New bioactive secondary metabolites from cyanobacteria

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ACADEMIC DISSERTATION

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Front cover: *Nostoc* sp. 159 (photo by Liwei Liu) and chemical structures of 4-methylproline, nostosins and nostoweipeptins.

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List of original publications

I **Liwei Liu**, Lars Herfindal, Jouni Jokela, Tania Keiko Shishido, Matti Wahlsten, Stein Ove Døskeland and Kaarina Sivonen. 2014. Cyanobacteria from terrestrial and marine sources contain apoptogens able to overcome chemoresistance in acute myeloid leukemia cells. Marine Drugs. 12(4): 2036-2053.

II **Liwei Liu**, Jouni Jokela, Matti Wahlsten, Bahareh Nowruzi, Perttu Permi, Yue Zhou Zhang, Henri Xhaard, David P. Fewer, and Kaarina Sivonen. 2014. Nostosins, trypsin inhibitors isolated from the terrestrial cyanobacterium *Nostoc* sp. strain FSN. Journal of Natural Products. 77(8):1784-1790.

III **Liwei Liu**, Jouni Jokela, Lars Herfindal, Matti Wahlsten, Jari Sinkkonen, Perttu Permi, David P Fewer, Stein Ove Døskeland, Kaarina Sivonen. 2014. 4-methylproline guided natural product discovery yielded novel cyclic nostoweipeptins and nostopeptolides. ACS Chemical Biology, in press.

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The Author's contribution

Liwei Liu participated in the design of the studies, performed most of the experiments, analyzed the results together with expert co-authors and wrote manuscripts.

Abbreviations

ABC transporter	ATP-binding cassette transporter
AML	acute myeloid leukemia
Arg	arginine
Asp	aspartic acid
Bcl-2	B-cell lymphoma-2
Choi	2-carboxy-6-hydroxyoctahydroindole
EC_{50}	half maximal effective concentration
ESI	electrospray ionization
Gly	glycine
HEK293	human embryonic kidney 293 cells with SV40 large T
Hhpba	2-hydroxy-4-(4-hydroxyphenyl)butanoic acid
IC_{50}	half minimal inhibitory concentration
Ile	isoleucine
LC-MS	liquid chromatography mass spectrometry
Leu	leucine
MS	mass spectrometry
NMR	nuclear magnetic resonance
NRPS	non-ribosomal peptide synthetase
OATP	organic anion-transporting polypeptide
orf	open reading frame
PAR	protease-activated receptors
PCR	polymerase chain reaction
Phe	phenylalanine
PKS	polyketide synthase
Pro	proline
QTOF	quadrupole-time-of-flight
RP-HPLC	reversed phase-high performance liquid chromatography
Ser	serine
Sp.	species
Thr	threonine
Tyr	tyrosine
UPLC	ultra performance liquid chromatography
Val	valine
3-mPro	3-methylproline
4-mPro	2S, 4S-4-methylproline
4-OH-Pro	4-hydroxyl-proline
4-OH-3-mPro	4-hydroxyl-3-methylproline

Abstract

Cyanobacteria are one of the most widespread microorganisms on earth, and they are found in almost all ecosystems, from fresh and marine water to terrestrial environments. Cyanobacteria received a great deal of attention as prolific producers of bioactive secondary metabolites. The aim of this study was to screen and characterize novel bioactive natural products, which present biological activity against acute myeloid leukemia (AML) cells, trypsin, and hepatotoxin microcystin/nodularin, from cyanobacteria isolated from different habitats.

Of 40 cyanobacteria strains isolated from the Baltic Sea shore and lichen symbiosis were used to look for anti-cancer compounds which could induce apoptosis of AML cells. Half of these cyanobacteria strains contain apoptosis-inducing anti-AML activity, and seven cell extracts contain potent apoptosis-induced activities against AML. Two of apoptogens were confirmed to be new variants of hassallidin and scytophycin.

Cyanobacteria produce a plethora of serine protease inhibitors with a broad range of chemical structures. Nostosins, new trypsin inhibitors, were discovered through the analysis of secondary metabolites in methanol extracts of *Nostoc* sp. FSN. The data showed that they were linear peptides with three residues: Hhpba, L-Ile and L-argininal/argininol. Nostosin A and B inhibited trypsin with IC_{50} values of 0.35 μM and 55 μM, respectively. Computer docking data indicated that the argininal aldehyde and guanidino groups played the most crucial role in the efficient inhibition of trypsin.

The bloom-forming cyanobacterium *Nodularia spumigena* has been reported to produce several bioactive peptides. In this study, *Nodularia spumigena* strains from the Baltic Sea were discovered to produce new hybrid peptides named as pseudoaeruginosins. Because of the low abundance in cyanobacteria strains, pseudoaeruginosins were chemically synthesized and the structure was confirmed with LC-MS and NMR. Pseudoaeruginosins contained all the structure blocks of aeruginosin NAL2 except that the Choi was replaced by 4-methylproline (4-mPro), which is a specific subunit of spumigins in *N. spumigena*. The aldehyde group

containing pseudoaeruginosin NS1 exhibited IC_{50} of 0.19 μM against porcine trypsin. The biosynthesis of pseudoaerugnosins was proposed to be a joint action of aeruginosin and spumigin biosynthesis pathways in *N. spumigena*.

4-mPro is a rare non-proteinogenic amino acid, which has been found in a small number of bioactive compounds identified from cyanobacteria. A combination of PCR and LC-MS were used to screen 116 cyanobacteria strains for the production of 4-mPro. A total of 12 strains were confirmed to produce 4-mPro, and eleven new cyclic peptides belonging to two groups were identified from two *Nostoc* spp. XPORK 5A and UK2aImI strains, respectively. Among 11 peptides, the tested five could inhibit the organic anion transporters OATP1B1/B3 to block the induction of apoptosis of hepatocytes by microcystin/nodularin.

In this research, a total of two new trypsin inhibitors and five new cyclic peptides with the antitoxic bioactivity against microcystin/nodularin were identified. It was shown that cyanobacteria from both marine and terrestrial environments are a good resource for discovering new bioactive compounds. These findings increased the diversity of bioactive secondary metabolites characterized from cyanobacteria and provide new leads for drug research.

1. Introduction

1.1 Cyanobacteria

Cyanobacteria are among the most successful microorganism on earth and were most likely present since 2600 million years ago (Hedges et al. 2001). The emergence of cyanobacteria is associated with the change of atmosphere from a reductive to oxidative state (Blankenship and Hartman 1998; Summons et al. 1999). Cyanobacteria are Gram-negative bacteria, which are the only bacteria capable of performing oxygenic photosynthesis. They were regarded as algae, but finding the typical bacterial cell structure finally led to their placement into prokaryotic kingdom (Stanier et al. 1978). Cyanobacteria are adaptive microorganisms that could be found almost all the ecosystems from terrestrial to aquatic habitats including extreme environments such as hot springs, hypersaline environments and glaciers (Garcia-Pichel et al. 1998; Papke et al. 2003; Comte et al. 2007).

1.2. Cyanobacterial secondary metabolites

With the emergence of antibiotic and chemotherapy resistance, more and more medicines gradually lose their effect. In 2013, more than 2 million infections were caused by antibiotic resistant bacteria, and 23 thousand people have died in US (CDC report. 2013). There is an urgent need for new pharmaceuticals. Even though more and more chemical libraries were built up for screening new drugs, the natural products remain an important resource for drug leads (Cragg and Newman 2013). The screening of new drugs could be from many different sources, such as plants, microbes and animals (Debnath et al. 2007; Jachak and Saklani 2007; Bah et al. 2013; Gomes et al. 2014). Most of those bioactive natural products are explored from plants and microbes (Berdy 2012). As one of the most successful and ancient microorganisms, cyanobacteria have shown a great potential in drug discovery (Singh et al. 2005; Tan 2007; Dixit and Suseela 2013).

Cyanobacteria have been considered as good resources to find novel bioactive natural products, which are mostly from the secondary metabolites pool (Welker

and von Döhren 2006; Sivonen and Börner 2008). The cyanobacterial secondary metabolites present diverse chemical structure, and many of them show different bioactivities, such as anti-cancer, anti-fungal, anti-bacteria, trypsin inhibitor, neurotoxic and hepatotoxic (Singh et al. 2005; Dixit and Suseela 2013). Most of these compounds are synthesized from the machinery of NPRS (non-ribosomal peptide synthetase) or mixed with PKS (polyketide synthase) (Welker and von Döhren 2006). However, some of them are from ribosomal pathways, such as microviridins and cyanobactins (Arnison et al. 2013). The role of these natural products is still not clear for the producers. They have been considered as potential drug leads (Tan 2007; Dixit and Suseela 2013).

1.2.1. Anti-cancer compounds in cyanobacteria

Cyanobacteria are well-known to produce a number of anti-cancer compounds. Many of these anti-cancer compounds target tubulin or actin filaments of eukaryotic cells, such as cryptophycin, curacin A and dolastatin 10 **(Figure 1)**. Dolastatin 10 was first isolated from *Dollabella auricularia*, a species of large sea slug (**Figure 1)** (Pettit et al. 1987), and found later in cyanobacterium *Symploca* sp. (Luesch et al. 2001). As a strong microtubule inhibitor, dolastatin 10 can arrest cells in mitotic division. Curacin A is an anti-cancer compound with a unique chemical structure which includes a cyclopropyl-substituted thiazoline heterocyclic ring isolated from *Lyngbya majuscula* (**Figure 1)**. It is a strong antiproliferative agent that inhibits microtubule assembly (Gerwick et al. 1994). Cryptophycin is an effective anti-fungal depsipeptide identified from *Nostoc* sp. GSV 224 (Schwartz et al. 1990). Later, it was proven that cryptophycin was a good cytotoxic agent against L1210 leukemia cells, the ovarian carcinoma and drug resistant breast cancer cells (Smith et al. 1994; Trimurtulu et al. 1994). A number of cryptophycin analogues were synthesized, the most potent of which is the cryptophycin 52, which has one methyl group on the C-terminal residue comparing with cryptophycin A (**Figure 1)**, which binds the microtubule ends to disturb the cell mitosis (Panda et al. 1998).

