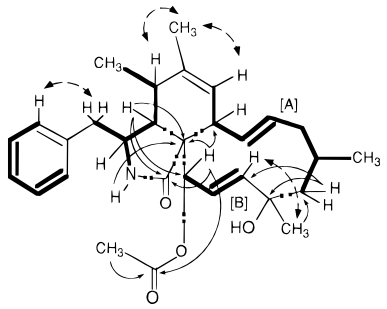
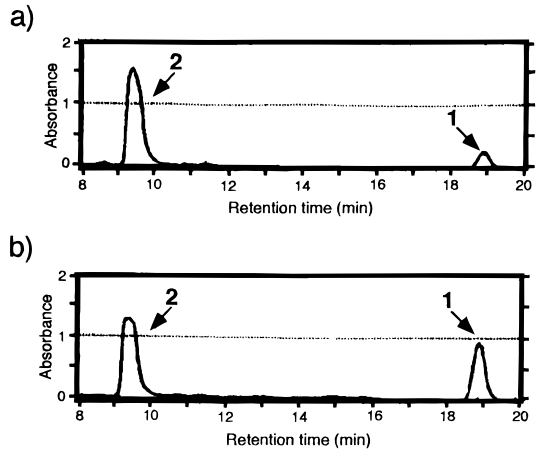


Figure 1. Partial structures (A, B) of **1** and connectivities among the partial structures. Bold lines indicate spin systems obtained by the analyses of $^{1}H-^{1}H$ COSY and PFG−HMQC spectrum. Dashed-line arrows indicate long-range $^{1}H-^{1}H$ couplings observed in the $^{1}H-^{1}H$ COSY spectra for the construction of the partial structures. Solid-line arrows indicate long-range $^{1}H-^{13}C$ couplings for the connectivities of the partial structures detected by the PFG−HMBC spectra.

HMQC) spectroscopy, coupled with the results of difference NOE experiments and the above physicochemical properties, enabled us to deduce partial structures A and B in **1** (Figure 1). The geometry at the 6 position was determined as the *E* configuration from the observation of NOEs between 7-CH and 12-CH_3, while the geometry of both the 13 and 19 positions was the *E* configuration from the large coupling constant ($J = 15.3$ and 16.5 Hz, respectively).

The PFG heteronuclear multiple bond correlation (PFG−HMBC) spectrum was measured in order to determine the total structure of **1**. In the PFG−HMBC spectrum, the methyl proton (δ 2.21, 25-CH_3) and methine proton (δ 5.65, 21-H) showed long-range coupling to the carbonyl carbon (δ 170.1, C-24), revealing that an acetoxyl group was connected to the 21 position. Connectivities of the partial structures A and B and other units were confirmed by the following cross peaks: (a) δ 1.34 (23-CH_3)/δ 53.4 (C-17), δ 1.53 and 1.89 (17-CH_2)/δ 74.4 (C-18) and δ 136.9 (C-19); (b) δ 2.17 (4-CH) and δ 5.65 (21-CH)/δ 175.2 (C-1), δ 5.65 (21-CH)/δ 43.0 (C-8), δ 5.45 (NH), δ 2.17 (4-CH), and δ 5.65 (21-CH)/δ 56.0 (C-9), δ 2.17 (4-CH)/δ 77.1 (C-21). Thus, the planar structure of **1** was deduced as shown in Figure 1. The relative stereochemistry of **1** was determined by difference NOE experiments. Significant NOEs were observed as follows: 4-H/5-H, 8H, 10H, and 21-H; 5-H/8-H; 20-H/21-H and 23-H; 22-H/ 23-H.



	1	2
control	100	100
metyrapone (1 mM)	310	66

Figure 2. Effect of a cytochrome *P*-450 inhibitor, metyrapone, on the production of **1** and **2** by *Phoma* sp. SNF-1778. (a) HPLC analysis in control; (b) HPLC analysis in 1 mM metyrapone treatment; (c) the ratio of the peak areas in HPLC analyses.

