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Yatakemycin, a Novel Antifungal Antibiotic Produced by *Streptomyces* sp. TP-A0356

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In the screening of novel antifungal compounds, yatakemycin was found in the culture broth of *Streptomyces* sp. TP-A0356. Yatakemycin was obtained by solvent extraction of the fermentation broth and chromatographic purification using ODS column and preparative HPLC. The structure of yatakemycin was elucidated by NMR and CID-MS/MS experiments as a novel antibiotic belonging to a family of CC-1065 and duocarmycins known to be DNA alkylating agents. Yatakemycin inhibited the growth of pathogenic fungi such as *Aspergillus fumigatus* and *Candida albicans* with the MIC values of 0.01~0.03 μg/ml, more potent than amphotericin B (MIC: 0.1~0.5 μg/ml) or itraconazole (MIC: 0.03~0.2 μg/ml). It also showed potent cytotoxicity against cancer cell lines with the IC_{50} of 0.01~0.3 μg/ml.

In our screening program for new antibiotics from microbial secondary metabolites, we isolated yatakemycin from the culture broth of an actinomycete. The producing-strain was isolated from a soil sample collected in Miyazaki, Japan and identified as *Streptomyces* sp. TP-A0356 based on the taxonomic study. The new antibiotic was found in the screening using paper disc assay against *Aspergillus fumigatus* and *Candida albicans*. The structure of yatakemycin was determined by analyzing NMR and MS/MS data to be a new member of cyclopropapyrrolo-indole antibiotics represented by CC-1065[1] and duocarmycins[2]. In this paper, we describe the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties, structure determination and biological properties of yatakemycin.

Materials and Methods

Microorganism

The producing microorganism, strain TP-A0356, was isolated from a soil sample collected in Yatake highland, Miyazaki, Japan. A pure culture of strain TP-A0356 was preserved in 20% glycerol at −80°C. It was also maintained on an ISP medium No. 4 slant at 10°C for laboratory use.

Taxonomy

Taxonomic characteristics of strain TP-A0356 were determined by cultivation on various media described by SHIRLING and GOTTLIEB[3], WAKSMAN[4] and ARAI[5]. 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

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utilization was determined by the method of SHIRLING and GOTTLIEB[3]. Cell wall composition was analyzed by the method of LECHEVALIER and LECHEVALIER[6], using thin layer chromatography plates as described by STANECK and ROBERTS[7].

Instrumental Analysis

Melting point was determined on a Yanagimoto apparatus and is uncorrected. NMR experiments were performed on a JEOL JNM-α500 NMR spectrometer in pyridine-d_5. The pyridine-d_5 signals (7.19 ppm for ^{1}H; 123.5 ppm for ^{13}C) were used as references. Tandem mass spectra (CID-MS/MS) were recorded on a JEOL JMS HX-110/HX-110 tandem mass spectrometer. HR-MS spectrum was measured on a JEOL JMS-HX110A spectrometer. UV spectrum was recorded on a Beckman DU 640 spectrophotometer. Optical rotation was measured on a Horiba SEPA-300 polarimeter.

Biological Assay

The antibiotic activity in purification samples was evaluated by the paper disc assay using *Candida albicans* A9540 as an indicator strain. MIC values against pathogenic fungi were determined by broth microdilution method. IC_{50} values against cancer cell lines were determined by MTT assay after incubation at 37°C for 24 hours with the inoculum size of 10^4 cells/ml.

Results and Discussion

Taxonomy of the Producing Strain

By scanning electron microscopy, strain TP-A0356 formed spiral type spores which were cylindrical, 0.5~0.6×0.7~0.8 μm in size, having smooth surface (Fig. 2). The cultural characteristics of TP-A0356 are summarized in Table 1. The color of aerial mycelia was medium gray and the color of reverse side was olive gray. Diffusible pigments were not formed. Strain TP-A0356 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, glycine and galactose. On the basis of these morphological and chemical characteristics, strain TP-A0356 was assigned to the genus *Streptomyces*.