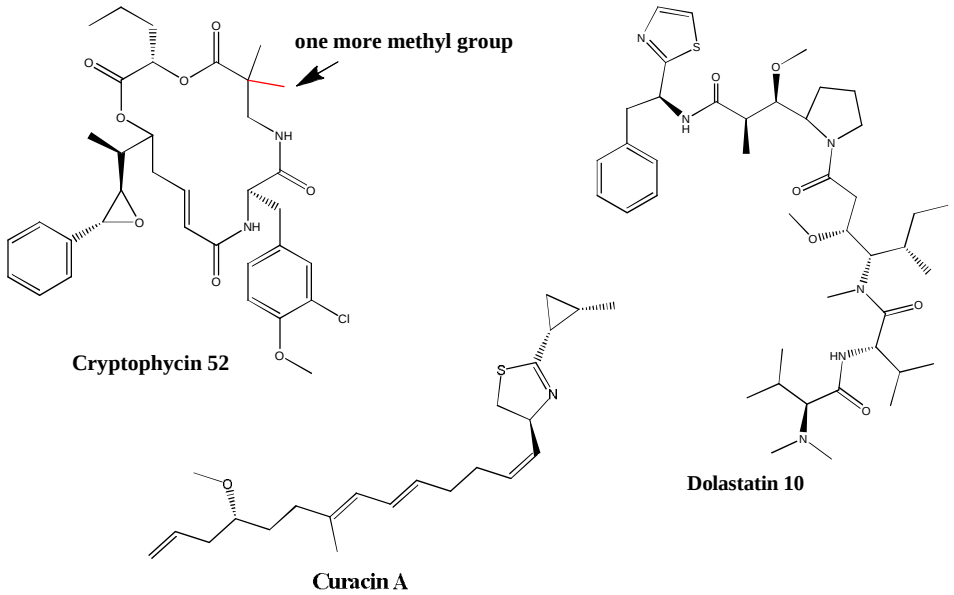


Figure 1. The chemical structures of cryptophycin 52, curacin A, and dolastatin 10, all well studied anti-cancer compounds from cyanobacteria (Pettit et al. 1987; Gerwick et al. 1994; Wagner et al. 1999).

Acute myeloid leukemia is one of the cancers with the characteristics of fast-growing abnormal white blood cells in the bone marrow. Until now, there is no very effective and safe treatment for this special disease. The common strategy is to use chemotherapy to kill the leukemia cells (Mehta and Hoffbrand 2010). However, the increasing trend of drug resistance has limited utility of anti-AML drugs (August et al. 2013; Craddock et al. 2013). Nowadays, marine cyanobacteria are emerging as new source to find bioactive natural products. Many new cytotoxic compounds against cancer cells were found from marine cyanobacteria, especially from *Lyngbya* spp. (Tan 2007). However, there is only one study which has explored anti-leukemia compounds from marine cyanobacteria. Oftedal et al. (2010) reported many cyanobacteria isolates from Baltic Sea contained anti-leukemia activity (Oftedal et al. 2010). Therefore, it is necessary to make use of marine cyanobacteria to find more effective anti-leukemia compounds in order to meet the increasing need of medicine in the treatment of leukemia diseases.

1.2.2. Trypsin inhibitors in cyanobacteria

As one member of protease family, trypsin has been shown to play an important role in a variety of cancers or brain development. The trypsin-like protease (or called trypsinogen) has been found in many types of carcinomas, such as ovarian neoplasm, pancreatic cancer, lung neoplasm and colorectal cancers (Nyberg et al. 2006). The high level of tumor-associated trypsinogen 2 is able to cause increased occurrence of tumors (Koivunen et al. 1990). In the brain, trypsin IV has shown wide distribution. Through the activation of PAR (protease-activated receptors)-1 or PAR-2, trypsin IV could perform neuroprotection from toxic insults in the brain (Wang et al. 2008). In addition, trypsin IV also proposed to contribute on neurogenic inflammation and pain by inducing PAR-2-dependent hyperalgeisia to thermal and mechanical stimuli (Wang et al. 2008). Therefore, trypsin or trypsin-like protease could be used as a good target to design new drugs, and trypsin inhibitors will be one of the idea drug leads.

As the good resources for looking for new natural products, cyanobacteria have been shown to synthesize a variety of trypsin inhibitors with diverse chemical structures (**Table 1)**. Among them, aeruginosins are one of the best-known trypsin inhibitors discovered from cyanobacteria. Up to now, more than 55 variants have been studied (Ersmark et al. 2008, Liu et al. 2014), and some new variants have been uncovered recently by Fewer et al. (2013). The biosynthetic gene cluster was first elucidated by Ishida et al. (2007). The aeruginosin gene cluster is composed of 9 genes. There are one PKS and two NRPS, which are responsible for incorporation of the subunits of aeruginosin 126 (**Figure 2)**. Another group of linear trypsin inhibitors, spumigins, was also discovered from cyanobacteria (Fujii et al. 1997; Fewer et al. 2009; Anas et al. 2012; Mazur-Marzec et al. 2013). The biosynthesis gene cluster of spumigin was identified by Fewer et al. (2009). There are six genes in the cluster (**Figure 2)**. The genes of *spuA* and *spuB* code for two non-ribosomal peptide synthetases, which are responsible for synthesis of spumigin E, a potent trypsin inhibitor. In addition, *spuD* and *spuE* show more than 90% identity to *nosE* and *nosF* genes, which code two enzymes, a zinc-dependent long-

chain dehydrogenase and a Δ^1-pyrroline-5-carboxylic acid (P5C) reductase (Luesch et al. 2003; Fewer et al. 2009).

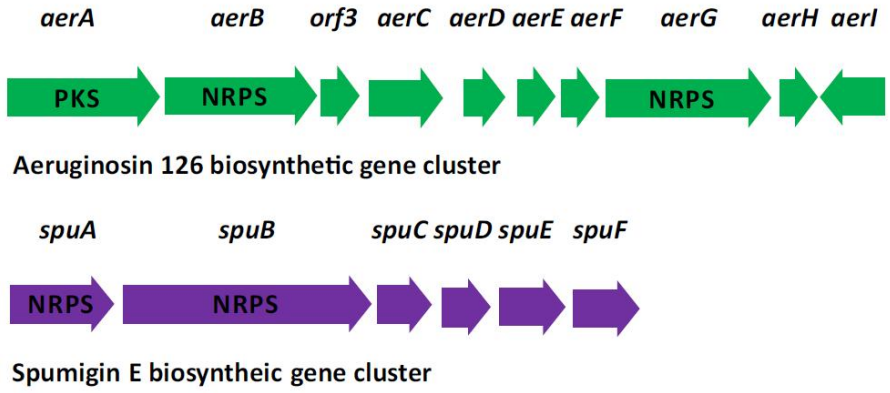


Figure 2. The biosynthesis gene clusters of aeruginosin 126 and spumigin E (Ishida et al. 2007; Fewer et al. 2009).

Cyanobacteria were shown to synthesize other trypsin inhibitors, such as microphycin AL828, A90720A, pompanopeptins, planktocyclin, banyasides and symplocamide A (**Figure 3** and **Table 1)**. Even though they show diverse chemical structures, they all have the same bioactivity. In order to study how the cyanobacteria trypsin inhibitor interact with trypsin, aeruginosin 98-B was cocrystallized with trypsin (Sandler et al. 1998). It was shown that Ser195, His57 and Asp102 in trypsin form strong interaction with the corresponding sites on aeruginosin 98-B. Because of the potent bioactivity and clear functional mechanism, aeruginosin 98-B was utilized as a lead structure to design new thrombin inhibitors used in the treatment of thrombosis (Radau et al. 2003). Besides trypsin inhibitors, cyanobacteria are also well-known to produce other protease inhibitors, such as the inhibitors of chymotrypsin, elastase, cathepsin, leucine and aminopeptidase (**Table 1)**.

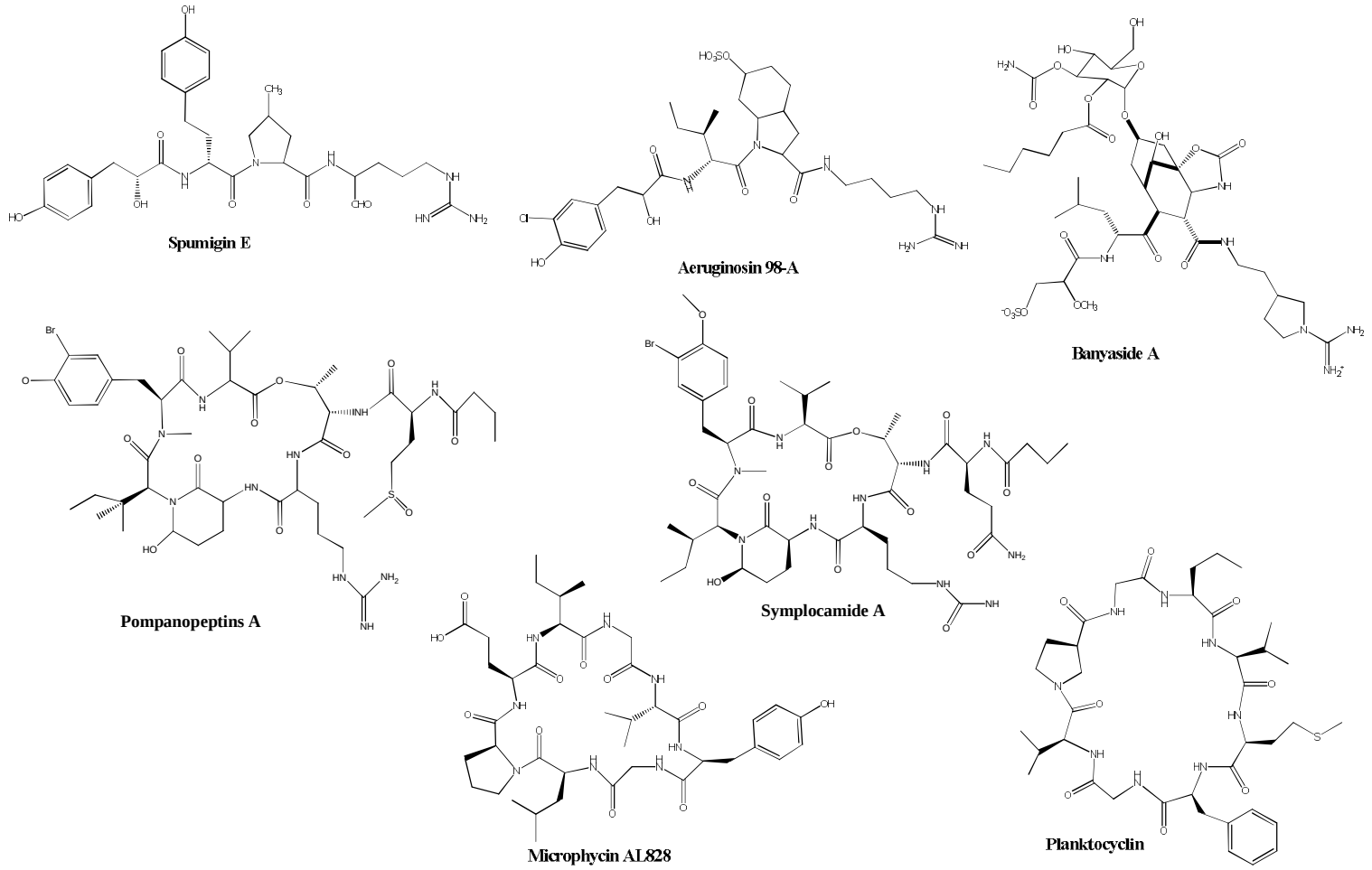


Figure 3. Examples of the trypsin inhibitors discovered from cyanobacteria.

Table 1. Protease inhibitors discovered from cyanobacteria show diverse bioactivities and structures. M: monoisotopic mass.

