YM-32890 A and B, New Types of Macrolide Antibiotics Produced by *Cytophaga* sp.

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Two new types of macrolide antibiotics, YM-32890 A and B, have been isolated from the fermentation broth of *Cytophaga* sp. YL-02905S. In this paper, the taxonomy of the producing strain, fermentation, isolation, structure elucidation, and biological activity of the antibiotics are reported. YM-32890 A inhibits the growth of staphylococci including a macrolide-resistant strain, but shows no antimicrobial activity against other Gram-positive, Gram-negative bacteria and yeast.

In the course of a screening program, novel 22-membered macrolide antibiotics, YM-32890 A (**1**) and B (**2**) were isolated from the culture broth of *Cytophaga* sp. YL-02905S (Fig. 1). Antibiotic **1** exhibited the potent antimicrobial activity against staphylococci including the macrolide-resistant strain. On the other hand, **2**, which differs from **1** in the position and the geometry of the double bonds in the side chain, indicated no antimicrobial activity. The present paper deals with the taxonomy of the producing strain, fermentation, isolation, structure elucidation and biological activity of the new antibiotics.

Materials and Methods

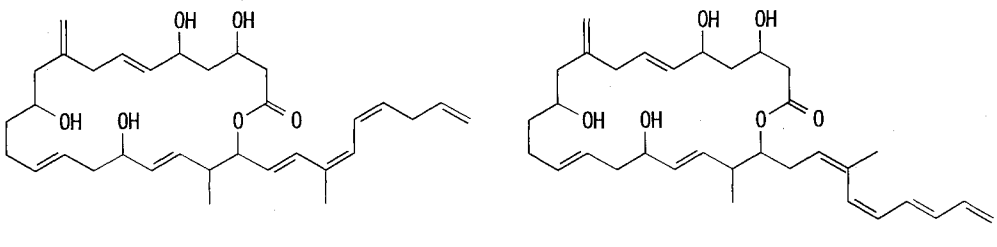
Isolation and Classification of Producing Organisms

The strain YL-02905S was freshly isolated from a forest soil sample collected in West Kalimantan, Indonesia. The strain YL-02905S was isolated on the GB-medium according to the method described in the previous paper[1]. Taxonomic study on the strain YL-02905S was carried out on the basis of methods of COWAN[2], CHRISTENSEN and COOK[3], DWORKI and GIBSON[4], GILARDI[5], MARMUR[6] and MARMUR and DOTY[7].

Fermentation

A thawed suspension of the producing organism was used to inoculated a 500-ml Erlenmeyer flask containing 100 ml of the seed medium consisting of poly peptone 1%, dry yeast 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCl_2$ 0.038%, pH 7.2. The flask was incubated at 27°C for 2 days on a rotary shaker at 220 rpm. The seed culture (2 ml) was transferred to 500-ml Erlenmeyer flasks containing 100 ml of the production medium consisting of potato starch 3%, S III meat (Ajinomoto) 1.5%,

Fig. 1. Structures of YM-32890 A (**1**) and B (**2**).



YM-32890 A (1) YM-32890 B (2)

pharma media 0.5%, carrot chip 0.5%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, NaCl 0.3% and $CoCl_2 \cdot 6H_2O$ 0.002%, pH 7.6. The fermentation was carried out at 27°C for 2 days on a rotary shaker at 220 rpm. The antibiotic production was monitored by HPLC analysis.

Physico-chemical Properties and Structure Elucidation

IR spectra were recorded on a Hitachi 260-50 infrared spectrophotometer. Fast atom bombardment mass spectra (FAB-MS) were obtained with a VG ZAB-VSE and a JEOL DX300 (positive) as matrix. ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-ALPHA500 FT NMR spectrometer.

Determination of Antimicrobial Activity and Cytotoxicity

MICs were determined by a conventional agar dilution method. The cytotoxicity of the antibiotics against L1210 cells was determined by using the trypan blue dye exclusion method. The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 20 mM HEPES buffer. The cells were incubated in the presence or absence of the antibiotics at 37°C for 3 days in a humidified atmosphere containing 5% CO_2.

