# Studies on antialgal compounds of algicidal bacteria

A Thesis Presented to The University of Tokyo
for
Doctor's Degree

by

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2002

# CONTENTS

# ABSTRACT

With the aim of eliminating water bloom, the screenings for the effective antialgal compounds were accomplished. The isolation and identification of the antialgal compounds from the algicidal bacteria was described. This doctoral thesis consists of 7 chapters as shown below.

Chapter I deals with the two different methods for isolating the algicidal bacteria. Firstly, the MeOH extracts of bacteria which grew on the nutrient media were subjected to the antialgal assay using the paper disk method. The second method is the screening of the algicidal bacteria with plaque-forming activity on algal lawn. As the result of the screenings, 30 strains of algicidal bacteria were obtained.

Chapter II deals with the identification of the strains by the analysis of 16S rDNA. About 1.5 kb fragment of each bacterium was amplified by the colony PCR method. Total or partial sequence of the fragment was analyzed by the DNA sequencer. Total 30 strains were classified in several groups including *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Arthrobacter*, and *Streptomyces* based on phylogenetic analysis.

Chapter III deals with the isolation and identification of L-Tyr as the antialgal compound from *Lysobacter* sp. LB-1 which was given by Dr. Mitsutani (Fukuyama University). The identification of L-Tyr was performed by the combination of amino acid analysis and Marfey's method. L-Tyr showed the antialgal activity at the concentration of 30 μg/disk against several cyanobacterial strains.

Chapter IV deals with the isolation and structure elucidation of harmane (1-methyl-β-carboline) as the antialgal compound from *Pesudomonas* sp. K44-1. The structure elucidation of harmane was accomplished by analyses of NMR and HRFABMS spectra data. Harmane and authentic norharmane (β-carboline) showed the antialgal activity against several cyanobacterial strains.

Chapter V deals with the examination of lytic activity of *Bacillus* sp. M1 strain and the structure elucidation of surfactin as the antialgal compound produced by M1 strain. M1 strain showed the potent plaque-forming activity on algal agar lawns and lytic activity in the liquid coculture. The identification of surfactin was accomplished by analyses of NMR and HRFABMS spectra data and Marfey's method. Surfactin showed the antialgal activity against several cyanobacterial strains.

Chapter VI deals with the isolation and structure elucidation of YM-28160 and permetin A as the antialgal compound from *Paenibacillus* sp. S4. The structure elucidation of YM-28160 and permetin A was accomplished by analyses of NMR and HRFABMS spectra data. The stereochemistries of amino acid moieties of YM-28160 and permetin A were determined by Marfey's method and analyses of OPA-derivatives. YM-28160 and permetin A showed the antialgal activity against several cyanobacterial strains.

Chapter VII gives the summary and the discussion based on the present studies.

# ACKNOWLEDGMENTS

I am very grateful to Associate Professor Masahiro Murakami for providing me with the opportunity to work on this subject and careful direction through my work. I am thankful to Professor Hiroki Abe for valuable discussion and suggestion. I would like to express thankfulness to the Professor Emeritus of The University of Tokyo, Katsumi Yamaguchi for valuable advice and suggestion about my work.

I wish to thank a large number of people whose help is necessary for accomplishing this work; Associate Professor Atsushi Mitsutani of Fukuyama University for kindly giving the algicidal bacterium and valuable suggestions through my phylogenetic work, Miss Akiko Imoto of the student of Fukuyama University for assistance in analysis of DNA sequence, Dr. Kazuo Furihata for assistance in measuring NMR spectra. Research Associate Youichi Nakao for assistance in measuring MS spectra.

I would especially like to thank all the members of the Laboratory of Marine Biochemistry for their collaboration and assistance. Especially, Dr. Keishi Ishida gave me useful advice for the measurement of NMR spectra. Research Associate Shigeru Okada gave me valuable advice for primer design and DNA cloning.

Finally, I am very thankful to my wife Shuko Kodani and my parents, Nobuyuki and Mariko Kodani, for supporting my daily life and their encouragement.

# ABBREVIATIONS

| | |
|---|---|
| COSY | Correlation Spectroscopy |
| FAB | Fast Atom Bombardment |
| HMBC | $^{1}$H-detected Multiple-bond Heteronuclear Multiple Quantum Coherence |
| HMQC | Heteronuclear Multiple Quantum Coherence |
| HPLC | High Performance Liquid Chromatography |
| HRFABMS | High Resolution Fast Atom Bombardment Mass Spectrometry |
| IAM | the Institute of Molecular and Cellular Biosciences, the University of Tokyo (formerly, Institute of Applied Microbiology) |
| NIES | National Institute for Environmental Studies |
| NMR | Nuclear Magnetic Resonance |
| NOESY | Nuclear Overhauser and Exchange Spectroscopy |
| ODS | Octadecylsilane |
| TLC | Thin Layer Chromatography |

# INTRODUCTION

Blooms of cyanobacteria are widespread in lakes and reservoirs, especially during the summer, and cause many problems for scenery, anxiety about toxicity[1] and unpleasant odors.[2,3] Cyanobacteria of the genera *Microcystis*, *Anabaena*, *Oscillatoria*, *Aphanizomenon*, and others can build up 'mat', visible aggregates that may cover large areas of a eutrophic lake. Some water blooms release substances such as microcystin, which is extremely toxic to human and domestic animals.[4] Blooms cause the killing of fish by excluding light necessary for photosynthesis in the lower water and preventing release of oxygen into the water, or by depleting the oxygen through decay or respiration within the bloom. Therefore, for water quality management, it is important to know what controls cyanobacterial dynamics in their natural habitats.[5,6] Changes in cyanobacterial populations have been attributed to many factors, including nutrient depletion, light, accumulation of metabolites, the pH–$CO_2$ system,[7] and predation of antagonistic microorganisms.

With the aim of eliminating water blooms, several approaches using antagonistic microorganisms have been tried. Various microorganisms, such as bacteria,[8,9,10] actinomycetes,[11] fungi, cyanophages,[12,13] and amoebae,[14] have been reported to kill the cyanobacteria. Cyanophages are ideal as biological control agents because they can selectively eliminate the toxic species in the water because of their high specificity for their hosts.[12,13] However, lytic bacteria have more valuable characteristics for algal control in natural environments. They can survive by organic carbon nutrient sources during non-bloom conditions, and mutation to no susceptible strains to the specific host is far less likely to occur because there are no unique attachment receptors. Non-obligate predator bacteria do not require the presence of prey cells for survival and they can grow on laboratory media in the absence of host cells. They attack and destroy host cells when nutrients in the environment become depleted. The bacteria classified in

the genera *Cytophaga* and *Myxobacteria* are mainly reported as non-obligate predators.[15,16,17]

Lysis of cyanobacteria by lytic bacteria was first reported by Shilo in 1967.[18] Cyanobacteria-lysing bacteria are usually present in waters of elevated productivity.[19] Lytic bacteria are particularly abundant in water where cyanobacteria blooms occur, and the number of lytic bacteria in direct association with the cyanobacterial colonies is always higher than the number free in the water column.[20] In particular, Yamamoto *et al.* studied the seasonal changes of a heterotrophic bacterial community in a eutrophic lake and found that the genera *Pseudomonas*, *Alcaligenes* and *Cytophaga-Flavobacterium* group accounted for the great majority of strains capable of lysing bacteria.[21] In numerous strains of lytic bacteria, members of the *Myxobacteria* and *Cytophaga* groups, have been isolated mainly.[22] *Myxobacteria* and *Cytophaga*-like bacteria were reported to lyse cyanobacteria by attachment and secretion of diffusible lytic substances. These bacteria produce a variety of different exoenzymes capable of hydrolyzing the cyanobacterial cell wall.[23,24]

Several different types of bacterial predation have been reported. Bacterial predators use two major mechanisms to kill their prey. The first mechanism, which is used by *Bdellovibrio* and *Daptobacter*, is to penetrate the cell wall of the prey and multiply within the periplasm or cytoplasm, eventually destroying the cell from within.[25] Other predators are extracellular predators that kill their prey through the use of antagonistic chemicals or enzymes. In most cases, these bacterial predators require contact with the prey cell in order to destroy it. This mechanism is used by *Cytophaga,*[15] *Myxobacteria,*[26] *Herpetosiphon,*[27] *Ensifer,*[28] and *Vampirovibrio.*[29]

Cyanobacterial-lysing bacteria have been shown to produce a variety of bacteriolytic exoenzymes such as proteases,[30,31] glucosaminidase,[32] lysozyme[33] and D-alanyl-N-lysine endopeptidase,[33] as well as antibiotics.[34] Each of these can affect the cell walls of cyanobacteria containing peptidoglycan and lipid

bilayer as their major structural component.[35] Recently, Mitsutani isolated protease from the culture broth of *Lysobacter* sp. LB-1 and reported its lytic activity against *Anabaena cylindrica* (NIES-19).[31] Interestingly, LB-1 protease have specific lytic activity against *Anabaena* cells in stationary phase.[31] However, the mechanism of its specific lytic activity have not been clear.

On the other hand, some chemical-screening programs from antagonistic bacteria have been reported as a result of increased emphasis on the ecological significance of algal controls.[36,37] The antibiotic or antibiotic-like materials produced by bacteria,[38,39] actinomycetes,[40] and fungi[41,42] were reported to kill cyanobacteria. The well known antibiotic, phenazine pigment produced by *Pseudomonas aeruginosa* was reported to have the potent antialgal activity.[38] The antibiotics containing gramicidin S, penicillin, bacitracin, terramycin, and chloramphenicol were reported to show the antialgal activity against the cyanobacterium *Plectonema boryanum*.[43,44,45]

Among the substances recently reported, there are several promising agents that have specific inhibitory activity to particular algal group. Yoshikawa *et al.* isolated the antialgal compound, β-cyanoalanine, from the culture broth of marine bacteria, and reported its specific antialgal activity against cyanobacteria.[46] The basic amino acids, L-Lys and L-Arg were isolated from the mycelia of *Streptomyces phaeofaciens* and reported to show the algal lysing activity.[47] Recently, Imamura *et al.* reported that argimicin A isolated from *Shingomonas* sp. showed the potent antialgal activity at the 100 ng/mL.[48] Argimicin A is thought to have the potential for algal controls in a natural environment. However, there has been few information about antialgal compounds, and more effective compounds were required for the application in lakes and reservoirs.

Under these backgrounds, two types of screening methods were accomplished for obtaining effective antialgal compounds in the present work. Firstly, the bacteria were obtained using the nutrient media from the eutrophic ponds, and MeOH extracts of the bacterial culture were subjected to the antialgal

screening. Another method is the screening for the algicidal bacteria by plaque-forming activity on algal lawn. Then, the isolation and structure elucidation of the antialgal compounds from the bacteria isolated by these screenings were described in detail.

# CHAPTER I

# Screening of algicidal bacteria

With the aim of obtaining the algicidal bacteria, 2 types of screening methods were performed. First method; the bacteria were isolated from the surface water of the eutrophic ponds and the MeOH extracts of cultured bacteria were subjected to the antialgal assay against the cyanobacterium *Anabaena cylindrica* (NIES-19) using the paper disk method. Second method; the bacteria were grown on the agar medium containing *A. cylindrica* (NIES-19). The bacteria which showed the plaque-forming activity were isolated from the agar plates.

## 1. Materials and methods

### 1.1 Organisms and culture conditions

*A. cylindrica* (NIES-19) was obtained from the National Institute for Environmental Science (NIES). *A. cylindrica* (NIES-19) was cultured in CB medium[49] under illumination of 250 μE/m$^2$s on a 12L:12D cycle.

### 1.2 Antialgal screening with MeOH extracts

The surface water samples were collected from the pond Teganuma (Chiba, Japan) and the pond Shinobazu (Tokyo, Japan) from March to May 1999. About 100 μL of surface water was spread onto the Waksman agar medium (Table I-1) and the plates were incubated at 25 °C for 3 days. After detectable colonies of bacteria had appeared, the clonal colonies were isolated by repeated restreaking. Isolates were maintained on Waksman agar medium at 25 °C. Each isolate was

cultured in 5 mL of Waksman liquid medium for 3 days. The whole culture broth was extracted with 15 ml of MeOH and centrifuged at 3000 rpm for 10 min. The supernatant was evaporated and redissolved in 1 mL of MeOH. The paper disk containing each MeOH extract was placed on the CB agar medium (Table I-2) containing *A. cylindrica* (NIES-19). The test agar plates were incubated for 5 days under illumination of 250 μE /$m^2$s on a 12L:12D cycle.

### 1.3 Plaque-forming assay

The surface water samples were collected from the pond Shinobazu, the pond Teganuma, and the moat Ote-bori (Tokyo, Japan) in every month from January to October 2000. The surface water samples and sediment samples were collected from the eutrophic pond Shinobazu in March 2001. About 100 μL sample water was spread onto 0.5% casitone CB agar medium or Waksman agar medium (Table I-1, I-2) and the plates were incubated at 25 °C for 3 days. After detectable colonies of bacteria had appeared, a small sample of each colony was inoculated on the plates of the CB agar medium containing *A. cylindrica* (NIES-19). The plates were incubated for 5 days at 25 °C under illumination of 250 μE /$m^2$s on a 12L:12D cycle. The colonies making plaques on the algal lawns were picked up and restreaked onto 0.5% casitone CB agar medium or Waksman agar medium. After repeated streaking, the clonal colonies were isolated and determined for their plaque-forming activities.

## 2. Results

### 2.1 Antialgal screening with MeOH extracts

Sixty-four strains of bacteria were obtained from surface water of the eutrophic pond Teganuma and Shinobazu in 1999 March to May. Each strain

was cultured for 3 days in Waksman medium and extracted with MeOH. These MeOH extracts were subjected to the antialgal assay against the cyanobacterium *A. cylindrica* (NIES-19) using the paper disk method.[40] As a result, the MeOH extract of the isolate designated M1 strain only showed the potent antialgal activity.

### 2.2 Plaque-forming assay

The samplings of the surface water and the sediment were performed in the eutrophic pond Teganuma (Chiba, Japan), the pond Shinobazu (Tokyo, Japan), and the moat Ote-bori (Tokyo, Japan) in which the water bloom forming was visually observed during all the year. The sample materials were spread on the 0.5% CB casitone agar medium or Waksman agar medium. The colonies appeared after 3 days incubation were directly inoculated onto the CB agar medium containing the cyanobacterium *A. cylindrica* (NIES-19), and the plaque forming colonies were isolated by restreaking (Fig. I-1). By using the CB medium, 14 bacterial strains were obtained from 9250 inoculated colonies in 2000. On the other hand, 15 bacterial strains designated S-1 to S-15 were obtained from 1250 colonies inoculated in March 2001 by using Waksman medium (Table I-3). The sampling date and colony colors of algicidal bacteria were summarized in Table I-4. The strain U38-6 made the orange transparent colonies on 0.5% casitone CB agar medium. The strains containing U25-2, U330-1, and U929-2 have the yellow pigment.

## 3. Discussion

So far, several methods to obtain algicidal bacteria have been reported.[30,37,38] Among these methods, the double layer method is most general for obtaining algicidal microbes.[30] In brief, water samples and living cyanobacterial cells are mixed and cocultured in cyanobacterial agar medium.

Microbes which feed on the cyanobacterial cells as nutrient can only grow on the plates. This methods are also applied for isolating other microbes such as cyanophages and amoebae. In addition, oligotrophic bacteria tend to be detected by this method. In the present study, to the purpose of obtaining broader range of bacteria, 0.5% CB medium was used for obtaining the bacterial colonies. On the other hand, Waksman medium was generally used for culture of *Actinomyces*.[50] There are several reports about *Actinomyces* which have the lytic activity against cyanobacteria, the purpose using Waksman medium in the present study is to isolate the filamentous bacteria including *Actinomyces*.

Only one bacterial strain out of 64 strains was isolated with Waksman medium by the screening with MeOH extracts. This method was thought to be ineffective for obtaining large number of the algicidal bacteria as compared with plaque-forming assay since it takes the several procedures of extraction. On the other hand, 29 bacterial strains were isolated as the result of plaque-forming assay.

Yamamoto *et al.* studied the seasonal changes of heterotrophic bacterial community in the eutrophic lake and found that the genera *Pseudomonas*, *Alcaligenes* and *Cytophaga-Flavobacterium* group accounted for the great majority of strains capable of lysing bacteria.[21] The ratio of lytic bacteria increased in September with the decrease of algal biomass in a eutrophic lake. Recently, Rashidan *et al.* also reported that the number of lytic *Cytophaga* sp. started to increase during the bloom and reached a peak within a week after its decline.[51] In the present study, largest number of algicidal bacteria were obtained in March 2001, but only 2 strains were obtained in September and November 2001. However these numbers would underestimate the real number of lytic bacteria since the culture condition may restrict to the growth of bacteria. Namely, the plaque-forming activities were determined by using the specific bacterial colonies which can grow on 0.5% casitone CB or Waksman media. For obtaining broader range of the algicidal bacteria, the further study to use the variety of the bacterial media should be performed.

Fig.I-1. Plaque-forming assay against *Anabaena cylindrica* (NIES-19)

Table I-1. Composition of 0.5% casitone CB medium

| | | |
|---|---|---|
| $Ca(NO_3)_2 \cdot 4H_2O$ | 150 | mg |
| $KNO_3$ | 100 | mg |
| β-$Na_2$·glycerophosphate | 50 | mg |
| $MgSO_4 \cdot 7H_2O$ | 40 | mg |
| Vitamine $B_{12}$ | 0.1 | mg |
| Biotin | 0.1 | mg |
| Thiamine HCl | 10 | mg |
| PIV metals | 3 | mg |
| BICINE | 0.5 | g |
| Casitone | 5 | g |
| Distilled water | 1 | L |
| Agar | 15 | g |
| pH | 8.6 | |

Table I-2. Composition of Waksman medium

| | | |
|---|---|---|
| Peptone | 5 | g |
| NaCl | 3 | g |
| Glucose | 5 | g |
| Beef extract | 5 | g |
| Distilled water | 1 | L |
| Agar | 15 | g |
| pH | 7.0 | |

Table I-3. The result of plaque-forming assay

| Month | Medium | Inoculated colonies | Isolates |
|---|---|---|---|
| January, 2000 | 0.5% casitone CB | 820 | 1 |
| February | 0.5% casitone CB | 900 | 3 |
| March | 0.5% casitone CB | 1830 | 7 |
| April | 0.5% casitone CB | 330 | 1 |
| May | 0.5% casitone CB | 900 | 0 |
| June | 0.5% casitone CB | 240 | 0 |
| July | 0.5% casitone CB | 240 | 0 |
| August | 0.5% casitone CB | 200 | 0 |
| September | 0.5% casitone CB | 2290 | 1 |
| October | 0.5% casitone CB | 700 | 1 |
| November | 0.5% casitone CB | 800 | 0 |
| March, 2001 | Waksman | 1250 | 15 |
| Total | | 10500 | 29 |

Table I-4. Isolates by algicidal screenings

| Strain | Date | Place | Source | Medium | Colony |
|---|---|---|---|---|---|
| M1 | 1 May 1999 | Shinobazu | Water | Waksman | white |
| U114-1 | 1 January 2000 | Shinobazu | Water | 0.5% casitone CB | white |
| U22-1 | 2 February 2000 | Ote-bori | Water | 0.5% casitone CB | white |
| U25-2 | 5 February 2000 | Shinobazu | Water | 0.5% casitone CB | yellow |
| U25-3 | 5 February 2000 | Shinobazu | Water | 0.5% casitone CB | white |
| U38-3 | 8 March 2000 | Shinobazu | Water | 0.5% casitone CB | white |
| U38-6 | 8 March 2000 | Shinobazu | Water | 0.5% casitone CB | orange |
| K311-1 | 11 March 2000 | Ote-bori | Water | 0.5% casitone CB | white |
| K328-3 | 28 March 2000 | Ote-bori | Water | 0.5% casitone CB | white |
| K328-4 | 28 March 2000 | Ote-bori | Water | 0.5% casitone CB | white |
| U330-1 | 30 March 2000 | Shinobazu | Water | 0.5% casitone CB | yellow |
| U330-4 | 30 March 2000 | Shinobazu | Water | 0.5% casitone CB | white |
| K44-1 | 4 April 2000 | Shinobazu | Water | 0.5% casitone CB | white |
| U929-2 | 29 September 2000 | Shinobazu | Water | 0.5% casitone CB | yellow |
| U1013-1 | 13 October 2000 | Shinobazu | Water | 0.5% casitone CB | white |
| S1 | 2 March 2001 | Shinobazu | Water | Waksman | white |
| S2 | 2 March 2001 | Shinobazu | Water | Waksman | white |
| S3 | 2 March 2001 | Shinobazu | Water | Waksman | white |
| S4 | 2 March 2001 | Shinobazu | Sediment | Waksman | white |
| S5 | 2 March 2001 | Shinobazu | Sediment | Waksman | white |
| S6 | 2 March 2001 | Shinobazu | Water | Waksman | white |
| S7 | 9 March 2001 | Shinobazu | Water | Waksman | white |
| S8 | 9 March 2001 | Shinobazu | Sediment | Waksman | white |
| S9 | 9 March 2001 | Shinobazu | Sediment | Waksman | white |
| S10 | 9 March 2001 | Shinobazu | Sediment | Waksman | white |
| S11 | 19 March 2001 | Shinobazu | Sediment | Waksman | white |
| S12 | 19 March 2001 | Shinobazu | Sediment | Waksman | white |
| S13 | 30 March 2001 | Shinobazu | Water | Waksman | white |
| S14 | 30 March 2001 | Shinobazu | Water | Waksman | white |
| S15 | 30 March 2001 | Shinobazu | Water | Waksman | white |

# CHAPTER II

# Identification of algicidal bacteria and secondary screening

In chapter I, total 30 strains of algicidal bacteria were obtained. To identify these algicidal bacteria, the 16S rDNA sequence of each bacterium was analyzed. The almost full-length of 16S rDNA were amplified with the colony PCR method. The sequences were analyzed with a DNA sequencer by using a set of primers complementary to conserved regions located within the bacterial 16S rDNA. The algicidal bacteria were classified on the basis of phylogenetic analysis.

Then, with the aim of obtaining antialgal compound, secondary screening for antialgal compound was performed using the paper disk method.

## 1. Materials and methods

### 1.1 PCR amplification of 16S rDNA and sequencing

Each isolate was grown on a 0.5% casitone CB agar medium for 24-48 h, and a small amount of cells from a colony were picked by a needle and suspended in 25 μL MilliQ $H_2O$ containing 20 nmol of dNTP, 100 pmol of PCR primer and 2.5 μL of PCR buffer. The primers used in this experiment were 27F and 1492R which correspond to positions 8 to 27 and 1510 to 1492 in the *Escherichia coli* 16S rDNA sequence, respectively.[52,53] The cells were lysed by heating at 96 °C for 10 min, immediately cooled on ice, and centrifuged. Then 0.25 μL Taq DNA polymerase was added to each mixture. PCR amplification was accomplished using the automatic cycler (PCR system 9600, Perkin-Elmer Co.), with 30 thermal cycles of denaturation (1 min for 94 °C), annealing (2 min 30 s at 55 °C), extension

(2 min 30 s for 72 °C), and with a final elongation step of 7 min at 72 °C. The molecular size of PCR amplicon was determined by agarose electrophoresis including a molecular marker at a constant voltage of 100 V. Sequencing was performed for the purified PCR products from each strain using BigDye$^{TM}$Terminator Cycle Sequencing Ready Reaction Kits (ABI Prism), and DNA sequencer 377 (ABI Prism), or using ThermoSequenase Cycle Sequencing Kit (SHIMADZU), and DNA sequencer DSQ-2000L (SHIMADZU) by using a set of primers complementary to conserved regions located within the bacterial 16S rDNA.[52]

1.2 Primers used in PCR amplification and sequencing

PCR amplification and sequencing were performed by using 8 primers as shown below.

27F :5'-AGAGTTTGATCATGGCTCAG-3'
357F :5'-ACTCCTACGGGAGGCAGCAG-3'
517R :5'-GTATTACCGCGGCTGCTGGC-3'
536F :5'-GCCAGCAGCCGCGGTAATAC-3'
682R :5'-TCTACGCATTTCACCGCTACAC-3'
704F :5'-GTGTAGCGGTGAAATGCGTAGA-3'
1246F :5'-GGGCTACACACGTGCTACAA-3'
1492R :5'-GGTTACCTTGTTACGACTT-3'

1.3 Phylogenetic analysis

The 16S rDNA sequences were aligned using CLUSTAL W software Ver.1.7.[54] Kimura's two parameter model[55] was applied to the calculation of evolutionary distance. A phylogenetic tree was constructed by the neighbor-

joining method.[56] Bootstrap analyses of 1000 replicates were carried out using CLUSTAL W Ver.1.7.

## 1.4 Databases of 16S rDNA sequences

The 16S rDNA sequences used for the phylogenetic analyses include the sequences of *Actinomyces viscosus* (M33908), *Alteromonas macleodii* (X82145), *Arthrobacter aurescens* (X83405), *Cytophaga johnsonae* (M59051), *Escherichia coli* (Z83204), *Flavobacterium thalpophilum* (D14020), *Halomonas elongata* (M93355), *Oceanospirillum beijerinckii* (AB006761), *Prochloron* sp. (X63141), *Pseudoalteromonas haloplanktis* (X67024), *Pseudomonas aeruginosa* (Z76651), *Pseudomonas elongata* (AB021368), *Vibrio fisheri* (X74702), *Vibrio marinus* (X82142), *Xanthomonas campestris* (AF188831), *Streptomyces albidoflavus* (Z76685), *Streptomyces lividans* (AB037566), *Paenibacillus koreensis* (AF130254), and *Bacillus subtilis* (AB065370) in the RDP database.

