VOL. 55 NO. 3, MAR. 2002 THE JOURNAL OF ANTIBIOTICS pp. 249 — 255

Watasemycins A and B, New Antibiotics Produced by *Streptomyces* sp. TP-A0597

TOMOMITSU SASAKI, YASUHIRO IGARASHI, NORIKO SAITO[a] and TAMOTSU FURUMAI*

Biotechnology Research Center, Toyama Prefectural University
5180 Kurokawa, Kosugi, Toyama 939-0398, Japan
[a] National Institute of Infectious Diseases
1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan

(Received for publication October 9, 2001)

Two novel antibiotics, watasemycins A and B, were isolated from the fermentation broth of an actinomycete strain. The producing strain TP-A0597 was isolated from the seawater sample collected in Toyama Bay, Japan and identified as *Streptomyces* sp. based on the taxonomic study. The new antibiotics were obtained by solvent extraction and chromatographic purification and spectroscopic analyses identified that they were new analogs of thiazostatins. Watasemycin possesses a methyl group at 5′-position of thiazostatin instead of a hydrogen atom. Watasemycins showed antibiotic activity against Gram-positive and negative bacteria and yeast.

Soil microorganisms have been utilized as a rich source of therapeutically important molecules over five decades[1]. However, the frequency of discovering structurally new compounds is obviously decreasing in these years, implying that we exhausted the easily accessible microbes in soil and have to seek them from unexplored sources different from conventional ones. The diversity of secondary metabolites relies more or less on the isolation source, namely, the habitat of the producers. Marine environment is one of the currently, intensively exploited sources in the natural product screening[2]. We examined the metabolites of actinomycetes isolated from the seawater samples collected in Toyama Bay, Japan, and found a couple of new antibitoics produced by *Micromonospora* sp.[3~6] Watasemycins are novel antibiotics produced by an actinomycete inhabiting the marine environment. The producing strain, TP-A0597, was identified as *Streptomyces* sp. based on the taxonomic study. Structural analyses revealed that watasemycins are new analogs of pyochelin[7] and thiazostatin[8].

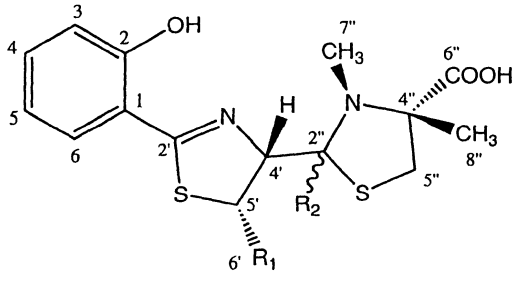
In this paper, we describe the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties, structure determination and biological properties of watasemycins A and B.

Materials and Methods

Microorganism

The producing microorganism, strain TP-A0597, was isolated from a seawater sample collected in Toyama Bay, Japan as described in our previous report[1]. The strain was isolated by the membrane filter method and cultured on an agar plate. A pure culture of strain TP-A0597 was

Fig. 1. Structures of watasemycins A and B, and thiazostatins A and B.



Watasemycin A : $R_1 = CH_3$ R_2 = ····H
B : $R_1 = CH_3$ R_2 = ◀H
Thiazostatin A : $R_1 = H$ R_2 = ····H
B : $R_1 = H$ R_2 = ◀H

* Corresponding author: furumai@pu-toyama.ac.jp

preserved in 20% glycerol at −80°C. It was also maintained on Bennett's agar slant at 10°C for laboratory use.

Taxonomy

Taxonomic characteristics of strain TP-A0597 were determined by cultivation on various media described by SHIRLING and GOTTLIEB[9], WAKSMAN[10] and ARAI[11]. Morphological characteristics were observed after incubation of the culture at 30°C for 14 days on oatmeal agar (ISP med 3). Cultural characteristics were determined after growth at 30°C for 14 days. The color names and hue numbers were assigned using the Manual of Color Names (Japan Color Enterprises Co., Ltd., 1987). The carbon utilization was determined by the method of SHIRLING and GOTTLIEB[9]. Cell wall composition was analyzed by the method of LECHEVALIER and LECHEVALIER[12], using thin layer chromatography plates as described by STANECK and ROBERTS[13].

