ACTIKETAL, A NEW MEMBER OF THE GLUTARIMIDE ANTIBIOTICS

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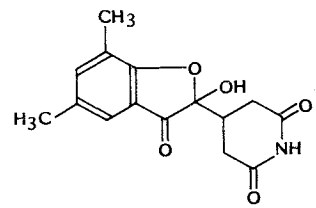
A new glutarimide antibiotic named actiketal was isolated from the culture fluid of the epiderstatin-producing streptomycete, *Streptomyces pulveraceus* subsp. *epiderstagenes*. The IC_{50} of the antibiotic for inhibition of the incorporation of [^{3}H]thymidine into epidermal growth factor-stimulated Balb/MK cells was 14.5 μM. The structure of this compound was determined by ^{1}H and ^{13}C NMR spectroscopic analyses using ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY, heteronuclear multiple-bond correlation spectroscopy, selective ^{13}C-{^{1}H} NOE's techniques.

Recently, we isolated epiderstatin[1,2] which inhibits the mitogenic activity induced by epidermal growth factor (EGF) in the culture fluid of *Streptomyces pulveraceus* subsp. *epiderstagenes*. Actiketal, a new member of the glutarimide antibiotics (Fig. 1), was isolated from the same culture fluid as a minor component. The compound inhibited the incorporation of [^{3}H]thymidine into Balb/MK cells stimulated by EGF. In this paper, isolation, physico-chemical properties, structure elucidation and biological activity of actiketal are described.

Fig. 1. Structure of actiketal.



Materials and Methods

Isolation

Actiketal was produced by *S. pulveraceus* subsp. *epiderstagenes* which is a producing strain of epiderstatin and its fermentation was carried out as described previously.[1] All the purification steps were monitored by measuring the inhibitory activity of incorporation of [^{3}H]thymidine into quiescent Balb/MK cells stimulated by EGF.[1]

Culture broth (72 liters) was filtered with the aid of Celite. The filtrate (55 liters) was extracted with the same volume of ethyl acetate. The organic layer dried over anhydrous sodium sulfate was evaporated under reduced pressure. The oily extract (21.8 g) was applied to a silica gel column (76 i.d. × 430 mm) and eluted with chloroform - methanol (92 : 8). The eluate was fractionated into each 300 ml of volume and the active fractions were combined and evaporated. The resulting oily material (13.1 g) was rechromatographed on a silica gel column (45 i.d. × 700 mm) with chloroform - methanol (95 : 5). The eluate was fractionated into each 23 ml of volume, and the active fractions were combined and evaporated. The resulting oily material (4.57 g) was dissolved in a small volume of methanol and was applied to gel filtration through a Sephadex LH-20 column equilibrated with 80% methanol.The eluate was fractionated into each 10 ml of volume and the active fractions were combined and evaporated. From the dried material (18.0 mg), actiketal (4.5 mg) was purified by the preparative HPLC using a reverse phase column (Senshu Pak ODS-5251-N, 20 i.d. × 250 mm, monitored by UV at 220 nm) with 50% methanol as a solvent.

Instrumental Analyses

Optical rotation was determined on a Perkin-Elmer 241MC polarimeter. MP was measured with a

Yanagimoto micro melting point apparatus. UV and IR spectra were taken on a Hitachi 220A spectrophotometer and Shimadzu IR27G recording IR spectrophotometer, respectively. MW and molecular formula were estimated by using a Hitachi M-80 mass spectrometer. ^{1}H NMR, ^{1}H-^{1}H COSY and ^{13}C-^{1}H COSY spectra were obtained on a Jeol GSX-500 spectrometer at 500 MHz in $CDCl_3$. Heteronuclear multiple-bond correlation spectroscopy (HMBC) spectrum was measured on a Jeol GX-400 spectrometer at 400 MHz in $CDCl_3$. Selective ^{13}C-$\{^{1}H\}$ NOE[3] spectrum was gained by running a Jeol FX-100 spectrometer at 25 MHz in $CDCl_3$.

