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YM-181741, a Novel Benz[*a*]anthraquinone Antibiotic with Anti-*Helicobacter pylori* Activity from *Streptomyces* sp.

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A novel benz[*a*]anthraquinone, YM-181741, was isolated from the culture broth of actinomycete strain Q57219. The strain was identified as *Streptomyces* sp. by morphological and chemotaxonomic characterization. YM-181741 was purified from the culture supernatant by serial column chromatography. The structure of YM-181741 was determined by spectroscopic analysis including one- and two-dimensional NMR experiments. YM-181741 showed selective anti-*Helicobacter pylori* activity with a MIC value of 0.2 μg/ml.

Many recent studies have shown that peptic ulcer diseases are mainly caused by *Helicobacter pylori* (*H. pylori*) infection[1,2]. *H. pylori* is a microaerophilic, Gram-negative, spiral-shaped and flagellated bacterium, which is found only on gastric mucosa. Eradication of this bacterium dramatically decreases the recurrence rate in peptic ulcer patients. Treatment regimens including a proton pump inhibitor and antimicrobial agents such as amoxicillin and clarithromycin are now recommended[3]. However, these therapies have associated problems including side effects (*e.g.* diarrhea), build-up of drug resistance, and poor compliance[4,5]. Therefore, the development of a new class of anti-*H. pylori* agents is needed.

In the course of our screening for anti-*H. pylori* agents, we have isolated *N*-acetyl aureothamine from the culture broth of *Streptomyces netropsis* JCM 4544[6]. *N*-acetyl aureothamine and other γ-pyrone compounds had highly selective anti-*H. pylori* activity. Our continuous screeninig for anti-*H. pylori* agents led to the discovery of a novel antibiotic, YM-181741 (**1**), in the culture broth of *Streptomyces* sp. Q57219. In this paper, we describe the taxonomy of the producing strain, fermentation, isolation, structure elucidation, and the biological properties of **1**.

Materials and Methods

Taxonomic Studies

Morphological, cultural and physiological properties were examined according to the methods described by SHIRLING and GOTTLIEB[7]. Morphological observations were made using scanning electron microscopy (JEOL T220). The chemical composition of the cells was determined using the methods of LECHEVALIER and LECHEVALIER[8] and BECKER *et al.*[9]. Color names were taken from the Guide to Color Standard by Nihon Shikisai Co., Ltd.

Spectral Analysis

Mass spectra were measured on a JEOL JMS-700T mass spectrometer. UV and IR spectra were recorded on a

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Table 1. Cultural characteristics of strain Q57219.

Medium	Growth	Aerial mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP No.2)	Good Pale yellowish brown	Abundant Light gray	None
Oatmeal agar (ISP No.3)	Good Pale yellowish brown	Abundant Light gray	None
Inorganic salts-starch agar (ISP No.4)	Good Pale yellowish brown	Abundant Light gray	None
Glycerol-asparagine agar (ISP No.5)	Moderate Pale yellowish brown	Moderate Grayish white-light gray	None
Tyrosine agar (ISP No.7)	Moderate Pale yellowish brown	Moderate Grayish white	None
Nutrient agar	Good Pale yellow	Poor Grayish white	None
Sucrose-nitrate agar	Poor	Moderate Bluish gray	None
Glucose-asparagine agar	Moderate Yellowish brown	Moderate Bluish gray	None

Shimadzu UV-2200 and PERKIN ELMER 2000 FT-IR spectrophotometers, respectively. Optical rotations were determined using a HORIBA SEPA-200 polarimeter and CD spectra were measured on a JASCO J-720W spectropolarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-LAMBDA500 FT NMR spectrometer.

Antimicrobial Activity

Antimicrobial activities were determined using a conventional agar dilution method. *H. pylori* were cultivated on Brucella agar at 37°C for 72 hours in an atmosphere of 80% N_2, 15% CO_2 and 5% O_2. Aerobic bacteria were cultivated on Mueller-Hinton agar at 37°C for 18 hours under air. Anaerobic bacteria were cultivated on GAM agar at 37°C for 18 hours in an atmosphere of 80% N_2, 10% CO_2 and 10% H_2.

