MK4588, A NEW ANTIBIOTIC RELATED TO XANTHOCILLIN

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A new antibiotic, MK4588, structurally related to xanthocillin, was isolated from the culture broth of *Leptosphaeria* sp. L-179. Antibiotic MK4588 exhibited inhibitory activity against a limited range of Gram-positive and Gram-negative bacteria. The antibiotic was degraded by alkali to a more active product. The structures of MK4588 and the degradation product were determined to be (1*R**,6*S**,7*S**)-7-(*Z*)-(1-isocyano-2-(4-methoxyphenyl))ethenyl-1-hydroxy-7-isocyanobicyclo-[4,2,0]oct-2-en-4-one and (*Z*)-2,3-diisocyano-1-(4-methoxyphenyl)buta-1,3-diene, respectively, by NMR spectral analyses coupled with X-ray crystallographic analysis of MK4588.

In the course of our screening program for new antibiotics of fungal origin, strain L-179 belonging to the genus *Leptosphaeria* was found to produce a new antibiotic, MK4588 (**1**)†. The antibiotic was moderately stable in neutral solution, but unstable in acidic or alkaline solution. In alkaline methanol, **1** was immediately converted into an antibacterial product (**2**). The biological activity of **1** and **2** appeared to be limited to Gram-positive and Gram-negative bacteria. The structures of **1** and **2** were established by means of spectral analyses and by X-ray diffraction analysis of **1**. These antibiotics possess vicinal isocyano groups and are structurally related to the fungal metabolites, xanthocillin[1] and xanthoascin[2].

In this paper, the taxonomy and fermentation of the producing strain, the isolation, chemical conversion, structural elucidation and biological activities of **1** and **2** are reported.

Taxonomy of the Producing Strain

The producing microorganism, strain L-179, was isolated from a dead stem of *Artemisia japonica* Thunb. collected at Oomi-island, Ehime Prefecture, Japan. The identification of strain L-179 was done as described by HOLM[3], LUTTRELL[4], MÜLLER[5], SIVANESAN[6], and VON ARX and MÜLLER[7].

Morphological Characteristics: Pseudothecia scattered or gregarious, immersed beneath the epidermis, flattened-spherical with flat base, 200~250 μm i.d., 138~150 μm high, ostiolar neck not distinctly differentiated, wall parenchymatous, 15.6~30 μm thick at the sides, composed of 3~6 layers of polyhedral cells, outermost cells dark and thick-walled, inner cells thinner-walled and almost colorless, walls at the base of pseudothecium about 7.8~16.5 μm thick, brownish colored, composed of 2~3 layers of thin walled cells. Asci numerous, clavate, 78~100 × 14~15 μm, containing 8 ascospores, bitunicate. Ascospores long clavate, yellowish brown, 37~47 × 6.2~8.0 μm, usually 5-septate, sometimes 6-septate, third cell swollen.

The above morphological characteristics of the producing strain indicate that this fungus belongs

† Antibiotic MK4588 has been published in Jpn. Kokai 110,658 ('89), Apr. 27, 1989 as leptocillin. The producing strain has been deposited with the Fermentation Research Institute of Agency of Industrial Science and Technology as accession No. FERM P-9661.

to the genus *Leptosphaeria* Ces. & de Not., in the Pleosporaceae, the Loculoascomycetes. Among the recognized species of *Leptosphaeria*, this fungus is similar to *Leptosphaeria ogilvensis*, *Leptosphaeria submaculans*, *Leptosphaeria galeopsidicola* and *Leptosphaeria maculans* in having 5- to 6-septate ascospores, but differed from these species in the size and shape of ascospores and the position of swollen cell of ascospores. Consequently, this fungus is considered to be a new species of *Leptosphaeria*. However, the definite nomenclature of this fungus will be reported after more strains are isolated and examined.

Fermentation

Ten 200-ml Erlenmeyer flasks each containing 40 ml of the seed medium consisting corn starch 3.0%, soybean meal 3.5%, soybean oil 0.2%, wheat germ 1.5%, K_2HPO_4 0.2% and $CaCO_3$ 0.5% were inoculated with the culture of strain L-179 grown on a potato-glucose agar slant. The inculated flasks were shaken at 26°C for 4 days on a rotary shaker at 210 rpm.

