# Action Mechanism of a Selective Anti-cyanobacterial Compound, Argimicin A

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Argimicin A is a potent anti-cyanobacterial compound produced by one of algae-lysing bacteria, Sphingomonas sp. M-17. Since the compound seemed to exhibit selective activities against cyanobacteria and such selectivity were considered to be quite rare, the mode of action of argimicin A was investigated. Argimicin A showed a unique delayed action, i.e., the cyanobacterial cell division continued until at least 36 hours treatment even though the decrement of oxygen evolution has been observed at 24 hours treatment. The compound is concluded to be a photosynthetic inhibitor which interrupts electron transport chain prior to photosystem II. From the preliminary fluorescent spectrum of argimicin A treated cyanobacterial cells, the site of action was speculated to be photo energy transfer from a cyanobacterial specific complex of accessory protein pigments, phycobilisome, to photosystem II.

Many problems caused by blooms of cyanobacteria have been reported[1] and the frequency of the blooms seems to be increasing. From investigation of aquatic ecosystems, it has been disclosed that microorganisms called algae-lysing bacteria have the ability to kill the organisms of water blooms[2]. In the course of our studies on the interactions between blue-green algae and algae-lysing bacteria[3], we found a new anti-cyanobacterial pentapeptide, argimicin A, produced by of Sphingomonas sp. M-17[4]. Argimicin A exhibits strong activity against the toxic cyanobacteria Microcystis viridis and M. aeruginosa, but it showed no effects on Escherichia coli, Bacillus subtilis, and chlorophyta Chlorella vulgaris[4].

Cyanobacteria are prokaryotes and fine structures of cells such as a multilayered cell wall, ribosome, and so on are resembled to be those of Gram-negative bacteria[5], and these structures are sites of actions of many anti-bacterial agents. On the other hand, cyanobacteria are phototroph that are capable of oxygen generating photosynthesis including photosystem I and II and electron transport chain like chloroplasts[5], and the chain is a good target of usual photosynthetic inhibitors. Therefore, if the anti-cyanobacterial compound argimicin A is an ordinary anti-bacterial agent or an ordinary photosynthetic inhibitor, it should be active against bacteria or eukaryotic phytoplankton. However, argimicin A seemed to be not active toward them, thus we thought the mode of action is interesting and should be unique one. We will describe the activities against cyanobacteria and the unveiled action mechanism of argimicin A in this paper.

## Methods and Materials

### Preparation of Argimicin A

The fermentation of a producer Sphingomonas sp. M-17 was carried out in 1/10 Trypto-soy medium (Tryptone 1.5 g, soypeptone 1.5 g, NaCl 5 g, distilled water 1 liter) at 30°C with agitation and aeration for 48 hours. Argimicin A was isolated and purified from the cultured broth as described previously[4].

### Microalgae and Its Cultivation Conditions

Cyanobacteria Microcystis viridis NIES-102, M. aeruginosa NIES-298, Merismopedia tenuissima NIES-230, Oscillatoria agardhii NIES-204, Spirulina platensis NIES-45, Aphanizomenon flos-aquae NIES-81, Fischerella major NIES-592, Anabaena circinalis NIES-41, and

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*Synechosystis* sp. PCC6803 were used for experiments. All cyanobacteria except *A. circinalis* NIES-41 were cultivated in MA medium[6] at 25°C under fluorescent illumination (40 $\mu E/m^2$/second, 12L-12D cycle), and *A. circinalis* NIES-41 was grown in CB medium[6] at 20°C under fluorescent illumination (23 $\mu E/m^2$/second, 12L-12D cycle). Rhodophyceae *Cyanidium caldarium* NIES-250 was cultured in Allen medium[6] at 20°C under fluorescent illumination (23 $\mu E/m^2$/second, 12L-12D cycle). Cells in the exponential growth stage ware used for the experiments. Following experiments using *M. viridis* were carried out under the same culture conditions unless otherwise specified.

## Measurements of Minimum Inhibitory Concentration (MIC)

Ten $\mu l$ of serial diluted solution of argimicin A was added to the subcultured fluid ($1\times10^6$ cells/ml, 190 $\mu l$) of a microalga in a well of a 96-well tissue culture plate (Bacton Dickinson & Co., Franklin Lakes, N.J.) and incubated at adequate conditions for a week. Duplicate experiments were carried out and the minimum concentration of argimicin A causing a colorless culture fluid were decided as MIC. The MIC values against *M. viridis* of several antibiotics, ampicillin, polymyxin B, rifampicin, and streptomycin, were also determined in the same manner.