## 1.5 Second screening of the algicidal bacteria

Each bacterial strain was cultured with 200 mL 0.5% casitone CB or Waksman media for 3 days. The whole culture broth was evaporated and extracted with 100 mL of MeOH. The MeOH extract was partitioned between $H_2O$ and EtOAc. The aqueous and lipophilic layers were subjected to the antialgal assay against *A. cylindrica* (NIES-19) using the paper disk method. The test plates were incubated for 5 days at 25 °C under illumination of 250 μE /$m^2$s on a 12L:12D cycle. The antialgal activity was determined by the diameter of the plaque formed around the disk.

## 2. Results

### 2.1 Phylogenetic analysis of algicidal bacteria

The almost full-length 16S rDNA sequences (about 1.5 k bp) of 16 bacterial strains containing 14 strains isolated in 2000, M1 strain, and S4 strain were amplified by the PCR method, and analyzed with a DNA sequencer (Fig. II-2~9). Figure II-1 shows the phylogenetic positions of these 16 algicidal bacterial strains. Phylogenetic analysis indicated that 12 strains fell into 3 clusters. Cluster A was comprised of strains U114-1, U25-3, U22-1, K44-1, U38-3, K328-3, K328-4, U330-4, K311-1 and relatively close to *Pseudomonas aeruginosa*. In cluster B, the sequences of strains U25-2 and U330-1 were identical to each other and 3 strain containing U929-2 were grouped in *Arthrobacter* family. The strain U38-6, which positioned in cluster C, was grouped with the *Cytophaga-Flavobacterium* family. The sequence data of M1 showed 99% homology with *Bacillus subtilis*, and M1 strain was thought to be grouped in *Bacillus* family. S4 strain was thought to be classified in *Paenibacillus* group, since the sequence data showed 96% homology with *Paenibacillus koreensis*. The strain U-1013 was grouped in *Streptomyces* family, because the sequence data showed 97% homology with *Streptomyces albidoflavus*.

The partial sequences of the rest 14 strains (S1 to S15 except for S4) were analyzed with DNA sequencer using the primer 682R in the same manner (Fig. II-10~12). These 14 strains were divided into 3 groups by homology of the partial 16S rDNA sequence data (about 600 bp). The first group was *Pseudomonas* group containing S2, S9, S10, S12, S13, S15, since each sequence of these strains showed at least more than 98% homology to that of *Pseudomonas aeruginosa*. The second group was *Bacillus* group containing S1,S3, S5, S6, S7, S8, S11, since each sequence of these strains showed at least more than 97% homology to that of *Bacillus subtilis*. The rest strain S14 was thought to fall within the genus

*Streptomyces*, because the sequence showed 97% homology with that of *Streptomyces lividans*.

2.2 Second screening of algicidal bacteria

Each bacterial strain was cultured in 200 mL of 0.5% casitone CB medium or Waksman medium. The whole culture broth was extracted with MeOH, and each extract was partitioned between $H_2O$ and EtOAc. Each fraction was subjected to the antialgal assay using the paper disk method. The results were summarized in Table II-1. The lipophilic fractions of U114-1, U22-1, U44-1, U38-3, and K311-1 in *Pseudomonas* group showed the antialgal activity. On the other hand, the antialgal activity was shown in the lipophilic fractions of all strains in *Bacillus* group. The lipophilic fraction of *Streptomyces* sp. S14 showed the antialgal activity at the concentration of 300 μg/disk. Among hydrophilic fractions, that of S4 strain only showed the antialgal activity at the concentration of 300 μg/disk.

## 3. Discussion

Yamamoto *et al.* reported the isolation of heterotrophic bacteria causing lysis of cyanobacteria in the eutrophic lake. They found that most of the cyanobacteria-lytic bacterial strains were consisted of *Alcaligenes, Pseudomonas,* and the *Flavobacterium/Cytophaga* group.[21] In the present study, 15 isolates out of total 30 strains were identified to be grouped in *Pseudomonas* genus by the analyses of 16S rDNA sequences and construct the major cluster in phylogenetic tree. These data enhanced the environmental role of the bacterium classified in *Pseudomonas* for algal control in the eutrophic pond.

Eight strains out of 15 strains, which were isolated using Waksman medium, were classified in *Bacillus* group. On the other hand, *Bacillus* was not

detected with plaque-forming assay by using 0.5% casitone CB medium. Using Waksman medium were isolated only 2 strains of *Streptomyces*, although it was expected that more streptomycetes would obtain by plaque-forming assay with Waksman medium.

Whyte *et al.* reported a method for isolating cyanobacteria-lysing streptomycetes from soil.[40] In brief, the cells of cyanobacteria which were precultured in liquid medium were filtered with filter paper, and the soil sample was spread onto the cyanobacterial lawn (filter paper). Within 4-6 days, cyanobacteria-lysing streptomycetes appeared with lytic zone. This method may be effective to isolate streptomycetes comparing with plaque-forming assay. On the other hand, 8 strains of *Bacillus* were obtained by using Waksman medium in the present study. The algicidal *Bacillus* were isolated from soil and surface water of the eutrophic pond Shinobazu. Considering that the bacteria classified in *Bacillus* genus is known to be usually present in any places such as soil, lake, and river,[57,58] these data showed the potential that the algicidal *Bacillus* would be a useful agent for algal control.

With the aim of obtaining the antialgal compound, the second screening of the algicidal bacteria was performed using the paper disk method. The lipophilic fraction of all strains in *Bacillus* group showed the antialgal activity. On the other hand, the lipophilic fractions of 5 strains in *Pseudomonas* group showed the antialgal activity. Each group consisted of genetically very closely related strains, so there is the possibility that these 2 groups would produce respectively the same antialgal compound.

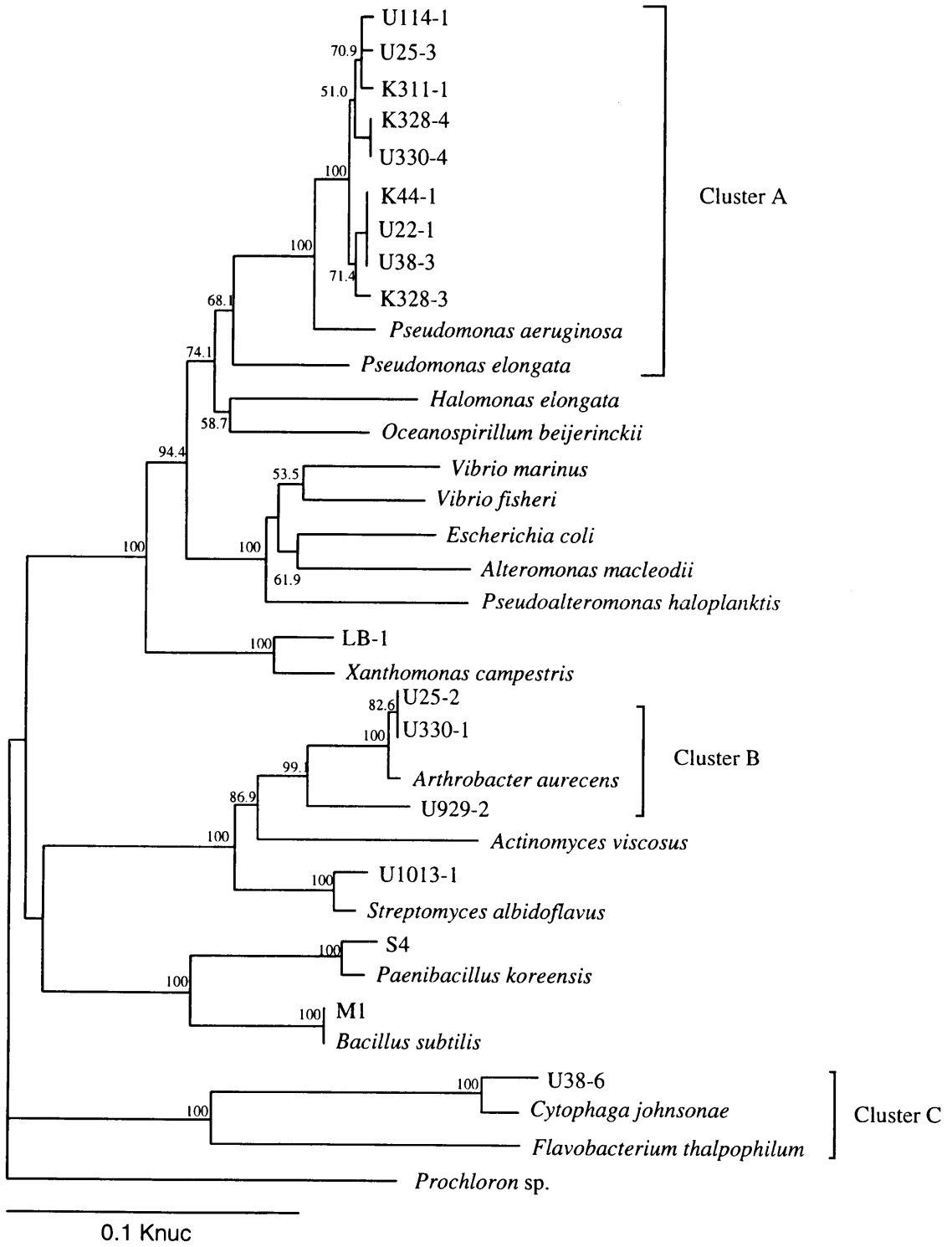



Fig. II-1. Phylogenetic positions of algicidal bacteria based on 16S rDNA sequences using neighbor-joining method

```
   1  AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATG
      CAAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTGGCGAGCGGCGGACG
 101  GGTGAGTAATGCCTAGGAATCTGCCTAGTAGTGGGGGATAACGTCCGGAA
      ACGGGCGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGATCTTCG
 201  GACCTCACGCTATTAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGT
      AATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGT
 301  CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG
      GAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGA
 401  AGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTA
      CCTAATACGTGATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAAC
 501  TCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAAT
      TACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGC
 601  CCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTATGG
      TAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGA
 701  AGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGG
      TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
 801  GTAAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAG
      CTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACT
 901  CAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
      CGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCT
1001  AGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTG
      TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAA
1101  CCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTG
      CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC
1201  TTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCC
      AAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGC
1301  AGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCA
      GAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA
1401  CCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGAC
      GGTTACCACGGTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACC
```

Fig.II-2. Full-length 16S rDNA sequence of U114-1 (U25-3)

```
   1  AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATG
      CAAGTCGAGCGGATGAAGTGAGCTTGCTCATGGATTCAGCGGCGGACGGG
 101  TGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAG
      GAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGG
 201  CCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAA
      TGGCTCACCAAGGCTACGATCCGTAACTGGTCTGAGAGGATGATCAGTCA
 301  CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA
      ATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG
 401  AAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGAT
      TAATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTC
 501  TGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTA
      CTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCC
 601  CGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTATGGTA
      GAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAG
 701  GAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTG
      CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT
 801  AAACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGCGCAGCT
      AACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
 901  AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
      AAGCAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCCAG
1001  AGATGGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTC
      GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACC
1101  CTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCC
      GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
1201  ACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAA
      GCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAG
1301  TCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGA
      ATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACC
1401  ATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGACGG
      TTACCACGGTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACC
```

Fig. II-3. Full-length 16S rDNA sequence of U22-1 (K44-1, U38-3)

```
   1  AGAGTTTGATCATGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATG
      CAAGTCGAACGATGATCCCAGCTTGCTGGGGGATTAGTGGCGAACGGGTG
 101  AGTAACACGTGAGTAACCTGCCCTTGACTCTGGGATAAGCCTGGGAAACT
      GGGTCTAATACCGGATACGACCATTCCACGCATGTGGTGGTGGTGGAAAG
 201  CTTTTGTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGGGGTA
      ATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCC
 301  ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG
      AATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGAT
 401  GACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGTAAGTGA
      CGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTA
 501  ATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGT
      AGGCGGTTTGTCGCGTCTGCTGTGAAAGACCGGGGCTCAACTCCGGTTCT
 601  GCAGTGGGTACGGGCAGACTAGAGTGCAGTAGGGGAGACTGGAATTCCTG
      GTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGC
 701  AGGTCTCTGGGCTGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAA
      CAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTG
 801  TGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCC
      GCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGC
 901  CCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACC
      TTACCAAGGCTTGACATGAACCGGAAAGACCTGGAAACAGGTGCCCCGCT
1001  TGCGGTCGGTTTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAG
      ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAG
1101  CGCGTGATGGCGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAA
      GGTGGGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGC
1201  ATGCTACAATGGCCGGTACAAAGGGTTGCGATACTGTGAGGTGGAGCTAA
      TCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCAT
1301  GAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACG
      TTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTTGGTAACAC
1401  CCGAAGCCGGTGGCCTAACCCTTGTGGGGGGAGCCGTCGAAGGTGGGACC
      GGCGATTGGGACTAAGTCGTAACAAGGTAACC
```

Fig. II-4. Full-length 16S rDNA sequence of U25-2 (U330-1)

```
   1  AGAGTTTGATCATGGCTCAGGATGAACGCTAGCGGCAGGCTTAACACATG
      CAAGTCGAGGGGTATATGTCTTCGGATATAGAGACCGGCGCACGGGTGCG
 101  TAACGCGTATGCAATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTG
      GATTAATACCTCATAGTATAGTGACTCGGCATCGAGATACTATTAAAGTC
 201  ACAACGGTAAAAGATGAGCATGCGTCCCATTAGCTAGTTGGTAAGGTAAC
      GGCTTACCAAGGCTACGATGGGTAGGGGTCCTGAGAGGGAGATCCCCCAC
 301  ACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAA
      TATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGA
 401  CGGTCCTATGGATTGTAAACTGCTTTTGTACGAGAAGAAACACTCCTACG
      TGTAGGAGCTTGACGGTATCGTAAGAATAAGGATCGGCTAACTCCGTGCC
 501  AGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGAATCATTGGGT
      TTAAAGGGTCCGTAGGCGGTTTAGTAAGTCAGTGGTGAAAGCCCATCGCT
 601  CAACGGTGGAACGGCCATTGATACTGCTAAACTTGAATTATTAGGAAGTA
      ACTAGAATATGTAGTGTAGCGGTGAAATGCTTAGAGATTACATGGAATAC
 701  CAATTGCGAAGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAG
      CGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA
 801  TGGATACTAGCTGTTGGAAGCAATTTCAGTGGCTAAGCGAAAGTGATAAG
      TATCCCACCTGGGGAGTACGTTCGCAAGAATGAAACTCAAAGGAATTGAC
 901  GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGATACGCGA
      GGAACCTTACCAAGGCTTAAATGTAGTTTGACCGATTTGGAAACAGATCT
1001  TTCGCAAGACAAATTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCG
      TGAGGTGTCAGGTTAAGTCCTATAACGAGCGCAACCCCTGTTGTTAGTTG
1101  CCAGCGAGTCAAGTCGGGAACTCTAACAAGACTGCCAGTGCAAACTGTGA
      GGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACGCCTTGGGCTAC
1201  ACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGTGACCAGGAG
      CGAATCTATAAAACCGGTCACAGTTCGGATCGGAGTCTGCAACTCGACTC
1301  CGTGAAGCTGGAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAAT
      ACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGCCATGGAAGCTGGGGG
1401  TGCCTGAAGTCGGTGACCGCAAGGAGCTGCCTAGGGTAAAACTGGTAACT
      AGGGCTAAGTCGTAACAAGGTAACC
```

Fig.II-5. Full-length 16S rDNA sequence of U38-6

```
   1  AGAGTTTGATCATGGCTCAGATGACGCTGGCGGCGTGCTTACACATGCAA
      GTCGAACGATGAAGCCCATACTTGCTGGGTGGATTAGTGGCGAACGGGTG
 101  ACGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTGGGAAAC
      TGGGTCTAATACCGGATAGGAGCGCCTACCGCATGGTGGGTGTTGGAAAG
 201  ACTTTATCGGTTTTGGATGGACTCGCGCCATATCAGCTTGTTGGTGAGGT
      AATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGC
 301  CACACTGGGACTGAGACACGGCCCAGACTCTACGGGAGGCAGCAGTGGGG
      AATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGAT
 401  GACGGCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGAC
      GGTACCTGCAGAAGAAGCACCGGCTAATACGTGCCAGCAGCCGCGGTAAT
 501  ACGTAGGGTGCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAG
      GCGGTTTGTCGCGTCTGTCGTGAAAGCTCCGGGCTTAACCCCGAATCTGC
 601  GGTGGGTACTGGGCAGACTAAAGTCATGTAAGGAGACTGGAATTTCCTGT
      GTAGCGGTGAAATGCGTAGATATCAGGAAGGAACACCGATGGCCGAAGGC
 701  AGCGTCTCTGGGCTGTAACTGACGCTGAGAACCAAAACATGGGGACCGAA
      CAGGATTAAATACCCTGGTAGTCCATCCCGTAAACGTTGGGCACTAGGTG
 801  TGGGGACCCATTCCACGGTTTCCGCGCCGCAGCTAACGCATTAAGTGCCC
      CGCCTGGGGAAGTACGGCCAGCAAGCCTAAAAACCAAAGGAATTGACGGG
 901  GGCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAA
      CCTTACCAAGGCTTGACATGTTCTCGATCGCCGTAGAGATACGGTTTCCC
1001  CTTTGGSGCGNGTTCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT
      GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGC
1101  CAGCACGTAATGGTGGGAACTCATGGGAGACTGCCGGGGTCAACTCGGAG
      GAAGGTGAGGACGACGTCAAATCATCATGCCCCTTATAGTCTTGGGCTTC
1201  ACGCATGCTCACAATGGCCGGTACAATGGGTTGCGATACTCGTGAGGTGG
      AGCTAAACCCAAAAAACCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGA
1301  CCCCACGAAGTCGGAGTCGCTATAATCGCAGATCAGCAACGCGCGGTGAA
      TACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTCGGTA
1401  ACACCCGAAGCCGGTGGCCTAACCCTTGTGGGGGGAGCCGCCGAAGGTGG
      GACCAGCGATTGGGACAAAGTCGTAACAAGGTCCAA
```

Fig. II-6. Full-length 16S rDNA sequence of U929-2

```
   1  AGAGTTTGATCATGGCTCCAGCAGAAAGTGCGGCTGCTAACACATGCAAG
      TGAGGATGAACATTCGTGGGATACGTGGCGAAACGGTGAGTAACACGTGG
 101  CAATCTCCTGCACTTGGACAAGCCTGAAACGGGTCTATGACCGATATGCT
      GCTGGCATCCGGGGGTCGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGC
 201  CTATCAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTAGC
      CGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACT
 301  CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATG
      CAGCGACGCCGCGTGAGGGATGACGGCTTCGGGTTGTAAACCTCTTTCAG
 401  CAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACT
      ACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATT
 501  ATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCC
      CGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAAGGCTAGAGTTTCC
 601  TTAGGGGAGATGGAATTCCTGGTTGTAGCGTGAAATTCGCAGATATCAGA
      AGAACACCGGTGGCGAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAC
 701  GAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACCCGTAA
      ACGGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAATA
 801  ACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAA
      AGGAATTGACGGGGGCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGAC
 901  GCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGA
      GACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCA
1001  GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTG
      TCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCG
1101  CCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCC
      TTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTCGA
1201  TACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGG
      GTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAG
1301  CATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACG
      TCACGAACAGTCGGTAACACCCGAAGCCGGTGGCCAACCCCTTGTGGGAG
1401  GGAGCTGTCGAAGGTGGGACTGGCGATTGGGACGAAGTCGTAACAAGGTA
      ACC
```

Fig. II-7. Full-length 16S rDNA sequence of U1013-1

```
   1  AGAGTTTGATCATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATG
      CAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACG
 101  GGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGA
      AACCGGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAA
 201  AAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAG
      TTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAG
 301  GGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
      CAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCC
 401  GCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGA
      ACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAG
 501  CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG
      TTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCT
 601  GATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAA
      CTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCG
 701  TAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAAC
      TGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGG
 801  TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCC
      TTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGC
 901  AAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
      TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC
1001  TCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGT
      GTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
1101  AACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTA
      AGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCA
1201  TCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAA
      AGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGT
1301  TCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAAT
      CGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
1401  CCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTT
      TNNGGAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAA
      CAAGGTAACC
```

Fig. II-8. Full-length 16S rDNA sequence of M1

```
   1  AGAGTTTGATCATGGCTCAGACGAACGCTGGCGGCGTGCCTAATACATGC
      AAGTCGAGCGGACTTCGGGGTTAGCAGCGGACGGGTGAGTAACACGTAGG
 101  CAACCTGCCTGTAAGACTGGGATAACTACCGGAAACGGTAGCTAAGACCG
      GATAAGTGATTCTCTCGCATGAGAGGATCAAGAAACACGGGGCAACCTGT
 201  GGCTTACAGATGGCTGCGGCGCATTAGCTAGTTGGTGGGGTAACGGCTCA
      CCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGG
 301  ACTGAGACACGGCCCAGACTCTACGGGAGGCAGCAGTAGGGAATCTTCCG
      CAATGGACGCAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTT
 401  CGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGTCGTGGAGAGTAACTGC
      TCTGCGAATGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAG
 501  CAGCCGCGGTACTACGTAGGGRGCAAGCGTTGTCCGGAATTATTGGGCGT
      AAAGCGCGCGCAGGCGGCCGCTTAATTCTGGTGTTTAAGCCCGAGGCTCA
 601  ACCTCGGTTCGCACTGGAAACTGGGTGCCTAGAGCACAAGAGAAAACCGA
      AATCCACGTGTAGCGGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTG
 701  GCGAAGGCGGCTTTCTGGCCTGTAACTGACGCTGAGGCGCGAAAGCGTGG
      GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGT
 801  GCTAGGTGTTAGGGGTTTCGATACCCTTGGTGCCGAAGTAAACACAATAA
      GCACTCCGCCTGGGGAGTACGCTCGCAAGAGTGAAACTCAAAGGAATTGA
 901  CGGGGACCCGCACAAGCAGTGGAGTATGTGGTTTAATTCGAAGCAACGCG
      AAAAACTTACCAGGTCTTGACATCCCTCTGAATATCCTAGAGATAGGGTA
1001  GGCCTTCGGGACAGAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
      TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAACTTAG
1101  TTGCCAGCATTGAAGTTGGGCACTCTAAGTTCACTGCCGGTGACAAACCG
      GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCT
1201  ACACACGTAGTGACAATGGCCGGTACAACGGGAAGCGAAGCGGAGATGGA
      GCCAATCTAAGAAAGCCGGTCTCAGTTCGGATTGCAGGCTGCAACTCGCC
1301  TGCATGAAGTCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA
      TACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTACA
1401  ACACCCGAAGTCGGTGGGGTAACCGCAAGAGCCAGCTGCTGAAGTGGGTA
      GATGATTGGGGTGAAGTTGTAACAAGGTAATCTGGAGGTAGAGTTTGATC
      ATGGTTCAGTAAGTCGTAACAAGGTAACC
```

Fig. II-9. Full-length 16S rDNA sequence of S4

```
  1  CGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAACAGAGAAGG
     AGCTTGCTCCTTTGACGTTAGCSGCGGACGGGTGAGTAACACGTGGGCAA
101  CCTACCTTATAGTTTGGGATAACTCCGGGAAACCGGGGCTAATACCGAAT
     AATCTTTTCCTCATGGTGAAATATTGAAAGACGGTTTCGGTGTCGCTATA
201  GGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAG
     GCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGA
301  GACACGGCCCAGACTCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATG
     GGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGATTTCGGTT
401  CGTAAAACTCTGTTGTAAGGGAAGAACAAGTACGTAGTAACTGGCTTACT
     TGACGGTACCTTATTAGAAAGCCACGGCTAATACGTGCCAGCAGCCGCGG
501  TAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGC
     GCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCACGGCTCAACCGTGGAGG
601  GT
```

Fig. II-10. Partial 16S rDNA sequence of S1

```
  1  GCCGATTGAAGTGAGCTTGCTCACGGATTCAGCCGCGCACGGGTGAGTAA
     TGCCTAGGAATCTCCCTGGTAGTGGCGGACAACGTTTCGAAAGGAACGCT
101  AATACCGCATACGTCTACGGGAGAAAGCAGGGGACTTCGCGCCTTGCGCT
     ATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACC
201  AGGCTACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACT
     GAGACACGGTCCAGACTCTACGGGAGGCAGCAGTGGGGAATATTGGACAA
301  TGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGG
     ATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGC
401  AATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAG
     CCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA
501  GCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACC
     TGGGAACTGCATCCAAAACTGGCGAGCTAGAGT
```

Fig. II-11. Partial 16S rDNA sequence of S2

```
  1   GAACCACTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCA
      ATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGA
101   TATGAGCCTGGGAGGCATCTCCCGGGTTGTAAAGCTCCGGCGGTGAAGGA
      TGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCG
201   ACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGAC
      ACGGCCCAGACTCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC
301   GAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCTTCGGGTTGTA
      AACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAG
401   CGCCGGCTAATACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGT
      TGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGA
501   TTGTGAAAGCCGAGGCTTAACCTCGGGTCTG
```

Fig. II-12. Partial 16S rDNA sequence of S14

Table II-1. Secondary screening of algicidal bacteria

| Group | Strain | Hydrophilic | Lipophilic |
|---|---|---|---|
| *Pseudomonas* | U114-1 | - | ++ |
| | U25-3 | - | - |
| | U22-1 | - | + |
| | K44-1 | - | ++ |
| | U38-3 | - | + |
| | K328-3 | - | - |
| | K328-4 | - | - |
| | U330-4 | - | - |
| | K311-1 | - | ++ |
| | S2 | - | - |
| | S9 | - | - |
| | S10 | - | - |
| | S12 | - | - |
| | S13 | - | - |
| | S15 | - | - |
| *Bacillus* | M1 | - | ++ |
| | S1 | - | + |
| | S3 | - | + |
| | S5 | - | + |
| | S6 | - | + |
| | S7 | - | + |
| | S8 | - | + |
| | S11 | - | + |
| *Paenibacillus* | S4 | + | + |
| *Arthrobacter* | U330-1 | - | - |
| | U25-2 | - | - |
| | U929-2 | - | - |
| *Streptomyces* | U1013-1 | - | - |
| | S14 | - | + |
| *Cytophaga-Flavobacterium* group | U38-6 | - | - |

++: active at the concentration of 30 μg/disk against *Anabaena cylindrica* (NIES-19);
+: active at the concentration of 300 μg/disk;
-: no activity.