Four ml of the seed culture was transferred to 80 ml of the production medium in a hundred 500-ml Erlenmeyer flasks. The production medium contained maltose syrup 2.0%, soybean meal 1.0%, soybean oil 0.15%, soluble vegetable protein 0.25%, Pharmamedia (Traders Oil Mill Co., Texas) 0.5%, $FeSO_4 \cdot 7H_2O$ 0.0005%, $NiCl_2 \cdot 6H_2O$ 0.00005%, $CoCl_2 \cdot 6H_2O$ 0.00005% and $CaCO_3$ 0.1%. Fermentation was carried out at 26°C for 5 days on a rotary shaker at 210 rpm. Antibacterial activity was assayed by the paper-disc method using *Micrococcus luteus* ATCC 9341 as a test organism.

Isolation

Fermentation broth of *Leptosphaeria* sp. L-179 (*ca.* 8 liters) was filtered and the mycelial cake was extracted with 70% aqueous acetone (1.8 liters). The extract was concentrated to 600 ml and the combined broth filtrate (3.4 liters) and concentrate were extracted with ethyl acetate (4 liters). The organic layer was dried over anhydrous sodium sulfate and evaporated to yield an oily residue (10.7 g). The residue was applied to a column of silica gel (Wakogel C-300, 100 g) packed in benzene - acetone (20 : 1) and the column was developed with the same solvent. Biologically active fractions were combined and concentrated to give a light yellow oil (250 mg). Further purification of the oil was achieved by Sephadex LH-20 (650 ml) column chromatography developed with methanol followed by crystallization from methanol to give pure MK4588 (60 mg) as colorless prismatic crystals.

Chemical Conversion

To a methanol solution (10 ml) of MK4588 (45.0 mg) was added 0.5 ml of 0.1 N NaOH and the mixture was kept at room temperature for 10 minutes. After neutralization of the solution with 0.1 N HCl, the reaction mixture was evaporated to dryness. The residue was chromatographed on a silica gel column (10 g) using benzene - acetone (20 : 1) as the developing solvent to give two fractions with Rf values of 0.70 and 0.32 (benzene - acetone, 4 : 1) on a TLC plate. The first eluate which contained the less polar product was concentrated to dryness and further purified by Sephadex LH-20 (200 ml) column chromatography developed with methanol to yield 20.5 mg of the degradation product (**2**) as colorless needles. The second eluate which contained the more polar product was purified in the same manner as described above to give 9.3 mg of crystalline hydroquinone: MP 173~174°C; HR-MS *m/z* 110.0372 (M^+), calcd for $C_6H_6O_2$ 110.0367; ^1H NMR (CD_3OD) δ 6.62 (s); ^{13}C NMR (CD_3OD) δ117.6 (d), 152.0 (s).

Physico-chemical Properties

Antibiotic MK4588 (**1**) and the degradation product (**2**) were neutral in nature and moderately soluble

in methanol, chloroform, ethyl acetate and acetone, but insoluble in water and *n*-hexane. The Rf values of **1** and **2** were 0.24 and 0.70 (benzene-acetone, 4:1), respectively, on TLC, and showed positive color reactions with H_2SO_4, $KMnO_4$ and Na_2MoO_4 reagents and a negative reaction with ninhydrin reagent. The other physico-chemical properties of **1** and **2** are listed in Table 1.

Structure of the Degradation Product (**2**)

Results of the elemental analysis, HR-MS (Table 1) and ^1H and ^{13}C NMR spectral data (Table 2) gave a molecular formula of $C_{13}H_{10}N_2O$ for **2**. The characteristic absorption bands at 2125 and 2100 cm^{-1} in the IR spectrum and two quaternary ^{13}C NMR signals at 168.5 and 173.5 ppm indicated the presence of two isocyano groups attached to sp^2 carbons. In the ^1H NMR spectrum of **2**, one set of 1,4-disubstituted benzene protons (2′-H, 3′-H, 5′-H, 6′-H), an olefinic proton (1-H), terminal vinyl (4-H) and methoxyl protons, and no exchangeable proton were observed. A long range ^1H-^{13}C COSY and long range selective proton decoupling (LSPD) experiments revealed the following structural

Table 1. Physico-chemical properties of MK4588 (**1**) and the degradation product (**2**).