Fermentation

A slant culture of the strain TP-A0356 grown on an ISP medium No. 4 was inoculated into two 500-ml K-1 flasks containing 100 ml of seed medium composed of soluble starch 3%, glucose 1%, soybean meal 2%, yeast extract 0.3%, corn steap liquor 0.5%, NaCl 0.3%, and $CaCO_3$ 0.3% (pH 7.0). The seed culture was incubated for 3 days at 32°C on a rotary shaker (200 rpm). Three ml each of the culture was inoculated into thirty 500-ml K-1 flasks containing 100 ml of production medium composed of glucose 1%, oatmeal 3%, yeast extract 0.2%, $(NH_4)_2SO_4$ 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, NaCl 0.1%, $CaCO_3$ 0.3%, $FeSO_4$ 0.0001%, $MnSO_4$ 0.0001%, $ZnSO_4$ 0.0001%, $CuSO_4$ 0.0001%, $CoSO_4$ 0.0001% and KI 0.0001%. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were incubated for 4 days at 32°C on a rotary shaker (200 rpm).

Fig. 1. Structure of yatakemycin.

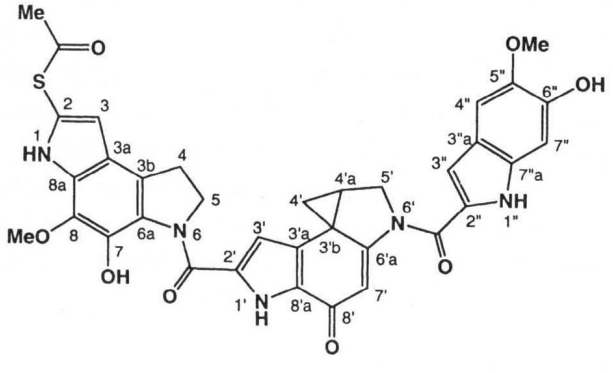
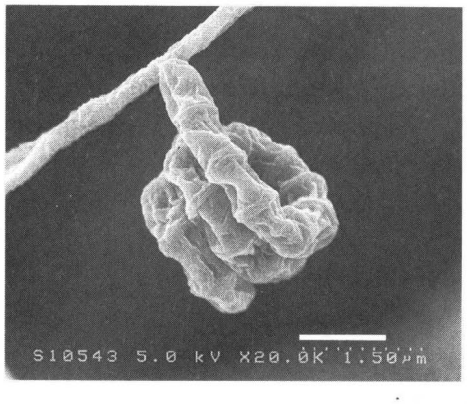


Fig. 2. Scanning electron micrograph of *Streptomyces* sp. TP-A0365.



Bar represents 1 μm.

Table 1. Cultural characteristics of strain TP-A0356.

Medium	Aerial mycelium	Reverse side	Diffusible pigment	Growth
Sucrose nitrate agar (Waksman med. 1)	Light grayish yellow green (105)	Light grayish yellow green (105)	None	Poor
Glycerol nitrate agar (Waksman med. 1)	—	Cream (67)	None	Medium
Glucose – asparagine agar (Waksman med. 2)	Snow white (201)	Colonial yellow (79)	None	Medium
Nutrient agar (Waksman med. 14)	—	Ivory white (204)	None	Poor
Bennett's agar (Waksman med. 30)	Snow white (201)	Colonial yellow (79)	None	Medium
Yeast extract - malt extract agar (ISP med. 2)	Sky gray (209)	Colonial yellow (79)	None	Medium
Oatmeal agar (ISP med. 3)	Sky gray (209)	Olive yellow (83)	None	Medium
Inorganic salts - starch agar (ISP med. 4)	Sky gray (209)	Melon yellow (80)	None	Good
Glycerol - asparagine agar (ISP med. 5)	—	Colonial yellow (79)	None	Medium
Tyrosine agar (ISP med. 7)	Sepia (62)	Frosty white (202)	Burnt umber (57)	Good
Yeast starch agar	Grayish yellow (156)	Brownish gold (97)	None	Good