Instrumental Analysis

Melting points were determined on a Yanagimoto apparatus and are uncorrected. NMR experiments were performed on JEOL JNM-LA400 NMR spectrometer in $CDCl_3$. The MS spectra were measured on a JEOL JMS-HX110A spectrometer. UV spectra were recorded on a Beckman DU 640 spectrophotometer. Optical rotations were measured on a Horiba SEPA-300 polarimeter.

Biological Assay

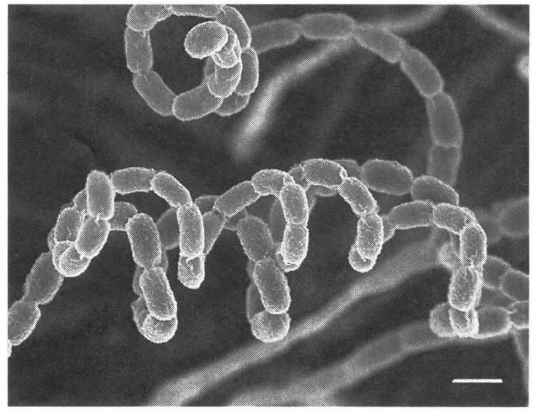
Antibiotic activity in fermentation broths and purification samples were monitored by the conventional paper disc assay using *Bacillus subtilis* M-45 as an indicator strain. MIC values were determined by the conventional serial two-fold dilution method against laboratory strains.

Results and Discussion

Taxonomy of the Producing Strain

By scanning electron microscopy, strain TP-A0597 formed spiral type spores which were cylindrical, 0.6~0.7×1.0~1.1 μm in size, having a smooth surface (Fig. 2). The cultural characteristics of TP-A0597 are summarized in Table 1. Color of aerial mycelia was medium gray and color of reverse side was olive gray. Diffusible pigments were not formed. Strain TP-A0597 utilized L-arabinose, sucrose, D-xylose, D-glucose, inositol, D-mannitol, D-fructose, L-rhamnose and raffinose for growth. Analysis of the whole-cell hydrolysates showed the presence of L,L-diaminopimelic acid and galactose. On the basis of these morphological and chemical characteristics, strain TP-A0597 was assigned to the genus *Streptomyces*.

Fig. 2. Scanning electron micrograph of *Streptomyces* sp. TP-A0597.



Bar represents 1.0 μm.

Table 1. Cultural characteristics of strain TP-A0597.

Medium	Growth	Aerial mycelium	Reverse side	Diffusible pigment
Yeast extract - malt extract agar (ISP med. 2)	good	Medium gray (406)	Brownish olive (163)	None
Oatmeal agar (ISP med. 3)	good	Medium gray (406)	Olive gray (411)	None
Inorganic salts - starch agar (ISP med. 4)	good	Medium gray (406)	Deep yellow (153)	None
Glycerol asparagine agar (ISP med. 5)	poor	Raddish gray (408)	Medium gray (406)	None
Tyrosine agar (ISP med. 7)	poor	Raddish gray (408)	Brownish gray (409)	None

Fermentation

A slant culture of the strain TP-A0597 grown on Bn-2 agar consisting of glucose 0.5%, soluble starch 0.5%, meat extract (Kyokuto) 0.1%, yeast extract (Difco Laboratories) 0.1%, NZ-case (Humco Scheffield Chemical Co.) 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% and agar 1.5% was inoculated into three 500-ml K-1 flasks containing 100 ml of seed medium composed of soluble starch 1.0%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, Tryptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0). The seed culture was incubated for 4 days at 30°C on a rotary shaker (200 rpm). Three ml each of the culture was inoculated into fifty of 500-ml K-1 flasks containing 100 ml of production medium composed of glucose 0.5%, glycerol 2.0%, soluble starch 2.0%, Pharmamedia (Trader's Protein) 1.5%, yeast extract 0.3% and Diaion HP-20 (Mitsubishi Chemical Co.) 1.0%. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were incubated for 7 days at 30°C on a rotary shaker (200 rpm).

