New Algicidal Compounds from a Marine Algicidal Bacterium against *Cochlodinium polykrikoides*

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1. Introduction

In recent years, large-scale harmful algal blooms (HABs) have frequently occurred and have been causing mass mortalities of cultured fishes and bivalves in coastal waters. The dinoflagellate, *Cochlodinium polykrikoides* is one of the most frequently appearing harmful species responsible for fish mortality in Korea. The alleged economic loss caused by this species in Korea was about $85.5 million in 1995 (Kim *et al*., 1997). Algicidal bacteria are considered to be one of the potential tools to regulate HABs. We isolated a marine bacterium *Bacillus* sp. SY-1 from Masan Bay of Korea. We could detect different types of algicidal compounds in culture broth of *Bacillus* sp. SY-1. Here we report the isolation, structure elucidation, and algicidal activities of MS 1056, MS 1070, and MS 1084. We also demonstrated the algicidal activities of these compounds against a wide range of HAB forming species.

2. Materials and Methods

Algicidal bacteria against *C. polykrikoides* were isolated from Masan Bay while blooms of this alga were occurring (Jeong *et al*., 2000). Among 110 strains isolated from the same seawater samples, one strain that showed the strongest algicidal activity against *C. polykrikoides* was designated *Bacillus* sp. SY-1.

The partial 16S rDNA sequences were aligned using CLUSTAL W software Ver. 1.7. A phylogenetic tree was constructed by the neighbor-joining method. Bootstrap analyses of 100 replicates were carried out using MEGAversion 2.0.

Axenic clones were obtained by repeated washing with capillary pipettes. Isolates were routinely maintained in f/2-Si medium (Guillard, 1975) made of seawater. The cultures were grown under an illumination of 140 μE m⁻² s⁻¹ on a 12-h light:12-h dark cy-
cle at 20°C.

Algicidal compounds were isolated by the following procedures: *Bacillus* sp. SY-1 cells were grown in a 5-liter flask containing 2-liter of PPES-II medium at 25°C on a rotary shaker at 150 rpm for 3 days. The culture broth was centrifuged (2,000×g for 20min at 4°C Sakuma 50A-IC, Japan), and then filtered (0.2μm pore-size membrane filter) to obtain cell-free culture filtrate, followed by partition with ethyl acetate. The ethyl acetate layer was concentrated by a rotary evaporator. The residue was subjected to ODS open column chromatography (YMC-GEL, 5×10cm) and eluted with a solvent system from 100% water to 100% MeOH with a stepwise increase of MeOH concentration by 20%. The 80% MeOH fraction was further purified by reversed-phase HPLC: column, cosmosil 5C18-MS (10×250mm) linear gradient of MeOHin H2O containing 0.05% trifluoroacetic acid, 70100% in 40min; flow rate, 2.0 ml/min; UV detection at 210nm. Three compounds, MS 1056, MS 1070, and MS 1084 were isolated and their structures were determined by FAB mass spectrometry and 1H, 13C, and 2D NMR spectroscopy.

3. Results and Discussion

A bacterial strain that showed a strong algicidal activity against *C. polykrikoides* was a gram-positive, spore-forming, rod-like, oval bacterium. This strain exhibited the highest level of 16S rDNA similarity to *B. subtilis* ATCC 6633T. *B. subtilis* ATCC 6633T has been reported as a producer of mycosubtilin. Thus we designated this strain as *Bacillus* sp. SY-1.

Bioassay-guided purification of algicidal compounds was conducted by determining the algicidal activity against *C. polykrikoides*. The algicidal compounds were found in the culture broth, not in the cells. Fractionations by solvent extraction, ODS open column chromatography, and reversed-phase HPLC gave three pure algicidal compounds. Their molecular weights were determined as 1056, 1070, and 1084 by FAB mass spectrometry. Thus we designated these compounds as MS 1056, MS 1070, and MS 1084, respectively. At first, we determined the chemical structure of the most abundant compound, MS 1084.