Biological Activity

The incorporation of [^{3}H]thymidine into Balb/MK cells stimulated by EGF was estimated in the absence or presence of the antibiotic as described previously.[1]

The antimicrobial activity was determined by the conventional paper disk-agar plate method. The paper disks contained 160 μg of the antibiotic.

Results and Discussion

Isolation

S. pulveraceus subsp. *epiderstagenes* produced various glutarimide antibiotics in addition to actiketal. Most of these antibiotics show both antifungal and inhibitory activity of mitogen response. Although actiketal had no antifungal activity, this activity was monitored in the culture broth to help differenciate it from other glutarimide antibiotics in the isolation. Therefore, both inhibitory activity of incorporation of [^{3}H]thymidine into quiescent Balb/MK cells induced by EGF and antifungal activity against *Pyricularia oryzae* were monitored to purify actiketal. Both activities reached the maximum at pH 7.5 in the culture after 72 hours-fermentation. When the pH of the culture reached over 8, the activity decreased rapidly.

Pure actiketal (4.5 mg) was obtained from 72 liters fermentation broth. The most effective purification step was the gel filtration through a Sephadex LH-20 column. In this step, the existence of actiketal was ascertained by being separated from the other glutarimides, *e.g.*, epiderstatin, cycloheximide, and acetoxycycloheximide. Actiphenol could be separated by the 2nd silica gel column chromatography.

Physico-chemical Properties

Actiketal was obtained as white amorphous powder with the mp 96~100°C. It was optically inactive when measured in methanol (*c* 0.1). The compound was soluble in methanol, ethanol, ethyl acetate, chloroform or DMSO but insoluble in water and hexane. The UV absorption maxima of actiketal in methanol were observed at 222 (ε 9,710), 264 (ε 7,630) and 350 nm (ε 2,485). This absorption was virtually unchanged in acidic methanol solution. In alkali methanol, the solution became turbid. The IR spectrum indicated the presence of hydroxyl (3380 cm^{-1}), imide (1710 cm^{-1}) and carbonyl (1688 cm^{-1}) groups. The MW of 289 was obtained from FD-MS and the molecular formula was calcd as $C_{15}H_{15}NO_5$ (289.0950) from *m/z* 289.0958 which was obtained by HREI-MS. The color reaction was positive to Rydon-Smith, Lemieux and ferric chloride. These results suggested the existence of imide, hydroxyl and phenol groups.

Structure Elucidation

The UV spectrum of actiketal was similar to that of actiphenol[4,5] or non-kang 101-G[6] which belong to the glutarimide antibiotics. However, the MW and ^{1}H NMR spectrum were different from those of both actiphenol and non-kang 101-G.

The structure of actiketal was elucidated from the ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY and ^{13}C-^{1}H COSY spectrum data and the results of the HMBC (Fig. 2) and selective ^{13}C-$\{^{1}H\}$ NOE experiments.

The ^{1}H NMR spectrum showed fifteen signals due to two methyl, two methylene, three methine, one NH and one OH protons. The ^{13}C NMR spectrum exhibited fifteen signals consisting of two methyl, two methylene, one methine, six aromatic, three carbonyl and one hemiacetal carbons.

The correlation between protons and carbons was elucidated from the ^{1}H-^{1}H COSY and the ^{13}C-^{1}H COSY spectra as shown in Table 1. The HMBC experiment showed the presence of an aromatic ring and a glutarimide ring (Fig. 2). The proton signals of 2- and 4-CH_3 substituted to the aromatic ring were assigned by HMBC experiment. In the selective ^{13}C-$\{^{1}H\}$ NOE experiment, the irradiation of 8-OH resulted in the increase of C-8 signal and the irradiation of 12-NH caused the signal enhancement of C-11 and C-13. Therefore, the existence of a hemiacetal and an imide were deduced.

The unsaturation of actiketal was estimated as

Fig. 2. Long range coupling observed by HMBC experiment.