Isolation

After the addition of acetone (100 ml each), the fermentation flasks were shaken on a rotary shaker (200 rpm) at 30°C for 2 hours. The aqueous acetone solution (10 liters) was centrifuged (8,000 rpm, 15 minutes) to separate into the mycelium and the supernatant. The supernatant was concentrated *in vacuo* and the resultant aqueous solution (5 liters) was applied to a column of Diaion HP-20 (1 liter). The column was washed with water (1 liter) and eluted consecutively with 20, 40, 60 and 80% aqueous acetone (1 liter each). The antibacterial activity was found in the fractions eluted with 60 and 80% acetone. The active fractions were combined and concentrated *in vacuo* and the remaining aqueous solution was extracted with ethyl acetate (1 liter×2). After drying over anhydrous Na_2SO_4, the organic layer was concentrated *in vacuo* to give crude extract (289 mg). This was subjected to a column of reversed phase silica gel column chromatography (YMC gel ODS-AM 120-S50; Yamamura Chemical Lab., 0.4 liter), which was eluted with acetonitrile - 0.15% KH_2PO_4, pH 3.5 (20 : 80~80 : 20). The active fractions were pooled, adjusted to pH 7.0 with $NaHCO_3$ solution and evaporated. The resulting aqueous solution was extracted with ethyl acetate (50 ml×2). The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give brown powder (9.8 mg). This material was subjected to a preparative HPLC using an ODS column (Cosmosil 5C18-AR-II, Nacalai Tesque Inc., 250×20 mm, i.d.) eluted with acetonitrile - 0.15% KH_2PO_4, pH 3.5 (70 : 30) at a flow rate of 8.0 ml/minute. Fractionation was guided by UV absorbance at 254 nm to afford four active peaks in the order of watasemycin A, thiazostatins A, watasemycin B and thiazostatin B. These active fractions were adjusted to pH 7.0 with $NaHCO_3$ solution and concentrated *in vacuo*. The resulting solutions were extracted with ethyl acetate (20 ml×2) respectively. Each organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give watasemycin A (4.3 mg), watasemycin B (1.0 mg), thiazostatin A (1.5 mg) and thiazostatine B (0.8 mg) as a pale yellow powder, respectively.

Structure Determination

Watasemycin A

Physico-chemical properties of watasemycins A (**1**) and B (**2**) are summarized in Table 2. The molecular formula of **1** was determined as $C_{16}H_{20}N_2O_3S_2$ on the basis of the HRFAB-MS, which gave a $[M+H]^+$ ion at *m/z* 353.0994 (Δ+0.1 mmu; calcd for $C_{16}H_{21}N_2O_3S_2$), and ^{13}C-NMR spectral data. The structure of **1** was determined by comparing its NMR spectra with those of thiazostatins. The major differences found in the ^{1}H- and ^{13}C-NMR spectra of **1** were the lack of the signal due to a methylene at C-5′ of thiazostatin and the appearance of signals due to a methyl group (δ_H 1.26, δ_C 17.0) and a methine group (δ_H 3.91, δ_C 46.1). This observation indicated that the methyl group was substituted at C-5′ position, which was confirmed by the detection of proton-spin system of the C-5′ methyl group/H-5′/H-4′/H-2″ in DQF-COSY and spin-decoupling experiments (Fig. 3).