Protease inhibitor	Cyanobacterial strain	M (Da)	Inhibitor against	Reference
Spumigin E	*Nodularia spumigena*	610	trypsin	Fewer et al. 2009
Aeruginosins 98A	*Microcystin aeruginosa*	687	trypsin	Murakami et al. 1995
Microphycin AL828	*Microcystis* sp.	828	trypsin	Gesner-Apter and Carmeli 2008
A90720A	*Microchaete loktakensis*	1040	trypsin	Bonjouklian et al. 1996
Pompanopeptins	*Lyngbya confervoides*	1069	trypsin	Matthew et al. 2008
Planktocyclin	*Planktothrix rubescens*	800	trypsin/chymostrypsin	Baumann et al. 2007
Banyasides A	*Nostoc* sp.	990	trypsin/thrombin	Pluotno and Carmeli 2005
Symplocamide A	*Symploca* sp.	1050	trypsin/chymostrypsin	Linington et al. 2008
Micropeptin MM836	*Microcystis* sp.	836	chymostrypsin	Zafrir-Ilan and Carmeli 2010
Cyanopeptolin 954	*Microcystis aeruginosa*	954	chymostrypsin	von Elert et al. 2005
Anabaenopeptin MM823	*Microcystis* sp.	823	chymotrypsin/elastase	Zafrir-Ilan and Carmeli 2010
Molassamide	*Dichothrix utahensis*	962	chymotrypsin/elastase	Gunasekera et al. 2010
Oscillapeptin A	*Oscillatoria agardhii*	1165	chymotrypsin/elastase	Shin et al. 1995
Largamide A	*Lyngbya confervoides*	841	elastase	Matthew et al. 2009
Scyptolin A	*Scytonema hofmanni*	980	elastase	Matern et al. 2001
Kempopeptins A	*Lyngbya* sp.	990	elastase	Taori et al. 2008
Microviridin G	*Nostoc minutum*	1805	elastase	Murakami et al. 1997
Grassypeptolide A	*Lyngbya confervoides*	989	protease	Kwan et al. 2014
Lyngbyastatin 5	*Lyngbya* sp.	1056	protease	Taori et al. 2007
Gallinamide A	*Schizothrix* sp.	592	cathepsin	Miller et al. 2014
Grassystatin A	*Lyngbya cf. confervoides*	1174	cathepsin	Kwan et al. 2009
Microginins 299-C/D	*Microcystis aeruginosa*	852/756	leucine aminopeptidase	Ishida et al. 1998

1.2.3. Bioactive compounds with 4-methylproline in cyanobacteria

The 4-mPro is a special non-proteinogenic amino acid, in which the methyl group is connected to the second carbon of the side chain. In cyanobacteria, it was discovered in nostopeptolide A and nostocycleopeptide A (Golakoti et al. 2000; Golakoti et al. 2001). However, both of these compounds showed no bioactivity during the discovery. The biosynthesis of 4-mPro has been revealed by Luesch et al. (2003). A zinc-dependent long-chain dehydrogenase and a Δ1-pyrroline-5-carboxylic acid (P5C) reductase, which are coded by genes *nosE* and *nosF* in *Nostoc sp*. GSV 224, convert L-Leu in to 4-mPro. Later on, 4-mPro biosynthetic genes were also found in the biosynthetic gene cluster of nostocycleopeptin A in *Nostoc* sp. ATCC 53789, nostopeptolide A in *Nostoc* sp. GSV 224, nostopeptolide in *Nostoc punctiforme* PCC 73102 and spumigin E in *Nodularia spumigena* CCY9414 (**Figures 4** and **5)** (Hoffmann et al. 2003; Becker et al. 2004; Hunsucker et al. 2004; Fewer et al. 2009).

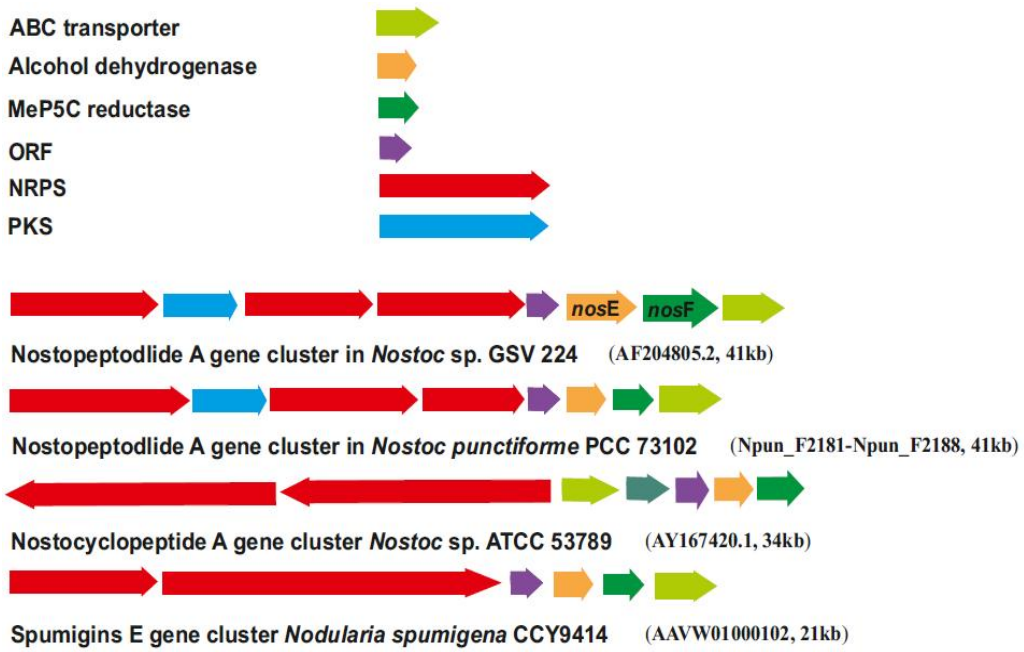


Figure 4. The biosynthetic gene clusters of 4-mPro-containing compounds discovered in cyanobacteria (Hoffmann et al. 2003; Becker et al. 2004; Hunsucker et al. 2004; Fewer et al. 2009).

The end products of these four gene clusters were also identified, but only spumigin E was shown to be a strong trypsin inhibitor (Fewer et al. 2009), while no biological activity were reported to the other three compounds.

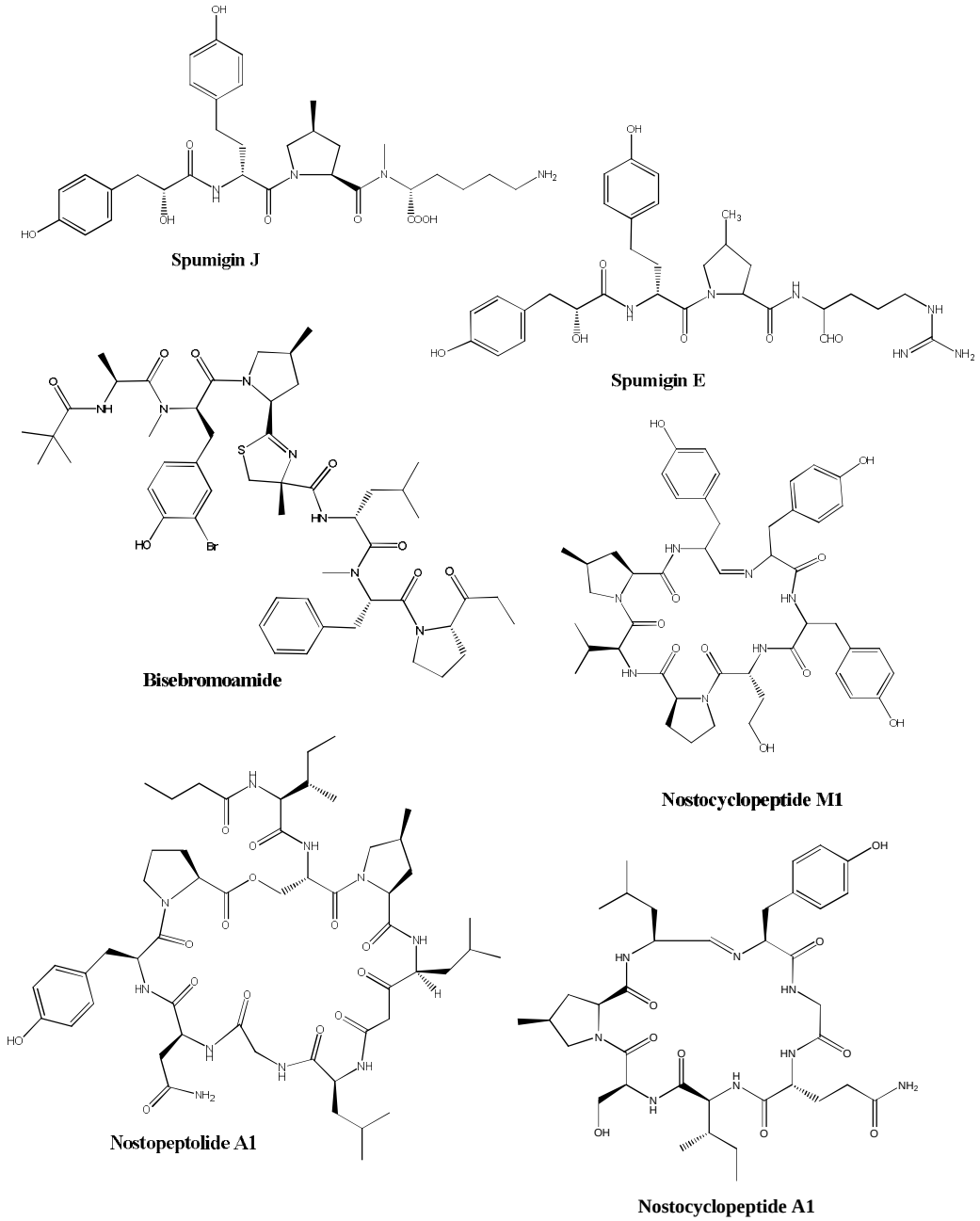


Figure 5. Examples of 4-mPro-containing compounds isolated from cyanobacteria.

In addition to 4-mPro, some other forms of methylproline have been discovered from the natural peptides produced from cyanobacteria, such as 3-OH-4-mPro, 4-OH-3-mPro and 3-mPro (**Table 2** and **Figure 6)** (Helms et al. 1988; Okino et al. 1997). 3-mPro was first found in scytonemin A. It is a cyclic peptide with a molecular mass 1462, which was found from cyanobacteria strain *Scytonema* sp. U-3-3. It contains one 3-mPro and two 4-OH-3-mPro (Helms et al. 1988). Though scytonemin A was discovered very early, its biosynthetic gene cluster has not been revealed. The 3-OH-4-mPro was explored from nostopeptins and insulapeptolides, which are elastase inhibitors (**Figure 6)** (Okino et al. 1997; Mehner et al. 2008). Both of them have a cyclic ring and a short branch chain. Besides the great difference on the side chain, the ring structures are almost the same.

