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Novel Triene-β-lactone Antibiotics, Oxazolomycin Derivative and Its Isomer, Produced by *Streptomyces* sp. KSM-2690

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In the course of our screening for novel antibacterial antibiotics produced by microorganisms, a strain termed KSM-2690 was found to produce a new complex of triene-β-lactone antibiotics in the culture filtrate. The active principle was recovered from the broth filtrate with ethyl acetate extraction and could be divided into six components, three major components KSM-2690 A (**1**), B (**2**) and C (**3**), and three minor components KSM-2690 D, E and F, on the basis of their respective order of elution under the reverse-phase HPLC. The characteristic UV and IR spectra of their components were similar to those of oxazolomycins[1,2], indicating that these new compounds are members of the triene-β-lactone group of antibiotics. Among them, the new antibiotics **2** and **3** presented here were an additional member of this group and its isomer, as shown in the gross structures. In this report, we describe preliminary taxonomic characteristics of the producing strain, fermentation, isolation, structural elucidation and biological activity of antibiotics **2** and **3**.

The producing strain KSM-2690 was isolated from a soil sample collected at Masuko-machi, Tochigi prefecture, Japan. According to the procedure of the International Streptomyces Project (ISP) as recommended by SHIRLING and GOTTLIEB[3], this strain exhibited the following cultural characteristics during incubation for 3 weeks at 28°C in various media. The aerial mass color was white to yellowish-gray and chocolate brown to dark brown, and the reverse-side color of vegetative growth was yellowish-gray to yellowish-brown; soluble pigment was not produced. Melanoid pigment was not produced on ISP medium 6 and 7. Optimum temperature for growth was 28~30°C. Microscopic observation showed that the mature sporophores formed spirales and had more than 20 spores per chain. The spore surface was spiny. By the method of PRIDHAM and GOTTLIEB[4], L-arabinose, D-glucose, D-fructose, inositol, D-mannitol and raffinose were utilized by this strain for growth and utilization of D-xylose was doubtful, but L-rhamnose and sucrose were not utilized. Based on the methods of BECKER *et al.*[5], the whole-cell hydrolysates of strain KSM-2690 contained LL-diaminopimelic acid and showed no characteristic sugar pattern. Therefore, the cell wall was type-I. These taxonomic characteristics of strain KSM-2690 indicated that the strain should be classified in the genus *Streptomyces*[6], and so we designated this strain as *Streptomyces* sp. KSM-2690. In addition, the phylogenic analysis of 16S ribosomal DNA sequences with MicroSeq Full Gene 16S rDNA Bacterial Sequence Kit (PE Corp.) revealed high sequence similarity (97.8%) against *Streptomyces nousei*. According to the published descriptions on *S. nousei*, this result supported the evidences of the morphological, cultural and chemotaxonomic properties of strain KSM-2690 as mentioned above, although the strain KSM-2690 was not directly compared with the type strain of *S. nousei*.

The producing strain KSM-2690 was maintained on YS slant consisting of soluble starch 1.0%, yeast extract 0.2% (Oriental Yeast Co.), and agar 2.0% (pH 7.0). One loopful of well-grown agar slant of strain KSM-2690 was used to inoculate 100-ml of medium containing glycerol 0.25%, polypeptone 1.0%, yeast extract 0.5%, beef extract 0.5% (Kyokuto), $MgSO_4 \cdot 7H_2O$ 0.05%, NaCl 0.2%, K_2HPO_4 0.05%, and $CaCO_3$ 0.32% (pH 7.0) in a 500-ml reciprocal flask. The determination of the activity of a complex of antibiotics KSM-2690 was made by a conventional paper-disc diffusion assay using spore suspension of *Bacillus*

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subtilis PCI 219 as test organism during the fermentation and isolation.

