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# Vinylamycin, a New Depsipeptide Antibiotic, from *Streptomyces* sp.

MASAYUKI IGARASHI, TETSUO SHIDA, YUKI SASAKI, NAOKO KINOSHITA, HIROSHI NAGANAWA, MASA HAMADA and TOMIO TAKEUCHI

Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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A new depsipeptide antibiotic, vinylamycin, was isolated from the culture broth of an actinomycete strain. The producing organism, designated MI982-63F1, was identified as a member of *Streptomyces*. Vinylamycin was isolated from the culture broth by extraction with EtOAc and purified by crystallization from EtOAc. The structure of vinylamycin was determined by spectroscopic analysis and degradation studies. Vinylamycin showed antimicrobial activities against Gram-positive bacteria including MRSA.

In the course of our screening program for new antibiotics, we found that a strain of *Streptomyces* sp. MI982-63F1 produced a new antibiotic, vinylamycin (**1**, Fig. 1). Vinylamycin showed antimicrobial activities against Gram-positive bacteria including MRSA.

In this paper, we describe the identification of the producing organism together with the isolation, fermentation, physico-chemical properties, structure elucidation and biological activities of vinylamycin.

## Materials and Methods

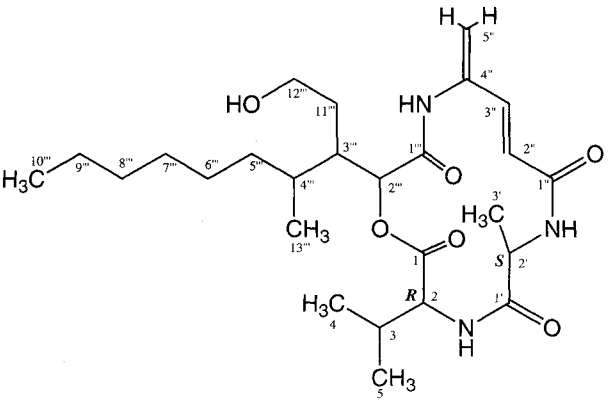
### General

Optical rotation was measured with a Perkin-Elmer model 241 polarimeter. UV spectra were recorded with a Hitachi 557 spectrophotometer. IR spectrum was recorded with a Horiba FT-210 fourier transform infrared spectrometer. The $^{1}H$ and $^{13}C$ NMR spectra were measured with a JEOL JNM-A500 spectrometer. The mass spectra were recorded with a JEOL JMS-SX102 mass spectrometer.

### Taxonomy

Vinylamycin producing organism, strain MI982-63F1, was isolated from a soil sample collected at the Institute of Microbial Chemistry, Shinagawa-ku, Tokyo, Japan in 1989. Morphological, cultural and physiological properties of the strain MI982-63F1 were examined according to the methods described by SHIRLING and GOTTLIEB[1], and WAKSMAN[2]. Detailed observation of mycelial morphologies was performed with the use of scanning electron microscope (Model S-570, Hitachi) after the strain MI982-63F1 was incubated on an inorganic salts - starch agar (ISP No.4) at 27°C for 5days. Chemical analyses of cell wall and menaquinones were performed with the methods of STANECK and ROBERTS[3] and TAMAOKA *et al.*[4], respectively.

Fig. 1. Structure of vinylamycin.



Fermentation

A slant culture of the vinylamycin-producing organism was inoculated into a 500-ml baffled Erlenmeyer flask containing 110 ml of a seed medium consisting of galactose 2%, dextrin 2%, glycerol 1.0%, Bacto-soytone (Difco) 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% in deionized water (pH 7.4 before sterilization). The culture was incubated on a rotary shaker (180 rpm) at 30°C for 3 days. The seed culture of the strain was transferred into a 500-ml baffled Erlenmeyer flask containing 110 ml of a producing medium which was consisting of starch 3%, soy bean meal 1.5%, corn steep liquor 0.5%, yeast extract 0.2%, NaCl 0.3%, $(NH_4)_2SO_4$ 0.05%, $CoCl_2$ 0.001% and $CaCO_3$ 0.3% in deionized water (pH 7.2). The fermentation was carried out at 27°C for 3 days on a rotary shaker (180 rpm).