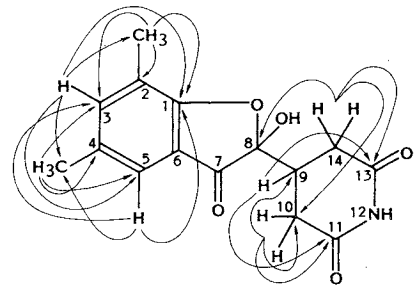
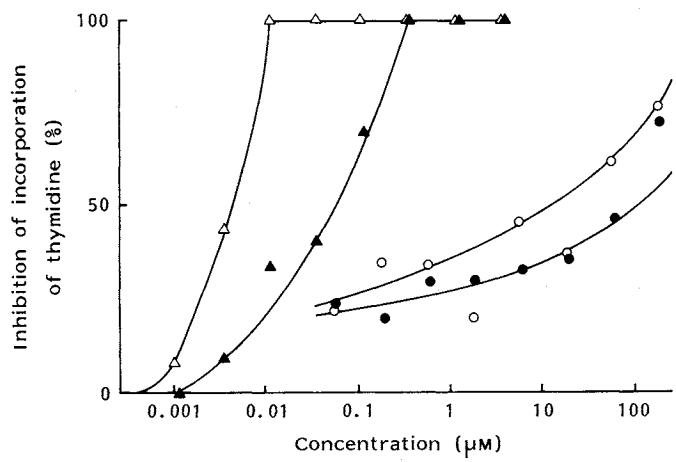


Table 1. ^{1}H and ^{13}C NMR data of actiketal.

Position	^{1}H NMR (ppm)	^{13}C NMR (ppm)	Position	^{1}H NMR (ppm)	^{13}C NMR (ppm)
1	—	167.5	10	2.79, 2.93 (2H, ddd, J=4, 10, 17 Hz)	30.8
2	—	123.1	11	—	171.5
3	7.31 (1H, s)	141.7	12-NH	7.91 (1H, s)	—
4	—	132.6	13	—	171.8
5	7.23 (1H, s)	121.6	14	2.55, 2.67 (2H, ddd, J=4, 10, 17 Hz)	31.7
6	—	118.3	8-OH	4.28 (1H, br s)	—
7	—	198.7	2-CH_3	2.25 (3H, s)	14.1
8	—	103.3	4-CH_3	2.31 (3H, s)	20.5
9	2.68 (1H, m)	35.7			

Fig. 3. Inhibitory activities of actiketal, actiphenol, epiderstatin and cycloheximide against incorporation of [^{3}H]thymidine into Balb/MK cells induced by EGF stimulation.

○ Actiketal, ● actiphenol, △ epiderstatin, ▲ cycloheximide.



nine from its molecular formula. Therefore, the structure of the antibiotic must contain six double bonds and three ring structures, which leads to the structure as shown in Fig. 1. The reason for the low-field shift of 10-H (2.79, 2.93 ppm, $J=17$ Hz) compared with 14-H (2.55, 2.67 ppm, $J=17$ Hz) may be explained by the restriction of free rotation of the glutarimide ring by the carbonyl group at the 7 position. In the ^{1}H NMR of actiphenol, the signals in the glutarimide ring corresponding to that of actiketal are observed as overlapping signals (2.43 and 2.86 ppm, $J=17$ Hz).

Biological Activity

Actiketal inhibited the incorporation of [^{3}H]thymidine into quiescent Balb/MK cells stimulated by EGF at the concentration of 14.5 μM (IC_{50}) as shown in Fig. 3. Actiphenol which is structurally related to actiketal showed 50% inhibition at the concentration of 109.6 μM. Compared with epiderstatin,[7] the activity of actiketal was about 3,000-fold weaker. The inhibition curves of actiketal and actiphenol which have an aromatic ring were less inclined than those of epiderstatin and cycloheximide as shown in Fig. 3. Actiketal did not show antifungal activity against *Alternaria mali* IFO 8984, *Colletotrichum lagenarium* IFO 7351, *Botryotinia fuckeliana* IFO 5365, and *P. oryzae* IFO 5994 at 160 μg/disk.

Acknowledgments

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