## Time Course of Action of Several Antibiotics and Argimicin A

Ten ml of MA medium containing an antibiotic or argimicin A of which concentration was eight to ten times of MIC was prepared. *M. viridis* was inoculated in to the medium (final approximately $5\times10^6$ cells/ml) and incubated under above mentioned condition or continuous dark light-condition. The cell numbers of *M. viridis* were counted every 24 hours till 72 hours by following methods. The fluid of *M. viridis* was placed on hemocytometer (depth 0.01 mm) and the image viewed with a supersensitive 0.4 mega-pixels CCD camera (Flovel HCC-600) equipped on a fluorescence microscope (Olympus BX50) was captured by a personal computer. The particles of fluorescence due to the photosynthetic pigments in living cells of *M. viridis* were counted using the public domain NIH image program[7].

## Time to Uptake of Argimicin A

*M. viridis* was inoculated in to 10 ml of MA medium (final approximately $5\times10^6$ cells/ml) and the medium containing argimicin A (0.2 $\mu g$/ml) and incubated for 4 hours. The fluid was filtered with membrane filter (Millipore UFC30HV00) and collected *M. viridis* cells were re-suspended in fresh MA medium (10 ml). After this procedure was repeated 10 times, the microalgal suspension was put into a well to 96-well tissue culture plate and was incubated. The cell densities were measured at an interval of 24 hours.

## Effects of Argimicin A on the Mixotrophic Growth of *Synechosystis* sp. PCC6803

Argimicin A (final concentration 0.2 $\mu g$/ml) was added to MA medium with and without an addition of glucose (final concentration 5 mM). *Synechosystis* sp. PCC6803 was inoculated these media (final approximately $5\times10^6$ cells/ml) and a time course of growth was monitored by cell counts at an interval of 24 hours. A photosynthetic inhibitor, dichlorophenyldimethylurea (DCMU; final concentration 0.23 $\mu g$/ml), was used as a control.

## Effects of Argimicin A on Oxygen Evolution

Two hundred ml of subcultured *M. viridis* in MA medium (approximately $9\times10^6$ cells/ml) was divided into10 test tubes, argimicin A (final concentration 0.2 $\mu g$/ml) was added to 5 tubes, and all of these test tubes were incubated. Every 12 hours, the cells of cultured *M. viridis* in a tube with and without argimicin A treatment were collected separately to measure cell densities, oxygen evolutional activities, and chlorophyll contents. Oxygen electrode (Rank Brothers Co.) was used for measurements of oxygen evolution. Collected cells were suspended in 2 ml of fresh MA medium, put into the electrode vessel and kept dark at 25°C until the dissolved oxygen was consumed adequately. Halogen lamp light (40 $\mu E/m^2$/second) was irradiated to the vessel, and recorded the concentration of dissolved oxygen. After the measurement of oxygen evolution, cells of 1.5 ml of the suspension were collected and extracted with 1.5 ml of acetone. The absorbance data at 630, 645, 663, and 750 nm of supernatant of the extract was allowed to estimate chlorophyll a contents[8].

## Effects of Argimicin A on Electron Transportation in Photosystem

Subcultured *M. viridis* (20 ml, approximately $5\times10^6$ cells/ml) was treated with argimicin A (final concentration 0.2 $\mu g$/ml) for 36 hours or DCMU (0.23 $\mu g$/ml) for 2 hours, separately. The cells were collected with a membrane filter and re-suspended in 2 ml of buffer (HEPES 50 mM, KCl 50 mM, MgCl$_2$ 5 mM, pH 7.0). By addition of following electron donor and acceptor to the cell suspension, activities of partial electron transportation around photosystem I (PSI) and II (PSII) were estimated from variation

of dissolved oxygen measured by oxygen electrode as mentioned above. Dichlorophenolindophenol (DCPIP: final concentration $50\,\mu M$) and methyl viologen ($50\,\mu M$) were added for measurement around PSI, and these reagents were the electron donor and acceptor, respectively, of the electron flow from cytochrome $b_6$-f complex to PSI (Exp. 1). An electron acceptor silicomolybdic acid (0.1 mM) was added for the electron flow from water to PSII[9] (Exp. 2).