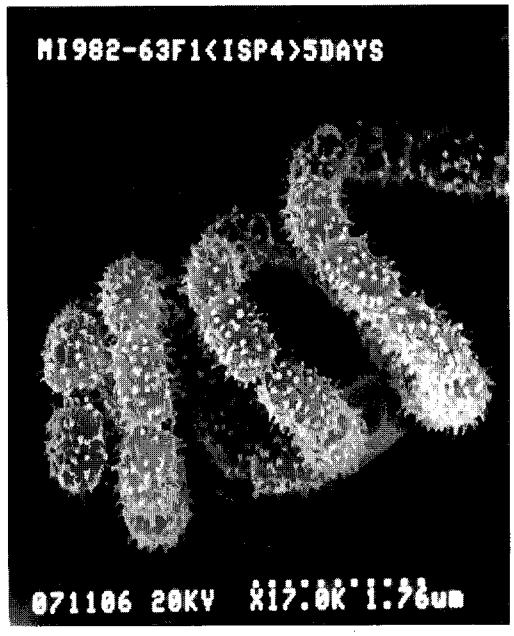
Analytical Procedure

Analyses of vinylamycin in the fermentation broth and its purification steps were performed using reversed phase HPLC and silica gel TLC. HPLC was performed using a CAPCELL PAK $C_{18}$ column (4.6×150 mm, Shiseido Co. Ltd., Japan; mobile phase, acetonitrile - $H_2O$=45 : 55; flow rate, 2.0 ml/minute; column temperature, 60°C; detection, UV at 258 nm). It was eluted at 5.9 minutes. TLC was performed with Kieselgel 60 $F_{254}$ (Art. No. 5715, Merck) developed with $CHCl_3$ - MeOH (4 : 1). Spot of the antibiotic on a TLC was detected by molybdophosphoric acid-sulfuric acid and UV quenting (254 nm). Rf value of vinylamycin was 0.56.

Acid Hydrolysis of Vinylamycin

Acid hydrolysis of vinylamycin (21 mg , 0.043 mmol) was carried out with 6 M HCl at 100°C for 4.5 hours. The products in the reaction mixture showed the presence of two ninhydrin-positive spots attributed to **2** and **3** on a cellulose TLC (AVICEL, Funakoshi Co. Ltd.) developing with *iso*PrOH - $H_2O$=7 : 3 as a solvent system. The Rf values of the two components **2** and **3** were 0.42 and 0.60, respectively. The reaction mixture of the acid hydrolysate was concentrated *in vacuo* to give a brownish syrup. The concentrate was charged on a column of DOWEX-50W ($H^+$, 1.5 ml, Dow Chemical Company). After washing the column with distilled water, the mixture of **2** and **3** was eluted with 1 M aq $NH_4OH$. The eluate was collected, neutralized and concentrated *in vacuo*. The mixture was purified by cellulose column chromatography (10 mm×200 mm, AVICEL, Funakoshi Co. Ltd., developing with *iso*PrOH - $H_2O$=7 : 3) to give **2** (0.7 mg) and **3** (1.3 mg), respectively. Chiralities of **2** and **3** were determined by chiral HPLC method. The HPLC was performed with a CROWN PAK CR(+) column (0.40×150 mm, Daicel Chemical Co. Ltd., Japan; mobile phase, $H_2O$ adjusted to pH 2.0 with $HClO_4$ ; flow rate, 0.4 ml/minute; column temperature, 25°C; detection, UV at 200 nm). Components **2** and **3** were eluted at 6.22 and 6.71 minutes and identified as D-valine and L-alanine by direct comparisons with authentic samples of D and L isomers, respectively.

Fig. 2. Scanning electron micrograph of spore chains of *Streptomyces* sp. MI982-63F1 grown on inorganic salts - starch agar (ISP4) for 5 days at 27°C.