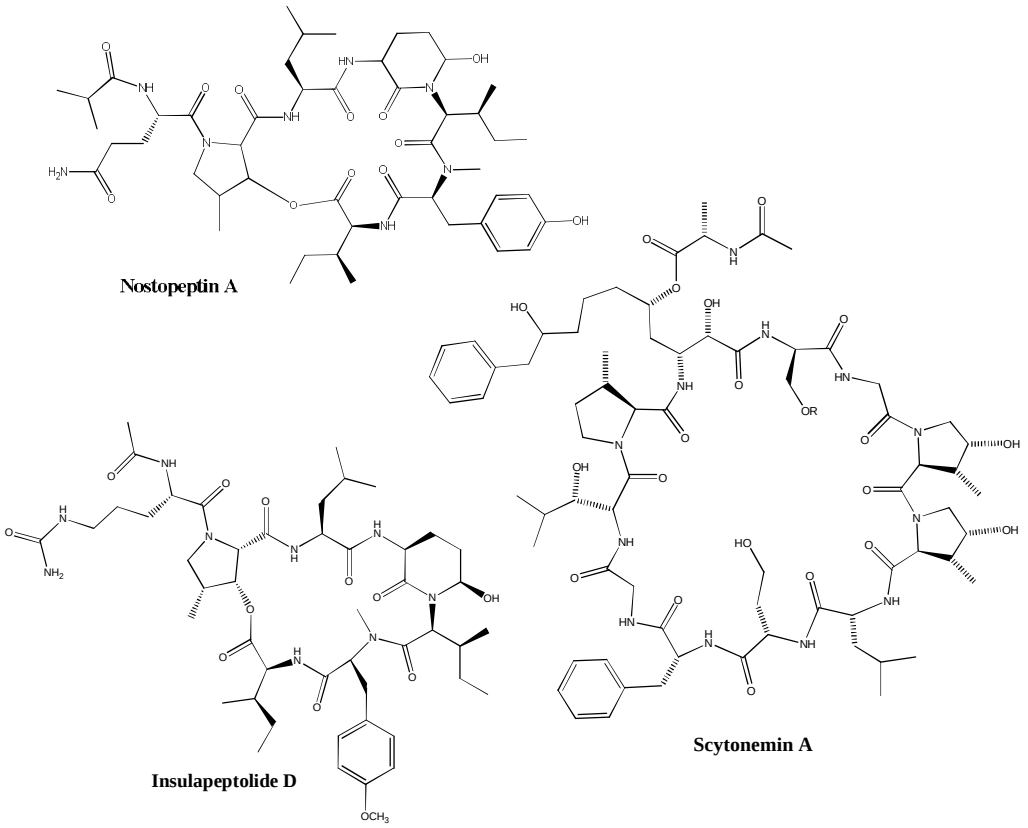


Figure 6. The chemical structures of scytonemin A, nostopeptin A and insulapeptolid D. (Helms et al. 1988; Okino et al. 1997; Mehner et al. 2008)

Methylproline amino acids were also found in natural products such as bottromycin D, perthamide, pteratides and leucinostatins synthesized by sponges, fungi and actinobacteria (**Table 2)**. Bottromycin D is a new anti-bacterial compound produced by *Streptomyes* spp. (Hou et al. 2012). Hou et al. (2012) used both structure and genome sequences to obtain its biosynthesis gene cluster. In cyanobacteria, the 4-mPro was synthesized by two enzymes, which are A zinc-dependent long-chain dehydrogenase and a Δ1-pyrroline-5-carboxylic acid (P5C) reductase (Luesch et al. 2003). The biosynthesis of methylproline in bottromycin is different from 4-mPro in cyanobacteria, because its methyl group is added by methyltransferase (Hou et al. 2012). The data revealed new source of methylproline amino acids.

Table 2. Methylproline-containing compounds isolated from cyanobacteria, sponges and other microbes.

Name	Strain	Bioactivity	mPro type	**Reference**
Scytonemin A	*Scytonema* sp.	calcium antagonistic property	3-mPro	Helms et al. 1988
Bottromycin D	*Streptomyces* spp.	anti-bacteria	3-mPro	Hou et al. 2012
Nostopeptins A/B	*Nostoc minutum*	anti-elastase/chymotrypsin	3-OH-4-mPro	Okino et al. 1997
Insulapeptolide A/B/C/D	*Nostoc insulare*	anti-elastase	3-OH-4-mPro	Mehner et al. 2008
Spumingin G/469/582/652*	*Nodularia spumigena*	-	4-mPro	Mazur-Marzec et al.
Spumingin E	*Nodularia spumigena*	anti-trypsin inhibitor	4-mPro	Fewer et al. 2009
Spumingin J	*Anabaena compacta*	anti-thrombin/cathepsin	4-mPro	Anas et al. 2012
Spumigin A/B	*Nodularia spumigena*	-	4-mPro	Fujii et al. 1997
Nostocylopeptide A1/A2	*Nostoc* sp.	-	4-mPro	Golakoti et al. 2001
Nostocyclopeptide M1	*Nostoc* sp.	antitoxin against microcystin	4-mPro	Jokela et al. 2010
Enopeptin A	*Streptococcus hawaiiensis*	anti-bacteria	4-mPro	Hinzen et al. 2006
Perthamide C/D	*Theonella swinhoei*	anti-inflammatory	4-mPro	Festa et al. 2009
PteratidesI/ II	*Pterula* sp.	anti-leukemia	4-mPro	Chen et al. 2006
Bisebromoamide	*Lyngbya* sp	anti-protein kinase	4-mPro	Teruya et al. 2009
Perthamide B	*Theonella* sp.	anti-1L-1b	4-mPro	Gulavita et al. 1994
Perthamide G-K	*Theonella swinhoei*	anti-inflammatory	4-mPro	Festa et al. 2012
Mutremdamide A	*Theonella species*	Peptide Inhibitors of HIV-1	4-mPro	Plaza et al. 2010
Perthamide E/F	*Theonella swinhoei*	anti-TNFa/IL-8	4-mPro	Festa et al. 2011
Nostopeptolide A1/A2/A3	*Nostoc* sp.	-	4-mPro	Golakoti et al. 2000
Leucinostatins A/B	*Paecilomyces lilacinus*	anti-bacteria/fungi/cancer	4-mPro	Fukushima et al. 1983

*: the methylproline amino acids in the compound have not been confirmed to be 4-methylproline or methylproline.

2. The aim of study

1. To screen benthic (and planktic) cyanobacteria from coastal environments and lichen symbiotic cyanobacteria for natural products with acute myeloid leukemia apoptosis-inducing activity (paper I)

2. To identify new trypsin inhibitors from cyanobacteria isolated from terrestrial and brackish water habitats with NMR and LC-MS (papers II, III).

3. To study the biosynthesis of pseudoaeruginosins in the bloom-forming cyanobacterium *Nodularia spumigena* (paper III).

4. To find new bioactive compounds containing 4-mPro from cyanobacteria. The structure and functional mechanism of new natural products were also in the study focus (paper IV).

3. Materials and Methods

The material and methods are described in detail in the four articles I-IV. A list of methods used in this study is shown in Table 3.

Table 3. The methods used in the thesis.

Methods	Paper
Mass cultivation of experimental cyanobacteria strains	I, II, III, IV
Isotope labeling experiment with ^{15}N and ^{34}S	II, V
PCR	I, III, IV
16S rRNA gene sequencing	I, IV
HPLC purification	II, III, IV
LC-MS analysis	I, II, III, IV
High resolution UPLC-ESI-QTOF mass spectrometry	II, III, IV
NMR	II, III, IV
Optical rotation	II
Infrared spectroscopy	II
UV spectrum measurement	II
Organic synthesis	III
Amino acid feeding experiment	III
Chiral analysis of amino acid	II, III, IV
Trypsin inhibition test	II, III
Microcystin apoptosis-inducing experiment	I, IV
Adenosine apoptosis-inducing experiment	I
The screening of apoptosis-inducing activity against IPC-81 cells	I
Green fluorescence protein expression	IV
The inhibition of protein OATP1B1/1B3 mediated transport of	I,IV
Computational docking analysis	II
Bioinformatic analysis	IV

4. Results and Discussion

4.1. Apoptogens against AML in cyanobacteria

Marine cyanobacteria have been shown to be a good resource to find anti-cancer compounds (Tan 2007; Dixit and Suseela 2013). In this research, forty cyanobacterial strains isolated from coastal, benthic and lichen-symbiotic environments were utilized to explore new anti-AML compounds. All the cyanobacteria strains were freeze dried and extracted with water, methanol and dichloromethane at the rate of 1:1:1. After the centrifugation, the upper layer (aqueous extracts) and lower layer (organic extracts) were separated and used in the cell apoptosis-inducing experiments. More than half of these strains contained the apoptosis-inducing activity (**Figure 7**).

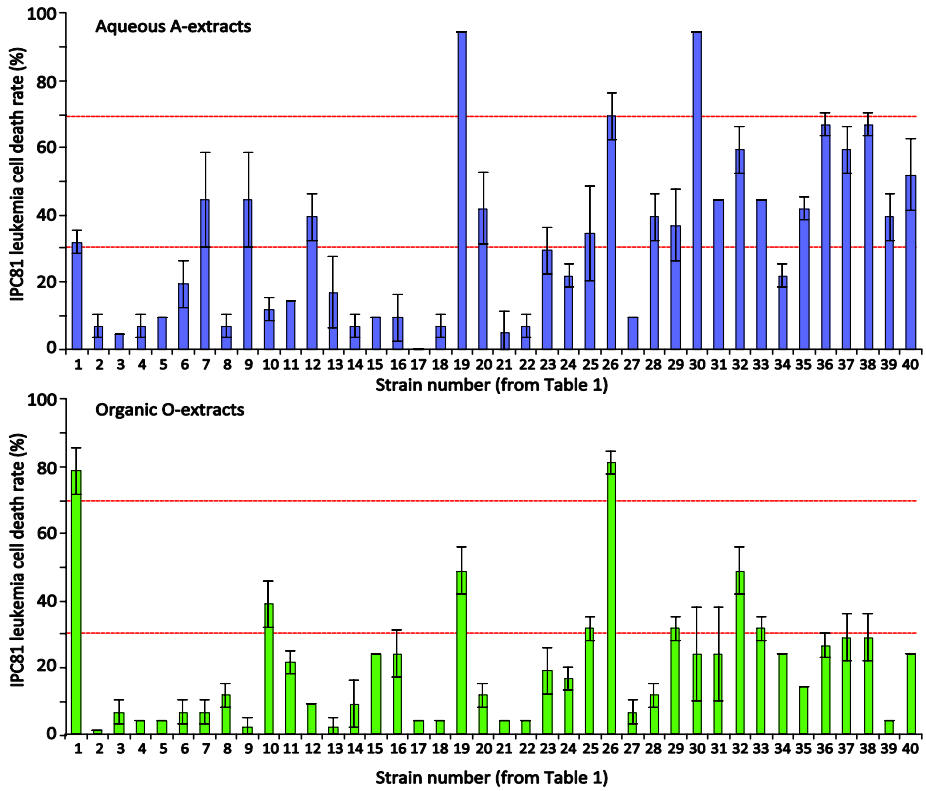


Figure 7. Leukemia cell death induced by cyanobacteria extracts. IPC-81 (the cell line IPC-81, isolated from brown Norwegian acute myelocytic leukemia rats) cells were incubated with extracts from 5 mg biomass/ml cell suspension for 24 h

before fixation in 2% buffered formaldehyde (pH 7.4). The X-axis gives the strain numbers. Cell death was assessed by microscopic assessment of the cell surface and nuclear morphology. The horizontal lines show 30% (low) and 70% (high) apoptosis levels.