# CHAPTER III

# Isolation and identification of L-Tyr from *Lysobacter* sp. LB-1

Mitsutani *et al.* isolated several algicidal bacteria classified in the genus *Lysobacter* by the agar double layer method from the surface water of the lake Biwa (Shiga, Japan) in 1985.[8] They reported that the protease produced by *Lysobacter* sp. LB-1 have the lytic activity against the cyanobacterium *Anabaena cylindrica* (NIES-19).[31] However, the antialgal screening for low molecular compound have not been performed yet. In this chapter, isolation and identification of the antialgal compound from *Lysobacter* sp. LB-1 are described.

## 1. Materials and methods

### 1.1 Organisms and culture conditions

The algicidal bacterium *Lysobacter* sp. LB-1 was given by Dr. Mitsutani (Fukuyama University). LB-1 strain was maintained in the 0.5% casitone CB medium at 25 °C. The cyanobacteria, *Anabaena cylindrica* (NIES-19), *A. variabilis* (NIES-23), and *Microcystis aeruginosa* (NIES-88) were obtained from the National Institute for Environmental Science (NIES). *A. cylindrica* (NIES-19) and *A. variabilis* (NIES-23) were cultured in CB medium[44] under illumination of 250 μE /$m^2$s on a 12L:12D cycle. *M. aeruginosa* (NIES-88) were cultured in MA medium[44] under the same conditions described above.

### 1.2 Preparation of crude extract

LB-1 strain was cultured in 5 L jar fermenter containing 0.5% casitone CB medium. After 4-5 days, the bacterial cells were harvested by continuous centrifugation at 10000 rpm. The yield of the lyophilized cells was 1.5 g from 5 L of culture. The harvested cells were stored at -20 °C until extraction. Freeze-dried bacterial cells was extracted with 80% MeOH (2 L × 2) and MeOH (2 L). The combined 80% MeOH and MeOH extracts was partitioned between $H_2O$ and $Et_2O$. The $H_2O$ and $Et_2O$ fractions were subjected to the antialgal assay against *A. cylindrica* (NIES-19) using the paper disk method.

## 1.3 Isolation of L-Tyr

The $H_2O$ fraction which showed the antialgal activity was partitioned between *n*-BuOH and $H_2O$. The $H_2O$ fraction was subjected to ODS column chromatography (YMC-ODS AM 120A, 10 × 10 cm), and eluted with $H_2O$, 20%MeOH, 40% MeOH, 60% MeOH, MeOH and $CH_2Cl_2$. The $H_2O$ fraction which showed the antialgal activity was subjected to preparative HPLC (COSMOSIL C18MS, 10 × 250 mm, eluted with $H_2O$, detector set at 210 nm) to obtain 2.1 mg of the antialgal compound, L-Tyr.

## 1.4 Amino acid analysis

L-Tyr isolated from LB-1 strain was redissolved in 0.02 N HCl to subject to the amino acid analyzer. The amino acid analysis was carried out with a Hitachi L-8500A amino acid analyzer. Retention time of amino acid (min): Tyr (48.66).

## 1.5 HPLC analysis of Marfey derivative

To 500 μg of Tyr which was isolated from LB-1 strain, 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA) in acetone (10 mg/mL) and 100

μL of 1 M $NaHCO_3$ were added, and the reaction mixture was kept at 80 °C for 3 min.[59] To the reaction mixture, 50 μL of 2N HCl and 300 μL of 50% MeCN were added and the reaction mixture was analyzed by reversed-phase ODS-HPLC: column Cosmosil 5C18MS (4.6 × 250 mm); gradient elution from $H_2O$/TFA (100:0.1) to MeCN/$H_2O$/TFA (60:40:0.1) in 60 min; UV detection 340 nm; flow rate 1.0 mL/min. Retention times of amino acids (min): L-Tyr (57.8), D-Tyr (60.8).

## 2. Results

### 2.1 Isolation and identification of L-Tyr

LB-1 strain was cultured in 5 L jar fermenter containing 0.5% casitone CB medium. After 4-5 days, the bacterial cells were harvested by continuous centrifugation. The lyophilized cells were extracted with MeOH. The MeOH extracts were partitioned between EtOAc and $H_2O$. The $H_2O$ layer which showed potent antialgal activity was subjected to ODS column chromatography followed by reversed-phase HPLC to yield L-Tyr (2.1 mg). The identification was performed with the amino acid analyzer. The retention time of the active compound from LB-1 strain corresponds with the retention time of the standard Tyr. The stereochemistry of Tyr isolated from LB-1 strain was determined by Marfey's method as L-form.[53]

### 2.2 Antialgal activity of L-Tyr

L-Tyr isolated from LB-1 strain showed the antialgal activities against *A. cylindrica* (NIES-19), *A. variavilis* (NIES-23), and *M. aeruginosa* (NIES-88) at the concentration of 30 μg/disk by using the paper disk method.

## 3. Discussion

So far, it has been reported that several amino acids have the antialgal activities against cyanobacteria. In particular, Yamamoto *et al.* reported L-Lys and L-Arg which were isolated from the mycelia of *Streptomyces phaeofaciens* have the antialgal activities against the cyanobacterium *M. aeruginosa.*[11] It is well known that the incorporation of basic amino acid into cyanobacterial cells occur at low inoculated concentration and the toxicity was caused at high inoculated concentration.[60,61] On the other hand, the aromatic amino acids were also reported to have the antialgal activities. [62,63]

Jensen *et al.* reported that the inoculation of L-Tyr and L-Trp inhibited the growth of *Anabaena cylindrica* at the concentration of 2 mM and reduced the nitrogenase activity.[62] Amino acid toxicity in bacterial autotroph has generally been attributed to imbalance of the metabolism. Hall *et al.* showed that 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase, the first step unique to the aromatic amino acid pathway, was inhibited in the presence of 0.5 mM Phe in *Anabaena* sp. Inhibition by L-Tyr was much less at 9%. Also a 58% inhibition of shikiminate/NADP dehydrogenase was caused by a mixture of L-Phe, L-Tyr, L-Trp, each at 0.5 mM.[63] The toxicities of L-Tyr against cyanobacteria can explain by the reductions in DAHP synthase and sikiminate dehydrogenase activities. In all cases, inhibitory effects were seen only at high concentration.

The algal lysis of LB-1 strain was reported to require for direct attachment. In the present study, L-Tyr was isolated as the antialgal compound from the culture of LB-1 strain. However it is not clear that L-Tyr involved actually the lytic stage in the mixed culture. Mitsutani *et al.* reported that the protease produced by LB-1 strain have the algal lytic activity against *A. cylindrica.* It was thought that enzymes such as protease and low molecular algicide may act compositely at lytic stage. However there are a few information about these lytic mode, further work is required for determination of the complete mechanism of lysis.

# CHAPTER IV

# Isolation and structure elucidation of harmane from *Pseudomonas* sp. K44-1

In chapter II, the lipophilic fraction of *Pseudomonas* sp. K44-1 showed the potent antialgal activity against the cyanobacterium *Anabaena cylindrica* (NIES-19). In this chapter, the isolation and identification of the antialgal compound from the whole broth of *Pseudomonas* sp. K44-1 was described.

## 1. Materials and methods

### 1.1 Organisms and culture conditions

The cyanobacteria, *A. cylindrica* (NIES-19), *A. variabilis* (NIES-23), *Oscillatoria agardhii* (NIES-506), *Microcystis aeruginosa* (NIES-299) and *M. viridis* (NIES-102), and the green algae, *Chlorella vulgaris* (NIES-227) and *Chlamydomonas tetragama* (NIES-446), were obtained from the National Institute for Environmental Science (NIES). The cyanobacterium *Anacystis marina* (IAM-122) was obtained from the Institute of Molecular and Cellular Biosciences, the University of Tokyo (formerly, Institute of Applied Microbiology, IAM). *A. cylindrica* (NIES-19), *O. agardhii* (NIES-506), and *A. marina* (IAM-122) were cultured in CB medium[44] under illumination of 250 $\mu E/m^2s$ on a 12L:12D cycle. *M. aeruginosa* (NIES-299) and *M. viridis* (NIES-102) were cultured in MA medium[44] under the same conditions described above. The green algae, *C. vulgaris* (NIES-227) and *C. tetragama* (NIES-446), were cultured in C medium[44] under the same conditions as described above.

### 1.2 Cultivation of K44-1 strain and isolation of harmane

K44-1 strain was cultured in 5 L glass flasks containing 2 L of 0.5% casitone CB medium using a rotary shaker at 30 °C for 4-5 days. The whole culture broth was ultrasonicated and extracted with EtOAc. Then the EtOAc layer was evaporated and stored at -20 °C until the next procedure. The EtOAc extract (1.52 g from 20 L culture) was subjected to silica gel chromatography and eluted with $CH_2Cl_2$, $CH_2Cl_2$-MeOH (9:1), $CH_2Cl_2$-MeOH (8:2), and $CH_2Cl_2$-MeOH (6:4). The $CH_2Cl_2$-MeOH (9:1) fraction (102 mg) showed the antialgal activity, and was subjected to preparative silica gel TLC (Kieselgel $60F_{254}$, 20 × 20 cm, Merck) using $CH_2Cl_2$-MeOH (8:2) as solvent. The active fraction (Rf 0.68, 5.2 mg) was subjected to preparative HPLC (Inertsil ODS-3, GL Sciences Inc., 10 × 250 mm, flow rate 2.0 mL/min, 35% MeOH, UV detector set at 240 nm) to obtain 1.5 mg of the antialgal compound, harmane.

### 1.3 General instrumentation

NMR spectra were recorded on a JEOL NM-A600 NMR spectrometer operating at 600 MHz using pyridine-$d_5$ as solvent at 27 °C. The resonance of residual pyridine-$d_5$ at $\delta_H$ 7.19 was used as internal reference for $^1H$ NMR spectra. FAB mass spectra were recorded by a JEOL JMS SX-102 mass spectrometer. The optical rotation was determined on a JASCO DIP-1000 digital polarimeter in MeOH. The UV spectrum was recorded on a SHIMADZU UV-VIS 1240 spectrophotometer.

## 2. Results

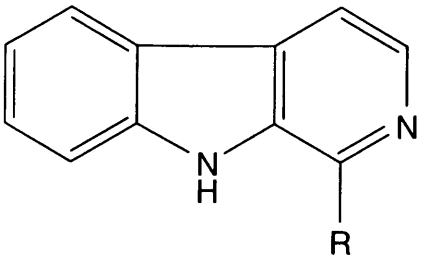
### 2.1. Isolation of harmane

K44-1 strain was cultured in 5 L glass flasks containing 2 L of 0.5%

casitone CB medium with rotary shaker. The whole culture broth was ultrasonicated and extracted with EtOAc. The EtOAc extract was subjected to the silica gel chromatography and eluted sequentially with $CH_2Cl_2$, $CH_2Cl_2$-MeOH (9:1), $CH_2Cl_2$-MeOH (8:2), and $CH_2Cl_2$-MeOH (6:4). The $CH_2Cl_2$-MeOH (9:1) fraction showed the potent antialgal activity against *A. cylindrica* (NIES-19). The bioautography with the silica gel TLC using $CH_2Cl_2$-MeOH (8:2) as solvent indicated that the spot (Rf value, 0.68) have the antialgal activity. The $CH_2Cl_2$-MeOH (9:1) fraction was subjected to the preparative silica gel TLC using $CH_2Cl_2$-MeOH (8:2) as solvent. Then the active fraction was subjected to the HPLC chromatography to obtain 1.5 mg of the antialgal compound, harmane (**1**, Fig. IV-1).[64,65]

## 2.2 Structure elucidation of harmane

Harmane (**1**) was isolated as amorphous powder: $[\alpha]_D$ + 2.6° (*c* 0.1, methanol); UV absorption in methanol at $\lambda_{max}$ 347 nm ($\varepsilon$ 3840), 287 nm ($\varepsilon$ 11070), and 237 nm ($\varepsilon$ 19580). The positive FABMS using glycerol as matrix showed $[M+H]^+$ ion at m/z 183 (Fig. IV-2). The molecular formula of **1** was determined as $C_{12}H_{10}N_2$ by the HRFABMS *m/z* 183.0903 $[M+H]^+$ calcd for $C_{12}H_{11}N_2$ (183.0922). The $^1H$ NMR (pyridine-$d_5$) spectrum of **1** showed 6 aromatic protons and one methyl group at $\delta_H$ 2.98 (Fig. IV-3, Table IV-1). The $^1H$-$^1H$ COSY spectrum revealed two spin-coupled networks (Fig. IV-1,4). The signals of protons H-3 and H-4 constituted an isolated vicinal pair, and the signals of protons H-5, H-6, H- 7, and H-8 constituted an adjacent four proton system. The extensive analyses of HMBC[66] and HMQC spectra[67] indicated the structure of 1-methyl-β-carboline (Fig. IV-5,6). The identity with harmane was confirmed by the comparison with literature data of the chemical shifts (Table IV-1) and ultraviolet spectra.[64,65]




Harmane (**1**) R=$CH_3$
Norharmane (**2**) R=H

## 2.3 Antialgal activities of harmane and norharmane

Harmane (**1**) and authentic norharmane (**2**, β-carboline) were subjected to the antialgal assay against the several strains of the cyanobacteria and the green algae using the paper disk method. Table IV-2 shows the results of the assay. Harmane (**1**) and norharmane (**2**) showed the antialgal activities against the cyanobacteria containing *A. cylindrica* (NIES-19), *A. variabilis* (NIES-23), *O. agardhii* (NIES-506), *A. marina* (IAM-122), *M. aeruginosa* (NIES-299), and *M. viridis* (NIES-102) at the concentration of 30 μg/disk. However, both did not show the activities at the concentration of 30 μg/disk against the green algae, *C. vulgaris* (NIES-227) and *C. tetragama* (NIES-446).

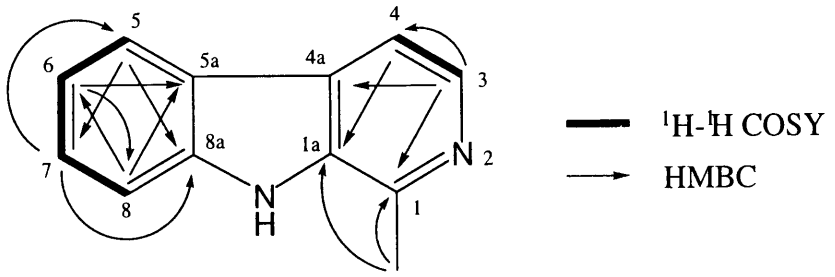
## 3. Discussion

The genus *Pseudomonas* was reported to be present in the eutrophic lake, and accounted for the great part of strains showing lytic activity against cyanobacteria.[21] The antialgal compound, harmane, was isolated from the whole culture broth of *Pseudomonas* sp. K44-1. However, the specific activity did not increase with each isolation procedure. Yamamoto *et al.* reported that volatile antialgal agents in the culture medium of *Streptomyces phaeofaciens* were lost through the fractionation procedure.[11] The crude extract of K44-1 strain also may contain volatile agents or other antialgal substances which are unstable.

Harmane and the related compounds occur naturally at high concentrations in plants such as *Peganum harmala* , *Banisteria caapi* and *Tribulus terrestris*.[68,69]

So far, the harmane have been reported from a widespread taxonomic distribution. Takeuchi *et al.* reported the isolation of harmane from the mushroom, *Criolus pogonomyces*.[65] Recently, the isolation from the gliding bacteria *Myxobacter* was reported.[70] To our best knowledge, this study is the first isolation of harmane from the genus *Pseudomonas*.

Harmane and norharmane showed the antialgal activity against the bloom-forming cyanobacteria such as *M. aeruginosa* and *M. viridis* and may become the useful agents for removing water bloom. In addition, harmane and norharmane did not show the antialgal activity against the green algae showed in Table IV-2. This data enhanced the potential of harmane in environmental usage from the ecological point of view. In conclusion, the present study indicates that *Pseudomonas* sp. K44-1 which produce the antialgal compound, harmane, is a useful algicidal agent. Further work is needed to the application in lakes and reservoirs.




**1**

Fig. IV-1. $^{1}H$-$^{1}H$-COSY and HMBC correlations of harmane

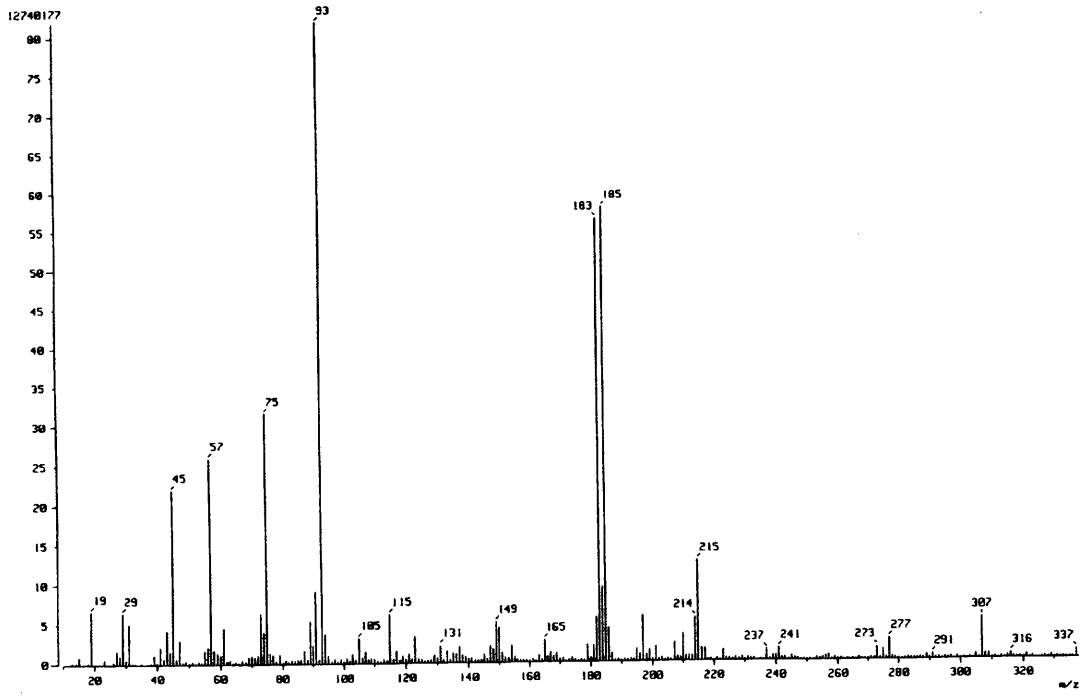



Fig. IV-2. Positive FABMS spectrum of harmane (matrix; glycerol)

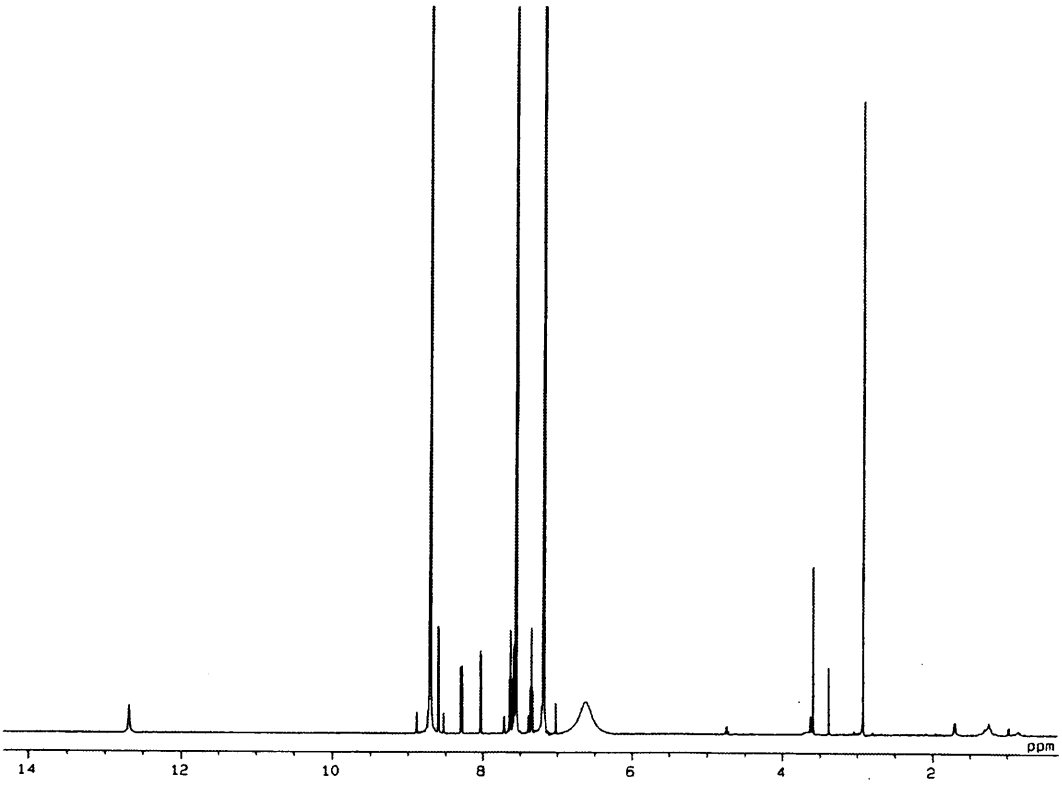



Fig. IV-3. $^{1}$H NMR spectrum of harmane (pyridine-$d_5$)

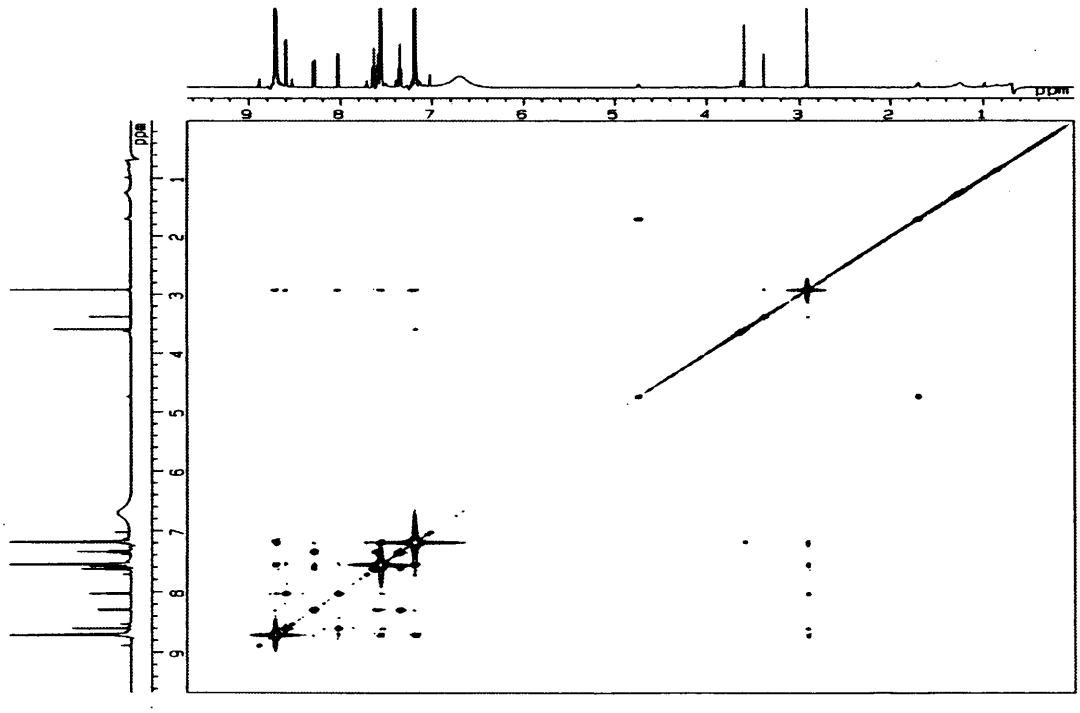



Fig. IV-4. $^{1}$H-$^{1}$H COSY NMR spectrum of harmane (pyridine-$d_5$)