MS 1084 was isolated as a colorless amorphous powder and showed UV maximum at 288nm(345). The IR(KBr) spectrum showed prominent broad peaks at 3319, 1655, and 1541cm⁻¹, that suggest the presence of amide carbonyl groups. Signals at wave numbers of 2925 to 2854 resulting from the CH stretching mode suggested the presence of an aliphatic chain. Ninhydrin assay revealed a negative reaction. However, the reaction was positive after acid hydrolysis, indicating the presence of a cyclic lipopeptide. The optical rotation of MS 1084 was also identified.

The positive FAB-MS spectrum (matrix:TEA) indicated an intense ion peak of [M+H]+ at m/z 1085. The molecular formula of MS 1084 was established as C₅₀H₈₈N₁₂O₁₄
by HRFAB-MS analysis [m/z 1085.5946 (M+H)', (4.9mmu)] and NMR spectral data. Its peptide nature was suggested by its $^1$H and $^{13}$C NMR spectra, and amino acid analysis of the hydrolysate indicated the presence of 1 mol each of Tyr, Glu, Pro, and Ser and 3 mol of Asp. However, Gln and Asn could not be distinguished from Glu and Asp, respectively. The $^1$H NMR (600 MHz, DMSO-$d_6$) of MS 1084 indicated the presence of amide NH protons ($^1$H 8.547.06) and $\beta$-protons ($^1$H 4.604.03) in a peptide, one para-substituted benzene ring ($^1$H 7.01, 6.64, each $d$, 2H), long methylene chain ($^1$H 1.431.12), and one terminal dimethyl group ($^1$H 0.85, $d$, 6H). The $^{13}$C NMR spectrum indicated the presence of 12 carbonyl groups (C 174.14169.98), one para-substituted benzene (C 155.8, 1C; 129.8, 2C; 127.8, 1C; 115.0, 2C), methylene carbons (C 41.3255.6), and two-methyl carbons (C 22.5).

Extensive NMR analyses including $^1$H-$^1$H COSY, HMQC, CT-HMBC, HOHAHA, and HSQC-TOCSY spectra revealed the spin systems of eight amino acids, containing $\alpha$-amino acid (AA). Most of the connections of amino acid residues in the sequence of MS 1084 were primarily deduced by the CT-HMBC correlations from $-H$ to C=O, however the correlation from $-H$ of Pro to C=O of Gln could not be observed. Finally, the NOESY correlations from $-H$ and $-H$ of Pro to $-H$ of Gln allowed the connection between Pro and Gln. Branching manner of the $\alpha$-amino acid side-chain was deduced from chemical shifts of CH$_3$ group in $^{13}$C NMR. Chemical shifts for CH$_3$ group of normal chain found in iturins are generally around 13.9 ppm, and those of anteiso CH$_3$ groups are 19.1 and 11.2 ppm. On the other hand, the $^{13}$C chemical shift for iso CH$_3$ groups in iturins or surfactin is 22.5 ppm. In MS 1084, there are CH$_3$ groups with the chemical shift at 22.5 ppm in a $\alpha$-amino acid unit. Therefore, the side chain of $\alpha$-amino acid in MS 1084 was deduced to be iso-form.

Moreover, the presence of twelve nitrogen atoms and the cyclic peptide nature of MS 1084 were also supported by the $^1$H-$^{15}$N HMQC and HMBC spectra. Therefore, the gross structure of MS 1084 was elucidated to be a cyclic peptide composed of eight amino acids with the following sequence: Asn Tyr Asn Gln Pro Ser Asn $\alpha$-amino acid with a (CH$_2$)$_4$CH(CH$_2$)$_2$ group as a side chain (iso-C$_{17}$, 3-$\alpha$-amino-15$^\circ$ methylhexadecanoic acid).