Biological Activity

The minimum inhibitory concentrations (MIC) of vinylamycin were examined by serial agar dilution method using Nutrient agar containing 1% glucose for yeast and fungi and Mueller-Hinton agar (Difco) for bacteria. The MIC was observed after an incubation for 42 hours at 27°C against yeast and fungi, and incubations for 18 or 42 hours at 37°C against bacteria, respectively.

## Results and Discussion

Taxonomic Features of Strain MI982-63F1

Strain MI982-63F1 produced well-branched vegetative mycelia. This strain formed long aerial hyphae which bore spirals. Mature spore chain consisted of 10 to 50 spores.

Table 1. Cultural characteristics of strain MI982-63F1.

| Medium | Growth | Aerial mycelium | Soluble pigment |
|---|---|---|---|
| Sucrose-nitrate agar | Colorless ~ pale yellow [2 gc, Bamboo] | None | None |
| Yeast extract-malt extract agar (ISP No.2) | Pale yellowish brown [3 le, Cinnamon ~ 3 ne, Topaz] | White ~ pale brown [4 ec, Bisque] | Brownish |
| Oatmeal agar (ISP No.3) | Pale yellow [2 gc, Bamboo ~ 2 ic, Honey Gold] | White ~ pale brown [4 ec, Bisque] | None |
| Inorganic salts-starch agar (ISP No.4) | Pale yellow [1 1/2 ca, Cream] | Pale brown [4 ec, Bisque ~ 4 ge, Lt Fawn] | None |
| Glycerol-asparagine agar (ISP No.5) | Pale yellow [2 gc, Bamboo] ~ pale yellowish brown [4 ne, Luggage Tan] | Pale brown [4 ec, Bisque ~ 4 ge, Lt Fawn] | None |
| Tyrosine agar (ISP No.7) | Light brownish gray [2 ec, Biscuit] ~ grayish yellow brown [2 ie, Lt Mustard Tan] | White ~ pale brown [4 ec, Bisque ~ 4 ge, Lt Fawn] | Black |

Observation after incubation at 27 °C for 21 days.

Color names and numbers from Color Harmony Manual, Container Corporation of America.[5]

The spores were oval with spiny surface and 0.5 to 0.7 by 0.8 to 1.0 $\mu$m in size (Fig. 2). No synnemata, sclerotia or sporangia were observed.

The cultural characteristics of strain MI982-63F1 on various agar media are shown in Table 1. The aerial mycelia were white to pale brown. The vegetative mycelia were pale yellow to pale yellowish brown. The soluble pigments were not formed. Physiological characteristics and carbohydrate utilizations are shown in Table 2. Permissive temperature range for growth of the strain was 20°C to 37°C. The optimum temperature for growth of the strain was 30°C.

Whole-cell hydrolysates of strain MI982-63F1 contained LL-diaminopimelic acid. The strain had MK-9($H_6$) and MK-9($H_8$) as the major components of menaquinones.

These taxonomic properties suggested that strain MI982-63F1 belonged to the genus *Streptomyces*. We searched the data of known *Streptomyces* species. In the results, strain MI982-63F1 was not closely related to the species. Therefore, the strain MI982-63F1 was designated *Streptomyces* sp. MI982-63F1. The strain MI982-63F1 has been deposited in the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology, Tsukuba, Japan, under the accession No. FERM P-16404.

## Fermentation and Isolation

A typical time course for production of vinylamycin in a 500-ml baffled Erlenmeyer flask was shown in Fig. 3. The production of vinylamycin in the broth started at 24 hours after inoculation and reached a maximum (*ca.* 200 mg/liter) at 72 hours. The fermentation was carried out at 27°C for 3 days on a rotary shaker. The fermentation broth (2 liters) was centrifuged for separating the mycelial cake and supernatant. The supernatant was extracted with EtOAc (3.0 liter) and dried with $Na_2SO_4$. The EtOAc solution was stood for 7 days at 5°C, and then **1** appeared as a colorless micro-crystalline (48.2 mg).