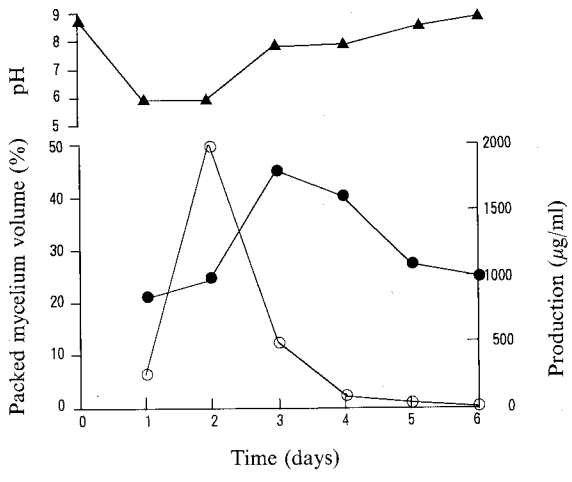
Results and Discussion

Producing Organism and Fermentation

The strain YL-02905S was freshly isolated from a forest soil collected in West Kalimantan, Indonesia. Colonies of the strain YL-02905S were semi-transparent, light yellow orange, circular, convex and entire-edged on nutrient agar. By light and electron microscopic observations, the strain YL-02905S was Gram negative, slender rods, usually 0.7~1.2 μm in width, 3.5~12.5 μm in length, without flagella. The strain YL-02905S exhibited gliding motility, but did not form fruiting bodies, intracellular granules, photosynthetic pigments, spores of microcysts. The temperature range for growth was 15 to 40°C. Oxidase, catalase and flexirubin reaction were positive. Nitrate was reduced to nitrite. The strain hydrolyzed starch, gelatin, casein and xanthine, but did not decompose cellulose, chitin and agar. The G+C content of DNA was 33.5 mol%. Major isoprenoid quinone was MK-7. Acid was formed observed from D-glucose, D-xylose, D-galactose, L-arabinose, sucrose, lactose and maltose. Based on the biological characteristics above, the strain YL-02905S was regarded as the genus *Cytophaga*, and designated *Cytophaga* sp. YL-02905S. The type strain was deposited in National Institute of Bioscience and Human-Technology, Ibaraki Prefecture, Japan, under the accession number FERM P-13370. A typical time course of the strain YL-02905S fermentation is presented in Fig. 2. The production of YM-32890 A was maximized after two days.

Fig. 2. Time course of the strain YL-02905S fermentation.

▲: pH, ●: Packed mycelium volume, ○: production of YM-32890 A.



Isolation

The purification procedure of **1** and **2** is outlined in Fig. 3. Acetone (2.5 liters) was added to the fermentation broth of *Cytophaga* sp. YL-02905S and the mixture was

Fig. 3. Purification procedure of YM-32890 A (**1**) and B (**2**).

Fermentation broth (2.5 liters)
- added to acetone (2.5 liters)
- shaked for 10 minutes
- centrifuged at 8,000 rpm

Supernatant
- concentrated *in vacuo* to remove acetone
- adjusted to pH 7.0
- extracted with EtOAc (3.0 liters)

Organic layer
- evaporated *in vacuo*

Brown syrup (2.64 g)
- Silica gel column chromatography
  eluted with $CHCl_3$ - MeOH (98 : 2)

Active fractions
- ODS-HPLC
  eluted with MeOH - CH_3CN - H_2O - THF (30 : 10 : 45 : 15)
  evaporated *in vacuo*

YM-32890 A (70 mg), B (30 mg)

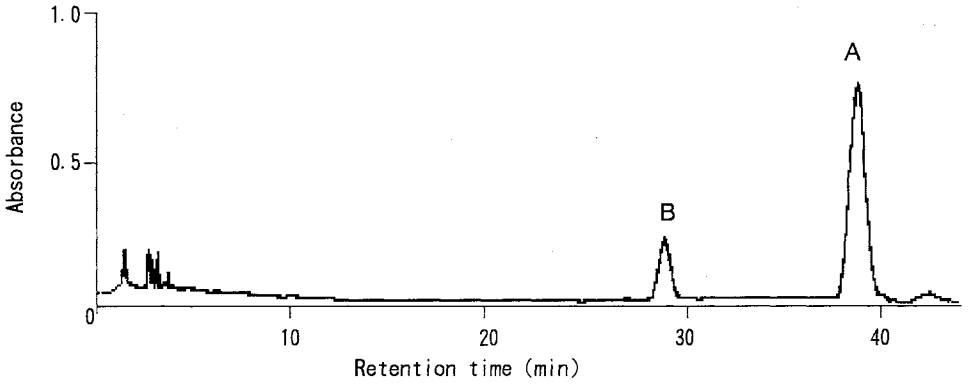
shaked for 10 minutes. The mixture was concentrated at 8,000 rpm for 15 minutes and then the supernatant was evaporated *in vacuo* to remove acetone. The aqueous solution was adjusted to pH 7.0 and extracted with 3.0 liters of ethyl acetate. The organic layer was evaporated *in vacuo* to obtain a brown syrup (2.64 g). The syrup was subjected to silica gel column chromatography (21 × 850 mm) and developed with $CHCl_3$-MeOH (98:2). The fractions exhibiting antibacterial activity against *Staphylococcus aureus* FAD 209P were collected and evaporated *in vacuo*. The yellow syrup was dissolved in methanol and applied to preparative HPLC (column; Simadzu STR-ODS, 20 × 250 mm, flow rate; 8.0 ml/minute). The column was eluted with methanol-acetonitrile-distilled water-tetrahydrofuran (30:10:45:15) to separate the peaks of **1** and **2** (Fig. 4). The yields of pure **1** and **2** were 70 and 30 mg respectively.