As a result of amino acids analysis on the hydrolysate of MS 1084, we found that MS 1084 is composed of seven usual amino acids: 1 mol each of Tyr, Glu, Pro, and Ser and 3 mol of Asp. The stereochemistry of these usual amino acids was determined by HPLC analysis of the acid hydrolysate derivatized with Marfey's reagent. MS 1084 was composed of 1 mol each of D-Tyr, L-Gln, L-Pro, and D-Asn and 2 mol of L-Asn. Since racemic Ser was not separated with this method, chiral GC analysis was performed. It revealed that D-Ser existed in MS 1084. Therefore, it is concluded that the absolute configuration of MS 1084 is identified: L-Asn D-Tyr D-Asn L-Gln L-Pro D-Ser L-Asn.
-amino acid with a (CH₂)₁₁CH(CH₃)₂ group as a side chain (3-amino-15-methylhexadecanoic acid).

A series of ¹H, ¹³C, and 2D NMR spectral analyses and amino acids analyses revealed that MS 1056 and MS 1070 have the same chemical structure as MS 1084, except for the side chain structure in the -amino acid unit. FAB-MS spectra of MS 1056 and MS 1070 showed an ion peak of [M+H]⁺ at m/z 1057 and 1071, respectively. ¹H NMR, ¹³C NMR, and CT-HMBC spectra of MS 1056 showed the presence of one terminal sec-butyl group, indicating that the side-chain of -amino acid was (CH₂)₉CH(CH₃)CH₂CH₃ (anteiso-C₁₅, 3-amino-12-methyloctadecanoic acid). This side chain has never been found from known mycosubtilins. Thus this compound was a new member of mycosubtilin families. Likewise, MS 1070 was shown to contain one terminal isopropyl group so that the side-chain of -amino acid was (CH₂)₁₀CH(CH₃)₂ (iso-C₁₆, 3-amino-14-methylpentadecanoic acid).

The number of this bacterial strain was at first small when the bloom of C. polykrikoides was dominant in Masan Bay, became the maximum just before the algal bloom disappeared, and fell down again. Thus there is a possibility that this algicidal bacteria is a playing an important role to control the bloom of C. polykrikoides in its natural habitat and that the compounds released by this bacteria would be useful as algicides that are harmless to the marine environments.

These lipopeptides, MS 1056, 1070, and 1084, would interact with the cytoplasmic membrane of fungi, increase ion permeability of the membrane by forming ion conducting pores, and lyse the fungal cells. We also examined the effects of MS 1084, on other organism than HAB-forming species. This compound was lethal to not only naked dinoflagellates and raphyophytes but also thecate dinoflagellates. Nevertheless, MS 1084 had quite selective algicidal activities against HAB-forming organisms and showed only weak growth inhibition of cyanobacteria. More studies on relation between algicidal activities and chemical structures would tell us more about the algicidal mechanism and give us some idea about compounds that could be used practically to control HABs in natural environment. Thus, this information will also be very useful for combinatorial chemistry to develop more desirable algicides.

4. Abstract

In screening of algicidal bacteria, we isolated a marine bacterium which had potent algicidal effects on harmful algal bloom (HAB) species. This organism was identified as a strain very close to Bacillus subtilis by 16S rRNA gene sequencing. This bacterium, Bacillus sp. SY-1, produces very active algicidal compounds against the harmful dinoflagellate Cochlodinium polykrikoides. We isolated three algicidal compounds (MS 1056,
1070, 1084) and identified them by amino acid analyses, fast atom bombardment mass spectrometry (FAB–MS), infrared spectroscopy (IR), $^1$H, $^{13}$C, and extensive two–dimensional nuclear magnetic resonance (2D NMR) techniques including $^1$H–$^{15}$N HMBC analysis. One of them, MS 1056, contains a b–amino acid residue with an alkyl side chain of C$_{15}$. MS 1056, 1070, and 1084 showed algicidal activities against C. polykrikoides with an LC$_{50}$ (6 hrs) of 2.3, 0.8, 0.6 µg/ml, respectively. These compounds also showed significant algicidal activities against other harmful dinoflagellates and raphidophytes. In contrast, MS 1084 showed no significant growth inhibition against various organisms coexisting with HAB species in natural environments, including bacteria, eukaryotic microalgae, and cyanobacteria, although it inhibited growth of some fungi and yeasts. These observations imply that algicidal bacterium Bacillus sp. SY–1 and its algicidal compounds could play an important role in regulating the onset and development of HABs in the natural environments.

References