Results and Discussion

Taxonomy of the Producing Strain

Strain Q57219 was isolated from a soil sample collected in Yakushima, Kagoshima, Japan. The vegetative mycelia and aerial mycelia were well developed and branched, and fragmentation of the vegetative mycelia was not observed. The spore chains were of the spiral type and each had 10 to 50 spores per chain. The spores were cylindrical, with a diameter of 1.0~1.9×0.6~1.2 μm, and had a smooth surface. No special morphology, such as the presence of sclerotia and sporangia, was observed. This strain grew well on yeast extract-malt extract agar, oatmeal agar, and inorganic salts-starch agar. The vegetative mycelia were pale yellowish brown and the aerial mycelia were grayish white to gray. No soluble pigment was formed on any media. The cultural characteristics of strain Q57219 are summarized in Table 1. The whole-cell hydrolysate contained LL-diaminopimelic acid, suggesting that this strain possesses a type I cell wall. The physiological properties and carbon utilization data are shown in Table 2. On the basis of these morphological and chemotaxonomic characteristics, the strain was assigned to the genus *Streptomyces*. Strain Q57219 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the name *Streptomyces* sp. Q57219 and under the accession number FERM P-17283.

Fermentation

A slant culture of strain Q57219 grown on Bennett's agar was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 1%, potato starch 2%, Polypepton (Nihon Pharmaceutical Co., Ltd.) 0.5%, yeast extract 0.5%, and $CaCO_3$ 0.4% (pH 7.0). The seed culture was incubated at 28°C for three days on a rotary shaker at 220 rpm. This culture (3 ml) was transferred to each of six 500-ml Erlenmeyer flasks containing 100 ml of the seed medium to produce a second seed culture that was then incubated similarly. The second seed culture (3 ml) was inoculated into each of two hundred 500-ml Erlenmeyer flasks containing 100 ml of a production medium consisting of dextrin 5%, corn steep liquor 3%, Polypepton (Nihon Pharmaceutical Co., Ltd.) 0.1%, and $CaCO_3$ 0.5% (pH 7.0). The fermentation was carried out at 28°C for five days on a rotary shaker at 220 rpm.

Table 2. Physiological properties and carbon utilization data of strain Q57219.

Temperature range for growth	15~37°C
Optimum temperature for growth	24~32°C
Formation of melanoid pigment	
Peptone-yeast extract-iron agar (ISP No.6)	−
Tyrosine agar (ISP No.7)	−
Liquefaction of gelatin	+
Coagulation of milk	−
Peptonization of milk	+
Hydrolysis of starch	+
Decomposition of cellulose	−
Reduction of nitrate	−
Utilization of carbon source	
L-Arabinose	−
D-Xylose	±
D-Glucose	+
D-Fructose	+
Sucrose	−
Inositol	+
Rhamnose	−
Raffinose	−
D-Mannitol	+

Isolation

The fermentation broth (20 liters) was filtered and the filtrate was applied to a Diaion HP-20 column. The column was washed with MeOH/H_2O (8 : 2) and MeOH, and then eluted with acetone. The acetone eluate was partitioned between hexane and MeOH/H_2O (9 : 1). The aqueous MeOH layer was subjected to silica gel column chromatography using $CHCl_3$/MeOH (20 : 1) as an eluent. The active fraction was then applied to a silica gel column and developed with hexane/EtOAc (1 : 2). The combined active fraction was finally purified by ODS HPLC on Cosmosil $5C_{18}$-AR with MeOH/H_2O (6 : 4) to yield 1.9 mg of **1**.

Structure Determination

The physico-chemical properties of **1** are listed in Table 3. The molecular formula of **1** was determined to be $C_{19}H_{14}O_5$ by high-resolution FAB-MS and NMR data that also indicated thirteen degrees of unsaturation. The IR spectrum suggested the presence of hydroxyl groups (3420 cm^{-1}) and conjugated carbonyl groups (1700, 1680 and 1640 cm^{-1}). The ^1H NMR and DEPT spectra revealed the presence of two methylenes, an oxygenated methylene, a methine, five aromatic methines, three conjugated

Table 3. Physico-chemical properties of YM-181741 (**1**).