Spectral data, including ^{1}H NMR, ^{13}C NMR, IR, and MS, of **2** corresponded to those of epoxycytochalasin H, which was originally isolated from a soybean pathogen, *Phomopsis sojae*.[11] Cell-cycle inhibitory activities for **1** and **2** were measured by using tsFT210 cells, and the bioassay was carried out as we have previously reported.[5] As seen from the results in both the asynchronous and the synchronous assays, **1** and **2** completely inhibited the cell-cycle progression of tsFT210 cells in the M phase at concentrations of 2.1 and 2.0 μM, respectively.

Finally, we investigated the involvement of cytochrome *P*-450 on the production of **1** and **2** by *Phoma* sp. SNF-1778. Cytochrome *P*-450 is a well-known oxygenase present from bacteria to mammals and plays an important role in the biosynthesis and degradation of various metabolites. In order to investigate the rela-

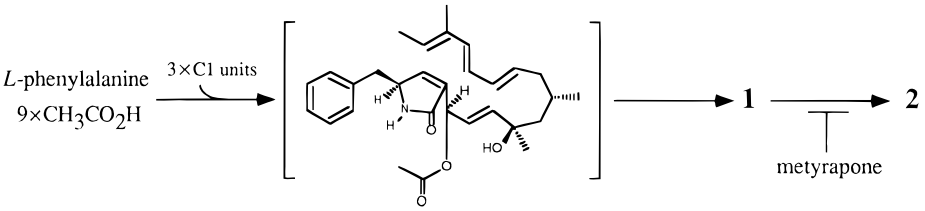


Figure 3. A possible biosynthetic pathway of **1** and **2**.

tionships between **1** and **2** in the biosynthesis, we have applied metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone),[12] an inhibitor of cytochrome *P*-450, for biosynthetic studies. After the producing strain was cultured with or without metyrapone, the broth filtrates were extracted with EtOAc and were analyzed by HPLC. Because the ϵ-values of UV absorption at 215 nm in **1** and **2** are nearly the same, the amounts of these compounds were estimated by the peak areas in the HPLC analysis. To the medium after 3 days' culture with *Phoma* sp. SNF-1778, metyrapone was added and fermented for an additional 2 days. Metyrapone exhibited superior effect for accumulation of the less oxidized compound, **1**. In the case of treatment with 1 mM metyrapone, the production of **2** was decreased to 66%, while that of **1** was increased three times more than the control experiment without metyrapone (Figure 2), suggesting that the inhibitor blocks metabolite flow. These observations allow us to propose the biosynthetic pathway of **2** as shown in Figure 3, together with several feeding experiments.[13] In addition, the isolation of **1** implies that the hypothetical hexaene is cyclized via [4 + 2]cycloaddition to afford **1**.

Although the possible involvement of a biosynthetic Diels−Alder reaction has been mentioned frequently, there is only one reported example of direct evidence for a biological Diels−Alder reaction[14] and only a few reports of catalytic antibodies that control the reaction pathways of Diels−Alder cycloaddition.[15] The present compound, RKS-1778, may be the biogenetic precursor of epoxycytochalasin H and may provide additional evidence for a cycloaddition reaction in the biosynthetic pathway for the [11]cytochalasin group.

Experimental Section

General Experimental Procedures. Melting points were measured on a Yanagimoto melting point apparatus and are uncorrected. Optical rotations were determined on a JASCO DIP-370. UV spectra were taken with a Hitachi 220A spectrometer, and IR spectra were recorded on a Shimadzu FTIR-8100M. FABMS were measured on a JEOL JMS DSX-300. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM-α-400 spectrometer with TMS as an internal standard, and chemical shifts are recorded in δ values.

Fungal Material. The producing strain SNF-1778 was isolated from a soil sample collected in Matsumotoshi, Nagano, Japan. The strain SNF-1778 has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the accession no. FERM P-15994.

Fermentation Conditions. The strain SNF-1778 from mature slant culture was inoculated into two 500-mL cylindrical flasks each containing 70 mL of an autoclaved medium consisting of glycerol 3%, soluble starch 3%, soybean meal 2%, yeast extract 0.3%, KCl 0.3%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7\ H_2O$ 0.05%, and $CaCO_3$ 0.2%. The medium was adjusted to pH 6.0 prior to sterilization. The flasks were shaken on a rotary shaker at 300 rpm for 2 days at 28 °C. Then, 3 mL each of the resultant seed medium was inoculated into 500-mL cylindrical flasks containing the same medium and cultured at 28 °C for 4 days.