The culture filtrate (22 liters, pH 7.7) obtained from the above fermentation was extracted 2 times with a half volume of ethyl acetate. The extract after dehydration with anhydrous Na_2SO_4 was concentrated *in vacuo* to give a yellowish-brown oil (8.38 g). The resulting oily material was applied to a silica gel column (Silica gel 60, Merck, 50×6.0 cm, i.d.) and the column was eluted with $CHCl_3$/MeOH (20 : 1). The fractions which were active against *B. subtilis* PCI 219 and rich in components **1**, **2** and **3**, as detected by the reverse-phase HPLC analysis, were combined to afford a pale yellow residue (1.34 g) after pooling and concentration *in vacuo*. One third of the residue obtained was chromatographed on a Sephadex LH-20 column (95×2.3 cm, i.d., Pharmacia) with methanol as elution solvent. The active fractions containing three components were collected and pooled with those from two similar runs with the remaining two portions of the crude residue to give a yellow oily residue (149 mg) after evaporation. Further isolation of components **1**, **2** and **3** was achieved in two parallel chromatographic separations using one half portion of the material on a medium pressure Develosil-ODS column (50×1.5 cm, i.d., Nomura Chemical) with CH_3CN/water (35 : 65) as elution solvent at a flow rate of 2.5 ml/minute. In this step, the components **1** and **3** did not show activity against *B. subtilis*. The eluants containing the respective components were evaporated to remove the organic solvent and then the concentrates were lyophilized to give pale yellow powders of **1**, **2** and **3** (6.9 mg, 26.2 mg and 9.2 mg), respectively. The individual purified materials gave single peaks by reverse-phase HPLC on Inertsil ODS-2 column (250×4.6 mm, i.d., GL Sciences) eluted with CH_3CN/water (50 : 50) as mobile phase at a flow rate of 0.8 ml/minute with detection by UV absorption at both 230 and 254 nm. **1**, **2** and **3** showed Rt 6.5, 6.8 and 7.2 minutes, respectively.

The UV spectrum of **2** showed absorption maxima at 230 and 278 nm with two shoulders at 267 and 288 nm. The characteristic absorption suggested the presence of conjugated diene and triene moieties. Furthermore, the IR bands at 1825, 1690 and 1640 cm^{-1} suggested the presence of β-lactone and amide structures in the molecule. These spectroscopic features were similar to those of the triene-β-lactone group of antibiotics such as oxazolomycins[1,2] and curromycins (triedimycins)[7,8], which possess the unusual structure of both oxazole and bicyclic β-lactone-γ-lactam rings linked through an olefinic chain. FAB-MS of **2** showed peaks at *m/z* 670 $[M+H]^+$ and *m/z* 668 $[M-H]^-$, respectively, and the nominal molecular weight of **2** was determined as 669, which is 14 mass unit larger than oxazolomycin. The ^{1}H-NMR spectrum of **2** was also similar to that of oxazolomycin (Table 1). However, it showed three doublet methyl signals (1.71, 1.05 and 0.87 ppm) in its aliphatic region. Its molecular weight considered, **2** was deduced to be a methyl-substituted oxazolomycin. Further, structural analysis was performed by means of 2D-NMR methods. The ^{1}H- and ^{13}C-NMR signals were completely assigned by combination of DQF-COSY, HOHAHA, HMQC and HMBC spectra (Table 1). The doublet methyl signal at 1.71 ppm was assigned to be a 16-methyl, and **2** was concluded to be a 16-methyl derivative of oxazolomycin. RYU *et al.*[9,10] already reported the structure of 16-methyloxazolomycin, and very recently, they also determined the absolute stereochemistry of 16-methyloxazolomycin. By the comparison of NMR data of **2** with that of 16-methyloxazolomycin, those were closely resembled, but slight differences in chemical shifts were observed at 16-methine proton signal (4.82 ppm; 16-methyloxazolomycin, 5.00 ppm; **2**) and 2-methyl ^{13}C signals (20.8 ppm; 16-methyloxazolomycin, 9.73 ppm; **2**). These differences suggested that **2** is a stereochemical isomer of 16-methyloxazolomycin reported very recently. To clarify the differences in stereochemistry, we analyzed ROESY spectrum (mixing time: 300 ms) of **2** in detail. The observed NOE correlations around the β-lactone-γ-lactam rings are displayed in Fig. 1. The results indicated the relative stereochemistry of **2** as 2R*, 3S*, 15S* and 16R* in contrast to that of 16-methyloxazolomycin (2R, 3R, 15S and 16S). Although the asymmetric centers (4, 6, 7, and 3′ positions) were not examined, the close similarity in ^{1}H-NMR data except for the 16 position imply the identical stereochemistry with 16-methyloxazolomycin. Consequently, **2** was determined to be a novel

Fig. 1. Correlations of NOE observed in ROESY spectrum (mixing = 300 ms) of KSM-2690 B (**2**).