Cyanobacteria are well-known producers of microcystin or nodularin, hepatotoxins common especially in cyanobacterial blooms; (Dittmann and Wiegand 2006; Sivonen 2009). More recently, they were found in terrestrial environment in lichen symbiotic cyanobacteria (Oksanen et al. 2004; Kaasalainen et al. 2012). Adenosine, which has been proven to be effective against leukemia cells *in vitro* (Tanaka et al. 1994), is often found in aquatic extracts of freeze-dried cyanobacteria cells. Here a new experimental strategy was used to exclude adenosine and microcystins/nodularins in order to increase the efficiency of finding new anti-AML compounds. The cyanobacterial cell extracts, which were treated with adenosine deaminase, were used to induce the apoptosis of AML cells. Both LC-MS and microcystin-inducing apoptosis experiment were introduced to test the existence and amount of microcystins in the cyanobacterial cell extracts. It was shown that many lichen-symbiotic cyanobacterial strains contain a large amount of microcystins, and adenosine (**Table 4** and **Figure 8)**. Those strains producing adenosine and high amounts of microcystin received more concern during the screening, because it was not clear whether new apoptogens or microcytin/adenosine lead to the apoptosis of leukemia cells.

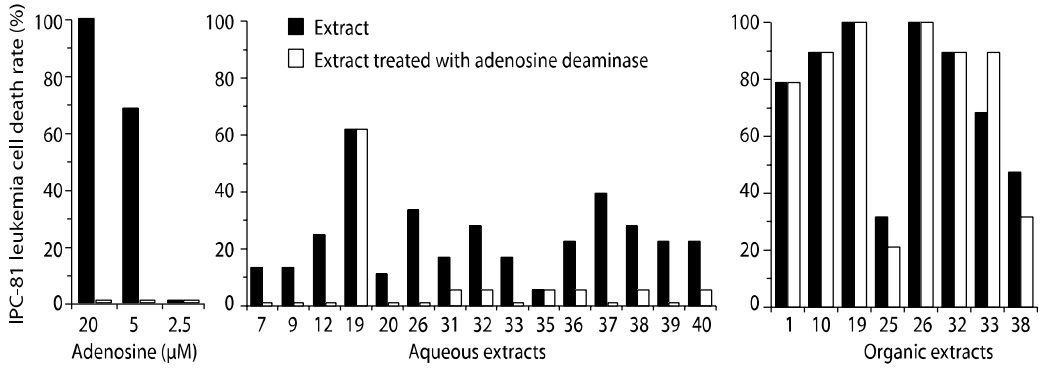


Figure 8. The presence of adenosine or deaminase-sensitive compounds in cyanobacterial extracts.

Table 4. The presence of microcystin and microcystin-like activity in selected cyanobacteria extracts.

Extract	Strains	Habitats	Microcystin-like activity in OATP-transfected HEK293 cells	Microcystins by LC-MS (nM)
L1-A	*Calothrix* HAN 24/1	Marine	–	–
L7-A	*Calothrix* HAN 24/1	Marine	–	3.9
L19-A	*Nodularia* HAN 37/1	Marine	–	–
L20-A	*Calothrix* HAN 16/1	Marine	–	–
L26-A	*Anabaena* HAN 21/1	Marine	–	–
L30 A	*Nostoc* 113.5	lichen-	ND	3.8
L31-A	*Nostoc* UK 92Ic	lichen-	+	2100
L32-A	*Nostoc* UK 89	lichen-	++	2700
L33-A	*Nostoc* UK 81I	lichen-	+	5100
L35-A	*Nostoc* UK 60II	lichen-	/	2600
L36-A	*Nostoc* N135.9.1	lichen-	–	620
L37-A	*Nostoc* N138	lichen-	+	13,000
L38-A	*Nostoc* UK 104	lichen-	–	54
L39-A	*Nostoc* UK 220Ib	lichen-	+	2200
L40-A	*Nostoc* N134.1	lichen-	+	1300
L1-O	*Calothrix* HAN 24/1	Marine	–	–
L10-O	*Calothrix* HAN 21/5	Marine	–	–
L19-O	*Nodularia* HAN 37/1	Marine	–	–
L25-O	*Anabaena* HAN 15/2	Marine	–	–
L26-O	*Anabaena* HAN 21/1	Marine	–	–
L32-O	*Nostoc* UK 89	lichen-	–	43
L33-O	*Nostoc* UK 81I	lichen-	–	89
L38-O	*Nostoc* UK 104	lichen-	–	5.6

The + signifies the presence of bioactivity-inducing microcystin-like apoptotic morphology in OATP1B3-transfected HEK293 cells (second column) or the detection of microcystin or nodularin by LC-MS (third column). +, present, but less than 30% apoptosis; ++, strong (30%–100% apoptosis); –, absent; /, unknown; ND = not determined due to necrotic morphology.

It was reported that many cyanobacterial stains isolated from Baltic Sea contained anti-leukemia activity, but no new natural products were reported (Oftedal et al. 2010). In this study, seven cell extracts showed good potential against leukemia cells, some of them can even overcome the chemoresistance caused by regulated proteins p53 (tumor suppressor) and Bcl-2 (B-cell lymphoma-2) (**Table 5)**.

Table 5. The involvement of the chemotherapy resistance gene Bcl-2 and p53 in apoptosis caused by potent cyanobacteria extracts. A comparison of the EC_{50} (half maximal effective concentration) values of potent cyanobacterial extracts against IPC-81 and Molm-13 cell lines.

Extract	Strains	EC_{50} (IPC-81)	Bcl-2 [a]	p53 [b]	EC_{50} (Molm-13)
L19-A	*Nodularia* HAN 37/1	2.4	-	0	2.1
L26-A	*Anabaena* HAN 21/1	>3.1	-	0	>3.1
L30-A	*Nostoc* 113.5	0.8	-	-	<0.5
L36-A	*Nostoc* N135.9.1	>4.8	-	0	>4.8
L1-O	*Calothrix* HAN 24/1	0.3	-	-	<0.3
L26-O	*Anabaena* HAN 21/1	<0.3	+	0	<<0.3
L30-O	*Nostoc* 113.5	ND	+	ND	ND

[a] -, extract not able to overcome resistance from Bcl-2; +, extract induces cell death also in cells overexpressing Bcl-2; [b] 0, no change in cell response dependent on the p53 status; −, absence of p53 protects cells from cyanobacterial extract; +, absence of P53 sensitizes cells to cyanobacterial extract; ND, not determined.

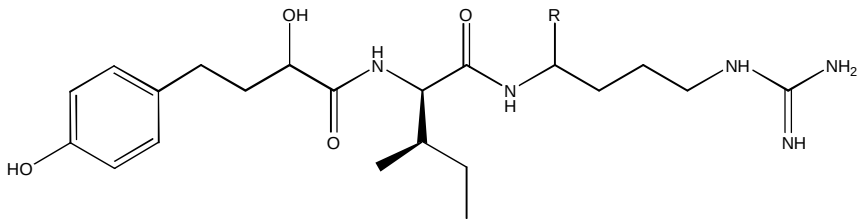
The active apoptogens in these positive cell extracts were also studied (**Table 4)**. The hassallidins were firstly discovered as antifungal compound against fungi *Aspergillus fumigatus* and *Candida albicans* (Neuhof et al. 2005). The new hassallidin variants were identified from *Nostoc* sp. 113.5 (L30) with LC-MS, and its biosynthesis genes were previously confirmed with PCR (Vestola et al. 2014). Scytophycins are well-known natural products with antifungal and cytotoxic activity. They were first identified from *Scytonema pseudohofmanni* (Ishibashi et al. 1986). Here, the new variant of scytophycins was found from *Anabaena* sp.

Han 21/1(L26). All these data indicated that the antifungal compounds identified in cyanobacteria might be a good resource to find new anti-cancer activities.

4.2. Nostosins-trypsin inhibitors

A pair of new trypsin inhibitors, nostosin A/B, was discovered from *Nostoc* sp. FSN. The nostosins were obtained using RP-HPLC with the guide of LC-MS. The MS, NMR and amino acid analysis showed they are composed of three residues, Hhpba, L-Ile, and L-argininal/argininol (**Figure 9)**.

Hhpba **L-Ile** **L-Argininal/Argininol**



Nostosin A: R= CHO
Nostosin B: R= CH_2OH

Figure 9. Chemical structures of nostosin A and nostosin B.

Nostosins inhibited porcine trypsin with an IC_{50} (half minimal inhibitory concentration) value of 0.35 µM (nostoin A) and 55 µM (nostosin B). Besides nostosins, many trypsin inhibitors containing the characterized subunit arigninal were identified, such as spumingin E, aeruginosin 102A, leupeptin, aeruginosin 89A (**Table 6**) (Aoyagi et al. 1969; Matsuda et al. 1996; Ishida et al. 1999; Fewer et al. 2009). All of them are potent trypsin inhibitors, which show an IC_{50} below 0.6 µM. However, some other trypsin inhibitors without argininal from cyanobacteria, such as aeruginosin 205A/B, also inhibit trypsin with a low IC_{50} (**Table 6)**.

This indicates that other subunits or whole structure of compounds are also important in trypsin inhibition. The cyanobacterial trypsin inhibitor, aeruginosin 98-B (argininol), was crystallized with trypsin together in order to study how linear trypsin inhibitor binds to the activity pocket of trypsin (Sandler et al. 1998). However, it is not clear how argininal interacts with trypsin.

Table 6. The potent linear trypsin inhibitors identified in cyanobacteria and actinomycete. They show an IC_{50} under 0.6 µM.

Name	**1**	**2**	**3**	**4**	IC_{50} (uM)	Reference
aeruginosin 205A	L-Pla	D-Cleu	L-Choi-Xyl-S	Agma	0.09	Shin et al. 1997
aeruginosin 205B	D-Pla	L-Cleu	L-Choi-Xyl-S	Agma	0.09	Shin et al. 1997
leupeptinΔ	Ac	L-Leu	L-Leu	L-Argal	0.5	Aoyagi et al. 1969
aeruginosin 89A	D-Hpla-Cl-S	D-Leu	L-Choi	L-Argal	0.56	Matsuda et al. 1996
aeruginosin 102A	D-Hpla-S	D-Tyr	L-Choi	L-Argal	0.27	Ishida et al. 1999
spumigin E	D-Hpla	D-Hty	L-mPro	L-Argal	<0.1	Fewer, et al. 2009

Ac = acetyl, Argal = argininal, Agma (agmatine) = 4-amidinobutylamide, Cleu = 3-chloro-Leu, Choi = 2-carboxy-6-hydroxyoctahydroindole, Choi-Xyl-S = Choi-6-O-(4-O-sulfate-xylose), Hpla-Cl-S = O-sulfated-m-chloro-Hpla, Hpla-S = O-sulfated-Hpla, Pla = phenyllactic acid. Δ: leupeptin was identified in actinomycete.