The relative configuration of **1** was determined by NOESY and difference NOE experiments (Fig. 4). The observed NOE between H-4′ and H-5′ confirmed that the C-2″ substituent and C-5′ methyl group were *syn*. The *anti*-relationship between H-4′ and H-2″ was confirmed by the large *J* coupling (10.7 Hz) between them and the detection of NOE between C-5′ methyl and H-2″. In addition, NOEs among H-4′, H-5″β, 3″-*N*-methyl and 4″-methyl groups established that the H-5″β, 3″-*N*-methyl and 4″-methyl groups were on the β-face of the molecule (Fig. 4). Based on these results, the structure of **1** was determined as shown in Fig. 1.

Watasemycin B

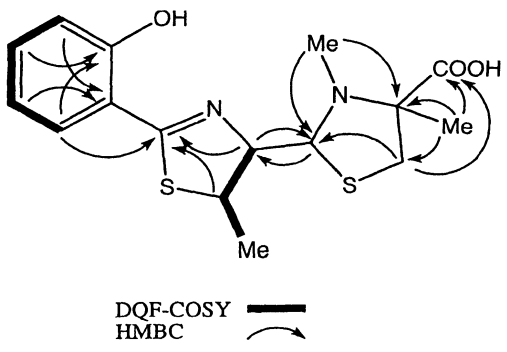
The molecular formula of watasemycin B (**2**) was assigned as $C_{16}H_{20}N_2O_3S_2$ on the basis of the HRFAB-MS, in which the parent ion peak $[M+H]^+$ was detected at *m/z*

Table 2. Physico-chemical properties of watasemycins A (**1**) and B (**2**).

	1	**2**
Appearance	pale yellow powder	pale yellow powder
Mp.	62~65°C	58~60°C
$[\alpha]_D^{28}$	+20.5 (*c* 0.20, $CHCl_3$)	-2.5 (*c* 0.20, $CHCl_3$)
HRFAB-MS		
Found:	353.0995 $[M+H]^+$	353.0998 $[M+H]^+$
Calcd:	353.0994 (for $C_{16}H_{21}N_2O_3S_2$)	353.0994 (for $C_{16}H_{21}N_2O_3S_2$)
Molecular formula	$C_{16}H_{20}N_2O_3S_2$	$C_{16}H_{20}N_2O_3S_2$
UV λ_{max} nm (log ε)		
in MeOH	212 (4.17), 252 (3.78), 319 (3.45)	216 (4.21), 286 (3.87), 322 (3.85)
in 0.01N HCl-MeOH (1:9)	215 (3.56), 259 (3.49), 320 (3.05)	220 (3.91), 288 (3.81), 323 (3.84)
in 0.01N NaOH-MeOH (1:9)	217 (3.82), 254 (3.55), 320 (3.05)	222 (3.97), 287 (3.81), 322 (3.80)
Solubility		
soluble in	methanol, $CHCl_3$	methanol, $CHCl_3$
insoluble in	*n*-hexane, H_2O	*n*-hexane, H_2O
TLC (Rf)[a]	0.47	0.20
HPLC (Rt)[b]	5.9 min	7.7 min

[a] Silica gel TLC (Merck Art 5715): ($CHCl_3$:MeOH=10:1)
[b] HPLC conditions: Cosmosil AR-II (250 × 4.6 mm, i.d.), mobile phase: CH_3CN-0.15% KH_2PO_4 (pH 3.5) (60:40), flow rate: 1.0 ml/min, detection: UV-254 nm.

Fig. 3. NMR analysis of watasemycin A.



353.0998 (Δ+0.4 mmu; calcd for $C_{16}H_{21}N_2O_3S_2$), and ^{13}C-NMR spectral data. The ^1H- and ^{13}C-NMR spectra of **2** were very similar to those of **1**. The HMBC spectrum revealed that the planar structure of **2** was identical with **1** as shown in Fig. 3.

The relative configuration of **2** was determined by analyzing the observed NOEs in comparison with **1**. The observation of NOESY correlations between H-5′ and H-4′, Hβ-5″ and H-8″, H-7″ and H-2″, H-4′ and H-7″, and H-7″ and H-8″ indicated that they were all on the β face of the molecule (Fig. 4). In addition, NOE was not detected between C-5′ methyl and H-2″. Thus, the structure of **2** was determined as the stereoisomer of **1** regarding to the configuration at C-2″. The absolute configuration of watasemycins is not determined yet.