Isolation

The whole culture broth (3 liter) was centrifuged (8,000 rpm, 15 minutes) to separate into the mycelium and the supernatant. The mycelium was extracted with 80% acetone (1.5 liters×3) and the aqueous acetone solution was evaporated. The remaining aqueous solution was combined with the supernatant and extracted with ethyl acetate (1.5 liters×3). The organic layer was evaporated to give a crude oily material (2.52 g). It was then dissolved in methanol (100 ml) and defatted by partitioning with *n*-hexane (100 ml) to give a solid extract (1.54 g). The defatted extract (200 mg) was subjected to reversed phase silica gel column chromatography (YMC gel ODS-AM 120-S50; Yamamura Chemical Lab., 0.4 liters) and the column was eluted with a gradient of acetonitrile - 0.15% KH_2PO_4, pH 3.5 (2 : 8~5 : 5). The active fractions were pooled, adjusted to pH 7.0 with $NaHCO_3$ solution and the acetonitrile of the combined fractions was evaporated. The resulting aqueous solution was extracted with a half volume of ethyl acetate twice. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give brown powder (42 mg). This powder 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 - distilled water (5 : 5) at a flow rate of 9.5 ml/minute. Fractionation was guided by UV absorbance at 400 nm to obtain the fraction containing yatakemycin. The acetonitrile of the fraction was evaporated and the remaining aqueous solution was extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give yatakemycin (13.4 mg).

Structure Determination

Physico-chemical properties of yatakemycin are summarized in Table 2. Yatakemycin was obtained as yellow powder, soluble in DMSO and pyridine and slightly soluble in ethyl acetate. The molecular formula of yatakemycin was determined as $C_{35}H_{29}N_5O_8S$ on the basis of the HRFAB-MS, which gave a $[M+H]^+$ ion at *m/z* 680.1816 (Δ +0.1 mmu; calcd for $C_{35}H_{30}N_5O_8S$), and ^1H- and ^{13}C-NMR spectral data. The presence of sulfur atom was confirmed by MS/MS experiments as described later.

From a combination of 2D-NMR experiments (DQF-COSY, HMQC, HMBC), four partial structures of yatakemycin were revealed (Fig. 3). The singlet methyl proton at 2.45 ppm had a long-range coupling to the carbon

Table 2. Physico-chemical properties of yatakemycin.

Appearance	Yellow powder
Melting point	>220°C (dec)
$[\alpha]_D^{24}$	+99.9 (*c* 0.39, DMF)
HRFAB-MS	
Found:	680.1816 $[M+H]^+$
Calcd:	682.1815 (for $C_{35}H_{32}N_5O_8S$)
Molecular formula	$C_{35}H_{31}N_5O_8S$
UV λ_{max}^{MeOH} nm (ε)	210 (31,200), 278 (sh), 310 (sh), 382 (32,600)
IR ν_{max} (cm^{-1})	3450, 1625, 1500, 1380, 1250
HPLC (Rt)	13.8 min

HPLC conditions: Cosmosil AR-II (250 × 4.6 mm, i.d.), mobile phase: 50% aqueous CH_3CN, flow rate: 0.7 ml/min.

Table 3. ^1H (500 MHz) and ^{13}C (125 MHz) NMR data for yatakemycin in pyridine-d_5.

Position	^{13}C	^1H
1-NH		13.66 (1H, s)
2	133.36	
3	107.77	7.50 (1H, d, 1.5 Hz)
3a	118.61	
3b	122.00	
4	28.22	3.22 (2H, t, 7.5 Hz)
5	53.85	4.43 (1H, d, 10.0 Hz)
		4.51 (1H, m)
6a	128.88	
7	140.50	
8	134.54	
8a	135.99	
2-S*C*OCH$_3$	183.45	
2-SCO*CH$_3$*	11.14	2.45 (3H, s)
7-OH		11.71 (1H, s)
8-OCH$_3$	60.45	4.03 (3H, s)
1'-NH		14.59* (1H, br.s)
2'	130.38	
3'	107.63	6.74 (1H, s)
3'a	130.53	
3'b	31.55	
4'	26.15	1.44 (1H, t, 5.0 Hz)
		1.77 (1H, dd, 4.5 and 7.5 Hz)
4'a	23.98	2.97 (1H, dd, 5.0 and 10.0 Hz)
5'	55.62	4.50 (2H, m)
6'a	161.89	
7'	112.85	7.72 (1H, s)
8'	178.54	
8'a	132.7	
2'-CO	161.89**	
1"-NH		12.68 (1H, s)
2"	129.21	
3"	107.63	7.18 (1H, s)
3"a	122.37	
4"	94.46	7.24 (1H, s)
5"	150.43	
6"	144.81	
7"	106.62	7.62 (1H, s)
7"a	133.08	
2"-CO	161.92**	
5"-OCH$_3$	55.89	3.84 (3H, s)
6"-OH		10.65* (1H, br.s)