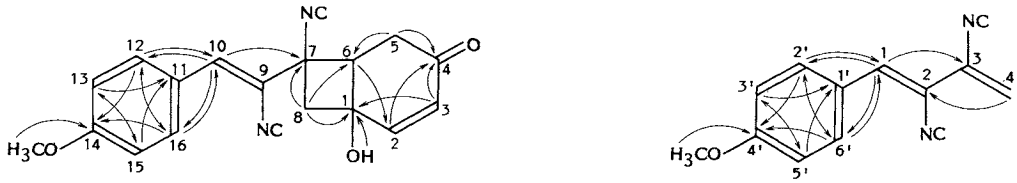
	1	**2**
Appearance	Colorless prisms	Colorless needles
Molecular formula	$C_{19}H_{16}N_2O_3$	$C_{13}H_{10}N_2O$
HR-MS (*m/z*)		
Calcd:	320.1160	210.0793
Found:	320.1084	210.0830
Anal Calcd:	C 71.24	C 74.27
	H 5.03	H 4.79
	N 8.74	N 13.32
Found:	C 71.00	C 74.18
	H 4.99	H 4.64
	N 8.67	N 13.35
$[\alpha]_D^{22}$ (*c* 1, MeOH)	+34.7°	—
MP (°C, dec)	126~127	96~97
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	222 (248), 300 (503)	238 (560), 328 (1,520)
IR (KBr) cm^{-1}	3250, 2110, 2090, 1655, 1605, 1510	2125, 2100, 1600, 1595, 1510

Table 2. ^1H and ^{13}C NMR data for MK4588 (**1**) and the degradation product (**2**).

Proton	**1**		Carbon	**1**		Proton	**2**		Carbon	**2**	
	δ	m		δ	m		δ	m		δ	m
1-OH	2.45	s	C-1	64.2	s	1-H	7.00	br s	C-1	129.5	d
2-H	6.87	dd	C-2	146.5	d	4-H_2	5.74	br d	C-2	114.4	br s
	(10.5,	2.1)	C-3	130.8	d		(1.3)		2-NC	173.5*	s
3-H	6.30	d	C-4	194.0	s		5.88	br d	C-3	128.1	br s
	(10.5)		C-5	32.3	t		(1.3)		3-NC	168.5*	s
5-H_2	2.66	dd	C-6	47.9	d	2′-H, 6′-H	7.79	d	C-4	115.9	t
	(19.2,	1.2)	C-7	57.6	br s		(9.0)		C-1′	124.1	s
	2.81	dd	7-NC	165.2	s	3′-H, 5′-H	6.98	d	C-2′, C-6′	132.1	d
	(19.2,	8.5)	C-8	46.7	t		(9.0)		C-3′, C-5′	114.5	d
6-H	3.52	m	C-9	118.1	br s	4′-OCH_3	3.87	s	C-4′	161.6	s
8-H_2	2.83	br d	9-NC	173.1	s				4′-OCH_3	55.4	q
	(13.3)		C-10	128.3	d						
	3.12	br dd	C-11	123.7	s						
	(13.3,	1.0)	C-12, C-16	131.4	d						
10-H	6.70	br s	C-13, C-15	114.4	d						
12-H, 16-H	7.70	d	C-14	161.3	s						
	(8.9)		14-OCH_3	55.4	q						
13-H, 15-H	6.97	d									
	(8.9)										
14-OCH_3	3.86	s									

δ: ppm from TMS (0 ppm) in $CDCl_3$.
m: Multiplicity.
* Interchangeable.
Coupling constants (J=Hz) are in parentheses.

Fig. 1. ^{1}H-^{13}C Correlation maps of MK4588 (**1**) and the degradation product (**2**).



1 **2**

information. A quaternary aromatic carbon (C-4') located as a multiplet at 161.6 ppm showed strong correlations to the methoxyl protons at 3.87 ppm and 2'-H, 6'-H at 7.79 ppm in the long range ^{1}H-^{13}C COSY spectrum. Another quaternary aromatic carbon at 124.1 ppm (C-1') was coupled to the 3'-H, 5'-H at 6.98 ppm (J=7.7 Hz). Furthermore, the long-range couplings were observed between 1-H at 7.00 ppm and C-2', C-6' at 132.1 ppm (J=5.8 Hz), and between C-1 at 129.5 ppm and 2'-H, 6'-H (J=4.5 Hz). Accordingly, **2** possess a 4-methoxyphenylvinylidene structure. The ^{13}C NMR signals at 114.4 and 128.1 ppm were very broadened, probably due to the spin coupling with an ^{14}N of isocyano group, so that they were assigned to the carbons linked isocyano group. In addition, by low power irradiation of 1-H and one of the exo-methylene protons at 5.74 ppm, remarkable enhancements of the ^{13}C signals were observed at 128.1 and 114.4 ppm, respectively. From these results, the structure of **2** was determined to be 2,3-diisocyano-1-(4-methoxyphenyl)buta-1,3-diene. The summary of the long range ^{1}H-^{13}C COSY and LSPD experiments on **2** is shown in Fig. 1.