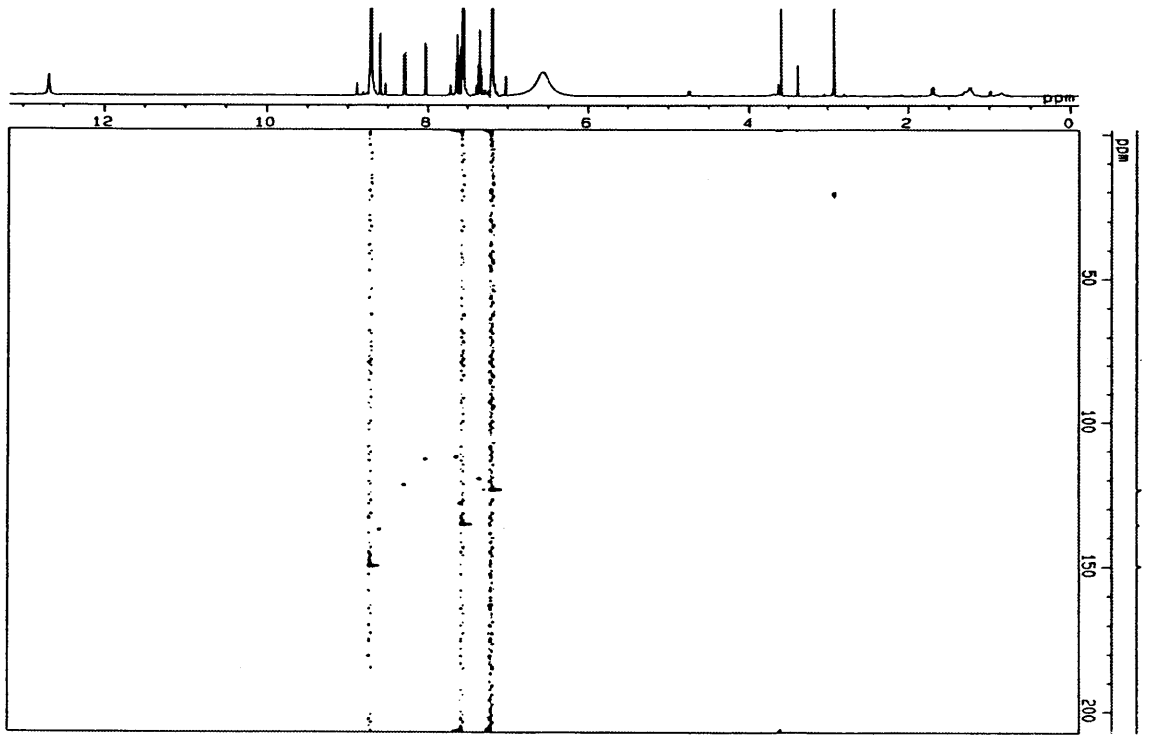



Fig. IV-5. HMQC NMR spectrum of harmane (pyridine-$d_5$)

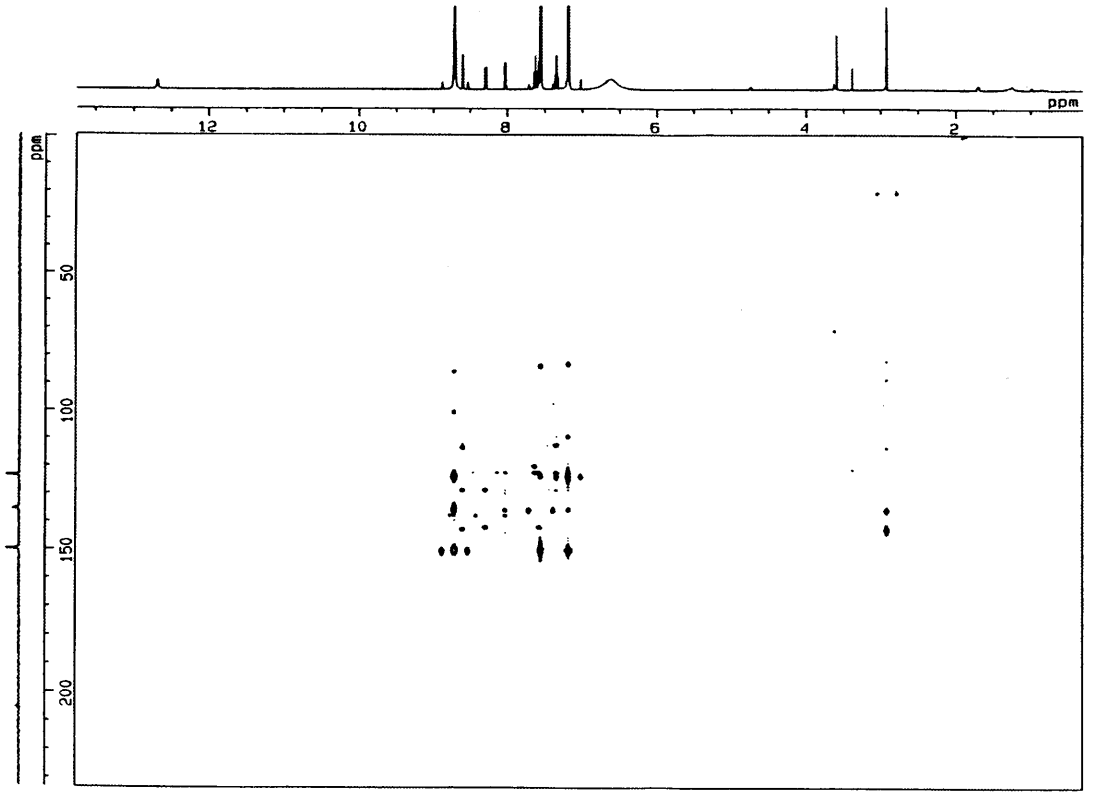



Fig. IV-6. HMBC NMR spectrum of harmane (pyridine-$d_5$)

Table IV-1. $^{1}H$ and $^{13}C$ NMR Data for harmane (**1**) in pyridine-$d_5$

| Position | $^{1}H$ | $J$ (Hz) | $^{13}C$ |
|---|---|---|---|
| 1 | | | 142.6 (s) |
| 1a | | | 135.9 (s) |
| 2 | | | |
| 3 | 8.60 | (d, 5.5) | 137.3 (d) |
| 4 | 8.05 | (d, 5.5) | 113.0 (d) |
| 4a | | | 128.5 (s) |
| 5 | 8.30 | (d, 8.1) | 122.0 (d) |
| 6 | 7.35 | (t, 8.1) | 122.2 (s) |
| 7 | 7.58 | (m) | 119.8 (d) |
| 8 | 7.63 | (d, 8.4) | 128.2 (d) |
| 8a | | | 112.2 (d) |
| 9 | | | 142.0 (s) |
| 1-Me | 2.98 | (s) | 20.2 (q) |

Table IV-2. Spectrum of the antialgal activities of harmane and norharmane

| Species | Harmane | Norharmane |
|---|---|---|
| Cyanobacteria | | |
| *Anabaena cylindrica* (NIES-19) | ++ | ++ |
| *Anabaena variabilis* (NIES-23) | + | + |
| *Anacystis marina* (IAM-122) | + | + |
| *Oscillatoria agardhii* (NIES-506) | + | + |
| *Microcystis aeruginosa* (NIES-299) | + | + |
| *Microcystis viridis* (NIES-102) | + | + |
| Green algae | | |
| *Chlorella vulgaris* (NIES-227) | - | - |
| *Chlamydomonas tetragama* (NIES-446) | - | - |

at the concentration of 30 μg/disk
++: > 5 mm of diameter of plaque
+: < 5 mm
-: no activity.

# CHAPTER V

# Isolation and structure elucidation of surfactin from *Bacillus* sp. M1

In chapter II, the lipophilic fraction of *Bacillus* sp. M1 showed the potent antialgal activity against the cyanobacterium *Anabaena cylindrica* (NIES-19). In this chapter, the algicidal activity of M1 strain was confirmed by the plaque-forming assay and mixed culture experiment. The isolation and identification of the antialgal compound from the whole broth of M1 strain was described.

## 1. Materials and methods

### 1.1 Organisms and culture conditions

The organisms and culture conditions used in this chapter were the same as described in chapter IV.

### 1.2 Plaque-forming activity of M1 strain

M1 strain was grown on Waksman agar medium for 2 days at 25 °C. The small amount of one colony was inoculated on the CB agar medium containing *A. cylindrica* (NIES-19) and plaque-forming activity was visually observed every day.

### 1.3 Mixed culture experiment

*Bacillus* sp. M1 strain was cultured in Waksman liquid medium for 24 h at 25 °C. The culture of M1 strain was centrifuged, and the cells were washed twice

with CB medium. The cells of M1 strain were diluted with CB medium, then the number of cells was counted under microscopic observation with blood corpuscles counting chamber (Fuchs-Rosenthal). The cells of M1 strain were inoculated at the concentration of $10^4$, $10^5$, $10^6$, and $10^7$ cells/mL into 5 ml of aqueous *A. cylindrica* culture which was precultured for 12 days. The 500 μL of mixed culture was sampled at 0, 3, 6, 9, and 12 days after inoculation. After lyophilization, each sample was extracted with 500 μL of MeOH. Each extract was centrifuged at 3000 rpm for 5 min. The 200 μL of supernatant was poured into a 96-well microplate and measured the optical density at 665 nm. All culture experiments were performed in triplicate at each inoculated concentration.

## 1.4 Preparation of crude extracts

M1 strain was cultured in 2 L glass flasks containing Waksman medium with rotary shaker. After 4-5 days, the bacterial cells were harvested by continuous centrifugation at 10000 rpm. The yield of the lyophilized cells was 9.8 g from 10 L of culture. The harvested cells were stored at -20 °C until extraction. Freeze-dried bacterial cells (9.8 g) were extracted with 80% MeOH (2 L × 3) and MeOH (2 L).

## 1.5 Isolation of surfactin

The combined 80% MeOH and MeOH extracts were concentrated to an aqueous suspension, and this was extracted with EtOAc. The EtOAc layer (5.2 g) was subjected to ODS flash chromatography (YMC-GEL, 5 × 10 cm), eluted with 20% MeOH, 50% MeOH, 70% MeOH, and MeOH. The MeOH fraction which showed the potent antialgal activity was subjected to reversed-phase HPLC (Cosmosil 5C18MS, 10 × 250 mm; 98% MeCN containing 0.05% TFA; flow rate, 2.0 mL/min; UV detection at 210 nm) to yield 22.8 mg of surfactin.

## 1.6 Amino acid analysis

Surfactin (1 mg) was hydrolyzed with 0.5 mL of 6N HCl at 105 °C for 24 h. The solution was evaporated in a stream of dry nitrogen with heating and redissolved in 0.02 N HCl to subject to the amino acid analyzer. Retention times of amino acids (min); Asp (10.6), Glu (21.0), Val (39.7), Leu (46.9).

## 1.7 HPLC analysis of Marfey derivative

The hydrolyzate of surfactin was treated with 10 % $Me_2CO$ solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (L-FDAA, Marfey's reagent) in 1M $NaHCO_3$ at 80-90 °C for 3 min followed by neutralization with 2N HCl. The reaction mixture was dissolved in 50% MeCN and subjected to reversed-phase HPLC: column, Cosmosil MS (4.6 × 250 mm), gradient elution from $H_2O$/TFA (100:0.1) to MeCN/$H_2O$/TFA (60:40:0.1) in 60 min, UV detector (340 nm). Retention times of amino acids (min); L-Asp (37.2), D-Asp (38.0), L-Glu (38.8), D-Glu (40.0), L-Val (47.0), D-Val (51.2), L-Leu (51.6), D-Leu (55.6).

## 1.8 General instrumentation

NMR spectra were recorded on a JEOL NM-A600 NMR spectrometer operating at 600 MHz using DMSO-$d_6$ containing 0.5% TFA as solvent at 27 °C. The resonance of residual DMSO-$d_6$ at $\delta_H$ 2.79 was used as internal reference for $^1H$ NMR spectra. FAB mass spectra were recorded by a JEOL JMS SX-102 mass spectrometer. The amino acid analysis was carried out with a Hitachi L-8500A amino acid analyzer. The optical rotation was determined on a JASCO DIP-1000 digital polarimeter in MeOH. The UV spectrum was recorded on a SHIMADZU UV-VIS 1240 spectrophotometer.

## 2. Results

### 2.1 Lytic activity of M1 strain

M1 strain have potent plaque-forming activity on the algal grown agar plate (Fig. V-1). After 7 days from inoculation, the diameter of plaque reached to about 5 cm. To determine the lytic efficiency in liquid culture, *A. cylindrica* was cocultured with M1 strain at several inoculated concentrations. Fig. V-2 showed the result of mixed culture. M1 strain lysed *A. cylindrica* effectively at a concentration of $10^5$ to $10^7$ cells/mL from 6 days after inoculation. The lysis of *A. cylindrica* was observed by the light microscopy at inoculated concentrations of $10^5$ to $10^7$ cells/mL. However, there are no effect on algal culture at a concentration of $10^4$ cells/mL.

### 2.2 Isolation of surfactin

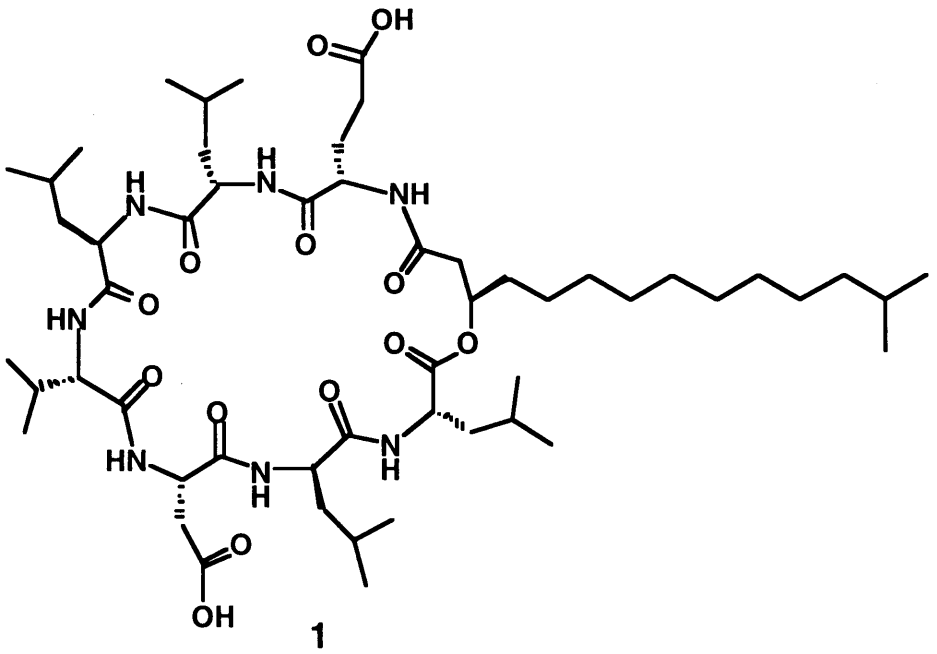
M1 strain was cultured in 2 L glass flasks containing Waksman medium with rotary shaker. After 4-5 days, the bacterial cells were harvested by continuous centrifugation. The lyophilized cells were extracted with MeOH. The MeOH extract was partitioned between EtOAc and $H_2O$. The EtOAc layer which showed potent antialgal activity was subjected to ODS column chromatography followed by reversed-phase HPLC to yield the antialgal compound, surfactin (**1**, Fig. V-3, 22.8 mg).

### 2.3 Structure elucidation of surfactin

Surfactin (**1**) was isolated as amorphous powder: $[\alpha]_D$ -23.9° (*c* 0.1, MeOH); UV absorption spectrum showed the absence of absorption maximum in the range from 230 nm to 400 nm. The positive FABMS using glycerol as matrix

showed $[M+H]^+$ ion at m/z 1036 (Fig. IV-4). The molecular formula of **1** was determined as $C_{53}H_{93}N_7O_{13}$ by the HRFABMS *m/z* 1036.6851 $[M+H]^+$ calcd for $C_{53}H_{94}N_7O_{13}$ (1036.6910). The amino acid analysis of the hydrolyzate gave 4 mole of Leu, 1 mol of Glu, and 1 mol of Asp. The $^1H$ NMR spectrum of **1** did not give sharp signals when DMSO-$d_6$ was used as the solvent. We found that the addition of a small amount of trifluoroacetic acid to the DMSO-$d_6$ solution afforded relatively sharp NMR signals, which allowed us to make the following structural elucidation (Fig. V-5). The extensive NMR analyses of **1** including $^1H$-$^1H$ COSY, HMQC[60], and HMBC[61] spectra indicated the presence of β-hydroxy fatty acid moiety (Fig. V-6~8). The sequences of each unit were determined by HMBC and NOESY correlations (Fig. V-8,9) as shown in Fig. V-3. Neither HMBC nor NOESY correlation between Leu (1) and (2), and between Leu (3) and (4) was observed, however the identity with surfactin was confirmed by the comparison with literature data of the chemical shifts (Table V-1).[71,72]

The stereochemistry of **1** was confirmed by Marfey's Method. As a result, L-Asp, L-Glu, L-Val, L-Leu, and D-Leu were detected at the ratio of 1:1:1:2:2 by HPLC analysis. The composition of these amino acids corresponds with literature data.[71]




**1**

### 2.4 Antialgal activity of surfactin

Surfactin (**1**) was subjected to antialgal assay against several strains of cyanobacteria and green algae using the paper disk method. The result was summarized in Table V-2. Surfactin (**1**) showed antialgal activities against the cyanobacteria *A. cylindrica* (NIES-19), *A. variabilis* (NIES-23), *O. agardhii* (NIES-506), *A. marina* (IAM-122), *M. aeruginosa* (NIES-299), and *M. viridis* (NIES-102) at a concentration of 30 μg/disk. However, **1** did not show activities at a concentration of 30 μg/disk against the green algae, *C. vulgaris* (NIES-227) and *C. tetragama* (NIES-446).

## 3. Discussion

Reim *et al.* reported that the lytic activity was found in the culture filtrate of *Bacillus brevis*.[43] They deduced that the lytic compounds produced by *B. brevis* were antibiotics such as gramicidin S and tyrocidine. However, the identification of the lytic compound was not accomplished. In the present study, we isolated the algicidal bacterium, *Bacillus* sp. M1, from the eutrophic pond and identified the antialgal compound as surfactin. Surfactin showed the antialgal activities against wide range of water bloom-forming cyanobacteria, but not against green algae. Surfactin is a type of biosurfactant and exhibits some antibiotic ability presumably as the result of interactions with lipid membrane.[73] On the other hand, it is well known that the structural appearance of cyanobacterial cell membrane is similar to that of Gram-negative bacteria, and they have the lipid bilayer outside the peptidoglycan.[74,75] Considering the effect of surfactin to the outer membrane, the specific activity against cyanobacteria is reasonable.[76]

M1 strain showed potent plaque-forming activity on the algal agar plate,

and lytic activity against *A. cylindrica* in liquid mixed culture. These activities showed that M1 strain would be the useful agent for the algal control. It is well known that the bacteria classified in the genus *Bacillus* are commonly presented in any places such as soils, lakes, and rivers.[57,58] This point is the advantage to the application in eutrophic lakes from the ecological point of view. So far, the gliding bacteria, mainly members of the *Myxobacteria* and *Cytophaga* groups, have been reported to lyse the cyanobacteria effectively.[16] The present study indicated that the genus *Bacillus,* which produces the antibiotic such as surfactin, is also thought to become the useful agent for algal control. However there are a little information about the algicidal bacteria classified in the genus *Bacillus*, further work is needed for determination of the algicidal efficiency in the eutrophic lakes.

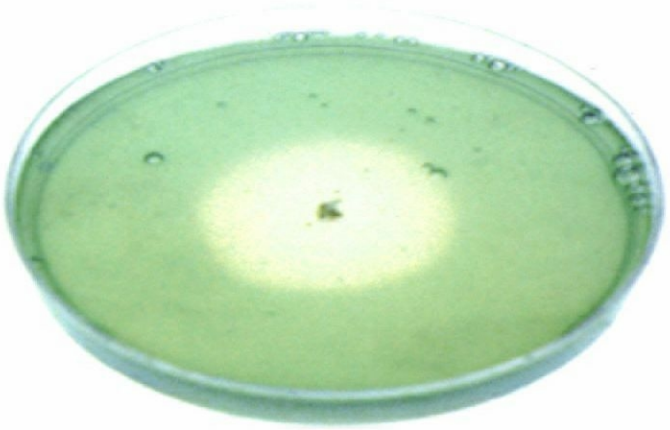


Fig. V-1. Plaque-forming activity of M1 strain

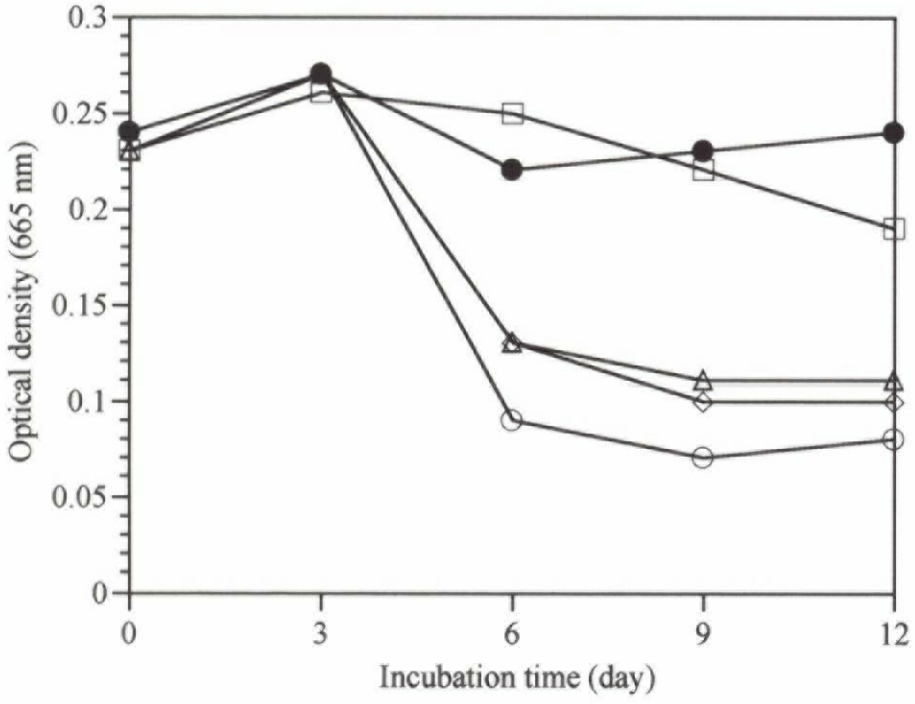



Fig. V-2. Lytic activity of M1 strain against *A. cylindrica* (NIES-19) at several inoculated concentration.; closed circle, no inoculated; open circle, $10^7$ cells/mL; open rhombus, $10^6$ cells/mL; open triangle, $10^5$ cells/mL ;open square, $10^4$ cells/mL