## Structure Elucidation

The physico-chemical properties of **1** are summarized in Table 3. The antibiotic is soluble in DMSO slightly soluble in MeOH, $CHCl_3$ and insoluble in water. The molecular formula of **1** was established as $C_{26}H_{43}N_3O_6$ on the basis of HRFAB-MS and NMR spectral analysis. The UV absorption maxima in MeOH were shown at 227 ($\varepsilon$ 14,100) and 261 nm ($\varepsilon$ 14,200), indicating a substituted dienone chromophore[6]. The IR spectrum of **1** indicated the presence of ester (1736 $cm^{-1}$) and amide (1670, 1660 and 1625 $cm^{-1}$) functions. It was suggested the antibiotic belonged to a class of depsipeptide.

The $^{13}C$ NMR spectral data of **1** showed 26 signals. All

Table 2. Physiological characteristics of strain MI982-63F1.

| | |
|---|---|
| Temperature range for growth(℃) | 20 ~37 |
| Optimum temperature(℃) | 30 |
| Formation of melanoid pigment | |
| ISP No.1 | Positive |
| ISP No.6 | Positive |
| ISP No.7 | Positive |
| Liquefaction of | |
| gelatin | Negative |
| glucose peptone gelatin | Negative |
| Coagulation of milk | Negative |
| Peptonization of milk | Positive |
| Hydrolysis of starch | Positive |
| Reduction of nitrate | Negative |
| Utilization of * | |
| D-Glucose | + |
| L-Arabinose | + |
| D-Xylose | d |
| D-Fructose | + |
| Sucrose | + |
| Inositol | + |
| Rhamnose | (+) |
| Raffinose | + |
| D-Mannitol | + |

* : + , Utilization; d , doubtful utilization; - , No utilization.

Fig. 3. A typical time - course of vinylamycin production.

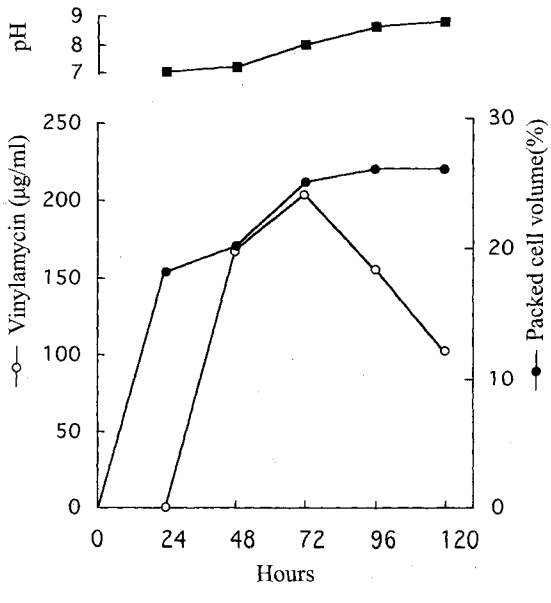



Table 3. Physico-chemical properties of vinylamycin.

| | |
|---|---|
| Molecular formula | $C_{26}H_{43}N_3O_6$ |
| FAB-MS (*m/z*) | 494 $(M+H)^+$<br>492 $(M-H)^-$ |
| HRFAB-MS (*m/z*) | |
| Calcd. for $C_{26}H_{44}N_3O_6$ : | 494.3230 |
| Found : | 494.3237 $(M+H)^+$ |
| M.P.(°C) | 182~184 |
| $[\alpha]_D^{25}$ | +173°(*c* 0.85, DMSO) |
| $UV\lambda_{max}^{MeOH}$ nm(ε) | 261 (14,200)<br>227 (14,100) |
| $IR\nu_{max}(KBr)cm^{-1}$ | 3400~3100, 2962, 2929, 2858, 1736, 1670,1660, 1625, 1533, 1466,1387, 1261, 1051, 978 |
| TLC(Rf) | 0.36* 0.25** |
| Color reaction positive | $I_2$, molybdophosphoric acid-sulfuric acid |

* The Rf values of **1** on silica gel TLC (Kieselgel 60 F254, art 5715, Merck) developed with $CHCl_3$-MeOH=4 : 1. ** The Rf values of **1** onsilica gel TLC (Kieselgel 60 F254, art 5715, Merck) developed with EtOAc-MeOH=4 : 1.