*, **: interchangeable

at 183.45 ppm, indicating the presence of an acetyl group bonding to a heteroatom (partial structure A). The heteroatom was speculated to be sulfur in order to fulfill the molecular formula obtained from the HRFAB-MS data. The pyrroloindole moiety (partial structure B) was confirmed by the HMBC correlations. The long-range couplings from the OH proton at 11.71 ppm to C-6a, C-7 and C-8 and from the methoxy proton to C-8 established the substituents at C-7 and C-8. The HMBC correlation from the methylene proton H-5 to the carbonyl carbon at 161.9 ppm indicated the peptide bonding at the 6-nitrogen. Partial structure C containing the cyclopropapyrroloindole unit, the most characteristic moiety in this molecule, was also deduced from the HMBC correlations. The methylene proton H-4′ in the cyclopropane ring showed the long-range couplings to C-3′b, C-4′a, C-5′ and C-6′a. In addition, significant correlations were observed from H-3′ and H-7′ to quaternary sp^2 carbons in the indole ring. Although the HMBC correlation was not detected from H-5′, the 6′-nitrogen was assumed to bond to a carbonyl carbon in consideration of the structures of related antibiotics. Partial structure D was confirmed by HMBC correlations from the NH proton at 12.68 ppm and three singlet sp^2 protons (H-3″, H-4″, H-7″) to the indole carbons as summarized in Fig. 3. The position of the methoxy and hydroxy groups was determined by comparing the relative intensity of cross peaks observed in the HMBC spectrum which was measured with the duration time of 60 msec ($^nJ_{C,H}$=6 Hz). The cross peaks derived from the couplings from H-4″ and H-7″ to the carbons at *meta*-positions were larger than those to the carbons at *ortho*-positions. Partial

Fig. 3. Partial structures of yatakemycin revealed by HMBC experiment.

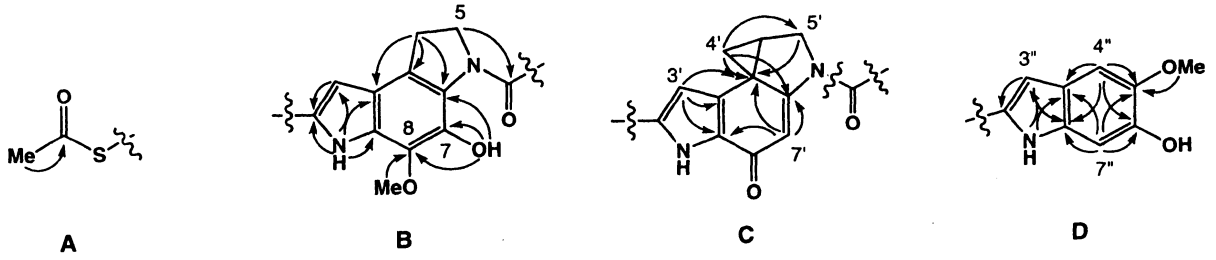
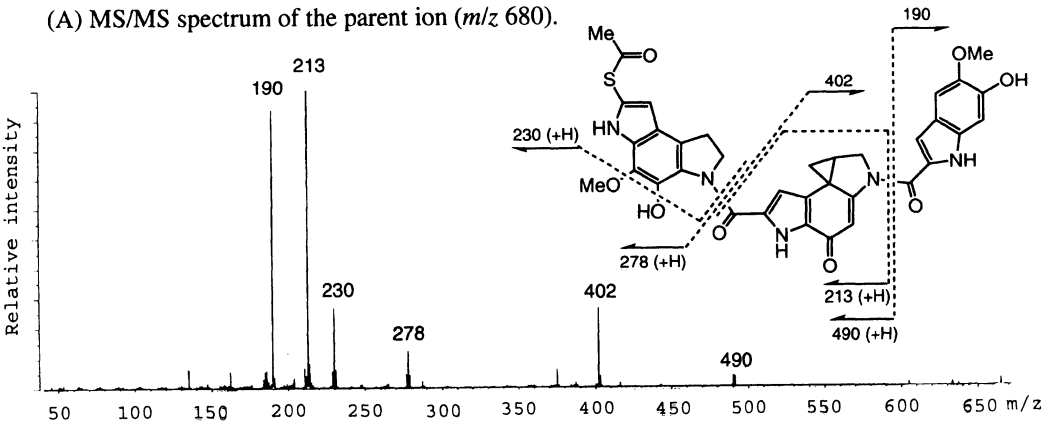
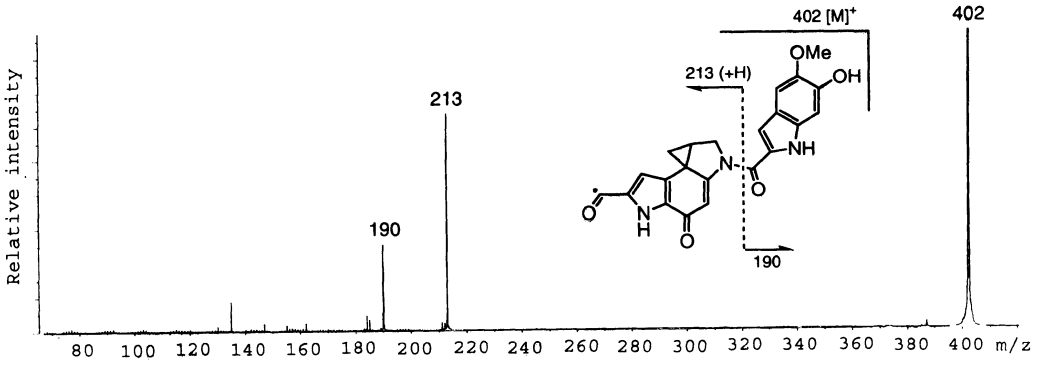