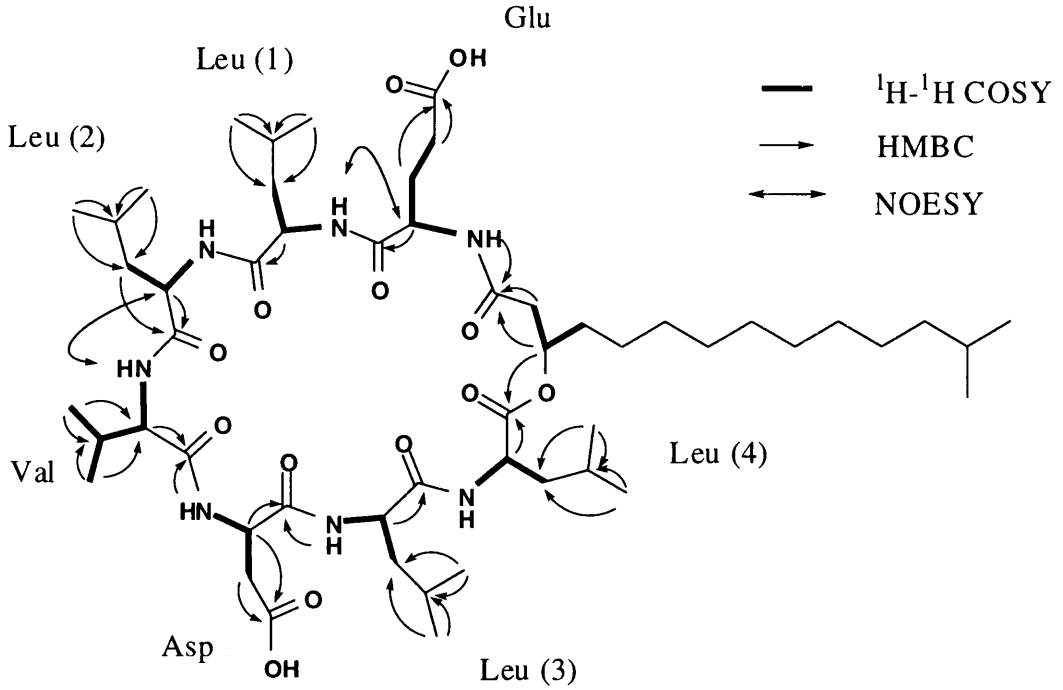



Fig. V-3. $^1$H-$^1$H COSY, NOESY, and HMBC correlations of surfactin

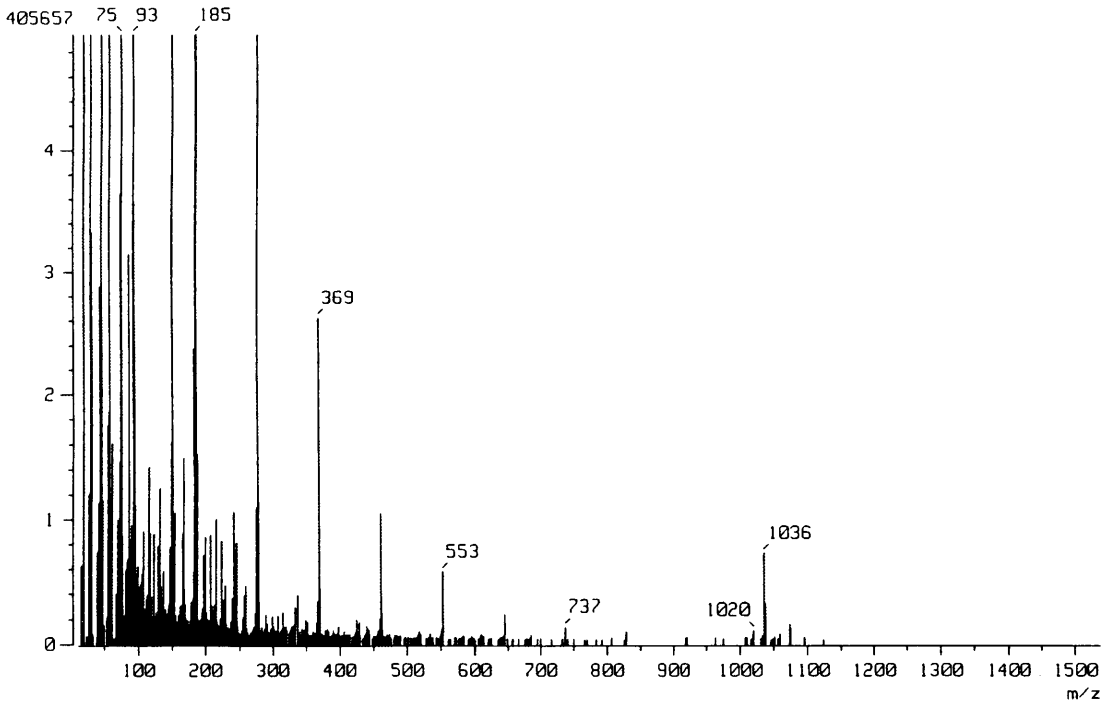



Fig. V-4. Positive FABMS spectrum of surfactin (matrix; glycerol)

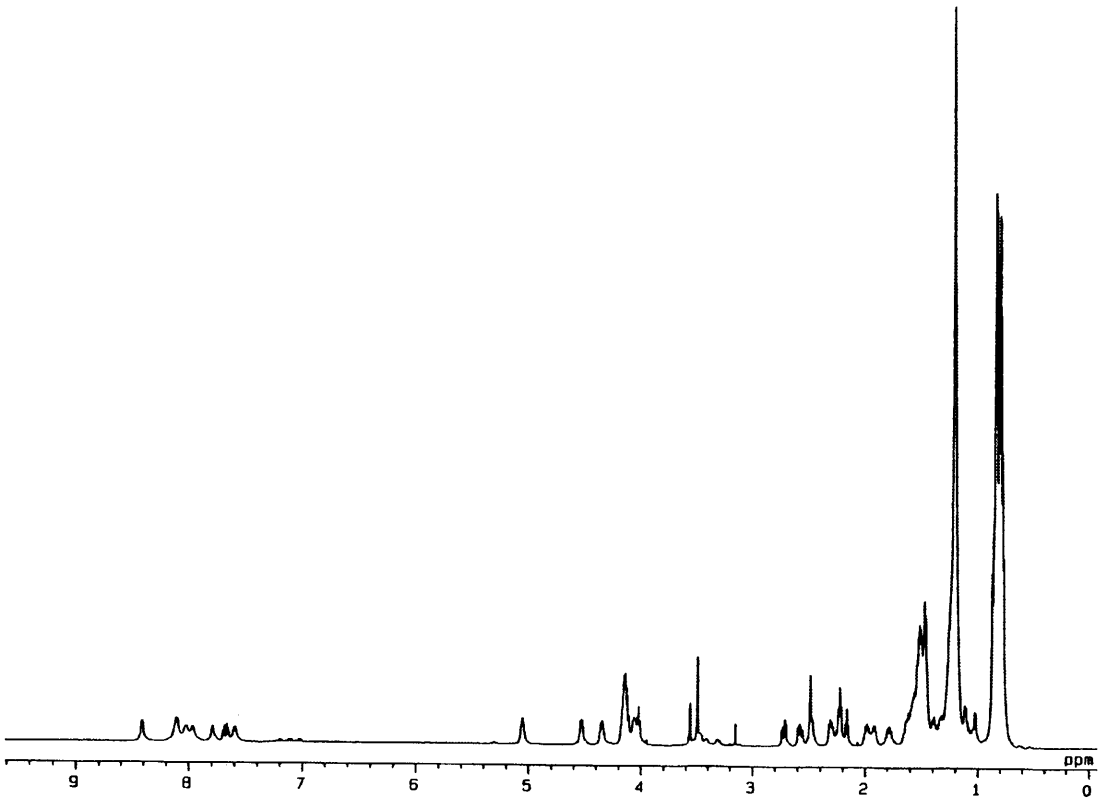



Fig. V-5. $^{1}H$ NMR spectrum of surfactin (DMSO-$d_6$ containing 0.5% TFA)

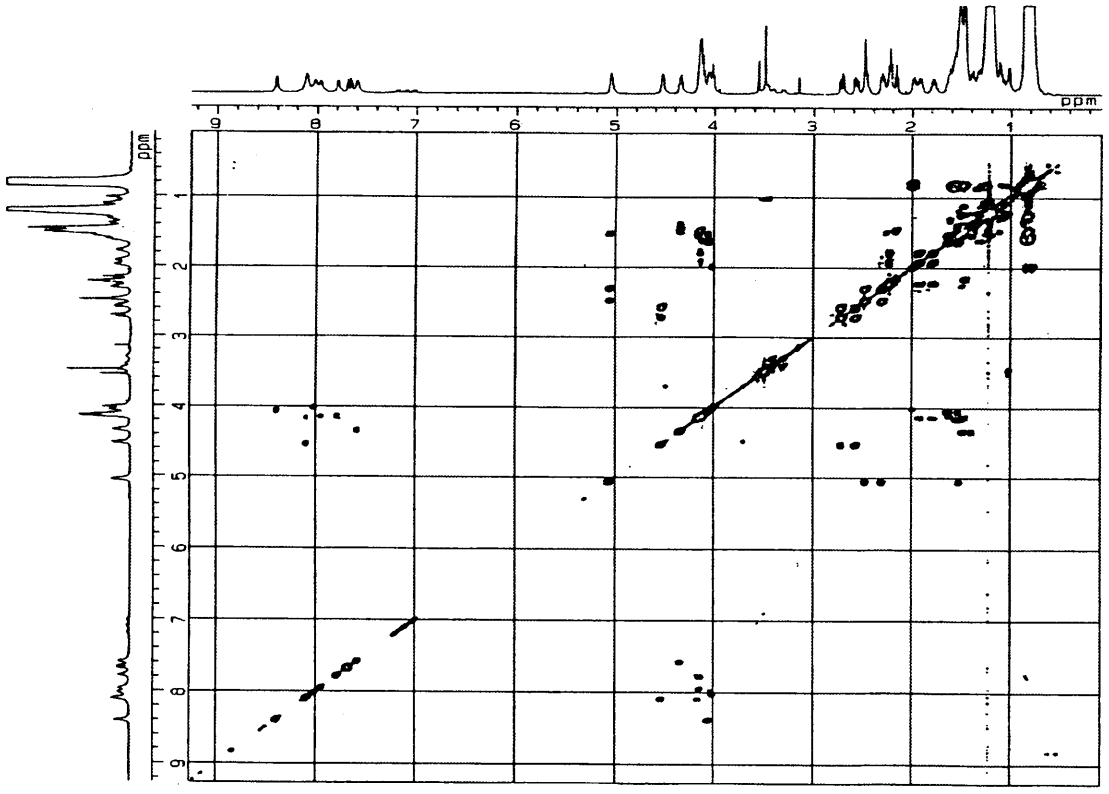



Fig. V-6. $^{1}H$-$^{1}H$ COSY NMR spectrum of surfactin (DMSO-$d_6$ containing 0.5% TFA)

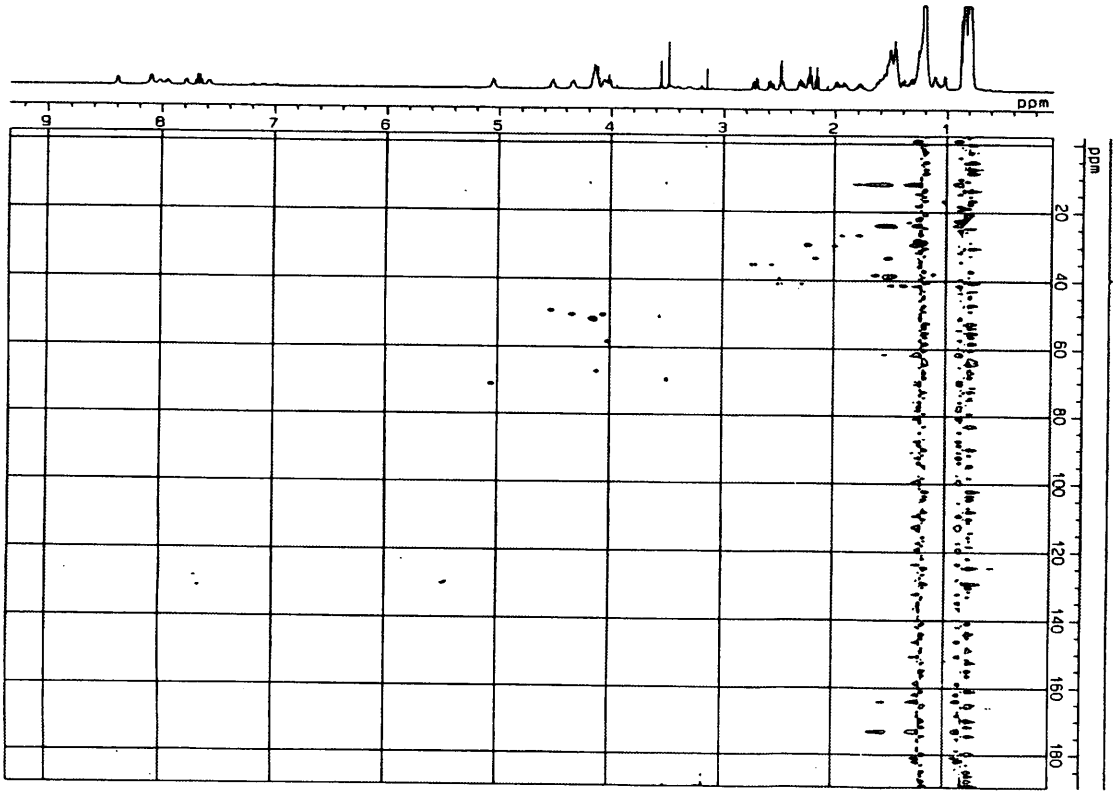



Fig. V-7. HMQC NMR spectrum of surfactin (DMSO-$d_6$ containing 0.5% TFA)

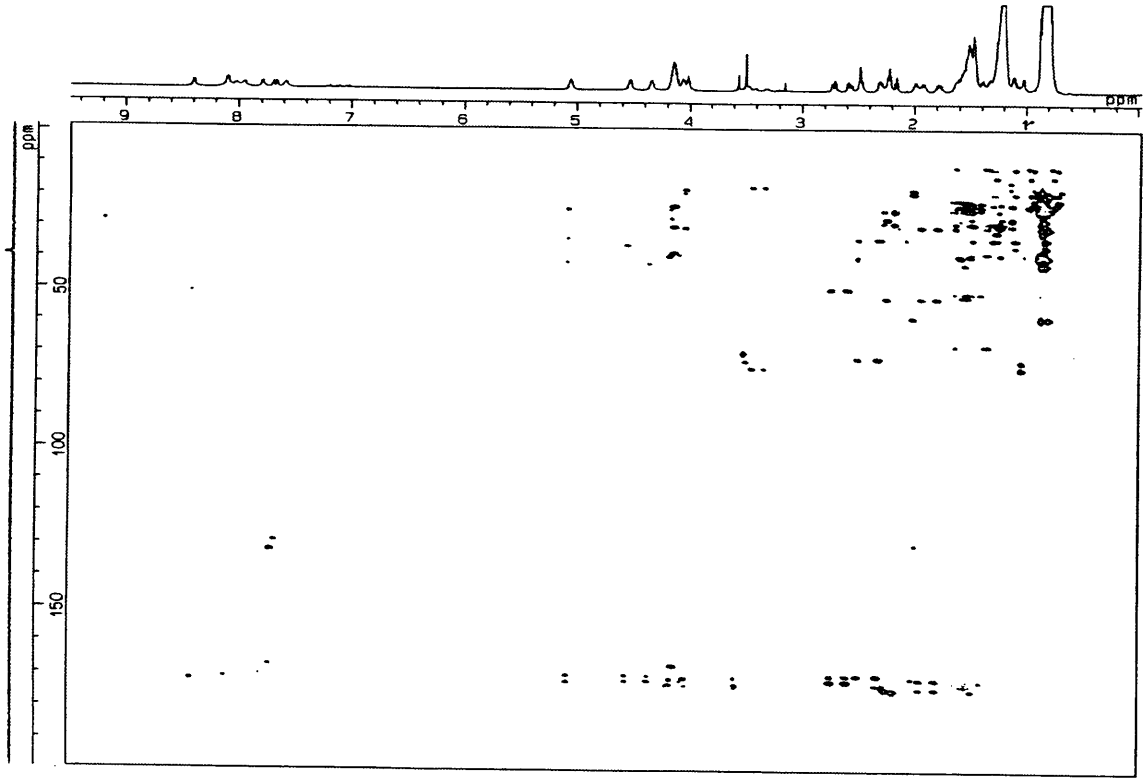



Fig. V-8. HMBC NMR spectrum of surfactin (DMSO-$d_6$ containing 0.5% TFA)

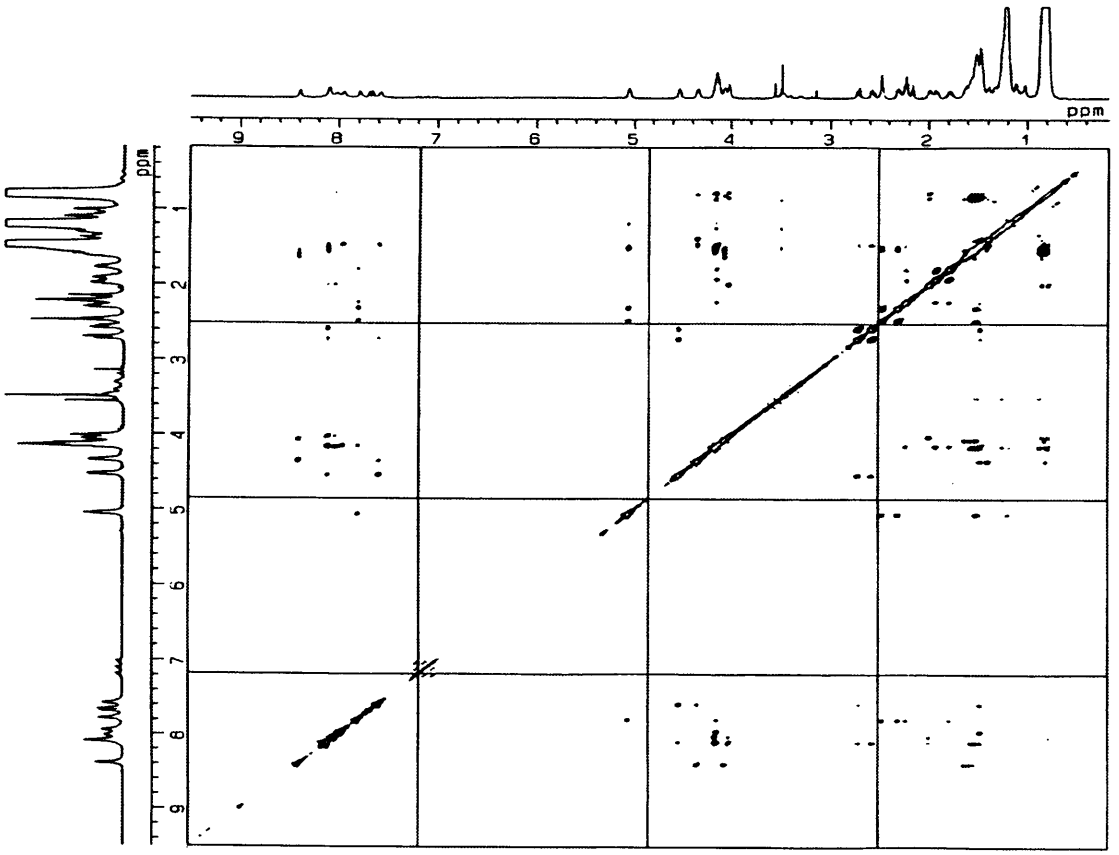



Fig. V-9. NOESY NMR spectrum of surfactin (DMSO-$d_6$ containing 0.5% TFA)

Table V-1. $^{1}H$ and $^{13}C$ NMR spectral data of surfactin in DMSO-$d_6$ containing 0.5% TFA

| Position | | $^{1}H$ | *J* (Hz) | $^{13}C$ | Position | | $^{1}H$ | *J* (Hz) | $^{13}C$ |
|---|---|---|---|---|---|---|---|---|---|
| Glu | 1 | | | 170.9 (s) | Leu (3) | 1 | | | 171.6 (s) |
| | 2 | 4.14 | m | 52.3 (d) | | 2 | 4.34 | m | 50.4 (d) |
| | 3 | 1.79 | m | 26.9 (t) | | 3 | 1.38 | m | 41.2 (t) |
| | | 1.93 | m | | | | 1.47 | m | |
| | 4 | 2.23 | dd, 8.6, 6.7 | 29.5 (t) | | 4 | *a | | |
| | 5 | | | 173.9 (s) | | 5 | *b | | |
| | NH | 7.80 | br | | | 6 | *a | | |
| Leu (1) | 1 | | | 170.0 (s) | | NH | 7.61 | br | |
| | 2 | 4.13 | m | 52.3 (d) | Leu (4) | 1 | | | 171.7 (s) |
| | 3 | 1.62 | m | 38.4 (t) | | 2 | 4.07 | m | 50.5 (d) |
| | 4 | *a | | | | 3 | 1.53 | m | 38.7 (t) |
| | 5 | *b | | | | | 1.64 | m | |
| | 6 | *a | | | | 4 | *a | | |
| | NH | 7.95 | br | | | 5 | *b | | |
| Leu (2) | | | | 172.9 (s) | | 6 | *a | | |
| | 2 | 4.17 | m | 52.2 (d) | | NH | 8.39 | d,7.3 | |
| | 3 | 1.54 | m | 38.6 (t) | Leu (5) | 1 | | | 172.0 (s) |
| | 4 | *a | | | | 2 | 4.15 | m | 52.3 (d) |
| | 5 | *b | | | | 3 | 1.50 | m | 38.6 (t) |
| | 6 | *a | | | | 4 | *a | | |
| | NH | 7.76 | br | | | 5 | *b | | |
| Val | | | | 170.7 (s) | | 6 | *a | | |
| | 2 | 4.03 | m | 58.3 (d) | | NH | 8.10 | br | |
| | 3 | 2.01 | m | 30.1 (d) | Lipid | 1 | | | 169.9 (s) |
| | 4 | 0.84 | m | 19.1 (q) | moiety | 2 | 2.31 | dd, 14.11, 7.3 | 41.0 (t) |
| | 5 | 0.78 | m | 18.0 (q) | | | 2.48 | m | |
| | NH | 8.03 | br | | | 3 | 5.05 | m | 71.0 (d) |
| Asp | 1 | | | 169.8 (s) | | 4 | 1.52 | m | 33.5 (t) |
| | 2 | 4.53 | | 49.3 (d) | | 5-12 | *c | | |
| | 3 | 2.58 | dd, 16.7, 9.5 | 35.2 (t) | | 13 | *a | | |
| | | 2.73 | dd, 16.7, 4.7 | | | 14 | *b | | |
| | 4 | | | 171.7 (s) | | 15 | *a | | |
| | NH | 8.11 | br | 7.61 | | | | | |

*a $^{1}H$ 0.79-0,95 ppm, $^{13}C$ 21-23 ppm; *b $^{1}H$ 1.50-1.55 ppm, $^{13}C$ 24.0-24.5 ppm;
*c $^{1}H$ 1.20-1.25 ppm, $^{13}C$ 28.0-29.0 ppm

Table. V-2. Spectrum of antialgal activities of surfactin against cyanobacteria

| Species | Antialgal activity |
|---|---|
| Cyanobacteria | |
| *Anabaena cylindrica* (NIES-19) | ++ |
| *Anabaena variabilis* (NIES-23) | ++ |
| *Anacystis marina* (IAM-122) | + |
| *Oscillatoria agardhii* (NIES-506) | + |
| *Microcystis aeruginosa* (NIES-299) | + |
| *Microcystis viridis* (NIES-102) | + |
| Green algae | |
| *Chlorella vulgaris* (NIES-227) | - |
| *Chlamydomonas tetragama* (NIES-446) | - |

at the concentration of 30 μg/disk; ++: > 5 mm of diameter of plaque, +: < 5 mm, -: no activity.

# CHAPTER VI

# Isolation and structure elucidation of YM-28160 and permetin A from *Paenibacillus* sp. S4

In chapter II, the hydrophilic and lipophilic fractions of *Paenibacillus* sp. S4 showed the potent antialgal activity against the cyanobacterium *Anabaena cylindrica* (NIES-19). In this chapter, the isolation and identification of the antialgal compounds, YM-28160 and permetin A from the agar culture of S4 strain was described.

## 1. Materials and methods

### 1.1 Organisms and culture conditions

The organisms and culture conditions were the same as described in chapter IV. S4 strain was isolated from the sediment of the pond Shinobazu at 2/March/2001 and identified as *Paenibacillus* by analysis of 16S rDNA sequence as described in chapter II.

### 1.2 Isolation of YM-28160 and permetin A

S4 strain was cultured on the Waksman agar medium at 25 °C. After 4-5 days cultivation, the agar media (5 L) containing cultured cells of S4 were homogenized and lyophilized. The lyophilized powder was extracted with MeOH (2 L × 3). The MeOH extract was concentrated to an aqueous suspension, and this was extracted with EtOAc. The $H_2O$ fraction was further partitioned with *n*-BuOH. The *n*-BuOH fraction (5.8 g) was subjected to ODS flash chromatography (YMC-GEL, 5 × 10 cm), eluted with 20% MeOH, 40% MeOH, 60% MeOH, 80%

MeOH, and MeOH. The 80% MeOH fraction (282.0 mg) which showed the potent antialgal activity was subjected to reversed-phase HPLC (Cosmosil 5C18MS, 10 × 250 mm; 40%-70% MeOH containing 0.05% TFA, linear gradient condition; flow rate, 2.0 mL/min; UV detection at 210 nm). The active fraction (23.3 mg) was concentrated by rotary evaporator, and subjected to reversed-phase HPLC (Cosmosil 5CN, 10 × 250 mm; 33% MeCN containing 0.05% TFA, isocratic condition; flow rate, 2.0 mL/min; UV detection at 210 nm) to obtained YM-28160 (4.4 mg) and permetin A (3.5 mg).

### 1.3 Amino acid analysis

YM-28160 and permetin A (each of 500 μg) were hydrolyzed with 0.5 mL of 6N HCl at 105 °C for 24 h. The solutions were evaporated in a stream of dry nitrogen with heating and redissolved in 0.02 N HCl to subject to the amino acid analyzer. Retention times of amino acids (min); Dab (18.3), Val (39.5), Ile (45.1), Val (49.8), Leu (46.4), Phe (52.5).

### 1.4 HPLC analysis of Marfey derivative

The hydrolyzate of each peptide was treated with 10% $Me_2CO$ solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (L-FDAA, Marfey's reagent) in 1 M $NaHCO_3$ at 80-90 °C for 3 min followed by neutralization with 2 N HCl. The reaction mixture was dissolved in 50% MeCN and subjected to reversed-phase HPLC: column, Cosmosil 5C18MS (4.6 × 250 mm), gradient elution from $H_2O$/TFA (100:0.1) to MeCN/$H_2O$/TFA (60:40:0.1) in 60 min, UV detector (340 nm). Retention times of amino acids (min); L-Dab (28.8), D-Dab (31.4), L-Val (46.0), D-Val (49.8), L-Ile (49.4), D-Ile (53.6), L-Leu (50.4), D-Leu (53.8), L-Phe (53.4), D-Phe (56.0).