Table 4. $^{13}C$ and $^{1}H$ NMR data of vinylamycin (**1**) in DMSO-$d_6$.

| | $\delta_C$[a] | | $\delta_H$[b] | | | | $\delta_C$[a] | | $\delta_H$[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *J* (Hz) | | | | | | *J* (Hz) | |
| 1 | 168.7 | s | | | | 1''' | 171.6 | s | | | | |
| 2 | 57.64 | d | 4.19 | 1H | dd 8.0, 10.0 | 2''' | 76.44 | d | 5.22 | 1H | dd | 1.0, 10.0 |
| | | NH | 8.09 | 1H | d 10.0 | 3''' | 44.97 | d | 2.97 | 1H | m | |
| 3 | 31.94 | d | 1.92 | 1H | m | 4''' | 33.58 | d | 1.77 | 1H | m | |
| 4 | 18.3 | q | 0.87 | 3H | d 7.0 | 5''' | 33.42 | t | 1.08 | 1H | m | |
| 5 | 19.37 | q | 1.28 | 3H | d 7.0 | | | | 1.25 | 1H | m | |
| 1' | 172.7 | s | | | | 6''' | 26.67 | t | 1.3~1.38 | 1H | m | |
| 2' | 50.96 | d | 4.29 | 1H | m | | | | 1.18~1.28 | 1H | m | |
| | | NH | 7.57 | 1H | d 5.0 | 7''' | 28.81 | t | 1.18~1.28 | 2H | m | |
| 3' | 18.08 | q | 0.87 | 3H | d 7.0 | 8''' | 31.12 | t | 1.18~1.28 | 2H | m | |
| 1'' | 166.2 | s | | | | 9''' | 22.00 | t | 1.2~1.3 | 2H | m | |
| 2'' | 118.8 | d | 6.28 | 1H | d 15.0 | 10''' | 13.92 | q | 0.85 | 3H | t | 6.0 |
| 3'' | 138.7 | d | 6.86 | 1H | d 15.0 | 11''' | 32.41 | t | 1.61 | 2H | m | |
| 4'' | 137.5 | s | | | | 12''' | 58.30 | t | 3.44 | 1H | m | |
| | | NH | 8.82 | 1H | s | | | | 3.3 | 1H | m | |
| 5'' | 115.6 | t | 5.39 | 1H | s | | | OH | 4.59 | 1H | t | 5.0 |
| | | | 5.52 | 1H | s | 13''' | 13.08 | q | 0.97 | 3H | d | 7.0 |

a 125MHz, chemical shifts in ppm, multiplicity.

b 500MHz, chemical shifts in ppm, multiplicity.