Fig. 4. CID-MS/MS analysis of yatakemycin.

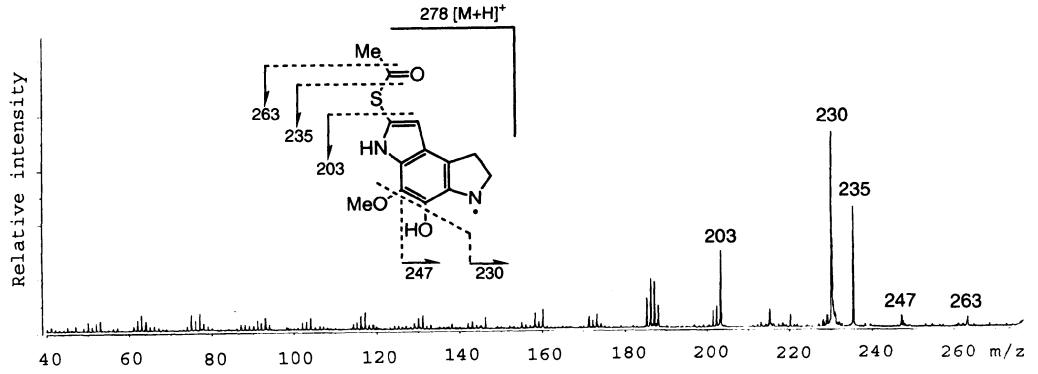
(A) MS/MS spectrum of the parent ion (*m/z* 680).



(B) MS/MS/MS spectrum of the precursor ion at *m/z* 402.



(C) MS/MS/MS spectrum of the precursor ion at *m/z* 278.



structures B and C are linked to another partial structures at two sites and partial structures A and D at one site. Therefore partial structures A and D were determined to be located at both end of the molecule.

In order to determine the connectivity between partial structures and the presence of thioester in partial structure A, yatkemycin was subjected to the collision-induced dissociation tandem mass spectrometry (CID-MS/MS). First, the molecular ion of yatakemycin $[M+H]^+$ at *m/z* 680 was introduced to CID-MS/MS (Fig. 4A). The ion peaks corresponding to the partial structures C and D were detected at *m/z* 213 and 190, respectively. The ion peak at *m/z* 402 was speculated to be the fragment composed of the partial structures C and D. In addition, the ion peak at *m/z* 278 was supposed to be the fragment derived from the partial structure A bonding to the partial structure B. Next, the fragment ion at *m/z* 402 generated by the first collision was further decomposed in the second collision cell (CID-MS/MS/MS) (Fig. 4B). In the product ion spectrum, the formation of two fragments corresponding to the partial structures C (*m/z* 213) and D (*m/z* 190) was observed. Therefore the connectivity between the partial structures C and D was established. Furthermore, the fragment ion at *m/z* 278 generated by the first collision was given to the CID-MS/MS/MS (Fig. 4C). Fragmentation occurred from the far end of the thioester side chain. Ions of the fragments obtained after the cleavage between CH_3 and C=O (*m/z* 263 after loss of CH_3), between CH_3C=O and S (*m/z* 235 after loss of CH_3CO), and between CH_3COS and C-2 (*m/z* 203 after loss of CH_3COS) were observed. It was therefore concluded that the acetyl group was linked to the 2-position of the indole *via* sulfur atom as a thioester. The ion peak at *m/z* 230 suggests the generation of benzyne which was produced by the loss of methoxy and hydroxyl groups from the fragment ion at *m/z* 278. Thus, the gross structure of yatakemycin was established as shown.