The nostosins A showed far more potent activity than nostosin B. These two inhibitors have a very similar structure and differ only at the third residue, which is argininal in nostosin A, and argininol in nostosin B (**Figure 9**). The argininal residue seems to be crucial for trypsin inhibition. Similar results were also reported in case of spumigins A and E (Fewer et al. 2009). The compounds of aeruginosin 89 A and B show that also the configuration of argininal is important so that L configuration in argininal makes the compound an efficient trypsin inhibitor (Ishida et al. 1999). In order to gain insight into nostosins inhibition mechanism, computational docking of nostosins with trypsin was carried out (**Figure 10).**

As the stereochemistry of Hhpba was not proven, two configuration of Hhpba was used to make computer docking of nostosins. From the docking prediction, nostosin A in D-L-L configuration binds in a similar manner to leupeptin, which is potent trypsin inhibitor discovered from actinomycetes (Aoyagi et al. 1969; Kurinov and Harrison. 1996).

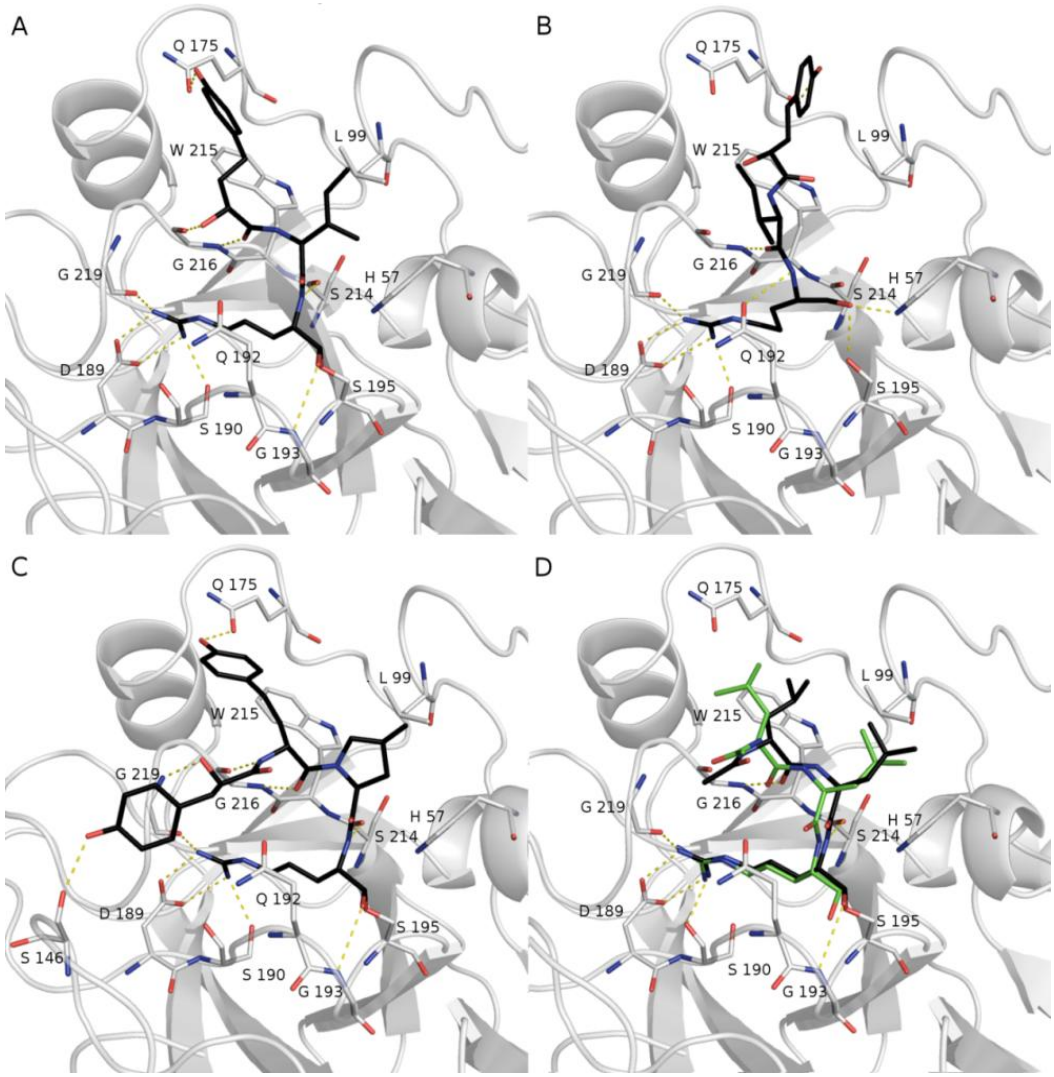


Figure 10. Binding poses of nostosins A and B, spumigin E and leupeptin. (A) Most favorable binding orientation of nostosin A in the binding site. (B) Most favorable binding orientation of nostosin B in the binding site. (C) Most favorable binding orientation of spumigin E in the binding site. (D) Most favorable binding orientation of leupeptin in the binding site (carbon atom labeled with black) and extracted from the X-ray crystal structure with trypsin (protein data bank code: 1jrs; carbon atom labeled with green).

The argininal of nostosin A and leupeptin could form covalent bond with serine Ser195. In nostosin B, the argininal was replace with argininol, therefore the absence of covalent bond prevents strong binding of nostosin B to the activity center of trypsin. In addition, the guanidino group of nostosins forms an ion-pairing with Asp189 and two strong hydrogen bonds with Ser190 and Gly219. These noncovalent bonds, which happen in the S1 selectivity pocket in trypsin, strengthen the inhibition on the trypsin.

4.3. Pseudoaeruginosins-trypsin inhibitors

Nodularia spumigena is a filamentous cyanobacterium, which could fix nitrogen. A few bioactive compounds have been found from *Nodularia* sp. such as spumigins, aeruginosins, nodularins (Fujii et al. 1997; Fewer et al. 2009; Fewer et al. 2013). In this study, the structures of pseudoaeruginosins NS1 and NS2 are similar to the new discovered trypsin inhibitor, aeruginosin NAL2/NOL3, with only difference that the Choi was replaced with 4-mPro (**Figure 11)**. It means pseudoaeruginosins might come from aeruginosin biosynthesis pathway.

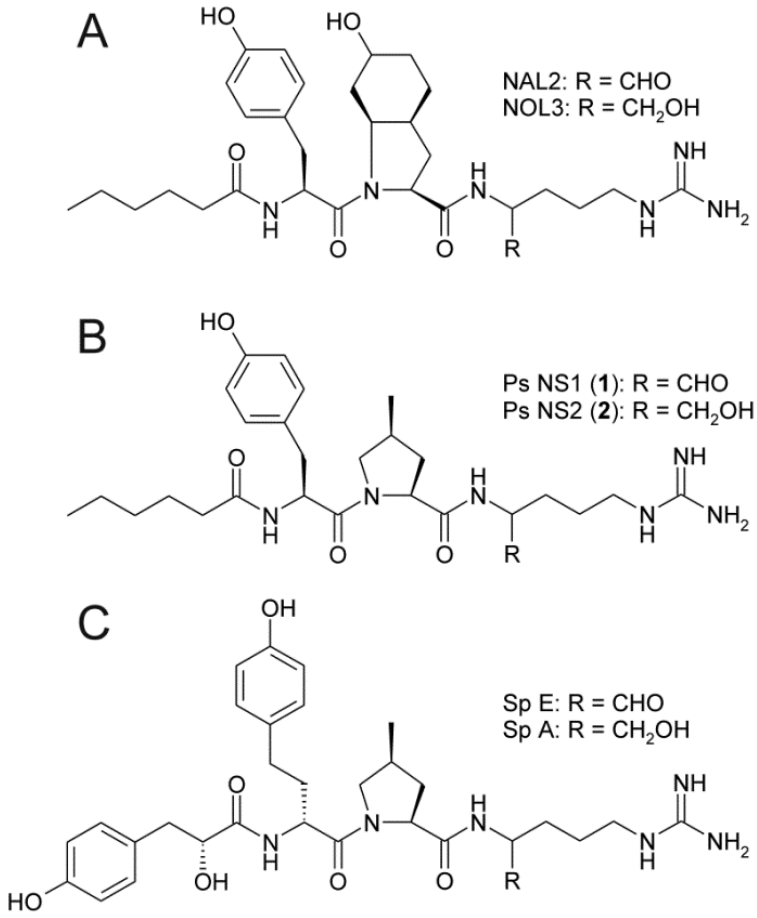


Figure 11. Aeruginosins and spumigin produced by strains of *Nodularia spumigena* isolated from brackish water bodies in Australia and the Baltic Sea. (A) aeruginosin NAL2, (B) methylproline-containing aeruginosin NS1, (C) spumigin E.

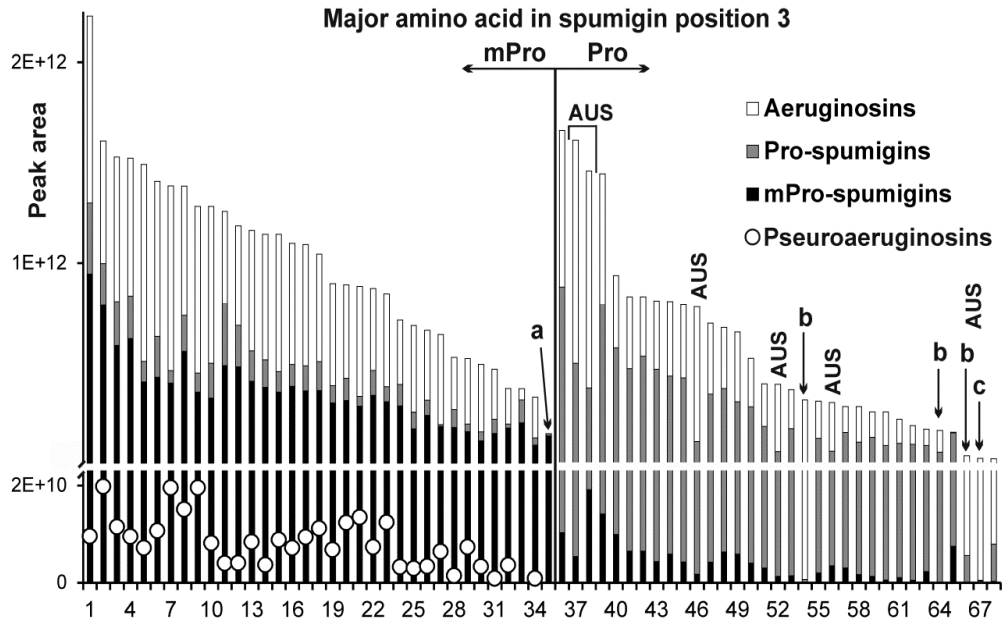


Figure 12. The peak areas from the extracted ion chromatograms of the protonated compounds of aeruginosins and spumigins. a = no detectable levels of aeruginosins, b = no detectable levels of mPro containing spumigins, c = no detectable levels of Pro containing spumigins, AUS = Australian strains.