bond connections between $^{1}H$ and $^{13}C$ signals were interpreted by DEPT and heteronuclear multiple quantum coherence (HMQC) experiments. The DEPT and HMQC spectra revealed the presence of five methyl, seven methylene, an olefinic methylene, six methine, two olefinic methine, an olefinic quaternary and four carbonyl carbons. The $^{1}H$ and $^{13}C$ NMR spectral data of **1** are shown in Table 4. The $^{1}H$-$^{1}H$ COSY and HMBC (Heteronuclear multiple bond correlation) spectra of **1** suggested that **1** consisted of four partial structures (**a**, **b**, **c** and **d**) as shown in Fig. 4. The partial structure for **a** was valyl moiety by analysis of the $^{1}H$-$^{1}H$ COSY spectrum. The $^{1}H$-$^{13}C$ long-range cross peaks in valyl moiety showed that a methine proton at $\delta_H$ 4.19 (2-H) was coupled to a carbonyl carbon at $\delta_C$ 168.7 (C-1). The partial structure for **b** was easily given from the $^{1}H$-$^{1}H$ COSY spectrum. The $^{1}H$-$^{13}C$ long-range coupling in **b** showed a methine proton at $\delta_H$ 4.29 (2'-H) was coupled to a carbonyl carbon at $\delta_C$ 172.7 (C-1'). As for 4-amino-penta-2,4-dienoyl moiety (**c**), a large spin coupling constant ($J$=15 Hz) between two olefinic protons of 2''-H and 3''-H indicated its geometry was *trans*. In the HMBC spectrum, the olefinic methine signal at $\delta_H$ 6.28 (2''-H) was coupled to an amide carbonyl carbon at $\delta_C$ 166.2 (C-1''). The olefinic methine signal at $\delta_H$ 6.86 (H-3'') and an amino $\delta_H$ 8.82 (4''-NH) were coupled to an olefinic quaternary carbon at $\delta_C$ 137.5 (C-4'') and an exomethylene carbon at $\delta_C$ 115.6 (C-5''). The partial structure for 2-hydoxy-3(2-hydroxyethyl)-4-methyl-decanoic acid (**d**) was depicted by the analysis of the $^{1}H$-$^{1}H$ COSY spectrum as shown in Fig. 4. In the HMBC spectrum, a methine signal at $\delta_H$ 5.22 (2'''-H) was coupled to a carbonyl carbon at $\delta_C$ 171.6 (C-1'''). The connectivities among the three partial structures in **1** were done by the following results of the HMBC spectrum. An amide proton at $\delta_H$ 8.09 (2-NH) was coupled to the carbonyl carbon at $\delta_C$ 172.7 (C-1'). An amide proton at $\delta_H$ 7.57 (2'-NH) was coupled to a carbonyl carbon at $\delta_C$ 166.2 (C-1''). An amide proton at $\delta_H$ 8.82 (4''-NH) was coupled to the carbonyl carbon at $\delta_C$ 171.6 (C-1'''). The methine signal at $\delta_H$ 5.22 (2'''-H) was coupled to the carbonyl carbon at $\delta_C$ 168.7 (C-1). Thus, the results of HMBC experiment of **1** are summarized in Fig. 5.

Acid hydrolysis of **1** with 6 M HCl at 110°C for 3.5 hours gave two amino acid components. They were identified to be valine (**2**) and alanine (**3**) by cellulose TLC. The chirality of these amino acids were determined by chiral HPLC. The retention time of **2** (6.22 minutes) and **3** (6.71 minutes) were agreed with those of D-valine and L-alanine, respectively. Therefore, configurations of C-2 and C-2' in

Fig. 4. Partial structures in vinylamycin.

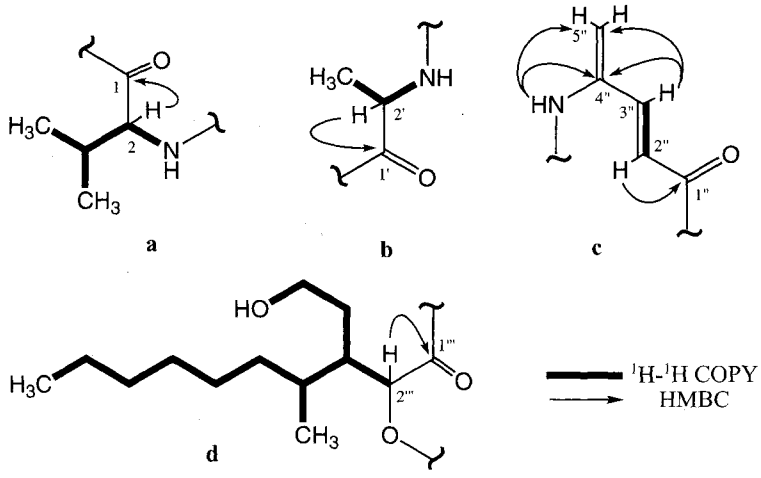
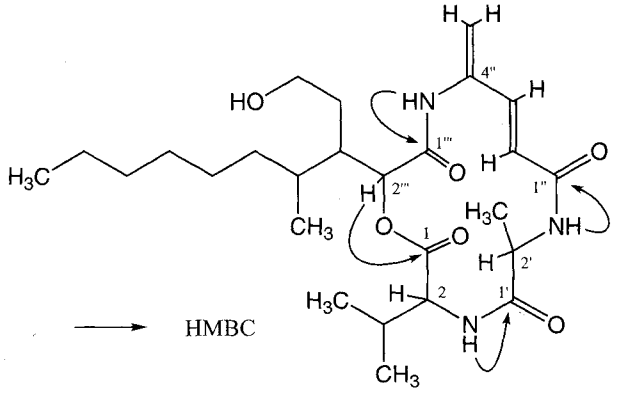