Appearance	Yellow powder
Molecular weight	322
Molecular formula	$C_{19}H_{14}O_5$
HRFAB-MS (m/z)	
Found:	323.0934 $(M+H)^+$
Calcd:	323.0919
$[\alpha]^{25}_D$	+ 29.6° (c 0.15, $CHCl_3$)
UV (MeOH) λ_{max} nm(ε)	265 (29700), 402(4800)
CD (MeOH) λ_{ext} nm (Δε)	494 (0.6), 412 (-0.1), 348 (2.3), 303 (0.1), 280 (3.9)
	249 (-2.0), 234 (0.9), 224 (-1.0), 208 (1.3)
IR νmax (film) cm^{-1}	3420, 2920, 1700, 1680, 1640, 1590, 1460, 1360
	1280, 1220, 1160, 1090, 1050

Table 4. ^{1}H and ^{13}C NMR data of YM-181741 (**1**) in $CDCl_3$.

No.	^{13}C[a]	^{1}H (m, J in Hz)[b]	HMBC[c]
1	198.6		
2	42.0	3.04 (dd, 15.9, 4.9)	C-1, C-3, C-4, C-12b, C-13
		2.72 (dd, 15.9, 10.4)	
3	37.7	2.58 (m)	C-1, C-2, C-4, C-4a, C-13
4	32.8	3.11 (dd, 16.5, 4.3)	C-2, C-3, C-4a, C-12b, C-13
		2.86 (dd, 16.5, 10.4)	
4a	149.7		
5	133.2	7.59 (d, 7.9)	C-4, C-6, C-6a, C-12b
6	129.1	8.32 (d, 7.9)	C-7, C-12, C-12a, C-4a
6a	133.6		
7	187.5		
7a	115.5		
8	162.2		
9	123.7	7.28 (dd, 7.9, 1.8)	C-7a, C-11
10	137.1	7.66 (dd, 7.9, 7.3)	C-8, C-11a
11	119.6	7.69 (dd, 7.3, 1.8)	C-7a, C-9, C-12
11a	135.1		
12	182.9		
12a	135.7		
12b	136.9		
13	65.9	3.78 (dd, 10.4, 4.9)	C-2, C-3, C-4
		3.70 (dd, 10.4, 6.7)	
8-OH		12.29 (br s)	

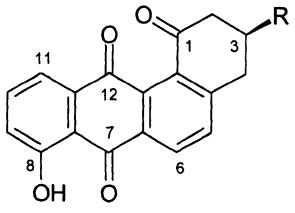
[a] 125 MHz. [b] 500MHz. [c] Parameters were optimized for J_{CH}=8 Hz.

carbonyls and seven other sp^2 quaternary carbons, which accounted for nine of the thirteen degrees of unsaturation. The remaining four degrees of unsaturation were attributed to four rings. The ^{1}H and ^{13}C NMR chemical shifts are shown in Table 4.

Analysis of one- and two-dimensional NMR spectra including COSY, HMQC and HMBC led to the assignment of two partial structures, **a** and **b**, as shown in Fig. 2. In partial structure **a**, a 1,2,3-trisubstituted benzene ring moiety was easily assigned by interpretation of the COSY and HMBC spectra. An aromatic proton H-11 was correlated with C-12 in the HMBC spectrum, indicating linkage between C-11a and C-12. Judging by the chemical shift of C-8 (δ_C 162.2), a phenolic hydroxyl group (δ_H 12.29) was attached to C-8. In partial structure **b**, the C^2–C^3(C^{13})–C^4 and C^5–C^6 portions were assigned by tracing of cross peaks in the COSY spectrum. A cyclohexenone ring moiety was disclosed by HMBC correlations (H-2/C-1, C-12b; H-3/C-1, C-4a; H-4/C-4a, C-12b) and the chemical shift of C-1 (δ_C 198.6). HMBC correlations (H5/C-4, C-6a, C-12b; H-6/C-4a, C-12a) indicated the presence of a benzene ring moiety fused with the cyclohexenone ring. A conjugated carbonyl at δ_C 187.5, which showed an HMBC correlation with H-6, could be located at C-7. Oxygenation of C-13 was inferred from its chemical shift at δ_C 65.9.