Physico-chemical Properties

Both **1** and **2** are unstable under light and aerobic conditions at room temperature and their color gradually turned brown. The molecular formula of **1** and **2** was determined to be $C_{33}H_{48}O_6$ on the basis of positive-ion high resolution FAB-MS data ($(M-H_2O)^+$ *m/z* calcd:

Fig. 4. HPLC chart of YM-32890 A (**1**) and B (**2**).

A: YM-32890 A, B: YM-32890 B.



HPLC condition
Column: Shimadzu STR-ODS (20 × 250 mm)
Mobile phase: MeOH-CH_3CN-H_2O-THF (30:10:45:15)
Flow rate: 8.0 ml/minute
Detection: UV 230 nm

Table 1. Pysico-chemical properties of YM-32890 A (**1**) and B (**2**).

Appearance	Colorless syrup	Colorless syrup
Molecular formula	$C_{33}H_{48}O_6$	$C_{33}H_{48}O_6$
HRFAB-MS (*m/z*)		
Calcd:	522.3345 $(M-H_2O)^+$	522.3345 $(M-H_2O)^+$
Found:	522.3348 $(M-H_2O)^+$	522.3350 $(M-H_2O)^+$
FAB-MS (*m/z*)	563 $(M+Na)^+$, 539 $(M-H)^+$	563 $(M+Na)^+$, 541 $(M+H)^+$, 539 $(M-H)^+$
UV λ_{max} nm (ε) (in MeOH)	278.4 (77,300)	277.6 (81,900), 285.6 (82,500), 299.0 (77.200), 313.4 (52.400)
IR ν_{max} (KBr) cm^{-1}	969, 1642, 1721, 2922, 3368	N.D.[a]
TLC Rf value[b]		
$CHCl_3$:MeOH (9:1)	0.36	0.36
EtOAc:MeOH (9:1)	0.55	0.55
Benzene:acetone (6:4)	0.24	0.24
Solubility		
Soluble:	MeOH, DMSO, EtOAc, $CHCl_3$	MeOH, DMSO, EtOAc, $CHCl_3$
Insoluble:	H_2O	H_2O
Color reaction		
Positive:	50% H_2SO_4[c]	50% H_2SO_4[c]
Negative:	Ninhydrin	Ninhydrin

[a] Not detected.
[b] TLC was performed with Merck Kieselgel $60F_{254}$.
[c] The color of the spot is blue.

522.3345, found: 522.3348 and 522.3350 respectively). The IR spectral data had absorption bands at 3368 cm^{-1} and 1721 cm^{-1} indicating the presence of hydroxyl group and carbonyl group respectively. The physico-chemical properties of **1** and **2** are shown in Table 1.

Structure Elucidation

The structure elucidation was mainly performed with

Fig. 5. ^{1}H-^{1}H DQF COSY, HMBC and NOE experiments of YM-32890 A (**1**) and B (**2**).