## 1.5 Derivatization with Boc-L-Cys-OPA and HPLC analysis

The derivatization reagents was prepared by dissolving 10 mg of *o*-phthaldialdehyde (OPA) and 10 mg of N-*tertiary*-butyloxycarbonyl-L-cysteine (Boc-L-Cys) in 1 mL of MeOH.[73] A borate buffer was made using 0.4 M boric acid adjusted to pH 9.0 with sodium hydroxide. A 10 μL volume of Boc-L-Cys-OPA reagent and 70 μL of the borate buffer were added to a vial containing 10 μL of the amino acid standard or the hydrolyzate of each peptide. After derivatization for 2 min at room temperature, an aliquot (20 μL) of the reaction mixture was analyzed by the HPLC system. The HPLC analysis was performed with ODS column (Superiorex ODS, 4.6 × 250 mm) by detection using spectrofluorometer (Excitation, 344 nm; emission 443 nm). A phosphate buffer was prepared using 0.1 M sodium dihydrogenphosphate adjusted to pH 6.0 with sodium hydroxide. Mobile phase A was the phosphate buffer with 5% MeCN and 5% tetrahydrofuran (THF) and mobile phase B was the phosphate buffer with 45% MeCN and 5% THF. The analyses of the Boc-L-Cys-OPA derivatizates were accomplished with a linear gradient from mobile phase A to B in 120 min. Retention times of amino acids (min); L-Ser (48.9), D-Ser (54.0).

## 1.6 General instrumentation

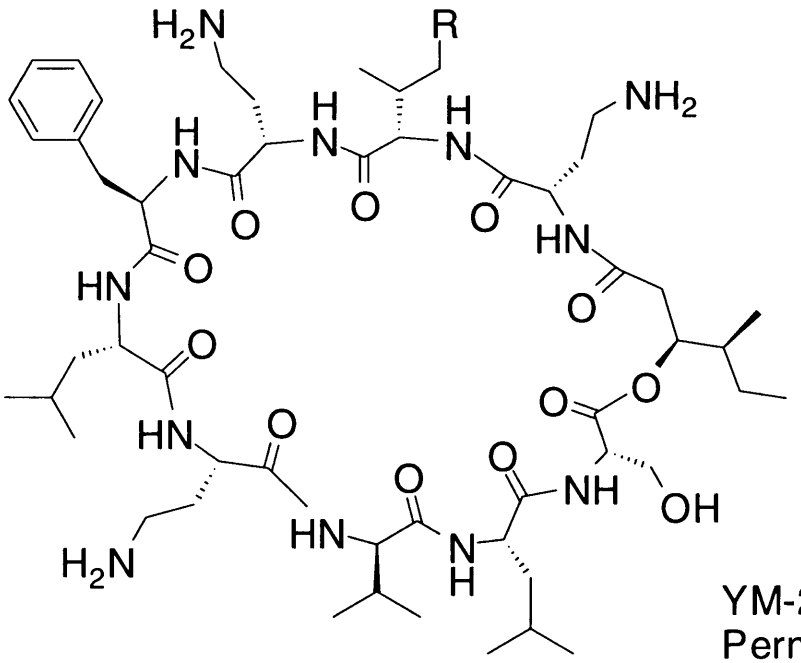
NMR spectra were recorded on a JEOL NM-A500 NMR spectrometer operating at 500 MHz using DMSO-$d_6$ as solvent at 27 °C. The resonance of residual DMSO-$d_6$ at $\delta_H$ 2.79 was used as internal reference for $^1$H NMR spectra. FAB mass spectra were recorded by a JEOL JMS SX-102 mass spectrometer. The amino acid analysis was carried out with a Hitachi L-8500A amino acid analyzer. The optical rotation was determined on a JASCO DIP-1000 digital polarimeter in MeOH. The UV spectrum was recorded on a SHIMADZU UV-

VIS 1240 spectrophotometer.

## 2. Results

### 2.1 Isolation of YM-28160 and permetin A

S4 strain was cultured on agar plates containing Waksman medium at 25 °C. After 4-5 days, the bacterial grown agar media were homogenized and lyophilized. The lyophilized powder was extracted with MeOH. The MeOH extract was evaporated to aqueous suspension and partitioned between EtOAc and $H_2O$. The $H_2O$ layer which showed potent antialgal activity was extracted with *n*-BuOH. The *n*-BuOH fraction was subjected to ODS column chromatography followed by reversed-phase HPLC to yield the antialgal compound, YM-28160 (**1**, 4.4 mg) and permetin A (**2**, 3.5 mg).



## 2.2 Structure elucidation of YM-28160 and permetin A

YM-28160 (**1**) was isolated as an amorphous powder: $[\alpha]_D$ -6.0° (*c* 0.1, MeOH); UV absorption spectrum in MeOH showed the absence of absorption maximum in the range from 230 nm to 400 nm. The positive FABMS using glycerol as matrix of **1** showed $[M+H]^+$ ion at m/z 1087 (Fig. VI-2). The molecular formula of permetin A was determined as $C_{53}H_{90}N_{12}O_{12}$ by the HRFABMS *m/z* 1087.6876 $[M+H]^+$ calcd for $C_{53}H_{91}N_{12}O_{12}$ (1087.6879). The amino acid analysis of the hydrolyzate gave 2 mole of Leu, 2 mole of Val, 1 mol of Phe, and about 3 mole of the unknown amino acid. The unknown amino acid was identified as 2,4-diamino-butylic acid (Dab) by comparing with retention time of authentic Dab. The extensive NMR analyses of **1** including $^1H$-$^1H$ COSY, HMQC[66], and HMBC[67] spectra indicated the presence of all amino acids which were detected by amino acid analysis (Fig. VI-3~6), and 3-hydroxy-4-methyl hexanoic acid moiety (HMHA). The sequences of all units were determined by the HMBC and NOESY correlations (Fig. VI-6, 7) as shown in Fig. VI-1. The linkages between Dab (1) and Leu (2), and between Ser and HMHA were not determined. However the identity with permetin A was confirmed by the comparison with literature data of the chemical shifts (Table VI-1).[78, 79]

Permetin A (**2**) was isolated as an amorphous powder: $[\alpha]_D$ -12.3° (*c* 0.1, MeOH); UV absorption spectrum in MeOH showed the absence of absorption maximum in the range from 230 nm to 400 nm. The positive FABMS using glycerol as matrix of **2** showed $[M+H]^+$ ion at m/z 1101 (Fig. VI-8). The molecular formula of **2** was determined as $C_{54}H_{93}N_{12}O_{12}$ by the HRFABMS *m/z* 1101.7065 $[M+H]^+$ calcd for $C_{54}H_{94}N_{12}O_{12}$ (1101.7036). The amino acid analysis of the hydrolyzate gave 2 mole of Leu, 3 mole of Dab, and 1 mol of Phe, Ile, and Val. The structure of **2** was determined by the NMR analyses including $^1H$-$^1H$ COSY, HMQC, HMBC, and NOESY spectra in the same manner as **1** (Fig. VI-9-13, Table VI-2). It was determined that **2** have the structure substituted Ile

for Val (2) in the structure of **1**.[80]

The stereochemistries of amino acids except for Ser in **2** were determined by the Marfey's method. The stereochemistries of Dab (3 mole), Leu (2 mole) and Ile were determined as L-form, and the stereochemistries of Phe and Val were determined as D-form. The stereochemistry of Ser in **2** was determined as L-form by HPLC analyses of Boc-L-Cys-OPA derivative.[77] As the results of subjecting **1** to Marfey's method, the amino acids containing L-Dab, L-Leu, D-Phe, L-Val and D-Val were detected in the ratio of 3:2:1:1:1 by HPLC analysis. The stereochemistry of Ser in **1** was determined as L-form in the same manner of **2**. All the stereochemistries of amino acids in **1** and **2** were identical with literature data except for Val in **1**.

### 2.3 Antialgal activity of YM-28160 and permetin A

YM-28160 (**1**) and permetin A (**2**) was subjected to antialgal assay against several strains of cyanobacteria and green algae using the paper disk method. The result was summarized in Table VI-3. YM-28160 (**1**) and permetin A (**2**) showed antialgal activities against the cyanobacteria *A. cylindrica* (NIES-19), *A. variabilis* (NIES-23), *O. agardhii* (NIES-506), *A. marina* (IAM-122), *M. aeruginosa* (NIES-299), and *M. viridis* (NIES-102) at a concentration of 30 μg/disk. However, **1** and **2** did not show activity at a concentration of 30 μg/disk against the green algae, *C. vulgaris* (NIES-227) and *C. tetragama* (NIES-446).

## 3. Discussion

In the present study, the positions of L-Val and D-Val in **1** were not determined by Marfey's method. However Takeuchi *et al.* reported that Val positioned between Dab and Leu was determined as D-form by the analyses of partial hydrolyzates.[78] Considering the biosynthesis of peptide antibiotics, It was

reasonable since the same positioned Val in **2** was D-form. Murai *et al.* reported that the stereochemistry of 3-methyl-4-hydroxy-hexanoic acid in permetin A was determined as 3S, 4S by the comparison with synthesized isomers.[81]

It was reported that permetin A had the broad spectrum of antibacterial activities against gram-negative and gram-positive bacteria.[78] The mechanism of antibacterial activity of permetin A is not clear. YM-28160 and permetin A did not show the antialgal activities against green algae as surfactin. These peptides may be the useful agent for removing the cyanobacterial bloom. However further study about the mode of action against cyanobacteria is required for application.

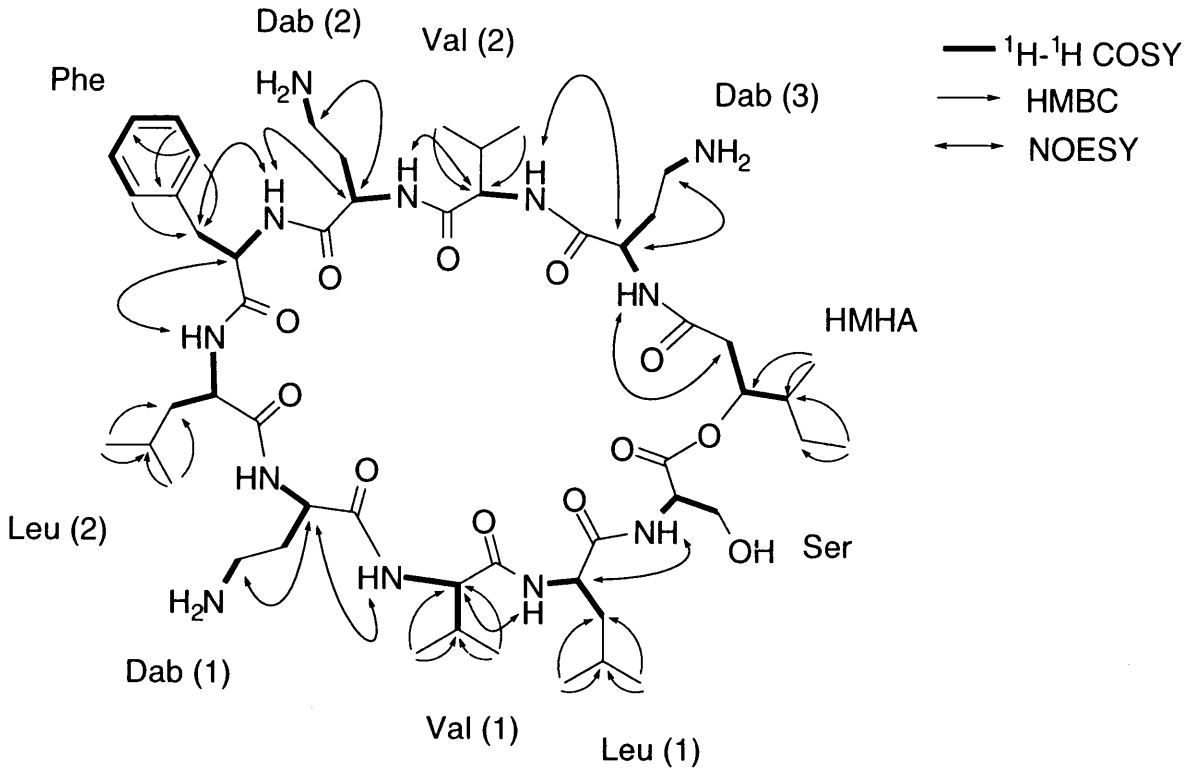



Fig. VI-1. $^1$H-$^1$H COSY, NOESY, and HMBC correlations of YM-28160

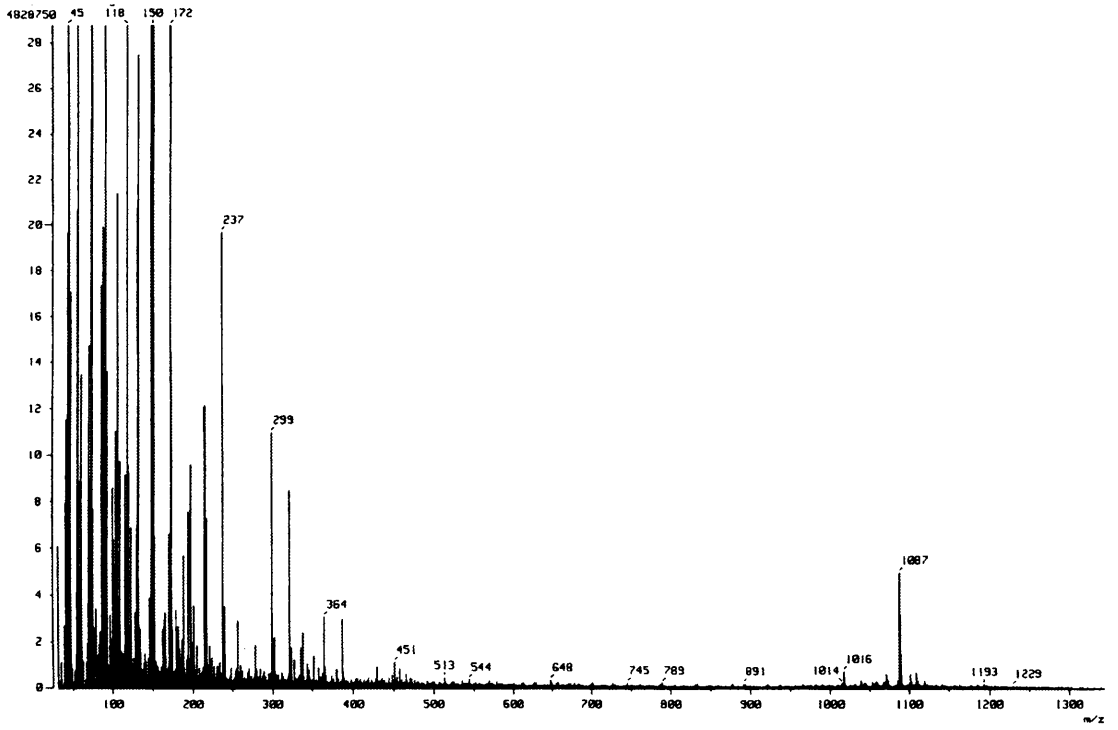



Fig. VI-2. Positive FABMS spectrum of YM-28160 (matrix; glycerol)

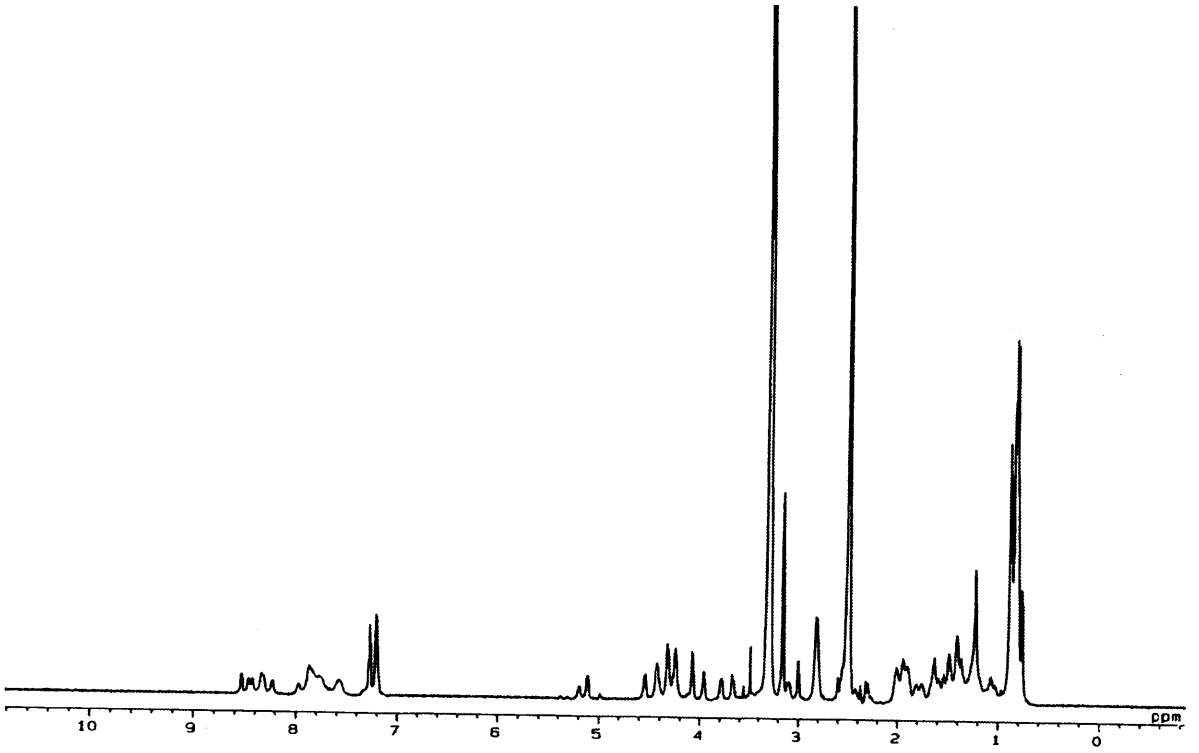



Fig. VI-3. $^{1}H$ NMR spectrum of YM-28160 (DMSO-$d_6$)

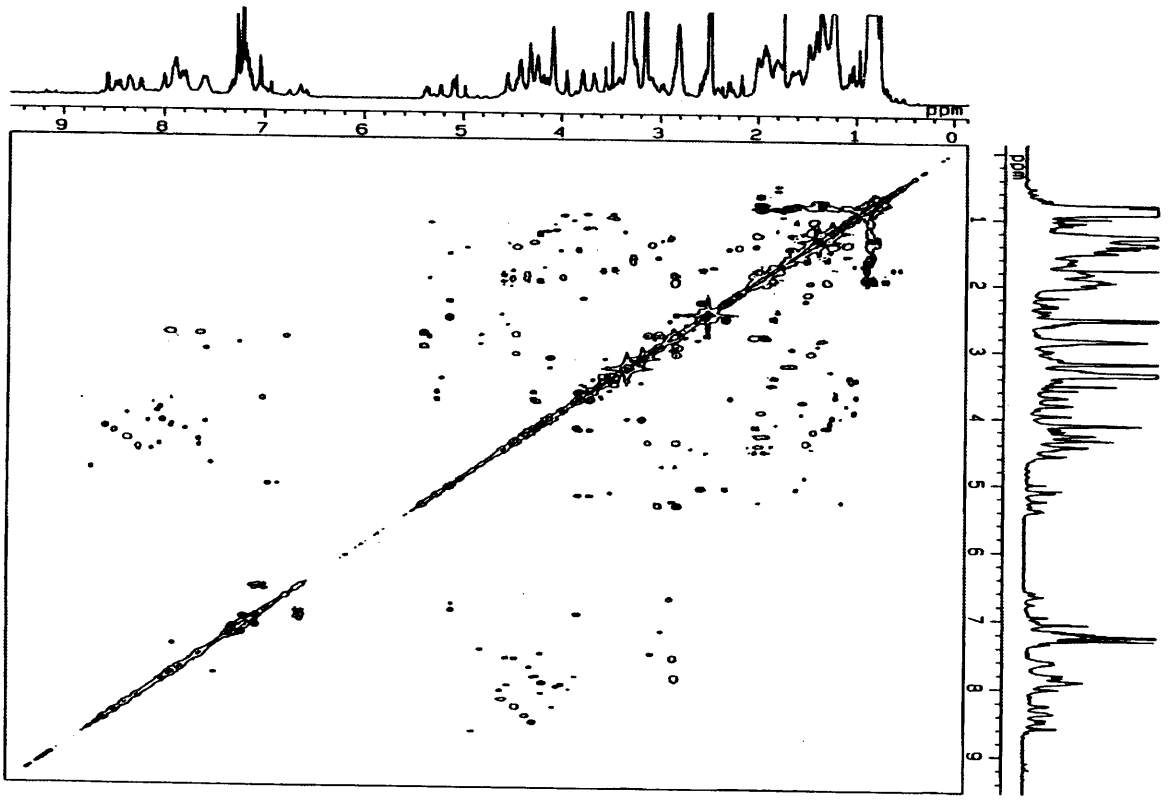



Fig. VI-4. $^{1}H$-$^{1}H$ COSY NMR spectrum of YM-28160 (DMSO-$d_6$)

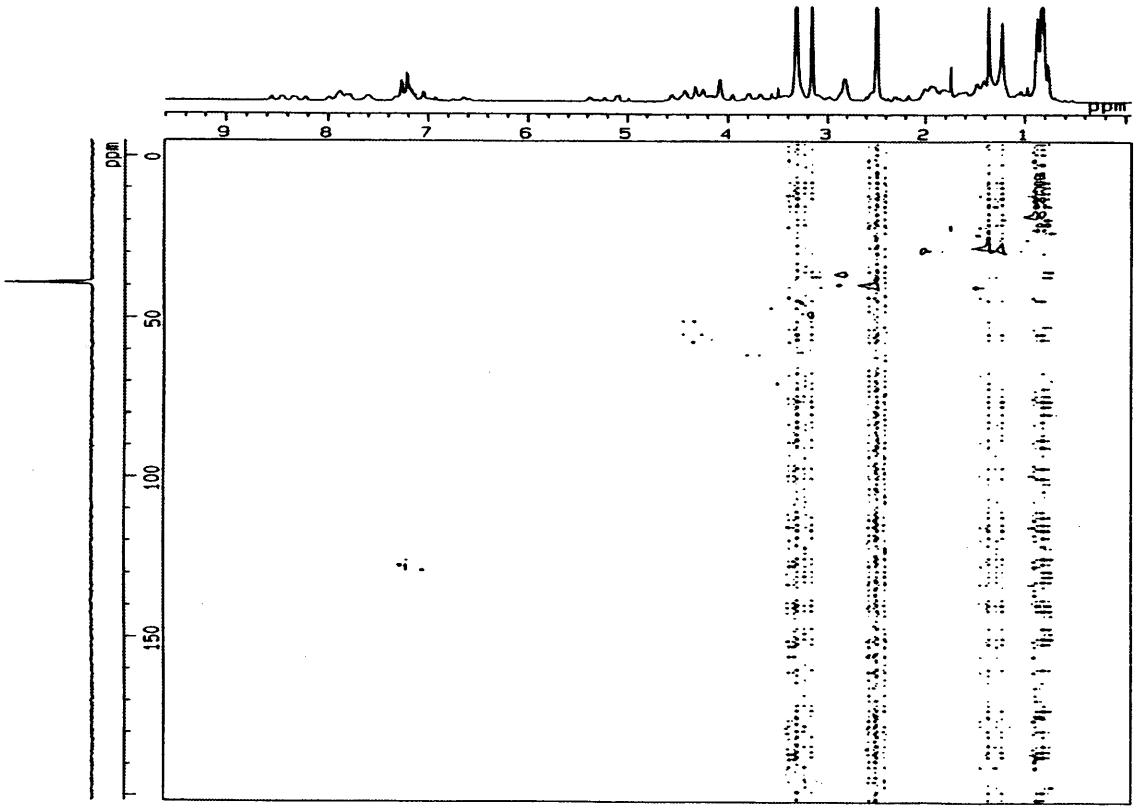



Fig. VI-5. HMQC NMR spectrum of YM-28160 (DMSO-$d_6$)

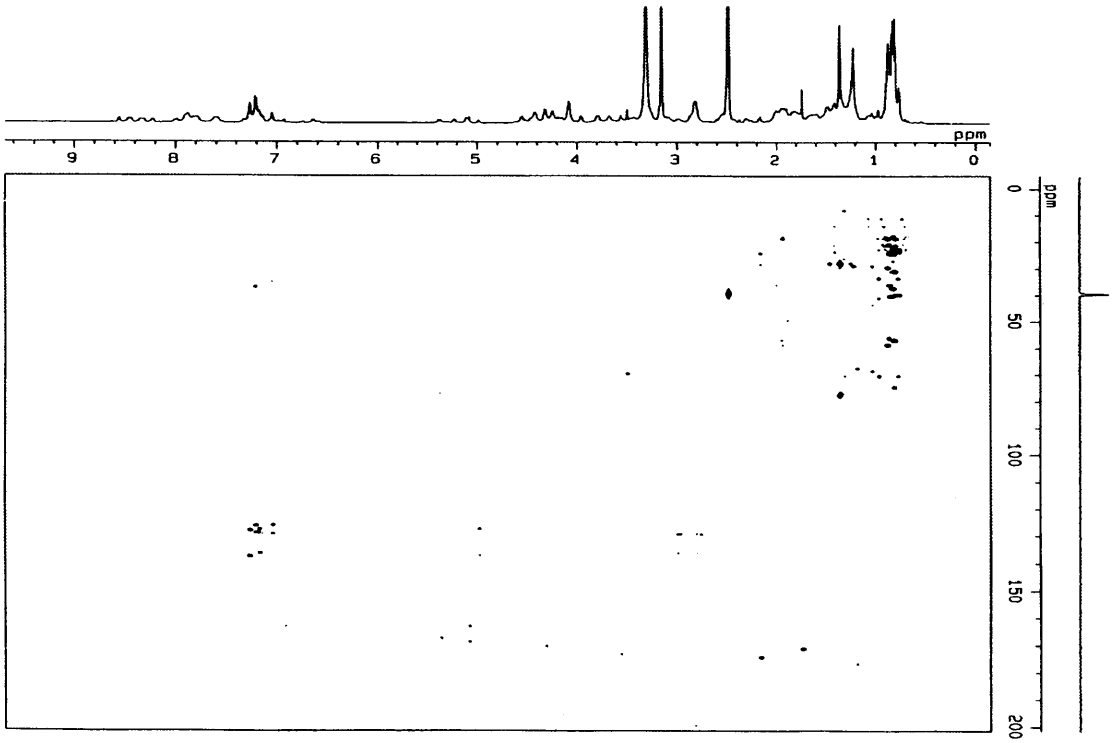



Fig. VI-6. HMBC NMR spectrum of YM-28160 (DMSO-$d_6$)