Structure of MK4588 (1)

A comparison of NMR data for **1** with those for **2** shown in Table 2 revealed a partial structure of **1** from C-9 to C-16 including the 9-isocyano and 14-methoxyl groups that is identical with the corresponding structure of **2**. The remaining moiety of **1** consists of two methylene groups, one of which is adjacent to a methine proton, one disubstituted *cis*-ethylene, one isocyano and one hydroxyl groups. By spin decoupling experiments, it was shown that a methine proton at 3.52 ppm (6-H) was long-range coupled to the three protons at 6.87 (2-H), 2.83 and 3.12 ppm (8-H_2). Also, a long-range coupling was observed between one of methylene protons at 2.66 ppm (5-H) and an olefinic proton at 6.30 ppm (3-H). The above-mentioned results and the molecular formula of **1** suggested the presence of a bicyclo[4,2,0]octane structure. In order to confirm this partial structure, long-range ^{1}H-^{13}C COSY, heteronuclear multiple bond correlation (HMBC) spectroscopy and LSPD experiments were accomplished and the results are summarized in Fig. 1. An α,β-unsaturated carbonyl carbon at 194.0 ppm was coupled to 2-H, 3-H and 5-H_2, and a quaternary carbon at 64.2 ppm was coupled to 1-OH, 3-H and 8-H_2. Furthermore, a broad carbon signal at 57.6 ppm was enhanced by low power irradiation of the protons at 3.12 (8-H) and 6.70 ppm (10-H). Therefore, the carbonyl carbon was assigned to C-4, and the hydroxyl and isocyano groups were located at C-1 and C-7 positions, respectively. From these results, the structure of **1** was deduced as shown in Fig. 1. This structure was supported by the fact that **1** was easily converted into **2** and hydroquinone in alkaline methanol.

The relative stereochemistry of **1** was finally determined by a single-crystal X-ray diffraction analysis. A computer-generated perspective drawing of **1** is shown in Fig. 2. The absolute configuration of **1** remains to be proved. Accordingly, structures **1** and **2** were determined to be (1*R**,6*S**,7*S**)-7-(*Z*)-(1-isocyano-2-(4-methoxyphenyl))ethenyl-1-hydroxy-7-isocyanobicyclo[4,2,0]oct-2-en-4-one and (*Z*)-2,3-diisocyano-1-(4-methoxyphenyl)buta-1,3-diene, respectively, as shown in Fig. 3.

Fig. 2. Molecular structure of MK4588 (**1**).

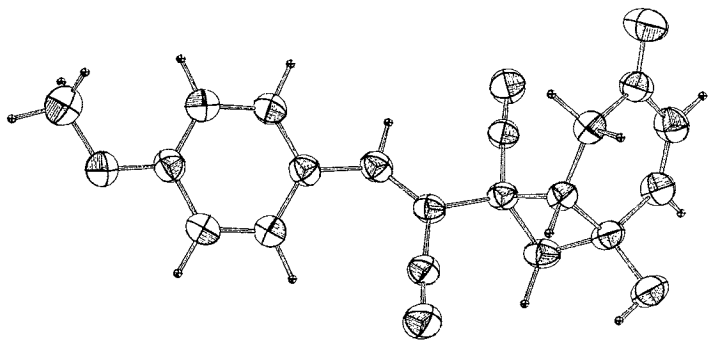
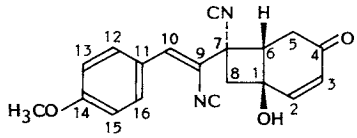
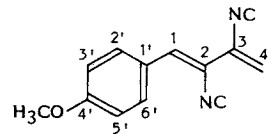


Fig. 3. Structures of MK4588 (**1**) and the degradation product (**2**).



1



2

Table 3. Antibacterial activities of MK4588 (**1**) and the degradation product (**2**).

