COMMUNICATIONS TO THE EDITOR

Factumycin and Its New Derivative RK-1009 Enhance Threonine-phosphorylation of a 60-kDa Protein in *Streptomyces griseus*

Sir:

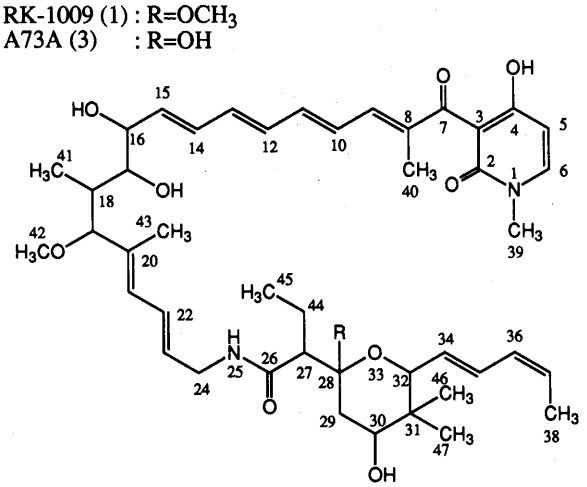
We have established a convenient and practical bioassay system for the screening of novel antiphage compounds.[1] In the course of the screening, we found pyrrolobenzodiazepine antibiotics, RK-1441A and B[1,2] and depsipeptide antibiotics, enopeptin A and B[3,4]. In continuation of our research, we isolated a new RK-1009 (**1**) and two known compounds, factumycin (A40A) (**2**)[5] and its stereoisomer at the 14,15-double bond, A73A (**3**)[6] produced by an actinomycete identified as *Streptomyces* sp. isolated from a soil sample collected in Tsuruoka City, Yamagata Prefecture, Japan. We describe herein the production, isolation, physico-chemical properties, and biological activity of RK-1009.

The producing organism was cultivated in a 500-ml cylindrical flask containing 70 ml of a medium consisting of glucose 2.0%, soybean meal 2.5%, soluble starch 1%, meat extract 0.1%, dried yeast 0.4%, and NaCl 0.2% (pH 7.0) on a rotary shaker at 28°C for 4 days. The culture broth (3 liters) was centrifuged and the supernatant was extracted with EtOAc. The mycelial extract was concentrated to a small volume and then extracted with EtOAc. The combined extract was subjected to silica gel column chromatography with $CHCl_3$-MeOH (100/5, 100/10, 100/20 and 50/50). The active elute was chromatographed on a Sephadex LH-20 column with *n*-hexane-CH_2Cl_2-MeOH (4/5/1) and further purified by HPLC using a PEGASIL ODS column with 42% CH_3CN (pH 3.5) for **1** (3.2 mg) or 37% CH_3CN (pH 3.5) for **2** (2.0 mg) and **3** (2.0 mg). Spectral data, including ^1H NMR, ^{13}C NMR, UV, IR, and MS, of **2** and **3** corresponded to those of factumycin and its stereoisomer, which were originally isolated from *S. lavendulae* and *S. viridifaciens*, respectively.[5,6]

RK-1009 (**1**) is an amorphous yellow powder: $[\alpha]_D^{27}$ +8.5° (*c* 0.1, MeOH). The molecular formula of **1** was determined as $C_{45}H_{64}O_{10}N_2$ by HR-FAB-MS [*m/z* 791.4498 $(M-H)^-$; calcd. 791.4483 for $C_{45}H_{63}O_{10}N_2$], ^1H NMR, and ^{13}C NMR spectra. The UV spectrum of **1** showed the presence of a diene moiety and a tetraenone-4-hydroxy-2-pyrridone chromophore, λ_{max} (MeOH) 232 (ε, 79365) and 350 (ε, 38095), respectively. The IR spectrum indicated the presence of amide carbonyl groups, 1635 ~ 1655 cm^{-1} and a hydroxy group, 3445 cm^{-1}.