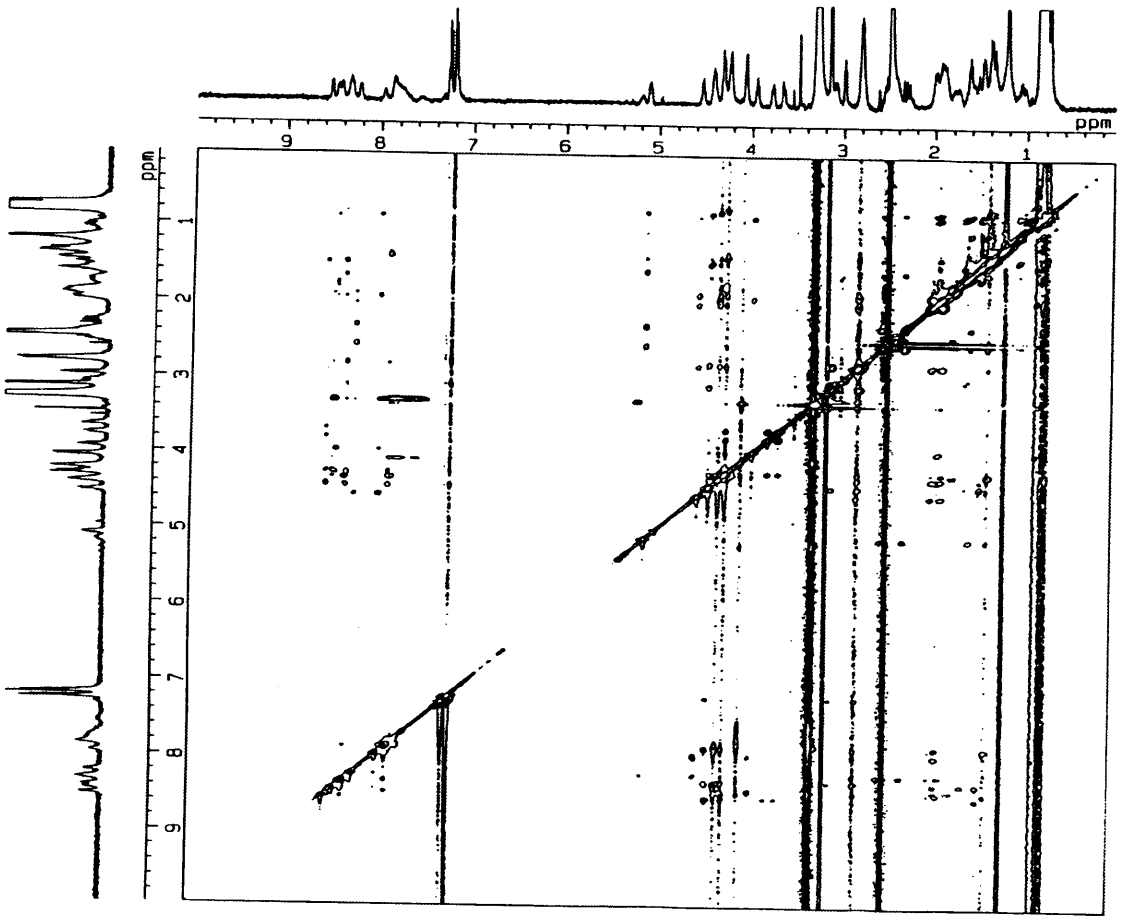



Fig. VI-7. NOESY NMR spectrum of YM-28160 (DMSO-$d_6$)

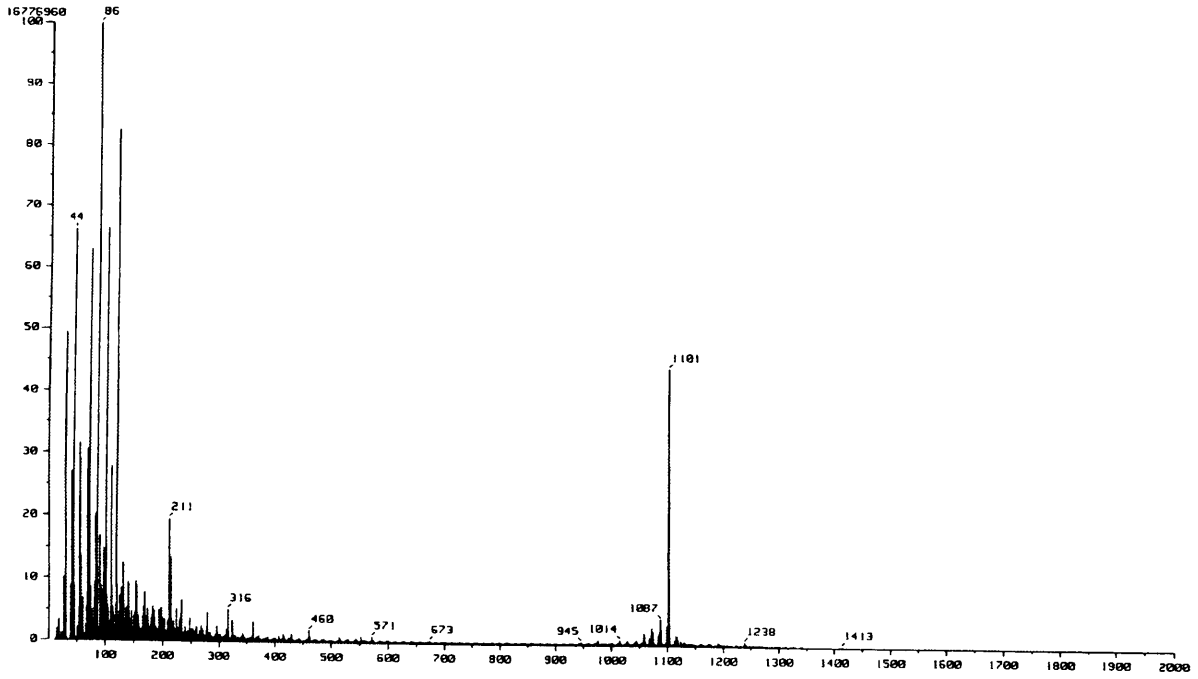



Fig. VI-8. Positive FABMS spectrum of permetin A (matrix; glycerol)

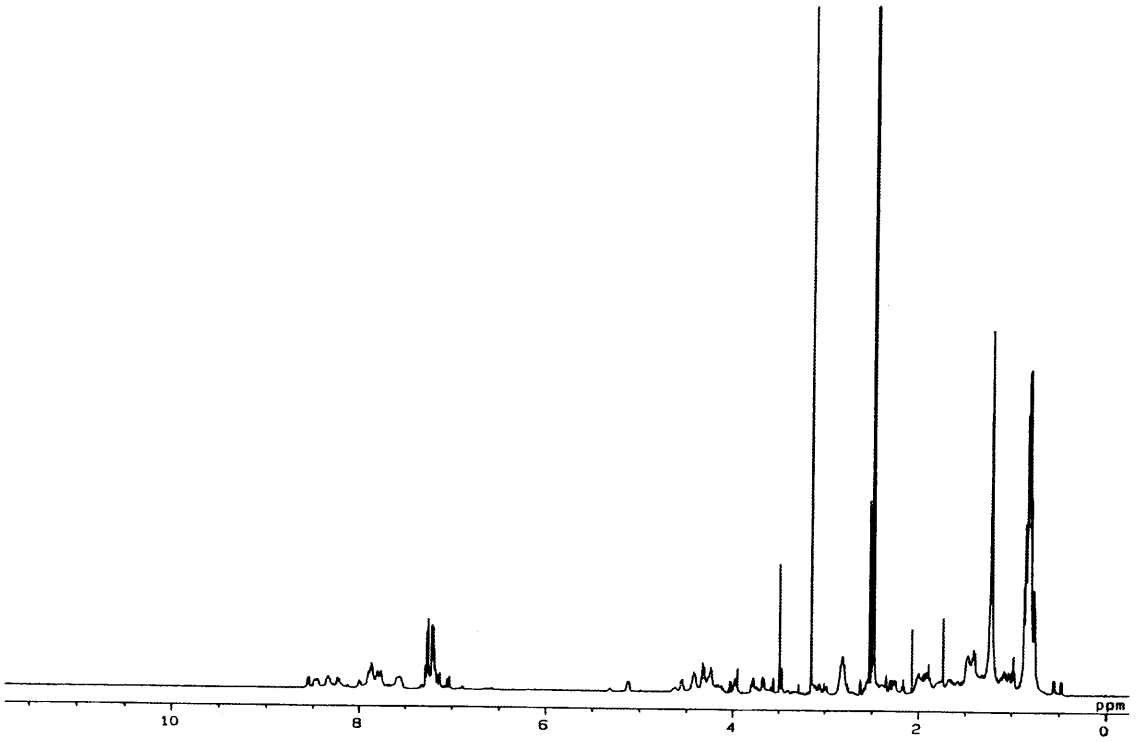



Fig. VI-9. $^{1}H$ NMR spectrum of permetin A (DMSO-$d_6$)

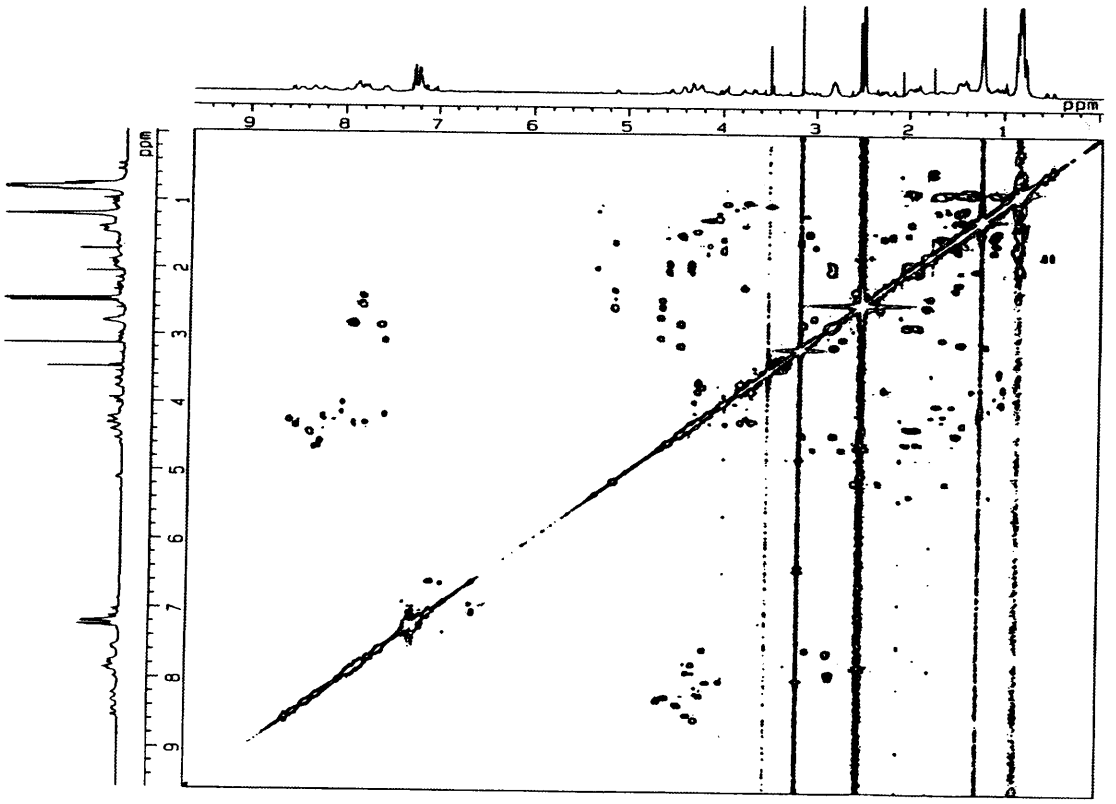



Fig. VI-10. $^{1}H$-$^{1}H$ COSY NMR spectrum of permetin A (DMSO-$d_6$)

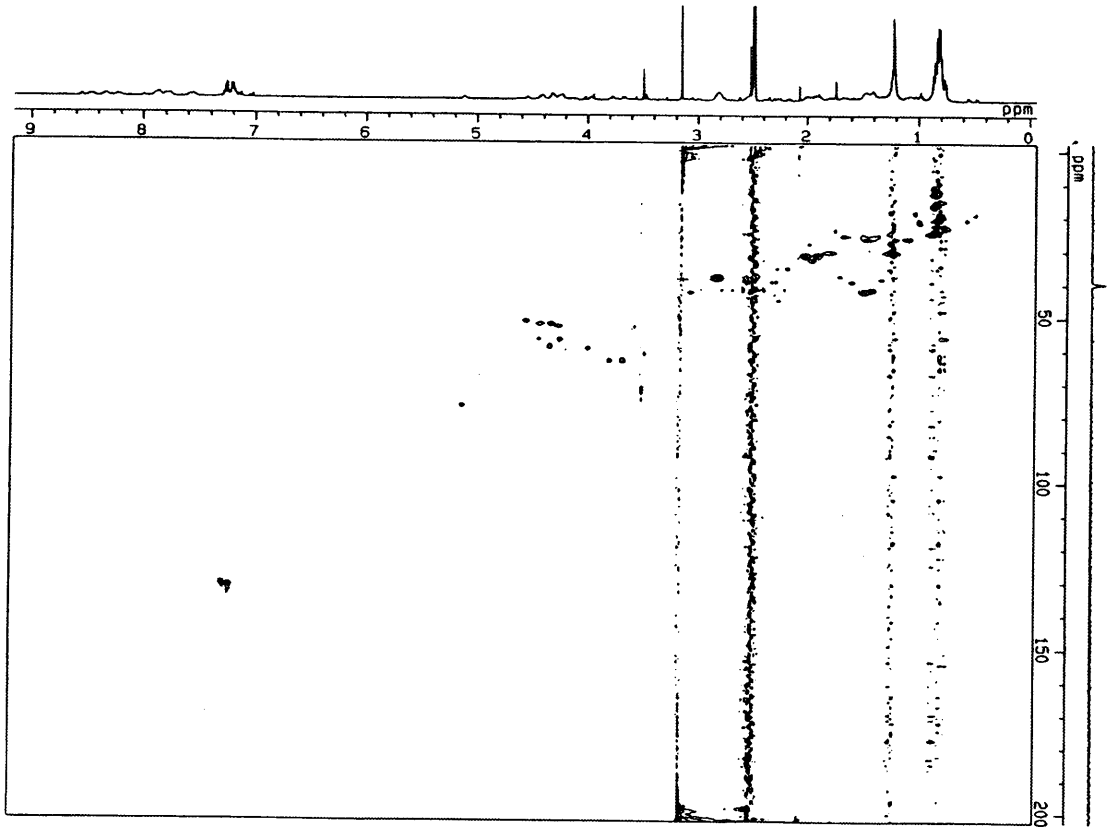



Fig. VI-11. HMQC NMR spectrum of permetin A (DMSO-$d_6$)

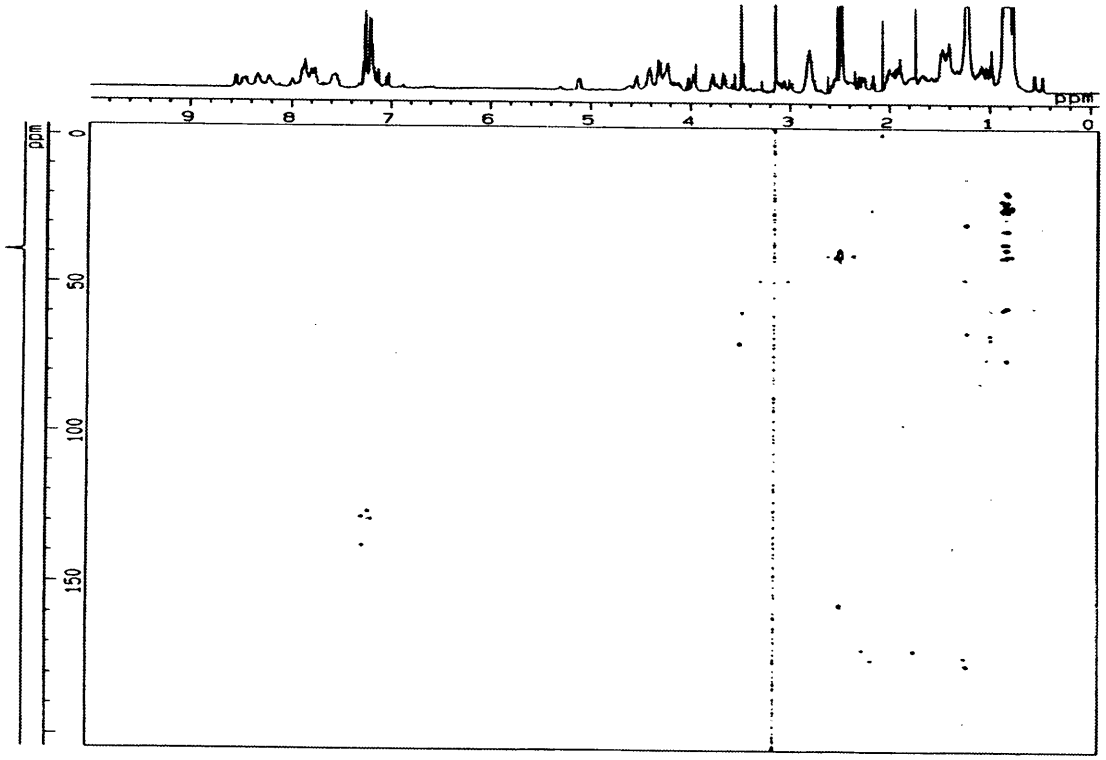



Fig. VI-12. HMBC NMR spectrum of permetin A (DMSO-$d_6$)

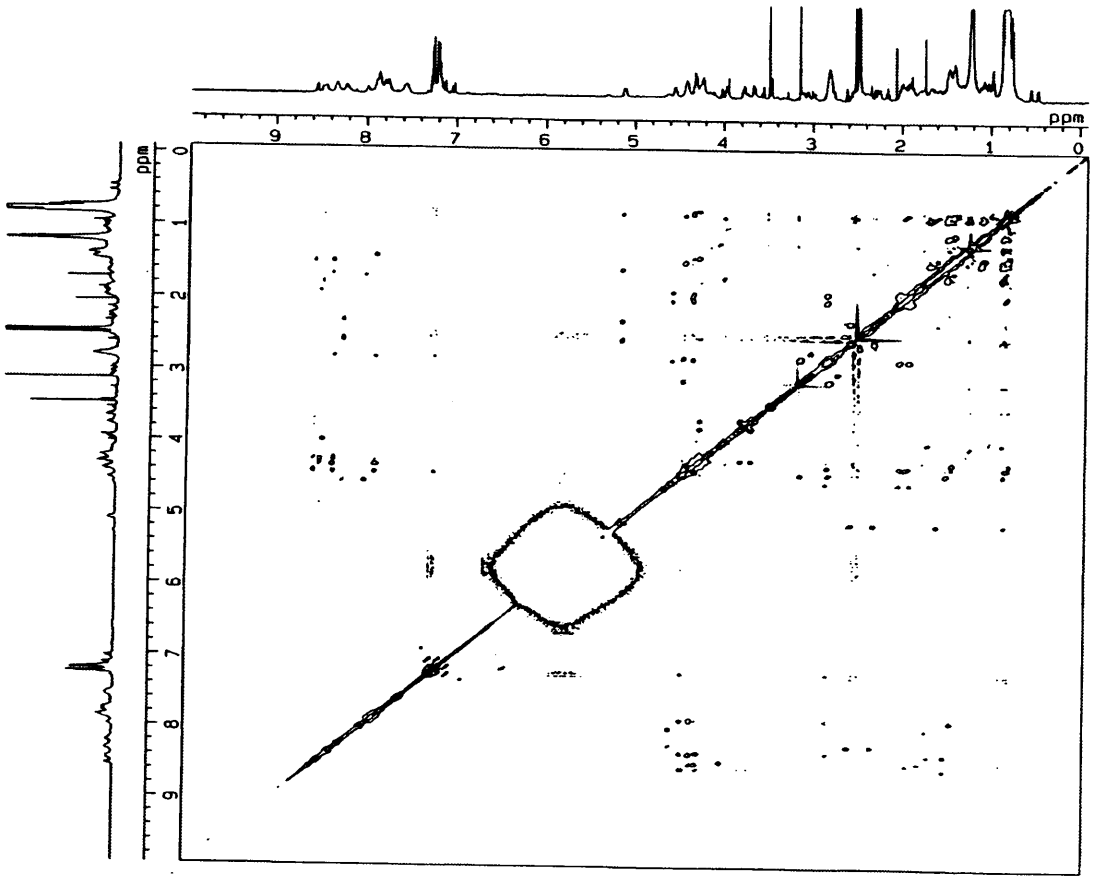



Fig. VI-13. NOESY NMR spectrum of permetin A (DMSO-$d_6$)

Table VI-1. $^{1}H$ and $^{13}C$ NMR Spectral data of YM-28160 in DMSO-$d_6$

| Position | | $^{1}H$ | $J$ (Hz) | $^{13}C$ | Position | | $^{1}H$ | $J$ (Hz) | $^{13}C$ |
|---|---|---|---|---|---|---|---|---|---|
| Dab (1) | 1 | | | *a | Dab (3) | 1 | | | *a |
| | 2 | 4.55 | m | 49.8 (d) | | 2 | 4.31 | m | 49.9 (d) |
| | 3 | 1.95 | m | 30.5 (t) | | 3 | 1.95 | m | 30.5 (t) |
| | | 2.05 | m | | | | 2.05 | m | |
| | 4 | 2.80 | m | 36.5 (t) | | 4 | 2.80 | m | 36.5 (t) |
| | $NH_2$ | 7.60 | br | | | $NH_2$ | 7.90 | br | |
| | NH | 8.24 | br | | | NH | 8.24 | br | |
| Val (1) | 1 | | | *a | Val (2) | 1 | | | *a |
| | 2 | 3.95 | m | 59.0 (d) | | 2 | 4.32 | m | 57.3 (d) |
| | 3 | 2.05 | m | 29.9 (t) | | 3 | 2.05 | m | 31.0 (t) |
| | 4 | 0.87 | m | 18.0 | | 4 | 0.81 | m | 17.9 (q) |
| | 5 | 0.89 | m | 18.9 | | 5 | 0.82 | m | 18.5 (q) |
| | NH | 7.98 | br | | | NH | 8.47 | br | |
| Dab (2) | | | | *a | Leu (2) | 1 | | | *a |
| | 2 | 4.25 | m | 50.6 (d) | | 2 | 4.42 | m | 50.5 (d) |
| | 3 | 1.75 | m | 30.5 (t) | | 3 | 1.40 | m | 40.5 (t) |
| | 4 | 2.40 | m | 36.5 | | 4 | 1.51 | m | 24.1 (d) |
| | | 2.61 | m | | | 5 | 0.83 | m | 21.0 (q) |
| | $NH_2$ | 7.75 | br | | | 6 | 0.87 | m | 23.3 (q) |
| | NH | 8.42 | br | | | NH | 8.34 | | |
| Phe | | | | *a | Ser | 1 | | | *a |
| | 2 | 4.44 | m | 54.8 (d) | | 2 | 4.22 | m | 50.1 (d) |
| | 3 | 2.82 | m | 37.2 (d) | | 3 | 3.67 | d, 3.7, 5.3 | 61.5 (t) |
| | | 3.10 | m | (q) | | | 3.78 | d, 5.3, 5.1 | |
| | 4 | | | 137.9 (q) | | NH | 8.53 | d, 7.0 | |
| | 5,9 | 7.21 | d, 6.8 | 129.0 (d) | HMHA | 1 | | | *a |
| | 6,8 | 7.27 | d, 7.2 | 128.3 (d) | | 2 | 2.30 | m | 37.8 (t) |
| | 7 | 7.18 | m | 127.0 (d) | | | 2.58 | | |
| | NH | 8.32 | br | | | 3 | 5.13 | m | 75.2 (d) |
| Leu (1) | 1 | | | *a | | 4 | 1.60 | m | 38.0 (d) |
| | 2 | 4.24 | m | 50.7 (d) | | 5 | 0.82 | m | 14.2 (q) |
| | 3 | 1.40 | m | 40.0 (t) | | 6 | 1.40 | m | 25.0 (t) |
| | 4 | 1.51 | m | 23.9 (d) | | 7 | 0.84 | m | 11.2 (q) |
| | 5 | 0.77 | m | 21.5 (q) | | | | | |
| | 6 | 0.81 | m | 23.4 (q) | | | | | |
| | NH | 7.88 | br | | | | | | |