Watasemycins A and B were easily isomerized to each other under acidic condition. Despite of the absence of experimental evidences, the isomerization mechanism is proposed as shown in Fig. 5. 7″-CH_3 of **1** may be fixed in the same side of 8″-CH_3 owing to the acid-base interaction between 4″-COOH and the *tert*-amine at 3″-nitrogen to form a pseudo five-membered ring. The generated cation at 3″-nitrogen causes the bond cleavage between 3″-nitrogen and 2″-carbon and the formation of sulfonium cation at 1″-position. The isomerization of double bond and the subsequent nucleophilic attack of the amino nitrogen generates **2**, C-2″ stereoisomer of **1**.

Biological Properties

The antimicrobial activities of watasemycins A (**1**) and B (**2**) are shown in Table 4. **1** exhibited weak antibacterial activities against Gram-positive bacteria, *Staphylococcus aureus* 209P JC-1 and *Bacillus subtilis* ATCC 6633 at 12.5 and 25.0 μg/ml, respectively, and potent activity against the

Fig. 4. NOEs observed with watasemycins A and B.

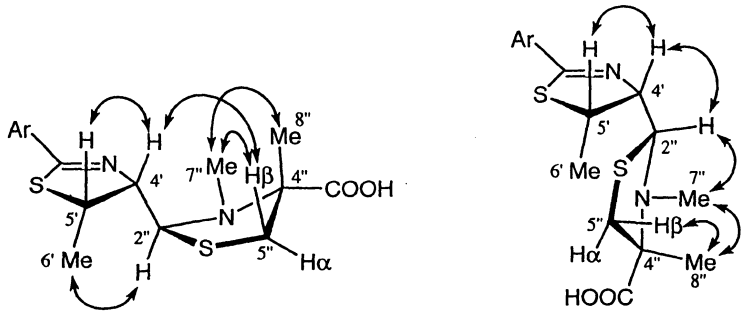


Table 3. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for watasemycins A (**1**) and B (**2**) in $CDCl_3$.

Position	1		2	
	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
1	116.4		116.4	
2	159.0		159.2	
3	117.1	7.00 (1H, dd, *J*=7.3, 1.5 Hz)	117.2	6.99 (1H, dd, *J*=8.3, 0.7 Hz)
4	133.7	7.39 (1H, ddd, *J*=8.7, 7.3, 1.5 Hz)	133.1	7.35 (1H, ddd, *J*=8.3, 7.1, 1.4 Hz)
5	119.2	6.91 (1H, t, *J*=7.8 Hz)	118.7	6.87 (1H, ddd, *J*=7.6, 7.4, 0.9 Hz)
6	130.6	7.45 (1H, dd, *J*=7.8, 1.5 Hz)	130.4	7.39 (1H, dd, *J*=7.6, 1.1 Hz)
2'	174.3		171.9	
4'	83.5	4.43 (1H, dd, *J*=10.7, 5.9 Hz)	78.3	4.79 (1H, t, *J*=6.3 Hz)
5'	46.1	3.91 (1H, q, *J*=6.8 Hz)	46.1	4.05 (1H, q, *J*=6.8 Hz)
6'	17.0	1.26 (3H, d, *J*=7.1 Hz)	17.6	1.38 (3H, d, *J*=7.1 Hz)
2"	73.5	4.37 (1H, d, *J*=10.7 Hz)	71.1	4.68 (1H, d, *J*=4.4 Hz)
4"	76.2		73.8	
5"	40.1	3.60 (1H, d, *J*=11.7 Hz)*	39.3	3.41 (1H, d, *J*=10.5 Hz)*
		2.96 (1H, d, *J*=12.0 Hz)**		2.88 (1H, d, *J*=10.8 Hz)**
6"	174.4		177.2	
7"	39.0	2.76 (3H, s)	32.6	2.62 (3H, s)
8"	18.6	1.63 (3H, s)	22.4	1.60 (3H, s)