In order to check the distribution of pseudoaeruginosins in the *Nodularia spumigena*, a total of 68 *N. spumigena* strains collected from Baltic Sea and Australia were screened to find pseudoaeruginosins. A total of 33 strains showed production of pseudoaeruginosins, but the others contain no pseudoaeruginosins or the amount is too low to detect **(Figure 12)**. It was found that pseudoaeruginosin successfully synthesized by those cyanobacteria strains which could produce a large amount of aeruginosin and mPro-spumigin. On the right panel of **Figure 12**, the production of mPro-supmigin is so low that the cyanobacteria strains have not enough 4-mPro as substrate for the synthesis of pseudoaeruginosin. These findings suggest that the spumigin biosynthesis pathway is involved in the synthesis of pseudoaeruginosins by providing 4-mPro to the aeruginosin synthetase.

Spumigins and aeruginosins were reported to have their own NRPS pathway. In *Nodularin spumigena* CCY9414, no other biosynthetic gene clusters, which code

for peptides resembling pseudoaeruginosin were discovered from this strain **(Fewer et al. 2009; Fewer et al. 2013)**. This suggests that pseudoaeruginosins are produced through the joint action of spumigin and aeruginosin biosynthesis pathways. This is first time in cyanobacteria to discover that two NRPS biosynthesis pathways function together to assemble new metabolites (**Figure 13)**.

Spumigin biosynthesis is most likely responsible for producing mPro for spumigin and pseudoaeruginosin biosyntheses. Some of the 4-mPro is proposed here to be activated by the AerG adenylation domain of aeruginosin synthetase leading to the incorporation of 4-mPro into the growing pseudoaeruginosin chain. It has been reported that NRPS of naphthyridinomycin and quinocarcin incorporate hydroxyethyl C_2 from central metabolic ketosugars by transketolases in *Streptomyces lusitanus* **(Peng et al. 2012)**. This is an example of joint work between central metabolites and NRPS biosynthesis pathway.

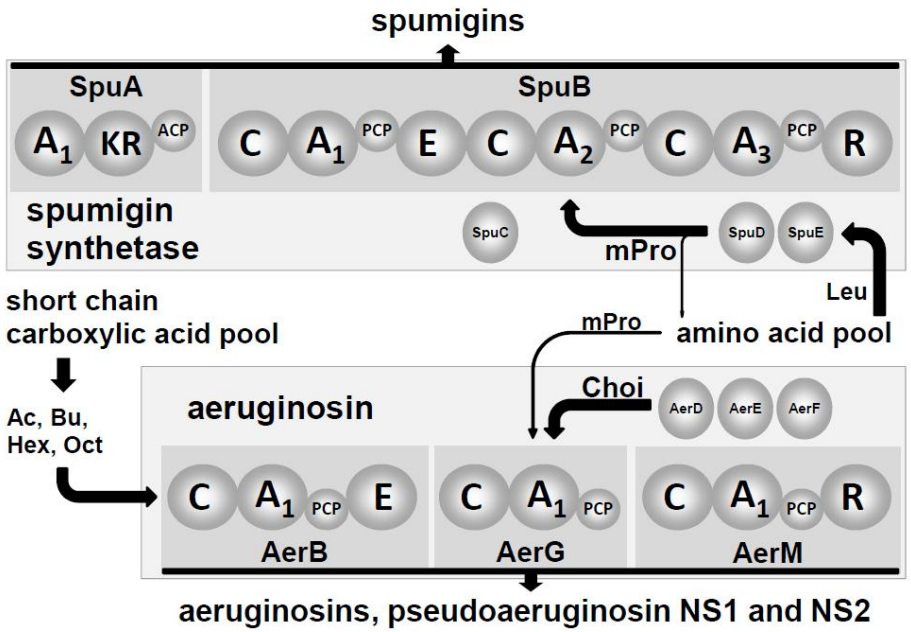


Figure 13. The proposed interaction between spumigin and aeruginosin biosynthetic pathways leading to the formation of pseudoaeruginosin NS1 and NS2. *spuD* and *spuE* are genes for 4-mPro synthesis, *spuC* function is unknown and *aerD*, *aerE* and *aerF* genes are for Choi synthesis **(Fewer et al. 2009 and 2013)**.

Abbreviations: A, adenylation domain; ACP, acyl carrier protein; C, condensation domain; PCP, peptidyl carrier protein; KR, keto reductase domain; E, epimerase domain; R, reductase domain.

4.4. Nostoweipeptins and nostopeptolides-antitoxins against microcystin

In cyanobacteria, the biosynthesis of the non-proteinogenic amino acid 4-mPro was first revealed by Luesch and coworkers (Luesch et al. 2003). Two genes, *nosE* and *nosF*, were confirmed to code two enzymes that are responsible for converting leucine to 4-mPro. In this study, genes *nosE* and *nosF* were used to find new gene clusters possibly coding for 4-mPro-containing compounds. The gene clusters examined came from national center for biotechnology information (NCBI) public genome database. Finally, eight gene clusters containing 4-mPro biosynthetic genes were found (**Figure 14)**.

Figure 14. The gene clusters of 4-mPro containing-compounds in cyanobacteria were blasted from national center for biotechnology information public cyanobacteria genome database.

In these gene clusters, 4-mPro genes have conserved distribution with open reading frame (orf) and one ATP-binding cassette transporter (ABC transporter) (**Figure 14**). Therefore, it is possible to make use of methylproline genes to screen new natural products from cyanobacteria with PCR. Even though only eight gene clusters with 4-mPro genes were found, it does not mean that 4-methylproline genes are rare in cyanobacteria since only limited genome data is available.

In order to explore more compounds with 4-mPro amino acid and check the distribution of 4-mPro in cyanobacteria, 116 cyanobacteria strains were screened with a combination of PCR and LC-MS (**Figure 15)**.

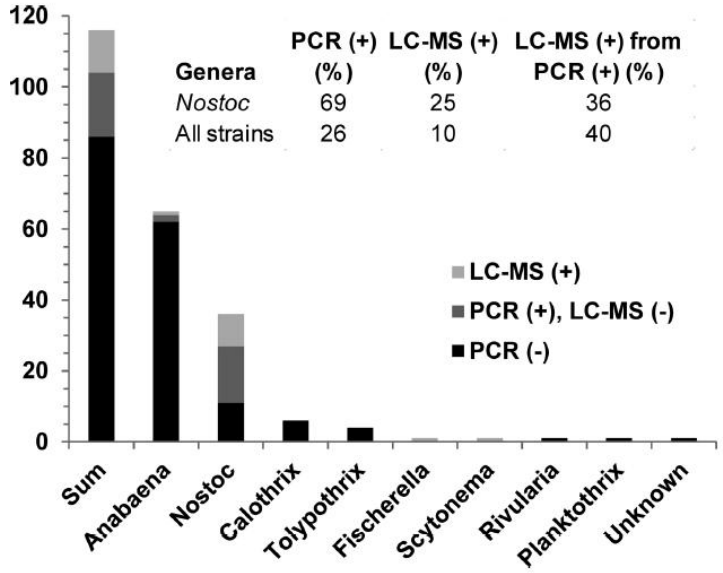
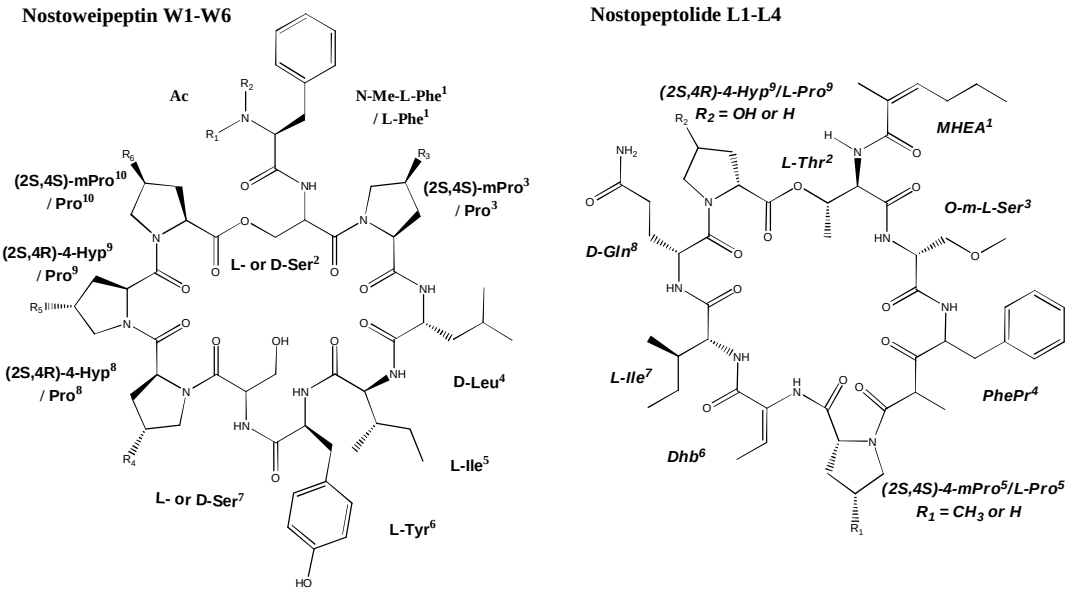


Figure 15. PCR screening of 4-mPro biosynthetic genes from 116 cyanobacterial strains, and LC-MS analysis of the presence of 4-mPro in the PCR positive strains.

The 4-mPro genes were found from genus of *Anabaena*, *Nostoc*, *Fischerella* and *Scytonema*. Most of the 4-mPro owners are from *Nostoc* sp. The frequency of 4-mPro genes in *Anabeana* sp. is low. Based on the screening results, the 4-mPro genes are not rare in cyanobacteria, especially in *Nostoc* strains. A total of 12 producers of 4-mPro were found. With the guidance of LC-MS analysis, two group of cyclic peptides, nostoweipeptins and nostopeptolides were identified from two

cyanobacteria strains, *Nostoc* sp. XPORK 5A and *Nostoc* sp. UK2aImI, respectively (**Figure 16)**.

In cyanobacteria, the nostopeptolide was first identified by Golakoti et al. (2000)**.** By comparing nostopeptolide A with the new nostopeptolide L1 discovered in this study (**Figures 16** and **17)** it is apparent that nostopeptolide A contains more proteinogenic amino acids, while more non-proteinogenic amino acids appear in nostopeptolide L1. In addition, two different proline derivatives, 4-mPro and 4-OH-Pro, were found in nostopeptolide L1. Unluckily, the nostopeptolide A was not checked for protection of hepatocytes from apoptosis during the discovery, so it is difficult to compare these two compounds from the aspect of biological function and structure.