The ^1H and ^{13}C NMR spectra of **1** are shown in Figs. 2 and 3, respectively. Comparison of the ^1H NMR spectra of **1** and **3** showed a clear difference in that one singlet methoxy signal was observed at 3.25 ppm in **1**, but not in **3**. In the ^{13}C NMR spectra of **1**, another methoxy carbon signal was observed at 48.01 ppm and the characteristic hemiacetal carbon signal (102.84 ppm) at the position 28 was observed at a low magnetic field compared with that of **3**. Based on the results of the 2D NMR spectra data including ^1H-^1H and ^{13}C-^1H COSY

Fig. 1. Structures of RK-1009 (**1**), factumycin (**2**) and A73A (**3**).



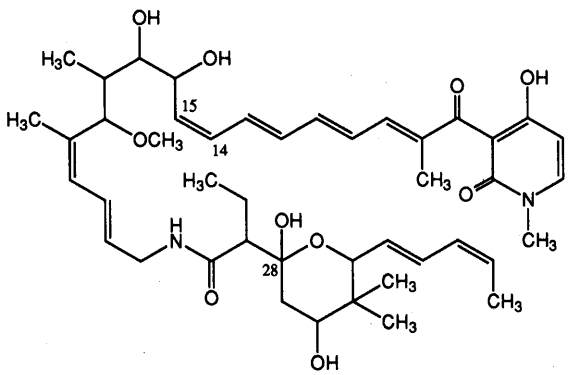


Fig. 2. ^{1}H NMR spectra of RK-1009 (**1**) (400 MHz, in CD_3OD).

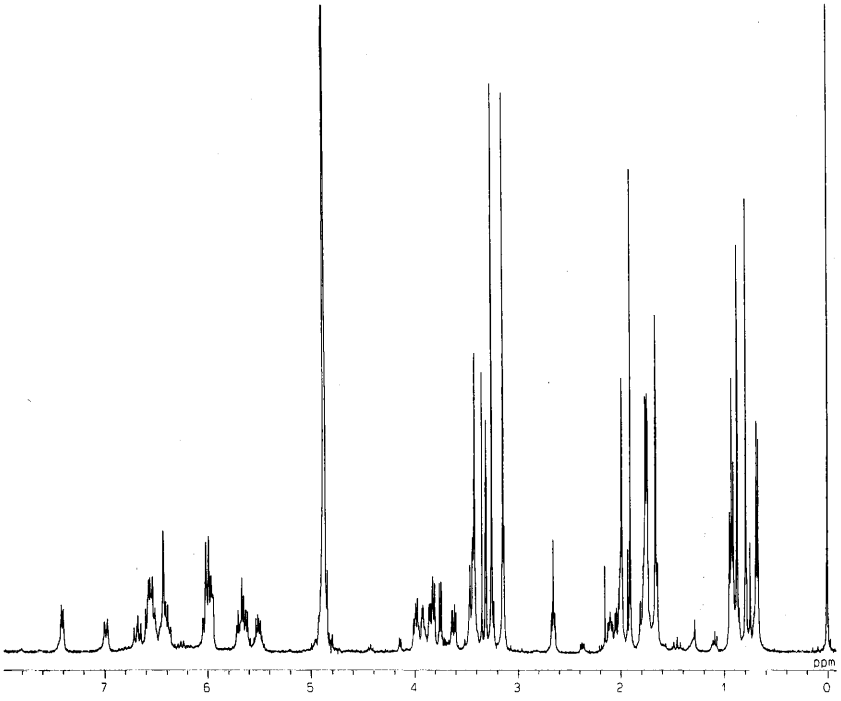
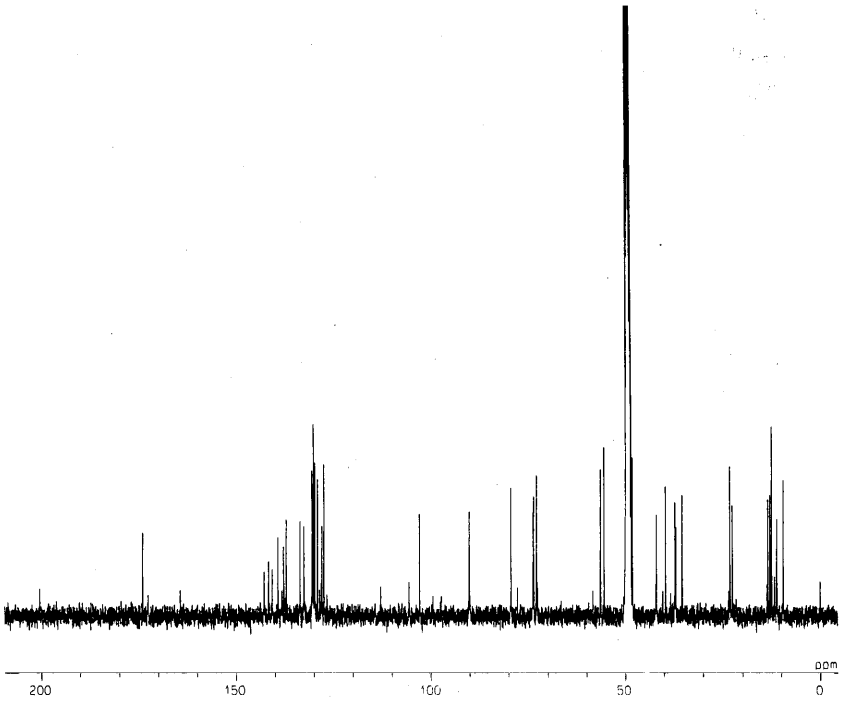