Treatment of *Phoma* sp. SNF-1778 with a Cytochrome *P*-450 Inhibitor, Metyrapone. A solution of metyrapone (final concentration: 1.0 mM) was added to the cultures on the third day after inoculation in the medium described above, and the incubation continued for an additional 2 days. Then, the cultured broth was extracted with EtOAc and concentrated *in vacuo* to afford the crude extracts for HPLC analysis. Analytical conditions were as follows: PEGASIL ODS (4.6 × 250 mm, Senshu Scientific Co. Ltd., Tokyo, Japan), UV 215 nm, flow rate 0.6 mL/min, solvent CH_3CN-H_2O (65:35) [t_R (min) **1** 18.9, **2** 9.4].

Extraction and Isolation. The whole broth was mixed with 80% Me_2CO to extract the active principles and concentrated *in vacuo* to remove the Me_2CO. The aqueous solution was adjusted to pH 7.0 and extracted with EtOAc. After concentration of the EtOAc layer *in vacuo*, the oily material was dissolved in a small volume of MeOH, applied onto a reversed-phase C18 column (Waters SEP-PAK cartridge, Millipore Co., Milford, MA), followed by elution with MeOH. The resultant active fractions were further purified by reversed-phase HPLC using a PEGASIL ODS column (10 × 250 mm, Senshu Scientific Co. Ltd., Tokyo) with 65% CH_3CN to give **1** (6.2 mg) and **2** (310 mg).

RKS-1778 (1). RKS-1778 was obtained as a white powder and was soluble in $CHCl_3$ or MeOH, but only slightly soluble in H_2O or *n*-hexane. The R_f value in the solvent system $CHCl_3$−MeOH (10:1) was 0.67: mp 117−120 °C; $[\alpha]_D$ −20° (*c* 0.05, MeOH, 23 °C); UV (MeOH) λ_{max} (ϵ) end absorption; IR (KBr) ν_{max} 3425, 2925, 1745, 1695, 1630, 1235, 965, 625 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; HRFABMS *m/z* 478.2920 $(M + H)^+$ (calcd for $C_{30}H_{40}NO_4$, 478.2958).

Epoxycytochalasin H (2). Epoxycytochalasin H (**2**) was obtained as a white powder and soluble in $CHCl_3$ or MeOH, but only slightly soluble in H_2O or *n*-hexane. The R_f value in the solvent system $CHCl_3$−MeOH (10:1) was 0.69: mp 134−136 °C; $[\alpha]_D$ −85.5° (*c* 0.1, $CHCl_3$, 21 °C) [lit.[11] $[\alpha]_D$ −84.7° (*c* 0.33, $CHCl_3$, 30 °C)]; IR (KBr) ν_{max} 3415, 2925, 1745, 1700, 1630, 1230, 965, 625 cm^{-1}; ^{1}H- and ^{13}C-NMR data, identical to that in the literature;[11] HRFABMS *m/z* 494.2910 $(M + H)^+$ (calcd for $C_{30}H_{40}NO_5$, 494.2906).

Bioassay for Cell-Cycle Inhibitory Activity. tsFT210 cells, a mouse temperature-sensitive (ts) *cdc2* mutant cell line of the mammary carcinoma cell line, FM3A, were routinely maintained at 32 °C in RPMI-

1640 medium supplemented with 5% calf serum (HyClone Inc., Logan, UT) 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. In the asynchronous-culture assay, cells cultured at the permissive temperature of 32 °C were seeded into a 24-well plate at a density of 1×10^5 cells/mL, test chemicals were added and cultured at 32 °C for 17 h. In the synchronous-culture assay, the cells seeded at a density of 1×10^5 cells/mL into a 24-well plate were preincubated at 39 °C for 17 h to obtain G2-arrested cells. Then, test chemicals were added and further incubated at 32 °C for 4 h.

In both cases, morphological characteristics of the cells were observed directly under a microscope after incubation. The DNA contents in each nucleus were determined by flow cytometry as previously reported.[5]

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