## Amounts of Phycobilisome Pigments

Ten ml of *M. viridis* culture fluid treated with argimicin A (final concentration $0.2\,\mu g/ml$) for 24 and 36 hours and untreated were filtered, and collected cells were suspended separately in phosphate buffer (pH 6.0, 0.02 M, 2 ml) and braked down with ultra-sonication. The extract was centrifuged at 4°C, $100,000\,g$ for 1 hour. The absorbance of supernatant at 565, 620, 650, and 750 nm were measured. From these data, concentrations of phycocyanin, allophycocyanin, and phycoerithrin were determined according to expressions[10].

## Fluorescence Spectrum of Argimicin A Treated Culture Fluid

Fluorescence spectra of argimicin A (final concentration $0.2\,\mu g/ml$) treated *M. viridis* culture fluid ($6.0\times10^6$ cells/ml) for 24 and 36 hours were measured by fluorescent spectroscopy (Hitachi F-4500). Excitation wavelength was 580 nm, and a fluorescent spectrum was recorded in the range from 600 to 800 nm[11]. Fluorescence spectra of DCMU (final concentration $0.23\,\mu g/ml$, 2 hours) treated cells was also measured.

## Results and Discussion

The anti-microbial activity of argimicin A was displayed in Table 1. Argimicin A exhibits strong activity against cyanobacteria at the concentrations in the range of 0.013 to $5\,\mu g/ml$. Argimicin A did not show activity against eukaryotic algae (*Chlorella* and rhodophyceae) and yeast. The MIC values of antibiotics against *M. viridis* were ampicillin $5\,\mu g/ml$, polymyxin B $25\,\mu g/ml$, rifampicin $5\,\mu g/ml$, and streptomycin $0.3\,\mu g/ml$.

The growth curves of *M. viridis* treated with antibiotics and argimicin A under 12L-12D light cycle and continuous dark conditions were shown in Fig. 1. Under light-dark cycle conditions, the cell numbers of cultures treated for 24 hours with an antibiotic were decreased or not increased from the initial cell density. On the other hand, argimicin A showed unique delayed action, *i.e.*, argimicin A did not

Table 1. Anti-microbial activities of argimicin A.

| | Test organisms | MIC ($\mu g/ml$) |
|---|---|---|
| Cyanobacteria | *Microcystis viridis* NIES-102 | 0. 025 |
| | *Microcystis aeruginosa* NIES-298 | 0. 100 |
| | *Synechocystis* sp. PCC6803 | 0. 013 |
| | *Merismopedia tenuissima* NIES-230 | 0. 600 |
| | *Oscillatoria agardhii* NIES-204 | 0. 080 |
| | *Spirulina platensis* NIES-45 | 0. 600 |
| | *Aphanizomenon flos-aquae* NIES-81 | 0. 080 |
| | *Fischerella major* NIES-592 | 5. 000 |
| | *Anabaena circinalis* NIES-41 | 0. 100 |
| Rodophceae | *Cyanidium caldarium* NIES-250 | >100 |
| Chlorophyceae | *Chlorella kessleri* IAM C-143 | >200 |
| Yeast | *Saccharomyces cerevisiae* DKD-5D | >1000 |
| Bacteria | *Bacillus subtillis* IFO3027 | >1000 |
| | *Escherichia coli* IAM12119 | >1000 |

inhibit *M. viridis* growth for initial 24 hours and then exhibited the activity. Under dark conditions, only polymyxin B which caused acute damage to membrane demonstrated activity against *M. viridis* but the remained compounds did not show any effects. These results indicated that the action mechanism of argimicin A should not be caused by membrane damage.

One of the reasons why argimicin A showed the delayed action was considered that uptake of the compound needed long hours. In that case, *M. viridis* seemed to have been imbibed argimicin A for at least several hours, thus, the 4 hour-treatment with the compound should be no effect. However, the brief exposure was potent enough to inhibit the growth of *M. viridis*. This result means that the delayed action of argimicin A is not caused by uptake time into the cell.

The effects of argimicin A and a photosynthetic inhibitor DCMU on the growth of *Synechosystis* sp. PCC6803 in MA medium with and without glucose were shown in Fig. 2. Under the autotrophic condition, DCMU inhibited the growth of the organism at 24 hours in analogy with antibiotics. However, since argimicin A and DCMU did not effect in mixotrophic medium, argimicin A was concluded to be a photosynthetic inhibitor.