The phenolic hydroxyl proton 8-OH was hydrogen-bonded and H-6 gave a weak HMBC cross peak with C-12, indicating a connection between partial structures **a** and **b**. The presence of a quinone moiety was supported by the chemical shifts of C-7 (δ_C 187.5) and C-12 (δ_C 182.9). Considering the molecular formula, the oxygen on C-13 had to be provided by a hydroxyl group. Thus, the planar structure of YM-181741 (**1**) was determined as shown in Fig. 1. Structurally, YM-181741 is a novel member of the benz[*a*]anthraquinone antibiotics and is closely related to ochromycinone (**2**)[10,11], which lacks a 13-hydroxyl group. By comparing the CD spectrum of **1** (λ_{ext} nm ($\Delta\varepsilon$): 494 (0.6), 412 (−0.1), 348 (2.3), 303 (0.1), 280 (3.9), 249 (−2.0), 234 (0.9), 224 (−1.0), 208 (1.3)) with that of ochromycinone ((λ_{ext} nm ($\Delta\varepsilon$): 495 (0.4), 412 (−0.4), 347 (2.4), 303 (−0.2), 277 (4.7), 249 (−1.8), 234 (0.4), 225 (−2.4), 208 (0.2)) possessing 3S-configuration, the absolute configuration of **1** was assigned as 3S. The absolute stereostructure of YM-181741 (**1**) was determined as shown in Fig. 1.

Fig. 1. Structures of YM-181741 (**1**) and ochromycinone (**2**).



YM-181741 (**1**) R= CH_2OH

Ochromycinone (**2**) R= CH_3

Fig. 2. Partial structures of YM-181741 (**1**).

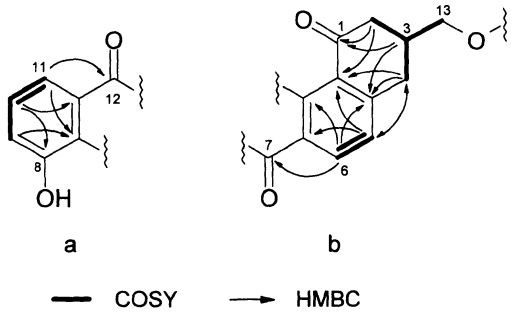


Table 5. Antimicrobial activities of YM-181741 (**1**), Ochromycinone (**2**) and the reference compounds.

Test organisms	MIC(μg/ml)			
	1	**2**	amoxicillin	clarithromycin
Helicobacter pylori ATCC 43504	0.2	0.1	0.025	0.013
Staphylococcus aureus FDA209P JC-1	>12.5	>12.5	0.39	0.2
Bacillus subtilis ATCC 6633	12.5	>12.5	0.2	0.2
Peptostreptococcus productus CAYA 12-2	>12.5	>12.5	0.05	0.025
Bifidobacterium bifidum CAYA21-1	>12.5	>12.5	0.39	0.1
Clostridium perfringens CAYA 39-1	>12.5	>12.5	0.1	0.78
Escherichia coli O-1	>12.5	>12.5	1.56	25
Klebsiella pneumoniae ATCC 10031	>12.5	>12.5	50	6.25
Pseudomonas aeruginosa ATCC 10490	>12.5	>12.5	>100	>100
Bacteroides fragilis GAI 5562	12.5	>12.5	12.5	0.78

Biological Properties

The antimicrobial activities of YM-181741 (**1**) are shown in Table 5. **1** exhibited anti-*H. pylori* activity with a MIC value of 0.2 μg/ml. The anti-*H. pylori* activity of another benz[*a*]anthraquinone, ochromycinone (**2**), was similar to that of **1** but weaker than that of amoxicillin and clarithromycin. Both benz[*a*]anthraquinones were inactive against other Gram-positive and Gram-negative bacteria that were tested, in contrast to amoxicillin and clarithromycin, which are active against a variety of microorganisms and therefore cause diarrhea as a side effect. These results suggest that benz[*a*]anthraquinones are selective anti-*H. pylori* agents with a low potential for causing diarrhea by the disturbance of intestinal microbial flora.

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