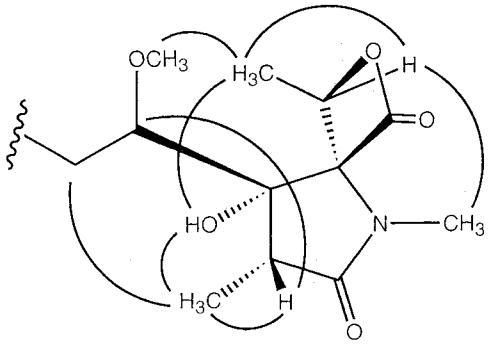


Table 1. ^{1}H- and ^{13}C-NMR data of KSM-2690 B (**2**) and KSM-2690 C (**3**) (400 MHz in DMSO-d_6, 30°C)

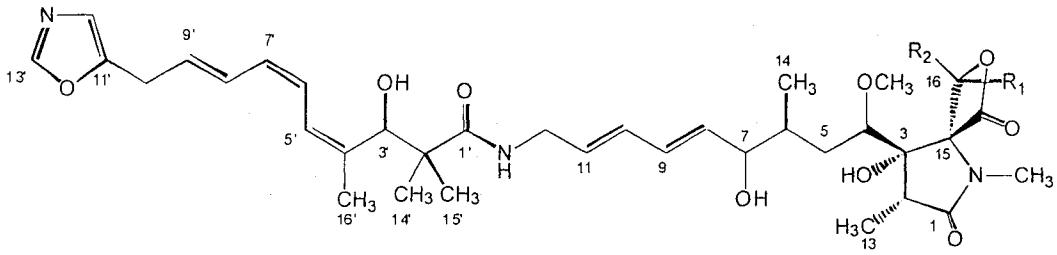
	KSM-2690 B (2)						KSM-2690 C (3)		
position	$\delta^{1}H$	multi.	NOE	$\delta^{13}C$	multi.	HMBC	$\delta^{1}H$	multi.	NOE
1				174.32	s	2, 13, NCH_3			
2	2.37	1H,q,7Hz	4, 6, 13	43.54	d	4, 13, 3-OH	2.36	1H,q,7Hz	4, 6, 13
3				80.67	s	4, 5, 13, 16, 3-OH			
4	3.37	1H,t,5Hz	2, 6, 7, 14, OCH_3	82.61	d	2, 5, 6, 3-OH, OCH_3	3.37	1H,t,5Hz	2, 6, 14, OCH_3
5	1.96	1H,dt,15,5Hz	5, 13	32.00	t	4, 6, 14	1.96	1H,m	5
	1.17	1H,m	5, 14, OCH_3				1.15	1H,m	5
6	1.61	1H,m	2, 4, 7, 13, 14, OCH_3	36.66	d	4, 5, 14, 7-OH	1.60	1H,m	2, 14
7	3.83	1H,m	4, 5, 6, 8, 9, 14	75.13	d	5, 6, 8, 9, 14	3.83	1H,m	6, 9
8	5.61	2H,overlapped	7, 10, 14	134.43	d	6	5.61	2H,overlapped	10
9	6.13	2H,d,15Hz	7, 11	129.75	d		6.12	2H,overlapped	7, 11
10	6.13	2H,d,15Hz	8, 12	130.21	d	8, 9or10	6.12	2H,overlapped	8
11	5.61	2H,overlapped	9, 12	129.81	d	7, 12	5.61	2H,overlapped	9
12	3.72	2H,m	11, 10, NH	40.36	t	11, 10, NH	3.71	2H,m	10
13	1.05	3H,d,7Hz	2, 5, 6, 3-OH	9.73	q	2	1.05	3H,d,7Hz	2, 5, 3-OH
14	0.87	3H,d,7Hz	4, 5, 6, 7, 8, OCH_3	16.06	q	5, 6, 7	0.87	3H,d,7Hz	4, 6
15				83.49	s	16, 16-CH_3, 3-OH, NCH_3			
16	5.00	1H,q,7Hz	16-CH_3, NCH_3	77.23	d	16-CH_3	5.00	2H,q,7Hz	16-CH_3, NCH_3
17				170.07	s	16			
1'				175.99	s	12, 3', 14', 15', NH			
2'				45.75	s	3', 14', 15', 3'-OH			
3'	4.63	1H,d,4Hz	5', 6', 14', 15', 16', 3'-OH, NH	73.07	d	4', 5', 14', 15', 3'-OH	4.59	1H,d,5Hz	6', 14'
4'				139.87	s	3', 5', 6', 16'			
5'	6.40	1H,d,12Hz	3', 8'	123.39	d	3', 7', 16'	5.93	1H,d,11Hz	7', 16'
6'	6.31	1H,t,11Hz	3'	124.36	d	8'	6.59	1H,dd15,11Hz	3'
7'	5.93	1H,t,11Hz	9'	127.03	d	5', 8', 9'	6.13	1H,overlapped	5'
8'	6.74	1H,dd,15,12Hz	5', 10'	127.97	d	6', 7', 10'	6.23	overlapped	
9'	5.79	1H,dt,15,7Hz	7', 10'	128.89	d	7', 10'	5.75	1H;dt,15,7Hz	
10'	3.55	2H,m	8', 9'	28.18	t	8', 9'	3.51	2H,d,7Hz	
11'				150.47	s	9', 10', 12', 13'			
12'	6.89	1H,s		121.95	d	10', 13'	6.89	1H,s	
13'	8.22	1H,s		151.23	d		8.22	1H,s	
14'	1.11	3H,s	3', 15', 16', NH	24.67	q	3', 15'	1.11	3H,s	3', 15'
15'	0.98	3H,s	3', 14', 16', NH	21.48	q	3', 14'	0.98	3H,s	3', 14'
16'	1.73	3H,s	3', 14', 15'	19.86	q	3', 5'	1.68	3H,s	5'
16-CH_3	1.71	3H,d,6Hz	16, 3-OH, OCH_3	16.79	q	16	1.71	3H,d,6Hz	16, 3-OH
NCH_3	2.79	3H,s	16	25.90	q		2.79	3H,s	16
OCH_3	3.17	3H,s	4, 5, 6, 14, 16-CH_3	55.91	q	4	3.16	3H,s	4
3-OH	5.31	1H,s	16-CH_3				5.31	1H,s	13, 16-CH_3
7-OH	4.80	1H,d,3Hz					4.80	1H,d,3Hz	
3'-OH	5.45	1H,d,5Hz	3'				5.44	1H,d,5Hz	3'
NH	7.64	1H,t,5Hz	12, 3', 14', 15'				7.64	1H,t,5Hz	