*: Hβ-5", **:Hα-5"

Gram-negative bacterium, *Proteus mirabilis* ATCC 21100 at 0.39 μg/ml but it had no activity against *Escherichia coli*. *Proteus mirabilis* is a common pathogen associated with urinary tract infections, an important cause of septicemia[14]. **2** exhibited similar antibacterial spectrum, but the activity was generally weaker than **1**. Both compounds exhibited weak activity against yeast. Other biological activities of watasemycin were not yet tested.

Watasemycin is structurally closely related to pyochelin and thiazostatin. The former was isolated from *Pseudomonas aeruginosa* as an endogenic growth promoter and the latter from *Streptomyces tolurosus* as an antioxidant. All these molecules are considered to be biosynthesized by the condensation of salicylic acid and two moles of cysteine[8,15]. Recently the biosynthetic mechanism of yersiniabactin, an another molecule belonging to this class of metabolites, was elucidated[15]. Watasemycin and thiazostatin have a methyl group at C-4″ position, which is presumably introduced by the nucleophilic methylation at the α-carbon of cysteine with *S*-adenosylmethionine. In contrast, the mechanism of introducing the methyl group at C-5′ of watasemycin is

Fig. 5. Proposed mechanism of isomerization between watasemycins A and B.

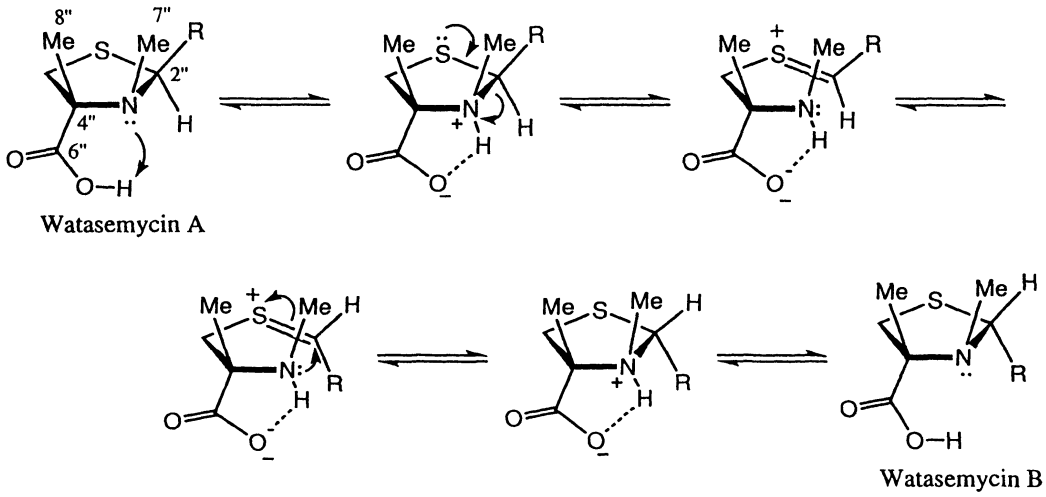


Table 4. *In vitro* antibacterial activities of watasemycins A (**1**) and B (**2**).

Organism	MIC (μg/ml)	
	1	**2**
Staphylococcus aureus 209P JC-1	12.5	25.0
Bacillus subtilis ATCC 6633	25.0	50.0
Escherichia coli NIHJ JC-2	100	>100
Proteus mirabilis ATCC 21100	0.39	6.25
Pseudomonas aeruginosa A3	>100	>100
Saccharomyces cerevisiae S-100	50.0	25.0
Candida albicans A9540	100	100
Candida tropicalis IFO 1400	100	100
Cryptococcus neoformans ATCC 90112	25.0	100

obscure if the nucleophilic substitution takes place.