Biological Properties

In vitro antifiungal activity of yatakemycin was evaluated in comparison with amphotericin B, itraconazole and aureobasidin A (Table 4). Yatakemycin inhibited the growth of *Aspergillus*, *Candida* and *Cryptococcus* 10~100-fold more potent than reference antibiotics. In addition, it showed strong cytotoxicity against cancer cell lines used in this study (Table 5). It was 1,000-fold more cytotoxic than mitomycin C, a DNA alkylating antitumor agent. The antifungal and cytotoxic activity of yatakemycin can be attributed to its ability of DNA alkylation and noncovalent binding affinity to duplex DNA. Studies using CC-1065 and duocarmycins have proved that the DNA alkylation occurs at the least substituted carbon in the cyclopropane ring (C-4′ in case of yatakemycin) with the addition of nitrogen at N-3 of adenine[8]. From the fermentation broth

Table 4. *In vitro* antifungal activities of yatakemycin and reference compounds.

Organism	MIC (μg/ml)			
	YTM	AMPH	ITCZ	AUR
Asperigillus fumigatus TIMM0069	0.0156	0.25	0.0625	4
A. fumigatus TIMM0070	0.0313	0.125	0.125	16
A. fumigatus MF-13	0.0078	0.125	0.25	1
A. flavus ATCC9643	0.0156	0.5	0.0625	4
A. flavus TIMM0057	0.0039	0.25	0.0625	32
A. niger MUD3625	0.0156	0.125	0.125	4
A. terreus TIMM2814	0.0039	0.0625	0.25	0.25
Candida albicans ATCC90018	0.0078	0.5	≤0.0313	0.25
C. glabrata ATCC90030	0.0625	0.125	0.125	0.125
C. parapsilosis ATCC90112	0.0313	0.25	≤0.0313	0.125
Cryptococcus neoformans ATCC10259	≤0.0009	0.0625	0.0625	25

Abbreveations: YTM, yatakemycin; AMPH, amphotericin B; ITCZ, itraconazole; AUR, aureobasidin A

MICs were deteremined by broth microdilution method using RPMI1640 medium buffered with MOPS in accordance with NCCLS document M27-T.

Incubation at 35°C for 24 hr, 48 hr and 72 hr for *Aspergillus*, *Candida* and *Cryptococcus*, respectively.

Table 5. *In vitro* cytotoxicity of yatakemycin and reference compounds.

Cell line	IC_{50} (μg/ml)			
	YTM	AMPH	5-FU	MMC
P388	<0.00001	0.739	0.107	0.045
HeLa	0.000278	>16	1.353	0.299
Huvec	0.000215	3.361	>16	0.252
HEL	0.000186	6.025	0.681	1.129

Abbreveations: YTM, yatakemycin; AMPH, amphotericin B; 5-FU, 5-fluorouracil; MMC, mitomycin C

of strain TP-A0356, a component showing UV-vis spectrum similar to that of yatakemycin was isolated but not antifungal. MS and MS/MS analysis of the component proved that it was water-adducted yatakemycin and that the water adduct site was in the cyclopropapyrroloindole unit, supporting the importance of the cyclopropane to the bioactivity. CC-1065, duocarmycins and gilvusmycin[9] are the members of this class of antibiotics reported so far, and they possess the cyclopropapyrroloindole unit at the left terminus of the molecule. Yatakemycin, in contrast, has the cyclopropane-containing unit in the center part of the molecule and thus may show different chemical and biological properties.

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