stereochemical isomer of 16-methyloxazolomycin.

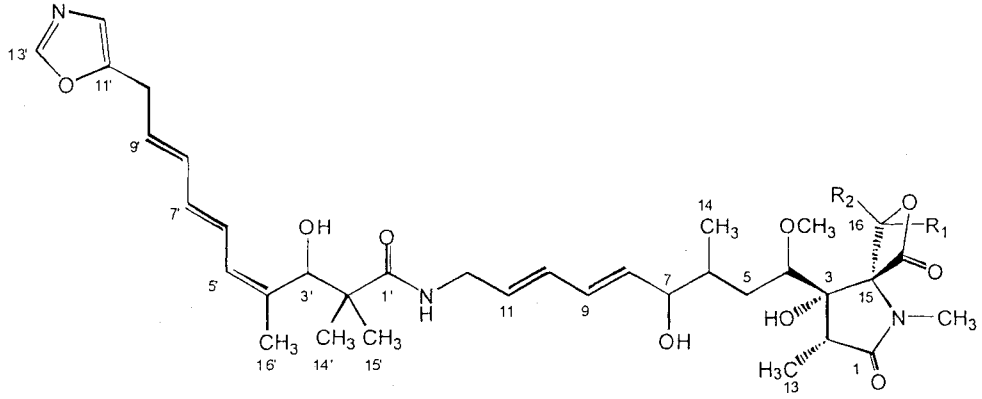
FAB-MS of **3** showed peaks at *m/z* 670 $[M+H]^+$ and *m/z* 668 $[M-H]^-$, respectively, and UV and IR spectra were closely similar to that of **2**. Thus, **3** was considered to be an isomer of **2**. The ^{1}H-NMR spectrum of **3** was analyzed by means of DQF-COSY, HOHAHA and ROESY spectrum (mixing time: 300 ms) to compare with that of **2** (Table 1). The obvious differences were found in the chemical shifts around the triene moiety (H5', 6', 7' and 8') of **3**. The coupling constants of H6'-7' and H8'-9' were determined to be both 15 Hz, whereas those were 11 Hz and 15 Hz, respectively, in **2**. KANZAKI *et al.*[2] found two oxazolomycin isomers, and further investigated the relation between triene configurations and proton chemical shifts. The chemical shifts of H3' to H9' of **3** was closely similar to that of oxazolomycin C. Thus, the triene configurations of **3** were determined to be 4'*Z*, 6'*E* and 8'*E*. The NOE correlations around the β-lactone and γ-lactam rings were same as that of **2**. Therefore, **3** was determined to be a configurational isomer of **2** at 6'-7' double bonds.