Test organisms	MIC (μg/ml)		Test organisms	MIC (μg/ml)	
	1	**2**		**1**	**2**
Staphylococcus aureus 209-P JC-1	25	6.25	*S. typhimurium* LT-2	>100	>100
S. aureus Smith S-424	25	12.5	*Salmonella* sp. D-0001	100	>100
S. aureus No. 26	>100	>100	*Shigella sonnei* EW 33 Type 1	>100	>100
S. epidermidis ATCC 14990	100	50	*Klebsiella pneumoniae* PCI 602	>100	>100
S. epidermidis 109	100	50	*K. pneumoniae* 22#3038	1.56	0.78
Enterococcus faecium ATCC 8043	>100	>100	*Proteus vulgaris* OX19	>100	>100
Bacillus anthracis No. 119	6.25	6.25	*P. mirabilis* GN310	1.56	0.78
Escherichia coli JC-2	>100	>100	*Providencia rettgeri* J-0026	12.5	1.56
E. coli No. 29	>100	>100	*Morganella morganii* Kono	3.13	1.56
E. coli W3630 RGN823	>100	>100	*Serratia marcescens* MB-3848	>100	>100
E. coli JR66/W677	>100	>100	*Pseudomonas aeruginosa* MB-3829	>100	>100
Citrobacter freundii GN346	>100	>100	*P. cepacia* M-0527	25	6.25
Salmonella typhi O-901-W	50	6.25	*Xanthomonas maltophilia* M-0627	50	>100
S. enteritidis No. 11	50	6.25			

Inoculum size: 10^6 cfu/ml.
Medium: Sensitivity Disk Agar-N (Nissui).

Biological Activities

The antibacterial activities of MK4588 (**1**) and its degradation product (**2**) are shown in Table 3. The antibiotics **1** and **2** showed moderate or strong activity against a limited range of Gram-positive and Gram-negative bacteria. It is very interesting that the antibiotics revealed inhibitory activity at a concentration of 0.78~12.5 μg/ml against *Klebsiella pneumoniae* 22#3038, *Proteus mirabilis* GN310, *Providencia rettgeri* J-0026 and *Morganella morganii* Kono, but no activity against four strains of *Escherichia coli* treated even at a concentration of 100 μg/ml. When tested in mice by the ip route, acute LD_{50} values for **1** and **2** were more than 40 mg/kg.

Experimental

General Procedure

UV and IR spectra were recorded on Shimadzu UV-260 and Hitachi 260-10 IR spectrophotometers, respectively. ^{1}H and ^{13}C NMR spectra were recorded on a Jeol JNM-GSX400 spectrometer with TMS as an internal standard in $CDCl_3$. The carbon numbers of MK4588 (**1**) and the degradation product (**2**) shown in Fig. 1 were used for the NMR assignments. MP's were determined with a Yanaco MP-S3 micro melting point apparatus and are uncorrected. Mass spectra were recorded with a Hitachi M-80B mass spectrometer. Optical rotations were measured with a Perkin Elmer model 241 polarimeter. TLC was done on a Silica gel 60 F_{254} plate, 0.25 mm thick (E. Merck, Art. No. 5715). Antibacterial activity was determined by the agar dilution method.

Single-crystal X-Ray Diffraction Analysis of MK4588

Antibiotic MK4588 was recrystallized from chloroform as transparent prismatic crystals. A crystal of approximate dimensions $0.15 \times 0.25 \times 0.25$ mm was mounted on a Philips PW-1100 X-ray diffractometer. All X-ray measurements were made using graphite monochromated CuKα radiation. The lattice constants were derived from setting angles of 15 higher angle ($\theta = 16.4° \sim 39.7°$) reflections.

Crystal data: $C_{19}H_{16}N_2O_3$, MW 320.35, orthorhombic, space group $P2_12_12_1$, $a = 10.480$ (5), $b = 21.810$ (11), $c = 7.245$ (4) Å, $U = 1656$ Å^3, $Z = 4$, $D_{calc} = 1.285$ gcm^{-3}, μ for CuKα radiation $= 1.70$ cm^{-1}.

Intensities were measured by a $2\theta - \omega$ scan method with a scan speed 0.1°/sec in ω. Backgrounds were measured at each end of the scan for half the total scan time. For weak reflections whose intensities were less than 3,000 counts during the single scan, the scans were repeated once more. A total of 1,595 reflections in the 2θ range $6° \sim 156°$ was measured. The phases of 196 strong reflections with $|E| > 1.45$ were determined by direct methods using MULTAN[8]. In the final refinement, the nonhydrogen atoms were refined anisotropically by block-diagonal least-squares. All the hydrogen atoms were located on the difference electron-density map and the structure was refined to an R value of 0.0476. In the present structure determination, no attempt has been made to assign the absolute configuration. The calculations were done on a IBM 3090 computer at the Meiji Information System Center, using the UNICS III program[9]. An ORTEP[10] drawing of the molecule is shown in Fig. 2†.

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† Atomic coordinates have been deposited with the Cambridge Crystallographic Data-base. The list of Fo, Fc and other data may be obtained from YASUO TAKEUCHI upon request.