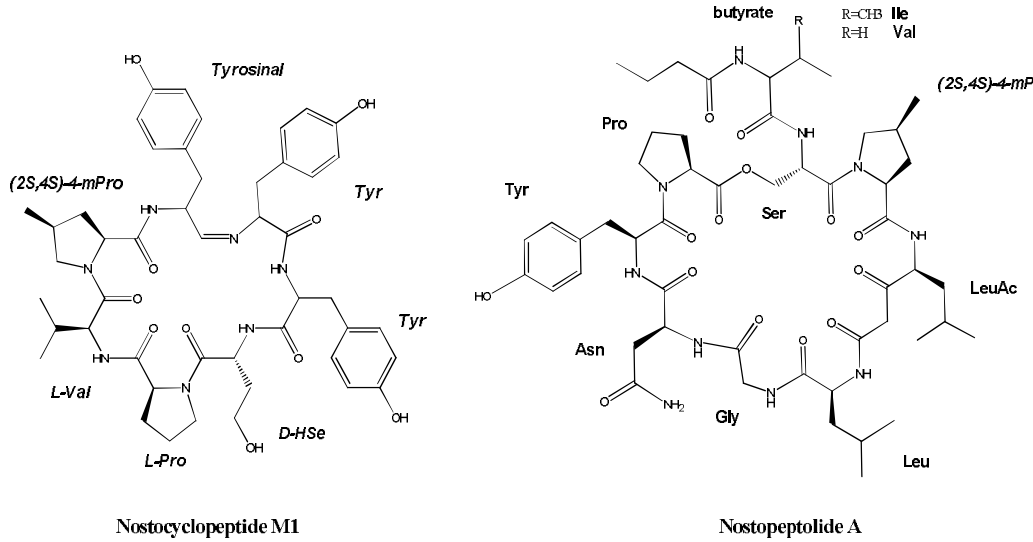
W1: R_1= CH_3CO , R_2 = CH_3, R_3= CH_3, R_4= OH, R_5= OH, R6= CH_3
W2: R_1= CH_3CO, R_2 = CH_3, R_3= CH_3, R_4= OH, R_5= H, R6= CH_3
W3: R_1= CH_3CO, R_2 = CH_3, R_3= CH_3, R_4= H, R_5= OH, R6= CH_3
W4: R_1= CH_3CO, R_2 = H, R_3= CH_3, R_4= OH, R_5= OH, R6= CH_3
W5: R_1= CH_3 CO, R_2 = CH_3, R_3= CH_3, R_4= OH, R_5= OH, R6= H
W6: R_1= CH_3 CO, R_2 = CH_3, R_3= H, R_4= OH, R_5= OH, R6= CH_3
W7: R_1= H, R_2 = CH_3, R_3= H, R_4= OH, R_5= OH, R6= CH_3

L1: R_1= CH_3 , R_2 = OH
L2: R_1= CH_3 , R_2 = H
L3: R_1= H, R_2 = OH
L4: R_1= H, R_2 = H

Figure 16. The chemical structures of nostoweipeptin W1 from *Nostoc* sp. XPORK 5A and nostopeptolides L1-L4 from *Nostoc* sp. UK2aImI.

The nostocyclopeptide M1 (**Figure 17**) was the first identified bioactive cyclic peptide with 4-mPro which inhibits apoptosis of hepatocytes caused by hepatotoxin microcystin by blocking the anion transporters OATP1B1/1B3 (Jokela et al. 2010). When nostocyclopeptin M1 was compared to nostopeptolides L and nostoweipeptins, the only similarity was that all of these three compounds have a ring structure with a side chain and 4-mPro. Herfindal et al. (**2011**) synthesized a analogue of M1, which is only composed of L proteinogenic amino acids. The new moleculehad lower bioactivity than M1 (Herfindal et al. 2011). It suggested the 4-mPro might play an important role in the blocking of ion transporters OATP1B3 and OATP1B1.

Nostoweipeptins are different from the nostocyclopeptide M1, nostopeptolide A and nostopeptolide L1-L4 peptides with 4-mPro. Nostoweipeptins contain two 4-mPro and two 4-OH-Pro. It indicates that the biosynthesis of nostoweipeptins differs from the others.



Nostocyclopeptide M1 **Nostopeptolide A**

Figure 17. The chemical structures of nostocycleopeptin M1 and nostopeptolide A (Golakoti et al. 2000; Jokela et al. 2010)

OATP1B1 and OATP1B3 are human organic anion transporters, which are responsible for transport of many endobiotics and drugs. They were confirmed to

deliver the hepatotoxin, microcystin/nodularin, into hepatocytes in order to cause apoptosis of hepatocytes (Fischer et al. 2005). Here, nostopeptolide L and nostoweipeptins were screened for their inhibition on OATP1B1/1B3 (**Figure 18)**. Among five cyclic peptides, nostoweipeptins show the greatest inhibition on OATP1B3, while nostopeptolide L2 is the best inhibitor on OATP1B1. The difference on bioactivities of cyclic peptides shows their different binding specificity on the organic anion transporters. To study the relationship between structure and activity in these cyclic peptides could help us to find good selective inhibitors on organic transporters OATP1B1 and OATP1B3.

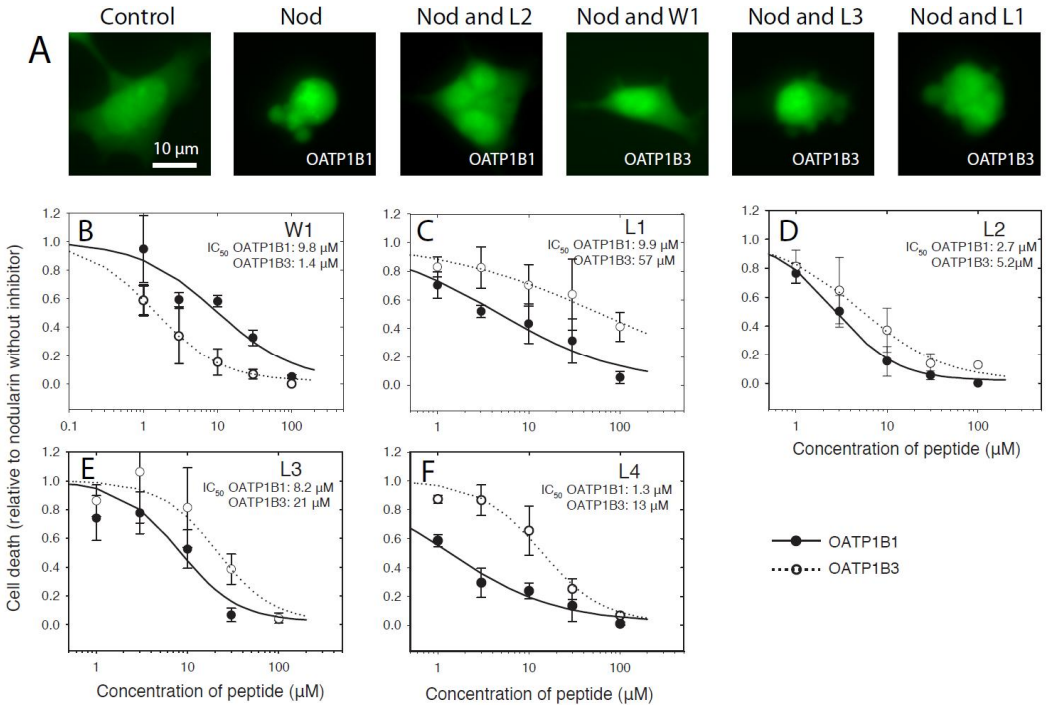


Figure 18. 4-mPro-containing nostopeptolides (Np) L1-L4 and nostoweipeptin (Nwp) W1 inhibit OATP1B1/1B3 mediated transport of nodularin (Nod) into transfected HEK293T cells. A: Fluorescent images of cells co-transfected with GFP and either OATP1B1 or OATP1B3. Note the round appearance and polarized blebbing of Nod-treated cells compared to control. Np L2 and Nwp W1 show complete protection whereas Np L3 and Np L1 have intermediate protection and

the cells were pro-apoptotic. B: HEK293T cells transfected with OATP1B1 or 1B3 were treated with increasing concentrations of peptides before addition of Nod and further incubations as in A. IC_{50} values were determined based on the ratio of green fluorescence protein-positive cells with apoptotic or normal morphology. The data are average of three independent experiments and scanning electron microscope. The curves are based on the IC_{50} values from the regression analyses.

5. Conclusion and Prospects

Of 40 benthic and lichen symbiotic cyanobacteria strains were studied for new anti-AML compounds. Half of them contained anti-leukemia activity. Two new kinds of anti-leukemia compounds, which are new variants of hassallidins and scytophycins, were identified. This study improved screening reliability of anti-cancer compound from cyanobacteria by excluding the interference of microcystins and adenosine. The marine cyanobacteria showed much more potential than lichen-symbiotic cyanobacteria to find new anti-AML activity.

A new pair of trypsin inhibitors, nostosinA/B, were identified from *Nostoc* sp. FSN. The computer docking showed that nostosin A binds to trypsin with a similar pose like commercial trypsin inhibitor leupeptin. This is the first time to find potent trypsin inhibitor with three residues from cyanobacteria. It not only revealed a new trypsin inhibitor, but also gave a further insight of how trypsin enzyme interacts with compact linear inhibitors. The nostosins could be used as a drug leads to test their bioactivity on human disease experiment *in vitro*. Their structures could be still optimized to improve their stability and activity with organic synthesis methods.

Pseudoaeruginosins are linear tetrapeptides, which were identified as potent trypsin inhibitors from *N. spumigena* collected from Baltic Sea. The biosynthesis of pseudoaeruginosins was revealed as a joint work of aeruginosin biosynthesis pathway and methylproline biosynthesis genes in spumigin gene cluster. This study demonstrated that NRPS pathway in cyanobacteria could use the enzymes in another NRPS pathway to produce more diverse secondary metabolites. It also exhibits a new way to synthesize antibiotics or drug leads with combined biosynthesis.

4-mPro was once considered a rare non-proteinogenic amino acid. In this study, eight 4-mPro-containing gene clusters were found in NCBI publicly available genome database, and 12 good producers of 4-mPro amino acid were found when 116 cyanobacteria were screened by LC-MS. Two new groups of cyclic peptides

with 4-mPro, nostopeptolides and nostoweipeptins, were identified as antitoxin against hepatocyte apoptosis induced by microcystin by blocking anion transporters OATP1B1/B3. The study of the genes coding for enzymes assembling the nonproteinogenic amino acids are needed to reveal details of their biosynthesis and possible biotechnological utilization of the enzymes in future. The additional studies are needed to understand how the new compounds block anion transporter OATP1B1/B3 and the role of 4-mPro in this process.

Nowadays, there is no doubt that antibiotic resistance and chemotherapy resistance have result in the loss of many useful medicines in the disease treatment. It is urgent to find new drugs to fulfill the need of patients. In this research, several bioactive compounds were identified. It was shown that cyanobacteria are good candidates to find novel compounds or drug leads. However, the bioassay was performed *in vitro*, so it is not clear whether they will function *in vivo* or be stable in the human metabolism. The new compounds could be evaluated in animal models. Since the natural products usually are relatively large and not each subunit play vital role in keeping the bioactivity, their structures could be optimized with organic synthesis methods combined with bioassays as a guide. In addition, the biosynthesis of these new identified compounds could be studied in the future. It not only lead us to have a deep insight how cyanobacteria synthesis secondary metabolites, but also provide some ideas for produce new compounds with synthetic biology.

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