*a, not determined by analyses of HMBC

Table VI-2. $^{1}H$ and $^{13}C$ NMR Spectral data of permetin A in DMSO-$d_6$

| Position | | $^{1}H$ | $J$ (Hz) | $^{13}C$ | Position | | $^{1}H$ | $J$ (Hz) | $^{13}C$ |
|---|---|---|---|---|---|---|---|---|---|
| Dab (1) | 1 | | | *a | Dab (3) | 1 | | | *a |
| | 2 | 4.55 | m | 49.8 (d) | | 2 | 4.32 | m | 49.8 (d) |
| | 3 | 1.95 | m | 30.5 (t) | | 3 | 1.95 | m | 30.5 (t) |
| | | 2.05 | m | | | | 2.06 | m | |
| | 4 | 2.82 | m | 36.5 (t) | | 4 | 2.80 | m | 36.7 (t) |
| | $NH_2$ | 7.61 | br | | | $NH_2$ | 7.90 | br | |
| | NH | 8.23 | br | | | NH | 8.24 | br | |
| Ile | 1 | | | *a | Val (2) | 1 | | | *a |
| | 2 | 3.95 | m | 59.0 (d) | | 2 | 4.32 | m | 57.4 (d) |
| | 3 | 2.05 | m | 31.9 (t) | | 3 | 2.05 | m | 31.0 (t) |
| | 4 | 0.87 | m | 18.0 | | 4 | 0.81 | m | 17.9 (q) |
| | 5 | 1.22 | m | 25.1 | | 5 | 0.82 | m | 18.5 (q) |
| | 6 | 0.90 | m | 18.9 | | NH | 8.46 | br | |
| | NH | 7.98 | br | | Leu (2) | 1 | | | *a |
| Dab (2) | | | | *a | | 2 | 4.42 | m | 50.6 (d) |
| | 2 | 4.25 | m | 50.6 (d) | | 3 | 1.40 | m | 40.5 (t) |
| | 3 | 1.75 | m | 30.5 (t) | | 4 | 1.51 | m | 24.1 (d) |
| | 4 | 2.40 | m | 36.5 | | 5 | 0.83 | m | 21.0 (q) |
| | | 2.61 | | | | 6 | 0.87 | m | 23.3 (q) |
| | $NH_2$ | 7.75 | br | | | NH | 8.34 | br | |
| | NH | 8.44 | br | | Ser | 1 | | | *a |
| Phe | | | | *a | | 2 | 4.22 | m | 50.2 |
| | 2 | 4.45 | m | 54.8 (d) | | 3 | 3.67 | d, 3.7, 5.2 | 61.5 |
| | 3 | 2.84 | m | 37.2 (d) | | | 3.78 | d, 5.2, 5.1 | |
| | | 3.12 | m | (q) | | NH | 8.53 | d, 7.0 | |
| | 4 | | | 138.0 (q) | HMHA | 1 | | | *a |
| | 5,9 | 7.20 | d, 6.8 | 129.0 (d) | | 2 | 2.30 | m | 37.5 (t) |
| | 6,8 | 7.27 | d, 7.2 | 128.5 (d) | | | 2.58 | m | |
| | 7 | 7.18 | m | | | 3 | 5.12 | m | 75.3 (d) |
| | NH | 8.34 | br | | | 4 | 1.61 | m | 37.9 (d) |
| Leu (1) | 1 | | | *a | | 5 | 0.82 | m | 14.2 (q) |
| | 2 | 4.25 | m | 50.7 (d) | | 6 | 1.41 | m | 25.1 (t) |
| | 3 | 1.40 | m | 40.0 (t) | | 7 | 0.83 | m | 11.3 (q) |
| | 4 | 1.51 | m | 23.9 (d) | | | | | |
| | 5 | 0.76 | m | 21.5 (q) | | | | | |
| | 6 | 0.81 | m | 23.4 (q) | | | | | |
| | NH | 7.88 | br | | | | | | |

*a, not determined by analyses of HMBC

Table VI-3. Spectrum of the antialgal activities of YM-28160 and permetin A

| Species | YM-28160 | permetin A |
|---|---|---|
| Cyanobacteria | | |
| *Anabaena cylindrica* (NIES-19) | ++ | + |
| *Anabaena variabilis* (NIES-23) | + | + |
| *Anacystis marina* (IAM-122) | + | ++ |
| *Oscillatoria agardhii* (NIES-506) | + | + |
| *Microcystis aeruginosa* (NIES-299) | + | + |
| *Microcystis viridis* (NIES-102) | + | + |
| Green algae | | |
| *Chlorella vulgaris* (NIES-227) | - | - |
| *Chlamydomonas tetragama* (NIES-446) | - | - |

at the concentration of 30 μg/disk
++: > 5 mm of diameter of plaque
+: < 5 mm
-: no activity.

# CHAPTER VII

## General discussion

In the present study, 30 bacterial strains were obtained by the algicidal screenings from the eutrophic ponds. The most part of 30 strains were classified in the genera *Pseudomonas* and *Bacillus*. Among reports of algicidal bacteria, there are many reports about algicidal *Cytophaga* and *Myxobacter*.[17,32,82] In the present study, only one strain out of 30 strains was identified as the bacterium classified in *Cytophaga-Flavobacterium* group. Stewart *et al.* reported that *Cytophaga* N-5 isolated from sewage lyses an array of living cyanobacteria.[16] *Cytophaga* N-5 grows in chemically defined medium or living algae in agar medium. They deduced that the plaque-forming activity was caused by extracellar lytic agents. Recently, Rashidan and Bird reported that the attempts to separate an extracellar lytic factor by use of filtered *Cytophaga*-cyanobacteria mixed cultures failed.[51] They indicated the possibility that *Cytophaga* required a direct contact with host cells in order to lyse. In the present study, the extracts of U38-6 strain classified in *Cytophaga-Flavobacterium* group did not show the antialgal activity. This strain may also require for the direct contacts with cyanobacteria.

About the antialgal compounds produced by *Bacillus* and *Pseudomonas*, there have been several reports so far. Reim *et al.* reported that the *Bacillus* isolated from the sewage oxidation pond lysed *Plectonema boryanum* effectively.[43] They deduced the antialgal compounds produced by the *Bacillus* may be two antibiotics, gramicidin S and tyrocidine. The mode of action of these antibiotics is thought to act upon the outer membrane of gram-negative bacterium. Generally, the bacterium classified in the genus *Bacillus* is known to produce variety of peptidic antibiotics such as colistin. In the present study, surfactin isolated from *Bacillus* sp. M1 have the specific antialgal activity against

cyanobacteria, the bacteria classified in *Bacillus* were thought to be useful agents for the control of cyanobacterial bloom.

Dakhama *et al.* reported 1-hydroxyphenazine and oxychloraphine isolated from *Pseudomonas aeruginosa* have strong antialgal activity.[38] These phenazine alkaloids act at the site of coenzyme Q or Co Q-cytochrome *b* by accepting electrons and transporting them to 'dead end'. So these compounds have the inhibitory activity against algae and higher plants. In the present study, harmane (1-methyl-β-carboline) was isolated as the antialgal compound from *Pseudomonas* sp. K44-1. This compound showed the specific antialgal activity against cyanobacteria. However, the mode of action was not clear. Further study is required for application in natural environments.

*Lysobacter* sp. LB-1 was reported to produce the protease which showed the algal lytic activity against *A. cylindrica*.[31] In the present study, L-Tyr was isolated as another algicidal agent. Mitsutani *et al.* reported that the lytic mode of LB-1 strain required the direct attachment to cyanobacteria. These data showed the possibility that protease may cowork with low molecular antialgal compound such as L-Tyr against cyanobacteria on the attached cyanobacterial membrane.

In the present study, two strains of *Streptomyces* were isolated by the plaque-forming assay. Whyte *et al.* reported the isolation of cyanobacteria-lysing streptomycetes from soil.[40] They determined no physical penetration of cyanobacterial cells by the streptomycete, under microscopic observation. They deduced that some extracellular agents may involve in lysis. Recently, Yamamoto *et al.* isolated L-Lys as the antialgal compound from *Streptomyces phaeofaciens*.[11] However, there are a few reports about the antialgal compounds of algicidal *Streptomyces*. In the present study, only 2 strains of *Streptomyces* were obtained by the plaque-forming assay. Since the bacteria grouped in the genus *Streptomyces* were thought to be useful agents for obtaining the antialgal compound, more effective method is required to obtain algicidal *Streptomyces*.

The bacteria classified in the genera, *Pseudomonas*, *Bacillus*, and

*Paenibacillus* which has the potent algicidal activity were obtained in present study. However, these algicidal acivity was not accounted only by the low molecular algicides obtained in the present study. It was thought that there may be more complex system involved in algal lysis. There was a little information about antialgal compounds produced by algicidal bacteria, further work is required for determination of algal lytic mechanism.

# References


1) Charmichael, W.W. (1992) *A status report of planktonic cyanobacteria* (Blue-green algae and their toxins.) United States Environmental Protection Agency/600/R-92/079: 32-33.

2) Kikuchi, T., Miura, T., Harimaya, K., Yano, H., Arimoto, T., Masada, Y., Inoue, T. (1973) Odorous metabolites of blue-green alga: *Schizothrix muelleri Nageli* collected in the southern basin of Lake Biwa. *Chem. Pharm Bull.*, 21: 2342-2343.

3) Tsuchiya, Y., Watanabe, M.F., Watanabe, M. (1992) Volatile organic sulfur compounds associated with blue-green algae from inland waters of Japan. *Wat. Sci. Technol.*, 25:123-130.

4) Harada, K-I. (1996) *Toxic Microcystis.* (eds) Watanabe, M.F., Harada, K-I., Charmichael, W.W., Fujiki, H. CRC Press., pp 103-148.

5) Reynolds, C.S. (1984) *The Ecology of Freshwater Phytoplankton.* Cambridge University Press., pp 384-395

6) Vincent, W.F. (1987) Special issue: Dominance of Bloom Forming Cyanobacteria (Blue-Green Algae). *NZ. J. Mar. Freshw. Res.*, 21: 361-542

7) Shapiro, J. (1990) Current beliefs regarding the dominance by blue-greens: The case for the importance of $CO_2$ and pH. *Verh. Internat. Verein. Limnol.*, 24: 38-54.

8) Mitsutani, A., Uchida, A., Ishida, Y. (1987) Occurrence of blue-green algae and algal lytic bacteria in Lake Biwa. *Bull. Jpn. Soc. Microbiol. Ecol.*, 2: 21-28.

9) Stewart, W.D.P., Daft, M.J. (1976) *Algal lysing agents of freshwater habitats.* (eds) Skinner, F.A., Carr, J.G. Academic Press, London. pp 63-90.

10) Gromov, B.V., Ivanov, O.G., Mamkaeva, K.A., Avilova, I.A. (1972) A *flexibacter* that lyses blue-green algae. *Microbiol.*, 41: 952-956.

11) Yamamoto, Y., Kouchiwa, T., Hodoki, Y., Hotta, K., Uchida, H., Harada, K-I. (1998) Distribution and identification of actinomycetes lysing cyanobacteria in a eutrophic lake. *J. Appl. Phycol.*, 10: 391-397.

12) Safferman, R.S., Morris, M.E. (1964) Growth characteristic of the blue-green algal

virus LPP-1. *J. Bacteriol.*, 88:771-775.

13) Daft, M.J., McCord, S.B., Stewart, W.D.P. (1975) Ecological studies on algal-lysing bacteria in fresh waters. *Freshw. Biol.*, 5: 577-596.

14) Yamamoto, Y, (1978) Detection of algal lysing biological agents in lakes by the soft-agar overlayer technique. *Jpn. J. Limnol.*, 39: 9-14. (in Japanese).

15) Burnham, J.C., Collart, S.A., Daft, M.J. (1984) Myxococcal predation of the cyanobacterium *Phormidium luridum* in aqueous environments. *Limnol. Oceanogr.*, 137: 220-225.

16) Stewart, J.R., Brown, R.M. (1969) *Cytophaga* that kills or lyses algae. *Science,* 164: 1523-1524.

17) Shilo, M. (1970) Lysis of blue-green algae by *Myxobacteria. J. Bacteriol.*, 104: 453-461

18) Shilo, M. (1967) Formation and mode of action of algal toxins. *Bacteriol. Rev.*, 31: 180-193.

19) Daft, M.J., Stewart, W.P.D. (1971) Bacterial pathogens of freshwater blue-green algae. *New Phytol.*, 70: 819-829.

20) Daft, M.J., McCord, S., Stewart, W.P.D. (1973) The occurrence of blue-green algae and lytic bacteria at a waterworks in Scotland. *Wat. Treat. Exam.*, 22: 114-124.

21) Yamamoto, Y., Niizuma, S., Kuroda, N., Sakamoto, M. (1993) Occurrence of heterotrophic bacteria causing lysis of cyanobacteria in a eutrophic lake. *Jpn. J. Phycol.*, 41: 215-220.

22) Christiensen, P.J. (1977) The history, biology and taxonomy of the *Cytophaga* group. *Can. J. Microbiol.*, 23: 1599-1653.

23) Gnosspelius, G. (1978) Purification and properties of an extracellular protease from *Myxococcus virescens. J. Bacteriol.*, 133: 17-25.

24) Sudo, S., Dworkin, M. (1972) Bacteriolytic enzymes produced by *Myxococcus xanthus. J. Bacteriol.*, 110: 236-245.

25) Shilo, M. (1969) Morphological and physiological aspects of the interaction of *Bdellovibrio* with host bacteria. *Curr. Top. Microbiol. Immunol.*, 50: 174-204.

26) Burnham, J.C., Collart, S.A., Highison, B.W. (1981) Entrapment and lysis of the cyanobacterium *Phormidium luridum* by aqueous colonies of *Myxococcus xanthus* $PCO_2$. *Arch. Microbiol.,* 129: 285-294.

27) Quinn, G.R., Skerman, V.D.B. (1980) *Herpetosiphon* nature's scavenger. *Curr. Microbiol.,* 4: 57-62.

28) Casida, Jr. L.E. (1982) *Einsfer adhaerens* gen. nov. sp.: a bacterial predator of bacteria in soil. *Int. J. Syst. Bacteriol.*, 32: 339-345.

29) Burnham, J.C., Conti, S.F. (1984) *Bergey's Manual of Systematic Bacteriology* vol 1. Williams & Wilkins Co., Baltimore, pp 118-124.

30) Gnosspelius, G. (1978) Purification and properties of an extracellular protease from *Myxococcus virescens. J. Bacteriol.,* 133: 17-25.

31) Mitsutani, A., Takei, K. (1993) Purification and properties of a protease from a cyanobacteria-lytic bacterium *Lysobacter* sp. LB-1. *J. Shimo. Univ. Fish.,* 41: 65-75. (in Japanese)

32) Stewart, J.R., Brown, Jr. R.M. (1971) Algicidal non-fruiting myxobacteria with high G + C ratios. *Arch. Mikrobiol.,* 80:176-190.

33) Sallal, A.K. (1994) Lysis of cyanobacteria with *Flexibacter* spp isolated from domestic sewage. *Microbios,* 77: 57-67.

34) Rosenberg, E., Vaks, B., Zuckerberg, A. (1973) Bacteriocidal actions of an antibiotic produced by *Myxococcus xanthus. Antimicrob. Agents. Chemother.,* 4: 507-513.

35) Lang, N.J. (1968) The fine structure of the blue-green algae. *Ann. Rev. Microbiol.,* 22: 15-46.

36) Palmer, C.M., Maloney, T.E. (1955) Preliminary screening for potential algicides. *Ohio J. Sci.,* 55: 1-8.

37) Safferman, R.S., Morris, M.E. (1962) Evaluation of natural products for algicidal properties. *Appl. Microbiol.,* 10: 289-292.

38) Dakhama, A., Noue, J., Lavoie, M.C. (1993) Isolation and identification of antialgal substances produced by *Pseudomonas aeruginosa. J. Appl. Phycol.,* 5: 297-306.

39) Granhall, U., Berg, B. (1972) Antimicrobial effects of *Cellvibrio* on blue-green algae.

*Arch. Microbiol.*, 84: 234-242.

40) Whyte, L.G., Maule, A., Cullimore, D.R. (1985) Method for isolating cyanobacteria-lysing streptomycetes from soil. *J. Appl. Bacteriol.*, 58: 195-197.

41) Redhead, K., Wright, S.J.L. (1978) Isolation and properties of fungi that lyse blue-green algae. *Appl. Environ. Microbiol.*, 35: 962-969.

42) Redhead, K., Wright, S.J.L. (1980) Lysis of the cyanobacterium *Anabaena flos-aquae* by antibiotic-producing fungi. *J. Gen. Microbiol.*, 119: 95-101.

43) Reim, L.R., Shane, M.S., Cannon, R.E. (1974) The characterization of a *Bacillus* capable of blue-green bacterial activity. *Can. J. Microbiol.*, 20: 981-986.

44) Krauss, R.W. (1962) *Physiology and Biochemistry of Algae* (ed) Lewin, R.A. Academic Press, New York, 673-685.

45) Srivastava, B.S. (1970) Sensitivity and resistance of a blue-green alga *Phormidium mucicola* to streptomycin and penicillin. *Arch. Mikrobiol.*, 72: 182-185.

46) Yoshikawa, K., Adachi, K., Nishijima, M., Takadera, T., Tamaki, S., Harada, K-I., Mochida, K., Sano, H. (2000) β-Cyanoalanine production by marine bacteria on cyanide-free medium and its specific inhibitory activity toward cyanobacteria. *Appl. Environ. Microbiol.*, 66: 718-722.

47) Yamamoto, Y., Kouchiwa, T., Hodoki, Y., Hotta, K., Uchida, H., Harada, K-I. (1998) Distribution and identification of actinomycetes lysing cyanobacteria in a eutrophic lake. *J. Appl. Phycol.*, 10: 391-397.

48) Imamura, N., Motoike, I., Noda, M., Adachi, K., Konno, A., Fukami, H. (2000) Argimicin A, a novel anti-cyanobacterial compound produced by an algae-lysing bacterium. *J. Antibiot.*, 53: 1317-1319.

49) Watanabe, M.M., Kawauchi, M., Hiroki, M., Kasai, F. (2000) *NIES-Collection, List of Strains sixth Edition Microalgae and Protozoa*; Microbial Culture Collection, Natl. Inst. Environ. Stud., Japan: 30-32.

50) Braun, D., Pauli, N., Sequin, U., Zaehner, H. (1995) New butenolides from the photoconductivity screening of *Streptomyces antibioticus* (Waksman and Woodruff). *FEMS Microbiol. Lett.*, 126: 37-42.

51) Rashidan, K.K., Bird, D.F. (2000) Role of predatory bacteria in the termination of a cyanobacterial bloom. *Microbial Ecol.,* 16: 97-105.

52) Neefs, J.M., Van de Peer,Y., Hendriks, L., Watcher, R. (1990) Compilation of small ribosomal subunit RNA sequences. *Nucleic. Acids Res.,* 18 Suppl: 2237-2317.

53) Maeda, T., Murakami, M., Ohsugi., Furushita, M., Mitsutani, A., Shiba, Y. (1998) Perspectives of the development of 16S rDNA probe specific for algicidal and/or algal-lytic gliding bacteria. *Fish. Sci.,* 64: 861-865.

54) Thompson, J.D., Higgin, D.G., Gibson, T.J. (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic. Acids Res.,* 22: 4673-4680.

55) Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.,* 16: 111-120.

56) Saitou, N., Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.,* 4: 406-425.

57) Brock, T.D. (1966) *Principles of microbial ecology.* Prentice-hall, Inc. Englewood cliffs, New Jersey. : 80-88.

58) Koleshko, O.I., Potaenko, Y.S., Sharangovich, L.N. (1982) Numbers and species composition of heterotrophic bacteria in lake Naroch. *Mikrobiologia,* 51: 143-145.

59) Marfey, P. (1984) Determination of D-amino acids 2; Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. *Carlsberg Res. Commun.,* 49: 591-596.

60) Labrre, J., Thuriaux, P., Chauvat, F. (1987) Genetic analysis of amino acid transport in the facultatively heterotrophic cyanobacterium *Synechocystis* sp. strain 6803. *J. Bacteriol.,* 169: 4668-4673.

61) Herrero, A., Flores, E. (1990) Transport of basic amino acids by the dinitrogen-fixing cyanobacterium *Anabaena* PCC7120. *J. Biol. Chem.,* 265: 3931-3935.

62) Jensen, R.A., Stenmark-cox, S., Ingram, L.O. (1974) Mis-regulation of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase does not account for growth inhibition

by phenylalanine in *Agmenellum quadruplicatum*. *J. Bacteriol.*, 120: 1124-1132.

63) Hall, G.C., Jensen, R.A. (1980) Enzymological basis for growth inhibition by L-phenylalanine in the cyanobacterium *Synechocystis* sp. 29108. *J. Bacteriol.*, 144: 1032-1042.

64) Takase, S., Murakami, H. (1966) Studies on the fluorescence of Sake. I. Fluorescence spectrum of Sake and identification of harmane. *Agr. Biol. Chem.*, 30: 869-876.

65) Takeuchi, T., Ogawa, K., Iinuma, H., Suda, H., Ukita, K., Nagamatsu, T., Kato, M., Umezawa, H. (1973) Monoamine oxidase inhibitors isolated from fermented broths. *J. Antibiot.*, 16: 162-177.

66) Bax, A., Subramanian, S. (1986) Sensitivity-enhanced two-dimensional heteronuclear shift correlation NMR spectroscopy. *J. Magn. Reson.*, 67: 565-569.

67) Bax, A., Summers, M.F. (1986) Complete proton and carbon-13 assignments of coenzyme B-12 through the use of new two-dimensional NMR experiments. *J. Am. Chem. Soc.*, 108: 2093-2094.

68) Allen, J.R.F., Holmstedt, B.R. (1980) The simple β-carboline alkaloids. *Phytochemistry*, 18: 1573-1582.

69) Cheng, J., Mitchelson, K.R. (1997) Improved separation of six harmane alkaloids by high-performance capillary electrophoresis. *J. Chromatogr.*, 761: 297-305.

70) Bohlendorf, B., Forche, E., Bedorf, N., Gerth, K., Irschik, H., Jansen, R., Kunze, B., TrowitzchKienast, W., Reichenbach, H., Hofle, G. (1996) Indole and quinoline derivatives as metabolites of tryptophan in myxobacteria. *Liebigs Annalen*, 1: 49-53.

71) Arima, K., Kakinuma, A., Tamura, G. (1968) Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: Isolation, characterization and its inhibition of fibrin clot formation. *Biochem. Biophy. Res. Comm.*, 31: 488-494.

72) Sandrin, C., Peypoux, F., Michel, G. (1990) Coproduction of surfactin and iturin A, lipopeptides with surfactant and antifungal properties, by *Bacillus subtilis*. *Biotech. Appl. Biochem.*, 12: 370-375.

73) Bernheimer, A.W., Avigad, L.S. (1970) Nature and properties of a cytolytic agent

produced by *Bacillus subtilis*. *J. Gen. Microbiol.*, 61: 361-369.

74) Goelecki, J.H. (1988) Analysis of the structure and development of bacterial membranes (outer, cytoplasmic and intracytoplasmic membranes). *Meth. Microbiol.*, 20: 61-77.

75) Jurgens, U.J., Benz, R. (1988) Pore-forming activity of outer membrane extracts from the unicellular cyanobacterium *Synechocystis* sp. PCC 6714. *Naturforsch,* 44c: 165-169.

76) Kitatsuji, K., Miyata, H., Fukase, T. (1996) Isolation of microorganisms that lyse filamentous bacteria and characterization of lytic substance secreted by *Bacillus polymyxa*. *J. Fer. Bioeng.*, 82: 323-327.

77) Hashimoto, A., Nishikawa, T., Oka, T., Takahashi, K. (1992) Determination of free amino acid enantiomers in rat brain and serum by high-performance liquid chromatography after derivatization with N-*tert.*-butyloxycarbonyl-L-cysteine and *o*-phthalaldehyde. *J. Chromatogr.*, 581: 41-48.

78) Takahara, Y., Takeuchi, Y., Komura, I., Hirose, Y., Murao, S. (1979) Isolation of a new peptide antibiotic permetin A, from *Bacillus circulans*. *J. Antibiot.*, 32: 115-120.

79) Takehuchi, Y., Murai, A., Takahara, Y., Kainosho, M. (1979) The structure of permetin A, a new polypeptin type antibiotic produced by *Bacillus circulans*. *J. Antibiot.*, 32: 121-129.

80) Sugawara, K., Konishi, M., Kawaguchi, H. (1984) BMY-28160, a new peptide antibiotic. *J. Antibiot.*, 37: 1257-1259.

81) Murai, A., Amino, Y., Ando, T. (1985) Absolute configuration of the β-hydroxy fatty acid constituent of permetin A. *J. Antibiot.*, 38: 1610-1613.

82) Daft, M.J., Stewart, W.D.P. (1973) Light and electron microscope observations on algal lysis by bacterium CP-1. *New Phycol.*, 72: 799-808.