On the other hand, structural elucidation of **1** has been unsuccessful because of its instability in NMR experiments, although **1** was another member of the same class of compounds with characteristic UV spectrum.

As shown in previous reports[2,8,9], oxazolomycin-group antibiotics were known to exhibit weak antimicrobial activity against only limited bacteria such as *B. subtilis*, *Micrococcus luteus* and *Agrobacterium tumefaciens*, and to have cytotoxic activity against P388 leukemia cells. Recently, TONEW *et al.*[11] reported that diffusomycin (oxazolomycin) had antiviral activity. Therefore, antibacterial activity of the new components **2** and **3** was examined by paper-disk (8 mm in diameter, thickness) diffusion method at the concentration of 50 μg/ml. Component **2** showed inhibition zones (diameter) against Gram-positive bacteria as follows: *B. subtilis* PCI 219; 23.0 mm, *Micrococcus luteus* ATCC 9341; 15.0 mm (hazy zone), *Staphylococcus aureus* smith; 25.0 mm (hazy zone). In contrast of **2**, no growth inhibition of **1** and **3** was observed. Components **1**, **2** and **3** against human bladder carcinoma T24 cells exhibited the same cytotoxicity with IC_{50} value of 10 μg/ml.



	R_1	R_2
oxazolomycin	H	H
16-methyloxazolomycin	CH_3	H
KSM-2690 B (2)	H	CH_3



	R_1	R_2
oxazolomycin C	H	H
KSM-2690 C (3)	H	CH_3

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References

1) MORI, T.; K. TAKAHASHI, M. KASHIWABARA, D. UEMURA, C. KATAYAMA, S. IWADARE, Y. SHIZURI, R. MITOMO, F. NAKANO & A. MATSUZAKI: Structure of oxazolomycin, a novel β-lactone antibiotic. Tetrahedron Lett. 26: 1073~1076, 1985
2) KANZAKI, H.; K.-I. WADA, T. NITODA & K. KAWAZU: Novel bioactive oxazolomycin isomers produced by *Streptomyces albus* JA3453. Biosci. Biotechnol. Biochem. 62: 438~442, 1998
3) SHIRLING, E. B. & D. GOTTLIEB: Methods for chracterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
4) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetes as an aid for species determination. J. Bacteriol. 56: 107~114, 1948
5) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
6) WILLIAM, S. T.; M. GOODFELLOW & G. ANDERSON: Genus *Streptomyces* Waksman and Henriei 1943. *In* BERGEY's Manual Systematic Bacteriology. Vol. 4. *Eds.*, S. T. WILLIAMS *et al.*, pp. 2452~2492, The Williams & Wilkins Co., 1989
7) OGURA, M.; H. NAKAYAMA, K. FURIHATA, H. SETO & N. OTAKE: Structure of a new antibiotic curromycin A produced by a genetically modified strain of *Streptomyces hygroscopicus*, a polyether antibiotic-producing organism. J. Antibiotics 38: 669~673, 1985
8) OGURA, M.; H. NAKAYAMA, K. FURIHATA, A. SHIMAZU, H. SETO & N. OTAKE: Isolation and structural determination of a new antibiotic curromycin B. Agric. Biol. Chem. 49: 1909~1910, 1985
9) RYU, G.; S. HWANG & S.-K. KIM: 16-Methyloxazolomycin, a new antimicrobial and cytotoxic substance produced by a *Streptomyces* sp. J. Antibiotics 50: 1064~1066, 1997
10) RYU, G. & S.-K. KIM: Absolute stereochemistry determination of 16-methyloxazolomycin produced by *Streptomyces* sp. J. Antibiotics 52: 193~197, 1999
11) TONEW, E.; M. TONEW, U. GRAFE & P. ZOPEL: On the antiviral activity of diffusomycin (oxazolomycin). Acta Virol. 36: 166~172, 1992