In summary, we identified watasemycin as a new antibiotic produced by a marine actinomycete and demonstrated the probability of finding additional new metabolites from the marine environment.

Acknowledgments

The authors are in debt to Drs. K. FUJII and K. HARADA at Meijo University for the HR-FABMS measurements.

References

1) NEWMAN, D. J.; G. M. CRAGG & K. M. SNADER: The influence of natural products upon drug discovery. Nat. Prod. Rep. 17: 215~234, 2000
2) PIETRA, F.: Secondary metabolites from marine microorganisms: bacteria, protozoa, algae and fungi. Achievements and prospects. Nat. Prod. Rep. 14: 453~464, 1997
3) FURUMAI, T.; K. TAKAGI, Y. IGARASHI, N. SAITO & T. OKI: Arisostatins A and B, new members of tetrocarcin class of antibiotics from *Micromonospora* sp. TP-A0316. I. Taxonomy, fermentation, isolation and biological properties. J. Antibiotics 53: 227~232, 2000
4) IGARASHI, Y.; K. TAKAGI, Y. KAN, K. FUJII, K. HARADA, T. FURUMAI & T. OKI: Arisostatins A and B, new

members of tetrocarcin class of antibiotics from *Micromonospora* sp. TP-A0316. II. Structure determination. J. Antibiotics 53: 233~240, 2000

5) FURUMAI, T.; Y. IGARASHI, H. HIGUCHI, N. SAITO & T. OKI: Kosinostatin, a new congener of isoquinocycline antibiotics, from *Micromonospora* sp. TP-A0468. I. Taxonomy, fermentation, isolation and biological properties. J. Antibiotics 55: 128~133, 2002
6) IGARASHI, Y.; H. HIGUCHI, T. OKI & T. FURUMAI: Kosinostatin, a new congener of isoquinocycline antibiotics, from *Micromonospora* sp. TP-A0468. II. Physico-chemical properties and structure determination. J. Antibiotics 55: 134~140, 2002
7) SHINDO, K.; A. TAKENAKA, T. NOGUCHI, Y. HAYAKAWA & H. SETO: Thiazostatin A and thiazostatin B, new antibiotics produced by *Streptomyces tolurosus*. J. Antibiotics 42: 1526~1529, 1989.
8) COX, C. D.; K. L. RINEHART, Jr., M. L. MOORE & J. C. COOK, Jr.: Pyochelin: novel structure of an iron-chelating growth promoter for *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA. 78: 4256~4260, 1981
9) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Intern. J. Syst. Bact. 16: 313~340, 1966
10) WAKSMAN, S. A. (*Ed.*): Classification, identification and description of genera and species. *In* The Actinomycetes. Vol. II. pp. 328~334. The Williams & Wilkins Co., Baltimore, 1961
11) ARAI, T. (*Ed.*): *In* Culture Media for Actinomycetes. The Society for Actinomycetes Japan, 1975
12) LECHEVALIER, H. A. & M. P. LECHEVALIER: A critical evaluation of the genera of aerobic actinomycetes. *In* The Actinomycetales, *Ed.*, H. PRAUSER, pp. 393~405, Jena, Gustav Fischer Verlag, 1970
13) STANECK, J. L. & G. D. ROBERTS: Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. Appl. Microbiol. 28: 226~231, 1974
14) MATHAI, D.; R. N. JONES & M. A. PFALLER: Epidemiology and frequency of resistance among pathogens causing urinary tract infections of 1,510 hospitalized patients: A report from the SENTRY Antimicrobial Surveillance Program (North America). Diagn. Microbiol. Infect. Dis. 40: 129~136, 2001
15) GEHRING, A. M.; I. MORI, R. D. PERRY & C. T. WALSH: The nonribosomal peptide synthetase HMWP2 forms a thiazoline ring during biogenesis of yersiniabactin, an iron-chelating virulence factor of *Yersinia pestis*. Biochemistry 37: 11637~11650, 1998