Fig. 3. ^{13}C NMR spectra of RK-1009 (**1**) (100 MHz, in CD_3OD).

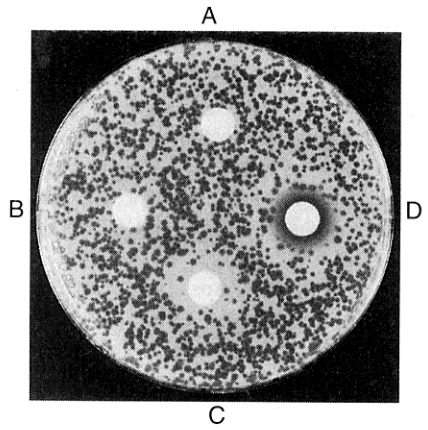


and HMBC, the structure of RK-1009 was confirmed as a methoxy derivative of **3** at the position 28 (Fig. 1).

RK-1009 (**1**) inhibited the plaque formation of bacteriophage B at the concentration of 0.4~40 μg/disk in the paper disk-agar plate method[1] as shown in Fig. 4. At the concentration of 40 μg/disk, **1** also exhibited the antimicrobial activity against *Streptomyces griseus*. In addition, the biological activities of **2** and **3** were almost the same as that shown by **1**. The characteristics based on the balance of the antiphage vs antibacterial

Fig. 4. Anti-bacteriophage activity of RK-1009 (**1**) in the assay system using bacteriophage B and *Streptomyces griseus*.

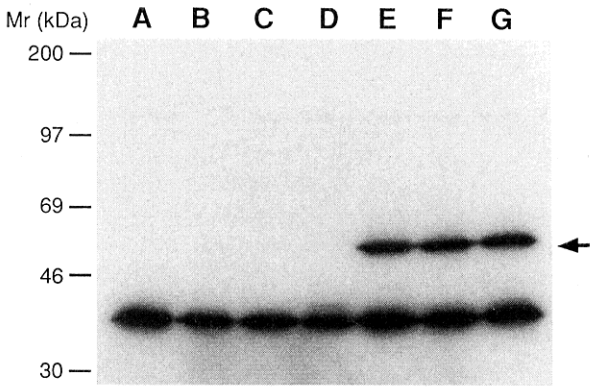
A, none; B, 0.4 μg/disk; C, 4 μg/disk; D, 40 μg/disk.



The assay using bacteriophage and *S. griseus* was carried out as described previously.[1] Briefly, test sample solutions were put on paper disks and laid on an agar plate containing bacteriophage B and *S. griseus*. After a 30-hour incubation at 28°C, observation was made of cell survival around the disks and a picture was taken.

Fig. 5. Effect of RK-1009 (**1**) on the *in vitro* protein phosphorylation in *Streptomyces griseus*.