Fig. 5. Summary of HMBC for each component in vinylamycin (DMSO-$d_6$).




vinylamycin were determined to be ***R*** and ***S***, respectively. The stereochemitry of the 2-hydroxy fatty acid residue is now in progress.

Table 5. Antimicrobial activities of vinylamycin.

| Test organisms | MIC(μg/ml) |
|---|---|
| *Staphylococcus aureus* FDA209P | 1.56 |
| *S. aureus* Smith | 3.13 |
| *S. aureus* MS9610 | 1.56 |
| *S. aureus* MS16526(MRSA) | 3.13 |
| *S. aureus* TY-04282(MRSA) | 6.25 |
| *Micrococcus luteus* IFO3333 | 1.56 |
| *M.luteus* PCI1001 | 3.13 |
| *Bacillus subtilis* NRRL B-558 | 12.5 |
| *B. cereus* ATCC10702 | 50 |
| *Corynebacterium bovis* 1810 | 3.13 |
| *Escherichia coli* NIHJ | >100 |
| *Shigella dysenteriae* JS11910 | > 50 |
| *Salmonella enteritidis* | > 50 |
| *Proteus mirabilis* IFM OM-9 | >100 |
| *Providencia rettgeri* GN466 | > 50 |
| *Serratia marcescens* | >100 |
| *Pseudomonas aeruginosa* A3 | > 50 |
| *Klebsiella pneumonie* PCI602 | > 50 |
| *Mycobacterium smegmatis* ATCC607[a] | >100 |
| *Candida albicans* 3147[b] | > 50 |

Mueller-Hinton agar, at 37 °C for 18 hours.
[a] At 37 °C for 42 hours.
[b] Nutrient agar +1% glucose, at 27 °C for 18 hours.

## Biological Activity

The antimicrobial activities of **1** is shown in Table 5. Vinylamycin (**1**) showed broad and moderate antimicrobial activities against Gram-positive bacteria including MRSA. Vinylamycin did not show acute toxicity in mice at a dose of 100 mg/kg when administered intraperitoneally. However, the therapeutic efficacy of vinylamycin against systemic bacterial infection using *Staphylococcus aureus* smith in mice was not active by subcutaneous injection at 25 mg/kg.

## Discussion

Our screening program for new antibiotics gave a new

antibacterial compound designated vinylamycin. Structural study revealed that vinylamycin is a fourteen membered ring depsipeptide consisting of one molecule of D-valine, L-alanine and 4-amino-penta-2,4-dienoic acid and 2-hydroxy fatty acid. Recently, rakacidins[6] were isolated from a strain of *Micromonospora* as cytotoxic depsipeptides. Although their structures are related to that of vinylamycin, they are fifteen membered ring compounds composed of sarcosine, 3-hydroxyasparagine, 4-amino-penta-2,4-dienoic acid and 3-hydroxy fatty acid. AM toxins[7] and LL-15G256s[8] compounds having 2-hydroxy fatty acid as a component in these cyclodepsipeptides were discovered by their phytotoxicities or antifungal activities. However, vinylamycin did not exhibit cytotoxicity, phytotoxicity and antifungal activity in spite of a member of these cyclodepsipeptides.

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