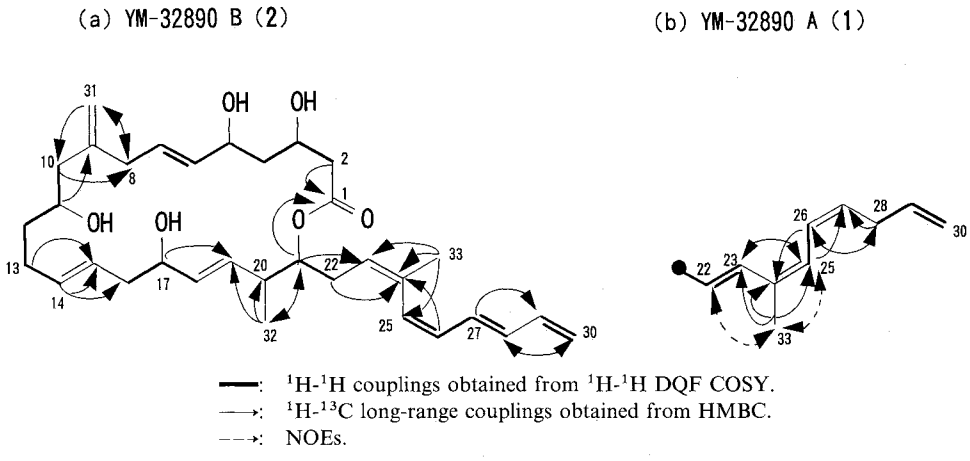


Table 2. ^{1}H NMR and ^{13}C NMR data of YM-32890 A (**1**) and B (**2**).

Position	YM-32890 A		YM32890 B	
	δ_C[a]	δ_H[b]	δ_C[a]	δ_H[b]
1	172.1		172.5	
2	44.2	2.46 (2H, m)	44.5	2.42 (dd, 14.3, 5.9), 2.49 (dd, 14.3, 8.1)
3	66.8	4.02 (m)	67.0	4.00 (m)
4	45.3	1.55, 1.74 (m)	45.2	1.59, 1.75 (m)
5	71.5	4.25 (m)	71.5	4.25 (m)
6	135.7	5.45 (d, 14.6)	135.7	5.46 (m)
7	131.4	5.61 (d, 14.6)	131.6	5.63 (dt, 14.6, 10.0)
8	40.3	2.82 (2H, m)	40.3	2.82 (2H, t, 10.0)
9	146.1		146.2	
10	44.3	2.06, 2.31 (m)	44.5	2.09, 2.29 (m)
11	68.9	3.73 (m)	69.0	3.73 (m)
12	38.1	1.50 (2H, m)	38.0	1.49 (2H, m)
13	30.0	2.07 (2H, m)	29.9	2.07 (2H, m)
14	133.6	5.49 (dt, 15.3, 10.0)	133.6	5.49 (dt, 15.3, 10.0)
15	127.3	5.36 (dt, 15.3, 10.0)	127.3	5.36 (dt, 15.3, 10.0)
16	41.4	2.18, 2.28 (m)	41.5	2.14, 2.28 (m)
17	73.7	3.98 (m)	74.0	3.95 (m)
18	135.1	5.49 (m)	135.2	5.42 (m)
19	133.9	5.49 (m)	133.7	5.40 (m)
20	42.7	2.43 (m)	42.1	2.31 (m)
21	80.0	5.20 (dd, 7.8, 7.8)	78.2	4.84 (m)
22	128.5	5.66 (m)	33.3	2.21, 2.28 (m)
23	131.6	6.89 (d, 15.5)	125.6	5.34 (m)
24	133.9		136.5	
25	126.4	6.29 (d, 11.6)	130.6	5.95 (d, 10.0)
26	125.3	6.52 (dd, 11.6, 11.6)	130.6	6.11 (dd, 10.0, 10.0)
27	130.1	5.49 (m)	131.5	6.44 (dd, 15.0, 10.0)
28	32.6	2.96 (2H, dd, 6.3, 6.3)	136.0	6.28 (dd, 15.0, 10.0)
29	137.6	5.83 (ddt, 17.0, 10.3, 6.3)	138.5	6.42 (ddd, 15.0, 10.5, 10.0)
30	115.5	4.98 (dd, 10.3, 1.8), 5.05 (dd, 17.0, 1.8)	118.2	5.10 (d, 10.5), 5.25 (d, 15.0)
31	114.0	4.81, 4.89 (br s)	113.9	4.83, 4.87 (br s)
32	17.5	1.00 (3H, d, 7.0)	17.9	0.97 (3H, d, 6.3)
33	20.9	1.90 (3H, s)	24.5	1.87 (3H, s)

[a] 125 MHz, CD_3OD as solvent.
[b] 500 MHz, CD_3OD as solvent.