The examinations about a time course of action toward the photosynthesis were carried out. The effects of argimicin A on oxygen evolution of treated *M. viridis* was shown in Fig. 3. Oxygen evolution of cell suspension treated with argimicin A did not discriminate from that of the control until 12 hours and then it dropped away from 24 hours ahead of decrement of the cell numbers. It was noteworthy that the cell division did not stop until 36 hours and oxygen evolution had not completely lost in the case of

Fig. 1.   Time course of action of several antibiotics and argimicin A.
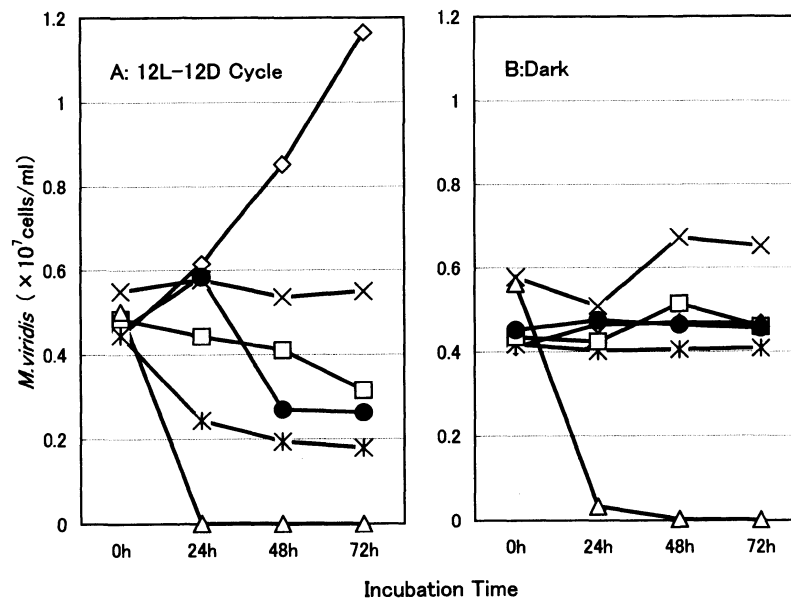


Light-condition A; 12L-12D cycle, B; continuous dark: Substances ●; argimicin A,
□; Streptomycin, ×; Rifampicin, ✳; Ampicillin, △; Polymyxin B, ◇; none (control).

Fig. 2.   Effects of argimicin A on the mixotrophic
growth of *Synechosystis* sp. PCC6803.
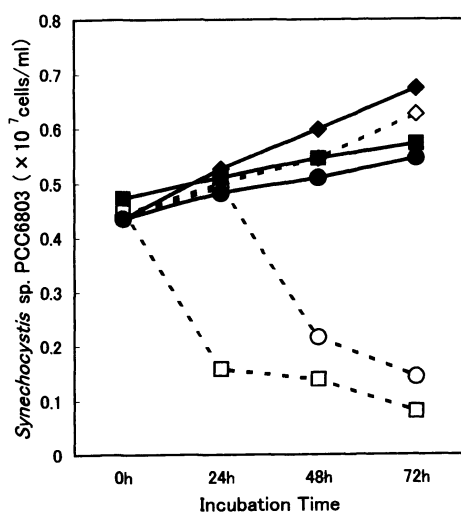


Solid lines; MA medium containing 5 mM glucose,
Broken line; MA medium: Substances ○ and ●;
argimicin A, □ and ■; DCMU, ◇ and ◆; none
(control).

argimicin A treatment, although the electron transport
inhibitor DCMU immediately restrains all of oxygen
evolution.