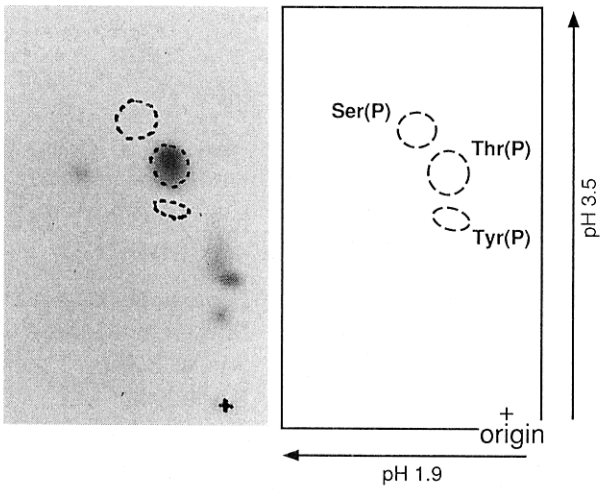
A, none; B, 10 μM staurosporine; C, 10 μM herbimycin; D, 100 μM tautomycin; E, 100 μM factumycin (**2**); F, 100 μM A73A (**3**); G, 100 μM RK-1009 (**1**).



The cell lysates were prepared from the early log-phase growing *S. griseus* by sonication in lysis buffer [20 mM Tris-HCl (pH7.0), 10% gricerol, 1 mM EDTA, 1 mM DTT, 2% aprotinin, 1 mM phenylmethylsulfonyl fluoride]. The reaction mixtures containing 80 μg of the cell lysate, 2 μCi of [γ-^{32}P]ATP in kinase reaction buffer [50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM $MnCl_2 \cdot 6H_2O$, 1 mM EDTA, 1 mM DTT], and the test chemicals were incubated for 10 minutes at 28°C. The samples were analyzed by 10% SDS-PAGE followed by autoradiography.

Fig. 6. Phosphoamino acid analysis of the ^{32}P-labeled 60-kDa protein by the treatment of RK-1009 (**1**).

Ser(P), phosphoserine; Thr(P), phosphothreonine; Tyr(P), phosphotyrosine.



The 60 kDa protein-containing gel band, which was significantly labeled with [γ-^{32}P]-ATP *in vitro* in the presence of RK-1009 (**1**) was excised, and the protein was eluted and analyzed by two-dimensional phosphoamino acid analysis; the electrophoresis at pH 1.9 and pH 3.5 in the first and second dimension, respectively, was carried out as described previously.[7]

The positions of the stained marker phosphoamino acids are indicated by broken lines. The minor spots are probably incompletely hydrolysed phosphopeptides.

activity was observed around the paper disk. Protein kinase or phosphatase inhibitors (staurosporine, herbimycin, and tautomycin) showed moderate antiphage and antibacterial activities, similar to **1**, **2**, and **3**. Therefore, we investigated the effect of **1** on the *in vitro* protein phosphorylation in *S. griseus* as the host cells. Incubation for 10 minutes of the cell extract and [γ-^{32}P]-ATP in the presence of **1** yielded several detectable labeled protein bands as shown in Fig. 5. Specifically, a protein with an apparent molecular mass of 60 kDa was significantly labeled with [γ-^{32}P]-ATP in the presence of **1**. This remarkable ^{32}P-labeled 60-kDa protein was also detected by the treatment with **2** or **3**; however, none of the kinase or phosphatase inhibitors tested, staurosporine, herbimycin, and tautomycin, induced that effect. These results suggest that the mode of action of factumycin-type antibiotics such as RK-1009 (**1**) is distinct from that of the well-known kinase or phosphatase inhibitors tested and these factumycin-type antibiotics have proved to be a

very useful tool for investigating the cellular responses in *S. griseus*. Moreover, two-dimensional phosphoamino acid analysis,[7] shown in Fig. 6, revealed that the incorporation of [γ-^{32}P]-ATP to this 60-kDa protein with treatment of **1** was mostly in the threonine residues. These results are well consistent with the recent findings that Ser/Thr-kinases/phosphatases cascades in mammalian cells exist in *S. griseus*.[8~10]

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