2. The ^{13}C NMR spectrum of **2** showed 33 carbon signals which were assigned to three methyl, nine methylene, eighteen methine and three quaternary carbons by a DEPT experiment. The ^{1}H and ^{13}C NMR spectral data of **2** are summarized in Table 2. A ^{1}H-^{1}H DQF COSY experiment of **2** revealed six spin networks, from 2-H to 8-H, from 10-H to 14-H, from 16-H to 18-H, from 20-H to 22-H, from 25-H to 28-H and from 29-H to 30-H as shown in Fig. 5a. The heteronuclear multiple-bond correlation (HMBC)[8] spectrum displayed ^{1}H-^{13}C long-range couplings indicating the following six connections: $-C^{8}H_2-(C^{9}=C^{31}H_2)-C^{10}H_2-$, $-C^{14}H=C^{15}H-C^{16}H_2-$, $-C^{18}H=C^{19}H-(C^{20}H-C^{32}H_3)-$, $-C^{22}H_2-C^{23}H=(C^{24}-C^{33}H_3)-C^{25}H=$, $=C^{28}H-C^{29}H=$ and $>C^{21}H-O-(C^{1}=O)-C^{2}H_2-$. The geometries of the two disubstituted double bonds, C-6, C-14, C-25 and C-27 were determined to be 6*E*, 14*E*, 25*Z* and 27*E* by the coupling constants, $J_{6,7}=14.6$ Hz, $J_{14,15}=15.3$ Hz, $J_{25,26}=10.0$ Hz and $J_{27,28}=15.0$ Hz. The geometrical configuration of C-23 was determined to be 23*Z* by a lower-field chemical shift for C-33 (δ_C 24.5)[9].

The lactone moiety of **1** was determined to be the same structure as that of **2** judging from the similarity of the ^{1}H-^{1}H DQF COSY and HMBC spectral data. The structure of the side-chain moiety of **1** was elucidated from the analysis of the ^{1}H-^{1}H DQF COSY, HMBC and NOE data. A ^{1}H-^{1}H DQF COSY experiment of **1** revealed three proton sequences, from 21-H to 23-H, from 25-H to 26-H and from 28-H to 30-H as shown in Fig. 5b. The HMBC spectrum displayed ^{1}H-^{13}C long-range couplings indicating the following connections: $=C^{23}H-(C^{24}-C^{33}H_3)=C^{25}H-$ and $-C^{26}H=C^{27}H-C^{28}H_2-$. The geometrical configurations of C-22 and C-26 were determined to be 22*E* and 26*Z* by $J_{22,23}=15.5$ Hz and $J_{26,27}=11.6$ Hz. The 22*E* configuration was confirmed by NOE observed between 22-H and 33-H. And the geometry of C-24 was shown to be 24*Z* by NOE observed between 25-H and 33-H.

Biological Activity

Antimicrobial activity of **1** and **2** is shown in Table 3. MICs were determined by the serial agar dilution method using Mueller-Hilton medium. Antibiotic **1** exhibited the potent activity against staphylococci including the macrolide-resistant strain, but showed no antimicrobial activity against other Gram-positive, Gram-negative bacteria and yeast. On the other hand, **2** indicated no antimicrobial activity against the microorganisms described above. Cytocidal activity of **1** and **2** was examined against L1210 cells *in vitro*. When the cells were exposed to the antibiotics for 3 days, the IC_{50} values of **1** and **2** were 15.7 and 70.0 μg/ml respectively. We consider the structure of the side chain in **1** plays an important role in anti-staphylococcal activity.

Table 3. Antimicrobial Activity of YM-32890 A (**1**) and B (**2**).

Test organism	MIC (μg/ml)	
	YM-32890 A	YM-32890 B
Staphylococcus aureus FDA 209P	0.05	>100
S. aureus (Mac^{R})[a]	0.4	>100
S. aureus (Meth^{R})[b]	0.2	>100
S. epidermidis IID886	1.6	>100
Bacillus subtilis ATCC 6633	>100	>100
Escherichia coli O-1	>100	>100
Pseudomonas aeruginosa NCTC 10490	>100	>100
Klebsiella pneumoniae ATCC 10031	>100	>100
Proteus vulgalis IFM OM-9	>100	>100
Mycobacterium smegmatis ATCC 607	>100	>100
Candida albicans ATCC 10231	>100	>100

[a] Resistant to macrolide.
[b] Resistant to methicillin.

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