It was well known that DCMU interrupts the electron
flow between PSII and cytochrome $b_6$-f complex and the
partial electron transportation around PSI or PSII can be
observed by addition of the adequate electron acceptor and
donor as shown in Fig. 4[9]. A recovery of partial electron
transportation of argimicin A treated cells was calculated
based on that of DCMU treated cells. The recoveries
around PSI (Exp. 2) and PSII (Exp. 1) of argimicin A
treated cells were 98% and 0% of that of DCMU,
respectively. These data clearly indicated that the electron
transport chain was cut off prior to PSII by argimicin A. In
this portion, there are two kinds of energy transportations in
the light reaction, one of them is the electron transport
chain from water to PSII and another is the photo energy
transfer from antenna pigments to reaction center
chlorophyll a. One of the most important differences
between cyanobacterial and eukaryotic photosystems is
accessory pigments of PSII[11]. In the case of cyanobacteria,
a complex of specific accessory protein pigments,
phycobilisome, presents on a thylakoid membrane in the
neighborhood of PSII complex. Photo energy collected at

Fig. 3. Effects of argimicin A on oxygen evolution.
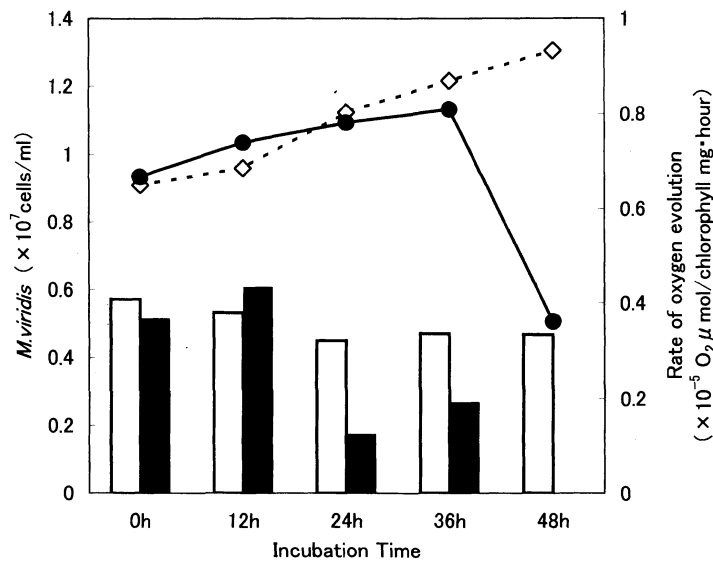


Lines are cell densities and bars are oxygen evolution. Solid line and black bars; argimicin A, Broken line and white bars; none (control).

Fig. 4. Schematic view of electron transfer in photosynthesis.[9]
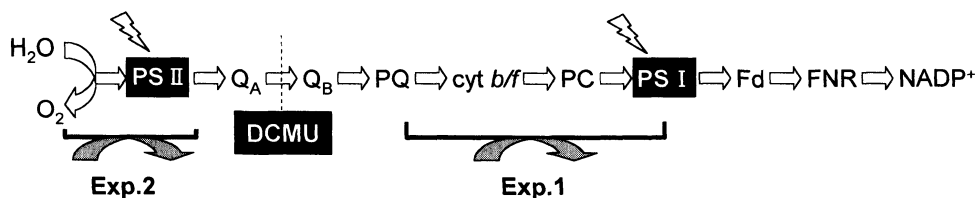


phycobilisome is transmitted to antenna chlorophyll a and then to reaction center chlorophyll a. Therefore, if argimicin A affected around phycobilisome, the selectivity of it against cyanobacteria was quite reasonable. However, the amounts of accessory protein pigments, phycocyanin, allophycocyanin, and phycoerithrin, of *M. viridis* cells that were treated with argimicin A for 24 and 36 hours were not significant differences from those of untreated cells, even though oxygen evolution of the treated cells had been decreased.

In the fluorescent spectrum of DCMU treated cells, an increment of fluorescence at 685 nm[12] which was caused by a photo energy overflow from PSII chlorophyll a was observed. On the other hand, in the fluorescent spectrum of argimicin A treated cells for 24 and 36 hours, a marked

increase of fluorescence at 655 nm was observed with elapsed time. The peak at the wave length was attributed to a characteristic fluorescence of allophycocyanin[13] and it suggested overflow of photo energy collected by phycobilisome. From these data, argimicin A was speculated to be a unique inhibitor of photo energy transfer from phycobilisome.

Argimicin A is a product of an algae-lysing bacterium. Algae-lysing bacteria in a water bloom might maintain the ecosystem balance in nature and it seems logical that a potent and selective active inhibitor of water bloom microbes would be produced. A selective anti-cyanobacterial compound is seemed to be quite rare, thus the action mechanism should be quite unique. The detailed mode of action of argimicin A including its delayed action and the

confirmation of photo energy transfer inhibition from phycobilisome are still under investigation.

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